Study of the interactions between milk proteins and hydroxyapatite particles

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Food Technology

at Massey University, Riddet Institute, Palmerston North,

New Zealand

MASSEY UNIVERSITY

Lucile Tercinier

2016

ABSTRACT

Hydroxyapatite (HA) and other insoluble calcium salts added to calcium-fortified milks are often described as inert, as they do not cause any protein aggregation and heat instability during heat treatment of the milk. However, it is well-known that proteins can interact with HA. The adsorption of milk proteins on HA has been demonstrated in many systems, for example in chromatography, bioceramic and dentistry applications, and has been shown to have consequence on the colloidal stability of HA, but has never been studied in food systems.

The main objective of the present study was therefore to explore the adsorption of milk proteins onto HA particles under a range of physico-chemical conditions. The consequences of these interactions on the colloidal properties of the HA particles and on the stability of the milk proteins were investigated.

It was shown that the five individual milk proteins α_S-casein, β -casein, κ-casein, β-lactoglobulin and α-lactalbumin adsorbed onto the HA particles. A Langmuir model was used to fit the adsorption data and determine the affinity constant and maximum surface loads of the different proteins. The adsorption of the different milk proteins onto HA particles was found to be of competive nature. β-casein and α_S-casein were always preferred for adsorption over κ-casein, β-lactoglobulin and α-lactalbumin. This was attributed to the presence of phosphoserine clusters in β-casein and α_S-casein, forming many anchor points capable of binding to the calcium sites of HA. β-casein and α_S-casein also adsorbed to higher maximum levels compared to κ-casein, β-lactoglobulin and α-lactalbumin. Both β-Casein and α_S-casein were considered to self-associate or associate together in the adsorbed layer, therefore forming a thick layer onto the HA surface. Conversely, κ-casein, β-lactoglobulin and α-lactalbumin adsorbed to lower maximum amounts and had lower affinities for HA, which was attributed to adsorption in a monolayer through their carboxyl groups binding to the calcium sites of HA.

The amount of protein adsorbing to the HA surface was affected by the physico-chemical properties of the solution such as pH and ionic strength, for all proteins. Decreasing pH and increasing ionic strength decreased the electrostatic repulsive forces between HA and the proteins and the electrostatic repulsive forces within the protein molecules, which allowed more protein to adsorb onto the HA surface. Milk serum ions such as calcium, phosphate

and citrate bound specifically onto HA particles, therefore competing with the milk proteins for adsorption.

In milk, it was shown the addition of HA in milk disrupted the mineral equilibrium and the milk protein phase. When HA particles were added to milk, the milk serum ions bound to the HA surface. This caused the colloidal calcium phosphate to be released from the casein micelles and the casein micelles to dissociate. Therefore the casein micelles did not bind as intact micelles but as individual molecules or small aggregates onto the HA particles.

The adsorption of milk proteins onto HA particles affected the colloidal properties of the HA particles in suspension. The adsorption of both caseins and whey proteins onto HA particles resulted in the particles becoming negatively charged, thus improving their suspension stability. Whey protein adsorption probably provided only electrostatic stabilisation, whereas casein adsorption also provided steric stabilisation.

Overall, this work has provided a detailed understanding of the interactions between milk proteins and HA particles. Calcium fortification of milk using insoluble calcium salts such as HA should be approached using an awareness of these interactions, as they may have consequences on the stability of calcium fortified milks.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisors, Dr Aiqian Ye, Professor Harjinder Singh and Anne Singh for their expert assistance, support, advice and encouragements.

I also would like to acknowledge Fonterra Co-operative group Ltd. for funding and hosting the research at the Fonterra Research and Development Centre (FRDC), and especially Mike Donkin and Andy Smith.

I am very grateful to Dr Skelte Anema for many hours of stimulating and valuable discussion and his contribution on my papers, Dr Ran Gao and Graeme Gillies for very good advice on chemistry and physical aspects of my topic, Dr Tim Coolbear for his mentoring role and precious friendship during the whole three years, Michael Loh for his help in microscopy and the RADA group for carrying out the mineral analysis.

Special thanks to all the people I have been working alongside with at FRDC, they make FRDC a great and friendly place to learn and work and a special thought for the people in the Food Science lab. Another big thank you to the close friends I have met in New Zealand who have now left Palmerston North to live somewhere else: Elise, Quentin, Guillaume and Marie-Pierre, Laureline and Mik, Christina, I miss you. Thanks to the ones who thankfully are still around: Orianne, Emmanuelle, Cyriele, Aurelie, Anne. Everyone, you have made my thesis years in New Zealand unforgettable! Thanks also to the many friends that have followed my adventures from France: Soso, Boubou, Harmo, Karen, Emeline, Clemence, Cloe, Anne, Marie, Marion, Melanie.

Finally and most importantly, a special thank you to my partner, Callum who has been amazingly patient and understanding during the whole thesis and who has never stopped to encourage me and believe in me, to my brother Valentin and my sister Juliette and to my parents who have been loving and supportive for a much longer time than just the thesis. Dad, I am sure you would have been very proud to see me completing my thesis and getting a job in NZ. To dad and mum, I dedicate this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

°C	Degree(s) Celsius
%	Percent
α-CN	α-Casein
α-La	α-Lactalbumin
β-CN	β-Casein
β-Lg	β-Lactoblobulin
κ-CN	κ-Casein
γ-CN	γ-Casein
µL	Microlitre(s)
µm	Micrometre(s)
BSA	Bovine serum albumin
ACP	Amorphous calcium phosphate
ANOVA	Analysis of variance
Asn	Asparagine
Asp	Aspartic acid
BSA	Bovine Serum Albumin
Ca	Calcium
$CaCl_2$	Calcium chloride
CCP	Colloidal calcium phosphate
Cit^{3-}	Citrate ions
Cl	Chlorine
Cl^-	Chloride ions
CMC	Carboxymethylcellulose
COO^-	Carboxyl group(s)
CPP	Caseinophosphopeptide(s)
C-site	Calcium site

DIC	Differential interference contrast
DF	Dilution factor
EC	Extinction coefficient (cm^2/g)
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier transform infrared
g	Gram(s)
g	Centrifugal force
Glu	Glutamic acid
h	Hour(s)
H^+	Protons
HA	Hydroxyapatite
HCl	Hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K	Langmuir equilibrium constant (100g/g)
K	Potassium
kDa	Kilodalton(s)
K_{LF}	Langmuir-Freundlich equilibrium constant ($(100g/g)^{1/n}$)
K_F	Freundlich affinity constant ($(100g/g)^N$)
kJ	Kilojoule(s)
kV	Kilovolt(s)
L	Litre(s)
Lys	Lysine
m_{abs}/S	Mass of protein per unit area (mg/m^2)
MCC	Microcrystalline cellulose
MF	Microfluidic or Microfiltration
Mg	Magnesium
m^2	Square metre(s)

mg	Milligram (s)
min	Minute(s)
mL	Millilitre(s)
mM	Millimolar (mmol.L^{-1})
mmol	Millimole(s)
mol	Mole(s)
n	Surface heterogeinity parameter of the Langmuir-Freundlich model
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_3^+	Amino groups
nm	Nanometre(s)
OH^-	Hydroxyl ions
[P]	Protein concentration at equilibrium
Pi	Inorganic phosphate
pI	Isoelectric point
pK	Dissociation constant
pKa	Acid dissociation constant
PO_4	Phosphate
P-site	Phosphate site
q_m	Maximum surface coverage
s	Second(s)
SEM	Surface electron microscopy
Ser	Serine
Ser-P	Phosphoserine groups
SC	Sodium caseinate
SDS–PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SM	Skim milk
SMUF	Simulated milk ultrafiltrate
T	Temperature
TCP	Tricalcium phosphate
TEM	Transmission electron microscopy
TN	Total nitrogen
TS	Total solids
UHT	Ultra-high temperature
UV	Ultraviolet
V	Volt(s)
WDP-SM	Whey protein-depleted skim milk
WDP-SMP	Whey protein-depleted skim milk powder
WPI	Whey protein isolate
w/w	Weight/weight
ZP	Zeta-potential

CHAPTER 1 Introduction

Bone health is an issue of growing importance, with osteoporosis on the increase largely due to an ageing global population (Gurr, 1999). Dietary calcium is a crucial factor in maintaining bone health (Heaney, 2000) and combating osteoporosis; therefore calcium fortification is a growing trend in the food industry. This is especially true for milk and dairy products, as they are naturally rich in calcium and already well-known for their benefits for bone health (Augustin & Williams, 2002), so consumer awareness and acceptance is already high. Milk in its natural state contains approximately 120 mg of calcium per 100 g (Lucey & Horne, 2009); to fortify milk and dairy products further with calcium, calcium salts such as calcium chloride, calcium phosphate, or calcium citrate need to be added to milk (Omoarukhe, On-Nom, Grandison, & Lewis, 2010; Singh et al., 2007). Selecting an appropriate source of calcium for fortification requires a good understanding of its physical and chemical properties and how it interacts with milk components (De la Fuente, Belloque, & Juarez, 2004). It is also important to take into account the impact of processing treatments on calcium-fortified milk and their consequences on sensory properties and shelf life stability (Philippe, Gaucheron, Le Graet, Michel, & Garem, 2003).

Ultra high temperature (UHT) treatment increases the shelf-life of liquid milks by rendering them sterile and bacteriologically stable for several months at ambient temperature (Datta & Deeth, 2001). UHT treatment of unfortified milk is rarely a problem, as it can withstand such heat treatment without any major issues. However, there are a number of practical issues related to the processing and formulation of calcium-fortified UHT milks, that make it challenging to maintain stability of the product over its intended shelf life (De la Fuente et al., 2004). For example, if soluble calcium salts such as calcium chloride or calcium lactate are used to fortify milk with calcium, their addition can lead to protein aggregation and heat instability during the UHT treatment (Omoarukhe et al., 2010). The milk mineral equilibrium is disrupted by the soluble salts, thereby reducing the electrostatic repulsion between the micelles, and leading to their aggregation on heat treatment. In commercial UHT calcium-fortified milks, insoluble calcium salts, such as tricalcium phosphate, hydroxyapatite (HA) and calcium carbonate, are usually used as calcium fortificants since they are considered to be chemically unreactive in milk, therefore not causing heat instability. However, shelf-life instability, such as phase separation and sedimentation, can occur when insoluble calcium salts are used (Gaucheron, 2003).

Calcium fortification is all the more challenging for calcium-fortified milks when they need to be stored at ambient and sometimes elevated temperatures for up to 9 months of shelf-life. Stabilisers are therefore required in the formulation to guarantee product stability during shelf-life (Gerstner, 2005). The choice of stabilisers in product development, however, is usually approached in a "trial and error" way, based on suppliers' recommendations; and the interactions between the different ingredients (milk proteins, calcium salts and stabilisers) in the products are not always fully understood.

The ideal situation is one where the interactions between a calcium fortification compound and milk components, and between these and stabilisers, are understood to the extent where the formulation of the products can be optimised to guarantee an optimum stability over both processing and shelf-life.

Calcium-fortified milk systems are, however, very complex, and it is therefore necessary to break the systems down into its component parts to understand the individual interactions, and subsequently build up an overall picture. This project therefore focussed on understanding the interactions between a model insoluble calcium salt used in calcium fortification (i.e., HA) and milk components (i.e., milk proteins, milk minerals) over a range of industrially relevant parameters (i.e., pH, ionic strength). Study of interactions with hydrocolloids and other stabilisers was deliberately placed out of scope for this thesis as it would then have become too large and complex.

It is well-known that HA does interact with proteins, as proteins adsorb strongly onto hydroxyapatite in a wide range of biological applications, such as chromatography and bioceramics materials (Kawasaki, 1991; Wang, Zhou, Hong, & Zhang, 2012). However, interactions between HA and milk proteins have never been studied in food systems, despite their industrial relevance. Milk protein adsorption onto HA particles may occur in calcium-fortified milks, and could potentially modify the colloidal stability of the HA particles in the products, or the stability of the milk protein fraction.

The main objectives of this research project were as follows:

(i) To develop an understanding of the interactions between milk proteins and HA in model systems and in milk.

(ii) To characterise the colloidal properties of HA particles in model systems and in milk, with a particular focus on the suspension stability of the particles.

(iii) To identify areas of stability/instability when fortifying milk with calcium salts and use this knowledge to optimise formulations of calcium-fortified beverages.

The overall goal of this project was to build a knowledge base in the area of calcium-fortified UHT milk, with the aim of understanding the interactions when HA particles are added to milk at neutral pH. Such an understanding could then be used to develop strategies to minimise calcium-induced instability of the milk during processing and shelf-life.

CHAPTER 2 Literature review

This thesis is concerned with the interactions of the insoluble crystalline mineral of calcium phosphate, known as hydroxyapatite (HA), with milk proteins. This is in the context of understanding, and potentially providing approaches for mitigation of the challenges faced in calcium supplementation of milk. This literature review therefore starts (Section 2.1) with the broad context of calcium supplementation – its importance in human nutrition and health. Then, to provide a background to milk components and structure that is affected by calcium supplementation, the review continues (Section 2.2) with an overview of milk itself and the features of the proteins and minerals that are essential to the study. The review then progresses to discuss calcium supplementation approaches in general, and with HA in particular (Section 2.3), before focussing on the interactions between HA and milk proteins (Section 2.4). The literature review itself concludes with further focus on the material, methods and models described in the literature to measure and characterise protein adsorption on HA (Section 2.5) and a final section on the aims of the experimental work in the thesis is provided.

2.1 Calcium supplementation of food

2.1.1 Calcium role and metabolism in the human body

The average human body contains 1.2 kg of calcium; 99% of this calcium is located in the skeleton, mainly as calcium phosphate in a form that provides the structure and mechanical properties of the bones and teeth (Gurr, 1999). The remainder is located in soft tissues and body fluids where it plays a role in physiological and regulation functions. Calcium also plays an important role in the catalytic activity of many enzymes, and in cell signalling, where it acts as a messenger in cellular events such as muscle contraction and cell division.

Bone is made of protein fibres (collagen) that are protected in a mineral phase comprising calcium phosphate, calcium hydroxide and, depending on bone type and a number of other factors, carbonate, citrate, sodium, magnesium and fluorine (Neuman & Neuman, 1953). The bone mineral phase provides strength and resistance to fracture, but it is not its only role. Calcium ions are located at the surface of the bones, and get exchanged with body fluids; therefore, bone can be seen as a reserve of calcium that can maintain a constant

concentration of calcium in blood, muscle and intercellular fluids and to fulfil metabolic needs (Gurr, 1999).

Bone is continually being formed and broken down by bone cells, called osteoblasts and osteoclasts. This process is referred to as bone turnover (Calvo, Eyre, & Gundberg, 1996). During early life, bone growth exceeds bone loss, but later in life bone resorption exceeds formation and it becomes very important to get enough calcium from food to allow bone remodelling. The fastest rate of bone growth is after birth and in the prepubertal growth spurt; peak bone mass is essentially achieved at the age of 25 years old. For about 10 years the bone mass remains approximately constant, but after this time it starts to decrease; in females bone loss accelerates after menopause, giving rise to the particular risk of bone fragility in older women (Gurr, 1999).

2.1.2 Calcium deficiency problems

Calcium is an essential nutrient for bone health. Adequate calcium intake has been shown to reduce the risk of osteoporosis, hypertension, colon and breast cancer and cardiovascular diseases (Gurr, 1999). Low calcium intake or calcium deficiency tends to increase the bone turnover process, leading to several health problems and diseases. The most well-known disease linked with calcium deficiency is osteoporosis, where bones become more fragile, causing pain, fractures and disabilities. It mainly affects older people and particularly postmenopausal women. Osteoporosis is mostly caused by a bone mass decrease and a deterioration of the bone tissues, which results in increased risk of fractures. Osteoporosis is affected by many factors, including life style and food consumption, and is consequently referred to as a disease of multifactorial characters: nutrition as well as exercise, hormonal status, heredity, weight fluctuations, or inactivity have an effect on bone mass (Heaney, 2000). Therefore, ensuring good nutrition and meeting the recommended calcium intakes even at an early stage of life is one of the multiple strategies to reduce osteoporosis; it has been shown that high calcium intake increases bone gain during growth and delays age-related bone loss, reducing the risk of fractures.

To prevent calcium deficiency problems, it is therefore important to ensure a good delivery of calcium from food to the body as an essential part of healthy nutrition. However, the ability of the body to gain calcium from food depends on the bioavailability of the calcium. The bioavailability of calcium in different foods varies widely, depending on other

components of the food that may prevent absorption (Levenson & Bockman, 1994), for example the presence of oxalates contained in rhubarb or spinach. In the case of dairy foods, one third of the calcium content is absorbed by the gastrointestinal tract, either by simple diffusion through the gut walls in the upper part of the small intestine or by a pumping mechanism that requires energy and a carrier molecule termed a vitamin D-dependent calcium binding protein, or a calbindin (Weaver & Heaney, 2005). Vitamin D is metabolically modified to generate calcitriol, a hormone that stimulates the production of a calbindin that carries the calcium through the gut walls. The remaining two thirds of the calcium in dairy foods are excreted in the faeces and urine; some that is absorbed is lost through the hair, skin and sweat (Gurr, 1999). Overall, calcium absorption decreases with age (Levenson & Bockman, 1994).

2.1.3 Recommended daily intake for calcium

Recommended daily intakes for calcium vary between countries and age groups. Recommended calcium allowances refer to the amount of calcium that each age group needs to consume to ensure that the calcium consumed compensates for the calcium excreted from the body each day. For teenagers, the allowances take into account the amount of calcium needed for skeletal growth. For elderly people, they take into account the lower intestinal absorption efficiency. Table 2.1 shows the FAO/WHO recommendations (WHO, 2004) based on data from different countries.

Table 2.1: Recommended daily calcium intakes by age group.

Age group	Calcium (mg/day)	Age group	Calcium (mg/day)
Infants and children		Women	
0-6 months	300-400	19 years to menopause	1000
7-12 months	400	Post menopause	1300
1-3 years	500	During pregnancy (last trimester)	1200
4-6 years	600	During lactation	1000
7-9 years	700		
		Men	
Adolescents		19-65 years	1000
10 to 18 years	1300	65+ years	1300

2.1.4 Factors to take into account in the selection of calcium fortificants to add in milk

Many foods are supplemented with different sources of calcium, such as calcium carbonate, calcium phosphate, calcium citrate, and calcium oxalates (Levenson & Bockman, 1994; Weaver, 1998). It is important to keep in mind that calcium bioavailability can vary depending on the nature of the supplemented calcium. The different calcium sources used for calcium supplementation have different solubilities. It is often believed that solubility is correlated with absorption and bioavailability but this is not the case. Most absorption studies carried out on adult subjects showed no obvious relationship between solubility and absorbability (Pak & Avioli, 1988; Patrick, 1999). For example, Heaney, Recker, and Weaver (1990) studied the absorption of seven calcium sources that had solubilities at neutral pH of between 0.04 and 1500 mM. They showed that there was no significant effect of solubility on absorption, especially in the common supplementation range (0.1 – 10 mM). Two very common calcium salts used for calcium fortification of food products, calcium citrate and calcium carbonate, have also been shown to absorb in the intestines to the same extent, even though calcium carbonate is less soluble than calcium citrate (Heaney, Dowell, & Barger-Lux, 1999). Some calcium salts have been shown to be more bioavailable than others, and therefore better absorbed by the gastro-intestinal tract, e.g., calcium citrate malate (Levenson & Bockman, 1994). However, absorption is only one parameter to consider for a calcium supplement, as studies on calcium sources should also involve evidence of positive effects on bone mass and fracture rates. Therefore, clinical studies (which are expensive and time-consuming) on animal and human subjects are usually needed to prove the long-term effect of the calcium fortificants on bone remodelling and remineralisation (Guéguen & Pointillart, 2000).

To meet the recommended daily intake for calcium and deliver extra calcium to people's diets, milk is the ideal source for supplementation; it is naturally rich in calcium, already well-known for its benefit on bone growth and health and consumed by most of the population of many industrialised countries. Milk has a natural level of calcium of approximately 120 mg/100 mL (Lucey & Horne, 2009) and additional calcium can be added to milk products using calcium salts. There is a wide range of calcium-enriched dairy products on the market, including milks and beverages, cheeses, yoghurts and milk powders. For example, calcium supplemented milk represents 6% of the world-wide milk market (Gaucheron, 2003).

However, supplementation of calcium in milk products can lead to instabilities and processing issues, as it modifies the physico-chemical properties of milk. This is especially the case when the level of calcium supplementation is high or when the product has to undergo severe industrial processing, such as high-temperature heat treatments or acidification. Sensory aspects also have to be taken into account in the choice of calcium fortificants, as additional calcium can alter the taste and texture of milk products. For example, adding calcium chloride to milk has been shown to cause bitter and acidic flavours, whereas calcium phosphate or calcium carbonate can cause chalky textures (Augustin & Williams, 2002).

To understand the challenges of fortifying milk and milk products with calcium, the following part of this review will therefore focus on the composition and physico-chemical properties of milk composition.

2.2 Milk composition

2.2.1 Milk: an overview

Milk is a fluid secreted by the female of all mammalian species to supply the complete nutritional requirements of the neonate for its growth; milk is a rich source of lipids, lactose, proteins, minerals, vitamins and water. As milk is perishable and susceptible to micro-organism growth, mankind has, over time, developed products from milks (mainly cows' milk for industrial products, but goats', sheep's, and buffaloes' milk are also traditionally used) that are more stable than milk, allowing its preservation (Fox, 2009). Today, the food products most produced from milk (40%) are beverage milks. However, dairy food products also include cheese, milk powders, concentrated milks, fermented milk products, butter, creams, and ice-creams. Technology and processing technique improvements have allowed the development of a huge variety of these products, including long-life products such as heat-treated products, and health-benefit products such as mineral or protein-enriched products. Calcium and iron supplementation has been of most interest in research and new dairy product development, as they provide solutions for osteoporosis and anaemia health problems (Gaucheron, 2000; Lynch, 2005; Prince et al., 1991).

Because this study was undertaken in the context of the New Zealand dairy industry, the remainder of this review will focus almost exclusively on bovine milk. The composition of

cows' milk can vary widely, reflecting the fact that the nutritional requirements of the new born calf depend on many conditions, including factors that can be genetic (e.g., between breeds or between individuals), physiological (e.g., stage of lactation, age of cow, maturity at birth, energy requirements) or environmental (e.g., feed, season, climate) (Fox, 2003; Walstra & Jenness, 1984).

Cows' milk is a complex liquid comprising about 87% water and 13% total solids, either dissolved in water or in suspension. The principal total solids are lactose (4.8%), fat (3.7%), and protein (3.4%). Fat and lactose provide energy to the new born. The proteins provide a source of essential amino acids and amino groups for synthesis of non-essential amino acids. (Fox, 2003). The mineral phase of milk is only a small fraction of milk (8-9 g/L) but is of major importance as minerals are essential for growth and development of bone and teeth and play a major role in the structure and stability of milk proteins (Gaucheron, 2005; Lucey & Horne, 2009). The constituents of milk can be either dissolved in the serum phase (e.g., lactose and most inorganic salts) or in a colloidal form (proteins) or as an emulsion (lipids) (Fox, 2009). To understand how addition of minerals can affect the milk properties, it is important to understand the structure and properties of both the protein and mineral phases. They are therefore detailed in the next part of this review.

2.2.2 Protein phase

The protein phase of milk is probably the best characterised food protein system and has attracted considerable research attention for years; milk proteins have unique technical properties and can be fractionated easily to be used as food ingredients. There are two main types of proteins in milk: caseins and whey proteins (Fox, 2009). Caseins are phosphoproteins that represent 80% of the milk proteins. Their isoelectric point is at pH 4.6, and so will precipitate in milk when it is acidified to pH 4.6. In contrast, whey proteins, which represent 20% of the milk proteins, remain in solution with the other soluble components (i.e., lactose and minerals). Therefore casein and whey can be separated from each other at pH 4.6 (Walstra & Jenness, 1984). Other methods are also possible to separate whey proteins from casein micelles, e.g., rennet-induced coagulation of the caseins (caused by selected proteinases and used for production of cheese), or sedimentation by ultracentrifugation (100,000 × g for 1 h), but they give slightly different fractions. The soluble component phase is often referred as the milk serum phase or soluble phase, whereas the casein phase is usually referred as the colloidal phase.

2.2.2.1 Caseins

Casein molecules, definition and surface properties

There are four types of caseins, characterised by different polypeptide chains: α_{S1}-, α_{S2}-, β- and κ-casein. The structure, chemical properties and functionality of the caseins have been extensively reviewed (Fox & McSweeney, 1998; Swaisgood, 2003; Wong, Camirand, Pavlath, Parris, & Friedman, 1996). Table 2.2 summarises the main characteristics of the different caseins.

Table 2.2: Principal characteristics of casein molecules (from Walstra and Jenness, 1984, and Fox, 2003).

Parameter	α_{S1}-casein	α_{S2}-casein	β-casein	κ -casein
Molecular weight (g/mol)	23614	25230	23983	19023
Amino acid residues (total)	199	207	209	169
Proline residues	17	10	35	20
Phosphoserine residues	8-9	11	5	1
Glutamic acid residues	25	24	19	12
Aspartic acid residues	7	4	4	3
Isoelectric pH	6	5.3	5.2	5.6
Total hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1
Calculated charge at pH 6.6	-21.9	-13.8	-13.3	-2

A high level of proline in the amino acid composition of the caseins, i.e., 17, 10, 35 and 20 moles of proline per mole of α_{S1}-, α_{S2}-, β- and κ-casein, respectively, explains their lack of secondary and tertiary structure and consequently their high flexibility. The sequence of the caseins is made of hydrophobic and hydrophilic amino acid residues that are not distributed uniformly in their sequences, conferring amphiphilic properties to casein molecules. α_{S1}-, α_{S2}-, and β-caseins have a high content of phosphate groups, with, respectively, 8–9, 11 and 5 phosphoserine residues per molecule. Figure 2.1 shows the chemistry of a phosphoserine residue; the amino acid serine is phosphorylated via a covalent bond between phosphorus and protein that can only be removed by severe heat treatment or enzymatic action. The phosphoserine residues are negatively charged, and they are the main contributors of hydrophilic groups. They are often found in clusters of two, three or four residues, referred

as phosphoseryl clusters. Therefore, they are able to bind cations, especially Ca^{2+}. The main consequence is that α_{S1}-, α_{S2}-, and β-caseins are insoluble and precipitate at calcium concentrations >6 mM; only κ-casein is not sensitive to calcium concentrations found in milk. The caseins are also rich in glutamic and aspartic acid residues; these residues carry side carboxyl groups and are usually grouped in clusters. The pKas of the carboxyl groups are comprised between 4.1 and 4.6, they are, therefore, negatively charged at the natural pH of milk, and consequently contribute to the negative charge of the caseins. Carboxyl groups can also bind Ca^{2+}, and have been shown to play an important role, along with the phosphoserine groups, in the ability of the caseins to bind to calcium (Byler & Farrell, 1989).

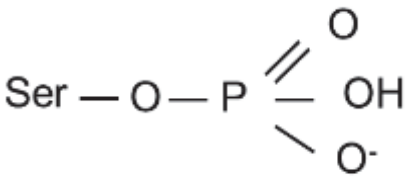

Figure 2.1: Representation of a phosphoserine residue; the phosphate is esterified to serine as a monoester (from Fox & McSweeney, 1998).

The flexible structure and amphiphilic nature of the caseins gives them very good surface activity and good adsorbing properties; they can therefore bind to different surfaces through hydrophobic or electrostatic interactions. They orientate at an interface in such a way that the non-polar regions stay in contact with the hydrophobic surface and the polar regions with the aqueous phase. For example, caseins are commonly used to stabilise oil-in-water emulsions, where they are spread out at the surface of the oil droplets through hydrophobic interactions (Fox, 2009; Horne, 2009).

The section below gives details on the structure and self-association of each casein, as these properties are important in the understanding of the adsorption mechanisms that will be studied in this thesis.

α_{S1}-Casein and α_{S2}-casein

α_{S1}-casein has one hydrophilic region found in between two hydrophobic regions. Figure 2.2A shows a schematic illustration of the distribution of charged and hydrophobic residues in the linear chain of the α_{S1}-casein molecule (Swaisgood, 2003). The train and loop model

(Horne, 1998) showing the distribution of the hydrophobic and hydrophilic groups along the molecule is also given (Figure 2.2Aiii). Two phosphoserine clusters are located in the hydrophilic charged region, and are rich in glutamic acid (amino acids 41 to 80). This region is highly charged (net charge of –20.6 at pH 6.6) whereas the rest of the molecule has very little charge (Swaisgood, 2003). The first cluster is made of two phosphoserine residues located in position 46 and 48 of the amino-acid chains, and the second cluster is made of four phosphoserine residues, located in positions 64, 66, 67, 68.

The distribution of hydrophilic and hydrophobic groups in α_{S1}-casein gives the protein a loose and flexible structure and the ability to self-associate or associate with other caseins (Horne, 1998; Schmidt, 1982). The self-association of α_{S1}-casein is driven by both hydrophobic and electrostatic interactions and is strongly dependent on pH and ionic strength. At ionic strength greater than 0.003 and pH 6.6, α_{S1}-casein can self-associate and form oligomers that exist in equilibrium with monomers (Schmidt, 1970; Schmidt & Van Markwijk, 1968). As ionic strength increases, the formation of oligomers become favourable and dimers or trimers can form. Srinivasan, Singh, and Munro (2000) showed that, upon addition of NaCl, the adsorption of α_{S1}-casein on emulsion droplets increased, and they related it to the formation of oligomers that led to more α_{S1}-casein adsorbed at the interface. As pH increases, it has been shown that α_{S1}-casein self-associates to a lesser amount, as the protein charge decreases and the electrostatic repulsive forces increase (Horne, 2009; Swaisgood, 2003).

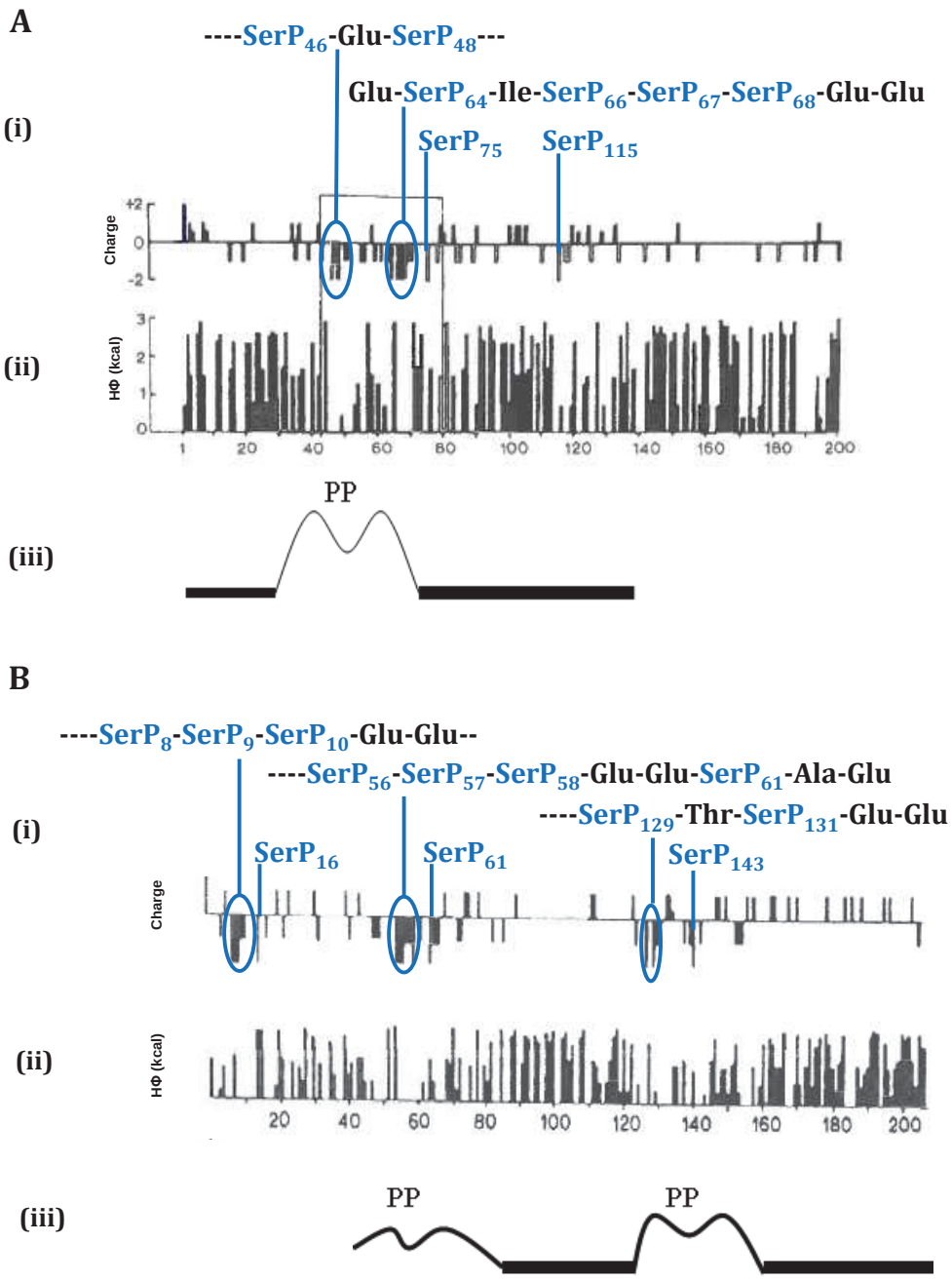

Figure 2.2: Schematic illustration of the distribution of charged, phosphoserine, and hydrophobic residues in the (A) α_{S1}- and (B) α_{S2}-casein primary sequences (from Horne (2009) and Swaisgood (2003)). The distribution of the charged (i) and hydrophobic residues (ii) along the protein chain and detailed amino acid sequence in the phosphoserine clusters are shown; the numbers on the x-axis are the numbered amino acids along the protein chain, the blue circles show the phosphoserine clusters and the phosphoserine residues are indicated in blue font in the amino-acids sequence. Also shown (iii) is the loop and train model: dark solid bars are the two hydrophobic regions, the loop is the hydrophilic region, and PP indicates the location of the phosphoseryl clusters.

α_{S2}-Casein is the most hydrophilic of the caseins and contains the most phosphoseryl residues. A schematic illustration of α_{S2}-casein is given in Figure 2.2B. Three clusters of phosphoseryl residues, also rich in glutamyl residues, are discernible, located along the amino acid chains in positions 8-12, 56-63 and 129-133 (Swaisgood, 2003). The molecule contains two hydrophobic regions, including residues 90-120 and 160-207, as shown by the train and loop model in Figure 2.2Biii. α_{S2}-Casein is characterised by distinct charged domains, with a negatively charged N-terminal domain of the molecule (net charge of –21 at pH 6.6) and a positively charged C-terminal domain (net charge of +9.5 at pH 6.6). α_{S2}-Casein can self-associate, via hydrophobic and electrostatic interactions between the oppositely charged N- and C-terminal domains of the molecule (Schmidt, 1982). As is the case for α_{S1}-casein, α_{S2}-casein self-association is very sensitive to ionic strength. Self-association of α_{S2}-casein reaches a maximum at ionic strength of about 0.2 M. Lower association occurs at lower ionic strength, as the electrostatic interactions between the charged domains are weaker (Swaisgood, 2003).

β-Casein

β-Casein has the largest number of proline residues (Table 2.2), which has a major effect on its structure. It is also the most hydrophobic of the casein molecules. Figure 2.3 shows the distribution of charged, hydrophobic and phosphoserine residues of β-casein. β-Casein has an amphiphilic nature, with a short polar head (N-terminal) and a hydrophobic tail (C-terminal). The polar N-terminal domain contains 4 of the 5 phosphoserine residues of the molecule, grouped in one cluster of sequence Glu-SerP-Leu-SerP-SerP-SerP-Glu-Glu. This region is therefore negatively charged (net charge of -11.5 at pH 6.6) and hydrophilic.

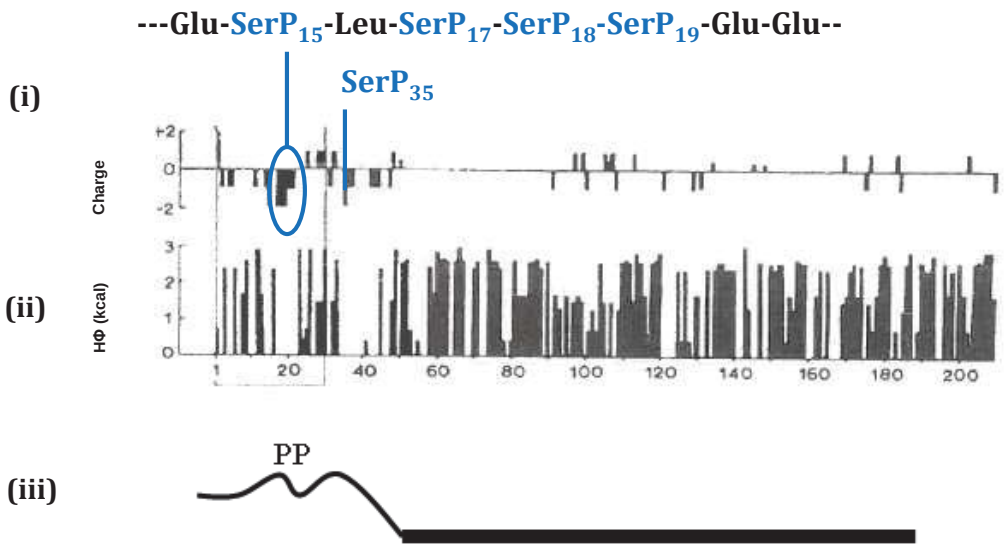

Figure 2.3: Schematic illustration of the distribution of charged, phosphoserine, and hydrophobic residues in the β-casein primary sequence (from Horne (2009) and Swaisgood (2003)). The distribution of the (i) charged and (ii) hydrophobic residues along the protein chain and detailed amino acid sequence in the phosphoserine clusters are shown; the numbers on the X-axis are the numbered amino-acids along the protein chain, the blue circles show the phosphoserine clusters and the phosphoserine residues are indicated in blue font in the amino-acid sequence. Also shown (iii) is the loop and train model: dark solid bars are the two hydrophobic regions, the loop is the hydrophilic region, and PP indicates the location of the phosphoseryl clusters.

It is interesting to note that the sequence found in the phosphoserine clusters of the three calcium-sensitive caseins, α_{S1}-, α_{S2}- and β-caseins are very similar (Swaisgood, 2003). The sequence of amino-acids Glu-SerP-X-SerP-SerP-SerP-Glu-Glu [where X is isoleucine (Ile) or leucine (Leu)] is found in both α_{S1}-casein and β-casein (Figure 2.2Ai and Figure 2.3i, respectively). In α_{S2}-casein, the two clusters are also rich in glutamic-acid (Figure 2.2Bi), with two different sequences, SerP-SerP-SerP-Glu-Glu and SerP-SerP-SerP-Glu-SerP-SerP-Ala-Glu. This similarity in sequence is known to play an important role in the binding of the caseins to calcium (Swaisgood, 2003).

β-Casein has been reported to self-associate and form micelle-like structures, with the hydrophobic tails of the molecule buried inside the micelle and the hydrophilic heads sticking out (Schmidt, 1982). Horne (1998) described the self-association of β-casein as an equilibrium between monomers and micelles, whereas recent authors suggested that the micelles are formed by the addition of β-casein monomers to already formed β-casein

polymers (De Kruif & Grinberg, 2002; O'Connell, Grinberg, & De Kruif, 2003). As the structure of β-casein is dominated by hydrophobic interactions, its self-association is less affected by ionic strength compared with the self-association of α_{S1}- or α_{S2}-casein, but is, however, extremely temperature-dependent. At 4 °C, no self-association occurs, due to the weakness of hydrophobic interactions at low temperatures (Payens & van Markwijk, 1963).

κ-Casein

κ-Casein has an amphiphilic structure similar to the structure of β-casein. The molecule is characterised by a negatively charged head (C-terminal domain, residues 105-109, net charge of about –10 at pH 6.6) and an hydrophobic tail (N-terminal domain, roughly between residues 0-105) (Figure 2.4). However, κ-casein is less hydrophobic and has less proline residues than β-casein (Table 2.2), and contains secondary structure (Swaisgood, 2003). Unlike the other caseins, κ-casein does not have any phosphoseryl clusters. It contains only one phosphoseryl residues (amino acid 149). Therefore κ-casein is less sensitive to calcium ions and does not precipitate in the presence of the amount of natural calcium contained in milk.

Like β-casein, κ-casein also self-associates in micelle-like structures (Vreeman, Brinkhuis, & Van der Spek, 1981). However, this self-association is less dominated by hydrophobic interactions with hydrogen and ion-pair bonding also involved. Therefore, the self-association of κ-casein is not very sensitive to temperature or ionic strength (Swaisgood, 2003).

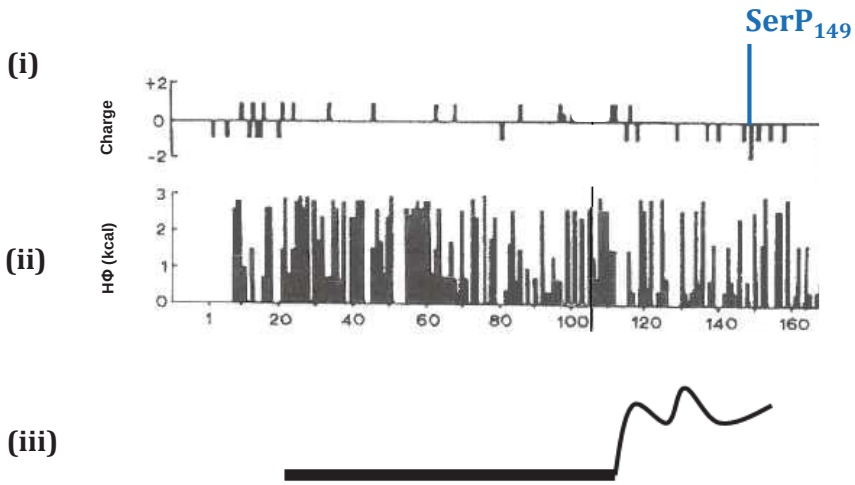

Figure 2.4: Schematic illustration of the distribution of charged, phosphoserine, and hydrophobic residues in the κ-casein primary sequence (from Horne (2009) and Swaisgood (2003)). The distribution of the (i) charged and (ii) hydrophobic residues of κ-casein along the protein chain and detailed amino acid sequence in the phosphoserine clusters are shown; the numbers on the X-axis are the numbered amino acids along the protein chain, the blue circles show the phosphoserine clusters and the phosphoserine residues are indicated in blue font in the amino acid sequence. Also shown (iii) is the loop and train model: dark solid bars are the two hydrophobic regions, the loop is the hydrophilic region, and PP indicates the location of the phosphoseryl clusters.

Casein micelles: definition and different models

The ability of caseins to bind calcium plays a crucially important role in the association of casein molecules in milk (Fox, 2009; Horne, 2009). As bovine milk contains about 30 mM calcium, α_{S1}-, α_{S2}-,and β-casein might be expected to precipitate in milk. The reason they do not precipitate is because κ-casein has the ability to stabilise the other casein molecules in milk. κ-Casein has only one phosphoserine residue and does not precipitate in the presence of calcium concentrations present in milk; it therefore plays a role of protection of the calcium-sensitive α_{S1}-, α_{S2}-, β-casein molecules in milk through the formation of large aggregates called casein micelles. The ratio of α_{S1}- to α_{S2}- to β-caseins to κ-casein in the casein micelle is about 3:1:3:1 (Wong et al., 1996).

The association of milk proteins into casein micelles involves mainly electrostatic and hydrophobic interactions. Changes in pH, temperature and ionic strength induce changes in the balance between those two types of interactions, and can lead to changes in casein

micelle structure (De la Fuente, 1998). The casein micelles are heat stable to high temperatures and can withstand a heat-treatment of 140 °C for 15-20 min at normal milk pH (6.7). Casein micelle stability is of major importance for many types of technological treatments used in dairy industry (Dalgleish & Corredig, 2012).

Casein micelles form a colloidal dispersion and scatter light, and they are therefore responsible for the high turbidity and white colour of skim milk (Fox, 2009; Schmidt, 1982). Their content comprises 94% protein; the remainder is mostly calcium and phosphate, with some magnesium and citrate, commonly referred to as colloidal calcium phosphate (CCP). CCP plays a major role in casein micelle structure and stability and its role will be detailed further in this review. The size of casein micelles ranges from 50 to 500 nm; their average size as measured by electron microscopy is around 150 nm, whereas dynamic light scattering measurements give an average diameter around 200 nm (Anema & Klostermeyer, 1996; De Kruif & Holt, 2003). The mass of casein micelles ranges from 10^6 to 10^9 Da (average 10^8 Da), their concentration in milk is 10^{14}–10^{16} micelles/mL and they have a natural hydration of 2.0 g of water per gram of protein (Fox, 2009)

Different models for the structure of casein micelles in milk have been proposed in the literature and are still debated. One common and well-accepted feature for the different models is that κ-casein molecules, which represent 15% of the total caseins, have to be located where they are able to stabilise the calcium sensitive caseins (α_{S1}-, α_{S2}-, and β-caseins, representing 85% of the total caseins). Another common feature concerns the role played by CCP in the casein micelle structure; since removal of CCP causes micelle disintegration into smaller particles, therefore CCP must hold the casein molecules together.

It has been often proposed that κ-casein forms an outer layer of protection on roughly spherical particles containing mainly α_{S1}-, α_{S2}-, and β-caseins, linked together by bridges of calcium phosphate (Fox, 2009; Walstra, 1999). The main model of this type is referred to as the sub-micellar model and is presented in Figure 2.5. Proposed first by C. V. Morr nearly fifty years ago (Morr, 1967), views of this model have evolved over the years and different refinements have been proposed (Schmidt, 1982; Walstra, 1999; Walstra & Jenness, 1984). However, this view of the micelles being composed of smaller sub-micelle units is questioned by many authors, as it relied mostly on electron microscopy studies, which are now believed to have generated artefacts (McMahon & McManus, 1998).

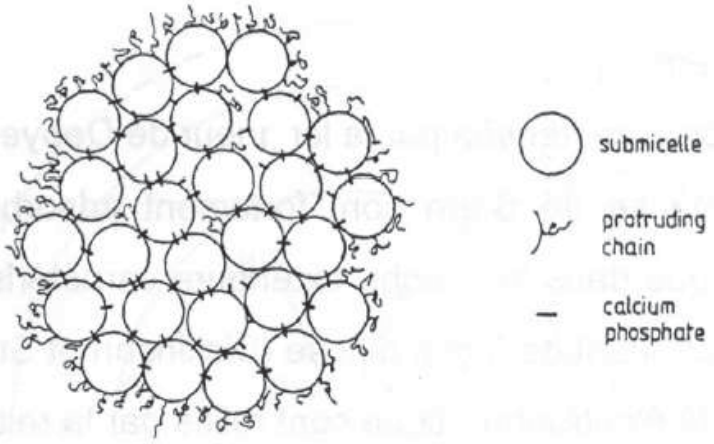

Figure 2.5: Sub-micelle model of casein micelles (Schmidt, 1982).

Recent authors have proposed a few different models for the casein micelle structure (Dalgleish, 2011; de Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, 1992; Holt, de Kruif, Tuinier, & Timmins, 2003; Horne, 1998, 2006). The most likely model does not involve any casein sub-micelles, but has an open structure, stabilised by calcium phosphate nanoclusters, as first proposed by the Holt model (De Kruif & Holt, 2003; Holt, 1992; Holt & Horne, 1996); see Figure 2.6. The nanoclusters are made of a core of calcium phosphate and a shell of casein phosphopeptide (De Kruif & Holt, 2003; Gaucheron, 2005), and the size of the calcium phosphate nanoclusters is a few nanometers.

A review by Horne (2006), illustrated the formation of the calcium phosphate nanoclusters and the growth of the protein network in the micelles, as described by Holt (1992, 2004). Both α_{S1}- and α_{S2}-caseins are described as multi-functional, since they contain, respectively, two and three clusters of phosphoserine residues per molecule (Figure 2.2Ai, Figure 2.2Bi and Figure 2.7), and β-casein as monofunctional, since it carries only one phosphoserine cluster (Figure 2.3i). The multi-functional caseins, α_{S1}- and α_{S2}-caseins, cross-link the calcium phosphate nanoclusters, allowing a three-dimensional protein network to grow and extend (Figure 2.7). The mono-functional β-casein binds to the network and prevents further growth in one direction. However, the original Holt model did not give a specific role to κ-caseins, and did not provide an explanation on how the micelle size is controlled, allowing an infinite growth of the casein micelle (Horne, 2006).

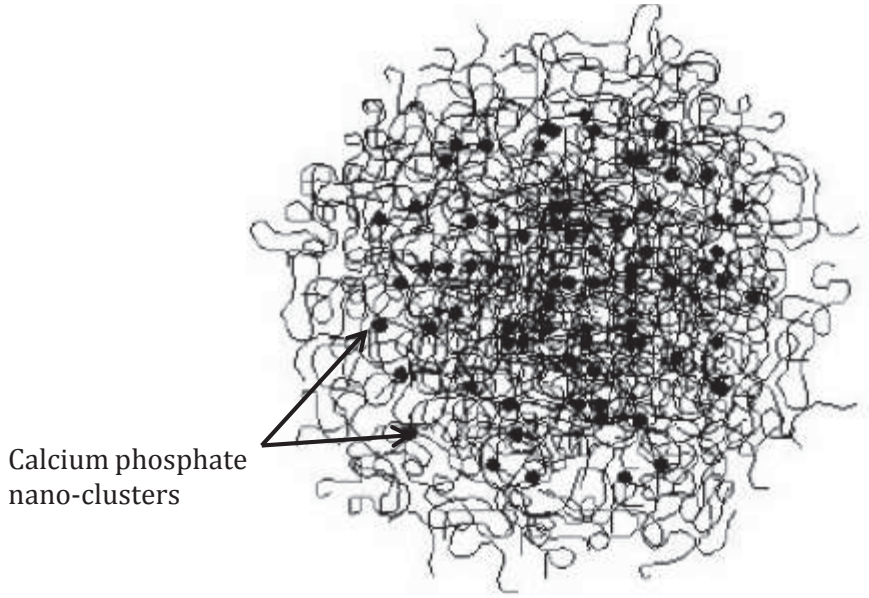

Figure 2.6: Calcium phosphate nanocluster model, as first proposed by Holt (1992)

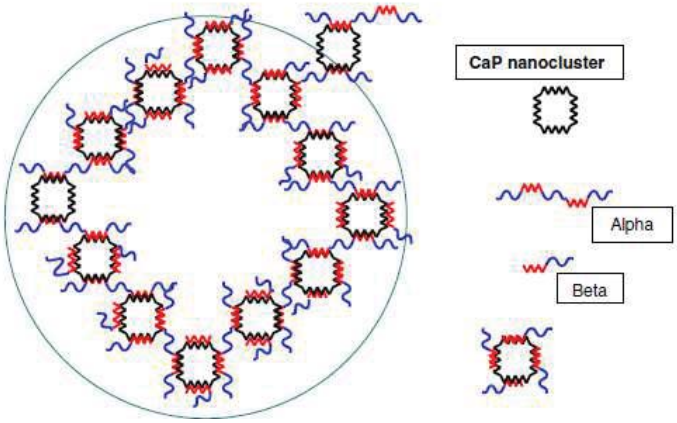

Figure 2.7: Illustration of the calcium phosphate nanocluster formation and the growth of the protein network in the casein micelles (Horne, 2006). Blue regions correspond to the hydrophobic regions of β- and α_S-caseins (α_{S1}- and α_{S2}-), red regions correspond to the hydrophilic regions of β and α_S-caseins, containing the clusters of phosphoserine residues.

All of the recent models confirm that only a model based on calcium phosphate nanoclusters is capable of explaining the structural and functional properties of casein micelles, but that the interactions involved in the nanoclusters themselves are not sufficient to explain the casein micelle structure (Horne, 2009). The most recent models for casein micelle structure

also rely on other forms of bonding between the caseins. In his dual-binding model, Horne (2006) includes a second type of bonding occurring by cross-linking through hydrophobic regions of casein molecules. The hydrophilic regions of the caseins, particularly phosphoseryl clusters, are negatively charged so they repel each other and usually prevent the caseins from polymerising and growing. However, when calcium phosphate nanoclusters are formed, the electrostatic repulsions between phosphoryl clusters are removed. Therefore, the possibility for hydrophobic interactions between the caseins is increased and more polymerisation of caseins occurs upstream and downstream from the nanocluster link, allowing a network to be built. κ-Casein molecules have then an important role in providing structure to the casein micelles. They cannot bind to the calcium phosphate nanoclusters as they do not contain phosphoserine clusters, but they can bind to other caseins via hydrophobic effect. Therefore they can bind to the surface of the micelles, forming a hairy layer and terminating the growth of the casein micelles.

Over the years, the dual-binding model of Horne has been reviewed and modified based on different scattering techniques and new insights from electron microscopy, but the main principles remain. The current accepted model is still often referred to as the nanocluster model, as first proposed by Holt and Horne (1996). However, the debate about the casein micelle still remains and the two most recent models have been proposed and discussed in recent reviews (Dalgleish, 2011; Dalgleish & Corredig, 2012; De Kruif et al., 2012). They are all derived from the original nanocluster model proposed by Holt and Horne (1996) and are illustrated in Figure 2.8. The common features of these two models are the presence of water channels, cavities and high densities nanoclusters within the micelles. The casein micelles are porous structures made of a matrix of casein molecules stabilised by calcium phosphate nanoclusters. On one hand, the phosphorylated residues of β- and α_S-caseins are bound to the calcium-phosphate nanoclusters and stabilise them, preventing uncontrolled growth and precipitation of calcium phosphate, whereas on the other hand, the tails of the caseins can associate through non-covalent interactions (hydrophobic interactions, as in the dual-binding model, but also hydrogen bonding, ion bonding, and electrostatic Van der Waals attractions) to form the protein network (Dalgleish, 2011). **κ**-Casein is believed to limit this self-association process and stabilise the surface of the casein micelles. A debate remains on how many phosphoserine residues in a cluster are necessary to bind to the calcium phosphate nanoclusters and this answer actually determines how many linkage sites are present per casein molecule (Horne, 2009). A debate also remains on the width of the water channels and the size of the pores inside the micelles (De Kruif et al., 2012).

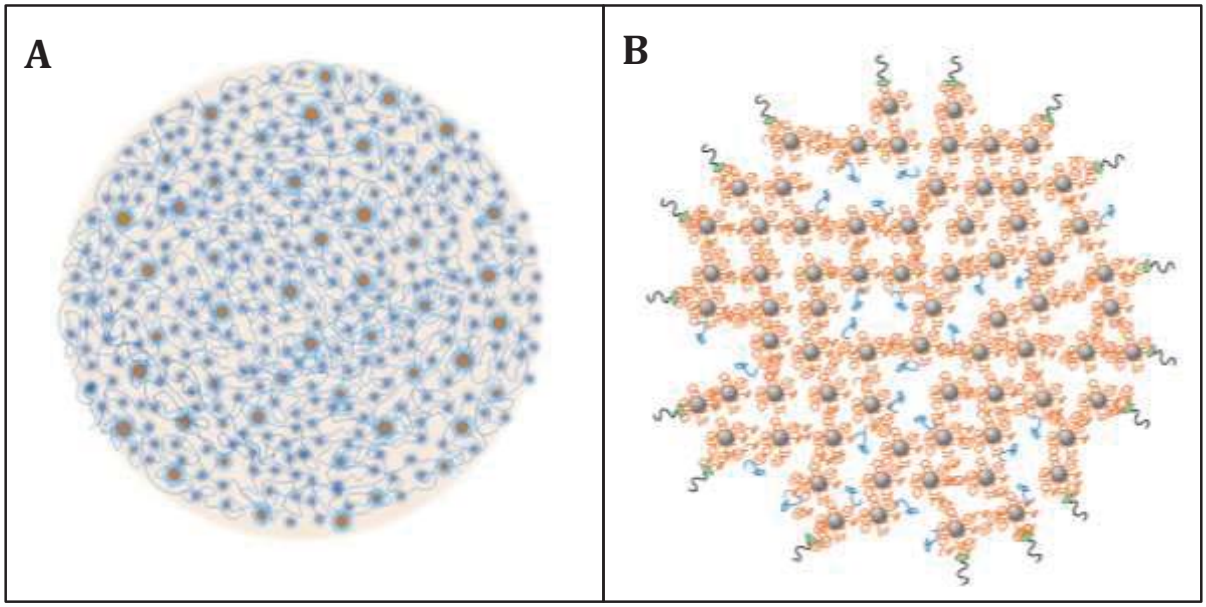

Figure 2.8: Two most recent models of casein micelles involving the concept of calcium phosphate nanoclusters surrounded by α_S- and β-caseins. Panel A: from De Kruif et al. (2012), the brown spheres represent the calcium phosphate nanoclusters, the blue strands represent the protein network. Panel B: from Dalgleish and Corredig (2012); in orange, β- and α_S-caseins are bound to the calcium phosphate nanoclusters (grey spheres); in blue, some β-casein are bound via hydrophobic interactions to other caseins; in black and green, the κ-casein molecules are bound on the surface of the casein micelles.

It is important to acknowledge that there is a debate in the literature on the importance of hydrophobic interactions in the self-association behaviour of the caseins and the structure of the casein micelles. Many authors consider hydrophobic interactions to be the force driving casein association (loop and train model, Horne (2009) and Swaisgood (1992)) and the casein micelle structure. However, many studies on unfolded proteins and model peptides seem to show that the association of the caseins may be driven mainly by backbone interactions between sequences of low sequence specificity, charge density and hydrophobicity. These sequences are called polar tracts and in caseins, they are depleted in hydrophobic residues and rich in proline and glutamic residues. These sequences are believed to explain the tendency of caseins to associate and act as chaperone molecules, which would mean hydrophobic interactions play only a minor role in casein association and casein micelle structure (Holt, Carver, & Ecroyd, 2013)

2.2.2.2 Whey proteins

About 20% of the milk proteins are whey proteins. They are typical globular proteins and have well-defined secondary and tertiary structures. The two major whey proteins are β-lactoglobulin (50% of whey proteins) and α-lactalbumin (20% of whey proteins). One of the important features of α-lactalbumin is its ability to bind calcium. The remaining whey proteins are bovine serum albumin (BSA, 6% of whey proteins), iron-binding proteins (lactoferrin and transferrin) and immunoglobulins (Fox, 2009). Whey proteins are subject to heat denaturation. This means that under appropriate heating conditions, whey proteins, in their native state, can denature, aggregate and form gels. Their non-covalent bonds are broken by heat, leading to unfolding of their compact globular structure. They can therefore aggregate by formation of both intermolecular covalent and non-covalent bonds.

As well as caseins, whey proteins have good surface properties and can be used to stabilise emulsions and colloidal systems. Both β-lactoglobulin and α-lactalbumin have been shown to stabilise food emulsions by spreading out at oil droplet surfaces (Ye & Singh, 2006). There are also many examples of encapsulation of bioactives by whey proteins (Livney, 2010).

The section below gives details on the structure of β-lactoglobulin and α-lactalbumin, as these properties are important to the understanding of binding studies and adsorption mechanisms.

β-Lactoglobulin

β-Lactoglobulin has a molecular mass of 18,281 kDa, contains 162 amino-acid residues and has a isoelectric point of ~5.35 (Farrell et al., 2004). It is a typical globular protein, with well-defined secondary, tertiary and quaternary structures. The structure of β-lactoglobulin has been extensively studied and reviewed (Edwards, Creamer, & Jameson, 2009; Sawyer, 2003). X-ray diffraction studies (Brownlow et al., 1997) revealed that the monomer of β-lactoglobulin contains nine β-strands. Eight of these strands are fold in an anti-parallel way, forming two β-sheets, with the first β-sheet formed by strands A to D, and the second β-sheet formed by strands E to H (Figure 2.9). The two β-sheets form a conical β-barrel or calyx at the N-terminus of the molecule, closed at one end by a tryptophan residue (Trp 19). The pocket formed by the barrel has been shown to be hydrophobic, therefore forming a buried binding site for various hydrophobic molecules (Kontopidis, Holt, & Sawyer, 2004). A three-turn α-helix flanks the outer surface of the calyx (Edwards et al., 2009).

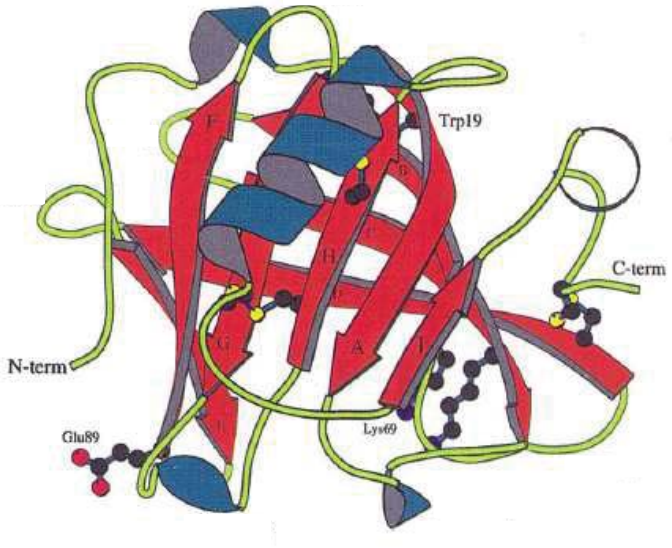

Figure 2.9: Schematic representation of a monomer of β-lactoglobulin; modified from Kraulis (1991)

β-Lactoglobulin can be in monomer, dimer or octamer form, depending on the conditions of pH, ionic strength and temperature (Sawyer, 2003; Verheul, Pedersen, Roefs, & de Kruif, 1999). For example, between pH 5.5 and 7.5, β-lactoglobulin exists mainly as dimers and the formation of dimers is enhanced by increasing ionic strength and decreasing pH. In the pH range between 3.5 and 5.5, β-lactoglobulin has been shown to form octamers and the formation of octamers is favoured by a decrease in ionic strength and temperature (Townend & Timasheff, 1960). β-lactoglobulin also dissociates to monomers at pH <3.5, because of electrostatic repulsions (Townend, Weinberger, & Timasheff, 1960). The self-association of β-lactoglobulin has been reported to affect its adsorption behaviour on solid surfaces (Elofsson, Paulsson, & Arnebrant, 1997; Luey, McGuire, & Sproull, 1991; Wahlgren, Arnebrant, & Paulsson, 1993) or oil droplets in emulsion systems (Das & Kinsella, 1989; Hunt & Dalgleish, 1994b).

α-Lactalbumin

α-Lactalbumin has a molecular mass of 14,178 kDa, contains 123 amino-acid residues and has a isoelectric point of ~4.80 (Farrell et al., 2004). It is a metalloprotein that has the ability to bind ionic calcium. The tertiary structure of the protein has an ellipsoid shape and is made of two lobes (α-lobe and β-lobe), as shown in Figure 2.10. The α-lobe contains three α-helices and two short 3_{10}-helices. The β-lobe is formed by three stranded β-sheets and a

short 3_{10}-helix. The two lobes are divided by a deep cleft that forms the binding site for calcium (Brew, 2003). The calcium site has been described as an elbow formed by five oxygen atoms: the side chain carboxyl oxygen atoms of three aspartic acid residues (Asp_{82}, Asp_{87} and Asp_{88}) and the peptide carbonyl oxygen atoms of a lysine and an aspartic acid (Lys_{79} and Asp_{84}). Two water molecules are also involved in the coordination of the calcium ion. The binding of the calcium ion in the calcium binding site stabilises the tertiary structure of α-lactalbumin. Without calcium, for example in the apo-protein, at low ionic strength and neutral pH, the repulsion between the carboxyl groups destabilises the protein, which then adopts a partially unfolded conformation called molten globular structure (Kuwajima, 1996).

Sodium ions have been shown to bind to α-lactalbumin and induce a conformational change of the protein (Desmet, Hanssens, & van Cauwelaert, 1987). Upon addition of sodium ions, α-lactalbumin loses entropy and hydrophobic groups are buried inside the molecule. The protein becomes more rigid and compact and less hydrophobic.

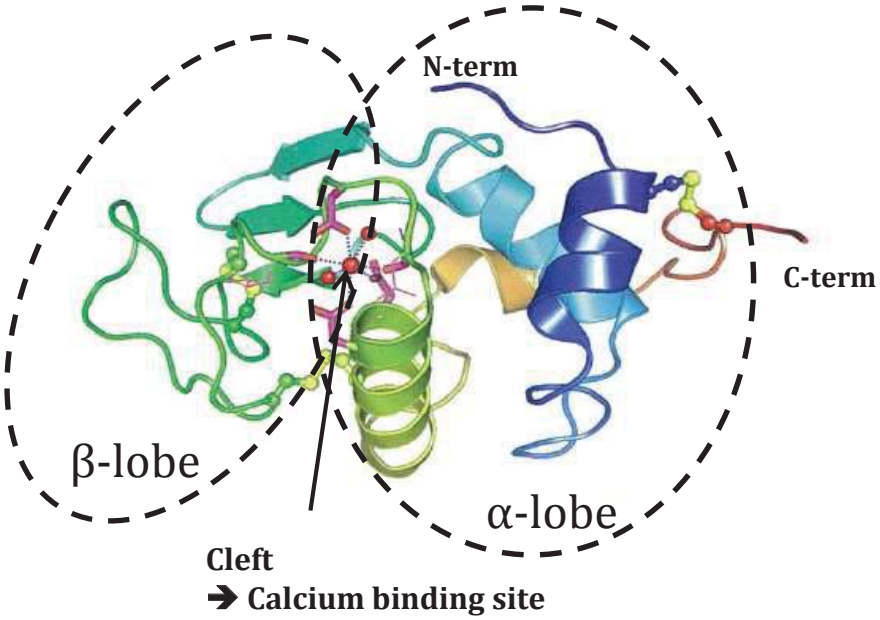

Figure 2.10: Schematic representation of a monomer of α-lactalbumin; modified from Kraulis (1991).

α-Lactalbumin has been shown to undergo conformational change with pH; it self-associates and slowly aggregates at acidic pH values <4.8 (Kronman & Andreotti, 1964; Kronman, Andreotti, & Vitols, 1964), but between pH 6 and pH 8, α-lactalbumin is mostly in the

monomer form and above pH 9.5, the protein expands without self-associating (Kronman, Holmes, & Robbins, 1967).

2.2.3 Mineral phase

2.2.3.1 Characterisation of the milk mineral phase

A good understanding of the properties of the milk mineral phase is crucial as this fraction interacts strongly with the protein fraction. The minerals of milk play a critical role in the formation and stability of casein micelles, as well as in milk protein stability during processing (e.g., heat treatment) and in the texture of many dairy products, such as yoghurt or cheese (Lucey & Horne, 2009).

The milk salts include the citrates, phosphates, chlorides, sulphates, carbonates and bicarbonates of sodium, potassium, calcium and magnesium. They also include minor and trace elements such as copper, iron, silicon, zinc and iodine. Strictly speaking, the milk proteins could be included in the salt system since they carry charges and can form salts with counter-ions; however, they are usually not considered in this way (Fox & McSweeney, 1998).

In milk, all the minerals are distributed differently between the colloidal and soluble phases (the latter is also referred to as the diffusible phase or serum phase). The partition of milk salts between the two phases depends on the solubility of the salts. Potassium, sodium and chlorides salts are sufficiently soluble to be essentially present in the serum phase. Other salts, like calcium phosphate and magnesium phosphate are at too high a concentration to stay in solution at normal milk pH and, therefore, they are partly bound to casein micelles and partly present in the soluble phase (Fox & McSweeney, 1998; Gaucheron, 2005).

In the serum phase, minerals can be found as free ions, such as K^+, Ca^{2+}, Na^+, or can associate with other ions, such as citrate and phosphates with calcium or magnesium to form complexes. For example, the concentration of ionic calcium in normal skim milk has been reported to be between 1.6 and 2 mM (Gaucheron, 2005; Holt, Dalgleish, & Jenness, 1981; Lin, Lewis, & Grandison, 2006; Philippe et al., 2003), but this represents only around 20% of the calcium in the serum phase. The rest of the calcium forms complexes with citrate (Cit^{3-}) and, to a lesser extent, with inorganic phosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) and with chloride.

Because the solubility constants of the calcium phosphate salts are low, the serum phase of milk at normal pH is supersaturated with respect to calcium and phosphate ions. Therefore calcium phosphate associates with the casein micelles, in CCP. Without casein micelles, there would be a precipitation of calcium phosphate, leading to a calcification of milk (Holt, Sørensen, & Clegg, 2009). The structure of the casein micelle allows the storage in milk of about three times more calcium and two times more phosphate than would be possible in milk without casein micelles, therefore providing enough intake of calcium and phosphate for the neonate growth (Lucey & Horne, 2009).

Table 2.3 summarises the partitioning of the main salts between the colloidal and serum phases at normal milk pH. The distribution depends strongly on environmental factors such as pH, temperature, concentration of milk and addition of other salts, sequestrants or chelating agents. Any change in one or several of these factors can strongly affect the dynamic milk salt equilibrium, i.e., a resulting change in the associated ions in the serum phase or a change in the partitioning between colloidal and soluble forms. These aspects are detailed in the next part of this review.

Table 2.3: **Salt partitioning in cows' milk (from Gaucheron, 2005).** [a]

Constituent	Concentration
Total Ca	29.4 mM
Soluble Ca	9.2 mM (31%)
Micellar Ca	20.2 mM
Micellar Ca/g casein	0.77 mM
Total Pi	20.9 mM
Soluble Pi	11.2 mM (54%)
Micellar Pi	9.7 mM
Total Mg	5.1 mM
Soluble Mg	3.3 mM (65%)
Micellar Mg	1.8 mM
Ester phosphate	3.5 mM
Total citrate	9.2 mM
Soluble citrate	8.2 mM (89%)
Soluble Na	24.2 mM
Soluble K	34.7 mM
Soluble Cl	30.2 mM

[a] Note that the casein concentration is 26.1 g/L (Gaucheron, 2005)

2.2.3.2 Definition of milk mineral equilibrium

The different ion associations in the serum phase depend mainly on the association constants of the different ions together (affinity) and the solubility of the salts (Gaucheron, 2003; Walstra & Jenness, 1984) . It is not possible to measure the proportion of the different salts in the serum phase as they cannot be separated. Therefore, models have had to be developed to calculate ion speciation in milk. The first model was proposed by Holt et al. (1981) and proposed a calculation for ion equilibrium in milk serum.

Many authors over the years have proposed different models to describe the ion equilibrium of milk-like systems. The first ion speciation models proposed in the 1980s did not take into account the protein fraction and were applied to a simulated milk salt solution or milk diffusate (Holt et al., 1981; Lyster, 1981; Wood, Reid, & Elvin, 1981). As there is a constant

mineral equilibrium between the micellar and serum phase, some more recent models were developed to describe the ion partitioning between the serum and micellar phases in milk or mineral-enriched milks (Holt, 2004; Mekmene, Le Graet, & Gaucheron, 2009). The effect of acidification has only been modelled very recently, as association constants needed to be recalculated for each different pH value (Mekmene, Le Graët, & Gaucheron, 2010). However, all models make assumptions and neglect some species or associations of secondary importance. For example, assumptions had to be made regarding the nature of the partition of minerals between the two phases and the nature of CCP.

Calcium phosphate chemistry is quite complex because it can have a lot of different phases depending on its Ca/P ratio and its crystallisation state (Gaucheron, 2005). Moreover, micellar calcium is partly associated with the colloidal inorganic phosphate, forming CCP, whereas another part of the calcium is directly bound to the phosphoserine residues of the caseins in the calcium phosphate nanoclusters (organic phosphate). These two types of colloidal calcium cannot be quantified separately. Therefore, the chemical nature of CCP has been studied and debated for many years, based on experimental determination of Ca/Pi molar ratios, titration studies or theoretical modelling, taking into account the acid-base properties of milk and pKa values for calcium phosphates (Gao, 2010; Lucey & Horne, 2009). In fact, at pH 6.7, i.e., the pH of milk, acidic and basic calcium phosphates can co-exist. Some authors found CCP to be a more acidic phase such as brushite-type forms $CaHPO_4 \cdot 2H_2O$ (Chaplin, 1984; Holt et al., 1989), whereas others found CCP to be more of a basic-type calcium phosphate like apatite or tricalcium phosphate $Ca_3(PO_4)_2$ (Lucey & Horne, 2009) or a mixture of both basic and acid form (Gao, 2010). Whether or not CCP has a crystalline structure and whether or not CCP includes citrate and magnesium is also debated. Most of the recent studies have suggested a basic form for CCP. For example, Gao (2010) found CCP to be a mixture of the basic form $Ca_4(PO_4)_2(OH)_2$, similar to HA, the acidic form $CaHPO_4 \cdot 2H_2O$ and magnesium phosphate $Mg_3(PO_4)_2$.

Schematically, the mineral equilibrium between the aqueous phase and the micellar phase has been summarised by Brule (1981) and Gaucheron (2004) and is shown in Figure 2.11.

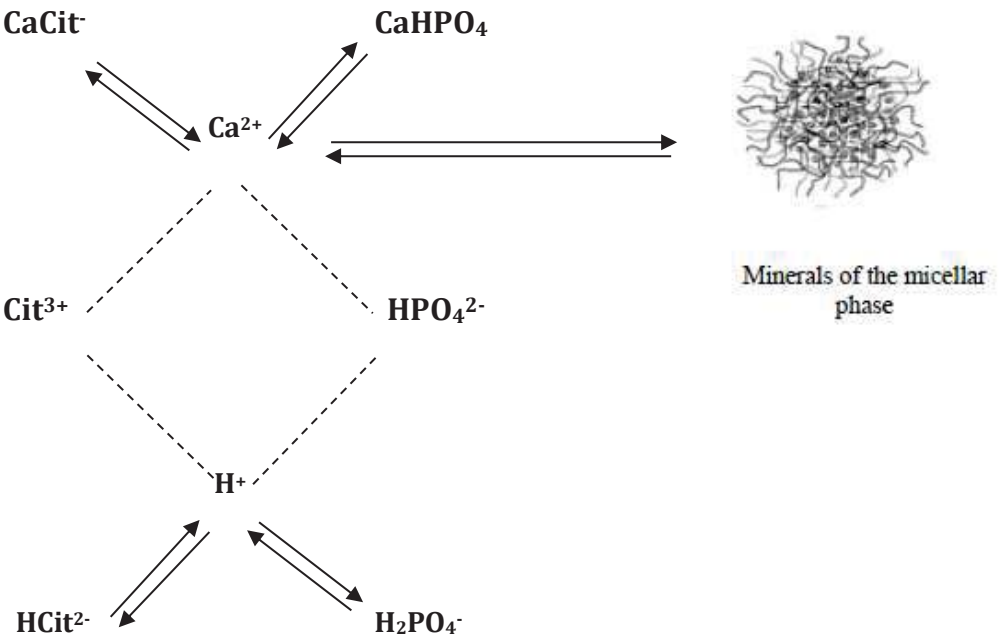

Figure 2.11: Schematic representation of mineral equilibrium in milk (from Brule, 1981 in Gaucheron, 2004).

2.2.3.3 Factors influencing the milk mineral equilibrium and casein micelle stability

Many factors have been shown to modify the mineral equilibrium in milk between the micellar phase and the serum phase. As described previously, micellar calcium phosphate plays a major role in the structure of the casein micelles. Therefore, any physico-chemical events that modify the milk mineral equilibrium can also have consequences on the stability of the casein micelles and the functional properties of milk products. This review will only detail the effect of pH, temperature, addition of calcium and calcium chelators on mineral equilibria and casein micelle stability, as they are the most important parameters to consider in the commercial processing of heat-treated calcium fortified milk products. However, other factors such as ionic strength, cooling or high-pressure are also known to have an impact (Gaucheron, 2005).

pH

Many authors studied the effect of milk acidification on casein micelle structure and mineral equilibrium and found the same trend in their results (Canabady-Rochelle et al., 2007; Le Graet & Gaucheron, 1999; Mekmene et al., 2010). Addition of HCl to milk releases proton ions that complex citrate ions (Cit^{3-}) and inorganic phosphate (HPO_4^{2-}) found in the soluble

phase. This protonation involves the dissociation of ion complexes such as calcium citrate, calcium phosphate and magnesium citrate, releasing calcium, citrate, phosphate and magnesium ions into the serum phase, therefore increasing the ionic calcium concentration. This change in mineral equilibrium upon acidification also induces a solubilisation of CCP from the micelles, leading to release of the CCP-bound calcium and magnesium into the serum phase, and increasing the ionic calcium concentration even further. For example, Gao (2010) acidified the milk with 6 M HCl and observed an increase of ionic calcium concentration up to 19.3 mmol/L at pH 4.5.

Different authors have found colloidal calcium phosphate to be fully solubilised around pH 5.2 (Gao, 2010; Le Graet & Brule, 1993; Le Graet & Gaucheron, 1999). Le Graet and Brule (1993) studied the effect of acidification on mineral balance in skim milk. They measured solubilisation of calcium and phosphate ions and showed that decreasing pH had the biggest effect on the solubilisation of colloidal calcium phosphate (CCP) up to pH 5.2. CCP was solubilised first upon acidification of skim milk and they also showed that at pH 5.2, all CCP was solubilised. At pH 6.0, the calcium bound to the casein via phosphoseryl and carboxyl groups starts to solubilise, probably due to the effect of decreasing pH on the pK of phosphoserines, leading to a slow increase of ionic calcium in the serum phase. Provided the pH was not reduced below pH 5.2, readjustment back to pH 6.7 results in the reformation of the casein micelles. As the structure of the casein micelles was observed to be modified considerably below pH 5.2, Le Graet and Brule (1993) suggested that the calcium directly associated to phosphoserine groups in casein micelles played a more important role in the casein micelle structure than the calcium that forms an integral part of the colloidal calcium phosphate (CCP).

Heat treatment

Casein micelles are known to be very stable when heat-treated. Commercial milks are often heat-treated at high temperature such as ultra-high temperature (UHT; i.e., between 120 °C and 140 °C for a few seconds) without major defects resulting in the products. However, the solubility of calcium phosphate decreases upon heating, leading to a decrease in calcium and inorganic phosphate in the milk serum phase (Gaucheron, 2005; Lucey & Horne, 2009).

Calcium phosphate precipitation occurs following the reaction (1) below and calcium phosphate is transferred from the serum phase to the micellar phase. As HPO_4^- is reduced in

the serum phase, a deprotonation of di-hydrogen phosphate $H_2PO_4^-$ occurs to re-establish the equilibrium in the serum phase (reaction (2) below), leading to a decrease in milk pH.

$Ca^{2+} + HPO_4^{2-} = CaHPO_4$ (1)

$H^+ + HPO_4^{2-} = H_2PO_4^-$ (2)

The mineral shift depends on the intensity of the heat treatment and can be more or less reversible. Under moderate heat treatment conditions (less than 95 °C for a few minutes), the modification of salt equilibrium is reversible upon cooling. However, more severe heating conditions, such as in UHT, can cause physico-chemical changes to the casein micelle. Dephosphorylation of the caseins and their dissociation from the micelles and the formation of complexes between denatured β-lactoglobulin complexes and κ-casein on the surface of the micelles have been reported (Singh, 2004; Singh & Fox, 1987;). Over time on storage, the β-lactoglobulin-κ-casein complexes get progressively released from the surface of the micelles to the serum phase, where they aggregate and form a network, which is believed to cause age gelation of UHT milks (Datta & Deeth, 2001; McMahon, 1996). An irreversible change of the micellar calcium phosphate phase to a more alkaline form (such as β-tricalcium phosphate or HA) upon UHT treatment has also been reported (Lucey & Horne, 2009).

Ionic calcium addition

The effect of calcium addition on mineral balance and heat stability has been extensively studied using the approach of adding calcium chloride ($CaCl_2$) to milk (Canabady-Rochelle et al., 2007; Lewis, Grandison, Lin, & Tsioulpas, 2011; Philippe, Le Graet, & Gaucheron, 2005).

Many authors (Canabady-Rochelle et al., 2007; Gao, 2010; Philippe et al., 2003; Udabage, McKinnon, & Augustin, 2000) have shown that part (around 30%) of the added calcium remains in the serum phase, either in the free form or in a complex form with citrate, whereas the other part must precipitate and be transferred to the micellar phase. Addition of $CaCl_2$ to milk is therefore observed to increase both micellar and serum calcium.

A decrease of pH upon calcium addition from $CaCl_2$ has been reported by many authors and has been explained by three main effects (Philippe et al., 2003):

(i) Formation of calcium citrate and calcium phosphate salts in the serum phase.

(ii) Exchanges between added calcium and micellar protons.

(iii) Acidity of the added calcium solution itself.

When $CaCl_2$ is added to milk, the increase in ionic calcium is due to two different effects: the effect of the addition of calcium itself, and the effect of the pH decrease caused by the addition of calcium. However, by readjusting the pH of the milk supplemented with $CaCl_2$ back to that of skim milk pH (6.7), the effect of the addition of calcium itself on ionic calcium concentration can be isolated. For example, Philippe et al. (2003) reported that addition of 13.5 mM $CaCl_2$ decreased the milk pH from 6.75 to 6.3 and increased ionic calcium from 1.56 to 6.94 mM. On pH adjustment, ionic calcium was reduced to 4.74 mM. They showed that, whatever the concentration of $CaCl_2$ added between 4.5 and 13.5 mM, when the pH was readjusted to milk pH, 80% of the added calcium was bound to casein micelles whereas 20% stayed in the ultra-filtrate. Also, Lin et al. (2006) added 10 mM $CaCl_2$ and found the pH decreased to 6.42 and the ionic calcium concentration increased to 4.88 mM, then reduced to 2.77 mM on pH adjustment. Canabady-Rochelle et al. (2007) observed that when calcium chloride was added, a decrease in pH and an increase in ionic calcium just after supplementation occurred, followed by a decrease in ionic calcium upon stabilisation. These authors determined this to be due to a re-equilibration of calcium between the different phases, likely involving the formation of complexes with citrates and phosphates in the soluble phase and transfer of ionic calcium from the serum phase to the micellar phase.

This mineral equilibrium modification, caused by the addition of calcium, has been shown to cause strong physico-chemical modifications of the casein micelles (Philippe et al., 2003). Association of ionic calcium with casein micelles is likely to shield their overall negative charge, resulting in a zeta-potential decrease. Philippe et al. (2003) also observed an increase in hydrophobicity and a release of water, probably caused by a reorganisation of the casein micellar internal structure. An increase in turbidity and some protein material incorporation (α_{S1}- and β-caseins from the supernatant) were also observed. As water was released and minerals and proteins were incorporated into the micellar structure, but no change in micelle size (hydrodynamic diameter) was observed, the authors concluded that addition of calcium caused an increase in the micellar density.

As a consequence of these physico-chemical changes, the heat stability of milk is decreased. At constant pH compared with non-supplemented skim milk, ionic calcium binds to the protein, causing the zeta-potential of the casein micelles to decrease. The electrostatic repulsions between the micelles are therefore weaker, leading to their aggregation (Sievanen, Huppertz, Kelly, & Fox, 2008).

Addition of calcium chelators

Calcium chelators such as ethylenediaminetetraacetic acid (EDTA), citrate or oxalates are salts that have a high affinity for calcium. They are able to form complexes with the free calcium contained in the milk serum. Therefore, they are used in industry to improve the heat and storage stability of UHT milks or to prevent fouling of heat-exchangers by calcium phosphate deposition. As serum ionic calcium is chelated, the milk mineral equilibrium is disrupted and calcium from CCP gets released from the micelles, and also forms complexes with the calcium-chelating salts (Kaliappan & Lucey, 2011) The addition of calcium chelators can therefore cause some physico-chemical changes in the casein micelles; for example, an increase in casein micelle voluminosity and hydration, and a partial dissociation of the micelles (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Mizuno & Lucey, 2005). The extent of micellar dissociation depends on the type and concentration of the calcium chelating agent. Different types of calcium chelating agents have different calcium-binding capacities (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2009); this determines the extent to which they affect the structure of the casein micelles when added to milk (de Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2012). When large amounts of CCP are released from the micelles, the changes in casein micelle size and the dissociation of the casein micelles are irreversible (Udabage et al., 2000).

In conclusion, it is important to emphasise that, with a serum phase saturated with calcium, adding more calcium to milk at normal pH is difficult without modifying the milk mineral equilibrium. The fact that calcium addition to milk leads to heat instability makes milk supplementation with calcium all the more challenging for commercial products that have to be heat-treated using high heat-treatments, such as UHT, which can also cause instability over processing and storage. While the effect of adding calcium to milk has been well reviewed, there is a lack of literature on calcium-enriched processed milks, especially when pasteurised or UHT-sterilised. Therefore, the next part of this review will detail the practical difficulties associated with enriching milk with calcium and the solutions that are usually recommended.

2.3 Calcium supplementation of cows' milk: an industrial challenge

2.3.1 Possible calcium sources: advantages and drawbacks

The options for adding calcium to milk are quite limited by the reactivity of calcium ions. Calcium salts such as calcium chloride, calcium lactate, calcium gluconate, calcium phosphate or calcium carbonate can be used for milk enrichment with calcium (Augustin & Williams, 2002). However, these calcium salts do not remain associated when added to milk and some dissociate to calcium cations, Ca^{2+}, and their associate anions, leading to a decrease in pH (De la Fuente et al., 2004) and an increase in ionic calcium. This dissociation depends on their solubility products. As explained in the previous section, any addition of salt that increases the ionic calcium of milk, or decreases the pH, or both, is likely to modify the casein micelle properties, leading to heat instability.

Calcium chloride is the most soluble salt to be added to milk, but it causes major heat instability over heat treatment and UHT processing, even for levels of calcium addition as low as 5 mM (Lewis et al., 2011). On the other hand, the addition of calcium lactate and calcium gluconate has also been shown to decrease pH and increase ionic calcium in milk, leading to heat instability (Singh et al., 2007; Vyas & Tong, 2004). Therefore Singh et al. (2007) recommended increasing the pH again to a more alkaline value than the natural pH of milk after supplementation with these calcium salts, using a buffer salt, such as disodium phosphate, to restore heat stability. When soluble calcium salts are used in heat-treated calcium-fortified milks, calcium chelating salts, such as trisodium citrates or sodium polyphosphates, are often used to reduce the ionic calcium concentration and improve the heat stability of the products (Augustin & Williams, 2002; de Kort et al., 2011; Kaliappan & Lucey, 2011). However, the addition of calcium chelating agents can also cause instability problems. Adding large amounts of calcium chelating salts causes the dissociation of the casein micelles (see previous section on calcium chelators). During heating, the dissociated casein micelles tend to re-associate and aggregate, which can lead to heat instability (Chandrapala, Augustin, McKinnon, & Udabage, 2010). Some calcium-chelating agents such as polyphosphate have also been reported to bind directly to positively charged amino acids of the caseins, causing cross-linking of the micelles that can lead to age gelation (Datta & Deeth, 2001; Mizuno & Lucey, 2005). Calcium chelating agents can also cause sensory

defects, such as browning over storage or taste defects, as reported for sodium dihydrogen phosphate by Tsioulpas, Koliandris, Grandison, and Lewis (2010).

The best way to avoid heat instability when adding calcium to milk is to use insoluble calcium salts, such as calcium carbonate or calcium phosphate, as they do not have an effect on the two parameters influencing heat stability, i.e., pH and ionic calcium. Omoarukhe et al. (2010) added 30 mM calcium from six different calcium salts, including three insoluble calcium salts. They showed that addition of calcium carbonate, calcium phosphate and calcium citrate did not affect heat stability as they all had low solubility. For this reason, these salts are often considered inert, since they do not cause heat aggregation of the casein micelles observed when soluble salts are added (Sievanen et al., 2008). The main problem with these salts, however, is their tendency to sediment during storage, leading to a phase separation and a layer of settled calcium salt at the bottom of containers. Insoluble calcium salts are also responsible for sensory defects such as chalkiness or grittiness or off-flavours such as bitter or metallic taste (Singh et al., 2007).

One of the most commonly used insoluble calcium salts in calcium-supplemented commercial products is hydroxyapatite, $Ca_5(PO4)_3(OH)$ (sometimes wrongly referred to as tricalcium phosphate (TCP) by the suppliers). HA is the most thermodynamically stable phase of the calcium phosphate phases and is almost insoluble in water (solubility constant $K_s = 3 \times 10^{-59}$ $(mol/l)^9$; Uskoković & Uskoković, 2010). Bones and teeth are actually also made of HA. Food-grade HA ingredients of different particle sizes are available on the market and have been shown to be well absorbed by the intestine (López-Huertas et al., 2006; Patrick, 1999). HA has quite a neutral taste and a less chalky mouth-feel than calcium carbonate.

Another source of calcium available for calcium fortification is calcium phosphate isolated from milk. The interesting feature of this milk-derived calcium phosphate is that it can be marketed as a natural dairy calcium source. Whilst the nature and composition of milk calcium phosphate ingredients are not fully understood, it has been shown that they are likely to be made of poorly crystalline HA, possibly with some amorphous calcium phosphate (ACP; Anema, 2009b). In the rest of this review, the focus will be on HA as a model salt for calcium supplementation of milk; this was a key proposition for the work in this thesis.

2.3.2 Ways of improving suspension of HA in products

Small size insoluble calcium salts suspended in a liquid such as water or milk can be considered as colloidal systems. A colloid state can be defined as "a state of matter in which finely divided particles of one substance (the dispersed phase) are suspended in another (the continuous phase) in such manner that the electrical and surface properties acquire special importance" (D. Dalgleish, personal communication); this definition has particular relevance for the work in this thesis.

The most common colloidal systems in food science are emulsions of oil in water (creams, beverages) or water in oil (butter, margarine). The primary challenge when it comes to emulsions and colloidal systems is to keep them stable over processing and shelf-life, so the two phases do not separate, but stay in a homogeneous state.

Sedimentation of particles can be explained with Stokes law. For dilute suspensions, the settling velocity (w) of small spheres in a fluid depends on the fluid and particle densities (ρ_f and ρ_p, respectively), the particle size (radius r), and the viscosity of the fluid (μ); it is influenced by *g*, the acceleration due to gravity. Thus Stokes law is represented by:

$$w = \frac{2\,(\rho_p - \rho_f) g\, r^2}{9\mu}$$

To avoid sedimentation of solid particles and improve their suspension stability in a liquid phase, the settling velocity has to be decreased. Three main ways are used commercially: reducing the particle size, increasing the viscosity of the continuous phase or using modified forms of calcium salts. These three options are detailed and reviewed below.

2.3.2.1 Particle size reduction

Producing insoluble calcium particles of small size is actually quite challenging. The most common way to produce HA is by wet chemical precipitation. HA is obtained from calcium hydroxide [$Ca(OH)_2$] and orthophosphoric acid (H_3PO_4) or from calcium nitrate [$Ca(NO_3)_2$], diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] and ammonium hydroxide (NH_4OH); the process involves nucleation and grain growth (Norton, Malik, Darr, & Rehman, 2006). The flocculation of particles cannot be controlled during the process, so the resulting product is

formed of large primary particles that are then dried and ground to micron-sizes to produce less agglomerated particles.

Figure 2.12 shows two pictures obtained by scanning electron microscopy (SEM) of a typical HA powder, produced by wet chemical precipitation and used in the calcium fortification of milk. The primary particles of HA are roughly spherical and have a diameter of a few microns (Figure 2.12A). Figure 2.12B shows that the primary particles are actually made of nano-sized smaller particles that are aggregated together in clumps. These aggregates are permanent and cannot be reverted. Therefore, these HA ingredients can have a very high surface area, up to around 100 m^2/g.

Many other chemical processing routes can be used to produce HA and the powder characteristics such as crystallinity, purity or particle size strongly depend on the process used (Koutsopoulos, 2002). However, the minimum size that can be obtained for a powdered HA ingredient is around a few micrometres, even when grinding steps are included. Such particles are still too large to be maintained for long periods in suspension and will inevitably settle out of the liquid if no additional stabilisation is used.

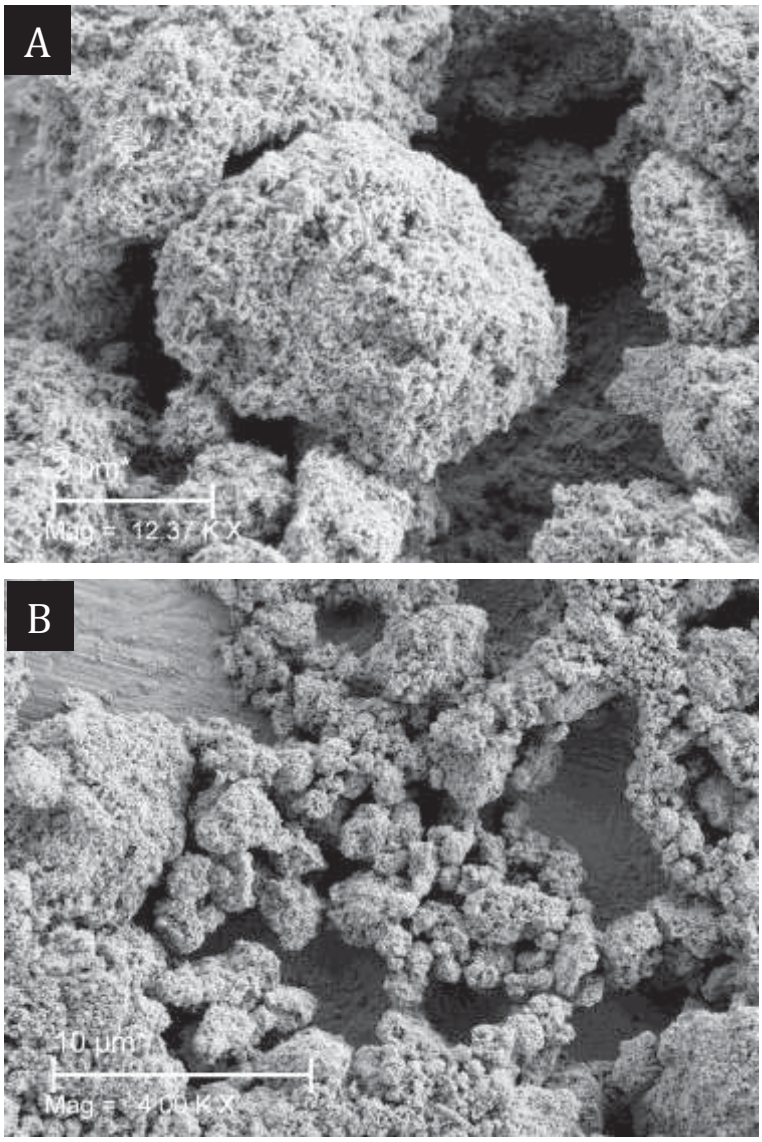

Figure 2.12: Typical SEM pictures of HA ingredients used for calcium fortification. SEM pictures kindly provided by Chemische Fabrik Budenheim KG, (Germany).

2.3.2.2 Viscosity increase and improvement of the continuous phase

To minimise the settling of the particles of insoluble calcium salts during processing and shelf-life, the main solution used in commercial calcium-fortified products is to incorporate hydrocolloids in the formulation. Hydrocolloids increase viscosity or induce the formation of a network between proteins and the hydrocolloid that improves the suspension of the calcium salt (Gerstner, 2005); the same technique is used to keep cocoa particles in suspension in UHT-treated chocolate milks (Dogan, Toker, & Goksel, 2011; Prakash, Huppertz, Karvchuk, & Deeth, 2010).

Some of the stabilisers used in commercial formulations have an effect only on viscosity. For example, guar gum is a non-gelling thickener that has been reported to be used in calcium-supplemented milk (Nelson, Crowley, & Weatherford). Sodium alginates are also commonly used in milk beverages for their thickening and gelling properties (Draget, 2009). However, thickeners increase viscosity of liquids and are usually not suitable for low viscosity beverages. Also, according to Stokes law, if the effect of the stabiliser is only on viscosity, the fine particles will still settle down, but at a slower rate.

To really have an effect on the suspension of solid insoluble particles, hydrocolloids that interact with milk proteins to give a weak gel matrix have to be used; in this respect carrageenan is the major hydrocolloid used in dairy products at neutral pH (Blakemore & Harpell, 2010). Many studies have shown the specific interactions between carrageenan at low concentrations and milk proteins, both in model systems such as milk protein solutions and carrageenan-milk mixtures (Dalgleish & Morris, 1988; Hemar, Hall, Munro, & Singh, 2002; Langendorff et al., 2000; Thaiudom & Goff, 2003) and in commercially relevant systems such as milk beverages formulations (Ramírez-Sucre & Vélez-Ruiz, 2010; Yanes, Duran, & Costell, 2002).

At neutral pH, both casein micelles and κ-carrageenans carry overall negative charges. However, specific electrostatic interactions are possible between the sulphate groups of κ-carrageenans and the positively charged regions of kappa-casein exposed on surface of the micelle (Snoeren, Both, & Schmidt, 1976). On cooling, the region of the carrageenan chains that are not involved in electrostatic interactions with the micelles can interact together to form helical structures. A weak gel matrix is then formed, entrapping insoluble particles, like cocoa or calcium particles.

Carrageenans are also widely used in UHT milks to reduce sedimentation caused by heat coagulation or fouling during processing (Sedlmeyer & Kulozik, 2006). The main problem with using carrageenans in UHT milks is that the weak gel formed by carrageenans can become firmer over shelf-life, leading to an undesirable gelation, sometimes referred as “gelation blurp”. Tijssen, Canabady-Rochelle, and Mellema (2007) found that this undesirable gelation was likely to be due to protein rearrangements over time, and perharps leading to protein-carrageenan rearrangements as well. They speculated that a change in calcium equilibrium occurred over shelf-life, with calcium migrating to the serum phase. More calcium in the serum phase would lead to a stronger gelation of carrageenans. However, they could not confirm this hypothesis.

Colloidal microcrystalline cellulose is another hydrocolloid commonly used in calcium-fortified milks. It is made from microcrystalline cellulose (MCC), usually co-processed with carboxymethylcellulose (CMC). When the colloidal MCC is well dispersed, the cellulose particulates set up a three-dimensional insoluble cellulose structural network that provides the functionality (Krawczyk, Venables, & Tuason, 2009). The gel formed has some elastic properties of a solid that exhibits relatively high yield stress and a thixotropic behaviour. It means it presents a gel structure at cold temperature when unsheared, but this structure will break down on shearing (i.e., when agitated or stressed), so the viscosity will decrease and the material will flow (liquid-like behaviour; Bell, 1993). These properties play a very effective role in suspending solids such as cocoa powder or insoluble calcium salts in beverages without impacting the viscosity. For this reason, colloidal MCC is often used in combination with other stabilisers in calcium-fortified milks to provide a long-term suspension of calcium salts at ambient temperature; for example MCC has been shown to improve the structure of the three-dimensional network formed by carrageenans in milk (Tuason, Krawczyk, & Buliga, 2009). Because MCC has shear-thinning properties, the weak gels that are formed can be easily thinned when the milks are shaken or poured out of the brik container. However, MCC can also cause sensory defects in the products, such as undesirable gelation.

Even though a lot of literature has been published on the stabilisation mechanisms of hydrocolloids, the choice of hydrocolloids in product development is approached in a rather "trial and error" way and possible synergies between different hydrocolloids are never explained in terms of mechanisms. Some of the commercial formulations are patented (Andersen, Keller, & Streiff, 1989; Kligerman, 1987), but published work on UHT formulations of milks or fortified-milks is rare (Prakash et al., 2010; Sedlmeyer & Kulozik, 2006; Tijssen et al., 2007). Only one published paper can be found that reports some work on optimising the ratio of different stabilisers in calcium-fortified milk (Cheng & Xie, 2001). A mixture design was used to study the efficiency of different hydrocolloids at suspending calcium salts and found that 3 g/L of a ratio 1:1 of carrageenans and MCC gave the best suspension of milk calcium (low viscosity, good thermal stability, and smallest amount of sediment). However, no precision was given on the nature of the MCC, and no explanation was provided on the mechanisms or possible synergies involved between carrageenan and MCC.

In conclusion, hydrocolloids provide solutions to keep insoluble particles in suspension, but they can also modify the texture or mouth-feel of the products, or cause some product

defects during storage such as undesirable gelation. Also, even though the use of hydrocolloids is very common in the food industry, the consumer's perception of additives and hydrocolloids can be quite negative (Varela & Fiszman, 2013), with some hydrocolloids having a very chemical connotation. It is therefore important to look for novel ways to stabilise products and keep insoluble particles suspended that do not involve the use of hydrocolloids.

2.3.2.3 Surface modification of calcium salts

Nano or micro-sized particles of HA are known to form spontaneous aggregates when suspended in aqueous media, due mainly to interparticle Van der Walls interactions and hydrogen bonding (Sadeghian, Heinrich, & Moztarzadeh, 2006). It means that particles will not necessarily remain as individual particles when added in a food product, but are likely to aggregate and form bigger particles that will sediment even faster during storage. The spontaneous aggregation of HA particles has been reported to create problems in the use of bioceramic nano-materials, such as instability in the processing and the use of bone implants in human bodies (Norton et al., 2006). Therefore, there have been extensive studies on the modification of the surface properties of HA particles to reduce particle aggregation and improve the stability of HA in bioceramic applications (Borum-Nicholas & Wilson, 2003; Borum & Wilson, 2003; Lee et al., 2007; Wilson, 2009), but none of the studies were extended to food systems.

One method of reducing particle aggregation is electrostatic stabilisation through adsorbing electrolytes such as ions (citrate, fluoride, phosphate or calcium ions) on the particles. This modifies the surface charge of the particles and renders them less prone to aggregation and can be monitored by measuring the change in zeta-potential. For example, the adsorption of fluoride ions on HA has been studied extensively in dentistry studies (Lin, Raghavan, & Fuerstenau, 1981; Spinelli, Brudevold, & Moreno, 1971).

Another method is steric stabilisation, which depends on the separation of the individual particles by adsorbing neutral or charged large chain polymers onto the surface. Dodecyl alcohol (Borum-Nicholas & Wilson, 2003), silica (Borum & Wilson, 2003), polyethylene glycol (Yu et al., 2007) and dextran (Rölla, 1971), are polymers that have all been used in the literature for this purpose. However, polymers that are used for steric stabilisation are usually not food-grade. Only a very few papers report surface modification of HA with

molecules that are also used in food systems. Chitosan (Wilson & Hull, 2008), xanthan gum and CMC (Barbour, Shellis, Parker, Allen, & Addy, 2005) have all been reported to adsorb onto and inhibit its dissolution, but no study was done on the consequences of the adsorption on the colloidal stability of HA. Gum Arabic, a natural gum that is already used in food products for its encapsulation and emulsification properties (McNamee, O'Riordan, & O'Sullivan, 1998), has been shown to adsorb on HA, encapsulating it and significantly improving its stability in suspension (Roque & Wilson, 2008; Wilson & Hull, 2008).

Proteins themselves are effectively large charged polymers; they have been showed to bind to HA and change its colloidal properties. A wide range of proteins or peptides have been previously used in the literature to bind to HA and stabilise it: collagen (Yin Hsu, Chueh, & Jiin Wang, 1999), gelatine (Chang, Ko, & Douglas, 2003), bovine serum albumin, egg lysozyme, bovine serum fibrinogen (Brandes, Welzel, Werner, & Kroh, 2006) and lactoferrin (Iafisco, Di Foggia, Bonora, Prat, & Roveri, 2011). However, none of the work reported on protein adsorption on HA has looked specifically at the main milk proteins, and none has looked at the effect of protein adsorption on the suspension stability of HA in food systems.

In conclusion, fortifying milk with HA particles (or other insoluble calcium salts) is challenging, as the particles aggregate and quickly sediment to the bottom of the products. Current methods of stabilising calcium-fortified milks involve the use of small micronised particles and hydrocolloids; however, the stability of the products over shelf-life is not always satisfactory, and functional defects can appear. There is probably an opportunity to use the studies carried out on non-food systems on surface modification of HA and apply it to food systems and find new ways to stabilise insoluble calcium particles in food products. However, from what can be found in the literature, this path does not seem to have yet been exploited.

To find novel methods to keep HA particles suspended in milk products, there is a need to understand further the colloidal behaviour of HA particles in a milk system. In this context, it seems important to reconsider a simple system of milk and HA particles, and understand the colloidal interactions in the system.

2.4 Interactions between hydroxyapatite and milk proteins

2.4.1 Hydroxyapatite properties

2.4.1.1 HA structural and surface properties

Hydroxyapatite, $Ca_5(PO_4)_3(OH)$, is a crystalline mineral of calcium phosphate. HA crystals are made of many unit cells that contain crystal ions. The unit cells of HA crystals have a hexagonal structure and are characterised by three vectors, *a*, *b* and *c*, and contain ten calcium ions (Ca^{2+}), six phosphate ions (PO_4^{3-}) and two hydroxyl ions (OH^-) (Kawasaki, 1991; Kawasaki et al., 1985). A computer representation of the spatial arrangements of the crystal ions in an unit cell of HA is shown in Figure 2.13A (Mostafa & Brown, 2007). After dispersing HA in an aqueous media, ions are expressed on the surface, due to various hydrolytic and dissolution reactions (Kandori, Kuroda, Togashi, & Katayama, 2011). Two types of main surfaces appear on the crystals and carry two types of adsorbing sites. Hydroxyl ions, (OH^-), are dissolved at the surface of HA, exposing calcium atoms and producing calcium ions at the surface (Kandori et al., 2011). Therefore, the surfaces called *a* and *b* surfaces (parallel to the *bc* and *ac* planes, respectively, see Figure 2.13) carry positively charged adsorbing sites, called C-sites, arranged in a rectangular manner and composed of two calcium ions.

The surface called *c* surface, parallel to the *ab* plane, lacks calcium atoms and therefore carries negatively charged adsorbing sites, P-sites. P-sites are arranged in a hexagonal manner and are made up of six oxygen atoms belonging to three phosphate crystal ions (Bernardi, Giro, & Gaillard, 1972; Gorbunoff & Timasheff, 1984a).

From a practical perspective, this means that the surface of HA particles is not homogeneously charged when suspended in aqueous media. HA particles are made of HA crystals, that contain C-sites and P-sites which are, respectively, positively and negatively charged, together with hydroxyl groups, distributed regularly on their surface (Figure 2.13B gives a schematic of this). C-sites and P-sites are binding sites for ions, proteins and other charged polymers (Gagnon, 1998).

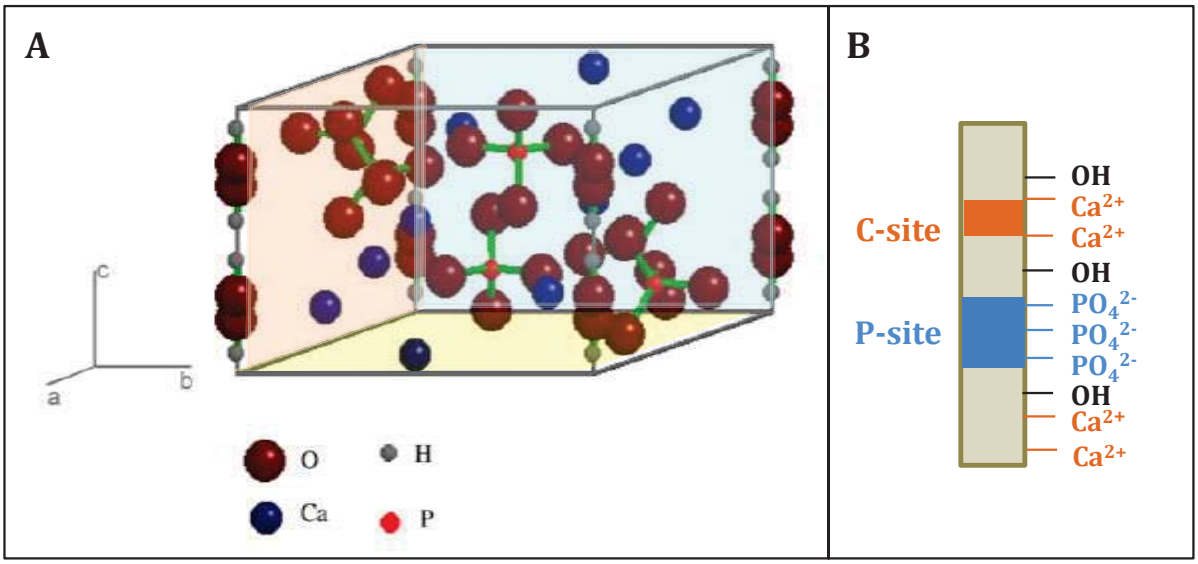

Figure 2.13: Structural properties of HA crystals. Panel A, unit cell of HA crystals, adapted from Mostafa & Brown (2007) showing the *a* surface in blue, the *b* surface in orange, and the *c* surface in yellow; Panel B, schematic illustration of the charged surface groups on HA crystals.

2.4.1.2 HA colloidal stability

Particles dispersed in a liquid phase naturally tend to aggregate together and form clusters, which leads to instability phenomena such as aggregation and flocculation. As the aggregates grow in size, they sediment to the bottom of the containers. The behaviour and aggregation of particles in suspension is usually explained by the "Derjaguin and Landau, Verwey and Overbeek" (DLVO) theory (Anema, 2009b; Liu & Nancollas, 1997; Walstra & Jenness, 1984). This theory considers the balance between the repulsive short range electrostatic interactions between particles through their electric double layers and the longer range Van der Waals attractive interactions between the particles. When the attractive forces are stronger than the repulsive forces, it leads to irreversible aggregation of the particles.

Zeta potential is the measurement parameter normally used to give an indication of the repulsive forces between the particles. It can predict whether or not particles will be stable in a colloidal system. The lower the absolute value of zeta-potential, the more likely the particles are to aggregate together. The zeta-potential of HA is determined by the

distribution of charges on its surface and depends on the properties of the surrounding medium. The only study on colloidal properties of HA in a food system was carried out by Anema (2009b), using milk-derived calcium phosphate particles. Milk-derived calcium phosphate is made of poorly crystalline HA and potentially amorphous calcium phosphate. The stability of milk-derived calcium phosphate suspensions was investigated by Anema (2009b) under different conditions of pH, ionic strength and mineral composition, by looking at the rate of aggregation of the particles. However, an extensive number of other studies have looked at the effect of pH, ionic strength and ionic composition on zeta-potential of HA in buffered solutions in non-food systems, and correlated the variation of zeta-potential with the rate of HA crystal growth (Harding, Rashid, & Hing, 2005; Huang et al., 2007; Johnsson, Richardson, Sallis, & Nancollas, 1991; López-Macipe, Gómez-Morales, & Rodriguez-Clemente, 1998; Martins, Santos, Almeida, & Costa, 2008; Yin, Liu, Zhan, Ding, & Yuan, 2002; Zhu, Fan, Li, Xiao, & Zhang, 2007).

Table 2.4 summarises the different studies. The general trends for pH, ionic strength and ionic composition are detailed below.

Table 2.4: Effect of physico-chemical parameters of the suspending solution on zeta-potential, aggregation and crystal growth behaviour of HA particles.

Parameters	Literature ranges (and an example from references)	Effect on magnitude of zeta-potential	Effect on aggregation rate or crystal growth rate	Mechanism (and references)
Decrease in pH	From 10 to 4 (e.g., from 9 to 5.5)	Decrease (less negative) (e.g., from –40 to –20 mV)	Not studied	Protonation of phosphate ions in P-sites (Martins et al., 2008; Yin et al., 2002; Zhu et al., 2007)
Increase in ionic strength (NaCl addition)	From 0 to 0.15 M (e.g., from 0 to 0.1 M)	Decrease (less negative) (e.g., from –9 mV to –5 mV)	Increase	Compression of electrical double layer / shielding of charges (Anema, 2009b; Huang et al., 2007; Yin et al., 2002; Zhu et al., 2007)
Addition of calcium ions	From 0 to 20 mM (e.g., from 0 to 5 mM)	Decrease (to 0 mV) and then increase (>0 mV) (e.g., from –9 mV to +8 mV)	Increase	Specific adsorption of calcium ions / calcium bridging (Anema, 2009b; Harding et al., 2005; Yin et al., 2002; Zhu et al., 2007)
Addition of phosphate ions	From 0 to 20 mM (e.g., from 0 to 5 mM)	Increase (more negative) (e.g., from –9 mV to –20 mV)	Decrease	Specific adsorption of phosphate ions on C-sites. (Anema, 2009b; Harding et al., 2005; Yin et al., 2002; Zhu et al., 2007)
Addition of citrate ions	From 0 to 4 mM (e.g., from 0 to 4 mM, pH 8)	Increase (more negative) (e.g., from –18 mV to –23 mV	Decrease	Specific adsorption of citrate ions on C-sites (Johnsson et al., 1991; Li et al., 2011; López-Macipe et al., 1998; Martins et al., 2008; Tan et al., 2009)

Effect of pH

Depending on the pH, the degree of protonation of the phosphate ions on the HA surface can change between PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^{-}$, which explains the variation in zeta-potential as a function of pH (Uskoković & Uskoković, 2010; Wassell, Hall, & Embery, 1995). The point at which the zeta-potential reaches 0 mV is usually called the point of zero charge (pzc). At pH = pzc, the surface of HA is still heterogeneously charged with positive and negative sites. However, HA carries the same amount of positive and negative sites, so the net charge is ~0 mV. The values reported in the literature for the point of zero charge vary widely between 4.3 to 7.6, depending on the hydroxyapatite studied and the method used for its determination (Bell, Posner, & Quirk, 1972).

Effect of ionic strength

When ionic strength is increased by addition of salts such as NaCl or KCl, it leads to a decrease in the magnitude of the zeta-potential of the particles. It is a typical salt effect, attributed to the charge-shielding effect of the salts, causing a compression of the electric double layer (Anema, 2009b), It has also been suggested that sodium ions can adsorb specifically to HA surfaces, whereas chloride ions cannot because of steric hindrance (Kawasaki, 1991). Sodium might be able to replace the protons on the phosphate groups in the P-sites (Bankston, Dattolo, & Carta, 2010; Dattolo, Keller, & Carta, 2010) or substitute for the calcium ions in the HA crystal structure (Bell, Posner, & Quirk, 1973; Shimabayashi, Tanaka, & Nakagaki, 1986). As ionic strength increases, the magnitude of the zeta-potential decreases so the electrostatic repulsion forces between the particles are lowered, and the particles tend to aggregate faster (Anema, 2009b).

Effect of potential-determining ions (calcium, phosphate and citrate)

Calcium, phosphate and citrate ions are potential-determining ions for HA. They have been reported to adsorb specifically on the charged sites of the HA crystals (Harding et al., 2005; Johnsson, Levine, & Nancollas, 1991; Leach, 1960; López-Macipe et al., 1998; Yin et al., 2002; Zhu et al., 2007). Calcium ions adsorb on the P-sites, neutralising the particle net charge and then charging the surface positively. Phosphate and citrate ions bind to the C-sites of HA, charging the particle negatively. Therefore, the adsorption of phosphate and citrate ions cause an increase of the repulsive forces between the particles, which explains the slower aggregation rate of the particles (Anema, 2009b) and the inhibition effect of citrate on the growth of HA crystals (Johnsson et al., 1991). The adsorption of citrate ions on HA has also been shown to improve the colloidal stability of HA particles in suspension, by providing a durable electrostatic stabilisation against sedimentation (Martins et al., 2008; Tan, Chen, & Xia, 2009).

In summary, under conditions where the magnitude of the zeta-potential increases, such as an increase of pH, a decrease in ionic strength, or the adsorption of negative potential-determining ions on the surface, the rate of aggregation and the rate of crystal growth decrease as the electrostatic repulsive forces between the particles are strengthened. Conversely, under conditions when the magnitude of zeta-potential decreases, such as a decrease of pH, an increase in ionic strength or the adsorption of positive potential-determining ions, the electrostatic forces between the particles are weakened, which leads to a faster rate of aggregation and crystal growth. However, the effect of the ions that determine potential on the magnitude of changes in zeta-potential is usually a lot greater

than the effect of pH or ionic strength, which leads to more pronounced effect on the rates of aggregation and crystal growth (Anema, 2009b).

2.4.2 Protein adsorption on HA

2.4.2.1 Definition of protein adsorption

The process of protein adsorption has been defined by Brandes et al. (2006) as "the first step after the contact of any biofluid with a solid surface". A practical example of protein adsorption in food manufacturing is the ability of food proteins contained in liquid foods to adhere to processing equipment such as membranes, bioreactors or pipes, of leading to fouling problems. Protein adsorption is mainly driven by electrostatic and hydrophobic interactions between the proteins and the surface. It is influenced by the properties of both the solid surface and the proteins, as well as by environmental conditions. The amount of protein adsorbed on a surface is usually in the order of a few milligrams per square meter and varies with the type of proteins and surface and the adsorption conditions (Nakanishi, Sakiyama, & Imamura, 2001)

It is well-known that HA interacts strongly with proteins in a wide range of applications:

(i) HA is used in analytical chemistry, where chromatography columns made of HA are used to separate proteins (Kawasaki, 1991). Compared with other chromatography methods such as anion-exchange chromatography or size exclusion, HA chromatography columns have a better selectivity for proteins, and therefore allow a better separation and purification of the proteins (Gagnon, 1997).

(ii) Interactions between proteins and synthetic HA crystals have been widely studied for nano-ceramics and bone replacement, as the binding of proteins to bone substitutes can have a dramatic effect on the clinical success of a bone implant (Wilson, 2009). HA and other calcium phosphate-based bioceramic materials are biomaterials with excellent biocompatibility. When HA biomaterials are implanted in the body, the protein contained in the body fluids adsorbs onto the implants, followed by cell attachment, proliferation and migration processed, allowing bone tissue regeneration around the bone implants (Wang et al., 2012). Therefore, the HA implants need a high adsorption capacity, so proteins can first adsorb to the implant, controlling the bone reconstruction and remineralisation processes

(Kandori, Mukai, Yasukawa, & Ishikawa, 2000). The studies on HA and bioceramic surfaces usually look at either the interactions between bioceramic materials and living tissues in-vivo, or attempt to understand the mechanism of HA growth and regeneration on the bone implants (HA bioactivity) (Wang et al., 2012). Both phenomena involve protein adsorption. A lot of studies also focus on developing new HA-based nano-material with better adsorption capacities. In this case, the adsorption of proteins found in body fluids is usually studied on synthetic HA nano-materials, to mimic the adsorption phenomena occurring in the body.

(iii) Tooth enamel is made of HA. The salivary proteins are known to bind to tooth enamel in a highly selective way, forming a pellicle that protects the enamel surface. The adsorbed proteins are also involved in tooth demineralisation and remineralisation processes taking place in the oral cavity, as they can control the growth or the dilution of tooth enamel (Juriaanse, Booij, Arends, & Bosch, 1981; Lamkin, Arancillo, & Oppenheim, 1996).

As milk contains proteins, protein adsorption is expected when HA particles are added to milk. The different aspects of protein adsorption to HA are therefore detailed in the following section of this review.

2.4.2.2 Binding mechanism

In chromatography, bioceramic or dental applications, the mechanism of protein adsorption on HA is the same, and involves specific protein domains. The adsorption mechanism was first studied by Bernardi and Kawasaki (1968), Gorbunoff (1984) and Gorbunoff and Timasheff (1984a,b) on chromatography columns. Their research led to an adsorption model that is today widely accepted in the literature, and that has been reviewed in detail by Kawasaki (1991) and Wang et al. (2012).

In theory, protein molecules are able to bind to both C-sites and P-sites of the HA crystals, since the adsorbing sites are located on different crystal surfaces. Negatively charged groups of the proteins, such as carboxyl and phosphate groups (in the case of phosphorylated proteins) can bind to the C-sites of HA, whereas positively charged groups of the proteins, such as amino groups, adsorb to the P-sites of HA (Bernardi & Kawasaki, 1968; Gorbunoff & Timasheff, 1984b). In protein sequences, negatively charged side chain carboxyl groups are

mostly carried by glutamic acid and aspartic acids (Byler & Farrell, 1989; Juriaanse, Arends, & Ten Bosch, 1980). A schematic illustration of the binding mechanism between proteins and HA is given in Figure 2.14. Carboxyl and phosphate groups bind to crystal calcium ions in the C-sites, whereas amino groups bind to crystal phosphate ions the P-sites.

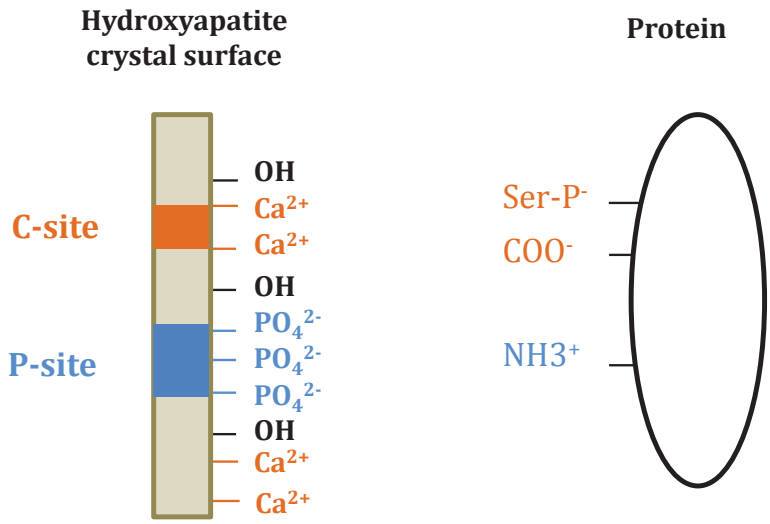

Figure 2.14: Schematic illustration of the binding interactions between HA and proteins.

However, in practice, the adsorption of proteins with an isoelectric point lower than 7 (referred to as acidic proteins) differs from that of proteins with an isoelectric point higher than 7 (referred to as basic proteins). The acidic proteins have been shown to predominantly adsorb mainly onto C-sites of the HA crystals, whereas the basic proteins adsorb mainly onto phosphate ions of the P-sites. The mechanisms involved in these two types of binding have been shown to be of different nature and different strength (Gorbunoff & Timasheff, 1984a). The two mechanisms are therefore detailed below.

Detailed mechanism of binding for acidic proteins (pI <7)

The binding between carboxyl groups of acidic proteins and calcium crystal ions in the C-sites of HA occurs via the formation of complexes (Gorbunoff & Timasheff, 1984a), and requires the presence of clusters of carboxyl groups, i.e., doublets or triplets of carboxyl groups (a single carboxyl is not enough for binding) on the protein surface (Gagnon, 1998; Gorbunoff, 1984; Kawasaki, 1991). The complexes between carboxyl groups and calcium

have been referred to as coordination complexes in the literature (Gagnon, 1998). However, from a chemistry point of view, the calcium atom does not have the orbital structure necessary to form a coordination complex. Strictly speaking, only transition metals such as iron, copper, and aluminium can form coordination complexes, as they have incomplete d-orbitals.

Phosphorylated proteins also bind via the formation of complexes between their phosphoryl groups and C-sites. The binding of phosphoryl groups to C -sites has been shown to be stronger than the binding of carboxyl groups to C-sites, as phosphate groups have a higher affinity for HA calcium than carboxyl groups (Benaziz, Barroug, Legrouri, Rey, & Lebugle, 2001; Bernardi et al., 1972; Gorbunoff & Timasheff, 1984a; Juriaanse et al., 1981). Only one phosphate groups is required to retain the proteins on HA and the affinity of phosphate groups for binding on HA has been reported to be 20 times greater than that of carboxyl groups (Moreno, Kresak, & Hay, 1984). For this reason, concentrated phosphate buffers are used to elute phosphorylated proteins from chromatography columns, as only free phosphate ions can displace them (Gagnon, 1998).

As well as the attractive forces between the carboxyl and phosphoserine groups of protein and the C-sites of HA, there is a repulsive force between the carboxyl and phosphoserine groups of proteins and the negative phosphate sites (P-sites) of HA.

Detailed mechanism of binding for alkaline proteins (pI >7)

The binding of basic proteins on HA is explained mostly by a classical cation exchange mechanism between the positively charged amino-acids (mostly amine groups) of the proteins and the P-sites of HA containing phosphate crystal ions. However, concurrent with the favourable electrostatic interaction between the negatively charged P-sites and the positively charged amino-acids of alkaline proteins, there is also a repulsive force between the positively charged amino-acids and C-sites.

Figure 2.15 summarises the different types of interactions involved in the adsorption or acidic and alkaline proteins on HA. The balance between attractive and repulsive interactions determines the amount of proteins that can adsorb on HA.

2.4.2.3 Factors affecting protein adsorption on HA

Many factors are known to affect protein adsorption onto HA. Table 2.5 summarises the main studies that have looked at the effect of different factors on protein adsorption. Bovine serum albumin (BSA) has been used as a model protein in most of the studies looking at the adsorption of acidic proteins on HA. Many studies have also looked at the interactions and protective effect of different salivary proteins on tooth enamel (Stayton, Drobny, Shaw, Long, & Gilbert, 2003). Lysozyme and lactoferrin have been used in most of the studies characterising the adsorption of alkaline proteins on HA. The physico-chemical conditions of the adsorption media, such as pH, ionic strength or mineral composition (calcium, phosphate and citrate ions) as well as the structural and physic-chemical properties of both proteins and HA have all been shown to affect the amount of protein that can adsorb on HA. In the section below, the effect of these different parameters on protein adsorption is detailed.

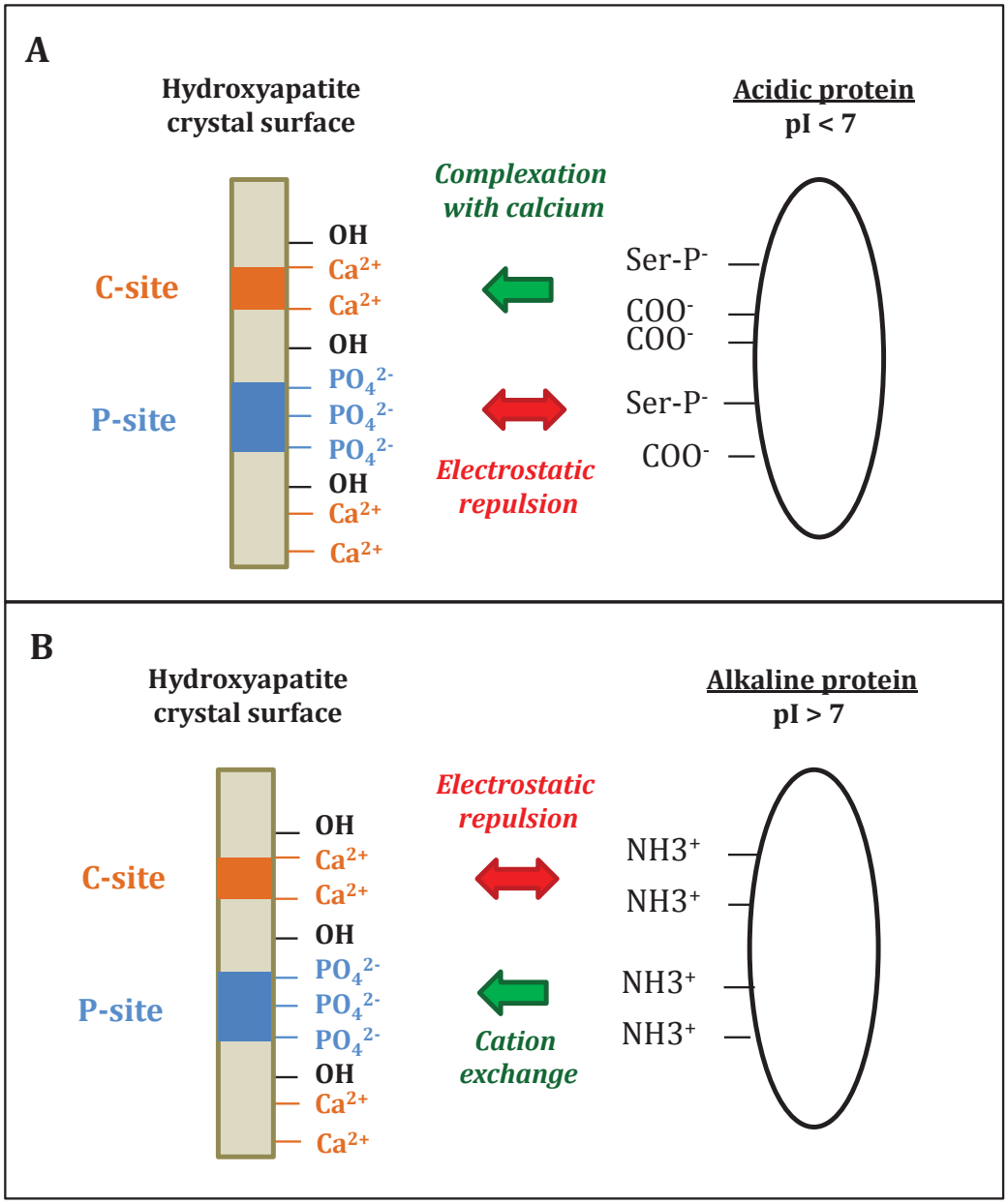

Figure 2.15: Schematic illustration of the interactions between HA and (A) acidic and (B) alkaline proteins. The formation of complexes between acidic proteins and HA require doublets or triplets of carboxyl groups, but for phosphoproteins, a single phosphate group is enough to form the complex.

Table 2.5: Summary of the main studies looking at the effect of various factors on protein adsorption on HA.

Protein	Parameter	References
Acidic proteins		
Bovine serum albumin (BSA)	Ionic strength pH	Sharpe et al. (1997); Wassell et al. (1995)
	HA properties (microstructure, porosity, size and surface area, processing methods)	He et al. (2008); Kandori et al. (2011); Wang et al. (2013); Zhu et al. (2007)
	Phosphate concentration	Kandori et al. (2008); Yin et al. (2002); Zhu et al. (2007); Mavropoulos et al. (2011)
	Ionic calcium concentration	Zhu, et al. (2007)
	Protein physico-chemical properties (pI, secondary and tertiary structures)	Brandes et al. (2006); Gorbunoff & Timasheff (1984b); Kandori et al. (2004)
Human serum albumin	Ionic strength pH	Hlady & Füredi-Milhofer (1979)
Statherin and other salivary proteins	Protein physico-chemical properties	Johnsson et al. (1993); Juriaanse et al. (1981); Lamkin et al. (1996); Stayton et al. (2003)
Alkaline proteins		
Lysozyme	Ionic strength pH Ionic calcium concentration	Luo & Andrade (1998); Barroug et al. (1989)
	Phosphate concentration	Barroug et al. (1989); Kandori et al. (2007, 2008)
	Protein physico-chemical properties	Kandori et al. (2000, 2004)
Lactoferrin	pH	Iafisco et al. (2011); Luo & Andrade (1998)
	Protein physico-chemical properties	Kandori et al. (2004)

pH

The effect of pH on protein adsorption on HA can only be studied for pH values >5, as HA dissolves at a more acidic pH (Gagnon, 1998).

The electrostatic interactions between proteins and HA are affected by both the HA surface charge and the protein net charge (Nakanishi et al., 2001; Wang et al., 2012). Any factor that has an effect on the charge of either HA or proteins will therefore have an effect on protein adsorption. As pH has an effect on both charges, it is known to affect the adsorption behaviour of proteins on HA.

For acidic proteins (pI <7), an increase in protein adsorption on HA has been reported when pH decreased (Yin et al., 2002; Zhu et al., 2007). As pH decreases, the negative charge on HA is reduced (see Section 2.4). The negative charge of the proteins is also reduced, as some negatively charged groups of the proteins are protonated Therefore, the electrostatic repulsion between the negatively charged proteins and the negatively charged groups on the HA surface (Figure 2.15) is reduced, allowing more protein to bind to the C-sites of the hydroxapatite particles.

Alkaline proteins such as lactoferrin (Iafisco et al., 2011) and lysozyme (Luo & Andrade, 1998) have been reported to bind more to HA as pH increases. As alkaline proteins bind to phosphate ions on HA through their positively charged groups, a pH increase is favourable to the binding of the proteins, since it increases the number of phosphate groups and the net negative charge of HA.

When the pH value is close to the isoelectric point (pI) of the proteins, the net charge of the protein is decreased. Less protein charged groups are therefore available for binding. However, it does not necessary mean that the amount of adsorbed protein decreases when pH values get close to the isoelectric point of the proteins. It has been shown that protein-protein interactions between the adsorbed proteins are usually favoured around pI values. Aggregates of protein can, therefore, still adsorb to the surface of HA, forming double or multi-layer of proteins on the surface of HA (Luo & Andrade, 1998).

Ionic strength

For adsorption mechanisms driven by electrostatic interactions, ionic strength is known to have an effect on the protein adsorption as increasing salt concentration neutralises charges on solid surfaces (Nakanishi et al., 2001).

For acidic proteins (pI<7), increasing ionic strength from 0 to 0.1 M has been shown to cause an increase in the amount of adsorbed proteins on HA and other calcium phosphate materials (Kandori, Miyagawa, & Ishikawa, 2004; Yin et al., 2002; Zhu et al., 2007). This increase in adsorption has been attributed to different effects of the salts on both the protein and the HA surface:

(i) As charges on HA particles are neutralised (lower magnitude of zeta-potential, see Section 2.4), the electrostatic repulsion between the negatively charged groups of the proteins and the phosphate ions on the P-sites of HA (Figure 2.15A) are reduced, therefore allowing more protein to adsorb to the C-sites.

(ii) As the electrostatic repulsions within the protein molecules are neutralised by the addition of salts, the protein conformations become more stable, therefore, proteins can adsorb in a more compact way on the surface, which allows a higher surface coverage (Kandori et al., 2004).

(iii) The lateral repulsion forces between protein molecules adsorbed on the surface are reduced, therefore more protein can access and bind to the surface (Kandori et al., 2004)

For alkaline proteins (pI >7), increasing ionic strength has been shown to reduce protein adsorption. As the alkaline protein binds to HA mostly through a cation exchange mechanism between the positively charged groups of the proteins and the phosphate groups on HA, there is competition for binding between the protein groups and the salt cations. In HA chromatography columns, sodium or potassium buffers are used to elute alkaline proteins, as sodium ions are able to desorb the positive groups of the proteins (Gagnon, 1998)

Effect of mineral composition and potential-determining ions

Phosphate ions: Potential-determining ions cause a strong change in zeta-potential by binding to either C-sites (for anions such as phosphate and citrate) or P-sites (for cations such calcium) of HA particles (see Section 2.4). Therefore, there can be a competition for binding between the acidic proteins and the ions. For example, BSA has been shown to bind less to HA in the presence of phosphate ions (PO_4^{3-}). As phosphate ions have a higher affinity than carboxyl groups for HA, they can compete with the carboxyl groups of the proteins for binding on the C-sites of HA (Mavropoulos et al., 2011; Yin et al., 2002; Zhu et al., 2007). Phosphate ions can even desorb proteins that are already adsorbed on the HA surface

(Gorbunoff & Timasheff, 1984a). Pyrophosphates ($P_2O_7^{4-}$) and tripolyphosphate ($P_3O_{10}^{5-}$) ions have also been shown to also decrease the amount of BSA adsorbed on HA because of their stronger affinity for HA than carboxyl groups.

Conversely, for alkaline protein such as lysozyme, phosphate ions increase the amount of adsorbed protein on HA (Kandori, Oda, & Tsuyama, 2008; Zhu et al., 2007). The increase in lysozyme adsorption upon addition of pyrophosphate ions was attributed to the effect of the ions on the structure of lysozyme. Pyrophosphate ions were believed to compress the electric double layer of the protein, reducing the electrostatic repulsion within the protein molecule. Therefore, lysozyme could bind in a more compact layer on HA, allowing more protein to bind. As phosphate ions increase the negative charge on the surface of HA particles, it could also create more negative binding P-sites for alkaline protein. However, this effect is not reported in the literature.

Citrate ions: There is no reported study in the literature looking at the effect of citrate ions on protein adsorption. However, as they adsorb to C-sites of HA (see Section 2.4), they must compete for adsorption with acidic proteins. As citrate ions have three carboxyl groups, they must be able to compete for adsorption with the carboxyl groups of the acidic proteins (Leach, 1960; Lee, Loo, Van, Zavgorodniy, & Rohanizadeh, 2011).

Calcium ions: Zhu et al. (2007) reported that the amount of BSA adsorbed on HA first increased upon addition of 0.01 M $CaCl_2$, as the adsorption of calcium ions on HA decreased the repulsion between the negatively charged HA and proteins, therefore enhancing the adsorption. However, when more than 0.01 M $CaCl_2$ was added, the amount of BSA adsorbed on HA decreased. It was probably due to the carboxyl groups of BSA binding calcium ions, rather than binding on the C-sites of HA (Zhu et al., 2007).

Protein characteristics

To study the adsorption behaviour of proteins on solid surfaces, it is important to know the structural properties of the proteins, such as size, charge, secondary and tertiary structure and flexibility as this will often determine which proteins bind preferentially on the surface (Wahlgren & Arnebrant, 1991; Wang et al., 2012).

As protein adsorption on HA is driven by electrostatic interactions, the number and distribution of the charged residues in the protein molecules are especially important (Nakanishi et al., 2001; Wahlgren & Arnebrant, 1991). Proteins that have domains of

clustered negatively charged groups have been reported to have much stronger affinity for HA. For example, satherin and proline-rich salivary phosphoproteins have been reported to have a very high affinity for HA, as they carry phosphoserine clusters on the N-terminal of the molecule (Johnsson et al., 1993). Proteins rich in amino-acids carrying side-chains carboxyl groups, such as glutamic acid and aspartic acids, also have a high affinity for HA. When these amino-acids are grouped in clusters, the affinity of the proteins for HA is even higher, as the presence of neighbouring carboxyl groups increase the probability of binding (cooperative adsorption) (Gorbunoff & Timasheff, 1984a). Larger proteins and unfolded proteins can also carry more binding sites, allowing more protein to adsorb on the surface (Wang et al., 2012).

The tertiary structure of the proteins can present favourable conformations for binding. If the spacing of the charged groups in the α-helix, β-sheets or β-turns structures are in phase with the spacing of the C-sites on HA, the binding between the proteins and HA is favoured. For example, the α-helix structure of osteocalcin has been shown to play an important role in the high affinity of osteocalcin for HA, as it provides a good alignment between the side chain carboxyl groups and the C-sites on HA (Goobes et al., 2007; Hauschka & Carr, 1982). When the tertiary structure of a protein is lost, for example in the case of denaturation followed by unfolding of the protein, it diminishes the ability or even prevents the protein from binding to HA, as shown by Gorbunoff and Timasheff (1984b) for lysosyme.

The adsorption behaviour of the proteins is also affected by the self-association behaviour of the proteins and by how physico-chemical parameters such as ionic strength or pH affect the protein structure. For example, proteins that can self-associate and form aggregates can adsorb in thick or multi-layers on solid surfaces (cooperative effect), whereas globular proteins, for example, usually adsorb in a close-packed monolayer (Wahlgren & Arnebrant, 1991).

HA particle characteristics

Much work has been done to produce HA nano-particles with different characteristics. HA can be processed by different chemical routes such as precipitation, hydrothermal reaction, sol-gel synthesis, and emulsification (Norton et al., 2006). Calcium or phosphate ions in the crystalline structure can be substituted by other ions such as magnesium, fluoride, or carbonate; ions can also be adsorbed to the surface of HA to modify its surface potential. The surface composition and potential will determine the type and strength of interactions with the proteins. Calcination and sintering of the powders are also steps that can be added to the

process, and modify parameters such as surface area, pore size, grain size, and chemical content (Norton et al., 2006) The surface topography can also be modified to obtain different levels of roughness, porosity, pore size and particle size (Wang et al., 2012), exposing more surface area for interacting with proteins. However, these methods are usually applied to HA nano-materials, but not to food-grade HA.

Food-grade particles are usually produced by a simple wet precipitation method, dried and micronised to micrometre sizes. The size and porosity of the powder are the two most important parameters to consider, as they have an effect on the surface area available for adsorption, therefore affecting the amount of protein that can adsorb to HA (He et al., 2008; Kandori et al., 2011; Rouahi, Champion, Gallet, Jada, & Anselme, 2006). It is generally accepted particles with a higher surface area and porosity can bind more protein (Wang et al., 2012). Usually, for the same volume fraction of particles of different sizes, smaller particles will have a larger surface area. However, the processing and grinding methods may also affect the porosity and therefore the surface area, so particles with the same average size might have a difference surface area. For example, spray-dried HA particles have a higher surface area than particles produced by a traditional drying method (Buddenheim, personal communication). The size of the particles can also limit the amount of protein that can adsorb on the particles. For example, the adsorption of large proteins on nano-size particles can be reduced, as the fraction of available C-sites and P-sites is reduced on the surface. Therefore, the available space is not enough to adsorb the large proteins, as shown for BSA by Kandori et al. (2011).

2.4.3 Milk protein binding to calcium phosphate and HA

Most of the studies reported in the literature on interactions between milk proteins and calcium have looked at milk protein binding and association with ionic calcium, in model systems such as purified milk protein solutions (Baumy & Brule, 1988a, b; Baumy, Guenot, Sinbandhit, & Brulé, 1989; Dalgleish & Parker, 1980; Holt, Parker, & Dalgleish, 1975; Mekmene & Gaucheron, 2011; Ono, Odagiri, Kaminogawa, & Yamauchi, 1976; Ono, Yahagi, & Odagiri, 1980), sodium caseinate (Gaucheron, Le Graet, Boyaval, & Piot, 1997; Zittle, DellaMonica, Rudd, & Custer, 1958), whey protein isolate (Zhu & Damodaran, 1994), and casein micelle suspensions (Le Ray et al., 1998; Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Philippe et al., 2005; Rollema & Brinkhuis, 1989) or in real milk systems (Le Graet & Gaucheron, 1999; Udabage et al., 2000). Studies using purified milk proteins usually

calculated the apparent association constants between the protein and calcium, and the number of calcium atoms fixed by protein molecules. However, calcium ions in HA are not in an ionic form, as they are involved in the HA crystal structure. Therefore, the binding of milk proteins on HA involves mechanisms different from those that are relevant to the binding of milk proteins to calcium ions, and will be influenced by different factors.

2.4.3.1 Milk protein interactions with HA

There is a lack of literature that looks specifically at the adsorption of milk proteins onto calcium phosphate or HA (except studies looking at BSA adsorption, which is a minor whey protein in milk). However, the adsorption of milk proteins on HA was demonstrated indirectly in studies looking at protein adsorption on HA chromatography, and in dentistry studies.

The studies explaining the adsorption behaviour of protein on HA chromatography columns looked at a variety of different proteins, including milk proteins. For example, β-lactoglobulin was shown to have an abnormality in its elution profile as it required a more concentrated fluoride buffer to be eluted, compared with other acidic proteins (Gorbunoff & Timasheff, 1984b). This was attributed to the presence in its primary structure of at least three clusters of side carboxyl residues located on 2 glutamic acids in positions 44-45, 2 glutamic acids and 1 aspartic acid in positions 129-131 and 2 glutamic acids in positions 157-158. β-lactoglobulin variants A and B also had different elution profiles, with β-lactoglobulin A binding stronger than β-lactoglobulin B (Gorbunoff & Timasheff, 1984b). This was attributed to a fourth cluster of carboxyl groups carried by β-lactoglobulin A in positions 62-65 (Glu-Asn-Asp-Glu). Donnelly (1977), Addeo, Chobert, and Ribadeau-Dumas (1977) and McGann, Kearney, and Donnelly (1979) studied the fractionation of the different caseins from milk on HA columns. It was shown that κ-casein could be eluted at low concentration of phosphate buffer (5 mM) but β- and α_S-caseins required higher concentration of phosphate buffer (up to 250 mM) to be eluted, due to their stronger binding through their phosphoseryl residues (Addeo et al., 1977).

Weiss and Bibby (1966) first showed that caseins reduced the solubility of tooth enamel by 20% under acidic conditions. The minor protein fraction proteose-peptone 5 of the milk was found to be the most efficient peptide fraction of the caseins at preventing demineralisation of tooth enamel under acidic conditions (Grenby, Andrews, Mistry, & Williams, 2001). As

this fraction, obtained by enzymatic action on β-casein, is made up of the charged end of the β-casein molecule, it contained all the phosphoserine residues of the molecule and was therefore very efficient at binding on tooth enamel and protecting against dissolution. A few other studies looked at the effect of casein on HA precipitation or dissolution. Van Kemenade and de Bruyn (1989) showed that HA formation was retarded in the presence of caseins, showing the inhibitory action of caseins on crystal growth and formation. The inhibitory action increased in the order κ-casein < β-casein < α_{S1}-casein. The authors related this order to a strong binding of the phosphoserine groups of α_{S1}-casein and β-casein to the growing calcium phosphate phase, but β-casein to a lower extent as α_{S1}-casein contains the most phosphate groups. Barbour et al. (2008) showed that caseins inhibited the dissolution of tooth enamel by citric acid. They attributed this effect to the strong adsorption of caseins on HA surface, stabilising the crystal surface and inhibiting the ion dissolution.

McDougall (1977) was the first to show that the presence of caseins can promote the remineralisation of tooth enamel lesions. The protective effect of caseins against lesions and caries when adsorbed onto the protein pellicle of the tooth enamel was then studied by other authors. Reynolds and Wong (1983) looked at a range of acidic and basic food proteins binding to HA and their effect on bacteria adherence. They showed that β-lactoglobulin, κ-casein, β-casein, α-lactalbumin and α_{S1}-casein all bound to HA and decreased the magnitude of the zeta-potential of HA, with zeta-potential values varying from –9.1 mV without any bound protein to –13.6, –18.5, –19.7,-21 and –24.5mV, respectively. The adsorption of all these acidic milk proteins reduced the adherence of the cariogenic bacteria cells that usually adhere on tooth enamel. Reynolds and Del Rio (1984) showed that both caseins and whey proteins had an anti-cariogenic effect when bound to rat tooth enamel, but the extent of fissures and caries reduction was a lot greater for caseins than for whey proteins, because the binding of caseins was stronger. It was also demonstrated that peptides contained in micellar casein (Schüpbach, Neeser, Golliard, Rouvet, & Guggenheim, 1996) and peptides contained in sodium caseinate (Reynolds, 1987) were incorporated in the pellicle formed by salivary proteins on tooth enamel, therefore contributing to the anticariogenic and protective effect of the pellicle. Another recent dentistry study looked at in-vitro adsorption of milk proteins on tooth enamel and studied their anti-cariogenic properties (Devold et al., 2006). They showed that at neutral pH, caseins mainly bound, forming protein pellicles, whereas at acid pH, BSA and lactoferrin preferentially bound and caseins remained unadsorbed, as pH was close to their isoelectric point.

However, none of the studies on milk proteins binding on HA was applied to food systems or looked at the effect of protein binding on the colloidal properties of HA. Only one report, a patent, has dealt with the effect of milk protein adsorption on the suspension stability of insoluble calcium salts particles (Knights & Kjelsberg, 1998); covering insoluble particles of calcium salts with milk proteins was reported to significantly improve the suspension of the particles in water.

2.4.3.2 Casein-phosphopeptide amorphous calcium-phosphate complexes

One of the only systems thoroughly described in the literature involving binding between milk proteins and calcium phosphate is the formation of complexes between caseinophosphopeptides (CPPs) and amorphous calcium phosphate (Cross, Huq, Palamara, Perich, & Reynolds, 2005; Holt, Wahlgren, & Drakenberg, 1996b; Little & Holt, 2004). The nature of the interactions between caseins and calcium phosphate was first demonstrated by Holt et al. (1996b) and Holt, Timmins, Errington, and Leaver (1998). They isolated and purified a phosphopeptide from β-casein by tryptic digestion, followed by chromatographic separation. When added to a solution of salt containing calcium and phosphate, the phosphopeptides allowed the spontaneous formation of calcium phosphate nanoclusters when the pH was increased from 5.5 to 6.7 (Holt et al., 1996b). The calcium phosphate nanoclusters are made of a core of amorphous calcium phosphate with a radius of 2.4 nm and a phosphopeptide shell of with an outer radius of 4 nm, containing about 50 peptides (Holt et al., 1998), as shown in Figure 2.16. Binding of the phosphopeptide to calcium phosphate prevents the uncontrolled precipitation of calcium phosphate. The study by Holt at al. (1998) led to the hypothesis that during the micelle formation in the mammary cells, the highly phosphorylated caseins, α_S- and β-caseins must prevent the precipitation of large calcium phosphate particles by binding to calcium phosphate and forming calcium phosphate nanoclusters (Dalgleish, 2011), in the same manner as during the formation of artificial calcium phosphate nanoclusters prepared with the β-casein CPPs. Calculations and small-angle X-ray and neutron scattering measurements have shown that about 800 nanoclusters are stabilised in a typical casein micelle with an average radius of 100 nm (Holt et al., 2003).

Four major CCPs have been isolated and purified by trypsic digestion of whole caseins. Their sequence have been identified to comprise the amino-acids 59-79 of α_{S1}-casein, 1-21 and 46-70 of α_{S2}-casein, and 1-25 of β-casein (Adamson, Riley, & Reynolds, 1993; Reynolds, Riley, &

Adamson, 1994). Cross et al. (2005) studied the structure of the complexes formed between the four main phosphopeptides and amorphous calcium phosphate using X-ray diffraction techniques, scanning electron microscopy and equilibrium binding analysis. They showed that the motif containing three contiguous phosphoserine residues and two glutamic acid (SerP-SerP-Ser-P-Glu-Glu) residues, found in α_{S1}-casein (residues 66 to 70, see Figure 2.2A), α_{S2}-casein (residues 8-12 and 56-60, see Figure 2.2B) and β-casein (residues 17-21, see Figure 2.3) was crucial to initialise binding to calcium phosphate.

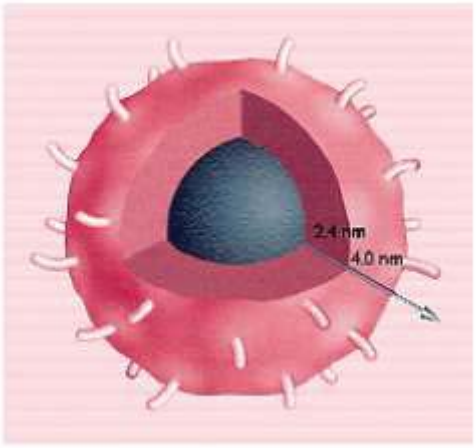

Figure 2.16: Schematic representation of a calcium phosphate nanocluster. Acore of calcium phosphate (in grey) is surrounded by a protein shell made of caseins (in pink); from Holt & Timmins (1997).

Much work has also been done on possible applications derived from the fact that selected CPPs can bind to calcium phosphate. As CPPs can solubilise minerals, they have been added to food products to enhance the bioavailability of the minerals (FitzGerald, 1998). They have also been complexed with amorphous calcium phosphate (ACP), to produce casein-phosphopeptide amorphous calcium-phosphate complexes (CPP-ACP) (Cross et al., 2005; Reynolds, 1998). CPP-ACP has been shown to bind to tooth enamel, providing anticariogenic and remineralisation effects for the enamel (Reynolds, 1997, 1998) and has been used in a lot of dentistry applications such as toothpaste or chewing-gums (Cross, Huq, & Reynolds, 2007).

2.5 Methods and models to measure and characterise protein adsorption on HA

2.5.1 Milk protein ingredients

Many studies have reported the adsorption of caseins and whey proteins onto hydrophilic and hydrophobic surfaces and have used sodium caseinate (for caseins) and whey protein isolate (for whey proteins) as convenient model systems to study their adsorption (Dickinson, 1999; Hunt & Dalgleish, 1994a; Noisuwan, Hemar, Wilkinson, & Bronlund, 2011; Sugiarto, Ye, & Singh, 2009).

Sodium caseinate (SC) is a spray-dried ingredient produced from skim milk, by precipitating the caseins at pH 4.6 and resolubilising them with sodium hydroxide (Kinsella & Morr, 1984; Wong et al., 1996). SC is water soluble and forms a translucent solution when redispersed in water. The proportions of the different caseins in SC is similar to those in skim milk, with α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein present in the ratio 3:1:3:1 (Wong et al., 1996). However, the caseins in SC are not in the native casein micelle form; they are soluble. Compared with milk, SC typically contains hardly any calcium (0.1%, w/w, on a powder basis) but a high amount of sodium ions (1.3%, w/w, on a powder basis) (Morr, 1982). Sodium caseinate is widely used in research studies to look at the adsorption behaviour of the different casein molecules onto hydrophobic and hydrophilic surfaces and is therefore used in this thesis as a model system to study the adsorption behaviour of caseins onto HA. Caseins in SC are not present as individual molecules; they can re-associate randomly to form self-aggregates. The composition of the SC aggregates with respect to the different caseins is not fully understood (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Lucey, Srinivasan, Singh, & Munro, 2000). However, the aggregation behaviour of the different caseins in sodium caseinate is very likely to follow the same mechanisms as the self-association of each of the caseins, described previously in this review (Section 2.2.2.1; Horne, 2009). Therefore, the interpretation of the adsorption results obtained with SC in this thesis will be mostly discussed based on the characteristics and self-association properties of the individual caseins.

Whey protein isolates (WPIs) are soluble spray-dried powders obtained by ion-exchange, containing a very high concentration of whey proteins (usually about 90% protein) (Wong et al., 1996). Compared with milk, WPI does not contain any lactose and has a very low salt

concentration, due to the manufacturing processes. However, the whey proteins in WPI are not denatured, and their properties and functionalities are largely unchanged (Wong et al., 1996). Therefore, WPI will be used in this thesis as a model system to study the behaviour of whey proteins onto HA.

2.5.2 Measurement methods

There are many different ways to measure the amount of proteins adsorbed on solid surfaces. Usually the amount of protein adsorbed onto solid surfaces is only about a few milligrams per unit square meter, so accurate methods need to be used to determine the adsorbed amounts of protein with precision (Nakanishi et al., 2001). The most common method to determine the amount of protein adsorbed on a solid surface is the depletion method (Nakanishi et al., 2001). The amount of adsorbed proteins is calculated by the difference between the initial protein concentration contained in a protein solution before adsorption and the amount of unadsorbed protein left in the solution after the solid has been in contact with the protein for a certain amount of time (until equilibrium has reached), and has been separated. This method is usually accurate for small solid particles that have a high surface area and that can be easily separated from the liquid solution, for example by low-speed centrifugation.

More complicated methods need to be used if information on the conformation or the thickness of the protein layer on the surface is required, for example ellipsometry, Fourier transform infrared spectroscopy, fluorescence spectroscopy or atomic force microscopy (Nakanishi et al., 2001).

2.5.3 Adsorption modelling

Many studies have used Langmuir, Langmuir-Freundlich or Freundlich models to characterise the adsorption characteristics of protein binding on HA (Iafisco et al., 2010, 2011; Lee et al., 2011; Mavropoulos et al., 2011; Mohsen-Nia, Bidgoli, Behrashi, & Nia, 2012; Wassell et al., 1995). The different models give physically interpretable parameters that can be used to compare the strength and levels of adsorption between different proteins, and obtain information on the structure of the adsorbed protein layer (Iafisco et al., 2011).

2.5.3.1 Langmuir model

The Langmuir model is the most common model used to describe protein adsorption processes on solid surfaces (Mura-Galelli, Voegel, Behr, Bres, & Schaaf, 1991). It quantifies the amount of proteins adsorbed onto a surface (in this case, HA) as a function of the unadsorbed protein concentration once equilibrium has been reached at a given temperature. This model assumes that the proteins bind to a series of distinct, empty sites on the surface to form a complex. The surface is energetically homogeneous with the same adsorption energy for all adsorption sites. This model only allows monolayer coverage and interactions between proteins at the surface are not allowed. The Langmuir model is given by the equation (1), as follows:

$$\frac{m_{abs}}{S} = q_m \times \frac{K[P]}{1 + K[P]} \quad (1)$$

where m_{abs}/S is the amount of protein bound to the surface expressed in mg of protein/m^2 of surface, [P] the protein concentration at equilibrium (g/100 g), q_m the maximum monolayer surface coverage and K is the Langmuir affinity constant (100g/g) (Iafisco et al., 2011).

The affinity constant of the Langmuir model characterises the initial slope of the adsorption isotherms and indicates a high protein affinity for HA (Iafisco et al., 2011).

Wassell et al. (1995) characterised the adsorption of bovine serum albumin (BSA) on HA using the Langmuir model. They showed that both K and q_m changed with pH and ionic strength, and they related these changes to the effect of salt and pH on the adsorption. Johnsson et al. (1993) compared different salivary proteins for their adsorption onto HA, using the maximum surface coverage and the affinity constants given by the Langmuir model. The Langmuir model can be useful to compare the adsorption characteristics of different proteins onto HA. However, the application of the Langmuir model to protein adsorption of HA is limited, as some of the assumptions of the model do not apply to the characteristics of protein adsorption onto HA. The Langmuir model assumes a reversible adsorption process, whereas the adsorption of proteins onto HA tends to be an irreversible process and does not necessarily reach an equilibrium state even after a few hours of adsorption (Mura-Galelli et al., 1991). The Langmuir model also assumes a homogeneously charged surface and no interaction between the adsorbed protein but the surface of HA is heterogeneously charged and cooperative adsorption lateral interactions are possible

between the adsorbed proteins (Luo & Andrade, 1998). It is therefore important to understand the limits of the Langmuir model and use other models or methods to characterise protein adsorption on HA.

2.5.3.2 Langmuir-Freundlich model

Langmuir-Freundlich also assumes a single-layer coverage, but takes into account that the adsorption energy is not equal for all sites, for example in the case of a heterogeneous surface or if some interactions between proteins at the surface occur. The Langmuir-Freundlich expression is given by expression (2).

$$\frac{m_{abs}}{S} = q_m \times \frac{(K_{LF}[P])^n}{1+(K_{LF}[P])^n} \quad (2)$$

K_{LF} is the Langmuir-Freundlich affinity constant ($(100g/g)^{1/n}$) and n is a surface heterogeneity parameter. For n = 1, the Langmuir-Freundlich equation (2) is similar to the Langmuir equation (1). As HA carries two types of adsorption sites, C-sites and P-sites, the surface of HA is often considered energetically heterogeneous, (Mohsen-Nia et al., 2012). When n deviates from 1, it means that either the surface heterogeneity has an effect on the protein adsorption (n <1), or that there are cooperative interactions between the proteins on the surface of the particles (n <1 negative cooperativity, or n >1, positive cooperativity between the proteins), so the probability of adsorption on different sites is not equal.

Iafisco et al. (2011) showed that the Langmuir-Freundlich model fitted the adsorption data of lactoferrin onto HA better than the Langmuir model. They attributed it to the heterogeneous distribution of the adsorption sites on the HA surface. The good fit of the Langmuir-Freundlich model for BSA adsorption on HA (Mavropoulos et al., 2011) with a parameter n >1 indicated that there must have been strong protein-protein interactions between the BSA molecules on the HA surface, enhancing the amount of BSA molecules that can adsorb on the surface.

2.5.3.3 Freundlich model

The Freundlich isotherm model does not limit adsorption to a monolayer and is often applicable to multiple-layer adsorptions. It follows the equation given in equation (3).

$$\frac{m_{abs}}{S} = K_F \times [P]^{1/N} \quad (3)$$

K_F is the Freundlich affinity constant ($(100g/g)^N$), while n is the surface heterogeneity parameter, similar to n in the Langmuir-Freundlich model. When the parameter 1/N is close to 1, it means the surface is energetically homogeneous.

Lee et al. (2011) showed that the Freundlich model was a better fit for the adsorption data of BSA and lysozyme on amino acid-functionalised HA better than did the Langmuir model. It was hypothesised to be due to the proteins aggregating and forming multiple layers on the surface of HA. The Freundlich model also best fitted the BSA adsorption on HA at high initial protein concentration in the study carried out by Mavropoulos et al. (2011), and this was attributed to the fact that large aggregates of BSA formed on the surface once the protein surface coverage had exceeded a first saturation value. Protein adsorbing in multiple layers often causes heterogeneity in surface energy, as different types of interactions can occur concurrently at the surface, for example protein-surface interactions and protein-protein interactions.

Usually, lateral interactions (cooperative effects that can be positive or negative) and conformational changes of the adsorbed proteins on HA surface, or adsorption in multiple layers explain the better fits of the Langmuir-Freundlich and Freundlich models compared with the Langmuir model for protein adsorption on HA (Mavropoulos et al., 2011; Mohsen-Nia et al., 2012)

2.6 Conclusion and positioning of the study

HA and other insoluble calcium salts that are added to calcium-fortified milks are often described as inert, as they do not cause any protein aggregation and heat instability during heat treatment of the milk. However, it is well-known that protein can interact with HA. The adsorption of milk proteins on HA has been demonstrated in different studies looking at

chromatography, bioceramic and dentistry applications. However, most of the research has been carried out on model systems and no one has looked at the interactions between protein and HA particles in food systems, and the consequences of these interactions on the colloidal stability of the particles.

Therefore, the aim of this thesis is to examine whether or not milk proteins from model protein solutions and from skim milk adsorb onto the surface of HA particles. The adsorption processes will be characterised and modelled and the adsorption mechanisms will be discussed. The effect on functional properties of HA will also be considered. If milk proteins bind to HA particles and change their surface properties, their colloidal properties are likely to be modified as well, potentially affecting their suspension stability in aqueous solution.

Characterising the surface interactions between HA and milk proteins will not only allow a better understanding of the colloidal system formed by insoluble calcium salts suspended in milk beverages, but could also lead to insights for new product development and new ways of improving the suspension stability of the particles over shelf-life, possibly without the use of hydrocolloids.

CHAPTER 3 Material and methods

3.1 Materials

3.1.1 Sources

3.1.1.1 Protein powders

Milk protein blends

Sodium caseinate (SC; Alanate 180), whey protein isolate (WPI; Alacen 895), and low-heat skim milk powder (SMP) were obtained from Fonterra Co-operative Group Limited, New Zealand. Fresh skim milk (SM) was obtained from a local supermarket. Whey protein-depleted skim milk powder (WPD-SMP) was prepared by microfiltration of fresh SM and spray-drying on a bench scale; the detailed method for WPD-SMP preparation is given in Section 3.7.1.

Isolated proteins

Freeze-dried powders of α_S-casein (#C6780, comprising a mix of α_{S1}- and α_{S2}-caseins), β-casein (#C6905), κ-casein (#C0406), β-lactoglobulin (L3908), and α-lactalbumin (L7269) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

3.1.1.2 Chemicals

Food-grade hydroxyapatite (HA) powder was purchased from Budenheim (TCP 53-83, Budenheim, Germany). All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich unless specified otherwise. Milli-Q water was used for all experiments.

3.1.2 Characterisation

3.1.2.1 Protein powders

Total protein content of milk protein blends

The total protein content of the milk protein blends (SC, WPI, SMP, WPD-SMP) was determined by the Kjeldahl method (see Section 3.5.2). The proportions of each type of protein contained in the blends of milk proteins were estimated based on the theoretical proportions of the individual proteins in skim milk (Wong et al., 1996). The total protein content and estimated percentages of each protein in the powders are given in Table 3.1.

Table 3.1: Protein composition of the blends of milk proteins

Protein powder [a]	Total protein content (g/100 g powder)	Percentage (%) by type [b]				
		α_S-CN	β-CN	κ-CN	β-Lg	α-La
SC	90	50	38	12	-	-
WPI	93	-	-	-	80	20
WPD-SMP	31	50	38	12		
SMP	33	40	30	10	12	3

[a] Abbreviations are: SC, sodium caseinate; WPI, whey protein isolate; WPD-SMP, whey protein-depleted skim milk powder; SMP, skim milk powder; CN, casein; Lg, lactoglobulin; La, lactalbumin. α_S-CN is a blend of α_{S1}- and α_{S2}-caseins.
[b] Percentages estimated from theoretical proportions of the different milk proteins in skim milk, from Wong et al. (1996).

Total protein content of isolated proteins

The purity of the isolated proteins powders (α_S-casein, β-casein, κ-casein, β-lactoglobulin, α-lactalbumin) were determined by UV spectroscopy at 280 nm (see Section 3.5.1). The purity was 80% for α_S-casein (α_{S1} + α_{S2}), 98% for β-casein, 68% for κ-casein, 85% for β-lactoglobulin, and 89% for α-lactalbumin.

Estimated ionic strength of SC and WPI

Ionic strength standard solutions were prepared by adding NaCl to water in the concentration range 1 to 10 mM. One millilitre of the standard solutions was added to disposable DTS 1060C zeta-potential cells (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The electrical conductivity of the standard solutions (in mS/cm) was

measured using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd) and plotted against the NaCl concentration to obtain a linear regression. The linear equation obtained for the regression was then used to convert the measured conductivity of 1% (w/w) SC and WPI solutions to equivalent ionic strength. The 1% (w/w) SC and WPI solutions had estimated ionic strengths of, respectively, 4.5 mM and 1 mM.

3.1.2.2 HA powder

Particle size and specific surface area

The particle size analysis of the HA powder was carried out using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) (see section 3.3.1). The median particle size $d_{(0.5)}$ was found to be 4.5 μm. The reported specific surface area of the powder, provided by the ingredient supplier and determined by the Brunauer–Emmett–Teller (BET) method, was 65 m^2/g.

Fourier-transform infra-red spectrometry

The crystal phase of the HA powder was characterised by Fourier transform infrared spectrometry (FTIR) using a Perkin Elmer Spotlight 200 spectrometer (Perkin Elmer-Cetus, Norwalk, CT, USA). The material was pressed into a potassium bromide (KBr) pellet and 25 scans were acquired between 4000 and 400 cm^{-1} at 4 cm^{-1} to obtain the spectrum.

The HA spectrum of the HA sample used in this thesis (TCP 53-83, see Section 3.1.1.2) is given in Figure 3.1A. The FTIR spectrum had sharp peaks at 630.7 cm^{-1} and 3572 cm^{-1}, belonging to the stretching vibrations of the OH^- ions, and considered to be characteristic peaks of crystallised HA (Kandori et al., 2011). The peaks present at 564.7, 602.8 and 472.1 cm^{-1} and the peaks at 962.6, 1033 and 1092.6 cm^{-1} belong, respectively, to the bending and stretching modes of the PO_4 groups in HA (Kandori et al., 2011). The peaks at 3436 cm^{-1} and 1640.3 cm^{-1} come from the OH bending and stretching modes of water and might be explained by some residual water in the HA powder (Kandori et al., 2011). The peaks at 1496.9, 1453.1, 1416.9 cm^{-1} are peaks belonging to the asymmetric CO stretching modes of carbonate ions, showing that carbonate ions must be present in the HA lattice of the sample or adsorbed on the HA surface (Rehman & Bonfield, 1997; Ren, Ding, & Leng, 2014). HA samples containing carbonate ions often come from HA powders that have been prepared by wet precipitation in aqueous solution, in the presence of CO_2 (Rehman & Bonfield, 1997).

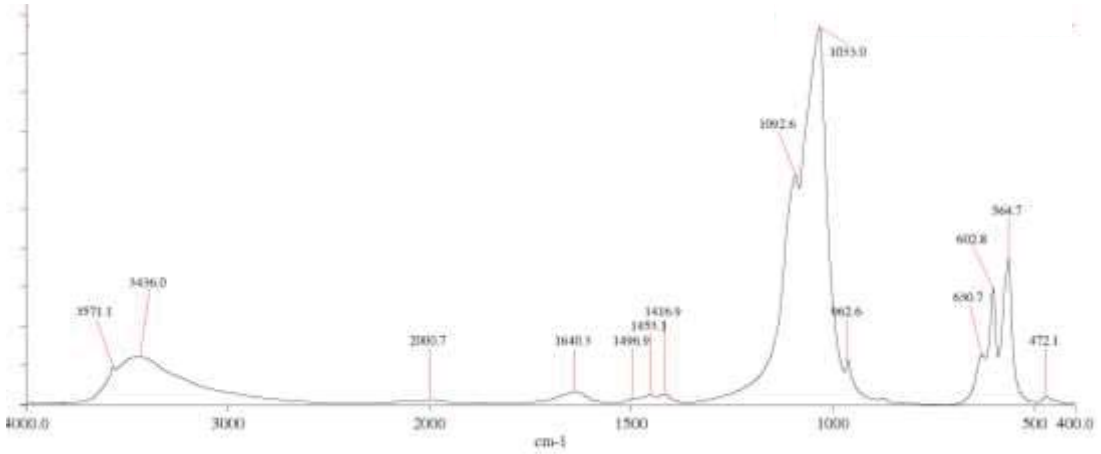

Figure 3.1: FTIR spectrum for HA powder used in this thesis (TCP-53-83).

3.2 Adsorption experiments

3.2.1 Preparation of stock protein solutions

In this study, the adsorption of a range of different proteins onto HA particles was studied under different physico-chemical conditions. The studied proteins were either blends of milk proteins or isolated proteins, and are all listed in the material sources (Section 3.1). Stock protein solutions were first prepared by reconstituting the protein powders in the suspending solutions (water or buffers) that were of interest for the experiment, and left overnight to equilibrate. The choice of suspending solutions was determined by the experiments and the type of proteins studied. Figure 3.1 summarises by chapter the proteins that were studied, the total solid concentrations of the prepared stock solutions (i.e., the weight of powder per 100 g of suspending solution), the suspending solutions that was used and the reason of choice for the protein sources and the suspending solutions. Section 3.2.2 gives details on the preparation of the suspending solutions.

Table 3.2: Summary of the adsorption experiments carried out in this thesis (protein sources, concentration and suspending solution used for the preparation of the stock solutions). [a]

Chapter	Protein source	TS of stock solution (%, w/w)	Suspending solution	Reason
4	SC WPI	6 6	Milli-Q water	Studying the adsorption of the two main types of milk proteins (caseins and whey proteins) in a simple media, with no additional effect of buffers or other ions
5	α_S-CN β-CN κ-CN β-Lg α-La	5	HEPES buffers, pH 6.8, at low ionic strength and 0.1 M ionic strength	Studying the adsorption of the individual milk proteins under two different conditions of ionic strength, at ~ milk pH
6	SC WPI	6 6	Milli-Q water, NaCl solutions (0-0.5 M) with pH adjustment	Studying the effect of pH and ionic strength on the adsorption behaviour of caseins and whey proteins
			SMUF, with and without added citrate, pH 6.7	Studying the effect of milk mineral composition on the adsorption behaviour of caseins and whey proteins
7	SMP WPD-SMP	10 10	Stock solutions prepared in water, then successive dilutions in SMUF	Studying the adsorption of casein micelles in their natural physico-chemical environment (milk serum), with the same milk mineral composition regardless of the protein concentration

[a] Abbreviations are: SC, sodium caseinate; WPI, whey protein isolate; α_S-CN, α_S-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin; SMP, skim milk powder; WPD-SMP, whey protein depleted skim milk powder; TS, total solids; SMUF, simulated milk ultrafiltrate.

3.2.2 Preparation of the suspending solutions

3.2.2.1 NaCl solutions

NaCl solutions of concentrations ranging from 0 to 0.5 M were prepared by diluting the appropriate amount of NaCl powder in Milli-Q water.

3.2.2.2 Simulated milk ultrafiltrate

Simulated milk ultrafiltrate (SMUF) was prepared according to Jenness & Koops (1962) with slight modifications. SMUF mimics the composition of the milk serum. The salts were weighed and added one by one into 1 L of water until completely diluted according to the order and amounts shown in Table 3.3. After the dilution of the last salt (KCl), the solution was stirred for 30 min. The pH was then adjusted to 6.7 using 1 M KOH. SMUF was stored in the fridge for up to one week, until needed.

Table 3.3: SMUF recipe. [a]

Salt	Molecular weight (g/mol)	Weight (g/kg of water)	Concentration (mM)
KH_2PO_4	136.09	1.58	11.6
$K_3Citrate.H_2O$	324.42	0.5	1.5
$Na_3Citrate.2H_2O$	294.16	1.794	6.1
K_2SO_4	174.87	0.18	1.0
$CaCl_2.2H_2O$	147.02	1.32	9.0
$Mg_3Citrate_2.14H_2O$	702	0.7722	1.1
K_2CO_3	138.21	0.3	2.2
KCl	74.56	1.081	14.5

[a] The salts were added to 1 L of water in the order indicated in the table.

SMUF with added citrate was prepared by modifying the recipe shown in Table 3.3. Whereas 1.794 g of sodium citrate was added in 1 L of water in the normal SMUF recipe, for SMUF

with added citrate, an extra 1.44 g, 2.88 g, 5.77 g or 11.5 g sodium citrate were added to obtain 1.5, 2, 3 or 5 times more citrate, respectively, than in the original SMUF.

3.2.2.3 HEPES buffers

Two 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffers (pH 6.8, two different ionic strengths) were prepared. A low ionic strength buffer (~7.10^{-3} M) was prepared by mixing 11.92 g of HEPES powder and 7.5 mL of 1 M NaOH in ~900 mL of water. A 0.1 M ionic strength buffer was prepared by mixing 11.92 g of HEPES powder, 7.5 mL of 1 M NaOH and 92.5 mL of 1 M NaCl in ~800 mL of water. The pH was adjusted to pH 6.8 if needed using 1 M NaOH and the solutions were made up to 1 L.

3.2.3 Preparation of suspensions of HA particles in protein solutions

Two main methods were used to prepare suspensions of HA particles in protein solutions with a range of different protein to HA ratios.

3.2.3.1 Constant amount of HA, variable protein concentration

A range of protein solutions at different concentrations were prepared. For the isolated protein solutions, SC solutions and WPI solutions, appropriate volumes of the protein stock solutions were diluted in the same suspending media as used for the stock solutions preparation (see Table 3.2). For the WPD-SM and SM solutions, diluted WPD-SM and SM solutions at different protein concentrations (0.15–2.8% (w/w) total protein) were prepared by diluting appropriate volumes of the WPD-SM and SM stock solutions (10% w/w, total solids in water, corresponding to 3.3% w/w, total proteins) in SMUF. SMUF was used to prepare the successive dilutions of the WPD-SM and SM solutions so that the casein micelles were suspended in a solution of similar pH, ionic strength, and mineral composition, for all samples. A constant amount of HA powder (0.05 g or 0.1 g) was added to aliquots of the protein solutions (0.95 g or 0.9 g) of different concentrations.

3.2.3.2 Constant concentration of protein, variable amount of HA

A constant amount of protein solution (100 µL) at a given concentration was added to 0.9 mL of HA suspensions at various concentrations (0-4%, w/w, total solids). The suspensions were stirred for 2 h at room temperature (approximately 20 °C). They were then centrifuged at 3000 × *g* for 2 min using a bench centrifuge (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany) to separate the HA pellet from the protein solutions. The supernatants were carefully poured from the pellet, and both supernatants and pellets were kept aside for analysis.

3.2.4 Determination of surface concentration and composition

The amount of protein adsorbed onto the surface of the HA particles was determined by measuring the protein concentration of the supernatants containing the unadsorbed proteins, and subtracting it from the initial protein concentration (depletion method), as detailed below.

3.2.4.1 Determination of protein concentration and composition of the supernatants

The protein concentration of the supernatants was determined using one of the four following methods:

(i) Ultra-violet (UV) spectroscopy at 280 nm using known extinction coefficients (see Section 3.5.1) for the adsorption experiments carried out with isolated protein solutions (Chapter 6).

(ii) Kjeldahl method (see Section 3.5.2) for the first adsorption experiments carried out with SC and WPI solutions.

(iii) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; see Section 3.5.3) for adsorption experiments carried out with SC, WPI, SM and WPD-SM (Chapters 4, 6, 7).

(iv) Micro fluidic chip electrophoresis (MF-electrophoresis; see Section 3.5.4) for adsorption experiments carried out with blends of isolated proteins (Chapter 5) and SM and WPD-SM (Chapter 7)

The three different methods (Kjeldahl, SDS-PAGE and MF-electrophoresis) used for protein quantifications of the SC, WPI and milk solutions all had advantages and disadvantages.
The Kjeldahl method is the official method of analysis for determination of protein concentration (AOAC, 1984). It provides a direct measurement of the total protein concentration of the sample, from the measurement of the total nitrogen (TN) content. A correction factor of 6.38 is applied to the TN results to convert it to the protein content. However, the conversion factor of 6.38, generally accepted and widely used in the literature to convert TN to total protein content for any type of milk protein, is an average factor. In reality, the conversion factor depends on the amino-acid sequence of the proteins and is not therefore constant for the different milk proteins. The correction factor has been shown to vary between 6.15 for κ-casein and 6.34 for β-casein and α_S-casein, and between 6.14 for α-lactalbumin and 6.38 for β-lactoglobulin (Karman & Van Boekel, 1986). Applying an average correction factor of 6.38 to convert TN to total protein on samples that vary in protein composition could induce errors in the determination of the amount of adsorbed protein, if the different proteins contained in a sample do not adsorb at the same rate. The main disadvantage of the Kjeldahl method is that it is a time-consuming method, which requires a large amount of sample and does not give any information on the composition of the different types of milk proteins contained in the supernatants, i.e., how much of each type of protein was unadsorbed: α_S-casein (α_{S1} + α_{S2}), β-casein and κ-casein for SC adsorption; β-lactoglobulin and α-lactalbumin for WPI adsorption; α_S-casein (α_{S1} + α_{S2}), β-casein, κ-casein, β-lactoglobulin and α-lactalbumin for SMP and WPD-SMP experiments.

The two electrophoresis methods (traditional SDS-PAGE and MF-electrophoresis) provided only a relative measurement of the protein concentration in the supernatant compared with a control. The measured data are the percentage of the initial protein remaining in the supernatants after adsorption. Therefore, an absolute value of protein concentration needed to be attributed to the controls loaded on the gels. In this study, the total protein content of the controls determined by Kjeldahl was chosen to be attributed to the integrated gel area of the sum of the three protein bands (α_S- + β- + κ-casein). The advantage of electrophoresis methods is that they provided extra data on the composition of the different types of milk proteins contained in the supernatants. Therefore, electrophoresis data gave extra information that was necessary to calculate the surface composition of the adsorbed protein layer, i.e., how much of each type of protein was adsorbed.

The percentage of protein remaining in the supernatants was measured by both Kjeldahl and SDS-PAGE in preliminary experiments looking at the adsorption of SC and WPI in water.

As there was a good correlation between the results obtained for the two methods, it was decided to only use SDS-PAGE results for all the adsorption results of SC and WPI reported in this thesis.

3.2.4.2 Calculation of surface protein concentration

The surface protein concentration of protein onto HA (in mg/m^2) was calculated from the difference between the amount of protein in the initial protein solutions, and that measured in the supernatant, divided by the surface area of the HA particles, using the following formula:

$[Mi \times (Ci / 100 - Csup / 100)] / [(M_{HA} \times SA_{HA}) \times 1000]$

where Mi is the mass of the initial protein solution, Ci is the protein concentration of the initial protein solution (g/100 g), calculated using the known protein content of the protein powder (see Section 3.1.2.1), Csup is the protein concentration of the supernatant (g/100 g) determined experimentally by one of the four methods mentioned above, M_{HA} is the initial mass of HA powder and SA_{HA} is the specific surface area per gram of HA (65 m^2/g).

The amount of adsorbed individual proteins for the milk protein blends (SC, WPI, WPD-SM and SM) was calculated from the percentage of each protein left in the supernatant, determined by SDS-PAGE and from an estimated composition of the initial SC, WPI, WDP-SM and SM solutions, based on the theoretical proportions of the individual proteins in skim milk and given in Table 3.1.

3.3 Characterisation of HA particles

3.3.1 Particle size

Particle size analysis of the HA powders was carried out by laser diffraction using a Malvern Mastersizer 2000 (Malvern Instruments Ltd.). The principle of the technique is based on the fact that diluted particles passing through a laser beam scatter the light at an angle that is proportional to their size. When a diluted suspension of particles is introduced in the dispersion unit of a Mastersizer, the laser beam light is diffracted and a series of receptors

collect the diffracted light at different scattering angles. The volume size distribution is then calculated from the intensities of the light collected at each angle, using the Lorenz-Mie theory. The calculation takes into account the refractive index and the absorbance of the particles and the refractive index of the dispersing media. To measure the particle size distribution of the HA particles, a suspension of HA particles (~1%, w/w) was first prepared in water and introduced drop by drop into the wet dispersion unit of the Mastersizer until the desired obscuration was achieved. The particle size distribution was calculated based on a refractive index of 1.63 for HA, 1.33 for water and an absorption factor of 0.001.

3.3.2 Zeta-potential

A Malvern Zetasizer Nano-ZS instrument and disposable DTS 1060C zeta-potential cells (Malvern Instruments Ltd) were used for determining the zeta-potential of the HA particles in suspension. The HA particles were suspended at a concentration of 0.05% (w/w) in the appropriate suspending solution. As the physico-chemical characteristics of the solution such as pH, ionic strength or mineral composition have a major effect on zeta-potential measurements, the HA particles were usually suspended in the same solution as used for the adsorption experiments. The samples were transferred to the measuring cell and the cell was placed in the instrument. The temperature of the cell was maintained at 20 ± 0.2 °C. An applied voltage of 50 V was used in all experiments. The zeta-potential was calculated from the measured electrophoretic mobility using the Smoluchowski model. An average of four measurements was used.

3.3.3 Microscopy

Different techniques of microscopy were used to characterise both the bare HA particles and the protein-coated HA particles. They are detailed below.

3.3.3.1 Confocal microscopy

A Leica (TCS 4D, Leica Lasertechnik, Heidelberg, Germany) confocal laser scanning microscope with a 100× oil immersion objective lens and an Ar/Kr laser with an excitation line of 488 nm was used to observe the HA particles. HA pellets obtained after

centrifugation were re-suspended in Milli-Q water to a 0.05% (w/w) concentration. Approximately 3 mL of this suspension was placed in a test tube, 0.5 mL of Fast Green (fluorescent dye for protein) was added to the mixture and the mixture was stirred. A sample was then placed on a microscope slide. The slide was covered with a coverslip and observed under the microscope. The images were acquired using two different channels of the microscope. The Fast Green fluorescence channel was used to visualise only the stained protein material. The differential interference contrast (DIC) channel was used to visualise the unstained calcium phosphate particles.

3.3.3.2 Transmission electron microscopy

HA particles were prepared for transmission electron microscopy (TEM) observation, using a method similar to that of McKenna, Lloyd, Munro, and Singh (1999). Suspensions of HA particles were mixed with warm 3% low-temperature gelling agarose at a ratio 1:1. This solution was poured onto a microscope slide, was allowed to set, and was chopped into 1 mm^3 cubes. Agarose embedded cubes were put into bijoux bottles containing 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 and left at 5 °C for 24 h. The glutaraldehyde was rinsed twice with 0.2 M sodium cacodylate buffer pH 7.2 over 2 h. The agarose embedded samples were then placed in 1% osmium tetroxide overnight at 5 °C. The samples were placed in 1% uranyl acetate for 30 min and then rinsed twice with distilled water before dehydration. The dehydration process consisted in successive rinses of the samples carried out at 5 °C in 25% acetone for 15 min, then 50%, 70% and 90% acetone for 30 min each, followed by 100% acetone (3 changes over 90 min). The acetone was then replaced by embedding resin (Procure 812) and the bottles were placed on a rotator for 36 h. A cube or a tube of the samples was then placed into an embedding capsule containing resin and baked at 60 °C for 48 h. The embedded samples were then sectioned to a thickness of 90 nm using the Reichert Ultracut microtome. These sections were mounted on 3 mm copper grids and stained with lead citrate before examination in a Philips transmission electron microscope (Philips, 201C, The Netherlands) at an accelerating voltage of 60 kV.

3.3.3.3 Light microscopy

Selected sections prepared for TEM microscopy were also observed under light microscopy, using an Olympus BX 60 light microscope (Olympus Optical Co Ltd, Tokyo, Japan) and Toluidine blue to specifically stain protein material.

3.3.4 Suspension stability

HA particles were suspended in Milli-Q water or in the relevant suspending solution (same solution as for the adsorption experiment, i.e. HEPES buffer or NaCl solutions or SMUF), to a 0.125% w/w concentration and absorbance measurements were performed using a Jasco V-560 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan) and 10 mm plastic cuvettes. Absorbance values at a wavelength of 900 nm were recorded over 200 min, as they indicated the change in the turbidity of the samples and were related to the suspension stability of the particles.

3.4 Characterisation of WPD-SM and SMP solutions

WPD-SM and SM contained casein micelles. Specific methods were used to characterise the casein micelles in these solutions. They are listed below.

3.4.1 Casein micelle size

One hundred microlitres of WPD-SM and SM solutions were diluted in approximately 5 mL of SMUF for casein micelle size measurements. Casein micelle size was measured by photon correlation spectroscopy using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd.); the temperature of the cell was maintained at 20 °C for the duration of the experiments. Milks (25 µL) were diluted with buffer (5mL of buffer containing 20 mM imidazole, 30 mM NaCl and 5 mM $CaCl_2.2H_2O$ at pH 6.7) that was first filtered through a 0.45-µm syringe filter to remove any dust particle, and 1 mL was transferred to a plastic cuvettes and placed in the instrument. Measurements of the dynamics of the scattered light were collected at a scattering angle of 173°, with automated attenuator settings. Average diffusion coefficients were determined by the method of cumulants and were translated into average particle diameters using the Stokes–Einstein relationship for spheres.

3.4.2 Non-micellar casein content

Non-micellar caseins were defined as those that did not sediment from the adsorption supernatants during centrifugation at 21,000 × g and 25 °C for 60 min using a bench centrifuge. The serum containing the non-micellar caseins was carefully removed from the pellet (containing the casein micelle) and the protein content was determined by MF-electrophoresis (see Section 3.5.4), and expressed as a percentage of the protein content in the adsorption supernatants.

3.4.3 Transmission electron microscopy

The WPD-SM and SM solutions were pipetted into agarose tubes, and the ends of the tubes were sealed with agarose. The agarose tubes were treated in the same way as the agarose embedded cubes used for TEM microscopy of HA particles (see method described in Section 3.3.3.).

3.5 Protein quantification methods

3.5.1 Absorbance at 280 nm

When only one type of protein is present in solution, the protein concentration of the solution can be determined by UV-spectrometry, using the protein extinction coefficient for calculation. The absorbance of the isolated protein solution was measured at 280 nm in a 1 cm quart cuvette, using a Jasco V580 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan). The protein solutions were diluted prior to the measurement in 1 mL of water or other suspending solutions, to obtain an absorbance value at 280 nm comprised between ~0.2 and 1.5. The absorbance of the solution was also measured at 320 nm (A_{320}), to correct for dust absorbance. The protein concentration was calculated as follows:

$$(A_{280} - A_{320} \times 1.7) / (EC \times 10 \times DF)$$

where A_{280} is the absorbance measured at 280 nm, A_{320} is the absorbance measured at 320 nm, EC is the extinction coefficient of the protein (cm^2/g), DF is the dilution factor.

The extinction coefficients were 0.46 cm^2/g for β-casein, 1.05 cm^2/g for α_S–casein, 1.02 cm^2/g for κ-casein, 0.97 cm^2/g for β-lactoglobulin, and 2.01 cm^2/g for α-lactalbumin (Swaisgood, 1982).

3.5.2 Kjeldahl method

The Kjeldahl method is the official method of analysis for determination of protein concentration (AOAC, 1984). The Kjeldahl method involved three different steps: digestion, distillation and titration and was performed using an automatic Kjeldahl distillation unit (Büchi Auto Kjeldahl Unit K-370, Büchi Labortechnik AG, Zürich, Schwitzerland). The total nitrogen of the samples was measured and expressed in g/100g, and a multiplication factor of 6.38 was used to convert total nitrogen to protein content.

3.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a traditional method used to quantify and separate proteins according to their molecular weight. It was carried out according to the method of Singh & Creamer (1991). The different steps for preparing and running gels are detailed below.

3.5.3.1 Buffer preparation

Tris-HCl buffer (0.5 M, pH 6.8; stacking buffer) and 1.5 M Tris-HCl buffer, pH 8.8 (resolving buffer), were prepared by weighing respectively 6 g and 18.15 g of Tris base in about 60 mL of Milli-Q water, adjusting the pH to, respectively, 6.8 and 8.8 using 6 M HCl and bringing the volume to 100 mL using a volumetric flask. These buffers were stored at 4 °C. A stock solution of acrylamide (30%, w/w) was prepared and stored at 4 °C. A stock solution of SDS (10%, w/w) was prepared and stored at room temperature.

Bromophenol blue (0.4%, w/v) was prepared by dissolving 1.6 g of bromophenol blue in 7 mL of 0.1 M NaOH, and the solution was made up to 400 mL using Milli-Q water. SDS-sample buffer was prepared by mixing 500 mL of Milli-Q water, 125 mL of 0.5 M Tris-HCl buffer,

100 mL of glycerol, 200 mL of 10% (w/w) SDS and 0.4 mL of 0.4% (w/v) bromophenol blue. The pH of the sample buffer was adjusted to 6.8.
A five-times concentrated electrode buffer was prepared by dissolving 15 g of Tris-base, 72 g of glycine and 5 g of SDS powder in 1 L of Milli-Q water. The pH was measured to check that it was 8.6 ± 0.2. If the pH was outside this range, the buffer was not used and a new solution was prepared. Before running a gel, the concentrated buffer was diluted five times (80 mL of buffer in 400 mL of Milli-Q water).

3.5.3.2 Gel preparation

A resolving gel solution was prepared by mixing 3 mL of water, 3.75 mL of resolving buffer, and 7.95 mL of 30% (w/w) acrylamide (quantities given for 4 gels). The solution was degased for 10 min. One hundred and fifty microlitres of 10% (w/w) SDS, 7.5 µL of TEMED, and 75 µL of freshly prepared 10% (w/w) ammonium persulphate (APS) were then immediately added to the degased solution. Three millilitres of this solution were poured between the two electrophoresis plates to form the resolving layer of the gel (Mini Protean II dual cell system, Bio-Rad Laboratories, Richmond, CA, USA). Three hundred microlitres of saturated butanol in water was added on top of the stacking layer to avoid drying of the gel, and the resolving layer was left to polymerise for an hour at room temperature. The butanol layer was then carefully poured out of the plates and the top of the gel dried with filter paper. A stacking gel solution was prepared by mixing 3 mL of water, 3.75 mL of stacking buffer and 7.95 mL of 30% acrylamide. The solution was degassed for 10 min. One hundred and fifty microlitres of 10% (w/w) SDS, 7.5 µL of TEMED (w/w), and 75 µL of freshly prepared 10% (w/w) APS were added to the solution, which was then poured on top of the resolving gel to form the stacking layer. A plastic comb (10 or 15 well comb) was immediately placed on top of the layer to form loading wells in the gel matrix. The gels were left overnight at room temperature to completely polymerise, and if not used the next day were stored until needed for up to a week at 4 °C in a box containing damp paper towels to prevent dehydration.

3.5.3.3 Protein sample preparation and reduction

The protein samples were diluted in 1 mL sample buffer in Eppendorf tubes, so the final protein concentration in the samples was comprised between 0.05% and 0.1% (w/w).

Twenty microlitres of β-mercaptoethanol was added to the tubes and the samples were heated at 100 °C in a heating block for 5 min to reduce the proteins.

3.5.3.4 Gel running, staining and destaining

The gels were put in a Bio-Rad power supply unit and immersed in 400 mL of diluted electrode buffer. Either 10 µL (in the 10 well-gels) or 6 µL (in the 15 well-gels) of the reduced samples were injected into the wells of the gel. The gels were run at 200 V and 70 mA for approximately 70 min, until the blue dye line had just diffused out of the bottom of the gels. The Amido Black staining solution was prepared by adding 1 g of Amido Black 10B dye in 250 mL of isopropanol, 100 mL of glacial acetic acid and making up to 5 L with Milli-Q water. The gels were transferred in a plastic container containing 50 mL of Amido Black staining solution and shaken for at least 4 h to stain. They were then destained in three steps, rinsing them with ~100 mL of 10% (v/v) acetic acid for a total of 6 h.

3.5.3.5 Gel scanning and integration

The destained gels were scanned using a computing laser densitometer (Molecular Dynamics, model P.D., Sunnyvale, CA, USA) and the protein bands for α_S-casein (α_{S1} + α_{S2}), β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin were integrated using the Image Quant TL software (Image Quant TL version 7.0, Molecular Dynamics, Sunnyvale, CA).

3.5.4 Microfluidic chip electrophoresis

3.5.4.1 Description of the technique

Microfluidic chip electrophoresis (MF-electrophoresis) is a miniaturised electrophoresis technique that uses capillary electrophoresis and works on the same principle as traditional SDS-PAGE. The proteins are moved across the chip in microfluidic channels that are filled with a polymer matrix and proteins are separated according to their molecular weight. The procedure includes similar steps as for the SDS-PAGE method, such as protein sample preparation, sample loading in the wells of the chip, electrophoretic separation, protein staining and destaining and detection and quantification of the proteins by integration. These steps are detailed below. The application of MF-electrophoresis for milk protein separation has been described in detail and compared with SDS-PAGE by Anema (2009a).

Two main advantages can be pointed out compared with SDS-PAGE: (i) MF-electrophoresis shortens the time of analysis and (ii) no gel making procedure is involved and the staining/destaining procedure is immediate. Therefore, a set of 10 samples can be analysed for protein content and composition within 30 min. It also has a larger linear range of detection than traditional SDS-PAGE, so samples containing low levels of proteins can be analysed with more accuracy and with further dilution needed.

3.5.4.2 Procedure

The MF-electrophoresis was performed using an Agilent 2100 Bioanalyzer system and the associated Protein 80 kit (Agilent Technologies, Waldbronn, Germany). The kit contained a gel matrix solution, a concentrated protein dye solution, and a concentrated ladder solution containing proteins of known molecular mass.

The following solutions were prepared:

Protein ladder
The diluted protein ladder was prepared by adding 84 µL of Milli-Q water to 6 µL of the protein ladder supplied in the kit.

Gel dye and destaining solution
Gel dye and destaining solution were prepared according to the protocols supplied with the chips. The gel matrix provided in the kit was added in an Eppendorf tube equipped with a disposable filter and centrifuged for 15 min at 2500 × *g*. The filtered solution was used as it is for destaining solution. 25 µL of concentrated dye was added to the filtered solution to make up the gel dye solution.

Sample buffer
Two hundred and fifty microlitres of Milli Q water, 62.5 mL of 0.5 M Tris-HCl buffer, pH 6.8, and 50 mL of 10% (w/v) SDS (50 mL) were mixed together, then 1 mL of 0.3 mg/mL lactoferrin was added to 150 mL of the sample buffer before further use. Lactoferrin was used as the upper marker in the integration step while SDS was the lower marker.

The protein samples were diluted in sample buffer in 1 mL Eppendorf tubes to obtain a final protein concentration comprised between 0.01% and 0.1%. 5% β-mercaptoethanol was

added and the samples were heated at 100 °C for 5 min, as explained for traditional SDS-PAGE.

Figure 3.2 shows the layout of a MF-electrophoresis chip. A chip contains 16 wells, of which three are for the gel dye (G1 to G3, G5), one is for the destaining solution, one is for the protein ladder and ten are for the protein samples. A chip was filled first with 12 µL of gel dye in G1, and air pressure was applied on the well with a syringe for 1 min mounted on a supplied loading station. The rest of the wells were then loaded with 12 µL of gel dye in G2, G3, G4, 12 µL of destaining solution in well DS, 6 µL of ladder in well L and 6 µL of samples in wells S1 to S10. Once loaded, the chip was inserted to the machine and the lid was closed. The electrophoresis was started and lasted for ~ 30 min.

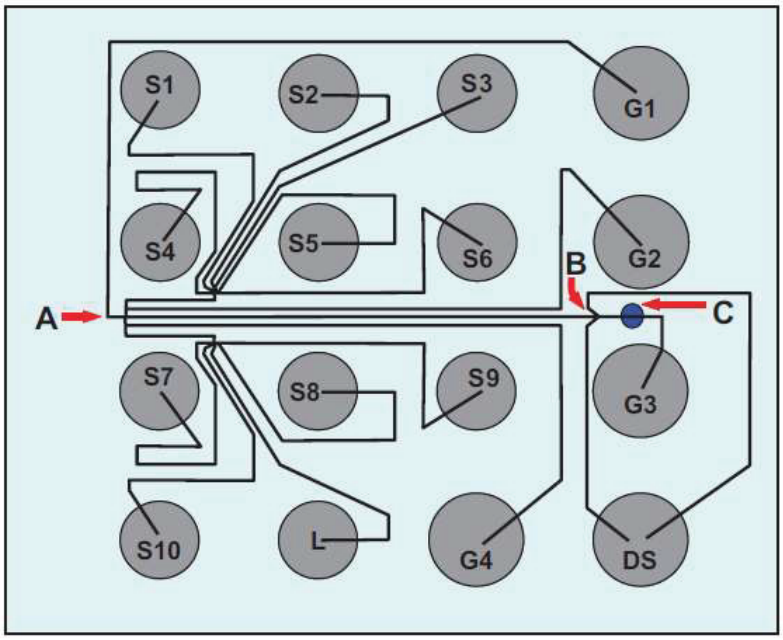

Figure 3.2: Layout of the wells and channels in a typical microfluidic electrophoresis chip. The separations channel (A), position of destaining (B) and position of the detection window (C) are highlighted with arrows. Source: Anema (2009a).

3.5.4.3 Interpretation of the results

A typical elution profile obtained for reduced SM by MF-electrophoresis is shown in Figure 3.3. From left to right of the elution time profile, the milk proteins appear in the order α-lactalbumin, β-lactoglobulin, β-casein, α_S-casein ($\alpha_{S1} + \alpha_{S2}$), κ-casein. Lower molecular weight proteins usually eluted faster, which was the case for all the proteins except κ-casein.

According to the molecular weight of the different proteins, the peak of κ-casein should have been on the left side of the peak of β-casein, as κ-casein has the lowest molecular weight of the caseins. A faster elution time corresponds to a longer distance of migration on the SDS-PAGE gels. On the SDS-PAGE gels, κ-casein migrated further than α_S-casein and β-casein (band lower on the gel). This anomalous behaviour of κ-casein in microfluidic chip electrophoresis has been reported before and has been attributed to potential artefacts affecting the elution position of κ-casein in the technique (Anema, 2009a). However, it does not affect the quantification of κ-casein. The Agilent 2100 expert software (Agilent Technologies) automatically integrated the protein peaks, but the integration usually had to be refined manually. The reported values for protein quantification are the peak areas.

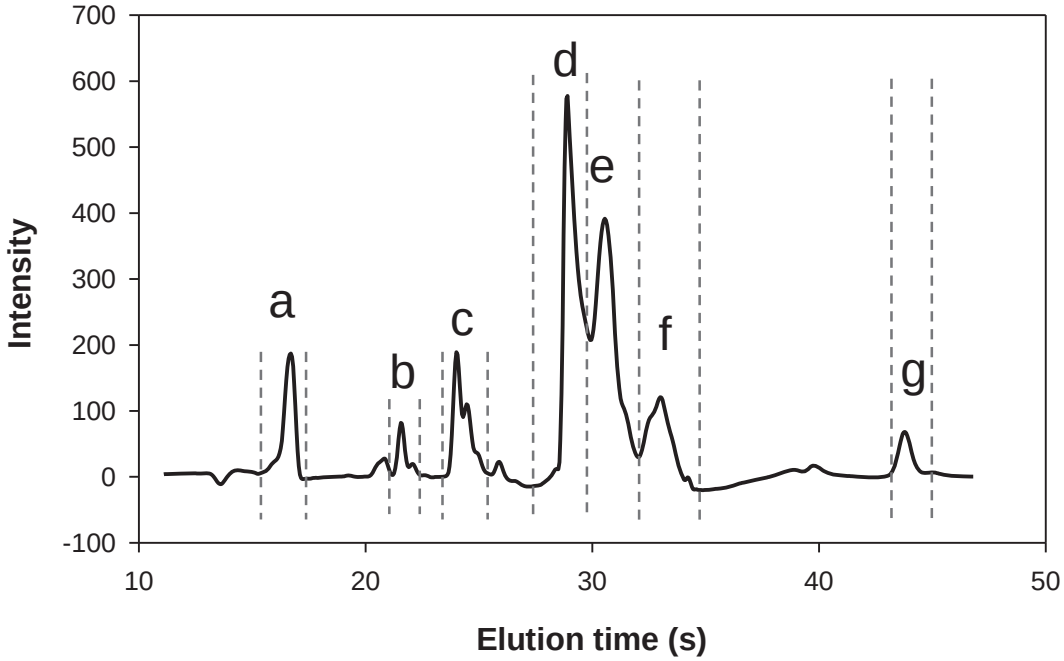

Figure 3.3: Typical elution profile obtained from MF-electrophoresis technique for reduced skim milk. The peaks were (a) SDS lower marker, (b) α-lactalbumin (c) β-lactoglobulin, (d) β-casein, (e) α_S-casein ($\alpha_{S1} + \alpha_{S2}$), (f) κ-casein, (g) lactoferrin upper marker. Skim milk was diluted in sample buffer at 1:20 ratio.

From the elution profile given in Figure 3.3, a computer generated gel image is given by the software. Figure 3.4 shows a typical gel image of skim milk analysed by MF-electrophoresis, at two different dilution ratios in sample buffer. The protein bands appear from bottom to top of the gel in order of increasing elution times, in the order α-lactalbumin, β-lactoglobulin, β-casein, α_S-casein ($\alpha_{S1} + \alpha_{S2}$), κ-casein. The order of the protein bands on the

gel is similar than that obtained on a traditional SDS-PAGE gel, and proportional to their molecular weight, except for κ-casein, that has a different migration position on MF-electrophoresis gels, where the κ-casein band is located on top of the other casein bands, compared with on traditional SDS-PAGE gels, where the κ-casein band is located under the β-casein band.

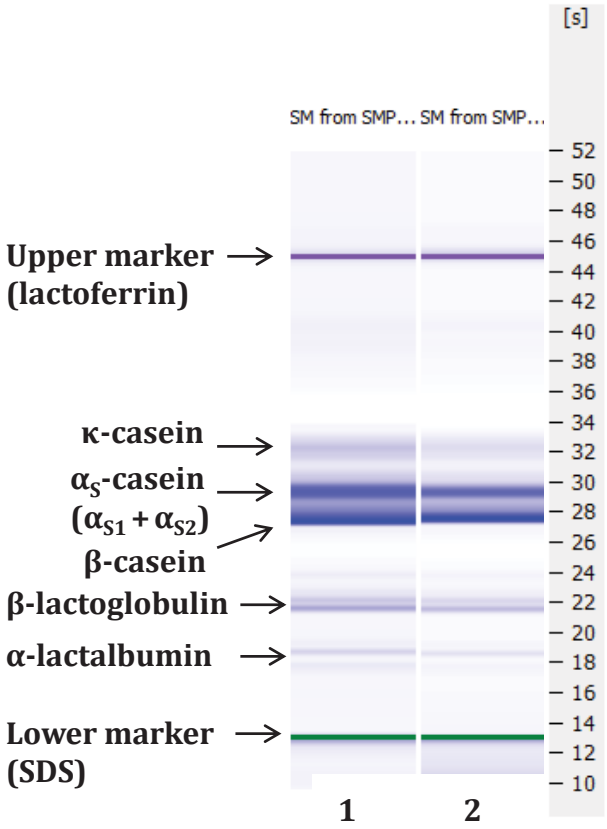

Figure 3.4: Computer-generated gel image from the elution profile of reduced skim milk obtained by MF-electrophoresis. Skim milk was diluted in sample buffer at the ratios 1 to 10 (lane 1) and 1 to 20 (lane 2); the axis on the left shows the elution times.

3.6 Mineral analysis

3.6.1 Total calcium

Total calcium concentration was measured by Inductively Coupled Plasma-Atomic emission Spectrometry (ICP-AES), using a Varian Vista CCD Simultaneous ICP-OES spectrometer (Varian Inc, Mulgrave, Victoria, Australia). An acidic solution of the samples was prepared by dry ashing followed by dissolution in nitric acid and hydrochloric acid. This solution is

injected into the ICP-OES using a glass concentric nebuliser. The wavelength used for calcium analysis was 317.9 nm.

3.6.2 Ionic calcium concentration

Ionic calcium concentration was measured using a Radiometer (Copenhagen, Denmark) type F2112 Ca calcium-specific electrode paired with an Orion (Beverly, Massachusetts, USA) Sure-Flow model 90-02 Ag/AgCl double junction electrode with 0.08 M KCl in the outer electrode chamber. A 5 mM stock solution of $CaCl_2$ was prepared in 0.08 M KCl and successive dilutions were carried out to obtain six calibration solutions in the Ca^{2+} concentration range of 0.5-5 mM (calibration solutions: 0.5, 1, 2, 3, 4 and 5 mM Ca^{2+}). The electrical output (mV) obtained from the electrode for the calibration solutions was read two times and plotted against the log of Ca^{2+} concentration of the standard solutions as a linear relationship exists between the two (Nernst equation). The theoretical slope for the linear equation should be around 29 mV (Lewis, 2011). The calibration was considered to be correct if the electrode gave a stable reading within a minute, and if the slope was comprised between 27 and 31 mV. The linear equation given by the standard was then used to convert measured electrode potentials to Ca^{2+} concentration.

3.6.3 Inorganic phosphate

Inorganic phosphate was determined by automated colorimetric analysis using a continuous-flow nutrient analyser (model Skalar San ++, Skalar Analytical, Breda, The Netherlands). The samples were pumped through a segmented flow injection system and inorganic phosphate was determined by a procedure involving measurement of the absorption at 420 nm of a complex formed by orthophosphate ions and a phosphate colour reagent, containing ammonium molybdate and ammonium metavanadate.

3.6.4 Citrate

Citrate was measured by an enzymatic method, involving successive enzymatic reactions with citrate lyase, malate dehydrogenase, lactate dehydrogenase and $NADH,H^+$. After

enzymatic reactions, the concentration of $NADH,H^+$, which was stoichiometric with the concentration of citric acid, was determined by measuring absorbance at 365 nm.

3.7 Preparation of modified milk protein ingredients

3.7.1 Preparation of whey protein-depleted skim milk powder

Whey protein-depleted skim milk powder (WPD-SMP) was prepared as follows. Fresh skim milk (SM) was obtained from a local supermarket and microfiltrated using a microfiltration (MF) membrane (Amicon hollow fibre membrane, H1P30-43, pore size 0.1 microns). This membrane allows the passage in the MF permeate of the two main whey proteins β-lactoglobulin and α-lactalbumin, the lactose and the milk minerals, but retains the casein micelles in the MF retentate. The detailed preparation method is represented in Figure 3.5.
1.3 L of fresh skim milk was run through the membrane, in three separate batches. The retentate containing the casein micelles was poured back into the beaker containing the original skim milk. The MF permeate was collected in a measuring cylinder. The volume of the MF permeate was monitored and an equivalent volume of SMUF (recipe given in Section 3.2.2) containing 5% (w/w) lactose was added back to the milk, to keep the protein concentration constant. 7 successive rinses with SMUF were carried out. The percentage of depletion in whey proteins of the whey protein-depleted skim milk (WDP-SM) was measured by MF-electrophoresis. There were less than 5% of the initial whey proteins remaining in the milk after 7 rinses. The WDP-SM was then spray-dried using a Yamato Pulvis spray-drier (Model GB-22, Yamato Scientific Co., Tokyo, Japan) to obtain a whey protein-depleted skim milk powder (WPD-SMP).

It is important to note that WPD-SMP is not a protein concentrate. It is similar to SM in terms of protein content. Casein micelles were not concentrated by the MF procedure and lactose was re-added in the original milk to allow an easy reconstitution of the spray-dried powder. WPD-SMP was stored in an air-tight container in the fridge until needed.

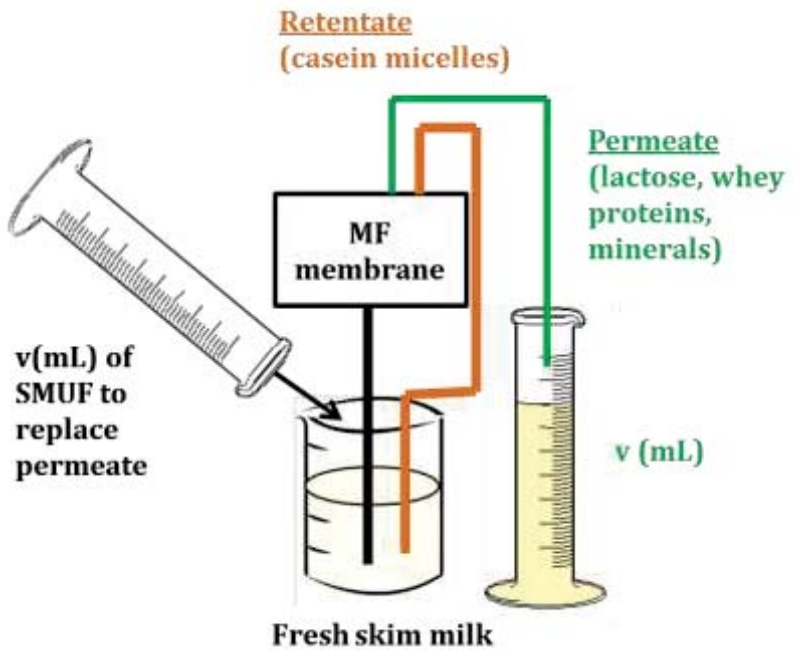

Figure 3.5: Preparation of whey protein-depleted skim milk by microfiltration; the obtained solution was then spray-dried to obtain whey protein-depleted skim milk powder (WPD-SMP)

3.7.2 Preparation of EDTA-treated skim milk

Skim milks with different levels of casein micelle dissociation were obtained using an EDTA/dialysis method, adapted from Griffin, Lyster, and Price (1988) and Ward, Goddard, Augustin, and McKinnon (1997). EDTA dissociates the casein micelles by solubilising the colloidal calcium phosphate (CCP) from the micelles. Appropriate volumes of a stock solution of 0.25 M Na_2H_2EDTA solutions were added drop by drop under stirring conditions to 100 mL samples of reconstituted SM from SMP (11.6%, w/w, total solids) to obtain four levels of EDTA additions (0, 5, 10, 20 mM of added EDTA). Water was added appropriately so the final concentration of all the samples was 10% (w/w) total solids. The samples were then dialysed overnight using a dialysis membrane of 10 kilodalton (kDa) cut-off against the original 10% (w/w) SM to remove the excess soluble calcium and phosphate and to restore the original pH.

3.8 Statistical analysis

3.8.1 Statistical tests

T-tests and analysis of variance (ANOVA) tests were carried out using the Minitab software (Minitab version 16, Minitab, Inc., State College, PA) to determine the significance of the differences. Significance was established at $P < 0.05$.

3.8.2 Modelling

The adsorption data were fitted using three different models (Langmuir, Langmuir–Freundlich and Freundlich, described in part 2.5.2 of the literature review) to obtain quantified interpretable parameters that described the adsorption processes. The model curves were fitted to the experimental data using the freeware computer program R (version 2.15.0) and a non-linear least-squares fitting algorithm was used to obtain the best-fit model parameters.

CHAPTER 4 Adsorption of caseins and whey proteins onto hydroxyapatite particles*

4.1 Abstract

The adsorption of caseins from sodium caseinate (SC) and whey proteins from whey protein isolate (WPI) onto particles of hydroxyapatite (HA) particles was studied. The ratio of HA to protein was varied by either adding a constant amount of HA particles (10%, w/w) in SC and WPI solutions of varying protein concentration (0 to 5%, w/w), or varying concentrations of HA particles (0 to 3%, w/w) were added to a constant amount of protein from SC and WPI (0.1%, w/w), and the amount of adsorbed protein was quantified using total nitrogen quantification (Kjeldahl) and sodium dodecyl sulphate polyacrylamide gel electrophoresis. Results show that the protein concentration and ionic strength of the initial protein solution had an effect on the amount of adsorbed caseins and the preferential adsorption between the different caseins. In water, there was a preference in the protein adsorption: in the order, β-casein > α_S-casein (α_{S1}-+α_{S2}) > κ-casein for SC-coated particles and in the order, β-lactoglobulin > α-lactalbumin for WPI-coated particles. The adsorption of caseins and whey proteins onto HA could be fitted using a simple Langmuir model and a Langmuir-Freundlich model, suggesting a single layer adsorption of caseins and whey proteins, with possible protein-protein interactions (cooperative effects) occurring between the adsorbed proteins on the HA surface. Confocal microscopy and zeta-potential measurements confirmed that both caseins and whey proteins bound to HA, resulting in an increase in the absolute value of the zeta-potential of the particles. This adsorption improved the suspension stability of the HA particles in water. Possible mechanisms involved in the interactions between milk proteins and HA are discussed in relation to the structure and the surface properties of both milk proteins and HA particles.

---

* Part of the content presented in this chapter has been published as an original paper: Tercinier, L., Ye, A., Anema, S., Singh, A., & Singh, H. (2013). *Journal of Colloid and Interface Science, 394,* 458–466.
Part of the content was also presented as a conference presentation at 5th International Symposium on Delivery of Functionality in Complex Food Systems, Israel, September 30th – October 3rd, 2013 and as a graduate student poster presentation at São Paulo School of Advanced Science Advances in Molecular Structuring of Food Materials, Pirassununga, April 1st – 5th, 2013 (Poster given in Appendix 2)

4.2 Introduction

Hydroxyapatite (HA) particles are widely used in calcium-fortified ultra-high temperature (UHT) treated milks, where they are often described as chemically unreactive. This view is taken because HA is insoluble at the pH of milk, so it can be added to milk and heat-treated without reacting with the milk proteins or causing heat instability, unlike soluble calcium salts (Omoarukhe et al., 2010). However, it is well-known that HA interacts strongly with proteins in a wide range of biological applications, such as chromatography, nano-ceramics and bone implants or tooth enamel (Juriaanse et al., 1981; Kawasaki, 1991; Wilson, 2009).

Despite its dairy industry relevance, there is a lack of literature on the interactions between HA and milk proteins, as highlighted in the literature review of this thesis (Chapter 2). Only a few studies have reported on the adsorption of milk proteins onto HA, mostly in relation to bone or dentistry topics. For example, Van Kemenade and de Bruyn (1989) showed that caseins were able to inhibit the precipitation of HA by binding to calcium in the order, κ-casein < β-casein < α_{S1}-casein. Devold et al. (2006) studied the in-vitro adsorption of milk proteins onto tooth enamel and showed that, at neutral pH, caseins were preferentially bound to tooth enamel forming protein pellicles. Reynolds and Wong (1983) showed that κ-casein, α_{S1}-casein , β-casein, β-lactoglobulin and α-lactalbumin all bound to HA and rendered the zeta-potential of HA more negative. This leads to the hypothesis that interactions may occur between milk proteins and HA particles when hydroxyapatite particles are suspended in milk, resulting in the adsorption of the milk proteins onto the surface of the HA particles, and a corresponding change in the zeta-potential and colloidal properties of the particles.

The adsorption of milk proteins onto the surface of HA is likely to be governed mainly by specific electrostatic interactions, not only between the carboxyl groups of the proteins and the calcium ions in the HA crystal lattice, but also between the phosphoserine residues of the casein molecules and the HA calcium ions (Bernardi & Kawasaki, 1968; Gorbunoff & Timasheff, 1984a). Caseins are flexible linear proteins, whereas whey proteins have a globular structure. Differences in structure and amino-acid sequences, as well as the phosphorylated nature of the caseins, are likely to cause different adsorption profiles for the two types of proteins (Gorbunoff & Timasheff, 1984b).

The objective of this chapter was to examine the adsorption of the two main milk proteins, caseins and whey proteins, on the surface of HA particles, using sodium caseinate (SC) and whey protein isolate (WPI) as model protein solutions. The adsorption experiments were

carried out in water, at the natural pH of the SC and WPI solutions, to avoid any complication from buffer salts affecting the adsorption behaviour. This approach would confirm whether or not milk proteins are able to bind to HA, and would provide a better understanding of the interfacial interactions between HA and milk proteins. This in turn would be useful for further studies of the system formed by HA particles suspended in milk, i.e., in a calcium-fortified milk.

4.3 Material and methods

Stock protein solutions of SC and WPI in water (6%, w/w, total solids on a powder basis) were prepared, as described in Section 3.2.1. Suspensions of 10% (w/w) HA powder were prepared in SC and WPI solutions of different concentrations (0 to 6%, w/w, total solids) to obtain different protein to HA ratios as described in Section 3.2.3, and stirred for 2 h. A kinetic study was also performed, by varying the stirring times between 1 min and 4 h. Another adsorption experiment was carried out by adding different amounts of HA particles (0.1 to 3%, w/w) to protein solutions of fixed concentration (SC and WPI; 0.1%, w/w, total solids). The HA powder was then separated from the protein solutions and the protein concentration and composition of the supernatants were measured using SDS-PAGE (Section 3.5.3). The amount and composition of adsorbed protein were determined by the difference between the protein concentration and composition in the initial solution and in the adsorption supernatants (depletion method), as described in Section 3.2.4.

The HA pellets, obtained after centrifugation, were suspended in water and characterised for zeta-potential (Section 3.3.2), suspension stability (3.3.4), and observed using confocal microscopy. The details for the preparation and observation of samples with confocal microscopy can be found in Section 3.3.3

4.4 Results and discussion

4.4.1 Protein adsorption

4.4.1.1 Constant amount of HA added to different concentrations of protein

The protein concentrations of the initial solutions were calculated from the protein concentrations of the original SC and WPI powders, as determined by the Kjeldahl method, and comprised between ~0.25 and ~5.5% (w/w, total protein). The supernatants containing the unadsorbed protein were analysed for protein content using SDS-PAGE.

Surface protein concentration determined using SDS-PAGE

With SDS-PAGE gels, the concentration of protein remaining in the supernatants after adsorption of the proteins onto HA was determined as a percentage of the protein contained in the initial SC or WPI solutions, with the initial protein solutions and the supernatants both loaded on the same gel. The SDS-PAGE patterns obtained for the initial protein solutions and the supernatants in the SC and WPI adsorption experiments are shown in Figure 4.1 and Figure 4.2, for four different initial protein concentrations. Each supernatant (lanes 2, 4, 6, and 8 on both figures) was loaded next to its corresponding initial solution (lanes 1, 3, 5, and 7 on both figures). However, to obtain bands that were in the measurable range of intensities for integration, the protein samples were diluted in SDS sample buffer to obtain a protein concentration of between ~0.05% and 0.1% (w/w). As the protein concentrations in the supernatants were much lower than those of the initial protein solutions, the supernatants were less diluted in SDS sample buffer than their respective initial solution controls, therefore not allowing a direct visual comparison between initial solutions and supernatants. However, for samples that were diluted by the same dilution factor for both initial protein solution and supernatant, e.g., for samples prepared with SC containing 3.6% (w/w) protein (Figure 4.1A), it can be seen that the intensities of the casein bands decreased between the initial solution (lane 1 in Figure 4.1A) and the supernatant (lane 2 in Figure 4.1A), showing that caseins did adsorb onto HA particles. A decrease in intensity could also be observed in the gels between the initial solutions and the supernatants of the samples prepared with WPI containing 3.72% and 2.79% (w/w) protein (lanes 1 and 2 in Figure 4.2A and lanes 3 and 4 in Figure 4.2B). For the other samples, the corrected band intensities that would have been obtained on the gels if the dilutions had been the same for initial solutions and supernatants were calculated.

The percentage of total protein left in the supernatants after adsorption was determined by calculation of the ratio between the sum of the band intensities (corrected for dilution factor if needed) of the three caseins α_S- (α_{S1}-+α_{S2}), β- and κ-casein (for SC) or the two whey proteins β-lactoglobulin and α-lactalbumin (for WPI) in the supernatants over the sum of the band intensities of the three caseins (for SC) or the two whey proteins (for WPI) in the initial solutions.

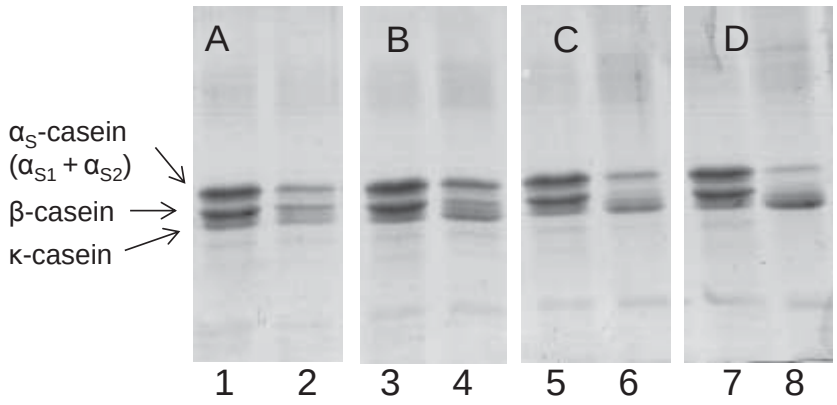

Figure 4.1: SDS-PAGE pattern of initial protein solutions and supernatants obtained for adsorption experiments carried out with sodium caseinate (SC) solutions at initial protein concentrations (w/w) of: A, 3.6%; B, 2.7%; C, 1.8%; D, 0.9%. Lanes 1, 3, 5 and 7, control samples (i.e., initial SC solutions); lanes 2, 4, 6 and 8, respective supernatant samples (i.e., containing the unadsorbed proteins). The samples were diluted in SDS sample buffer at the following ratios: 1 to 60 (lanes 1 and 2), 1 to 40 (lanes 3), 1 to 20 (lane 4), 1 to 30 (lane 5), 1 to 10 (lane 6), 1 to 15 (lane 7), 1 to 2 (lane 8).

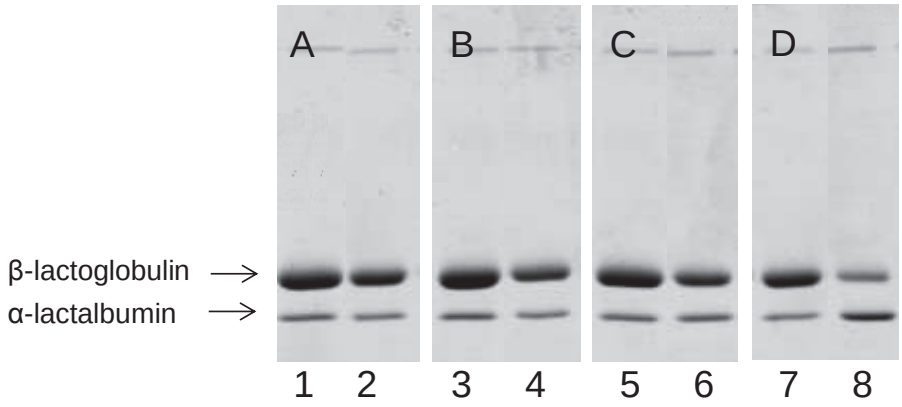

Figure 4.2: SDS-PAGE pattern of initial protein solutions and supernatants obtained for adsorption experiments carried out with whey protein isolate (WPI) solutions at initial protein concentrations (w/w) of: A, 3.72%; B, 2.79%; C, 1.86%; D, 0.93%. Lanes 1, 3, 5 and 7, control samples (i.e., initial WPI solutions); lanes 2, 4, 6 and 8, respective supernatant samples (i.e., containing the unadsorbed proteins). The samples were diluted in SDS sample buffer at the following ratios: 1 to 60 (lanes 1 and 2), 1 to 40 (lanes 3 and 4), 1 to 20 (lane 4), 1 to 30 (lane 5), 1 to 20 (lane 6 and 7), 1 to 4 (lane 8).

Figure 4.3 shows the amount of SC or WPI adsorbed to the surface of the HA particles as a function of the initial protein concentration. For both SC and WPI, as the initial protein concentration increased, the amount of adsorbed caseins and whey proteins also increased until a plateau was reached. For HA particles mixed with SC, the amount of adsorbed protein increased gradually with SC concentration up to about 3% (w/w, total protein) and then reached a maximum coverage value of ~2.7 mg/m^2. The surface protein concentration of the HA particles mixed with WPI was lower than that of the HA particles mixed with SC. The surface protein concentration increased gradually with WPI concentration up to 1.5% (w/w, total protein) to a maximum coverage value of ~1.3 mg/m^2. As the surface protein coverage values increased up to a maximum value (threshold of adsorption), a saturation state of the HA surface with respect to protein must have been reached.

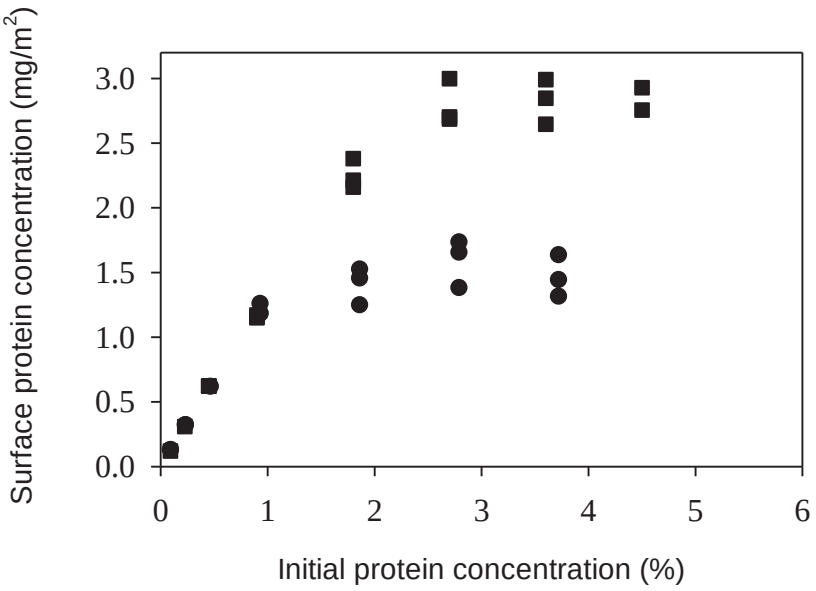

Figure 4.3: Surface protein concentration (mg/m²) of (■) caseins from SC and (●) whey proteins from WPI on HA particles. Values calculated from SDS-PAGE data, as a function of initial protein concentration; all experimental data points are shown from three replica experiments.

Kinetics

A kinetic experiment was carried out with SC (3%, w/w) to verify that the initial chosen stirring time of 2 h was sufficient for the proteins to absorb and for equilibrium to occur. Ten percent (w/w) HA was added to 3% (w/w) SC. The suspensions were stirred for different times between 1 min and 4 h. Immediately after stirring ceased, the HA particles were centrifuged (3000 × *g*, 2 min), and the supernatants separated from the particles and added directly to SDS-PAGE sample buffer to quantify the unadsorbed protein concentration.

The SDS-PAGE pattern obtained for the initial SC solution and the supernatants obtained after different stirring times is shown in Figure 4.4. The intensity of the bands decreased during the first hour of adsorption (lanes 1 to 7) and then remained unchanged (lanes 7 to 11). Therefore, after one hour, an equilibrium had been reached and no more protein was adsorbed onto the HA particles. A stirring time of 2 h was used for the rest of the study. These results were consistent with other studies of protein adsorption onto HA that reported stirring times of only a few hours (Sharpe et al., 1997; Zhu et al., 2007).

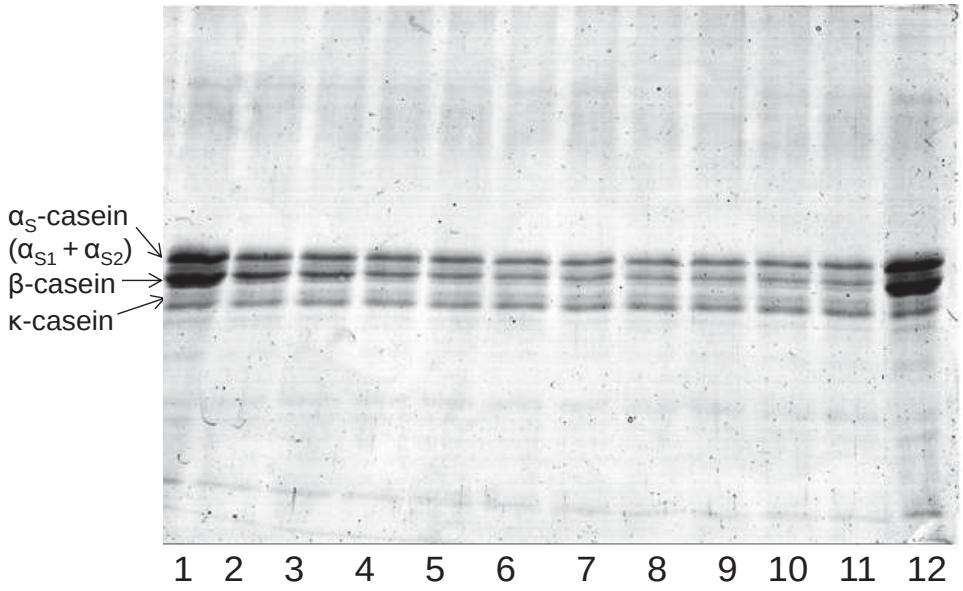

Figure 4.4: SDS-PAGE pattern of the adsorption supernatants obtained after different stirring times. HA particles (10%, w/w) were added to 3% (w/w) SC, stirred for various times from 1 min to 4 h, and separated from SC by centrifugation; the supernatants were added to SDS-PAGE sample buffer just after centrifugation. Lanes 1 to 11: samples were stirred for 1 min, 2 min, 5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, respectively; lane 12, initial SC (3%, w/w) solution, no HA added.

Adsorption modelling

The Langmuir, Langmuir-Freundlich and Freundlich models are often used in studies on the adsorption of protein onto HA, to compare the adsorption characteristics of different types of protein (Iafisco et al., 2010, 2011; Mohsen-Nia et al., 2012), as detailed in Section 2.5.2 of the literature review. All three models were therefore fitted to the adsorption data for SC and WPI to obtain physically interpretable parameters to compare the adsorption characteristics of caseins and whey proteins. It is important to note that the parameters obtained for each model and the interpretation of these parameters are slightly different in this chapter compared with those described in Tercinier, Ye, Anema, Singh, and Singh (2013). The models in Tercinier et al. (2013) were fitted using data for the protein content of the supernatant obtained with the Kjeldahl method, whereas in this chapter the models were fitted using the SDS-PAGE data, and the modelling method was refined further after the publication of Tercinier et al. (2013).

The Langmuir model quantifies the amount of protein adsorbed onto HA as a function of the protein concentration once equilibrium has been reached (in this case, the unadsorbed

protein concentration) at a given temperature. This model assumes that the proteins bind to a series of distinct empty sites on the HA surface. The surface is assumed to be energetically homogeneous and the model allows only monolayer coverage. The Langmuir model is given by Eq. (1), as follows:

$$\frac{m_{abs}}{S} = q_m \times \frac{K[P]}{1 + K[P]} \quad (1)]$$

Where, m_{abs}/S is the amount of protein bound to the surface expressed in mg/m^2, [P] is the concentration of protein at equilibrium (g/100 g), q_m is the maximum monolayer surface coverage (mg/m^2), and K is the Langmuir affinity constant (100g/g).

The Langmuir–Freundlich model takes into account some heterogeneity of the surface (thus all sites are not equal), as well as some possible interactions between the proteins once they are adsorbed at the surface. The Langmuir–Freundlich model is given by Eq. (2). K_{LF} is the Langmuir–Freundlich affinity constant ((100g/g)$^{1/n}$) and n is a surface heterogeneity parameter. It still assumes monolayer coverage, but takes into account that the adsorption energy is not equal for all sites. For example, in the case of a heterogeneous surface, which would have a negative effect on the adsorption of the proteins, the n value is lower than 1. Vice versa, if some cooperative interactions between proteins at the surface occur, the n value is greater than 1. The closer the n value is to 1, the less impact the surface and the interactions between adsorbed proteins have on the adsorption process.

$$\frac{m_{abs}}{S} = q_m \times \frac{(K_{LF}[P])^n}{1+(K_{LF}[P])^n} \quad (2)$$

The Freundlich isotherm model does not limit adsorption to a monolayer and is often applicable to multiple-layer adsorptions. It follows the equation given in expression (3). K_F is the Freundlich affinity constant ((100g/g)^N)

$$\frac{m_{abs}}{S} = K_F \times [P]^{1/N} \quad (3)$$

The Langmuir, Langmuir-Freundlich and Freundlich isotherm curves for casein and whey protein adsorption onto HA particles are given in Figure 4.5. Table 4.1 gives the calculated constants for each of the isotherms.

For both SC and WPI, the Langmuir and the Langmuir–Freundlich models gave very similar results, as the two curves almost overlapped (Figure 4.5). The Freundlich model curves did not fit as well to the adsorption data, compared with the Langmuir and the Langmuir–Freundlich curves, for both SC (Figure 4.5A) and WPI (Figure 4.5B), which suggested that there was no multiple layer adsorption of caseins and whey proteins onto HA particles. Therefore, the Freundlich model will not be discussed further and the interpretation of the adsorption parameters will be based only on the Langmuir and the Langmuir–Freundlich models.

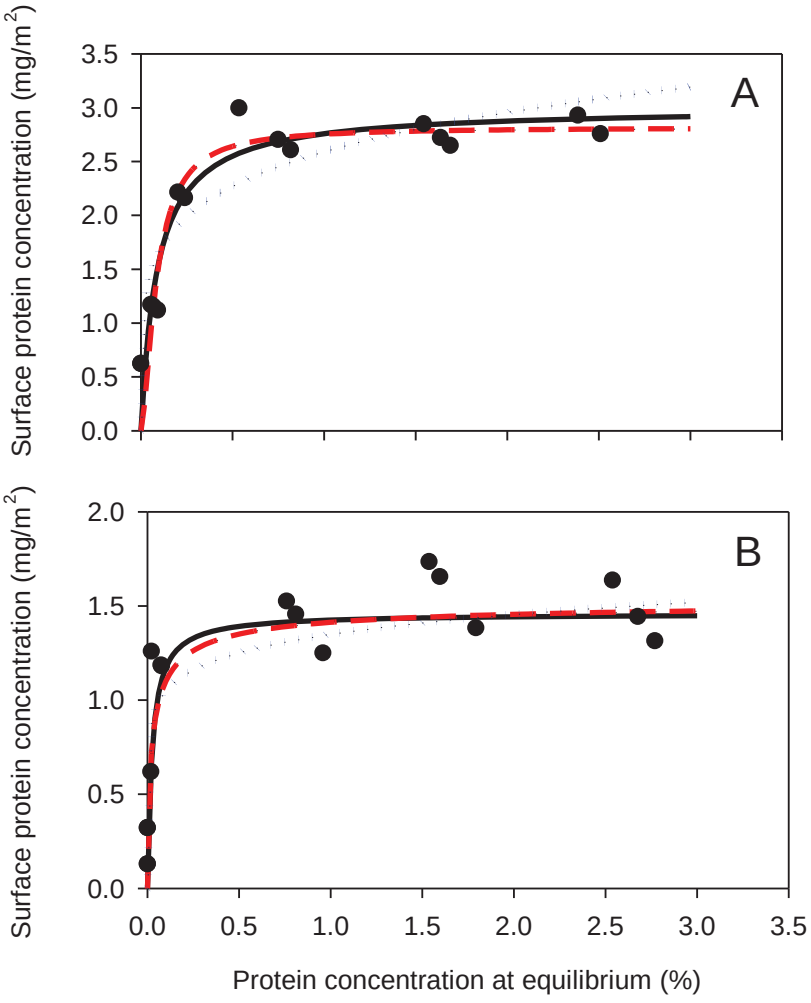

Figure 4.5: Isotherms of milk proteins adsorbed onto HA and the different best-fit model curves for (A) SC and (B) WPI. Experimental data, ●; Langmuir model, solid black line; Langmuir–Freundlich model, dashed red line; Freundlich model, dotted blue line.

Table 4.1: Parameters for the adsorption of SC and WPI onto HA particles calculated according to the Langmuir, Langmuir–Freundlich and Freundlich models.

Protein	Model	Maximum surface coverage, q_m (mg/m^2)	Affinity constant, K (100g/g) $K_{LF}(100g/g)^{1/n}$ and $K_F(100g/g)^N$	n[a]	N
SC	Langmuir	3.0 ± 0.2	11.3 ± 3.6	–	–
	Langmuir-Freundlich	2.8 ± 0.1	11.3 ± 3.4	1.5 ± 0.3	–
	Freundlich	–	2.6 ± 0.1	–	5.3 ±0.9
WPI	Langmuir	1.5 ± 0.2	40.7 ± 3.6	–	–
	Langmuir-Freundlich	1.5 ± 0.1	40.9 ± 13.1	0.7 ± 0.1	–
	Freundlich	–	1.4 ± 0.2	–	9.2 ± 3.9

[a] n is a surface heterogeneity parameter.

To understand the adsorption characteristics of caseins and whey proteins, it is important to consider the general mechanism of protein binding to HA that has been well studied and described in details in Section 2.4.2 of the literature review. The surface of HA particles is heterogeneously charged and carries two types of binding sites on the crystal surface of HA, usually called C-sites and P-sites (Kandori et al., 2011; Luo & Andrade, 1998). After dispersion in an aqueous medium, the C-sites are rich in calcium ions and therefore positively charged, whereas the P-sites are rich in phosphate ions, and therefore negatively charged. As a consequence, proteins can potentially interact with both positive and negative sites on HA. C-sites have been shown to interact with the carboxyl groups of proteins and P-sites have been shown to interact with the amino groups of proteins (Gorbunoff, 1984; Gorbunoff & Timasheff, 1984a). However, proteins that have an isoelectric point lower than 7 (acidic proteins) have been shown to mostly interact with the C-sites on HA. The side carboxylic groups on glutamic and aspartic acids residues of the proteins form complexes with the calcium ions on HA and those complexes are held together by strong specific electrostatic interactions (Wang et al., 2012). Therefore, the binding of both caseins and whey proteins to the surface of HA particles is likely to be irreversible, and driven by a strong complexation between the C-sites of HA and the carboxyl groups of the milk proteins.

In addition, casein molecules are phosphorylated proteins. α_{S1}-Casein, α_{S2}-casein and β-casein have a high content of phosphate groups (phosphoserine residues) that are not uniformly distributed in their sequences and are grouped in clusters (Fox, 2003). Phosphoserine residues play a crucial role in the structure of casein micelles because they are able to bind cations, especially Ca^{2+} (Horne, 2009). Because of their strong calcium-binding ability, phosphoproteins have also been shown to bind to the C-sites of HA (Bernardi & Kawasaki, 1968). Phosphoserine residues on proteins can interact with C-sites even more strongly than carboxyl groups (Kawasaki, 1991). Therefore, for SC, the adsorption mechanism must be explained mostly by specific interaction between the negative phosphoserine groups of the casein molecules and the positive C-sites of HA.

The modelling approach was useful to characterise and quantify the adsorption of SC and WPI onto HA particles. The maximum surface protein concentrations obtained for the Langmuir and Langmuir-Freundlich models were about the same (Figure 4.5 and Table 4.1), and close to those observed experimentally (Figure 4.3), with caseins binding to a maximum of about 2.9 mg/m^2 (Figure 4.5A and Table 4.1) and WPI to a maximum of about 1.5 mg/m^2 (Figure 4.5B and Table 4.1). Caseins bound twice as much as whey proteins onto HA. As protein adsorption onto HA is driven by mainly specific electrostatic interactions, the maximum amount of proteins that can bind to HA is usually determined by the number and distribution of the charged residues along the protein molecules (Nakanishi et al., 2001; Wahlgren & Arnebrant, 1991). As the phosphoserine groups of the caseins are grouped in clusters, they may have had more anchor points than the whey proteins and aligned better with the C-sites of the HA particles (Johnsson et al., 1993), which would partly explain the higher surface load of caseins compared with whey proteins. However, the different adsorption levels of caseins and whey proteins may be also due to the different structures of the two adsorbed proteins on the HA surface.

Globular proteins have been shown to adsorb in a close-packed monolayer onto HA (Wahlgren & Arnebrant, 1991). As whey proteins have a small compact globular structure (Dickinson, 2001), they probably form only a monolayer made up of individual proteins on the HA surface. Conversely, caseins in SC might not bind as individual molecules, but rather as small aggregates. The disordered linear and flexible structures of the caseins allow them to either self-associate to form oligomers (Swaisgood, 1982), or to associate with each other to form self-assembled particles made of different caseins (Srinivasan, Singh, & Munro, 1999). The association and aggregation behaviour of the caseins in SC is not completely understood, and might depend on the concentration of caseins present in solution. However,

it is hypothesised that caseins might not be present on the HA surface as individual molecules. They might associate and form small aggregates, resulting in a thicker layer and a higher surface load.

The surface heterogeneity parameter, n, of the Langmuir-Freundlich model is a good indicator of whether or not protein-protein interactions occur between the adsorbed proteins. The difference reported between the heterogeneity parameter (n) in the Langmuir–Freundlich model for the WPI data (n <1) and the SC data (n >1) may be explained by differences in the protein adsorption characteristics. The parameter n was lower than 1 for whey proteins (n = 0.7; see Table 4.1), which may mean that some negative interactions between the adsorbed proteins occurred on the surface, preventing the binding of more proteins (negative co-operative interactions). As whey proteins have a globular structure, adsorbed whey proteins might undergo conformational changes, causing steric hindrance of the other adsorbing C-sites available for adsorption (Wahlgren & Arnebrant, 1991), and thus preventing access of other whey proteins to the adsorbing sites. The negative charge of the adsorbed whey proteins might also cause electrostatic repulsions within the protein layer, preventing some more whey proteins from binding (Luo & Andrade, 1998). In contrast, the surface heterogeneity parameter (n) for caseins was higher than 1 (n = 1.5; see Table 4.1), which may mean that some lateral interactions between the adsorbed caseins enhanced the binding of the caseins onto the HA surface. Positive co-operative interactions between adsorbed caseins are very likely to occur on the HA surface since they are believed to either self-associate or associate with each other to form aggregates on the HA surface. Also, the presence of neighbouring phosphoseryl groups on the casein molecules that are already adsorbed on the HA surface could increase the probability of casein binding (co-operative positive adsorption), as shown previously by Gorbunoff (1984).

It was not clear why the affinity constants for the Langmuir and Langmuir-Freundlich models were larger for the WPI data than for the SC data (about 40 for WPI versus about 11 for SC). As the binding of phosphoserine residues to C-sites is much stronger than the binding of carboxyl groups to C-sites, it was expected that higher affinity constants would be obtained for SC than for WPI. For example, Johnsson et al. (1993) reported that phosphoproteins with domains of clustered negatively charged phosphoserine residues, such as satherin and proline-rich salivary phosphoproteins, had higher affinity constant values than non-phosphorylated proteins. The affinity constant of the Langmuir model characterises the initial slope of the adsorption isotherms (Iafisco et al., 2011). One

explanation of the difference between the constants for WPI and SC could be that the carboxyl groups on the whey proteins in WPI are initially more accessible for adsorption than the phosphoserine groups of the caseins in SC, allowing the whey proteins to have a better initial adsorption rate than caseins. It could also be due to SC and WPI containing different proteins that may have different affinities for HA, and this will be discussed later in this chapter. It is also important to emphasise that the initial slope of the adsorption isotherms is very dependent on the first few points of the adsorption isotherms, and that a lack of precision on the measurements of the amount of adsorbed protein in this region of the curve might explain the inconclusive results regarding the affinity constants for SC and WPI.

In conclusion, the simple Langmuir model showed a good fit for both the SC data and the WPI data, and was useful in comparing the adsorption of caseins and whey proteins. However, the results on the affinity constants were inconclusive, and require further investigation. Also, some of the assumptions of the Langmuir model, such as the homogeneity of the surface and the absence of interactions between adsorbed proteins, might not be applicable to the adsorption of proteins on HA (Luo & Andrade, 1998; Mura-Galelli et al., 1991). Therefore, applying other models, such as the Langmuir-Freundlich model, to characterise the adsorption of milk proteins on HA can be useful, as it allows interactions between proteins, and can therefore give further information on the co-operative interactions occurring between adsorbed proteins at the HA surface.

Surface protein composition and preferential adsorption

By looking at the relative composition of the supernatants in the different caseins or whey proteins, it is possible to show which proteins bind preferentially. For example, for the SC samples prepared at low initial protein concentration (1.86%, w/w, Figure 4.1C and 0.93%, w/w, Figure 4.1D), the relative intensity of the κ-casein band was higher in proportion in the supernatants (lanes 6 and 8) than in the controls (lanes 5 and 7). This means the supernatants were enriched in κ-casein, and therefore shows that κ-casein must have bound less than α_S- and β-caseins. For the WPI sample prepared at the lowest protein concentration (0.93%, w/w, Figure 4.2D), the supernatant (lane 8) contained more α-lactalbumin than β-lactoglobulin, as shown by the higher intensity of the α-lactalbumin band compared with the β-lactoglobulin band, whereas the initial solution control (lane 7) contained more β-lactoglobulin than α-lactalbumin. As the supernatant was enriched in α-lactalbumin compared with the control, it can be concluded that β-lactoglobulin was adsorbed preferentially over α-lactalbumin onto HA particles.

The amount of adsorbed individual proteins was calculated from the composition of the supernatants in the different individual proteins determined by SDS-PAGE (gels shown in Figure 4.1 and Figure 4.2), using an estimated composition of the initial SC and WPI solutions, based on the theoretical proportions of the individual proteins in skim milk (Wong et al., 1996). The initial SC solution contained approximately 50% α_S-casein (α_{S1} + α_{S2}), 38% β-casein and 12% κ-casein, and the initial WPI solution contained approximately 80% β-lactoglobulin and 20% α-lactalbumin.

Figure 4.6 shows the average amounts (from three repeated experiments) of the individual caseins and whey proteins adsorbed onto HA particles when the adsorption study was carried out with SC and WPI in water, as a function of the initial protein concentration, for SC (Figure 4.6A) and WPI (Figure 4.6B). The amounts of adsorbed α_S-casein (α_{S1} + α_{S2}) and β-casein increased gradually with initial protein concentration up to about 2.8% (w/w) and reached maximum coverage values of ~1.4 mg/m^2 for α_S-casein (α_{S1} + α_{S2}) and ~1.2 mg/m^2 for β-casein (Figure 4.6A). κ-Casein bound to a maximum amount of ~0.2 mg/m^2. For WPI, the amount of adsorbed β-lactoglobulin and α-lactalbumin increased gradually until they reached a maximum of respectively ~1.2 and 0.2 mg/m^2 (Figure 4.6B).

The percentages of each casein or whey protein in the adsorbed layer were calculated from the adsorption data represented in Figure 4.6, and are given in Table 4.2 and Table 4.3. The percentages of adsorbed proteins were compared with the percentage of proteins in the SC and WPI controls, to determine whether the proteins adsorbed in the same ratio as in the original protein solutions or whether there was a preferential adsorption of some of the proteins.

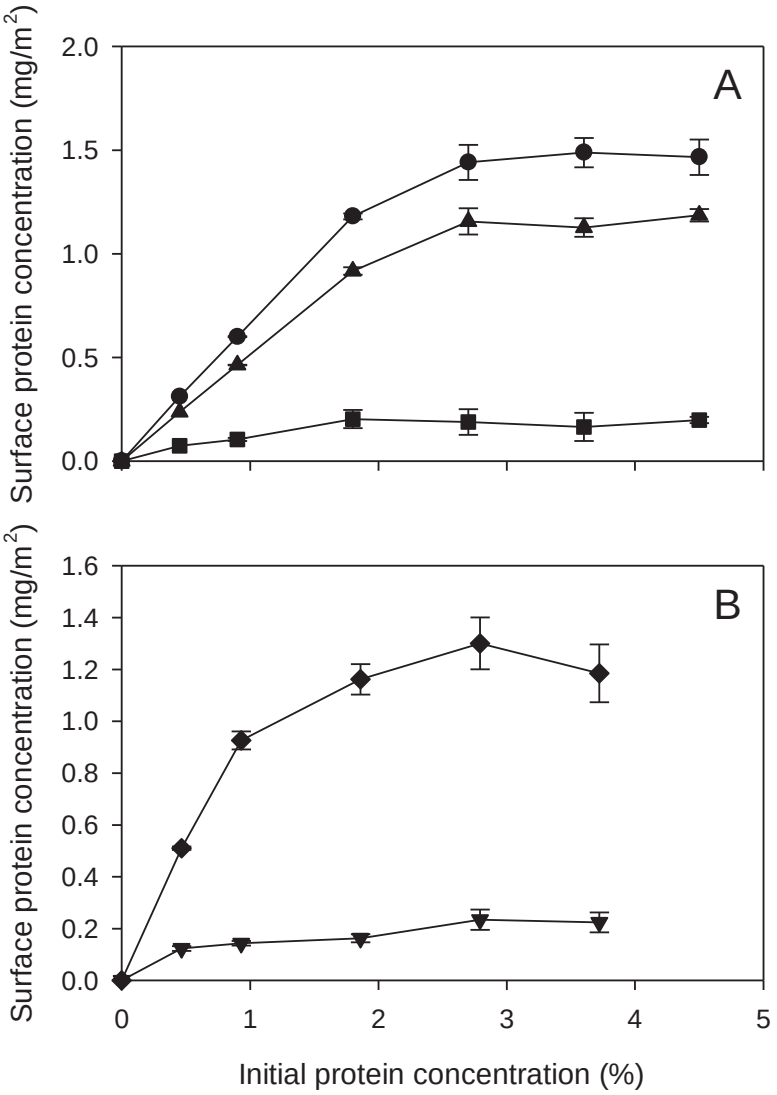

Figure 4.6: Estimated surface protein concentrations (mg/m²) of (A) individual caseins from SC and (B) individual whey proteins from WPI, as a function of initial protein concentration: ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin. Each data point is the average of two to three replicates; error bars represent standard deviation.

Table 4.2: Relative proportions of individual caseins adsorbed onto HA particles at different initial protein concentration of SC. [a]

Initial protein concentration (%)	α_S-casein (α_{S1} + α_{S2}) (%)	β-casein (%)	κ-casein (%)
SC control*	50	38	12
0.45	50.0 ± 0	38.0 ± 0	12.0 ± 0
0.90	51.4 ± 0.27	39.7* ±0 .3	8.9* ± 0.6
1.80	51.3 ± 1.06	39.9 *± 0.77	8.8* ± 1.6
2.70	51.8 ± 1.96	41.5* ± 0.83	6.7* ± 1.8
3.60	53.5* ± 1.53	40.6 ± 2.7	5.9* ± 2.2
4.50	51.4 ± 0.64	41.6* ± 0.9	7.0* ± 0.2

[a] Means with asterisk within a column differ significantly from the control (P <0.05). A 1-sample T-test was used to calculate the 95% confidence interval of the percentage values. If the confidence interval of a percentage for one given adsorbed protein did not include the percentage value of the same given protein in the control, the percentage of adsorbed protein was considered significantly different from the respective percentage in the control.

* Known values were used to characterise the proportions of the different proteins in the SC control: the SDS-PAGE bands of the control for α_s-, β- and κ-casein were attributed the theoretical percentages of the different caseins in milk. Therefore, no error is associated with the control data.

Table 4.3: Relative proportions of individual whey proteins adsorbed onto HA particles at varying initial protein concentration of WPI. [a]

Initial protein concentration (%)	β-Lactoglobulin (%)	α-Lactoglobulin (%)
WPI control*	80	20
0.47	80.9 ± 1.22	20 ± 1.22
0.93	86.4* ± 0.29	13.6* ± 0.29
1.86	87.4* ± 0.63	12.6* ± 0.63
2.79	84.1* ± 1.34	15.9* ± 1.34
3.72	84.1* ± 1.65	15.4* ±1.65

[a] Means with asterisk within a column differ significantly from the control (P <0.05). A 1-sample T-test was used to calculate the 95% confidence interval of the percentage values. If the confidence interval of a percentage for one given adsorbed protein did not include the percentage value of the same given protein in the control, the percentage of adsorbed protein was considered significantly different from the respective percentage in the control.

* Known values were used to characterise the proportions of the two whey proteins in the WPI control: the SDS-PAGE bands of the control for β-lactoglobulin and α-lactalbumin were attributed the theoretical percentages of these proteins in whey. Therefore, no error is associated with the control data.

At low initial protein concentration, the relative proportions of adsorbed α_S- (α_{S1} + α_{S2}), β- and κ-casein were similar to the proportions of the individual caseins in the SC control, and were, respectively, ~50%, 38% and 12% for α_S- (α_{S1} + α_{S2}), β- and κ-casein (Table 4.2). This is because at low protein concentration, protein concentration is the limiting factor for adsorption, and all the proteins present in solution adsorb onto the particles. As the initial protein concentration increased, the relative proportion of adsorbed κ-casein significantly decreased to a minimum of about ~6%, and therefore became lower than its relative proportion in the initial SC (12%), whereas the relative proportion of adsorbed β-casein significantly increased to a maximum of ~42% and therefore became higher than its relative proportion in the initial SC (38%). α_S-Casein (α_{S1} + α_{S2}) adsorbed in the same proportion as its respective proportion in the SC control (~50%) for all initial protein concentrations. As the proportion of adsorbed β-casein increased at the expense of adsorbed κ-casein, it means that β-casein was adsorbed preferentially to κ-casein. As there was no change in the proportion of adsorbed α_S-casein, it appears that there was no preference for adsorption between β-casein and α_S- casein.

The preferential adsorption of β-casein and α_S-casein onto the HA surface may be related to the number and distribution of the phosphoserine residues on the casein molecules. α_{S1}-casein, α_{S2}-casein, and β-casein contain, respectively, 7–9, 10–13, and 5 phosphoserine residues, that are grouped in clusters, whereas κ-casein contains only one phosphoserine residue (Walstra & Jenness, 1984). As α_S- and β-caseins carry more residues capable of binding to the C-sites on HA particles than does κ-casein, they must be able to bind to a greater extent than κ-casein. The grouping of the residues in clusters may also explain the preferential adsorption of β-casein and α_S-casein, compared with κ-casein, as proteins that have domains of clustered negatively charged residues have been reported to have much stronger affinities for hydroxyapatite (Gorbunoff & Timasheff, 1984a; Johnsson et al., 1993). It was interesting to note from these first results, it did not look like there was a preferential adsorption between αS- and β-caseins, even though the differences in the number and distribution of the phosphoserine residues in their sequences were different.

For WPI experiments, at low initial protein concentration, the relative proportions of adsorbed β-lactoglobulin and α-lactalbumin were similar to the proportions of the individual whey proteins in the WPI control, and were ~80% and 20%, respectively, for β-lactoglobulin and α-lactalbumin (Table 4.3). However, with increasing initial protein concentration, the proportion of adsorbed β-lactoglobulin increased to >80%, whereas the proportion of adsorbed α-lactalbumin decreased to <20%. As the proportion of adsorbed β-

lactoglobulin increased at the expense of adsorbed α-lactalbumin, it could be concluded that β-lactoglobulin was adsorbed preferentially to α-lactalbumin.

The preferential adsorption of β-lactoglobulin over α-lactalbumin must be related to the differences in the number and distribution of the charged residues within the whey protein molecules. It has been shown previously that β-lactoglobulin has a stronger affinity for HA compared with other acidic proteins (Gorbunoff & Timasheff, 1984b). This was attributed to the presence in its sequence of clusters of carboxyl groups, located on two glutamic acids in amino acids 44-45, two glutamic acids and one aspartic acid in amino acid residues 129-131 and two glutamic acids in amino acid residues 157-158. The first two clusters are located on the β-strand B and the α-helix of the protein (Sawyer, 2003), and are therefore easily accessible for binding onto HA, since they are located on its outer surface (See Figure 2.10 in the literature review). The self-association properties of β-lactoglobulin and α-lactalbumin could also explain the preferential adsorption of β-lactoglobulin over α-lactalbumin. At the pH value of this study (milk pH, about 6.8), β-lactoglobulin exists mostly as dimers (Sawyer, 2003; Verheul et al., 1999), whereas α-lactalbumin exists mostly in monomer form (Kronman et al., 1967). Therefore, if β-lactoglobulin and α-lactalbumin bind onto HA in their respective monomeric and dimeric forms, twice as much β-lactoglobulin would bind compared with α-lactalbumin to each binding site.

Kinetics aspects could also explain the preferential adsorption of β-lactoglobulin over α-lactalbumin, or of α_S- and β-caseins over κ-casein. As there is more β-lactoglobulin than α-lactalbumin in WPI, and more α_S- and β-caseins than κ-casein in SC, β-lactoglobulin and α- and β-caseins may access the HA surface first. In this case, the adsorption of each protein would be determined primarily by the order and the rate of arrival at the interface, rather than by a difference in their molecular structures (Dickinson, 2011). Further investigation of the adsorption of individual proteins is therefore needed to fully understand the differences in levels of surface coverage and the preference in adsorption between the different milk proteins on HA. The adsorption of individual caseins and whey proteins will therefore be studied in Chapter 5.

The ionic strengths of SC and WPI solutions were different. It was estimated that 1% (w/w, total solids) SC and WPI solutions had ionic strengths of respectively 4.5 mM and 1 mM, by measuring their electrical conductivity against standard solutions of NaCl. One problem with studying the adsorption of SC onto HA by adding a constant amount of HA in SC solutions of varying concentrations was that the ionic strength might have varied between

the different samples. SC and WPI protein solutions in the range 0.25 to 5% (w/w, total solids) were used; therefore the ionic strengths were estimated to vary from about 1.1 mM to 23 mM for SC and 0.25 to 6 mM for WPI, between the solutions at the lowest and the highest concentration.

It is well-known that ionic strength has an effect on the amount of protein adsorbing onto HA (Kandori et al., 2004; Nakanishi et al., 2001) and on the self-association behaviour of the caseins (Schmidt & Van Markwijk, 1968; Swaisgood, 2003), which can also affect the surface protein concentration and composition (Mulvihill & Murphy, 1991; Srinivasan et al., 2000). Another experiment on preferential adsorption of caseins in SC and whey proteins in WPI was therefore carried out by adding a constant amount of SC or WPI in various amounts of HA, to verify the characteristics of adsorption observed in the first adsorption experiment.

4.4.1.2 Different amounts of HA added to a constant amount of protein

The second adsorption experiment was carried out to characterise the preferential adsorption between the different proteins in SC and WPI onto HA particles. Instead of keeping the amount of HA constant and varying the protein concentration as in the first experiment, various amounts of HA (0.1 to 3%, w/w) were added to protein solutions containing a constant amount of SC or WPI (0.1%, w/w, total solids). Figure 4.7 shows the SDS-PAGE gels obtained from these supernatant samples. For a constant amount of protein in solution, the amount of protein in the supernatant decreased for both SC samples (Figure 4.7A) and WPI samples (Figure 4.7B), as the ratio of HA to protein increased, as shown by the decrease of the protein band intensities with increasing HA concentration from lane 1 to lane 9 in both figures. The SDS-PAGE results also showed that the ratios between the band intensities of the different caseins or whey proteins were different in the samples containing HA (Figure 4.7A and Figure 4.7B, lanes 2 to 9) compared with the sample containing only protein (controls, in lane 1). For SC, the intensity of the β-casein band decreased faster than the intensities of the α_S-casein and κ-casein bands, showing that β-casein was preferentially adsorbed onto HA particles. β-Casein had almost entirely disappeared from the supernatant of the sample made with 1.25% (w/w) HA (Figure 4.7A, lane 7) whereas α_S-casein (α_{S1} + α_{S2}) was still present. All the available α_S-casein was bound to HA at HA concentrations greater than 1.5% (w/w). A significant amount of κ-casein remained in the supernatant of the sample made with 2% (w/w) HA, as shown by the κ-casein band intensity (Figure 4.7A, lane 9), indicating that κ-casein was the least adsorbed among the caseins.

Similarly, there was a preferential adsorption of β-lactoglobulin compared with α-lactalbumin for samples prepared with WPI, as the band intensities of β-lactoglobulin decreased faster than the band intensities of α-lactalbumin (Figure 4.7B). Almost all of the available β-lactoglobulin was bound to the HA surface when 2% (w/w) HA was added to the WPI control, as shown by the very faint intensity of the β-lactoglobulin band (Figure 4.7B, lane 9), whereas some α-lactalbumin remained in the supernatants. This experiment confirmed the preferential adsorption of β-lactoglobulin over α-lactalbumin already observed in the previous experiment.

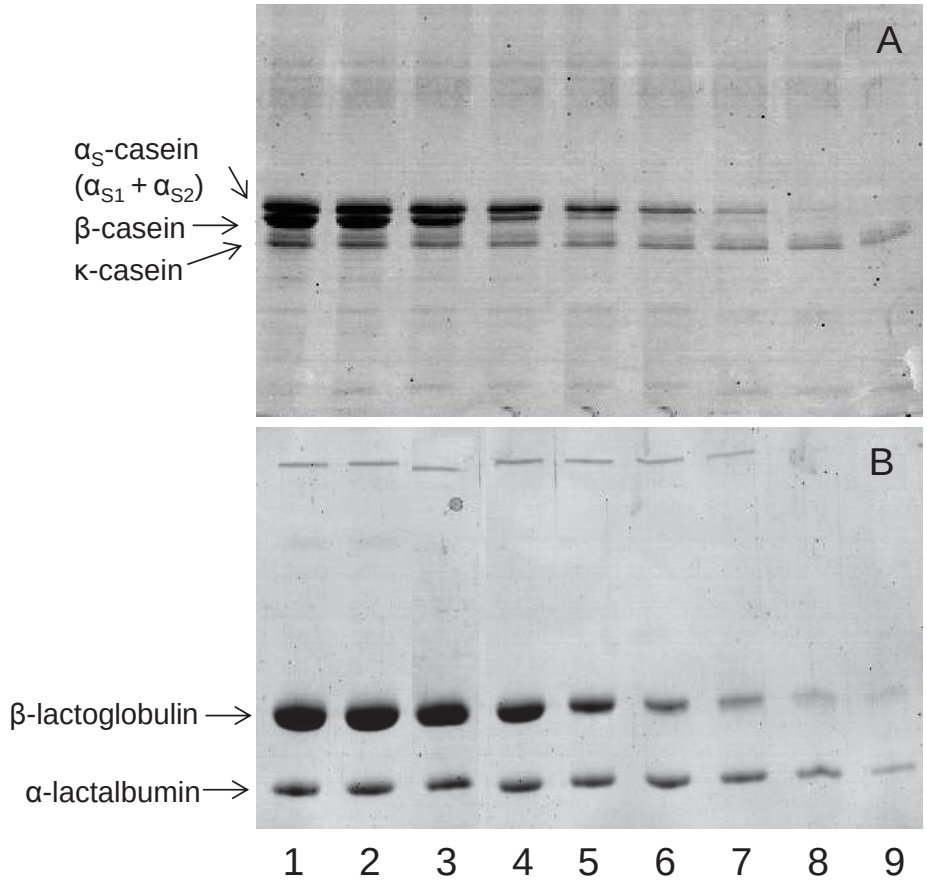

Figure 4.7: SDS-PAGE gels of the supernatants of samples containing (A) 0.1% (w/w) SC and (B) 0.1% (w/w) WPI and different HA concentrations. Lane 1, no added HA; lanes 2–9, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2% (w/w) added HA, respectively.

The amount of SC or WPI adsorbed to the surface of the HA particles was calculated by differences between the protein concentration in the control (lanes 1) and the protein concentration of the supernatants (lanes 2 to 9), for each HA concentration. Figure 4.8

shows the surface protein concentrations of SC (Figure 4.8A) and WPI (Figure 4.8B) onto HA particles, as a function of the initial HA concentration, when varying concentrations of HA particles were added in a constant amount of SC and WPI (0.1%, w/w, total solids). As the concentration of HA particles decreased from 2% to 0.1% (w/w), the amount of adsorbed protein per square meter of HA surface increased gradually for SC (Figure 4.8A) and WPI (Figure 4.8B). For high HA to protein ratios (right side of the curve), a large amount of HA surface was available for adsorption and the protein could not fully cover the HA particles; therefore protein concentration was the limiting factor. As the HA to protein ratio decreased, less HA surface was available, and maximum coverage values of approximately 2.3 mg/m^2 and 1.3 mg/m^2 were reached for SC and WPI respectively, for HA concentrations of respectively 0.25% (w/w) for SC and 0.75% (w/w) for WPI. However, the plateau value was variable between repeated experiments, and comprised between approximately 1.9 to 2.5 mg/m^2 for SC (Figure 4.8A) and 0.7 to 1.5 mg/m^2 for WPI (Figure 4.8B).

The absolute value of the maximum of adsorption was difficult to determine accurately, because the level of unadsorbed protein in the supernatants reached levels that were higher than 90% of the initial protein (see first three lanes of the SDS-PAGE gels in Figure 4.7). At these levels of unadsorbed protein, the depletion method used to calculate the surface protein concentration becomes imprecise and a difference of even one percentage unit in the determination of the percentage of unadsorbed protein can lead to large differences in the calculated amount of adsorbed protein. However, despite the imprecision in the determination of the plateau value, the maximum amount of adsorbed WPI obtained in this experiment was very close to the value obtained in the first adsorption experiment (constant amount of HA, varying concentrations of WPI, Figure 4.3), with values of respectively 1.5 mg/m^2 in the first experiment and 1.3 mg/m^2 in this experiment. On the other hand, the maximum amount of SC adsorbed onto HA particles obtained in this experiment (2.3 mg/m^2) was lower than the maximum amount obtained previously in the first experiment (approximately 2.9 mg/m^2, Figure 4.3A).

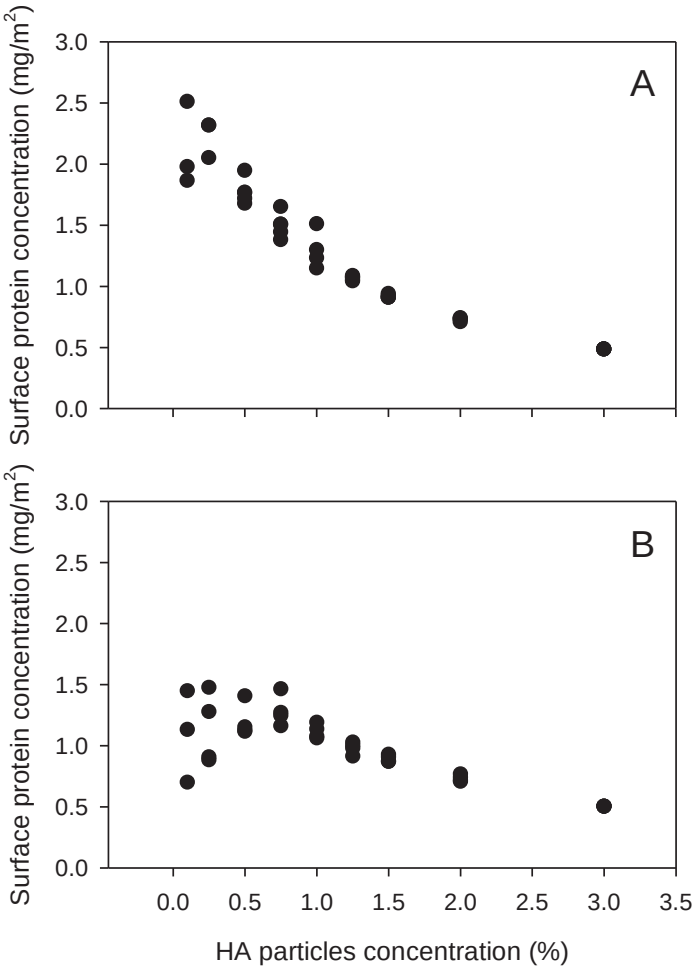

Figure 4.8: Surface protein concentration (mg/m²) of (A) caseins from SC and (B) whey proteins from WPI on HA particles. Values calculated from SDS-PAGE data, as a function of initial HA concentration; all experimental data points are shown, from three repeated experiments.

The first experiment used concentrations of SC ranging from 0.25% to 5% (w/w, total solids), corresponding to ionic strength varying from 5 to 23 mM, whereas the second experiment used a very low concentration of SC, of 0.1% (w/w, total solids), corresponding to a very low ionic strength (<1 mM). These differences in protein concentration and ionic strength may explain the difference observed in the maximum amounts of adsorbed caseins obtained for both experiments. It has been shown that the amount of caseins from SC that can adsorb onto surfaces depends on both the protein concentration and ionic strength (Lucey et al., 2000; Mulvihill & Murphy, 1991; Srinivasan, Singh, & Munro, 1996). It is well-known that caseins in SC can form large casein particles or aggregates, consisting of a mixture of partially aggregated β-casein, α_S-casein and κ-casein (Srinivasan et al., 1996). The composition of these aggregates is not fully understood but is believed to vary with ionic

strength and protein concentration (Lucey et al., 2000; Srinivasan et al., 1996). Depending on the initial protein concentration, caseins may either bind onto HA as individual molecules or as aggregates of associated caseins of different composition. For example, at low protein concentrations, caseins may possibly adsorb as individual molecules, whereas at high protein concentration, they may adsorb as small aggregates, thereby causing an increase in the maximum amount of proteins that can bind to HA. The difference in ionic strengths between the two experiments might also explain the difference in the maximum amount of adsorbed protein. Acidic proteins have been shown to bind more onto HA as ionic strength increases, because of more favourable electrostatic interactions between HA and protein (Yin et al., 2002; Zhu et al., 2007). The difference between the two maximum values obtained for SC adsorption in the two experiments carried out with SC could therefore have been due to differences in protein concentration and/or ionic strength between the two experiments. The effect of ionic strength and protein concentration on casein and whey protein adsorption on HA will be studied in detail and discussed in Chapters 5 and 6.

Figure 4.9 shows the average amounts of the individual caseins and whey proteins adsorbed onto HA particles as a function of the initial HA particle concentration, when the adsorption study was carried out with a constant amount of SC and WPI in water and varying amounts of HA particles, for SC (Figure 4.9A) and WPI (Figure 4.9B). The percentages of each casein or whey protein in the adsorbed layer were calculated from the adsorption data represented in Figure 4.9 for the different HA particle concentrations used in the experiment, and are given in Table 4.4 for SC and Table 4.5 for WPI. The amounts of adsorbed α_S-casein (α_{S1} + α_{S2}) and β-casein increased gradually with HA particle concentration decreasing from 3% to 1% (w/w) and reached a coverage values of ~1 mg/m^2 for both α_S-casein (α_{S1} + α_{S2}) and β-casein (Figure 4.9A). κ-Casein adsorbed to a maximum amount of ~0.2 mg/m^2 for HA concentration of 0.1% (w/w).

The variability of the surface protein concentration values could again be due to the concentration having been determined using the depletion method. The relative percentages of each casein or whey protein in the adsorbed layer were calculated from the adsorption data represented in Figure 4.9 and are given in Table 4.4. The relative proportion of adsorbed β-casein increased from 39% at 3% (w/w) HA to about 50% for low HA concentrations (e.g., 0.25 and 0.5% (w/w) HA), at the expense of both the proportions of adsorbed α_S-casein and κ-casein, that decreased from, respectively, 50% to 46% α_S-casein and 11.5% to about 7% for κ-casein when HA concentration decreased from 3% to 0.25% (w/w) (Table 4.4). At low HA concentrations, in the range where protein concentration was

not the limiting factor for adsorption, the proportions of adsorbed β-casein onto HA particles were significantly higher than its original proportion in SC, i.e., 38% (w/w), whereas the proportions of adsorbed α_S-casein and κ-casein were significantly lower than their original proportions in SC, i.e., 50% and 12% (Table 4.4). This confirmed that in this second adsorption experiment, β-casein adsorbed preferentially over α_S-casein (α_{S1} + α_{S2}) and κ-casein, as already observed on the SDS-PAGE gels (Figure 4.7A).

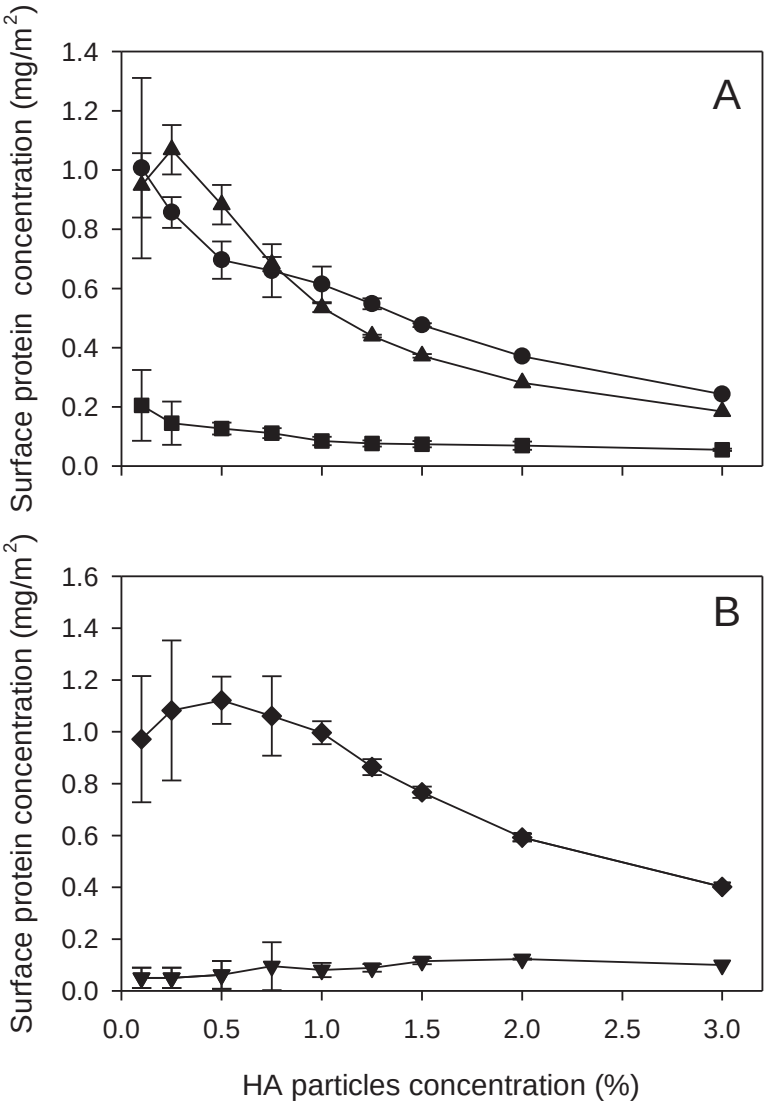

Figure 4.9: Estimated surface protein concentrations (mg/m²) of (A) individual caseins from SC and (B) individual whey proteins from WPI, as a function of initial HA concentration. ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin. Each data point is the average of two to three replicates; error bars represent standard deviation.

The geometric distribution of the phosphoserine residues of α_S-casein and β-casein is different, which may explain the preferential adsorption of β-casein observed in this experiment (Luo & Andrade, 1998). Even though α_S-casein contains more phosphoserine residues than β-casein, the phosphoserine residues of β-casein are all grouped at the N-terminus end of the molecule, making it highly negatively charged, compared with the rest of the molecule that carries no net charge. This type of distribution may therefore favour the adsorption of β-casein.

Table 4.4: Relative proportion of individual caseins adsorbed onto HA particles at different initial HA concentration, with a constant initial SC protein concentration (0.1%, w/w). [a]

Initial HA concentration (%)	α_S-Casein (α_{S1} + α_{S2}) (%)	β-Casein (%)	κ-Casein (%)
0 (SC control)	50	38	12
0.1	46.3* ± 4.8	46.1* ± 4.2	8.0* ± 2.4
0.25	41.4* ± 1.4	51.7* ± 4.1	7.6* ± 3.2
0.5	40.8* ± 0.8	51.7* ± 1.5	6.9* ± 1.4
0.75	45.3* ± 2.5	47.1* ± 2.3	7.7* ± 0.8
1	49.7* ± 1.8	43.4* ± 1.8	6.9* ± 1
1.25	51.5* ± 0.64	41.3* ± 0.9	7.2* ± 0.9
1.5	51.6* ± 0.5	40.4* ± 0.7	8.0* ± 1.1
2	51.4 ± 1.0	39.1 ± 0.8	9.6 ± 1.8
3	50.3 ± 3.3	39.5 ± 2.5	11.5 ± 0.7

[a] Means with asterisk within a column differ significantly from the control (P <0.05). A 1-sample T-test was used to calculate the 95% confidence interval of the percentage values. If the confidence interval of a percentage for one given adsorbed protein did not include the percentage value of the same given protein in the control, the percentage of adsorbed protein was considered significantly different from the respective percentage in the control.

It was also interesting to note that the results on preferential adsorption between β-casein and α_S-casein were different for both adsorption experiments. β-Casein was preferred for adsorption onto HA over α_S-casein when the protein concentration and ionic strength were low and maintained constant between the different samples (Table 4.4), whereas there was no preference for adsorption between β-casein and α_S-casein when the ionic strength levels were higher and varied between samples at both low and high protein concentration (Table

4.2). This may be due to β-casein and α_S-casein adsorbing onto HA as self-associated molecules at low initial protein concentration, whereas at high initial protein concentration, SC aggregates may be present. β-casein is known to self-associate to form a micelle-like structure, with the hydrophobic tails of the molecule buried inside the micelle and the hydrophilic heads sticking out (Schmidt, 1982). As the hydrophilic heads carry the phosphoserine residues, the phosphoserine residues in the β-casein micelles may be readily available for adsorption, leading to a high load of the HA surface with β-casein. However, it is also possible the caseins may bind onto HA as SC aggregates in both cases (low and high initial protein concentrations and ionic strengths), but that the composition of the aggregates might change (HadjSadok et al., 2008). β-Casein-rich aggregates may bind at low levels of protein concentration and ionic strength (as observed in the second experiment), whereas α_S-casein-rich aggregates may bind at higher levels of protein concentration and ionic strength (as observed in the first experiment). It was hypothesised from these results that an increase in ionic strength might favour the adsorption of α_S-casein at the expense of β-casein. This hypothesis will be further investigated in Chapters 5 and 6.

For WPI, the amount of adsorbed β-lactoglobulin and α-lactalbumin increased gradually with HA concentration decreasing from 3% to 1% (w/w), until they reached a maximum of ~1.1 and 0.1 mg/m^2, respectively (Figure 4.9B). These maximum levels of adsorption were very close to the maximum levels obtained in the first experiment, i.e., ~1.2 and 0.2 mg/m^2 for β-lactoglobulin and α-lactalbumin, respectively (Figure 4.3B). The relative percentage of adsorbed β-lactoglobulin increased as HA particle concentration decreased, and was higher than its original percentage in WPI (80%) for HA concentrations between 0.1 and 2% (w/w) (Table 4.5), showing again that β-lactoglobulin was preferred for adsorption over α-lactalbumin. As WPI has a low ionic strength, there was no major difference in ionic strength between the two experiments, and therefore no major differences in the adsorption results.

Table 4.5: Relative proportion of individual caseins adsorbed onto HA particles at different initial HA concentrations, with a constant initial WPI protein concentration (0.1%, w/w). [a]

Initial HA concentration (%)	β-Lactoglobulin (%)	α-Lactoglobulin (%)
0 (WPI control)	80	20
0.1	94.6* ± 4.2	5.4* ± 4.2
0.25	95.1* ± 3.4	4.9* ± 3.4
0.5	95.1* ± 3.5	4.9* ±3.5
0.75	91.4* ± 5.7	8.6* ± 5.7
1	92.5* ± 2.7	7.5* ± 2.7
1.25	90.7* ± 1.4	9.3* ± 1.4
1.5	87.0* ±0.8	13.0* ± 0.8
2	87.0* ± 0.4	17.2* ± 0.4
3	80.0 ± 0	20.0 ± 0

[a] Means with asterisk within a column differ significantly from the control (P <0.05). A 1-sample T-test was used to calculate the 95% confidence interval of the percentage values. If the confidence interval of a percentage for one given adsorbed protein did not include the percentage value of the same given protein in the control, the percentage of adsorbed protein was considered significantly different from the respective percentage in the control.

4.4.2 Characterisation of the protein coated HA particles

4.4.2.1 Confocal microscopy

The HA pellets prepared with 0.5% (w/w) SC or WPI solutions were rinsed, re-suspended in water and observed by confocal microscopy using the Fast Green fluorescence channel and the DIC channel of the microscope. The samples were analysed both by using the Fast Green fluorescence channel only and by overlapping the fluorescence images with the DIC images. Fast Green dye specifically stains proteins.

Figure 4.10 shows the micrographs obtained for the re-suspended stained HA particles prepared with water (control, no protein), 0.5% (w/w) SC solution or a 0.5% (w/w) WPI solution. The control HA sample (a suspension of 0.05%, w/w, HA powder in water) did not show any signal (black screen), showing that Fast Green could not stain HA (Figure 4.10A), and confirming that no protein was present originally on the HA particles. However, the HA particles could still be seen on the DIC channel of the confocal microscope (Figure 4.10B). They looked roughly spherical, comprised between 1 and 10 μm in diameter, and their

surface topography was uneven and heterogeneous. The presence of surface "craters" on HA is a feature often observed on HA material, and is referred to as surface roughness (Müller-Mai, Voigt, & Gross, 1990; Stanciu et al., 2007).

Figure 4.10C and Figure 4.10E show typical images of HA particles prepared with SC and WPI, obtained on the Fast Green fluorescence channel. The HA particles were roughly spherical, with a black core surrounded by a green ring. As no green was observed in the control prepared with water (Figure 4.10A), it was concluded that the green colour must be due to the adsorbed casein and whey protein layers on the particles. Therefore, the confocal microscopy images clearly confirmed that milk proteins from both SC and WPI adsorbed at the surface of HA particles. The SC and WPI-coated particles looked similar. However, the intensity of the green rings was lower in the micrographs of the WPI-coated HA particles (Figure 4.10E) compared with that of the SC-coated HA particles (Figure 4.10C), probably because a lower amount of whey proteins was adsorbed on the surface of the HA particles, as shown in Section 4.4.1, therefore forming a thinner layer.

Figure 4.10D and Figure 4.10F were obtained by overlapping the Fast Green channel signal and the DIC channel signal. The presence of green patches on the white surface of the SC-coated HA particles suggested that the surface was not covered homogeneously (Figure 4.10D) and that proteins might be bound preferentially to certain regions of the surface or might have formed aggregates in certain regions, as suggested before in Section 4.4.1. The WPI-coated particles (Figure 4.10F) showed a more uniform coverage of the proteins, which is consistent with the characteristics of a monolayer coverage by globular proteins (Wahlgren & Arnebrant, 1991).

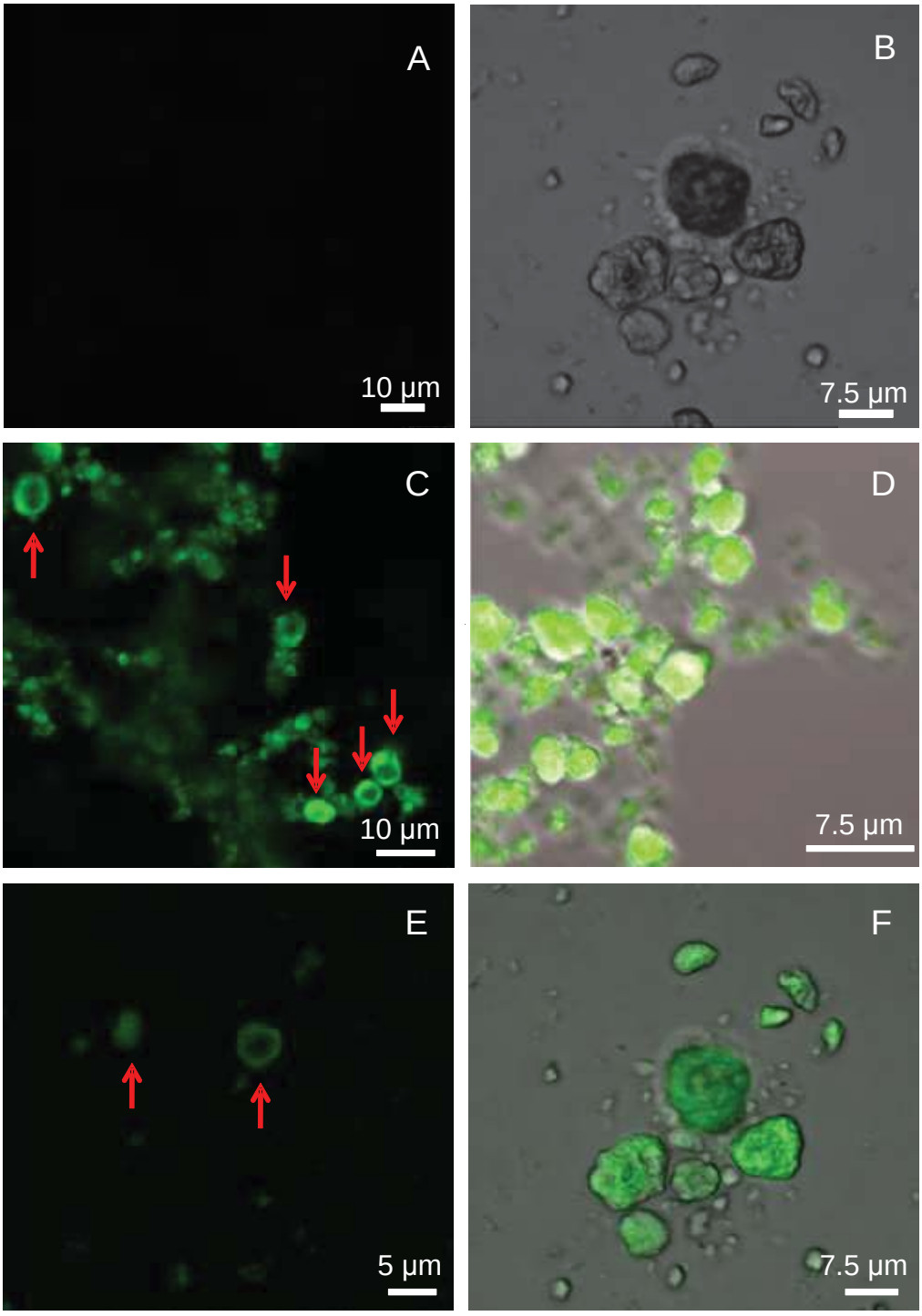

Figure 4.10: Confocal micrographs obtained for hydroxyapatite pellets prepared with water, SC or WPI solutions (0.5%, w/w, total solids), rinsed and re-suspended in water (0.05%, w/w) and stained with fast green. The HA particles were prepared with: A, water (no protein), fast green channel; B, water (no protein), DIC channel; C, SC, fast green channel; D, SC, overlapping of DIC and fast green channel; E, WPI, fast green channel; F, WPI, overlapping of DIC and fast green channel. The arrows show the HA particles covered by proteins.

4.4.2.2 Zeta-potential

Zeta-potential is a measure of the surface charge properties of the HA particles when suspended in aqueous solution (Yin et al., 2002). In this study, the term "increase in zeta-potential" is used to describe whenever the magnitude of the zeta-potential increases regardless of sign, i.e., when a negative zeta-potential becomes more negative, or a positive zeta-potential becomes more positive.

The HA particles were suspended in SC and WPI solutions of protein concentrations 0 to ~5.5% (w/w) and were stirred for 2 h to allow adsorption to occur. The suspensions were then centrifuged, and the pellets were removed, rinsed three times with water and re-suspended in water. HA was slightly negatively charged when suspended in water (about −11 mV). The changes in zeta-potential of the suspended pellets as a function of the initial protein concentration of the solutions are shown in Figure 4.11. After mixing with SC, the magnitude of the zeta-potential of the HA progressively increased from −11 to ~−28 mV with SC addition up to about 2% protein, but did not change further at higher addition levels of SC (Figure 4.11A). Similarly, for WPI, the magnitude of the zeta-potential progressively increased from −11 to ~−22 mV with the addition of up to 1.5% protein, with no further change at higher addition levels of WPI (Figure 4.11B).

The increase in the magnitude of the zeta-potential is probably a consequence of the protein adsorption on the HA particles. With respect to the initial protein concentration, the points at which the zeta-potential and the surface protein concentration reached a plateau value were coincident, as shown by comparing Figure 4.3 and Figure 4.11; the zeta-potential and surface protein concentration values reached their respective maximum values for the same initial protein concentration of about 2% protein for SC and ~1.5% protein for WPI. The correlation between zeta-potential and surface protein concentration was confirmed by the linear relationship between the two for both SC-coated particles and WPI-coated particles (Figure 4.12). The R^2 coefficients of 0.81 and 0.89 for SC and WPI data, respectively, showed that the experimental data were close to the fitted linear regression line. This correlation between the amount of adsorbed protein and the zeta-potential value proves that protein adsorption is responsible for the increase in surface charge of the HA particles. As both caseins and whey proteins are negatively charged at neutral pH, their adsorption on the surface of HA particles must increase the negative surface charge of the particles. It was shown before by Reynolds and Wong (1983) that the adsorption of milk proteins onto HA causes an increase of the HA surface charge. α_{S1}-Casein, β-casein, κ-caseins, β-lactoglobulin

and α-lactalbumin were shown to all adsorb onto HA at pH 7, increasing the zeta-potential from −9.1 mV to more negative values between −13.1 mV (for β-lactoglobulin) and −24.1 mV (for α_{S1}-casein).

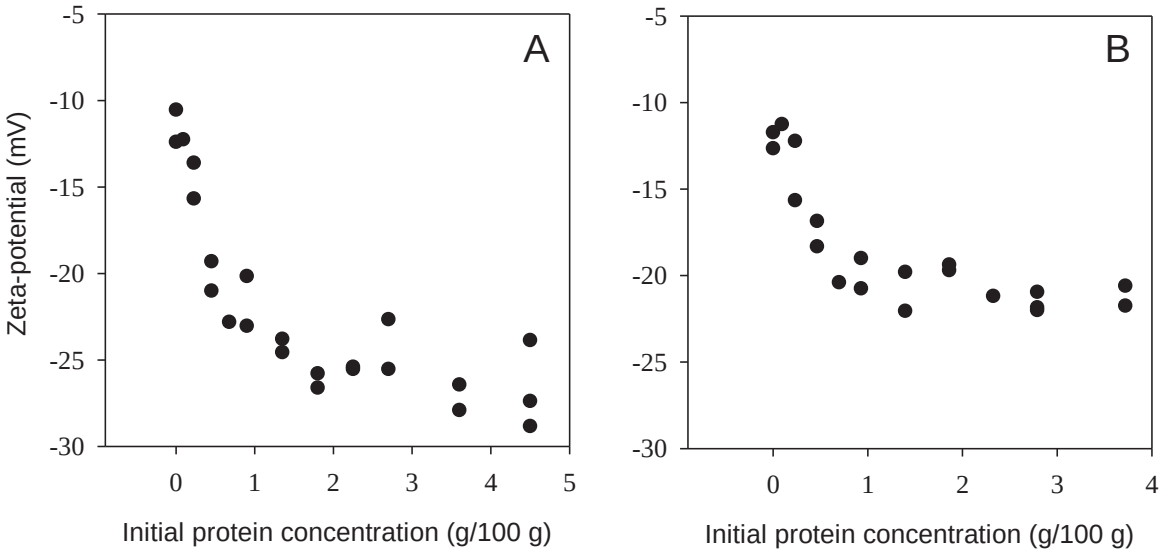

Figure 4.11: Effect of protein concentration on the zeta-potential of HA particles suspended in (A) SC or (B) WPI solutions of different initial concentrations. Suspensions were stirred for 2 h and centrifuged, and the pellets were then rinsed with water and re-suspended in water; all experimental data points are shown, from three repeated experiments.

As only a few negatively charged residues of the proteins are involved in the binding to the C-sites of the particles (Kawasaki et al., 1985), many negatively charged groups are still present in the adsorbed protein layer, resulting in a negative charge on the surface of the particles (Johnsson et al., 1993). The plateau value for pellets prepared with WPI (−22 mV) was lower in magnitude than that for pellets prepared with SC (−28 mV), which may reflect differences in the charge densities of the protein molecules. At neutral pH, the net charge of the caseins is more negative than that of the whey proteins (Chu, Ichikawa, Kanafusa, & Nakajima, 2008), which possibly explains the difference in zeta-potential once the proteins are adsorbed onto HA particles.

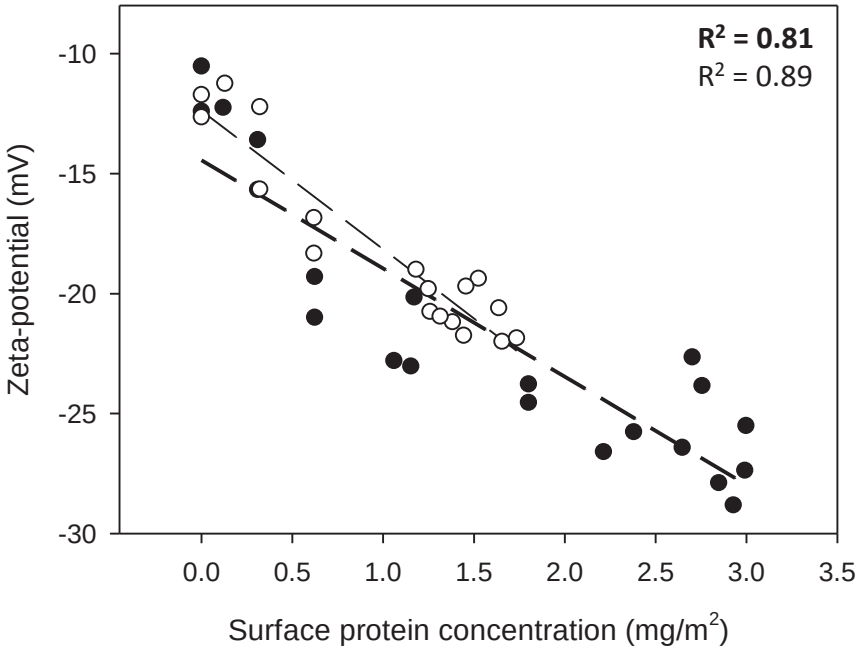

Figure 4.12: Linear relationship between the surface protein coverage of the protein-coated particles and the zeta-potential of the corresponding particles suspended in water. ●, SC-coated particles; ○, WPI-coated particles. The lines are linear regressions of the experimental points, bold line: SC, thin line: WPI.

4.4.2.3 Suspension stability

The pellets obtained after centrifugation of the particles prepared from a constant amount of HA particles added in 0.5 to 3% (w/w) SC solutions were rinsed twice with water then re-suspended in water (10%, w/w) and left undisturbed for 24 h at room temperature. Figure 4.13 shows photographs of the suspensions of these HA particles after 24 h. The number of particles remaining in suspension after 24 h increased with increasing initial SC concentration, as shown by increase in visual turbidity between the samples A to E in Figure 4.13. A similar pattern was observed for WPI-coated particles (images not shown).

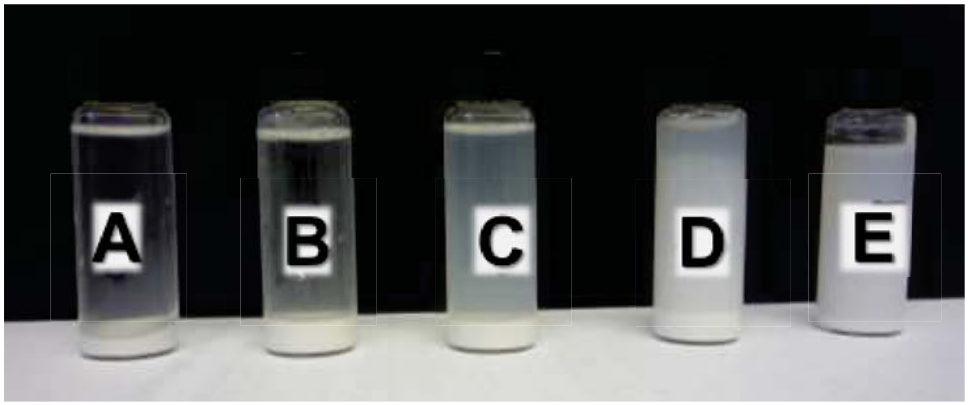

Figure 4.13: Suspension stability of SC-coated HA particles prepared with increasing concentrations of SC. Particles were mixed with SC, the HA pellets were centrifuged to obtain a pellet, the pellets were rinsed with water and resuspended in water. The initial SC concentrations (w/w) used to prepare the particles were: A, 0 (water); B, 0.5%; C, 1%; D, 2%; E, 3%.

This improvement in particle suspension was characterised by spectrophotometric measurements as a function of time. The particles prepared with different initial concentrations of SC and WPI were re-suspended in water at 0.125% (w/w) and the absorbance of the suspension was measured at 900 nm for 200 min. Figure 4.14 shows the reduction in absorbance with time of suspensions of HA particles made with 0, 0.5, 1, 2 and 3% (w/w) SC (Figure 4.14A) and WPI (Figure 4.14B). The reduction in absorbance was calculated using $(A_t/A_0) \times 100$, where A_0 is the initial absorbance and A_t is the absorbance at time t.

Within 200 min, the absorbance of a suspension of HA powder (control with no protein bound) decreased to less than 5% of its original value. All of the particles had sedimented to the bottom and the absorbance was close to the absorbance of the suspending medium (water). The absorbance of HA suspensions prepared with increasing amounts of SC or WPI (0.5 to 3%, w/w) decreased less rapidly compared with the control, and this effect increased with increasing surface protein concentrations. For samples prepared with low protein concentration (0.5%, w/w, SC and WPI, corresponding to surface protein concentration of about 0.6 mg/m^2 for both, see Figure 4.3), the improvement in suspension stability was better for WPI-coated particles than for SC-coated particles (Figure 4.14), as the suspensions had an absorbance of, respectively, 8% and 20% of the original absorbance value. However, the suspension stability of the HA particles that were fully covered by

proteins (maximum of adsorption reached for HA particles made with 3%, w/w, SC or WPI, see Figure 4.3) was better for particles covered with SC than for particles covered with WPI, as after 200 min, the absorbance of the suspension was respectively 45% and 35% of the original absorbance value for SC and WPI (Figure 4.14)

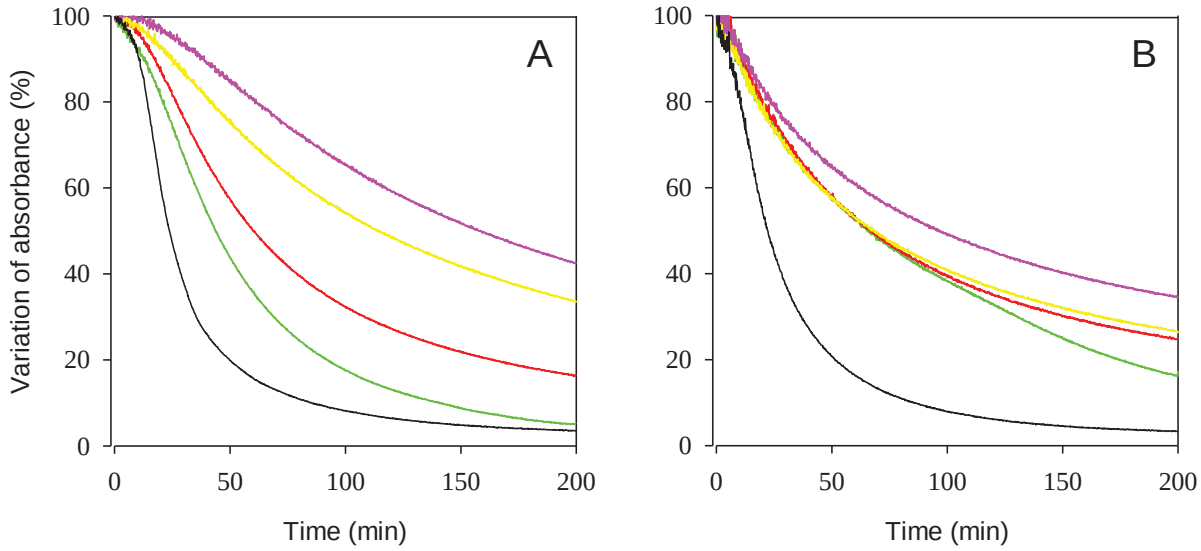

Figure 4.14: Variation of absorbance (% of initial absorbance) as a function of time of suspensions of HA particles (0.125%, w/w) made with water or solutions of different (A) SC or (B) WPI concentrations. Black line, water; green line, 0.5% (w/w); red line, 1% (w/w); yellow line, 2% (w/w); pink line, 3% (w/w).

Nano or micro-sized particles of HA are known to form spontaneous aggregates when suspended in aqueous media, mainly because of inter-particle van der Waals interactions and hydrogen bonding (Sadeghian et al., 2006). As the zeta-potential in water of the particles used in this study was low (−11 mV, see Figure 4.11), some aggregation between particles must have occurred, leading to an increase in their average size and a rapid sedimentation. The binding of proteins to the surface of the HA particles led to an increase in their surface charge, as shown by the correlation between zeta-potential and surface protein concentration (Figure 4.12). As the surface charge became more negative, the repulsive forces between the particles increased, rendering them less prone to aggregation and sedimentation over time (Figure 4.13 and Figure 4.14). This improvement in suspension stability can be referred to as electrostatic stabilisation. However, in the case of SC, steric stabilisation may also play a role. The difference in structure between caseins (linear flexible molecules) and whey proteins (globular molecules) usually explains why the adsorption of caseins can stabilise colloidal systems more against aggregation compared

with whey proteins (Dickinson, 2001; Marinova et al., 2009). By forming aggregates on adsorbing surfaces, caseins in SC have been shown to form thicker and denser layers than whey proteins, therefore providing steric stabilisation as well as an electrostatic stabilisation (Marinova et al., 2009). When caseins adsorb onto HA particles, the hydrophobic regions of the caseins may interact with each other, forming large aggregates on the particle surface. The negatively charged groups of the caseins that are not involved in the binding may protrude from the surface of the aggregates, forming a hairy layer that can provides steric hindrance between the particles, therefore providing a steric stabilisation against aggregation (Dickinson, 2001). The layer of SC on the HA particles has probably provided both electrostatic and electrosteric stabilisation to the particles, which may explain why the improvement in suspension stability was slightly better for SC-coated particles (Figure 4.14).

4.5 Conclusions

This chapter confirmed that both caseins and whey proteins adsorbed onto HA particles, using model protein solutions of SC and WPI in water. Methods to quantify and characterise the adsorption were developed. SDS-PAGE was proven to be a good technique for measuring by depletion the amount of adsorbed protein and the composition of the protein layer. The modelling approach (Langmuir and Langmuir-Freundlich models) was useful for characterising the adsorption with physically interpretable parameters that gave extra information on the structure of the adsorbed protein layer. The proposed adsorption mechanism was the formation of electrostatic complexes between the calcium ions expressed at the surface of the hydroxyapatite crystals (C-sites) and the negatively charged residues of the proteins, i.e., the side carboxyl groups of the whey proteins (on glutamic and aspartic acid residues) and the phosphoserine residues of the caseins, as illustrated in Figure 4.15.

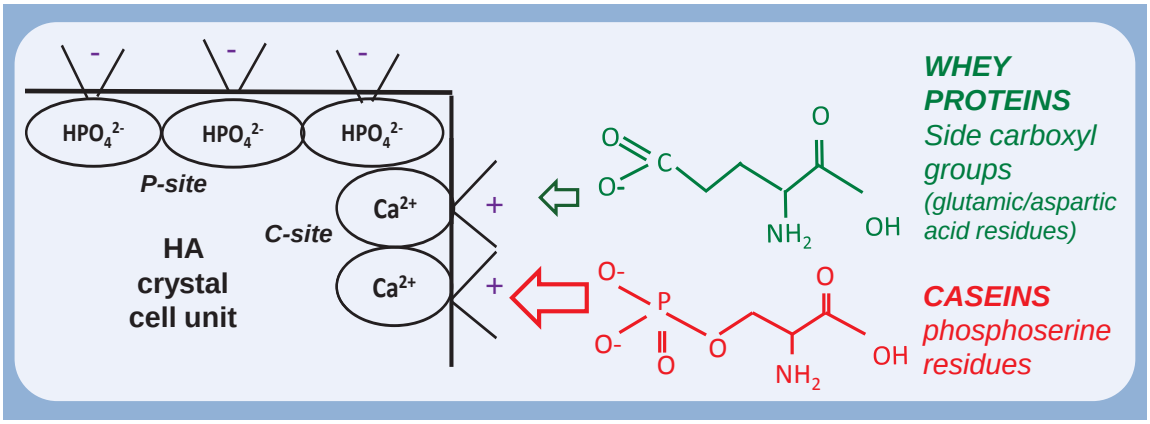

Figure 4.15: Proposed mechanism explaining the adsorption of whey proteins and caseins onto HA particles. Electrostatic complexes are formed between the protein negatively charged residues and the calcium ions on HA; the size of the arrows represents the strength of the interactions; the residue represented for whey protein is aspartic acid.

The adsorption of the milk proteins onto the HA particles changed the colloidal and suspension properties of the particles. The protein-coated particles were more negatively charged and had a slower sedimentation rate in water. Caseins were able to adsorb more at the HA surface, and stabilised the particles better against aggregation, compared with whey proteins, probably due to their combined electrostatic and steric stabilisation (electrosteric stabilisation), whereas whey proteins stabilised the particles only electrostatically, as illustrated in Figure 4.16. This suggested different structures and association behaviour of the adsorbed proteins in the adsorbed layers. Adsorbed caseins probably form aggregates or associate on the HA surface, therefore forming a thicker hairy layer on the surface of HA, compared with whey proteins, which probably adsorb in a close compact monolayer.

Not only do these initial results allow a greater understanding of the system formed by the hydroxyapatite salt when mixed in a simple model system containing milk proteins, they also demonstrate the possibility of modifying the surface properties of HA particles by adsorbing milk proteins on their surface. As the suspension stability of the particles was shown to improve, this could be potentially of interest, for example, as a way to increase the stability of HA particles when added to dairy (and non-dairy) beverages or in the development of solutions to control sediment formation and fouling on heat exchangers. For example, a functionalised HA ingredient stabilised by milk proteins could possibly be developed. The fabrication of milk protein-coated HA nanoparticles was briefly investigated

later in this study. No conclusive results could be drawn but some preliminary results are given in Appendix 1.

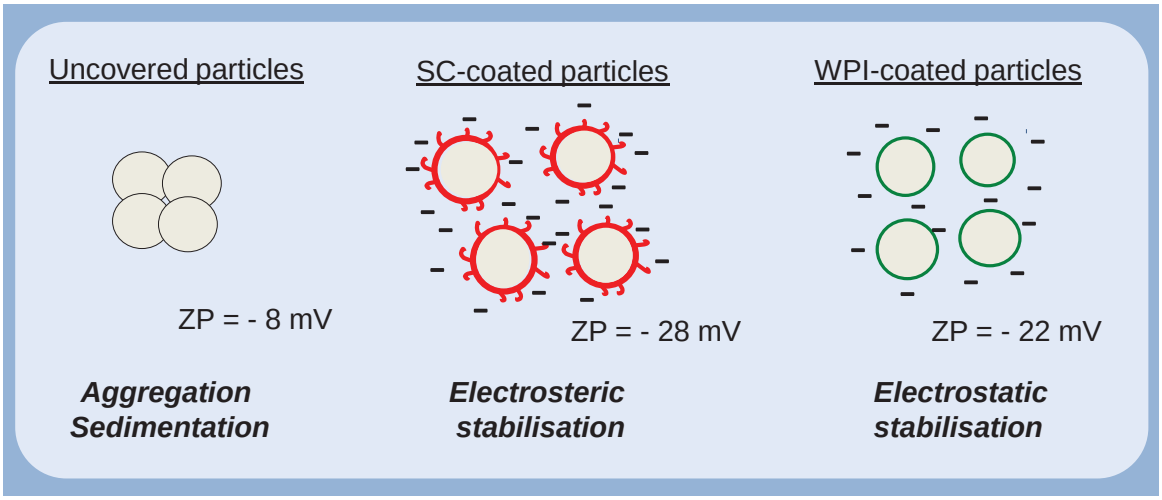

Figure 4.16: Proposed mechanism explaining the improvement of suspension stability observed for HA particles when caseins and whey proteins were adsorbed on the surface of the particles. Zeta-potential (ZP) values of the fully covered particles are indicated.

The aim of this research is to gain an understanding of the interactions occurring between milk proteins and HA particles, and to apply the knowledge obtained from model systems to milk systems. The next chapter will therefore look at the adsorption onto HA of the main individual milk proteins (α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin) both individually and mixed together in pure model systems to fully characterise and understand the adsorption mechanisms of each milk protein onto HA. The affinities of the individual milk proteins for HA will be compared, and any competitive adsorption between the different milk proteins when mixed together will be characterised. This will provide some fundamental understanding of the adsorption behaviour of milk proteins onto HA, that will underpin the ability to understand the interactions between milk proteins and HA in more complex systems that have physico-chemical characteristics closer to those of milk.

CHAPTER 5 Adsorption of individual milk proteins onto hydroxyapatite particles and competitive adsorption between milk proteins.

5.1 Abstract

The adsorption behaviour onto hydroxyapatite (HA) particles of the main types of milk proteins, α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin, was characterised both individually and in combinations. Individually, all proteins were able to bind onto HA, resulting in a decrease of the magnitude of the zeta-potential of the HA particles. The maximum amounts of protein that could bind onto HA and their affinity for HA were quantified using a Langmuir model. α_S-Casein and β-casein could bind onto HA at higher amounts than all of the other proteins onto HA and had a higher affinity for HA, probably because of the clusters of phosphoserine residues present in their structure. When the five proteins were mixed together, there was a preference for protein adsorption in the order: β-casein ≅ α_S-casein > κ-casein > β-lactoglobulin > α-lactalbumin. β-Casein displaced adsorbed β-lactoglobulin from the HA surface when added to a suspension of β-lactoglobulin-covered particles and adsorbed instead, most likely because the affinity of the casein phosphate groups for HA was stronger than that of the side carboxyl groups of the whey protein.

Ionic strength had a small effect on the adsorption of the individual milk proteins onto HA, with an increase in ionic strength leading to more protein adsorbing onto HA for all of the proteins studied. However, the increase in protein adsorption upon an increase of ionic strength was larger for α_S-casein than for the other proteins, probably due to a change in the aggregation state of α_S-casein with increasing ionic strength.

5.2 Introduction

The work carried out with sodium caseinate (SC) and whey protein isolate (WPI) in Chapter 4 demonstrated that the proteins contained in SC (α_S-, β- and κ-casein) and WPI (β-lactoglobulin and α-lactalbumin) adsorbed to a different extent onto hydroxyapatite (HA). Caseins in SC adsorbed to HA in water approximately twice as much as whey proteins in WPI. α_S-Casein (α_{S1}-casein + α_{S2}-casein) and β-casein were preferentially adsorbed over κ-casein, but the relative proportions of adsorbed α_S-casein and β-casein seemed to vary, depending on the initial protein concentration of SC, and on the ionic strength of the initial solution. For WPI, β-lactoglobulin was preferentially adsorbed over α-lactalbumin.

However, the caseins in SC and the whey proteins in WPI are present as mixed proteins in the same solution, in unequal proportions, and can interact with each other: for example, caseins in SC associate together to form aggregates (HadjSadok et al., 2008). Thus, the adsorption behaviour of a mixture of proteins is likely to be different from that of the individual protein components (Chu, Zhou, Wu, & Farrell, 1995). For example, the amount and type of proteins that can bind onto HA are likely to be different when using individual proteins than when using SC or WPI, as some competitive adsorption between the mixed proteins or some cooperative adsorption involving associations between the different adsorbed proteins on the HA surface might be involved (Dickinson, 2011). Also, when starting with a protein solution containing different proteins in unequal proportions, the adsorption of each protein from the protein solution may be determined primarily by the order and the rate of arrival at the interface (kinetics aspects), rather than by a difference in the structures and affinity of the proteins (Dickinson, 2011). In this case, the proteins present in lower initial amounts may reach the interface at a slower rate. Therefore, to fully understand the mechanisms of adsorption of milk proteins onto HA, it is logical to study the binding of purified isolated milk proteins onto HA, both individually and in mixtures containing equal amounts of each protein, so that each has the same initial probability of reaching the HA surface.

As α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin have different structural and physico-chemical properties, they are likely to have different binding affinities for HA, or/and bind to different maximum amounts (Nakanishi et al., 2001; Wahlgren & Arnebrant, 1991). For example, since the binding of proteins onto HA is mainly driven by specific electrostatic interactions between the carboxyl or phosphoserine groups of the proteins and the calcium groups on the HA, the net charge of the proteins, the type of charged residues

(side carboxyl groups or phosphoserine groups) and the distribution of these charged residues within the protein molecules, as determined by their primary, secondary and tertiary structures, may all play an important role in the binding of the different milk proteins onto HA (Wang et al., 2012). Also, for caseins in pure protein solutions, the individual caseins are expected to exhibit self-association behaviour, whereas in mixtures they might associate with each other rather than self-associating, and this may impact the amount and type of caseins that adsorb onto HA.

The first objective of this chapter was therefore to characterise the adsorption of the different types of caseins and whey proteins onto HA particles, both individually and in mixtures of equal amounts of caseins or whey proteins (a third of each casein, half of each whey protein), and to relate the results to the physico-chemical characteristics of the proteins. The isolated protein solutions, when reconstituted in water, had different pH values, comprised between 5.2 and 5.6 (measured data), i.e., lower than the natural pH of SC, WPI and milk (about 6.8). It was therefore decided to reconstitute the isolated protein powders in 50 mM HEPES buffers at pH 6.8, and at two different levels of ionic strengths (low ionic strength, ~7 mM, and high ionic strength, 100 mM). The low level of ionic strength (7 mM) was chosen so that the results could be compared with the results obtained for SC and WPI in water in Chapter 4. The high level of ionic strength (100 mM) was chosen to obtain physico-chemical conditions close to that of milk in terms of pH and ionic strength for all proteins, as the ionic strength of milk is approximately 80 mM (Gaucheron, 2005).

The second objective of this chapter was to look at the competitive adsorption between the different milk proteins. In the studies described in the previous chapters, the adsorption of caseins and whey proteins were studied separately, using SC for caseins and WPI for whey proteins, and SC and WPI were not mixed together. In the studies described in this chapter, the adsorption of α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin were studied from a protein solution containing each protein in equal amounts. The ability of caseins to displace the adsorbed whey proteins from the HA surface or of whey proteins to displace adsorbed caseins from the HA surface was also tested, by adsorbing first one type of protein (casein or whey protein) onto HA, and then adding the other type of protein.

The insights gained from these model systems would underpin the ability to understand interactions between milk proteins and HA in more complex systems and could therefore be the foundation for future studies in milk.

5.3 Material and methods

5.3.1 Adsorption of individual milk proteins

The adsorption of α_S-casein (α_{S1}-+ α_{S2}-), β-casein, κ-casein, β-lactoglobulin or α-lactalbumin onto HA was studied by adding 5% (w/w) HA particles to solutions containing different concentrations (0.1% to 5%, w/w, total solids) of the isolated protein powders (see Section 3.1.1.1), reconstituted in 50 mM HEPES buffer, pH 6.8, and adjusted to 7 mM or 100 mM ionic strength (see Section 3.2.2.3 for buffer preparation). The protein concentrations of the initial solutions were calculated based on the purity of the protein powders, as determined by UV spectroscopy at 280 nm (Sections 3.1.2.1 and 3.5.1). The protein concentration of the supernatants obtained after centrifugation of the HA particles was measured by UV spectroscopy at 280 nm (Section 3.5.1) and the amount of adsorbed protein was determined by difference between the protein concentration of the initial solution and that of the adsorption supernatants, as described in Section 3.2.4.2. The Langmuir model was fitted to the adsorption data, as detailed in Section 3.10.2. The HA pellets, obtained after protein adsorption and centrifugation of the HA particles, were re-suspended in the same HEPES buffer used for the adsorption experiment, and characterised for zeta-potential (Section 3.3.2).

To discuss the adsorption results in relation to the net charge of the protein, the protein sequences of the individual proteins (α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein, β-lactoglobulin or α-lactalbumin) were downloaded from the Universal Protein Resource database and the net charge of each was calculated at pH 6.8 using the Protein Calculator web-based program, as described in Section 3.9.

5.3.2 Adsorption of milk proteins from solutions containing equal amounts of different isolated proteins

To characterise the preferential adsorption between the isolated proteins in solution, 100 μL of solutions of fixed total protein concentration, containing a fixed amount of milk proteins, were added to 0.9 mL suspensions containing different amounts of HA particles (0.1 to up to 8%, w/w), in 50 mM HEPES pH 6.8 (7 mM or 100 mM ionic strength). The compositions of the protein solutions were as follows:

(i) Casein solution, containing 2% (w/w) total protein, with equal amounts of α_S-casein (α_{S1}-+ α_{S2}-), β-casein and κ-casein (33.3% of each).

(ii) Whey protein solution, containing 0.8% (w/w) total protein, with equal amounts of β-lactoglobulin and α-lactalbumin (50% of each).

(iii) Mixed solution containing 2% (w/w) total protein, with equal amounts of α_S-casein (α_{S1}-+ α_{S2}-), β-casein, κ-casein, β-lactoglobulin and α-lactalbumin (20% of each).

The protein composition of the supernatants obtained after centrifugation of the HA particles was determined using microfluidic chip- (MF-) electrophoresis (Section 3.5.4). MF-electrophoresis was selected over traditional sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein quantification, because of the rapidity of the method, and its greater precision for quantifying whey proteins.

5.3.3 Displacement of proteins

Only β-casein and β-lactoglobulin were studied in this experiment. β-Casein or β-lactoglobulin-coated HA particles were prepared by adding 5% (w/w) HA particles to 2% (w/w, total solids) β-casein or β-lactoglobulin solutions, in 50 mM HEPES buffer, pH 6.8, 100 mM ionic strength. The HA particles were centrifuged and rinsed three times with 50 mM HEPES buffer, pH 6.8, 100 mM ionic strength, to remove all the unadsorbed protein. To determine whether β-casein could displace adsorbed β-lactoglobulin from the HA surface, and whether β-lactoglobulin could displace adsorbed β-casein from the HA surface, 5% (w/w) β-lactoglobulin-coated HA particles were added to 50 mM HEPES buffer, pH 6.8, 100 mM ionic strength, containing various concentrations of β-casein (0.5–2%, w/w, total solids). Conversely, 5% (w/w) β-casein-coated HA particles were added to 50 mM HEPES buffer, pH 6.8, 100 mM ionic strength, containing various concentrations of β-lactoglobulin (0.5–2%, w/w, total solids). The suspensions were stirred for two hours, after which the particles were centrifuged and the supernatants containing the unadsorbed and displaced proteins were analysed qualitatively for protein composition using MF-electrophoresis (Section 3.5.4).

5.3.4 Calculation of protein net charge

The net charges of the protein at given pH values were predicted using a web-based computer program (Protein Calculator v3.4, http://protcalc.sourceforge.net/). In this program, the amino-acid sequence of a protein is entered as an input, and the net charge of the proteins is calculated as a function of pH. The amino-acid sequences were obtained from the Universal Protein Resource database (http://www.uniprot.org/) for bovine β-lactoglobulin (P02754), α-lactalbumin (P00711), α_{S1}-casein (P02662), α_{S2}-casein (P02663), β-casein (P02666), κ-casein (P02668), lactoferrin (P24627) and lysozyme C (P00698).

The net charge calculated with Protein Calculator v3.4 did not take into account the charge related to the phosphorylated or glycosylated residues of the caseins. Therefore, for caseins, the net charge due to these residues was calculated separately and added to the net charge given by the Protein Calculator from the protein sequences. The net charge of one phosphorylated and glycosylated residue was calculated as a function of pH using a freeware available online for pH calculation (http://www2.iq.usp.br/docente/gutz/Curtipot.html, CurTiPlot V4.1), using the pKa of the residues as inputs. The pKa values used for the phosphoserine residues of the caseins were taken from the literature (Baumy et al., 1989; Mekmene, 2010), and were 1.5 for pK_{a1} and 6.5 for pK_{a2}. The main carbohydrate contained in the glycomacropeptide of κ-casein is N-acetylneuraminic acid (Swaisgood, 2003), therefore the pKa of N-acetylneuraminic acid (2.6) (Dawson, Elliott, Elliott, & Jones, 1986) was used for the glycosylated residues. To calculate the net charge of the phosphorylated and glycosylated residues carried by the caseins at a given pH, the charge of one residue calculated with the CurtiPlot freeware was multiplied by the number of residues carried out by the caseins, respectively 8, 11, 5 and 1 phosphoserine residues per molecule of α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein and 5 glycosylated residues per molecule of κ-casein (Walstra & Jenness, 1984).

5.4 Results and discussion

5.4.1 Adsorption of individual milk proteins

5.4.1.1 Surface protein concentration and Langmuir modelling

Figure 5.1 shows the experimental results for the adsorption experiments carried out with different initial protein concentrations of individual proteins, α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin, in 50 mM HEPES buffer (pH 6.8) at two different ionic strengths; 7 mM (Figure 5.1A) and 100 mM (Figure 5.1B). The calculated surface protein concentrations for each protein are shown as a function of the initial protein concentration used in the experiment. At each ionic strength, as the initial protein concentration increased, the amount of adsorbed proteins increased until it reached a maximum coverage, corresponding to a saturation state of the HA surface with respect to protein. The maximum values were different for each protein, with β-casein and α_S-casein adsorbing to higher maximum amounts than κ-casein, β-lactoglobulin and α-lactalbumin, at both levels of ionic strength.

The Langmuir model was fitted to the adsorption results shown in Figure 5.1. Figure 5.2 shows the Langmuir representation of the adsorption results for the individual milk proteins, for experiments carried out in 50 mM HEPES buffer (pH 6.8) at two different ionic strengths, i.e., 7 mM (Figure 5.2A) and 100 mM (Figure 5.2B). The surface protein concentration of the different types of individual caseins and whey proteins was plotted against the equilibrium protein concentration (i.e., the unadsorbed protein left in the supernatants after adsorption, determined by UV spectroscopy). The best-fit Langmuir isotherm curves are shown for each protein, and the calculated constants for the Langmuir isotherms are given in Table 5.1.

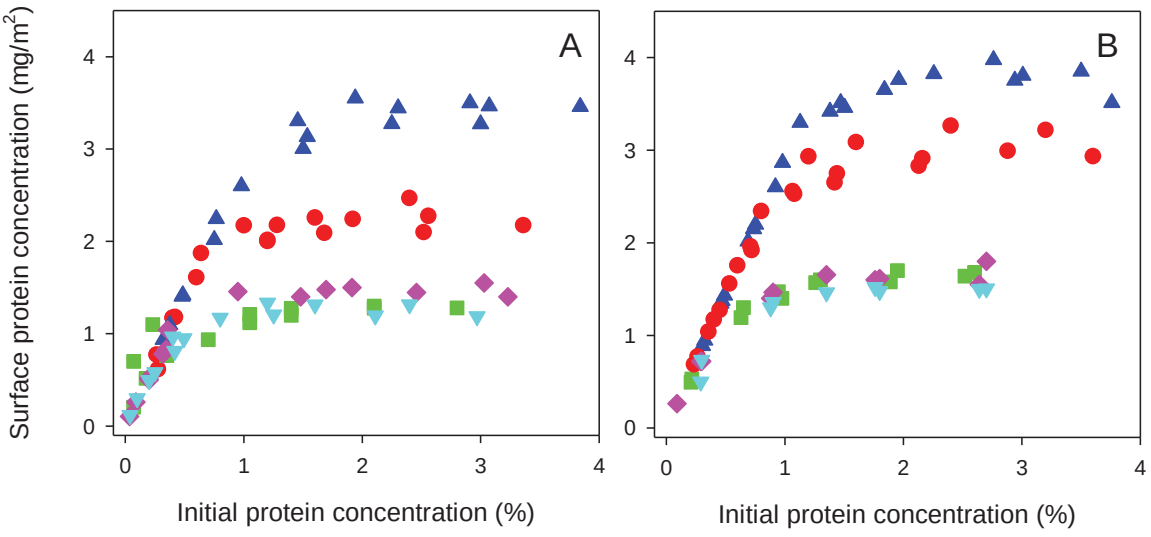

Figure 5.1: Surface protein concentration of individual milk proteins as a function of initial protein concentration, in 50 mM HEPES buffer (pH 6.8) of 7 mM ionic strength (panel A) and 100 mM ionic strength (panel B). ●, α_S-casein; , ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin; all experimental data points are shown from either two or three replica experiments.

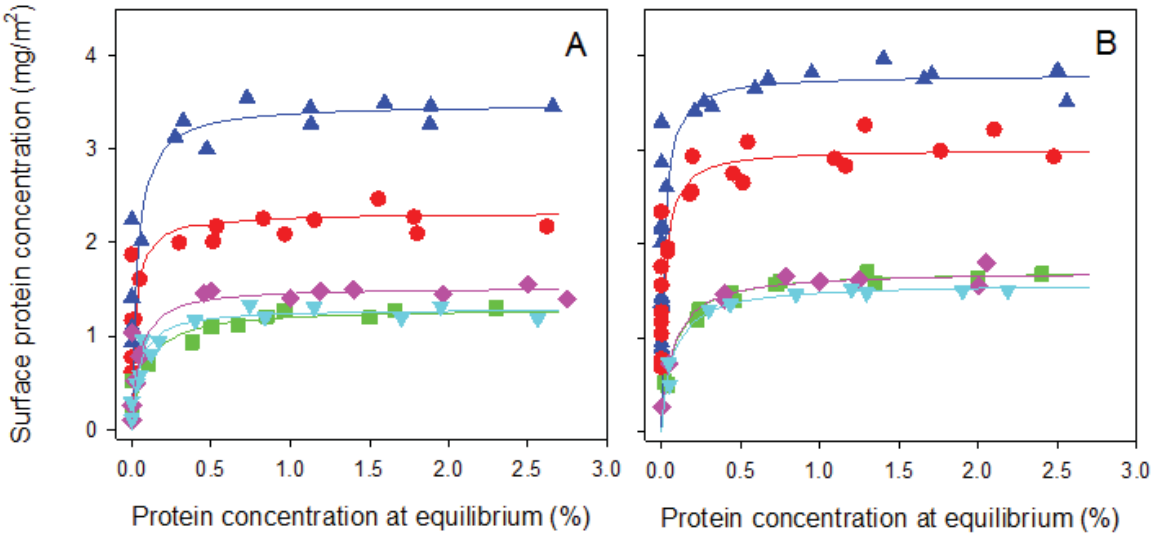

Figure 5.2: Surface protein concentration of individual milk proteins as a function of protein concentration at equilibrium, in 50 mM HEPES buffer (pH 6.8) of 7 mM ionic strength (panel A) and 100 mM ionic strength (panel B). ●, α_S-casein; ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin; all experimental data points are shown from either two or three replica experiments, the solid lines represent the best-fit model curves of the Langmuir model for each protein.

The adsorption curves of α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin all followed a Langmuir adsorption (Figure 5.2). The maximum surface coverage values for α_S-casein and β-casein, 2.32 and 3.49 mg/m^2, respectively, at 7 mM ionic strength and 3.01 and 3.80 mg/m^2, respectively, at 100 mM ionic strength were significantly higher than the maximum surface coverage values reached by κ-casein, β-lactoglobulin and α-lactalbumin: 1.30, 1.52 and 1.30 mg/m^2, respectively, at 7 mM ionic strength (Figure 5.2A and Table 5.1) and 1.70, 1.71 and 1.58 mg/m^2, respectively, at 100 mM ionic strength (Figure 5.2B and Table 5.1). All five proteins adsorbed slightly more at higher ionic strength, but the increase in the amount of adsorbed protein was only significant for α_S-casein, which adsorbed up to a maximum of 2.32 mg/m^2 at 7 mM ionic strength and 3.01 mg/m^2 at 100 mM ionic strength (Table 5.1).

Table 5.1: Affinity constant and maximum surface concentration for the adsorption of α_S-casein (α_{S1} +α_{S2}), β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) onto HA particles in 50 mM HEPES buffer (pH 6.8) of 7 mM and 100 mM ionic strengths (I), calculated according to the Langmuir model. [a]

Parameter	α_S-casein	β-casein	κ-casein	β-Lg	α-La
Maximum surface coverage q_m (mg/m^2)					
I=7 mM	2.32^{aA} ±0.1	3.49^{b} ± 0.14	1.3^{c} ± 0.03	1.52^{c} ± 0.05	1.30^{c} ± 0.05
I=100 mM	3.01^{aB} ± 0.2	3.80^{b} ± 0.05	1.7^{c} ± 0.04	1.71^{c} ± 0.1	1.58^{c} ± 0.06
Affinity constant *K* (100g/g)					
I=7 mM	42.7^{a} ± 15.1	31^{ab} ± 6.6	12.1^{b} ±1.3	21.2^{ab} ± 4.9	22^{ab} ± 3.9
I=100 mM	46.6^{a} ± 9.4	51.7^{a} ± 13.9	13.4^{b} ± 4.6	14.3^{b} ± 1.9	14.1^{b} ± 5.4

[a] Means and standard deviations are given, and were calculated from at least two repeated experiments. Means with different lower case superscript letters differ significantly between the different proteins (P <0.05; horizontal comparison in rows). Means with different upper case superscript letters differ significantly between the two ionic strength levels (P <0.05, vertical comparison in columns, α_S-casein only).

These results confirmed that α_S-casein and β-casein are able to adsorb more onto HA than κ-casein, and also more than β-lactoglobulin and α-lactalbumin. This was already observed in Chapter 4 when using SC and WPI in water, where α_S-casein and β-casein represented 95% of the total caseins from SC bound onto HA (Table 4.2) and where the sum of the maximum

amounts of adsorbed α_S-casein and β-casein (2.6 mg/m^2, of which 1.4 mg/m^2 were from α_S-casein and 1.2 mg/m^2 from β-casein , see Figure 4.6A) was about two times higher than the sum of the maximum amounts of adsorbed β-lactoglobulin and α-lactalbumin (1.4 mg/m^2, of which 1.2 mg/m^2 were from β-lactoglobulin and 0.2 mg/m^2 from α-lactalbumin, see Figure 4.6B).

The maximum amount of proteins that can bind to HA is usually determined by the number and distribution of the charged residues along the protein molecules (Nakanishi et al., 2001; Wahlgren & Arnebrant, 1991), and by the structure of the adsorbed protein layer on the HA surface (Wang et al., 2012). The number and distribution of protein residues than can bind onto HA differs between the different milk proteins, which could partly explain the higher surface coverage observed for β- and α_S-casein, compared with κ-casein, β-lactoglobulin and α-lactalbumin. If proteins contain many residues that can bind onto HA, and if these residues are grouped in clusters, they can provide many anchor points and a good alignment of the protein binding sites with the C-sites on HA, leading to high surface protein concentration values (Gorbunoff & Timasheff, 1984b; Wang et al., 2012). This is the case for α_{S1}-, α_{S2}- and β-caseins, which carry 7–9, 10–13, and 5 phosphoserine residues in their respective sequences, and these residues are grouped in clusters (Walstra & Jenness, 1984). In contrast, κ-casein carries only 1 phosphoserine residue, which may explain its lower surface coverage compared with α_S- and β-casein. Also, α_S- and β-casein are probably able to self-associate on the HA surface, forming thicker protein layers compared with the other proteins. β-Casein has been reported to self-associate and form micelle-like structures, with the hydrophobic groups of the molecule buried inside the micelle and the hydrophilic heads, carrying the phosphoserine residues, sticking out of the micelle (Schmidt, 1982). This conformation is probably favourable for β-casein binding onto HA. A thick layer of β-casein micelles may therefore be formed on the HA surface, which would explain why β-casein adsorbed to the highest maximum surface protein concentration of all the proteins, at both ionic strengths (Figure 5.1 and Figure 5.2). α_S-Casein (α_{S1}- + α_{S2}-casein) is also known to self-associate in solution. At ionic strength greater than 3 mM, both α_{S1}-casein and α_{S2}-casein can form oligomers that exist in equilibrium with monomers in solution (Schmidt, 1970; Schmidt & Van Markwijk, 1968; Swaisgood, 2003). As ionic strength increases, the formation of oligomers becomes favourable and dimers or trimers can form. At low ionic strength, the α_S-casein layer may be formed by mostly monomers of α_S-casein (α_{S1}- + α_{S2}-casein), whereas at 100 mM ionic strength, oligomers of α_S-casein might adsorb onto HA, leading to more α_S-casein adsorbing on the HA surface, and therefore explaining the

significant difference in the maximum amount of adsorbed α_S-casein observed in this study at two ionic strength levels.

β-Lactoglobulin and α-lactalbumin are globular folded proteins (Dickinson, 2001), and are believed to bind onto HA mostly though their side carboxyl groups. Globular folded proteins have been shown to usually bind less than linear flexible proteins onto HA, as the carboxyl residues in the globular proteins that bind onto HA are not as accessible and often not grouped in clusters, and can be buried inside the molecule (Wahlgren & Arnebrant, 1991). Therefore, globular proteins usually only form a closed packed monolayer on the HA surface, and do not interact with each other once adsorbed (Wahlgren & Arnebrant, 1991). This would explain why β-lactoglobulin and α-lactalbumin adsorbed less than α_S-casein and β-casein (Figure 5.1, Figure 5.2 and Table 5.1)

The variability of the Langmuir affinity constants was quite high between repeated experiments, as shown by the standard deviations reported in Table 5.1. The affinity constant of the Langmuir model characterises the initial slope of the adsorption isotherms, with a large slope value corresponding to a high affinity of the protein for HA (Iafisco et al., 2011). The affinity constant is therefore mostly determined by the first points of the adsorption isotherms, which are hard to determine with precision experimentally as they correspond to very low equilibrium concentrations, i.e., very low concentrations of unadsorbed proteins (Figure 5.2): a small variation in the measurement of these low concentrations can lead to a high variation of the affinity constants. However, it is interesting to note that the mean affinity constants for β-casein and α_S-casein were higher than those of κ-casein, β-lactoglobulin and α-lactalbumin, at both ionic strength levels (Table 5.1). For example, at 100 mM ionic strength, the affinity constants of β-casein and α_S-casein were, respectively, 46.6 and 51.7, and were significantly higher than the affinity constants of κ-casein, β-lactoglobulin and α-lactalbumin that were, respectively, 13.4, 14.3 and 14.1 (Table 5.1). Phosphorylated proteins have been reported to have much stronger affinity for HA than non-phosphorylated acidic proteins, because the phosphate groups of the protein have a higher affinity for C-sites on HA than the carboxyl groups (Benaziz et al., 2001; Bernardi et al., 1972; Gorbunoff & Timasheff, 1984a; Juriaanse et al., 1981). When the phosphate groups are grouped in clusters, the affinity of the phosphorylated proteins for HA is even higher, since the presence of neighbouring phosphorylated residues increase the probability of binding (Gorbunoff & Timasheff, 1984b; Johnsson et al., 1993). Therefore, the phosphorylated nature of β-casein and α_S-casein, and the grouping of their phosphate groups in clusters, may explain the higher affinity constants of β-casein and α_S-casein for HA

in this study, compared with β-lactoglobulin, α-lactalbumin and κ-casein, at both ionic strength levels (Table 5.1)

5.4.1.2 Zeta-potential

The zeta-potential of selected samples of HA particles obtained in the adsorption experiments carried out in 50 mM HEPES buffer, pH 6.8, 7 mM ionic strength with different initial concentrations of α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin was measured and the values are reported in Figure 5.3 as a function of the initial protein concentration. As the initial protein concentrations increased, the magnitude of the zeta-potential of the HA particles decreased from –15 mV when no protein was bound to maximum plateau values of about –28, –27, –25, –27 and –20 mV for α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin respectively (Figure 5.3)

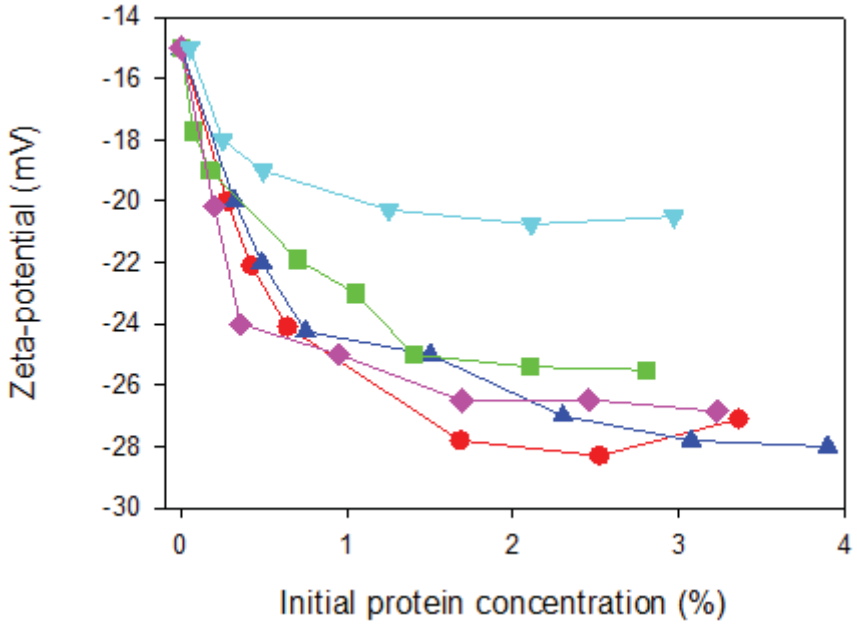

Figure 5.3: Zeta-potential of protein-coated HA particles, as a function of the initial protein concentration used in the adsorption experiments. HA particles were suspended in 50 mM HEPES buffer, pH 6.8, at 7 mM ionic strength containing (●) α_S-casein, (▲) β-casein, (■) κ-casein, (◆) β-lactoglobulin and (▼) α-lactalbumin solutions of different initial concentrations, stirred for 2 h and centrifuged, and the pellets were then resuspended in 50 mM HEPES buffer, pH 6.8, at 7 mM ionic strength for zeta-potential measurements.

In Chapter 4, it was shown that the zeta-potential of SC- and WPI-covered HA particles was correlated with the amount of adsorbed SC or WPI onto particles. This correlation was also observed with the adsorption results obtained for the individual proteins. The measured zeta-potential values obtained for each initial protein concentration, shown in Figure 5.3 were plotted against the corresponding surface protein concentrations, shown in Figure 5.1 and a linear regression was fitted to the results (Figure 5.4). For all proteins, there was a good linear correlation between the zeta-potential of the protein-coated HA particles, and the amount of adsorbed protein (R^2 coefficients >0.9 for each set of data). As the surface protein concentration of each protein increased, the zeta-potential of the HA particles became more negative, and the maximum zeta-potential values of respectively –28 mV, –27 mV, –25 mV, –27 mV and –20 mV for α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin, corresponded to the maximum surface protein concentrations of, respectively, 2.3, 3.5, 1.3, 1.5 and 1.3 mg/m^2. This linear relationship between surface protein concentration and zeta-potential for each protein (Figure 5.4) confirms that protein adsorption was responsible for the increase in surface charge of the HA particles, as already reported for the adsorption of α_{S1}-casein, β-casein, κ-caseins, β-lactoglobulin and α-lactalbumin by Reynolds and Wong (1983).

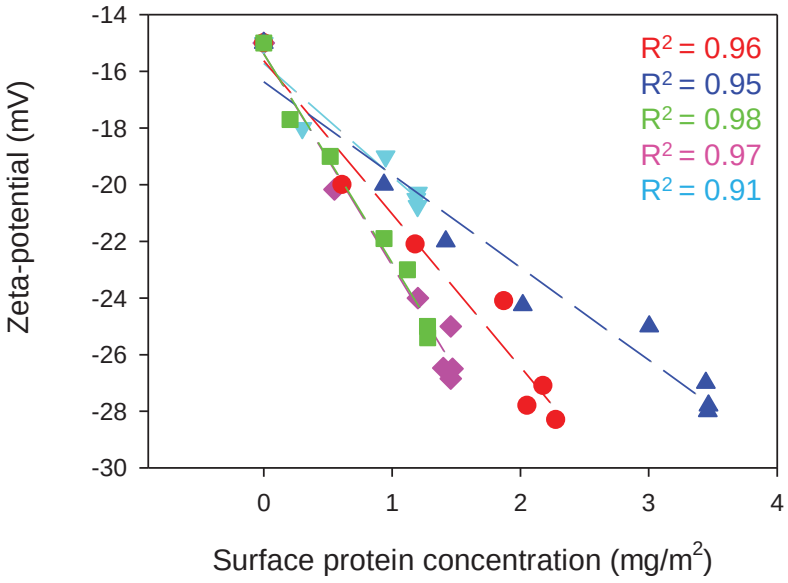

Figure 5.4: Linear relationship between the surface protein concentration of (●) α_S-casein, (▲) β-casein, (■) κ-casein, (◆) β-lactoglobulin and (▼) α-lactalbumin, and the zeta-potential of the corresponding particles. The experiments were carried out in 50 mM HEPES buffer, pH 6.8, at 7 mM ionic strength, and the HA particles were re-suspended in the same buffer for zeta-potential measurement.

The net charges of α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin were calculated at pH 6.8 and in water (Table 5.2), based on their amino-acid sequences and on the number and charge of their charged residues (phosphoserine residues for α_S-casein and β-casein and glycosylated residues for κ-casein). At pH 6.8, α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin are all negatively charged, but the magnitudes of their net charge differ in the order: κ-casein (–6e) < α-lactalbumin (–6.1e) < β-lactoglobulin (–7.4e) < β-casein (–14.6e) < α_{S2}-casein (–15e) < α_{S1}-casein (–23.6e) (Table 5.2). These calculated values were very close to those reported for the caseins at pH 6.6 by Fox (2003) (see Table 2.2 in literature review). It was shown that there was no correlation observed between the net charge of the milk proteins at pH 6.8 and the maximum zeta-potential of the protein-coated particles, reached for a saturation state of the HA surface with respect to protein (Figure 5.4 and Table 5.2). Only the magnitude of the zeta-potential of HA particles covered with α-lactalbumin was significantly lower (–20 mV) than that of the HA particles covered with the other milk proteins. The particles covered with κ-casein, β-lactoglobulin, β-casein, and α_S-casein had maximum zeta-potential values all comprised between –25 and –28 mV, regardless of their difference in net charge at pH 6.8.

Table 5.2: Estimated calculated net charge at pH 6.8 and in water of the individual caseins and whey proteins used in this chapter. [a]

Protein	α_{S1}-CN	α_{S2}-CN	β-CN	κ-CN	β-Lg	α-La
Net charge	–23.6e	–15e	–14.6e	–6e	–7.4e	–6.1e

[a] The protein sequences were downloaded from the Universal Protein Resource database and the protein net charge was calculated using a web-based program (Protein Calculator v3.4). Abbreviations are: CN, casein; Lg, lactoglobulin; La, lactalbumin.

The change of surface charge of HA due to protein adsorption is likely to depend on the binding affinity of the protein for HA, rather than on the protein net charge. The protein affinity is usually determined by the type and number of groups that will bind onto HA and by the geometrical arrangement of these groups within the local protein surface (Kawasaki et al., 1985). The negative zeta-potential of the HA particles after adsorption of the proteins is therefore due to the net charge of the adsorbed protein layer after adsorption (Johnsson

et al., 1993), and is not correlated with the initial net charge of the proteins before adsorption.

5.4.2 Adsorption from mixtures of individual proteins

5.4.2.1 Casein mixtures

Various amounts of HA (0 to 4%, w/w) were added to protein solutions containing 0.2% (w/w) protein, comprising a third of each casein (α_S-casein, β-casein and κ-casein), at two different ionic strengths (7 mM and 100 mM). After mixing for 2 h, the HA particles were centrifuged and the supernatants were analysed for residual protein content by MF-electrophoresis. Figure 5.5 shows the MF-electrophoresis computer generated gel images obtained from the supernatants and the quantitative data that was calculated from the gels as a function of the HA concentration added to the protein solution. The β-, α_S- and κ-casein concentrations in the supernatants are given as a percentage of their initial concentrations in the control (0.2%, w/w, protein, no added HA).

The amount of protein remaining in the supernatant decreased as the concentration of added HA increased from 0 to 4% (w/w). For both ionic strength levels, it can be seen that the ratio of the band intensities of the different caseins in the control sample containing only protein (lane 1 in Figure 5.5) is different to the ratio of the band intensities of the different caseins in the supernatant samples containing HA (lanes 2 to 9 in Figure 5.5, κ-casein represents more and more of the total band intensity as the HA concentration increases). This suggests that the relative proportions of different caseins adsorbing onto HA were different from the proportions of the individual caseins present in the initial solution. The intensities of the α_S-casein and β-casein bands decreased faster than that of the κ-casein bands, indicating that α_S-casein and β-casein were preferentially adsorbed onto HA particles at both ionic strength levels. For example, when 1% (w/w) HA particles were added, none of the initial β-casein remained unadsorbed (which means the initial β-casein had entirely adsorbed on the HA particles) at both ionic strength levels, and only 15% and 10% of the initial α_S-casein remained unadsorbed respectively at 7 mM and 100 mM ionic strength. In contrast, about 75% of κ-casein was still unadsorbed at both ionic strength levels (Figure 5.5), indicating that κ-casein was the least adsorbed among the caseins. The ratio of adsorbed β- to α_S-casein seemed to vary slightly between the two ionic strength levels. At 7

mM ionic strength, the band intensity of β-casein decreased faster than that of α_S-casein, but this difference became smaller at 100 mM ionic strength (Figure 5.5).

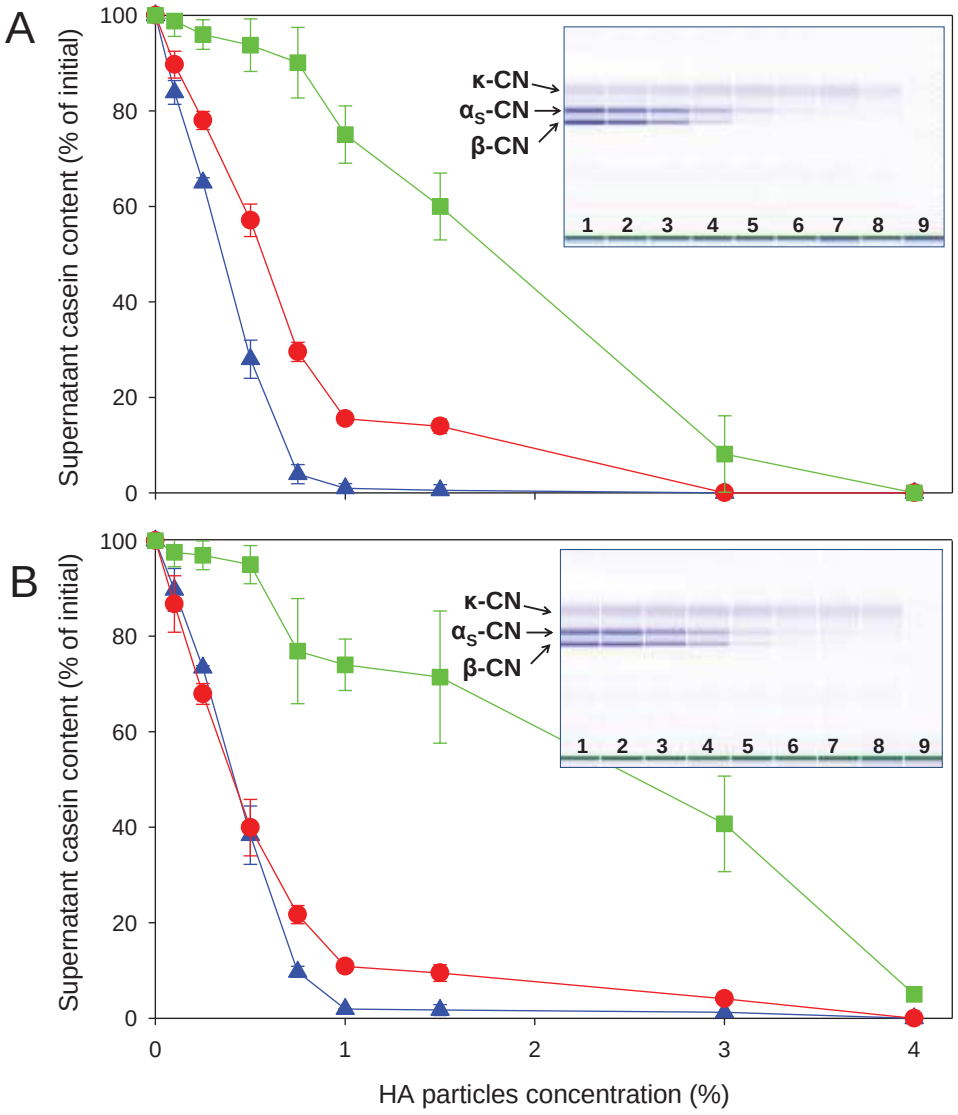

Figure 5.5: Integrated data of the MF electrophoresis gel patterns (inserts) of the supernatants of samples containing different concentrations of HA particles (0.1% to 4%, w/w) and 0.2% (w/w) total protein comprising equal amounts of α_S-casein (α_S-CN; α_{S1} + α_{S2}), β-casein (β-CN) and κ-casein (κ-CN). Experiments were carried out in 50 mM HEPES buffer (pH 6.8) at 7 mM (panel A) and 100 mM ionic strengths (panel B). Graphs show the percentage of the initial α_S-casein (●), β-casein (▲), and κ-casein (■) remaining in the supernatants after adsorption, as a function of the HA concentration. MF electrophoresis gels show in lanes 1–9: control (no added HA), 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4% (w/w) added HA, respectively.

Figure 5.6 shows the total amount of protein adsorbed onto HA particles as a function of the initial HA particle concentration, calculated from the MF-electrophoresis data given in Figure 5.5, at 7 mM ionic strength (Figure 5.6A) and 100 mM ionic strength (Figure 5.6B). At low ionic strength, the total amount of adsorbed protein was approximately 2.7 mg/m^2 (Figure 5.6A), of which approximately 1.5, 1 and 0.2 mg/m^2 were made of β-casein, α_S-casein and κ-casein respectively. β-Casein was therefore preferred over α_S-casein for adsorption, as β-casein represented about 53% of the total adsorbed casein. These results were very similar to the results obtained for SC in water in chapter 4, where the total amount of adsorbed protein from SC was approximately 2.5 mg/m^2, with a preferential adsorption of β-casein over α_S-casein and κ-casein (Figure 4.8A and Figure 4.9A). At 100 mM ionic strength, the total amount of adsorbed caseins was of approximately 3 mg/ m^2 of which more than 90% was made of β-casein and α_S-casein, since they both adsorbed to approximately the same maximum amounts, of approximately 1.4 mg/m^2 each (Figure 5.6B). κ-Casein still adsorbed to a lesser amount of approximately 0.3 mg/m^2 (Figure 5.6B). At 100 mM ionic strength, β-casein was no longer preferred anymore for adsorption over α_S-casein, since it adsorbed slightly less, and α_S-casein adsorbed significantly more. Overall, it appears that when all three caseins were present together in the initial solution, the maximum amounts of proteins that could bind to the HA particles were of about the same as when β-casein or α_S-casein were studied individually (values close to 3 mg/ m^2, see Figure 5.1) but the adsorption surface was shared between β-and α_S-casein.

These results are consistent with the observations on the adsorption of the individual caseins, reported in the previous section (Section 5.4.1.1), where only the amount of adsorbed α_S-casein significantly increased between the two ionic strength levels. When caseins are mixed together in solution, it is not really certain whether they remain as individual molecules and adsorb onto HA as such, or whether they associate with each other to form small aggregates that then adsorb onto HA (Horne, 2009; Morris, 2002). However, regardless of whether caseins bind onto HA as individual molecules or as small aggregates, the increase in the proportion of adsorbed α_S-casein as ionic strength increases must be due to some change in its association behaviour (either self-association or association with the other caseins). α_S-Casein must bind onto HA as mostly monomers at low ionic strength, but must form oligomers as ionic strength increases, therefore binding to a larger amount onto HA (Schmidt, 1970; Schmidt & Van Markwijk, 1968; Srinivasan et al., 2000). β-Casein and κ-casein also self-associate into subunits, but their self-association is not very sensitive to ionic strength (Schmidt, 1982), which would explain why their surface concentration onto HA does not change much between the two levels of ionic strengths (Figure 5.6).

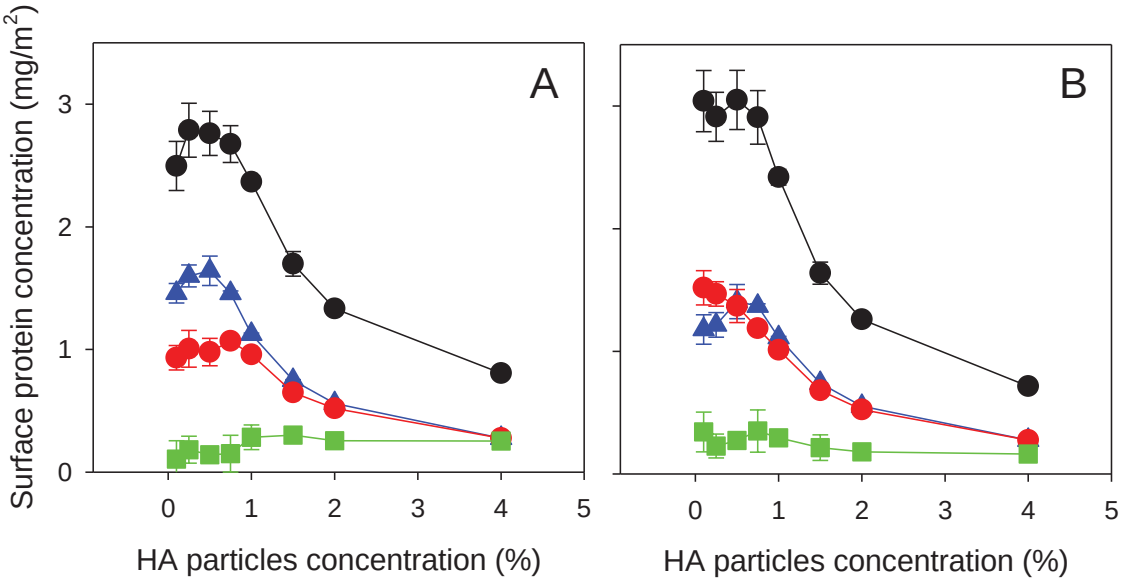

Figure 5.6: Estimated surface protein concentrations (mg/m²) of total protein and individual caseins from an initial solution containing 0.2% (w/w) total protein comprising equal amounts of α_S-casein (α_{S1} + α_{S2}), β-casein and κ-casein, in 50 mM HEPES buffer, pH 6.8, at 7 mM (panel A) and 100 mM ionic strengths (panel B), as a function of HA concentration. ●, total protein; ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein. Each data point is the average of two to three replicates; error bars represent standard deviation.

5.4.2.2 Whey protein mixtures

The same experiment undertaken with caseins was repeated with whey proteins, using solutions containing 0 to 4% (w/w) HA particles and a constant amount of whey protein (0.08%, w/w), comprising half of β-lactoglobulin and half of α-lactalbumin, at two different ionic strengths (7 mM and 100 mM). Figure 5.7 shows the MF-electrophoresis computer-generated gel images obtained from the supernatant of the whey protein solution and the quantitative data that were calculated from these gels. The amount of whey protein remaining in the supernatant decreased as the concentration of added HA increased from 0 to 4% (w/w) However, at both ionic strength levels, the intensity of the β-lactoglobulin bands decreased faster than that of the α-lactalbumin bands, indicating that β-lactoglobulin was preferentially adsorbed over α-lactalbumin.

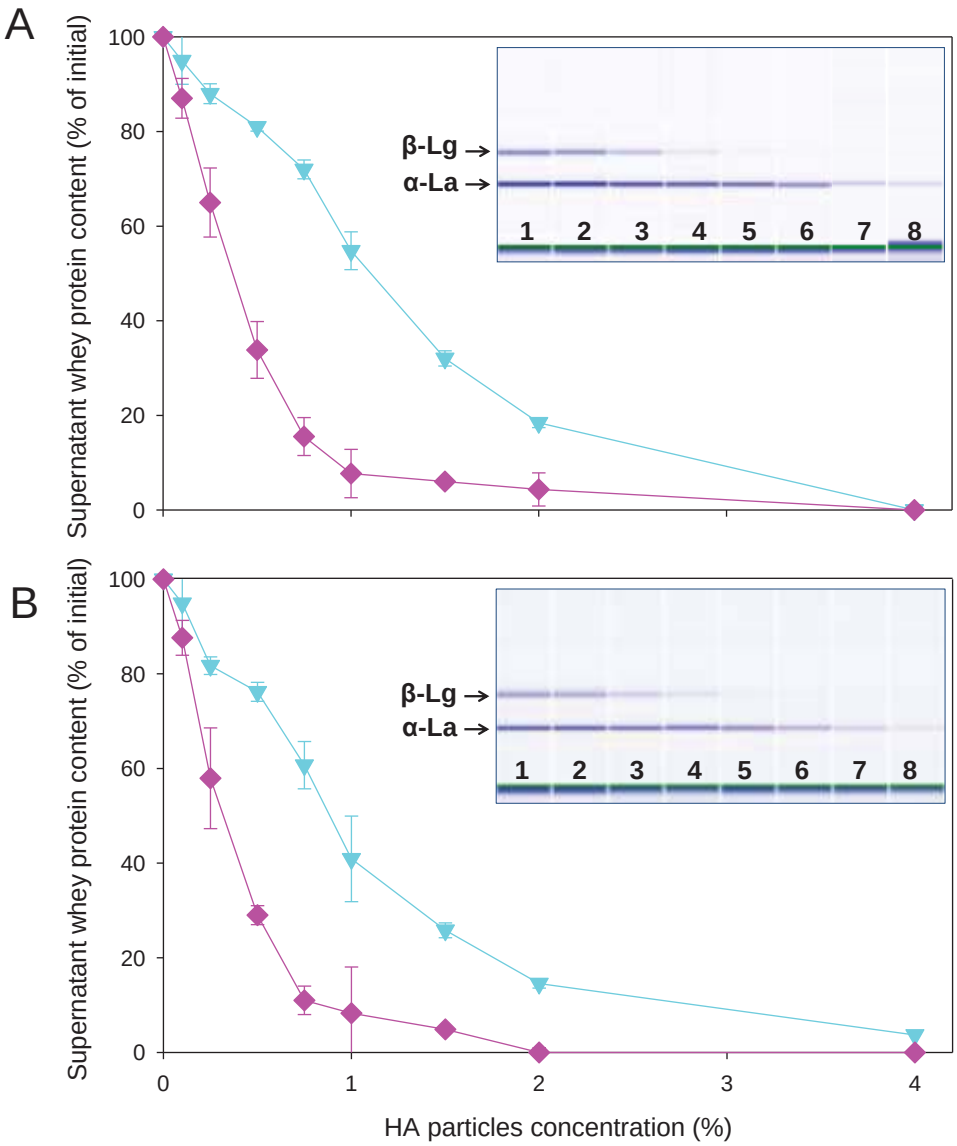

Figure 5.7: Integrated data of the MF electrophoresis gel patterns (inserts) of the supernatants of samples containing different concentrations of HA particles (0.1% to 4%, w/w) and 0.08% (w/w) total protein comprising equal amounts of β-lactoglobulin andα-lactalbumin. Experiments were carried out in 50 mM HEPES buffer, pH 6.8, at 7 mM (panel A) and 100 mM ionic strength (panel B). Graphs show the percentage of the initial β-lactoglobulin (β-Lg; ◆) and α-lactalbumin (α-La; ▼) remaining in the supernatants after adsorption, as a function of the HA concentration. MF electrophoresis gels show in lanes 1–9: control (no added HA), 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4% (w/w) added HA, respectively.

Figure 5.8 shows the amount of protein adsorbed onto HA from the initial solution containing 50% of β-lactoglobulin and 50% of α-lactalbumin, calculated from the MF-electrophoresis data given in Figure 5.7, at 7 mM ionic strength (Figure 5.8A) and 100 mM ionic strength (Figure 5.8B). The proteins in the initial solution containing 50:50 β-lactoglobulin:α-lactalbumin adsorbed onto HA up to a total maximum surface concentrations of about 1.2 and 1.4 mg/m^2 at 7 mM and 100 mM ionic strength respectively. These maximum values were very similar to the maximum values obtained for WPI in water in Chapter 4 (approximately 1.3 mg/m^2, see Figure 4.8B), and to the maximum values obtained for the individual β-lactoglobulin and α-lactalbumin proteins at both ionic strengths, shown in Figure 5.1. This means that regardless of the composition of the initial whey protein solutions (pure β-lactoglobulin, pure α-lactalbumin or a mixture of both β-lactoglobulin and α-lactalbumin), whey proteins always adsorb to the same maximum value. However, when both whey proteins were mixed together in the initial solution, α-lactalbumin always adsorbed a lot less than β-lactoglobulin onto HA. In this chapter, β-lactoglobulin adsorbed to maximum values of approximately 1 mg/m^2 at both ionic strength levels, whereas α-lactalbumin adsorbed to maximum values of only approximately 0.3 mg/m^2 at 7 mM ionic strength (Figure 5.8A) and 0.4 mg/m^2 at 100 mM ionic strength (Figure 5.8B). In Chapter 4, β-lactoglobulin and α-lactalbumin adsorbed to respective maximum values of approximately 1 mg/m^2 and 0.1 mg/m^2 (Figure 4.9B) for WPI in water. Even though the whey protein solution used in this experiment contained 50% of α-lactalbumin, rather than only 20% present in WPI, β-lactoglobulin was still largely preferred for adsorption onto HA, compared with α-lactalbumin at both ionic strengths, as β-lactoglobulin represented between 70% and 80% of the total adsorbed whey protein (Figure 5.8). It was noted that the amounts of adsorbed β-lactoglobulin and α-lactalbumin slightly increased when the ionic strength was increased from 7 mM to 100 mM, but the standard deviation of the measurements showed this to be not significant.

The preferential adsorption of β-lactoglobulin over α-lactalbumin can probably be explained by a more favourable distribution and location of the charged residues of β-lactoglobulin for binding onto HA, with β-lactoglobulin carrying clusters of carboxyl residues in its sequence (Gorbunoff & Timasheff, 1984b), as discussed in Chapter 4.

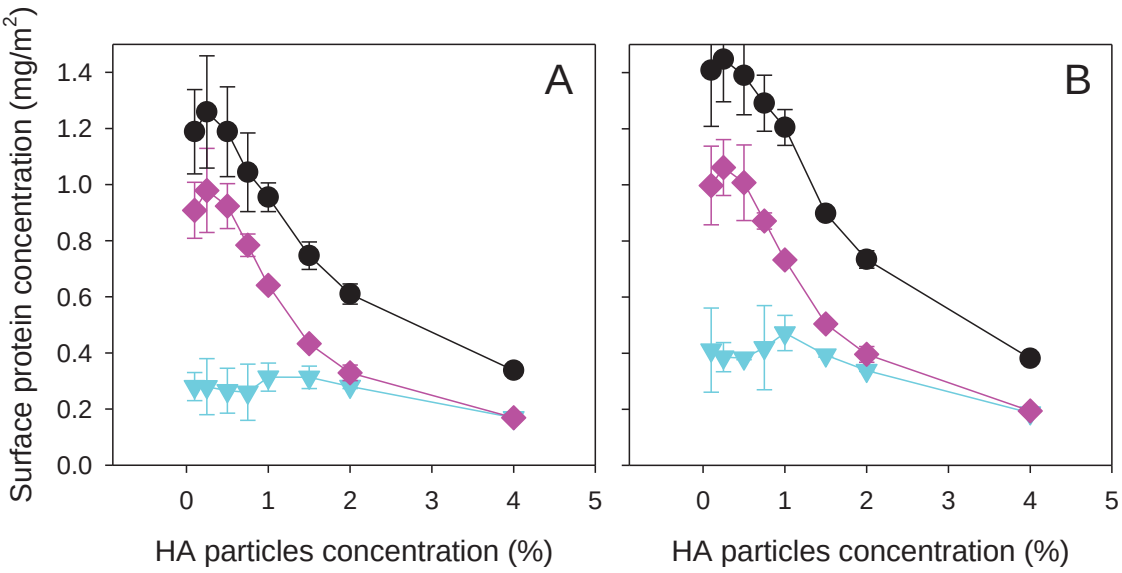

Figure 5.8: Estimated surface protein concentrations (mg/m²) of individual whey proteins from an initial solution containing 0.08% (w/w) total protein comprising equal amounts of β-lactoglobulin and α-lactalbumin, in 50 mM HEPES buffer, pH 6.8, at 7 mM (panel A) and 100 mM ionic strength (panel B), as a function of HA concentration. ●, total protein; ◆, β-lactoglobulin; ▼, α-lactalbumin. Each data point is the average of two to three replicates; error bars represent standard deviation.

5.4.2.3 Mixtures containing equal proportions of five milk proteins

A protein solution containing 0.2% (w/w) total protein comprising equal proportions of α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin (20% of each) was added to HA suspensions containing different amounts of HA particles (0 to 8%, w/w). The experiment was only carried out in 50 mM HEPES buffer, pH 6.8, at 100 mM ionic strength, corresponding approximately to the ionic strength of milk.

Figure 5.9 shows the MF-electrophoresis computer-generated gel images obtained from the supernatant of the protein solution after adsorption, and the quantitative data that were calculated from these gels, as a function of the added HA concentration. The intensities of the β-casein and α_S-casein bands in the supernatants decreased faster than those of κ-casein, β-lactoglobulin and α-lactalbumin. Almost all the available α_S-casein and β-casein had bound onto HA from the supernatant of the sample prepared with 1% (w/w) HA, whereas approximately respectively 70%, 80% and 90% of the initial κ-casein, β-lactoglobulin and α-lactalbumin remained unadsorbed in the supernatants (Figure 5.9). It

was only when there was no more α_S- and β-casein available for adsorption that binding of κ-casein, β-lactoglobulin and α-lactalbumin could occur. These results therefore indicate that there was a preference in the protein adsorption in the order β-casein ≅ α_S-casein (approximately similar adsorption rate) > κ-casein > β-lactoglobulin > α-lactalbumin, from a mixture containing equal proportions of these proteins.

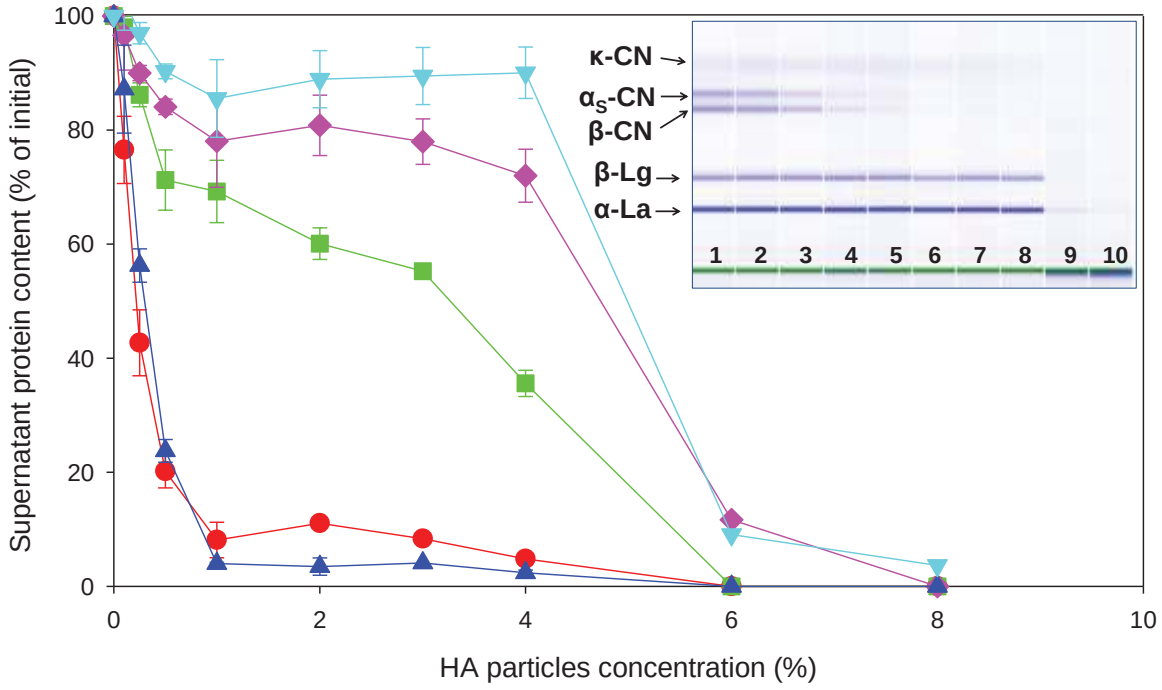

Figure 5.9: Integrated data of the MF electrophoresis gel patterns of the supernatants (inserts) of samples containing different concentrations of HA particles (0.1% to 8% w/w) and 0.08% (w/w) total protein comprising equal amounts of β-lactoglobulin and α-lactalbumin. Experiments were carried out in 50 mM HEPES buffer, pH 6.8, at 100 mM ionic strength. Graphs show the percentage of the initial α_S-casein (α_S-CN; ●), β-casein (β-CN; ▲), κ-casein (κ-CN; ■), β-lactoglobulin (β-Lg; ◆) and α-lactalbumin (α-La; ▼) remaining in the supernatants after adsorption, as a function of the HA concentration. MF electrophoresis gels show in lanes 1–10: control (no added HA), 0.1, 0.25, 0.5, 1, 2, 3, 4, 6 and 8% (w/w) added HA, respectively.

Figure 5.10 shows the estimated amounts of total protein and individual protein adsorbed onto HA particles, as a function of the HA particles concentration, calculated from the MF-electrophoresis data given in Figure 5.9, at 100 mM ionic strength. The maximum amount of total adsorbed protein was of approximately 3 mg/m^2, and the adsorbed protein layer was made up mostly of α_S-casein and β-casein, which adsorbed to maximum amounts of 1.4 and 1.2 mg/m^2 respectively. These maximum values were similar to those obtained when the initial protein solution was made up of a mixture of the three caseins in 50 mM HEPES buffer, pH 6.8, at 100 mM ionic strength, where the total amount of adsorbed casein was approximately 3 mg/m^2, made up mostly of α_S-casein and β-casein, with each adsorbing to maximum values of approximately 1.4 mg/m^2 (Figure 5.6B). The maximum amounts of adsorbed κ-casein, β-lactoglobulin and α-lactalbumin could not be determined with precision for HA concentrations between 0 and 2%, due to the high value and standard deviations of the percentage of unadsorbed protein remaining in the supernatant (Figure 5.9), but were negligible compared with the amount of adsorbed α_S-casein and β-casein.

Again, these results show that the adsorption of milk proteins onto HA particles is competitive between the different types of proteins. Individually, each of the five proteins studied in this chapter could bind onto HA in significant amounts, comprising between 1.3 and 3.5 mg/m^2 (Figure 5.2 and Table 5.1). However, when the five proteins were mixed together, regardless of their ratios in solution, α_S-casein and β-casein were the only proteins that adsorbed in significant amounts, in preference to κ-casein, and to both whey proteins (β-lactoglobulin and α-lactalbumin), and the maximum amount of adsorbed protein was of approximately 3 mg/m^2. As α_S-casein and β-casein carry several phosphoserine residues grouped in clusters, their binding onto HA is expected to be stronger, compared with the binding of κ-casein, which only carries one phosphoserine residue, and with the binding of whey proteins, which bind through their side carboxyl groups (Gorbunoff & Timasheff, 1984a, 1984b; Kawasaki, 1991). The binding of carboxyl groups has been shown to be weaker than that of phosphoserine groups, because phosphate groups on the proteins have a higher affinity for the C-sites of HA compared with carboxyl groups (Benaziz et al., 2001; Bernardi et al., 1972; Gorbunoff & Timasheff, 1984a).

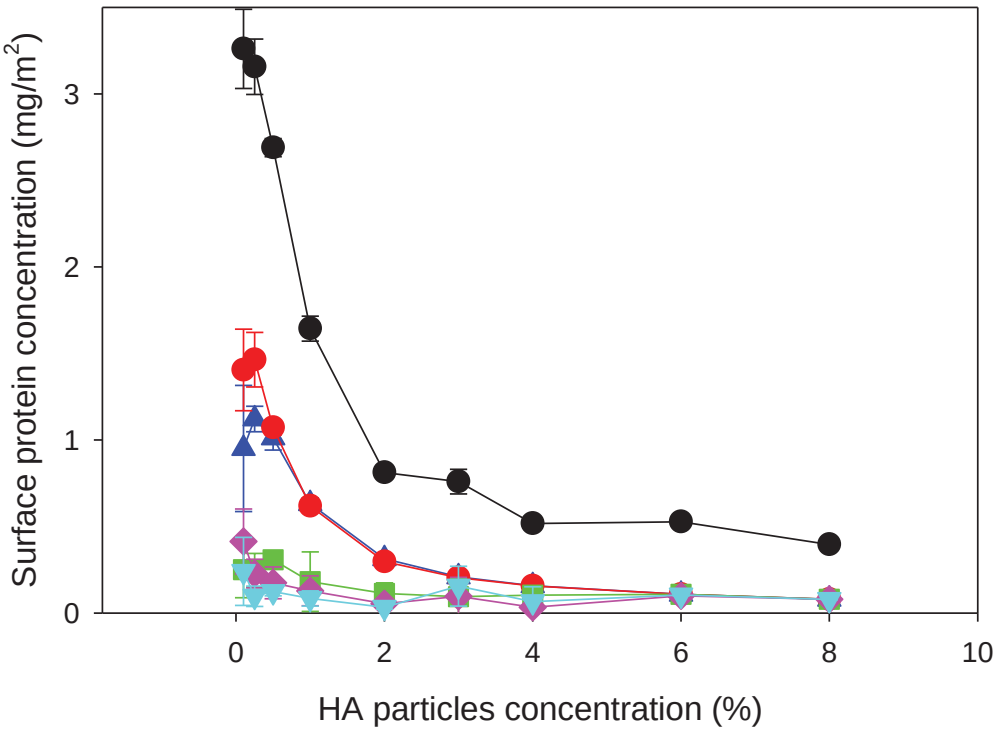

Figure 5.10: Estimated surface protein concentrations (mg/m²) of total protein and individual milk proteins from an initial solution containing 0.2% (w/w) total protein comprising equal amounts of α_S-casein (α_{S1} + α_{S2}), β-casein, κ-casein, β-lactoglobulin and α-lactalbumin, in 50 mM HEPES buffer (pH 6.8) at 100 mM ionic strength. ●, total protein; ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin. Each data point is the average of two to three replicates; error bars represent standard deviation.

5.4.3 Protein displacement

An experiment was designed to check whether caseins were able to displace adsorbed whey proteins, and whether whey proteins were able to displace any of the adsorbed caseins at the HA surface. β-Lactoglobulin-coated HA particles were added to β-casein solutions at different concentrations (0.25 to 2%, w/w, total solids), and β-casein-coated HA particles were added to β-lactoglobulin solutions at different concentrations (0.5 to 2%, w/w, total solids).

Figure 5.11 shows the MF-electrophoresis gel obtained from the initial protein solution and supernatants of the adsorption experiment carried out with β-lactoglobulin-covered HA particles added to β-casein solutions at different concentrations. For HA particles added to

β-casein solutions of concentrations 0.25, 0.5 and 1% (w/w) (lanes 2 to 4 in Figure 5.11), there was no β-casein left in the supernatants after adsorption, and an increasing amount of β-lactoglobulin was present in the supernatant, as shown by the increase in the intensity of the β-lactoglobulin bands. This shows that β-casein adsorbed onto the β-lactoglobulin-coated HA, and concurrently caused the release of β-lactoglobulin into the supernatant. The presence of the β-casein band in the supernatant of HA particles added to 2% (w/w) β-casein solution (lane 5 in Figure 5.11), shows that unadsorbed β-casein started to accumulate in the supernatant, probably because the HA surface was saturated with this protein. These results confirm that β-casein is able to displace the adsorbed β-lactoglobulin from the HA surface. This was expected, since it has been shown previously that phosphorylated proteins are able to displace non-phosphorylated proteins at the HA interface (Juriaanse et al., 1981), because of the higher affinity of phosphate groups for HA compared to carboxyl groups.

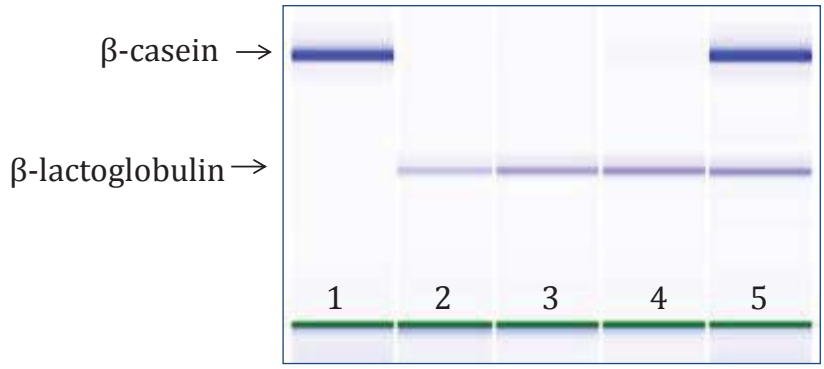

Figure 5.11: MF electrophoresis gel patterns of β-casein initial solution and supernatants obtained from adsorption experiments carried out with β-lactoglobulin-covered HA particles added to β-casein solutions at different initial concentrations. Lane 1, β-casein initial solution (1%, w/w, dilution ratio 1:20 in sample buffer); lanes 2 to 5, supernatant obtained after β-lactoglobulin-covered HA particles added to 0.25, 0.5, 1 and 2% (w/w) β-casein solutions, respectively (dilution ratio 1:10 in sample buffer).

Figure 5.12 shows the initial solutions and the supernatants obtained when β-casein-coated particles were added to β-lactoglobulin solutions at different concentrations. The intensity of the β-lactoglobulin bands in the supernatant (lanes 4 to 6 in Figure 5.12) were similar to the intensity of the β-lactoglobulin band in the initial solutions (lanes 1 to 3 in Figure 5.12), showing that no β-lactoglobulin was able to adsorb onto the HA particles. There was no β-

casein released in the supernatants, which shows that β-lactoglobulin was not able to displace β-casein from the HA surface, because of its lower affinity for HA.

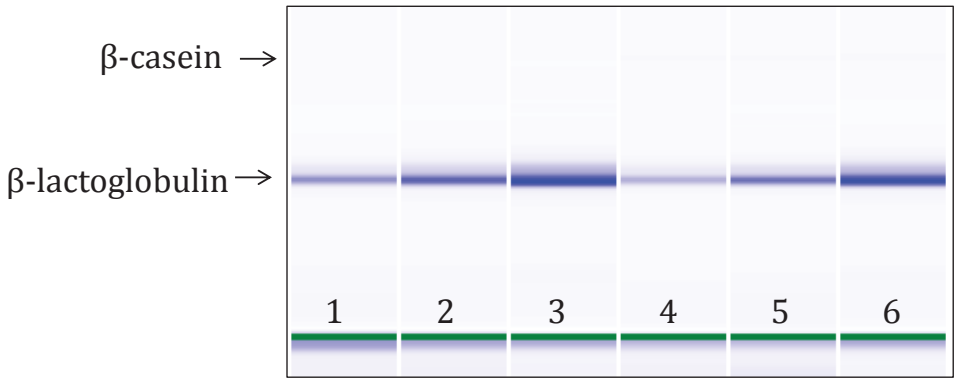

Figure 5.12: MF electrophoresis gel patterns of initial solutions and respective supernatants obtained for adsorption experiments carried out with β-casein-covered HA particles added to β-lactoglobulin solutions at different initial concentrations. Lanes 1 to 3, initial solutions of 0.5, 1 and 2% (w/w) β-lactoglobulin, respectively; lanes 4 to 6, supernatants obtained from initial solutions of 0.5, 1 and 2% (w/w) β-lactoglobulin, respectively, after addition of β-casein-covered HA particles.

5.5 Conclusions

This chapter characterised in detail the adsorption of the individual milk proteins, α_S-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin both individually, and mixed together, in pure model systems. A modelling approach using the Langmuir model enabled the differentiation of the proteins in terms of their affinity for HA and their maximum surface concentration. Individually, α_S-casein and β-casein adsorbed more onto HA, and had a higher affinity for HA, compared with κ-casein, β-lactoglobulin and α-lactalbumin, and this was attributed to the presence in their structure of clusters of phosphoserine residues. It was suggested that α_S-casein and β-casein might have been able to complex into oligomers (for α_S-casein) or micelle-type structure (for β-casein), therefore forming a thicker layer of adsorbed protein on the HA particles, compared to the whey proteins, which probably only formed a close-packed monolayer of protein on the HA surface. When all the proteins were mixed together, α_S-casein and β-casein were preferred for adsorption compared with κ-casein, β-lactoglobulin and α-lactalbumin, and this was related to the stronger nature of

their binding. It was not clear whether the different caseins from a mixture bound as individual molecules, or formed some aggregated structures on the HA surface.

The effect of ionic strength on the adsorption of the individual proteins and the proteins mixed together was also investigated. An increase of ionic strength from 7 mM to 100 mM led to more protein adsorbing onto HA, for all proteins. However, the increase in adsorption was larger for α_S-casein than for the other proteins, and this was considered to be due to a change in the aggregation state of α_S-casein on the HA surface with increasing ionic strength. It is well known that ionic strength has an effect on protein adsorption, because it affects both the HA surface properties and the properties of the adsorbed proteins (Wang et al., 2012; Zhu et al., 2007). Other physico-chemical variables such as pH or ionic composition of the suspending medium are also known to affect protein adsorption onto HA (Kandori et al., 2008; Sharpe et al., 1997; Yin et al., 2002). The effect of these different variables on protein adsorption and on the colloidal properties of HA particles need to be studied further to ultimately understand the protein adsorption phenomena and the colloidal properties of HA particles in milk. Therefore the next chapter will use the findings on the adsorption of the different individual milk proteins obtained in this chapter, to study in detail the effect of pH, ionic strength, ionic composition and milk serum composition on protein adsorption, and on the colloidal properties of the HA particles, using again SC and WPI as model milk protein solutions.

CHAPTER 6 Effect of pH, ionic strength and milk serum composition on the adsorption of milk proteins onto hydroxyapatite particles *

6.1 Abstract

The effects of ionic strength, pH and milk mineral composition on the colloidal properties of hydroxyapatite (HA) particles and on the adsorption of caseins and whey proteins onto HA particles were studied by zeta-potential measurements, turbidity, and determination of the amounts of adsorbed proteins on HA using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). NaCl addition, pH and addition of specific ions such as calcium, phosphate and citrate modified the zeta-potential of the HA particles, and, in the case of citrate and calcium ions, the suspension stability of the particles. The amount of adsorbed proteins increased with increasing ionic strength from 0 to 0.1 M and decreasing pH from 8 to 6, for both sodium caseinate (SC) and whey protein isolate (WPI). In general, when the absolute value of the zeta-potential decreased (because of changes in pH or ionic strength), more protein was found to adsorb onto the HA particles. This was attributed to a decrease in the electrostatic repulsions between the HA particles and the protein species. The effects of the composition of the milk serum were investigated using simulated milk ultrafiltrate (SMUF). Both caseins and whey proteins adsorbed less onto HA particles in SMUF than in water. However, the decrease in protein adsorption to HA in SMUF was less pronounced for caseins than for whey proteins and α_S-casein adsorption was the least affected. As the phosphoserine groups of the caseins bind strongly to HA, the caseins could compete better with the phosphate and citrate ions in the SMUF for adsorption. The binding of the caseins onto HA particles provided mostly a steric stabilisation to the particles against aggregation, and was independent of the zeta-potential magnitude, whereas the stabilisation provided by whey proteins was only of electrostatic nature, and therefore dependent on the zeta-potential magnitude.

---

* Part of the content presented in this chapter has been published as an original paper: Tercinier, L., Ye, A., Anema, S. G., Singh, A., & Singh, H. (2014a). *Food Biophysics*, DOI: 10.1007/s11483-014-9360-5
Part of the content was also presented as a conference presentation at the 5th International Symposium on Delivery of Functionality in Complex Food Systems, Tel Aviv, Israel, September 30th–October 3rd, 2013 and at the 2nd Mineral and Dairy Products Symposium, Auckland, New Zealand, February 26th–27th, 2014.

6.2 Introduction

The ability of milk proteins to bind to hydroxyapatite (HA) particles was demonstrated in Chapter 4 and Chapter 5. Casein and whey proteins in pure milk protein solutions, sodium caseinate (SC) and whey protein isolate (WPI) were able to bind onto HA particles, changing their zeta-potential and improving their suspension stability. Whey proteins were believed to bind by forming complexes between their side chain carboxyl groups and the calcium sites on HA, whereas caseins were believed to bind through a much stronger interactions between their phosphoserine residues and the HA calcium sites. However, these initial binding studies were only carried out in water or HEPES buffers. As the adsorption of proteins onto HA is driven by electrostatic interactions, the physico-chemical characteristics of the solution, especially its pH, ionic strength and salt composition, will affect the adsorption process. A number of studies has shown the effects of pH and ionic strength on the modification of the surface charge of HA (or other calcium phosphates) and its impact on the adsorption of different proteins, for example bovine serum albumin (Yin et al., 2002; Zhu et al., 2007), human serum albumin (Hlady & Füredi-Milhofer, 1979), lysozyme (Luo & Andrade, 1998) and lactoferrin (Iafisco et al., 2011). Specific ions, such as calcium, citrate and phosphate, have also been shown to adsorb strongly to the surface of HA particles, modifying the surface charge of HA, and therefore affecting the binding of proteins onto HA (Kawasaki, 1991; Leach, 1960; Lee et al., 2011; López-Macipe et al., 1998; Skwarek, Janusz, & Sternik, 2014; Yin et al., 2002; Zhu et al., 2007). As some calcium, phosphate and citrate are present as free ions in the serum phase of milk, they are likely to interact with HA particles, potentially affecting the binding of the milk proteins onto HA particles.

To date, there have been no studies undertaken on the interactions between HA particles and milk proteins in milk. Given the complexity of the milk system, a thorough understanding of the interactions between HA particles and individual milk proteins, studied using model protein systems, is first necessary. The objective of this chapter is therefore to examine the effect of ionic strength (indicated by NaCl concentration), pH and ionic composition of the milk serum on HA particles alone first, and secondly on the adsorption of caseins from SC and whey proteins from WPI onto HA particles. The insights gained from these model systems will be the foundation for further studies in milk that will be reported in Chapter 7.

6.3 Material and methods

6.3.1 Protein adsorption onto HA particles, under different physico-chemical conditions

Protein solutions of SC and WPI were prepared at different concentrations (w/w, total solids) and under different conditions of ionic strength, pH and mineral composition (see Table 3.2 for details), using NaCl solutions at concentrations of 0 to 0.5 M (see Section 3.2.2), SMUF, and SMUF with added citrate (see Section 3.2.2 and Table 3.3 for preparation of the suspending solutions). HA powder (10%, w/w) was added to the different SC and WPI solutions and stirred for 2 h, and the HA powder was then separated from the protein solutions by centrifugation. The protein concentration and composition of the adsorption supernatants containing the unadsorbed protein was determined using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; see Section 3.5.3) and the amount and composition of adsorbed protein was calculated by difference between the protein concentration and composition in the initial solution and in the adsorption supernatants (depletion method), as described in Section 3.2.4.

6.3.2 Characterisation of HA particles

HA particles were suspended in solutions of different physico-chemical conditions of pH, ionic strength and mineral composition (in calcium chloride, sodium phosphate buffer, sodium citrate buffer and SMUF) and characterised for zeta-potential (see Section 3.3.2), visual turbidity (see Section 3.3.4.1) and suspension stability using absorbance measurements (see Section 3.3.4.2).

6.4 Results and discussion

6.4.1 Effect of the pH and composition of the suspending solution on colloidal properties of HA particles

6.4.1.1 Ionic strength and pH effect

Zeta-potential of HA particles

HA particles were suspended in 0 to 0.5 M NaCl solutions, and the pH of which was adjusted to values between pH 5 and pH 10 using 1 M HCl and NaOH solutions, to measure the effect of ionic strength and pH on the zeta-potential of the particles.

Figure 6.1A shows the variation in the zeta-potential of the HA particles as a function of NaCl concentration, while Figure 5.1B shows the variation as a function of pH.

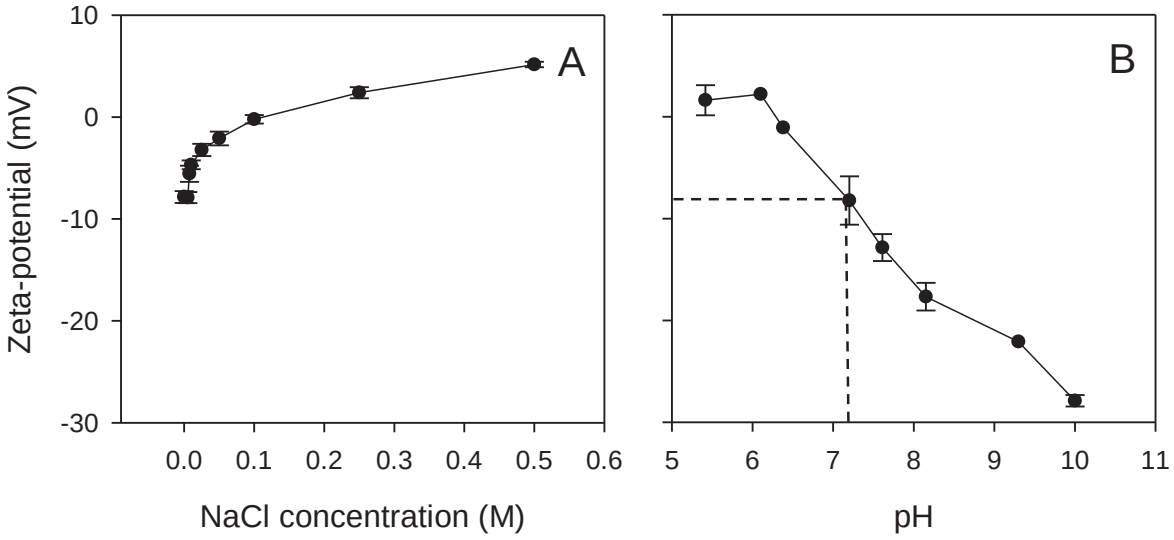

Figure 6.1: Effect of (A) NaCl concentration and (B) pH on the zeta-potential of suspensions of HA particles (0.05%, w/w). The dashed line indicates the pH and zeta-potential of the particles in water, without NaOH or HCl addition.

When the NaCl concentration was increased from 0 to 0.12 M, the zeta-potential had negative values, and the absolute value of the zeta-potential decreased from –8 to 0 mV; the zeta-potential values became positive above 0.12 M, reaching about +6 mV at a NaCl concentration of 0.5 M. As discussed in previous chapters, the surface of HA carries two types of binding sites on the crystal surface of HA: C-sites containing crystal calcium ions, P-

sites containing negatively charged oxygen atoms associated with crystal phosphate ions (Bernardi & Kawasaki, 1968; Kawasaki et al., 1985). The number and charge states of P-sites and C-sites on the HA surface depends on the physico-chemical conditions of the suspending media, such as pH, ionic strength, ionic composition, and determines the net charge of the particles (Kawasaki, 1991). In water (no NaCl added), HA had a net charge of the surface of the particles that was slightly negative (−8 mV, see Figure 6.1). This value was consistent with other zeta-potential values found in the literature for HA in water (Anema, 2009b; Huang et al., 2007; Kandori, Oda, Fukusumi, & Morisada, 2009), and has been explained by various hydrolytic and dissolution reactions at the surface of HA (Kandori et al., 2011), leading to a greater number of exposed surface P-sites than C-sites at neutral pH, and therefore a total negative charge at pH value around neutrality (Kandori, Masunari, & Ishikawa, 2005; Kandori et al., 2009).

A decrease in the magnitude of the zeta-potential of HA particles upon addition of NaCl has been observed in many other studies (Anema, 2009b; Huang et al., 2007; Yin et al., 2002; Zhu et al., 2007) and has been attributed to the charge-shielding effect of the salts. A net charge on the surface of any particle affects the distribution of ions in the surrounding interfacial region, called the electrical double layer, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the particle surface. The thickness of the electrical double layer (κ^{-1}, in nm, also called the Debye length) describes the distance at which the charge of the particle can be "felt" by the surrounding ions. The Debye length is inversely proportional to the square root of the ionic strength, which means that when salts are added to a solution, the Debye length decreases, and the double layer is compressed. Consequently, the magnitude of the zeta-potential of charged particles decreases upon addition of salts (Kirby & Hasselbrink, 2004).

If the monovalent salt ions (Na^+ and Cl^-) had no specific interactions with the particle surface (indifferent electrolytes), the zeta-potential value would be expected to reach a plateau at zero; all the charges would be shielded by the salt. However, the charge reversal observed above 0.12 M NaCl might indicate specific sodium ion adsorption onto the surface of the HA particles. It has been shown that sodium ions can adsorb specifically to HA surfaces, whereas chloride ions cannot because of steric hindrance (Kawasaki et al., 1985; Kawasaki, 1991), as the sodium ionic radius, of 95 picometres (Correia, Magalhaes, Marques, & Senos, 1996), is smaller than that of chloride ions, of 181 picometres (Koutsopoulos, 2002). It has been suggested that sodium ions might be able to replace the protons of the exposed phosphate groups on HA (Dattolo et al., 2010).

Figure 6.1B shows the variation in the zeta-potential of HA particles as a function of pH; the zeta-potential became more negative, from −8 to −30 mV, with increasing pH from 7.2 to 10 and became less negative, from −8 to 0 mV, with decreasing pH from 7.2 to 6.4. The zeta-potential became slightly positive at pH values below 6.4. Both OH^- and H^+ are potential-determining ions and bind to the phosphate ions of HA (Kawasaki, 1991; Wassell et al., 1995). Depending on the pH, the degree of protonation of the phosphate ions on the HA surface can change between PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, which explains the variation in zeta-potential as a function of pH. The point at which the zeta-potential reaches 0 mV is usually called the point of zero charge and was found to be around pH 6.5 in this study. This value is in accordance with the literature, where the point of zero charge is usually reported to be between 6.4 and 8 (Wassell et al., 1995).

Suspension stability of HA particles

As shown in Chapter 4, the zeta-potential of HA particles is a good predictor of the aggregation and sedimentation rates of the particles. The more negative the zeta-potential, the stronger the repulsive forces between the particles, the less the particles aggregate and the slower they sediment over time. As the addition of NaCl and the change in pH modified the magnitude of the zeta-potential (Figure 6.1), it was expected that differences in the suspension stability of the particles suspended in solutions of different NaCl concentrations or different pH values would be observed. For example, Anema (2009b) studied the aggregation rate of small calcium phosphate particles (of average diameter D[3,2] of about 0.5 μm) in NaCl solutions of different concentrations. He observed that the size increase of the particles suspended in 0.08 M NaCl over 70 min was greater than that of particles suspended in water, causing a faster aggregation rate of the particles in NaCl than in water. As far as can be ascertained from the literature, there is no study looking at the effect of pH on the aggregation and sedimentation rates of HA.

There was no obvious difference in the visual sedimentation rate of particles suspended in water and NaCl solutions of concentrations 0.05 M–0.5 M, or in water at pH 6, 7.2 and 10. Figure 6.2A shows photographs of suspensions of HA particles suspended in 0, 0.05, 0.1 and 0.5 M NaCl, with, respectively, zeta-potentials of about –8, –2, 0 and +6 mV (Figure 6.1A) after about an hour left undisturbed on the bench at room temperature. For all samples, the particles were completely sedimented at the bottom of the tubes, and visually, it was impossible to distinguish whether the particles in salt solutions (having a less negative zeta-potential) sedimented faster than the particles in water. Between particles suspended at different pH, there was a greater difference of zeta-potential between the different samples,

of about 30 mV between HA particles at pH 6 and pH 10 (Figure 6.1B). Therefore, it was expected that observe a difference in the sedimentation rate of the particles suspended at different pH would be observed. However, as was the case with ionic strength, after about an hour, the HA particles at pH 6, 7.2 and 10 had all sedimented to the bottom of the tubes (Figure 6.2B) As for NaCl concentration, it was hypothesised that the change in the magnitude of zeta-potential caused by a change of pH from 6 to 10 might have been too small, or might not have provided a sufficient electrostatic stabilisation to observe a decrease in the sedimentation rate of the particles, especially since the HA particles used in this study had micrometre sizes.

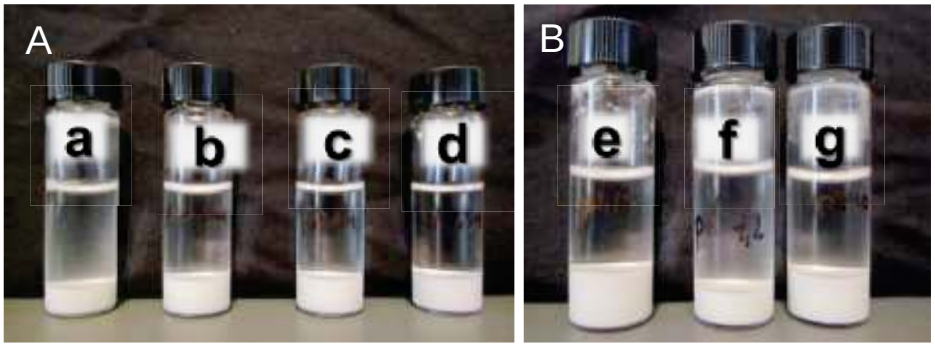

Figure 6.2: Effect of (A) NaCl concentration and (B) pH on the suspension stability of HA particles. HA particles were suspended at a concentration of 5% (w/w) in (a) water, (b) 0.05 M NaCl, (c) 0.1 M NaCl, (d) 0.5 M NaCl, (e) water at pH 6, (f) water at 7.2, (g) water at pH 10 and left undisturbed on the bench for one hour.

The suspension stability of the particles in salt solutions was further characterised by spectrophotometric measurements as a function of time (Figure 6.2), to check whether small differences in the sedimentation rate could be detected by a more precise instrumental technique using a less concentrated suspension of HA particles. The particles were suspended in NaCl solutions of concentrations 0–0.5 M and the absorbance of the suspension was measured at 900 nm for 200 min. Figure 6.3 shows the reduction in absorbance with time of suspensions of HA particles suspended in 0, 0.05, 0.1, and 0.5 M NaCl solutions. Within about 150 min, the absorbance of all the HA suspensions decreased to less than 5% of their original value, indicating that all the particles had sedimented to the bottom of the cuvette. There was no difference between the slopes of the different curves (Figure 6.3), indicating that the particles suspended in solutions of different salt concentrations sedimented roughly at the same rate.

No spectrophotometric measurement was carried out on HA suspension at different pH values, as it was shown for NaCl concentrations that measuring the variation of absorbance overtime did not provide more information than the simple observation over time of the HA particles in suspension.

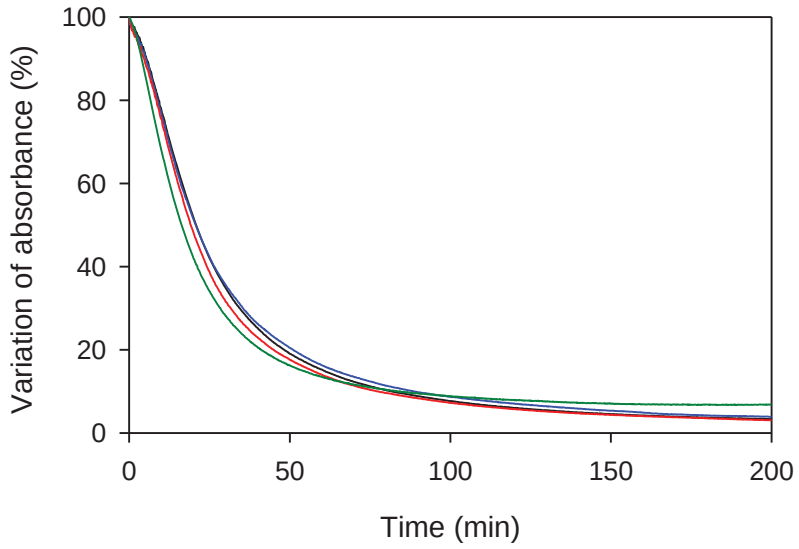

Figure 6.3: Variation of absorbance (expressed in percentage of initial absorbance) as a function of time of suspensions of HA particles (0.125%, w/w) in solutions of different NaCl concentrations. HA particles were suspended in water (black line), 0.05 M NaCl (red line), 0.1M NaCl (blue line) and 0.5 M NaCl (green line).

In conclusion, the visual observation of suspension stability and the spectrophotometric method did not enable differences to be observed in sedimentation rates of HA particles suspended in solutions of different NaCl concentrations or different pH. Some small differences in the sedimentation rate of the particles might have occurred, but the two methods were not precise enough to give measurable differences with respect to sedimentation rate, using particles of micrometre size. Small changes in aggregation and sedimentation rate of calcium phosphate particles due to NaCl addition and correlated with zeta-potential variations have been shown before by Anema (2009b)and Huang et al. (2007), but the authors used smaller particles, of nanometre size. Therefore, the aggregation and sedimentation of the particles occurred at a slower rate, which enabled the measurement of small differences between different suspending solutions.

6.4.1.2 Calcium, phosphate and citrate ions effect

Zeta-potential of HA particles

0.05% HA particles were suspended in sodium phosphate buffers (pH 7), sodium citrate buffers (pH 7) and calcium chloride solution ($CaCl_2$) of different concentrations. The zeta-potentials of the HA particle were measured, and reported as a function of the concentration of the phosphate, citrate and calcium (Figure 6.4).

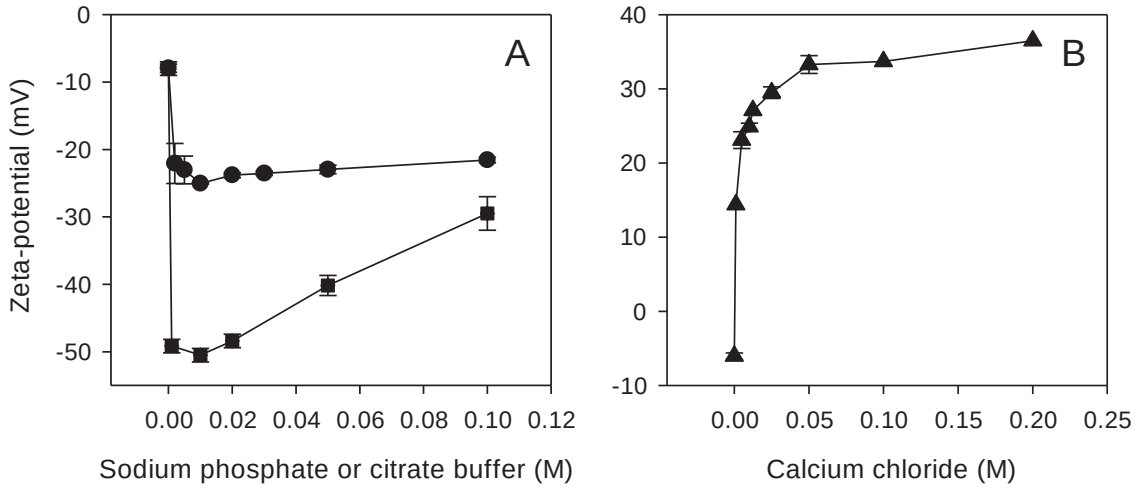

Figure 6.4: Effect of various ions on the zeta-potential of suspensions of HA particles (0.05%, w/w): (A) phosphate and citrate ions, (B) calcium ions. HA particles were suspended in sodium phosphate buffer (●), sodium citrate buffer (■), and calcium chloride solutions (▲).

The addition of phosphate and citrate buffers caused the absolute value of the zeta-potential to decrease. As the phosphate and citrate buffer concentrations increased from 0 to 0.01 M, the zeta-potential of the particles became more negative, from –8 mV to –26 mV for phosphate buffer addition (Figure 6.4A) and from –8 mV to –50 mV for citrate buffer addition(Figure 6.4B). In contrast, as the calcium chloride concentration increased from 0 to 0.05 M, the zeta-potential of the particles increased from –8 mV to +32 mV. Initially, the negative zeta-potential was neutralised by calcium ions, and then the HA surface became positively charged (Figure 6.4C). This shows that phosphate, citrate and calcium ions must bind onto the HA surface. Phosphate, citrate and calcium ions are potential-determining ions for HA. They have been reported to bind specifically on charged sites of HA, therefore affecting their surface charge (Harding et al., 2005; Johnsson et al., 1991; Leach, 1960;

López-Macipe et al., 1998; Yin et al., 2002). Calcium ions specifically complex with the phosphate ions on HA (P-sites) and charge the HA surface positively, whereas phosphate ions bind onto the calcium sites of HA (C-sites), charging the HA surface negatively. The adsorption of citrate ions onto HA has also been demonstrated before (Johnsson et al., 1991; López-Macipe et al., 1998; Martins et al., 2008; Skwarek et al., 2014; Tan et al., 2009) and two different adsorption mechanisms have been proposed. The citrate ions might either bind to the surface calcium ions (C-sites) (Johnsson et al., 1991; Martins et al., 2008), or they might replace the phosphate ions at the HA surface (ionic exchange of phosphate by citrate ions at the solid/solution interface), which would be explained by a higher affinity of the citrate species than phosphate species for the HA calcium ions (López-Macipe et al., 1998; Skwarek et al., 2014).

The maximum absolute values of the zeta-potential of -26 mV, -50 mV, and -32 mV, reached for calcium, citrate and calcium ions, respectively at concentrations of 0.01 M for phosphate and citrate, and 0.05 M for calcium, must correspond to a saturation state of the surface with respect to the ions. These maximum magnitudes increased in the order: phosphate (26 mV) < calcium (32 mV) < citrate (50 mV). Citrate ions have been reported previously to produce a larger negative zeta-potential compared with phosphate ions (Leach, 1960). The differences of magnitude between the different ions might be due to the difference in the net charge of the different ions at pH 7. Calcium is a divalent ion (Ca^{2+}) and has therefore a net charge of 2e at any pH. Sodium citrate and sodium phosphate buffers are polyprotic acids, therefore the charge of the citrate and phosphate ions depends on the pH of the solution, which determines the distribution of their respective ionic species between their monovalent, divalent and trivalent forms (Skwarek et al., 2014). Knowing the dissociation constants (pKas) of sodium phosphate and sodium citrate buffers, it is possible to calculate the ionisation state of the citrate and phosphate ions as a function of pH (López-Macipe et al., 1998; Skwarek et al., 2014). At pH 7, about 75% of the citrate is present in its trivalent form Cit^{3-} and 25% in its divalent form $HCit^{2-}$ (pKa_3 of sodium citrate = 6.4), whereas for the phosphate ions, about 70% of the phosphate is present as the divalent form HPO_4^{2-} and 30% as the monovalent form $H_2PO_4^-$ (pKa_2 of sodium phosphate = 6.8). Therefore, the net charge of the citrate ions is higher than the net charge of the calcium ions, which in turn is higher than the net charge of the phosphate ions. This must explain the order of magnitude of the zeta-potentials (phosphate < calcium < citrate).

As the phosphate and citrate concentrations were increased further from 0.01 M to 0.1 M, the zeta-potential values became less negative, from –26 mV to –22 mV for phosphate and

from –50 mV to –30 mV for citrate (Figure 6.4A). This was probably due to the diffusion of the phosphate and citrate ions in the double layer (charge shielding effect), as explained for the effect of NaCl on the zeta-potential of the particles. However, this was not observed for calcium ions, where further addition of calcium from 0.05 to 0.2 M did not lead to any significant change in the zeta-potential of the particles.

Suspension stability of HA particles

The suspension stability of the particles in the different buffers was assessed by looking at the turbidity of the HA suspensions over 24 hours (Figure 6.5).

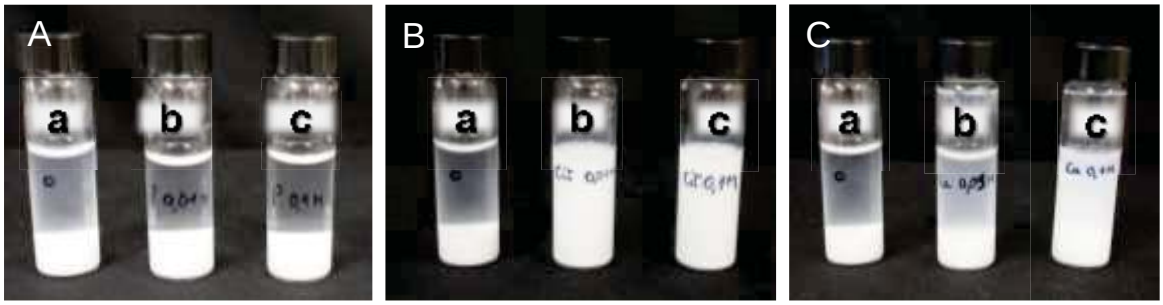

Figure 6.5: Effect of (A) phosphate ions, (B) citrate ions, and (C) calcium ions on the suspension stability of 5% (w/w) HA particles. Ionic concentrations were (a) 0 (i.e., water), (b) 0.01 M, (c) 0.1 M.

The suspension stability of the particles suspended in phosphate buffer did not improve, even though the addition of phosphate ions caused a decrease in the absolute value of the zeta-potential; after 24 h the particles were all sedimented to the bottom of the tubes, regardless of the phosphate concentration (Figure 6.5A). However, the particles suspended in the citrate buffer remained fully suspended after 24 h at the two citrate concentrations tested (0.01 M and 0.1 M, see Figure 6.5B, b and c, respectively), indicating that the binding of citrate ions to HA particles significantly improved their suspension stability. It should be noted that the citrate-coated particles were still well suspended after a week left undisturbed on the bench. The suspension stability of the particles suspended in the calcium buffer was also improved compared with the control suspended in water (Figure 6.5C), and this improvement was better for the suspension at the higher calcium concentration (0.1 M calcium; Figure 6.5C, sample c, corresponding to a zeta-potential of about +30 mV) than for the suspension at the lower calcium concentration (0.01 M calcium; Figure 6.5C, sample b,

corresponding to a zeta-potential of about +20 mV). Both citrate and calcium ions seemed to provide electrostatic stability to the particles. However, the electrostatic stability provided by calcium ions was weaker than that by citrate ions, as after about 2 days, all the particles in calcium buffers had all sedimented. This might be due to the higher magnitude of the zeta-potential of citrate-coated particles compared with calcium-coated particles (maximum values of, respectively, –50 mV and +30 mV). The significant improvement of the colloidal stability of HA particles in the presence on citrate has been observed before (Martins et al., 2008; Tan et al., 2009) and has been mostly explained by an electrostatic stabilisation against aggregation. As citrate ions carry three carboxyl groups per ion, it is possible that the citrate ions also provide some steric stabilisation (Wang et al., 2007).

Overall, these results show that the adsorption of potential-determining ions such as calcium, phosphate and citrate onto HA particles modify their zeta-potential, and can provide electrostatic stabilisation against aggregation. However, these results also suggest that the change in zeta-potential caused by the adsorption of the different ions is not the sole determinant of the improvement in suspension stability, since citrate ions provided a much better stabilisation than calcium and phosphate ions. In the presence of ions and salts, some other surface phenomena such as an alteration of the HA surface properties or some ion bridging between the particles might also occur, and affect the aggregation behaviour of the particles, as suggested before by Anema (2009b) and Martins et al. (2008).

6.4.1.3 Effect of SMUF composition on HA particles properties

SMUF mimics the composition of the milk serum in terms of pH, ionic strength, ionic calcium activity and salt concentration (Jenness & Koops, 1962). The zeta-potential of HA particles in SMUF (at pH 6.67) was –20 ± 2 mV, compared with –8 mV in water. The milk serum phase contains minerals that are either present as free ions or associated with other minerals as complexes. The concentrations of the different mineral species in the milk serum have been calculated by Holt et al. (Holt, 2004). The milk serum phase contains about 10 mM calcium, of which about 2 mM is present as free calcium ions (Ca^{2+}) and 8 mM is associated with other ions. It also contains about 12.4 mM phosphate, of which about 10 mM is present as free phosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) and 2 mM is complexed with other ions, and 9.2 mM citrate, of which about 0.3 mM is as free ions (Cit^{3-} and $HCit^{2-}$) and the rest is complexed with other ions, mostly in the form of calcium citrate complexes. Phosphate, citrate and calcium ions have been shown to be potential-determining ions for HA in the previous

section (Section 6.4.1.2). They specifically adsorb onto the P-sites (for calcium ions) or C-sites (for phosphate and citrate ions) of HA, affecting the zeta-potential of the particles. As the zeta-potential of the HA particles became more negative in SMUF, it can be hypothesised that phosphate and citrate ions from the SMUF solution are able to bind to the C-sites of HA particles.

The suspension stability of the HA particles suspended in SMUF was considerably better than in water, as the particles remained well suspended for a few days. This means that the adsorption of the ions from the SMUF solution provided some electrostatic stabilisation to the HA particles, as demonstrated for citrate ions in Section 6.4.1.2.

6.4.2 Effect of pH and composition of the suspending solution on milk protein adsorption onto HA particles

6.4.2.1 Effect of NaCl addition of milk protein adsorption onto HA particles

In the previous section, NaCl concentration was shown to affect the zeta-potential of HA particles (Figure 6.1A). NaCl concentration is also known to affect the structure and self-association of caseins (Schmidt & Van Markwijk, 1968; Swaisgood, 2003) and whey proteins (Kronman et al., 1967; Townend & Timasheff, 1960), therefore affecting their binding on different types of surfaces, for example on oil droplets in emulsion systems (Hunt & Dalgleish, 1995; Srinivasan et al., 2000) or on solid surfaces (Elofsson et al., 1997; Kull, Nylander, Tiberg, & Wahlgren, 1997; Wahlgren et al., 1993). Ionic strength is therefore expected to affect the binding of milk proteins onto HA particles, by having an effect on both the electrostatic interactions between HA and the proteins, and the structure of the adsorbed protein at the interface (Nakanishi et al., 2001; Wassell et al., 1995; Yin et al., 2002).

To study the effect of ionic strength on milk protein binding to HA, 10% (w/w) HA particles were added in SC or WPI solutions at two different initial concentrations (2% and 4%, w/w, for SC and 1% and 2%, w/w, for WPI) and six NaCl concentrations (0, 0.025, 0.05, 0.1, 0.25, and 0.5 M added NaCl). The higher initial concentration of both protein sources was chosen to correspond to the adsorption plateau, at which the maximum amount of adsorbed protein was reached (i.e., the proteins form a saturated protein layer on the surface of HA particles, as shown previously in Chapter 4). The lower initial concentration of both protein sources

corresponded to a medium level of protein coverage. As NaCl addition to the protein solutions caused a slight decrease in pH, the pH was readjusted to 6.8 for all the SC and WPI solutions, before adding the HA particles. The HA particles were stirred with the protein solutions for two hours and separated by centrifugation to obtain the adsorption supernatants containing the unadsorbed proteins. The initial SC and WPI solutions and their respective supernatants were analysed for protein content using SDS-PAGE.

Figure 6.6 shows SDS-PAGE patterns for the initial protein solutions and their respective supernatants, obtained at different NaCl concentrations, for SC (Figure 6.6A) and WPI (Figure 6.6B).

The intensities of the bands obtained for the initial solutions containing 0, 0.1, 0.25 and 0.5 M of added NaCl were similar (lanes 1, 2, 3 and 4, respectively, in Figure 6.6A,B), indicating that the addition of NaCl did not affect the solubility of SC and WPI. Therefore, an average intensity was calculated from the band intensities of the four initial solutions, and used as the control intensity for all the initial solutions. However, in the supernatants obtained at different NaCl concentrations (lanes 5 to 9 in Figure 6.6A,B), the intensities of the protein bands were different between different salt concentrations. For SC, the protein band intensities of the supernatants decreased as NaCl concentration increased from 0 to 0.1 M (lanes 5 to 7 in Figure 6.6A), but seemed constant with further addition of NaCl (0.25 M and 0.5 M: lanes 8 and 9, respectively, in Figure 6.6A).

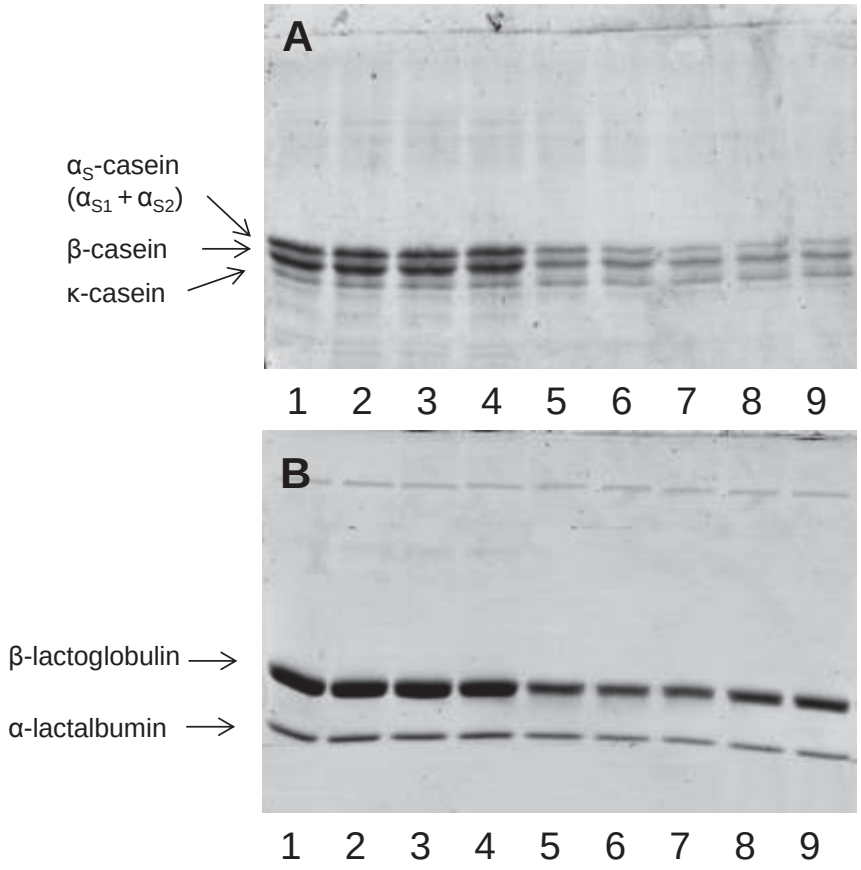

Figure 6.6: SDS-PAGE pattern of initial protein solutions (lanes 1-4) and supernatants (i.e., containing the unadsorbed protein; lanes 5-9) obtained for adsorption experiments carried out with (A) 4% (w/w) SC solutions and (B) 2% (w/w) WPI solutions prepared with different concentrations of added NaCl. Lanes 1 and 5, no added NaCl; lanes 2 and 7, 0.1 M NaCl; lanes 3 and 8, 0.25 M NaCl; lanes 4 and 9, 0.5 M NaCl; lane 6, 0.05 M NaCl.

As the supernatants contained the unadsorbed protein, the decrease in protein band intensities upon addition of salt shows that more protein must have adsorbed onto the HA particles as NaCl concentration increased from 0 to 0.1 M. It can be seen that it is mostly the intensity of the α_S-casein band that decreased as NaCl concentration increased from 0 to 0.1 M (Figure 6.6A). This shows that the addition of NaCl probably caused an increase mostly in the amount of adsorbed α_S-casein, rather than in the amount of all adsorbed caseins, as already shown in Chapter 5 when using a mix of the three caseins in 50 mM HEPES buffer (pH 6.8) at 0.1 M ionic strength. For WPI, the protein band intensities of the supernatants in water (no NaCl added, lane 5 in Figure 6.6B) were slightly higher than those of the supernatants in 0.05 M and 0.1 M NaCl (lanes 6 and 7, respectively, in Figure 6.6B). As was

the case for caseins, this indicates that more whey proteins must have adsorbed onto HA as NaCl concentration increased from 0 to 0.1 M. However, the protein band intensities of the supernatants containing 0.25 and 0.5 M added NaCl (lanes 8 and 9, respectively, in Figure 6.6B) were slightly higher than the protein band intensities in the supernatant containing 0.1 M of added NaCl (lane 7). This indicates that for NaCl concentrations higher than 0.1 M, the amount of adsorbed whey protein may have slightly decreased.

Figure 6.7 shows the surface protein concentrations of SC and WPI, calculated from repeated SDS-PAGE gels similar to those shown in Figure 6.6, for each NaCl concentration, and for two different initial concentrations of SC (2% and 4%, w/w) and WPI (1% and 2%, w/w). The maximum amount of protein bound to the HA particles was higher for SC than for WPI for all NaCl concentrations, as expected, since caseins were shown in Chapter 4 to bind more than whey proteins onto HA. Increasing the NaCl concentration from 0 to 0.1 M resulted in an increase in protein adsorption for both SC and WPI for the two chosen initial concentrations. The maximum amount of adsorbed caseins and whey proteins increased by 22% and 32%, respectively, when the concentration of NaCl was increased from 0 to 0.1 M, from about 2.7 to 3.3 mg/m^2 for SC (Figure 6.7A) and from about 1.3 to 1.7 mg/m^2 for WPI (Figure 6.7B). A further increase in NaCl concentration had no effect on the adsorption for SC and resulted in a slight decrease in adsorption for WPI.

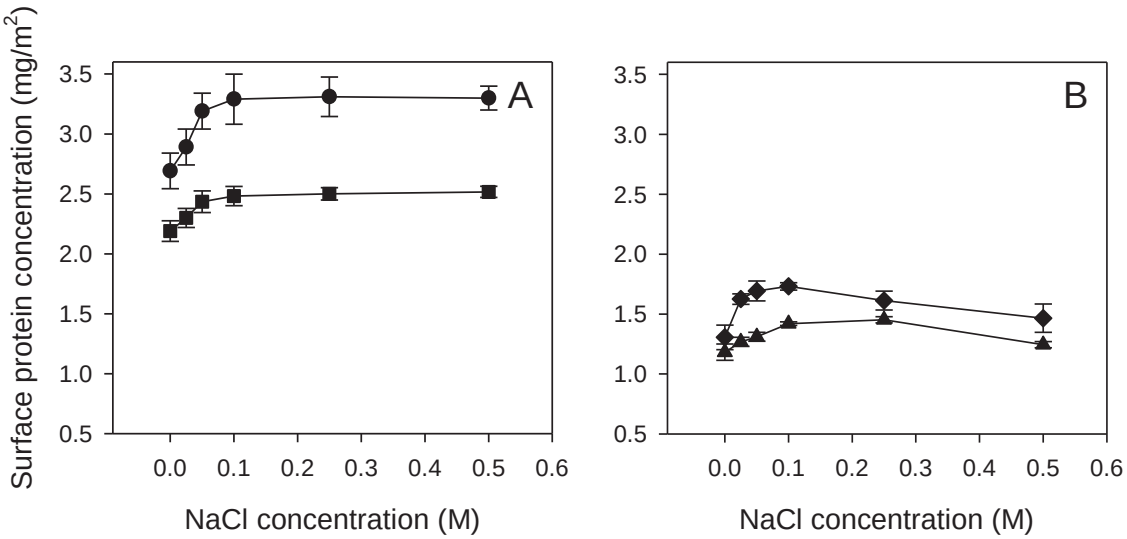

Figure 6.7: Effect of NaCl concentration on the surface protein concentration of (A) SC and (B) WPI onto HA particles. ■, initial SC concentration 4% (w/w); ●, initial SC concentration 2% (w/w); ◆, initial WPI concentration 2%; ▲ initial WPI concentration 1%. Error bars represent standard deviation.

The increase in the amount of protein adsorbed onto HA with increasing salt concentration observed in this study was in agreement with previous studies on the binding of acidic proteins onto HA (Gorbunoff & Timasheff, 1984a; Kawasaki, 1991; Yin et al., 2002). Both caseins and whey proteins are considered to be acidic proteins as they exhibit a net negative charge at pH 6.8. The mechanism of adsorption of acidic proteins onto HA has been explained mainly by the attractive force between the side carboxyl groups or phosphate groups of the protein and the calcium sites (C-sites) of HA, leading to the formation of complexes (Bernardi & Kawasaki, 1968; Gorbunoff, 1984; Gorbunoff & Timasheff, 1984a). However, as well as the attractive forces between carboxyl and phosphate groups of the proteins and the C-sites of HA, there is a repulsive force between the carboxyl and phosphate groups of proteins and the negative phosphate sites (P-sites) of HA. With increasing ionic strength, the zeta-potential of HA is reduced (Figure 6.1A). Therefore, the repulsive forces between the negatively charged proteins groups and the negative sites of HA are reduced, allowing more proteins to bind to the HA particles (Hlady & Füredi-Milhofer, 1979; Yin et al., 2002; Zhu et al., 2007). The main studies looking at the effect of NaCl concentration on the adsorption of acidic proteins onto HA have been done with bovine serum albumin (BSA) (Yin et al., 2002; Zhu et al., 2007), and the authors suggested that the reduced charge repulsion was the sole reason for the enhanced adsorption of BSA onto HA when the ionic strength was increased. However, to explain the effects of ionic strength on protein adsorption, the electrostatic interactions among the protein molecules and within the protein chains as well as the interactions between the proteins and the HA particles must be taken into account.

Increasing the salt concentration has an effect on the ionisation states of the protein residues and therefore on the structure and self-association of the milk proteins (Dickinson, 1998; Srinivasan et al., 2000; Syrbe, Bauer, & Klostermeyer, 1998). At low salt concentration, the electrostatic repulsions between the protein molecules or the negatively charged groups within the protein chains on the same molecule must limit the amount of protein that can bind to the HA particles. Increasing the salt concentration tends to shield the charges on the protein chains, therefore reducing the electrostatic repulsion between the adsorbed proteins (Kandori et al., 2004). This could lead to a more compact packing of the adsorbed caseins and whey proteins at the HA surface (Jones & O'Melia, 2000; Srinivasan et al., 2000), possibly contributing to the increase in the amount of adsorbed protein that was observed for both caseins and whey proteins when the NaCl concentration was increased from 0 to 0.1 M (Figure 6.7).

Possible differences in the aggregation behaviour of SC when salts are added could also explain the higher surface load of SC on HA particles. HadjSadok et al. (2008) showed that, at relatively low protein concentrations (<5%, w/w), SC was present as individual molecules at low salt concentration (3 mM) when electrostatic repulsions were strong, but formed small sized aggregates when NaCl was added because of the screening of the electrostatic interactions. The aggregation was maximal at a NaCl concentration of 0.1 M. The increase in casein binding upon addition of NaCl may therefore be due to SC binding onto the HA particles as individual molecules at low salt concentrations and as aggregates at high salt concentrations.

For NaCl concentrations greater than 0.1 M, even though the zeta-potential became positive (Figure 6.1), there was no further increase in casein adsorption (Figure 6.7A) and there was a small reduction in whey protein adsorption (Figure 6.7B). At high concentrations (usually >0.1 M), salts can alter the conformation and solubility of proteins. This is usually referred to as the salting-out effect and may lead to decreased protein adsorption (Zhang, Dalgleish, & Goff, 2004).

Surface protein composition

To further understand the effect of NaCl on protein adsorption, the estimated amounts of the main individual caseins and whey proteins bound to the HA particles were calculated, for the two higher SC and WPI initial concentrations used in the experiment (4%, w/w, for SC and 2%, w/w, for WPI). The amount of adsorbed individual proteins was calculated from the proportions of adsorbed caseins, determined by SDS-PAGE and from an estimated composition of the initial SC and WPI solutions, based on the theoretical proportions of the individual proteins in skim milk. The initial SC solution contained approximately 50% α_S-casein (α_{S1} + α_{S2}), 38% β-casein and 12% κ-casein and the initial WPI solution contained approximately 80% β-lactoglobulin and 20% α-lactalbumin (Wong et al., 1996). When the concentration of NaCl was increased from 0 to 0.1 M, the amount of adsorbed α_S-casein increased by 30%, from about 1.5 to 2 mg/m^2 (Figure 6.8A) whereas there were no significant changes in the surface concentrations of β-casein and κ-casein (P <0.05). This was also observed in Chapter 5 when using a mixture of the three caseins; α_S-casein was the only caseins that adsorbed significantly more at 100 mM ionic strength than at 7 mM ionic strength.

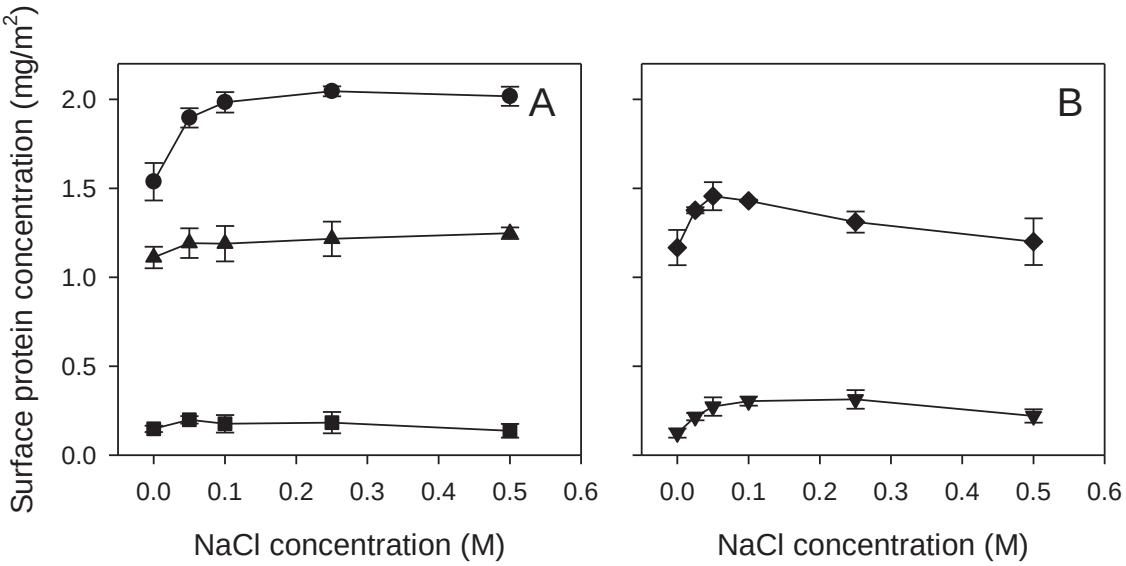

Figure 6.8: Changes in the estimated surface concentration (mg/m²) of (A) individual caseins from 4% (w/w) SC and (B) individual whey proteins from 2% (w/w) WPI, as a function of NaCl concentration. ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein; ◆, β-lactoglobulin; ▼, α-lactalbumin. Error bars represent standard deviation.

NaCl has been shown to affect the association behaviour of the individual caseins in SC, consequently affecting their adsorption behaviour (Srinivasan et al., 2000). At ionic strengths greater than 0.003 M and pH 6.6, α_{S1}-casein can self-associate and form oligomers (Schmidt & Van Markwijk, 1968; Schmidt, 1970; Swaisgood, 2003). As ionic strength increases, the formation of α_{S1}-casein oligomers become favourable and dimers or trimers can form (Schmidt & Van Markwijk, 1968; Schmidt, 1970; Srinivasan et al., 2000). α_{S2}-Casein also self-associates and form oligomers. The self-association of α_{S2}-casein is sensitive to ionic strength, and is maximal at ionic strength of about 0.2 M (Swaisgood, 2003). The increase in NaCl concentration in this experiment may have caused α_{S1}-casein and α_{S2}-casein in SC to self-associate and form oligomers, leading to a greater adsorption of α_S-casein (α_{S1} + α_{S2}) onto the HA particles , contributing mostly to the increased amount of adsorbed caseins observed in Figure 6.7. The same hypothesis was given in Chapter 5 to explain the significant increase of α_S-casein in HEPES buffer (pH 6.8) as the ionic strength increased from 7 mM to 100 mM. β-Casein and κ-casein also self-associate to form micelle-like structures (De Kruif & Grinberg, 2002; Schmidt, 1982; Vreeman et al., 1981), but their self-association involves mostly hydrophobic interactions and is not very sensitive to ionic strength (Swaisgood, 2003). It would therefore explain why when using SC, the amount of

adsorbed β-casein and κ-casein onto HA was not affected significantly by the increase in NaCl concentration.

The composition of the SC aggregates with respect to the different caseins is not fully understood (HadjSadok et al., 2008; Lucey et al., 2000). However, the aggregation behaviour of the different caseins in SC is very likely to follow the same mechanisms as the self-association of each of the caseins, and therefore be affected by ionic strength (Horne, 1998). At low salt concentration, caseins in SC might form β-casein-rich aggregates, whereas at high salt concentrations, they might form α_S-casein-rich aggregates. The SC aggregates would be adsorbed onto the HA particles, and the differences in their composition would explain the difference in composition of the adsorbed casein layer, observed upon salt addition.

When the concentration of NaCl was increased from 0 to 0.1 M, the increase in the amount of α-lactalbumin was greater than that of β-lactoglobulin. The adsorbed amount of β-lactoglobulin increased by 23%, from about 1.2 to about 1.4 mg/m^2, and the adsorbed amount of α-lactalbumin increased by 200%, from about 0.1 to 0.3 mg/m^2 (Figure 6.8B). No report in the literature could be found showing a change of ratio between adsorbed β-lactoglobulin and α-lactalbumin upon addition of NaCl, on either hydrophobic or hydrophilic surfaces. It is therefore unclear why the increase in adsorbed α-lactalbumin was greater than that of β-lactoglobulin when NaCl increased from 0 to 0.1 M. It could be that sodium ions affect the conformations of the two proteins differently. For example, sodium ions have been shown to induce a conformational change to α-lactalbumin; α-lactalbumin can lose entropy and become more rigid and compact when NaCl is added (Desmet et al., 1987). α-Lactalbumin might therefore be able to adsorb more onto HA surface, which could explain the relative enrichment of α-lactalbumin compared with β-lactoglobulin in the adsorbed protein layer. However, it is important to note that in 0.1 M NaCl, the relative percentage of adsorbed β-lactoglobulin was still in the order of about 82% of the total amount of adsorbed whey proteins (versus about 90% in water), so β-lactoglobulin was still preferred over α-lactalbumin for adsorption onto HA at 0.1 M ionic strength. This preference in adsorption of β-lactoglobulin over α-lactalbumin was already observed in Chapter 5, at low ionic strength (7 mM) and high ionic strength (100 mM) when starting from a protein solution containing half of each whey protein.

Zeta-potential and suspension stability of SC and WPI-coated particles

Table 6.1 shows the variation of the zeta-potential of the SC or WPI-coated particles with NaCl concentration. The HA pellets obtained after the adsorption of 4% (w/w) SC and 2% (w/w) WPI in 0 to 0.5 M NaCl solutions were re-suspended at 0.05% (w/w) in NaCl solutions at the same concentrations used for the adsorption experiment. The increase in the absolute value of the zeta-potential was calculated by difference between the zeta-potential of the uncoated particles (initial value) and the zeta-potential of the coated particles (final value) suspended in the same NaCl solution. The measurements were carried out on suspensions of HA particles that were fully coated by proteins; therefore, the final zeta-potential values obtained correspond to the charge of the particles, the surface of which is saturated with proteins.

From the data in Table 6.1, it can be seen that the change in the absolute value of the zeta-potential between uncoated HA particles and particles coated with SC or WPI was independent of ionic strength, and was approximately -18 mV for SC-coated particles (values between 15 and 20) and -12 mV for WPI (values between 10 and 14 mV). It was the magnitude of the initial zeta-potential that decreased as NaCl concentration increased, as discussed in Section 6.4.1, where it was attributed to the compression of the electrical double layer upon addition of salts. The same shielding effect was observed on the protein-coated particles, decreasing the magnitude of the zeta-potential of the SC- and WPI-coated particles as the salt concentration increased. However, the change in zeta-potential between the uncoated and the coated particles caused by the protein adsorption remained the same regardless of the ionic strength.

Zeta-potential values of particles coated by proteins at different salt concentrations can also give information on the structure of the adsorbed protein layer (Ohshima, 2007). In the case of particles coated by a tightly adsorbed layer, the zeta-potential values would be expected to reach 0 at high salt concentration, as a result of the charge shielding effect (Ohshima, 2007). In this study, the zeta-potential of the protein-coated particles did not reach 0. It reached a plateau value at –12 mV for the SC-coated particles and at –5 mV for the WPI-coated particles when NaCl was increased from 0 to 0.5 M. This could be due to the fact that the protein layer formed by SC (and to a lesser extent by WPI) is more of a soft penetrable layer, rather than a hard layer type (Ohshima, 2007).

Table 6.1: Effect of NaCl concentration on zeta-potential of HA particles coated with SC or WPI, and calculated change between the initial zeta-potential (uncoated particles) and the final zeta-potential (coated particles). [a]

NaCl concentration (M)	Zeta-potential (mV)		
	Initial	Final	Change
Particles coated with SC			
0 (water)	–8	–28	20
0.05	–2	–20	18
0.1	0	–16	16
0.25	+2	–13	15
0.5	+5	–12	17
Particles coated with WPI			
0 (water)	–8	–22	14
0.05	–2	–13	11
0.1	0	–12	12
0.25	+2	–8	10
0.5	+5	–5	10

[a] HA particles were suspended in 4% (w/w) SC or 2% (w/w) WPI solutions reconstituted in NaCl solutions at different concentrations (0-0.5 M), stirred for 2 h, then centrifuged and the pellets re-suspended in the same NaCl solution for zeta-potential measurements.

It was shown in Chapter 4 that the suspension stability in water of the particles coated with SC and WPI improved, and this was correlated with the negative value of their zeta-potential. The adsorbed proteins were believed to prevent the aggregation of the particles by providing an electrostatic (for WPI) and electrosteric (for SC) stabilisation to the particles (Figure 4.17). However, in NaCl solutions, the absolute values of the zeta-potential for SC- and WPI-coated particles were lower than in water. For example, in 0.5 M NaCl solution, the HA particles zeta-potential was only -5 mV for WPI-coated particles (versus –22 mV in water) and –12 mV for SC-coated particles (versus –28 mV in water) (Table 6.1). The stability of the HA suspensions was therefore assessed by visual observation to check whether the improvement in suspension stability of the particles was still observed in NaCl solutions, even though the absolute values of their zeta-potential were lower.

Figure 6.9 shows photographs of suspensions of SC-coated HA particles (Figure 6.9A) and WPI-coated HA particles (Figure 6.9B), prepared and re-suspended in NaCl solutions of concentrations 0, 0.05, 0.1, 0.25 and 0.5 M. The suspensions were left undisturbed on the bench at room temperature for about 24 h. The suspension stability of the SC-coated

particles in NaCl solutions of concentrations 0, 0.05, 0.1, 0.25 and 0.5 M did not vary, even though their zeta-potential values varied and were respectively –28, –20, –16, –13 and –12 mV (Table 6.1). The four suspensions remained turbid for at least a week. This confirms that the stabilisation provided by the adsorbed caseins against aggregation is not only of electrostatic nature. The adsorbed caseins must provide a steric stabilisation, probably forming a hairy layer around the particles, and preventing them from aggregating and sedimenting, regardless of the zeta-potential value of the particles, as hypothesised in Chapter 4. In contrast with SC-coated particles, the suspension stability of the WPI-coated particles varied depending on the salt concentration of the suspending solution. Only the suspension in water (corresponding to particles with a zeta-potential of –22 mV) stayed turbid for about 48 hours (Figure 6.9B). The WPI-coated particles in 0.05, 0.1, 0.25 and 0.5 M NaCl had respectively zeta-potential values of –13, –12, –8 and –5 mV, which did not seem to be enough to prevent their rapid aggregation and sedimentation. This confirms that the stabilisation provided by whey proteins is probably only of electrostatic nature, and seems to require a zeta-potential >20 mV to prevent particle aggregation.

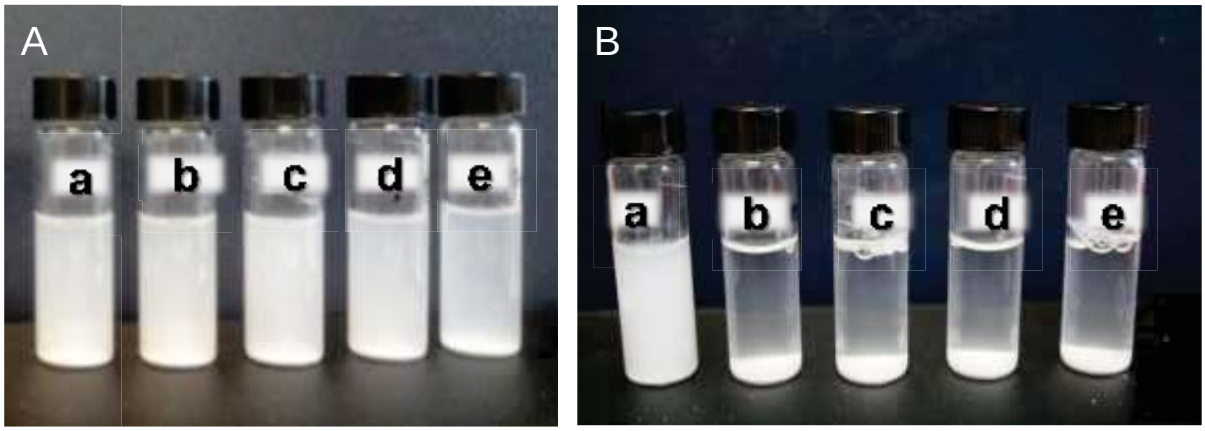

Figure 6.9: Suspension stability of (A) SC-coated HA particles and (B) WPI-coated particles, suspended in solutions of varying ionic strength. HA particles were suspended in: a, water; b, 0.05 M NaCl; c, 0.1 M NaCl; d, 0.25 M NaCl; e, 0.5 M NaCl.

6.4.2.2 Effect of pH on milk protein adsorption onto HA particles

Surface protein concentration

The effect of pH on the adsorption of milk proteins onto HA particles was investigated at different salt concentrations, because mutual effects between the two variables are possible. The experiment was carried out using initial concentrations of 4% (w/w) SC and 2% (w/w)

WPI. A NaCl concentration range of 0–0.1 M was chosen as it was observed previously that the adsorption increased with increasing NaCl concentration in this range (Figure 6.7). A pH range of 6–8 was chosen as this is relevant to milk products in the neutral pH range. It was not possible to study the binding at lower pH, as HA particles start to dissolve at pH <6 (Dattolo et al., 2010).

Figure 6.10 shows a SDS-PAGE pattern obtained for initial 4% (w/w) SC solutions and their respective supernatants containing the unadsorbed proteins, obtained at three different NaCl concentrations (0, 0.05 and 0.1 M) and three different pH values: pH 6 (Figure 6.10A), pH 6.8 (Figure 6.10 B) and pH 8 (Figure 6.10C). As shown previously, the intensities of the protein bands decreased as the salt concentration increased; this was true at all three pH values. However, what is also shown here is that at pH 8.0 (Figure 6.10C), the intensities of the protein bands in lanes 2-4 were greater than the corresponding bands at pH 6.8 (Figure 6.10B, lanes 2-4), and in turn these bands had greater intensity than those at pH 6.0 (Figure 6.10A, lanes 2-4). This shows that, while the amount of unadsorbed protein in each supernatant decreased in proportion to the level of NaCl, it also increased with an increase in pH. This indicates that more caseins must have adsorbed onto HA as pH increased from 6 to 8, for each salt concentration. There was no obvious difference in the ratio of protein band intensities at different pH values, suggesting that a change of pH must affect all the caseins in the same manner, rather than one type of casein more than another.

Figure 6.11 shows the same type of SDS-PAGE pattern as Figure 6.10, obtained for initial 2% (w/w) WPI solutions and their respective supernatants at two NaCl concentrations (0 and 0.1 M) and three different pH values: pH 6 (Figure 6.11A), pH 6.8 (Figure 6.11B) and pH 8 (Figure 6.11C). For low salt concentration (no added NaCl), at pH 8.0 (lane 2 in Figure 6.11C), the intensity of the protein band was greater than the corresponding band at pH 6.8 (lane 2 in Figure 6.11C) and in turn this band had a greater intensity than the band at pH 6.0 (lane 2 in Figure 6.11A). However, the differences in band intensities between the different pH values for supernatants containing 0.1 M of NaCl were not as obvious (comparison between lanes 3 in Figure 6.11A, Figure 6.11B and Figure 6.11C) as for supernatants containing no added NaCl. This indicated that, as for caseins, a decrease in pH from 8 to 6 at low salt concentration seemed to cause an increase in the amount of adsorbed whey proteins onto HA. However, at higher salt concentration, the pH effect on the amount of adsorbed whey proteins seemed to be less pronounced.

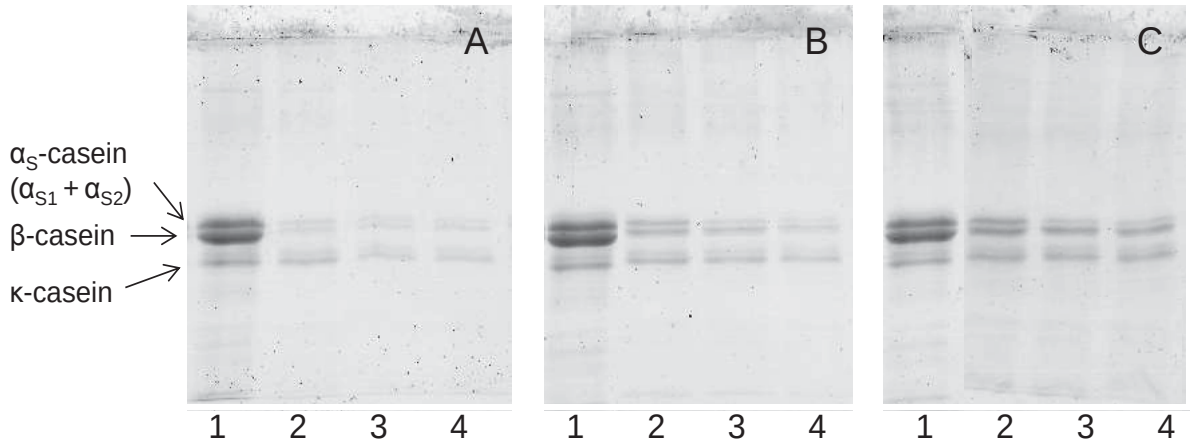

Figure 6.10: SDS-PAGE pattern of initial 4% (w/w) SC solutions and supernatants (i.e., containing the unadsorbed protein) obtained for adsorption experiments carried out at (A) pH 6, (B) pH 6.8, and (C) pH 8, with different levels of NaCl addition. Lane 1, initial solution in water; lane 2, supernatant, in water, no NaCl added; lane 3, supernatant, 0.05 M NaCl; lane 4, supernatant, 0.1 M NaCl.

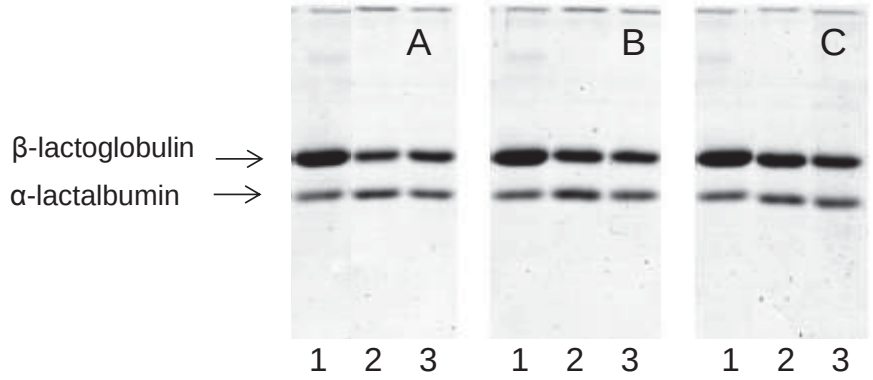

Figure 6.11: SDS-PAGE pattern of initial 2% w/w WPI solutions and supernatants (i.e., containing the unadsorbed protein) obtained for adsorption experiments carried out at (A) pH 6, (B) pH 6.8, and (C) pH 8, with different levels of NaCl addition. Lane 1, initial solution in water; lane 2, supernatants, in water, no NaCl added; lane 3, supernatant, 0.1 M NaCl.

Figure 6.12 shows the surface protein concentration of SC and WPI as a function of pH for three different levels of NaCl addition (0, 0.05, and 0.1 M), calculated from repeated SDS-PAGE gels similar to those shown in Figure 6.10 and Figure 6.11. As the pH increased from 6 to 8, the amount of adsorbed protein decreased for both SC (Figure 6.12A) and WPI (Figure 6.12B), but the effect of pH was less pronounced for WPI. When no NaCl was added, at pH

values of 6, 6.8 and 8, the maximum amounts of adsorbed caseins were about 3.4, 2.8 and 2.1 mg/m^2, respectively, and the maximum amounts of adsorbed whey proteins were about 1.6, 1.3 and 1.1 mg/m^2, respectively. A similar trend was observed for SC in the presence of NaCl. However, almost no differences in the amount of adsorbed protein were observed between the three pH values for WPI in the presence of NaCl.

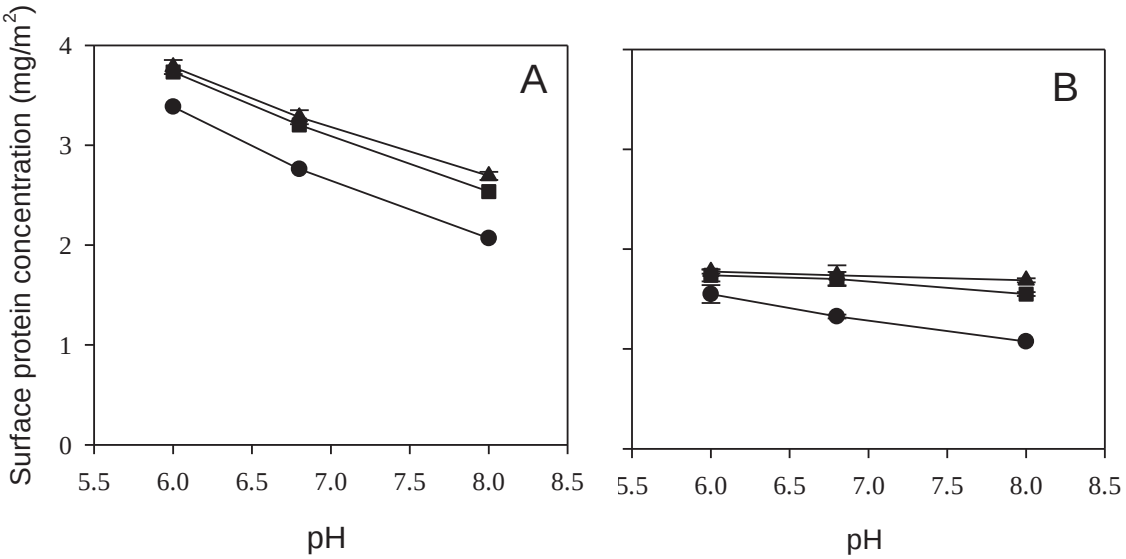

Figure 6.12: Effect of pH on the surface protein concentration of (A) SC and (B) WPI onto HA particles at three different NaCl concentrations. ●, no NaCl addition; ■, 0.05 M NaCl; ▲, 0.1 M NaCl. Error bars represent standard deviation.

The increase in the adsorption of acidic proteins onto HA particles as the pH decreases can be explained by a reduction in the electrostatic repulsions between the proteins and the HA surface caused by the drop in pH (Yin et al., 2002; Zhu et al., 2007). When the pH decreases, the phosphate ions on HA particles are protonated and their negative charges are therefore reduced, as shown by the less negative zeta-potential value (Figure 6.1B). Therefore, the electrostatic repulsion between the negatively charged proteins and the negatively charged groups on the HA surface is also reduced, allowing more binding of caseins and whey proteins to the C-sites of the HA particles.

However, the pH also has an effect on the net charge of the proteins and on the ionisation states of their carboxyl and phosphoserine residues, which is also expected to affect the binding of the milk proteins onto HA particles. As the pH decreases and becomes closer to the isoelectric point of the proteins, the net charge of the proteins decreases. Therefore, the

protonation of the carboxyl groups and phosphoserine residues of the proteins could potentially reduce their ability to bind to calcium ions on the HA particles. Under the conditions used in the present study (pH between 6 and 8), the pH is far removed from the pKas of the carboxyl residues, which is approximately 4.6 (Swaisgood, 2003). Therefore, the carboxyl groups would still be able to bind to the C-sites of the HA particles. However, pH values between 6 and 8 are in the range expected for the pKas of the phosphoserine residues of the caseins, which have been reported to range between 6.4 and 7.2 (Baumy et al., 1989). The protonation of phosphoserine residues at pH around 6 could therefore reduce the extent of binding of SC to HA particles. However, this could be compensated for by the changes in the aggregation state of the caseins with a decrease in pH. By screening some of the charges on the caseins, a decrease in pH could also potentially lead to the formation of bigger SC aggregates (HadjSadok et al., 2008). An increase in size of the SC aggregates binding to HA particles might therefore also contribute to the increase in the amount of adsorbed proteins upon pH decrease.

Surface protein composition

Figure 6.13 shows the estimated amounts of individual caseins adsorbed as a function of pH, for two levels of NaCl addition (0 M, Figure 6.13A and 0.1 M, Figure 6.13B). When the pH was increased from 6.8 to 8 and decreased from 6.8 to 6, the amounts of adsorbed α_S-casein (α_{S1} + α_{S2}), β-casein and κ-casein all varied in the same proportions, which means that a change of pH had a similar effect on the adsorption of the different caseins. This result was expected, as a change of pH has been reported to have an effect mainly on the size of the SC aggregates, but not on their composition (HadjSadok et al., 2008). The amount of adsorbed β-lactoglobulin and α-lactalbumin as a function of pH was not quantified, as the effect of pH on the total adsorbed protein was too small (Figure 6.12) to obtain any meaningful differences for the individual proteins with SDS-PAGE.

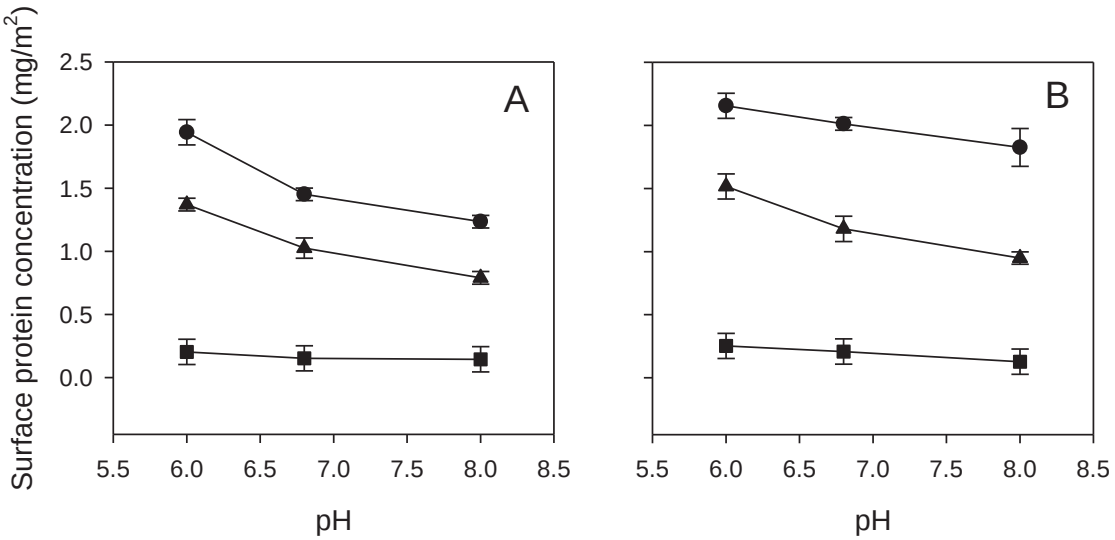

Figure 6.13: Changes in the estimated surface concentration (mg/m²) of individual caseins from SC as a function of pH in (A) water, no NaCl and (B) 0.1 M NaCl. ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■, κ-casein.

Zeta-potential and suspension stability of SC and WPI-coated particles

The zeta-potential and suspension stability of SC and WPI-coated particles obtained at different pH values were not studied, as re-suspending the HA particles in water would have modified the pH and therefore the zeta-potential of the particles. However, it was confirmed that the stabilisation obtained at pH 6.8 for both SC-coated particles and WPI-coated particles (and described in the previous chapter (Figure 4.14)) was also observed for the particles prepared at pH 6 and pH 8.

6.4.2.3 Effect of milk mineral composition (using SMUF) on adsorption of SC and WPI onto HA particles

Surface protein concentration

To evaluate whether the mineral composition of the milk serum affects the adsorption of milk proteins onto HA, experiments were carried out with SC and WPI solutions reconstituted in SMUF at different initial protein concentrations, and compared with the results obtained in water in Chapter 4. Figure 6.14 shows the SDS-PAGE gels obtained from the initial protein solutions and the supernatants of SC and WPI solutions in water (Figure 6.14A and Figure 6.14C, respectively) and in SMUF (Figure 6.14B and Figure 6.14D, respectively). To obtain bands that were in the measurable range of intensities for

integration, the protein samples were diluted in SDS sample buffer at different sample to buffer ratios (these ratios are indicated in the caption of the figure). The controls (initial protein solutions) were diluted at a ratio that was proportional to their concentration to obtain the same protein band intensities for all the controls.

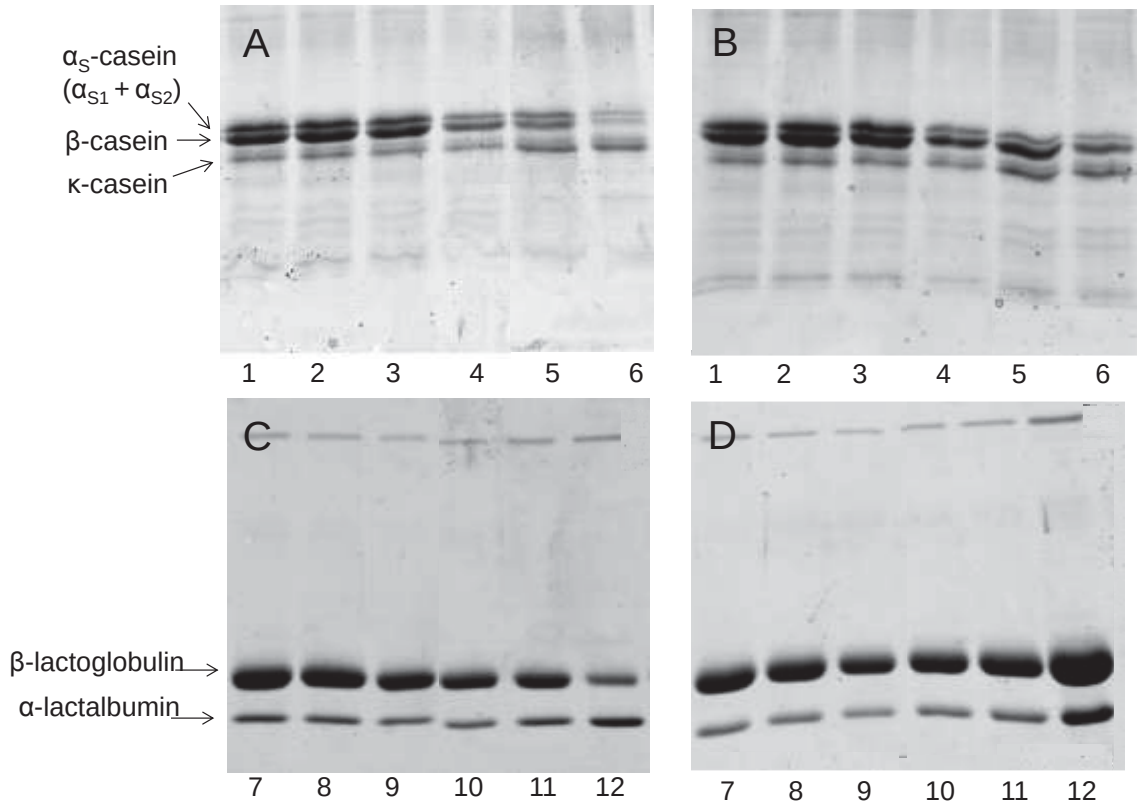

Figure 6.14: SDS-PAGE pattern of initial protein solutions and supernatants obtained for adsorption experiments carried out with SC and WPI solutions in water and in SMUF: (A) SC in water; (B) SC in SMUF; (C) WPI in water; (D) WPI in SMUF. Lanes 1, 2, and 3, SC control samples (i.e., initial solutions) at initial protein concentrations (w/w) of 3.6%, 2.7% , 1.8%, respectively; lanes 4, 5, 6, SC supernatants samples (i.e., containing the unadsorbed proteins) from initial protein concentrations (w/w) of 3.6%, 2.7%, 1.8%, respectively; lanes 7, 8, and 9, WPI control samples at initial protein concentrations (w/w) of 2.8%, 1.9%, 0.93%, respectively; lanes 10, 11, and 12, supernatant samples from initial protein concentrations (w/w) of 2.8%, 1.9%, 0.93%,respectively. The samples were diluted in SDS sample buffer at the following ratios: lanes 1 and 4, 1 to 60; lanes 2 and 7, 1 to 40; lanes 3 and 8, 1 to 30; lanes 5, 9 and 10, 1 to 20; lanes 6 and 11, 1 to 10; lane 12, 1 to 2.

The intensities of the controls were similar in SMUF and in water (lanes 1 to 3 for SC, lanes 7 to 9 for WPI) (Figure 6.14), indicating that SMUF did not affect the solubility of the protein powders. The intensities of the protein bands in the supernatants of SC solutions prepared at initial protein concentrations (w/w) of 3.6%, 2.7% and 1.8% were higher for samples prepared in SMUF (lanes 4, 5 and 6, respectively, in Figure 6.14B) than for those prepared in water (lanes 4, 5 and 6, respectively, in Figure 6.14A). The supernatants prepared at the same initial concentrations contained more unadsorbed caseins (mostly more unadsorbed β-casein) in SMUF than in water. This means that caseins adsorbed less onto HA particles in SMUF than in water, and this was mostly due to a lower adsorption of β-casein. Similarly, the intensities of the protein bands in the supernatants of WPI solutions prepared at initial protein concentrations (w/w) of 2.8%, 1.9% and 0.93% were greater for samples prepared in SMUF (lanes 10, 11 and 12, respectively, in Figure 6.14D) than for those prepared in water (lanes 10, 11 and 12, respectively, in Figure 6.14C). As the supernatants in SMUF contained more unadsorbed whey proteins than the respective supernatants in water, this shows that whey proteins adsorbed less onto HA particles in SMUF than in water.

Figure 6.15 shows the surface protein concentrations of SC and WPI onto HA particles as a function of the initial protein concentration, in water and in SMUF. For both protein sources, the amount of adsorbed protein increased gradually and then reached a maximum coverage value, corresponding to a saturated layer of protein on the surface of the HA particles. However, for both SC and WPI, a lower maximum amount of proteins was adsorbed onto HA particles in SMUF than in water. The binding of SC to HA particles was less affected by the SMUF composition (Figure 6.15A) compared with the binding of WPI to HA particles (Figure 6.15B). The maximum protein coverage in SMUF compared with that in water was reduced by 13% for SC (from about 2.75 mg/m^2 to about 2.4 mg/m^2), and by 62% for WPI (from about 1.4 mg/m^2 to about 0.5 mg/m^2).

The Langmuir model was fitted to the adsorption data obtained for SC and WPI in SMUF, and compared with the Langmuir model obtained for SC and WPI in water, described in Chapter 4. The Langmuir isotherm curves for casein and whey protein adsorption onto HA particles in water and in SMUF are shown inFigure 6.16. Table 6.2 gives the calculated constants for each of the isotherms.

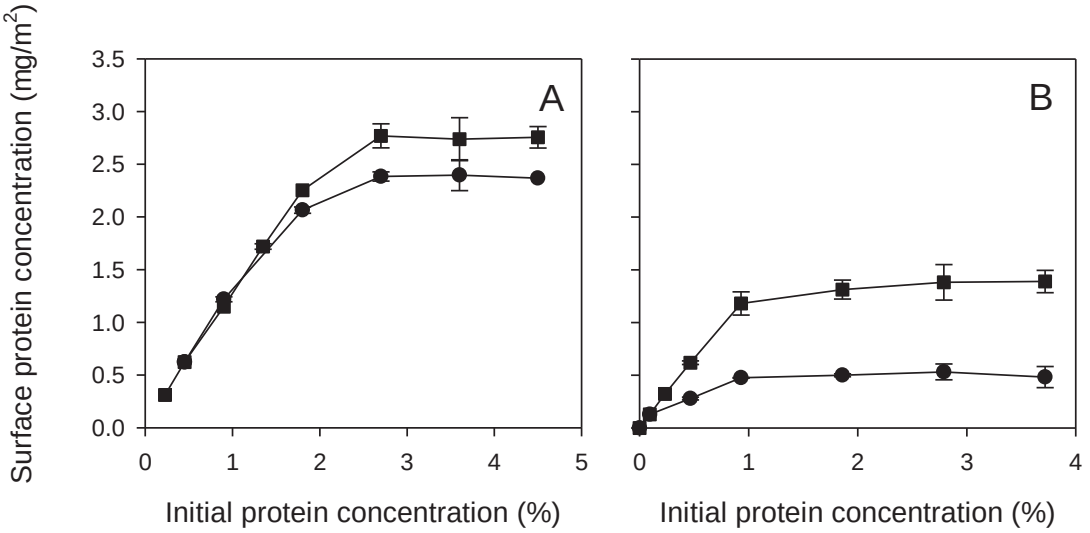

Figure 6.15: Effect of milk serum composition using SMUF on the surface protein concentration of (A) SC and (B) WPI, compared with adsorption in water. ■, adsorption in water; ●, adsorption in SMUF. Error bars represent standard deviation.

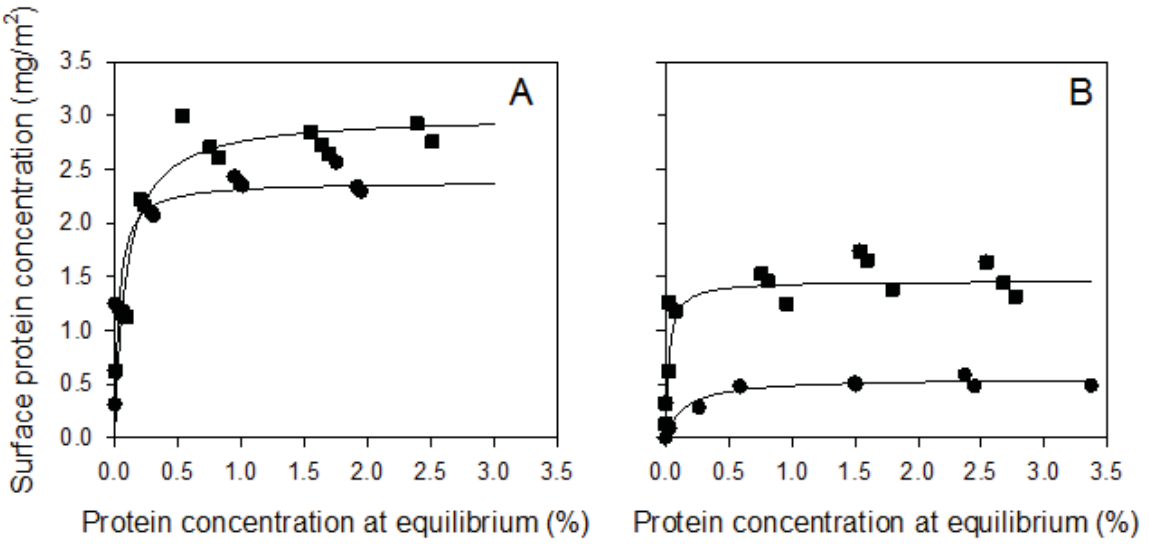

Figure 6.16: Comparison between Langmuir isotherms of (A) SC and (B) WPI adsorption onto HA particles. Experimental data points in water (■) and in SMUF (●); the solid lines show the best-fit curves for the Langmuir model.

Table 6.2: Parameters for the adsorption of SC and WPI onto HA particles in SMUF and in water, calculated according to the Langmuir model. [a]

Protein	Suspending medium	Maximum surface coverage, q_m (mg/m^2)	Affinity constant, K (100g/g)
SC	Water	$3.0^{a} \pm 0.2$	$11.3^{a} \pm 3.6$
	SMUF	$2.4^{b} \pm 0.01$	$31^{b} \pm 3.7$
WPI	Water	$1.5^{c} \pm 0.2$	$40.7^{c} \pm 3.6$
	SMUF	$0.56^{d} \pm 0.1$	$6.7^{ad} \pm 1.9$

[a] Means with different superscript letters within a column differ significantly (P <0.05).

The Langmuir model fitted well with the SC and WPI adsorption data in SMUF. The maximum surface protein concentrations obtained for the Langmuir models for SC and WPI in SMUF were 2.4 mg/m^2 for SC (Figure 6.16A and Table 6.2), and 0.56 mg/m^2 for WPI (Figure 6.16B and Table 6.2). These values were close to those observed experimentally (Figure 6.15), and significantly smaller than that obtained for SC and WPI in water, i.e., 3 mg/m^2 for SC and 1.5 mg/m^2 for WPI (Table 6.2). The Langmuir affinity constant K decreased from 40.7 in water to 6.7 in SMUF for WPI, whereas it increased from 11.3 in water to 31 in SMUF for SC, showing that the SMUF composition affected the affinity of the proteins for HA.

In Section 6.4.1.2, it was shown that citrate and phosphate ions bind to the C-sites of HA particles, charging the HA particles negatively. Both SC and WPI bound less onto HA particles in SMUF than in water, probably because the protein groups binding to the C-sites of HA have to compete with the milk serum ions for binding. The binding of whey proteins onto HA is believed to occur through the formation of complexes between carboxyl groups and C-sites on HA, whereas caseins bind mostly on the C-sites of HA through their phosphoserine groups. It has been demonstrated that phosphate ions have a higher affinity for HA than carboxyl groups of proteins. For this reason, phosphate buffers are used in HA chromatography columns to elute the acidic proteins from the columns (Kawasaki, 1991). The affinity of the phosphate and citrate ions in SMUF for the C-sites on HA particles must therefore be stronger than the affinity of the carboxyl groups of the whey proteins for the same binding sites. Therefore, a number of the binding C-sites on HA surface would be taken

up by the phosphate and citrate ions. By binding to the same sites as whey proteins and by charging the surface of the particles negatively (which must increase the electrostatic repulsion between the HA surface and the carboxyl groups of the whey proteins), the milk serum ions must prevent the whey proteins from binding. This would explain the lower Langmuir affinity constant for WPI in SMUF, compared with in water (Table 6.2).

The binding through the phosphate groups of proteins has been shown to be stronger than the binding through the carboxyl groups of proteins (Bernardi et al., 1972; Gorbunoff & Timasheff, 1984a; Juriaanse et al., 1981), with the affinity of phosphate groups for HA estimated to be 20 times greater than that of carboxyl groups (Moreno et al., 1984). Caseins must therefore be able to compete better for adsorption with the milk serum ions, which would explain why their binding was less affected by the SMUF composition compared with whey proteins. It is unclear why the affinity constant for SC was about three times (from 11.3 to 31, see Table 6.3) higher in SMUF than in water. As milk serum ions must compete with the phosphoserine groups of the caseins for binding, it was expected that a decrease in the affinity constant in SMUF would be observed, as was the case for whey proteins. However, it is possible that the phosphoserine groups of the caseins have a better affinity for the C-sites on HA than the milk serum ions, because they are grouped in clusters (Johnsson et al., 1993). In this case, the affinity constant would not be affected in SMUF. It is also possible that the SMUF composition affects the binding of the different types of caseins differently. This aspect is discussed in the section below.

Surface protein composition of HA particles in SMUF compared with in water

Figure 6.17 compares the estimated amounts of the individual caseins and whey proteins adsorbed onto HA particles in water and in SMUF, as a function of the initial protein concentration of SC or WPI added. For SC (Figure 6.17A), the maximum amount of adsorbed β-casein in SMUF compared with that in water decreased significantly (P <0.05) (from about 1.2 mg/m^2 in water to 0.7 mg/m^2). The maximum amount of adsorbed κ-casein decreased slightly, but this decrease was not significant. The adsorption of α_S-casein (α_{S1} + α_{S2}) did not seem to be affected by the SMUF composition as α_S-casein bound to the same maximum amount in SMUF and in water, of about 1.5 mg/m^2. α_{S1}-Casein contains 7–9 phosphoserine residues and α_{S2}-casein contains 10–13 phosphoserine residues, whereas β-casein and κ-casein contain, respectively, only 5 and 1 phosphoserine residues (Walstra & Jenness, 1984). As α_S-casein (α_{S1} + α_{S2}) contains the most phosphoserine residues of the caseins, it must be

able to compete better for adsorption onto HA particles with the phosphate and citrate ions in SMUF.

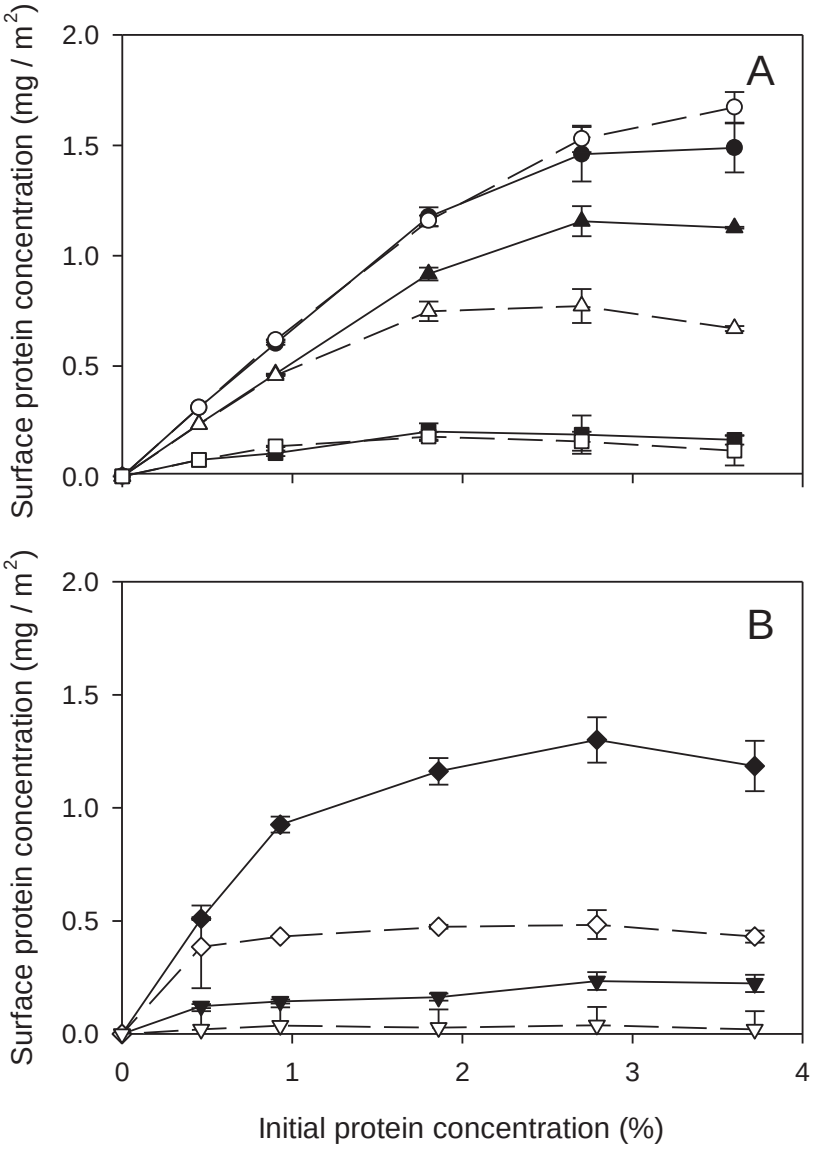

Figure 6.17: Comparison between estimated surface concentration (mg/m²) of (A) individual caseins from SC and (B) individual whey proteins from WPI, on HA particles in water (closed symbols, solid lines) and in SMUF (open symbols, dashed lines) as a function of initial protein concentration. ●,○, α_S-casein (α_{S1} + α_{S2}); ▲, △, β-casein; ■,□, κ-casein; ◆,◇, β-lactoglobulin; ▼,▽, α-lactalbumin. Error bars represent standard deviation.

For WPI (Figure 6.17B), the maximum amount of β-lactoglobulin and α-lactalbumin both decreased significantly. The maximum amount of β-lactoglobulin decreased by about 60%, from 1.2 mg/m^2 in water to 0.45 mg/m^2 in SMUF. The amount of adsorbed α-lactalbumin was difficult to determine with precision using SDS-PAGE, since almost all of the protein remained in the supernatant, and was therefore not quantified.

Adsorption of SC in SMUF containing added citrate

To verify the hypothesis that milk serum ions compete with milk proteins for adsorption onto HA, an adsorption experiment was carried out using SC reconstituted in SMUF solutions containing different amounts of citrate.

Figure 6.18 shows a SDS-PAGE pattern obtained for initial solutions of 2% (w/w) SC reconstituted in SMUF containing 1, 1.5, 2, 3 or 5 times the concentration of citrate in normal SMUF, and their respective supernatants. The intensities of the protein bands in the SC controls in SMUF containing different concentrations of citrate (lanes 1 to 5 in Figure 6.18) were similar, indicating that the added citrate in SMUF did not affect the solubility of the SC powder. However, the intensities of the protein bands in the supernatants increased as the citrate concentration in SMUF increased from 1 to 5 times the concentration of citrate in normal SMUF (lanes 6 to 10 in Figure 6.18).

The increase in the protein band intensities of the supernatants upon addition of citrate in SMUF shows that less caseins adsorbed onto HA particles as the citrate concentration increased. The total surface protein concentration and the relative proportions of each individual casein in the adsorbed layer were calculated from the SDS-PAGE data for each citrate concentration, and are reported in Table 6.3. When the citrate concentration was increased, the total amount of adsorbed proteins from 2% (w/w) SC decreased from 2.14 mg/m^2 to 0.84 mg/m^2. However, the relative proportion of α_S-casein (α_{S1}- + α_{S2}-) in the adsorbed layer increased from 55% to 72%, whereas the relative proportion of both β-casein and κ-casein decreased from, respectively, 37% to 25% and 7.7% to 3.4%. This shows that the binding of α_S-casein was less affected by the increase of citrate concentration in SMUF than the binding of β-casein and κ-casein. It was calculated that the amount of adsorbed α_S-casein decreased only 48% upon addition of 5 times more citrate, whereas the amount of adsorbed β-casein and κ-casein decreased 74% and 82%, respectively. This result confirms that α_S-casein was able to compete better with citrate ions (and therefore probably with other milk serum ions such as phosphate) for adsorption onto HA than β-casein and κ-

casein, probably because it contains the most phosphoserine residues of all the caseins (Walstra & Jenness, 1984).

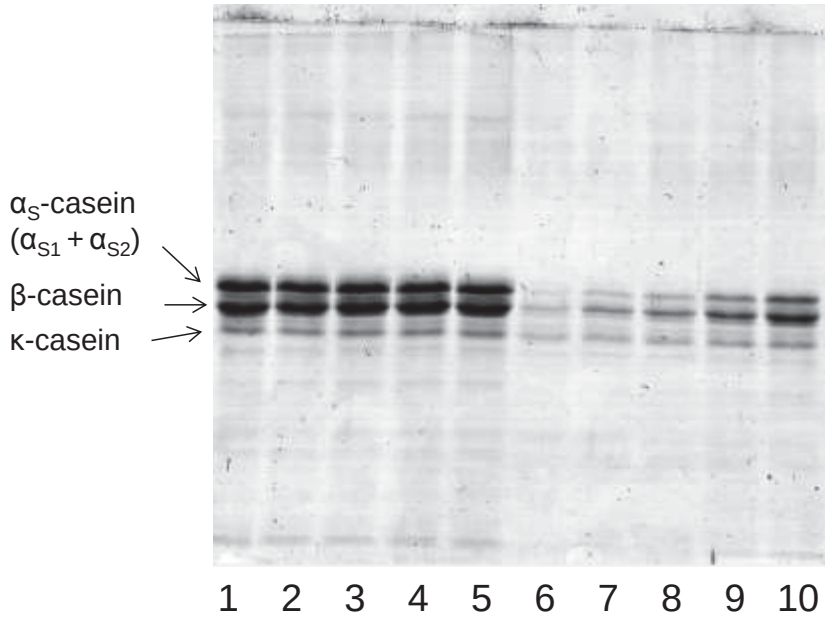

Figure 6.18: SDS-PAGE pattern of initial 2% (w/w) SC solutions (lanes 1–5) and supernatants (i.e., containing the unadsorbed protein; lanes 6–10) obtained for adsorption experiments carried out in SMUF containing different amounts of citrate. Lanes 1 and 6, control (normal citrate level in SMUF recipe); lanes 2 and 7, 1.5 times normal; lanes 3 and 8, 2 times normal; lanes 4 and 9, 3 times normal; lanes 5 and 10, 5 times normal.

Table 6.3: Relative proportions of individual caseins adsorbed onto HA particles when the adsorption experiment was carried out from 2% (w/w) SC in SMUF containing different amounts of citrate.

Citrate concentration (× normal SMUF concentration)	Total surface protein concentration (mg/m²)	Percentage (%) by casein type		
		α_S-casein	β-casein	κ-casein
1	2.14	55	37	7.7
1.5	1.91	58	35	7.3
2	1.76	59	34	7.0
3	1.35	63	30	6.9
5	0.85	72	25	3.4

Zeta-potential and suspension stability of SC- and WPI-coated particles in SMUF

The HA pellets obtained in the adsorption of SC and WPI in SMUF at different initial protein concentrations were re-suspended at 0.05% (w/w) in SMUF for zeta-potential measurements. Figure 6.19 shows the zeta-potential of the SC- and WPI-coated particles measured in SMUF, as a function of the initial protein concentration of SC or WPI used in the adsorption experiment in SMUF. The zeta-potential of uncoated HA particles suspended in SMUF was –22 mV. As discussed in Section 6.4.1.3, this negative value of the zeta-potential of HA particles in SMUF was likely caused by the adsorption of citrate or phosphate ions from SMUF on the surface of the HA particles. When SC or WPI were adsorbed onto the particles, the zeta-potential of the particles became slightly less negative for both SC and WPI, and reached a plateau value, probably corresponding to a saturated layer of adsorbed protein on the surface. After mixing with SC in SMUF, the magnitude of the zeta-potential decreased from –22 mV to –16 mV with SC addition up to 1% and did not change further at higher addition of SC (Figure 6.19). For WPI, the zeta-potential magnitude decreased slightly, from –22 mV to –19 mV with WPI addition up to 2% and no further change was observed for higher addition of WPI (Figure 6.19).

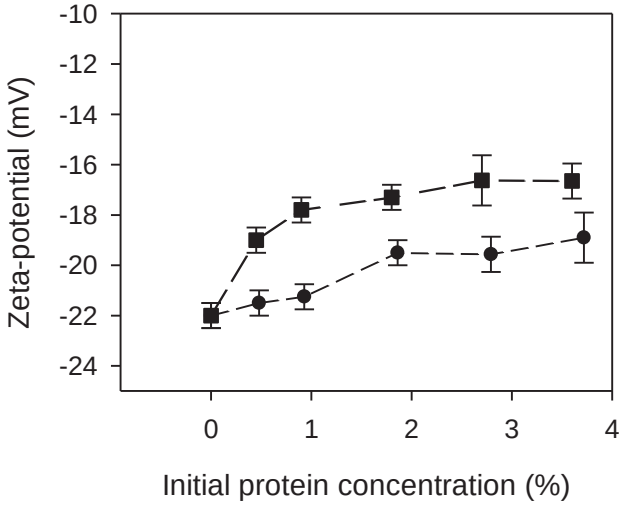

Figure 6.19: Effect of initial protein concentration on the zeta-potential of HA particles that were suspended in solutions of SC (■) or WPI (●) reconstituted in SMUF at different initial concentrations. The suspensions were stirred for 2 h and centrifuged, and the pellets were then rinsed with SMUF and re-suspended in SMUF.

In Chapter 4, it was shown that the adsorption of SC and WPI in water led to an increase in the magnitude of the zeta-potential. However, in water, the magnitude of the zeta-potential of the suspended particles alone was smaller than in SMUF (–8 mV versus –22 mV in SMUF). When negatively charged proteins were adsorbed, the particles became therefore more negatively charged. In contrast to the case in water, in SMUF the adsorption of casein and whey proteins led to only a small change in the absolute value of the zeta-potential of the particles, because the magnitude of the zeta-potential of the particles suspended in SMUF was already high (–22 mV).

The HA pellets prepared with 0 to 3% (w/w) initial SC and WPI in SMUF were re-suspended in SMUF and left undisturbed on the bench for 24 h at room temperature. Figure 6.20 shows photographs of these suspensions after 24 h. The particles prepared with no protein (bare particles suspended in SMUF, sample a in Figure 6.19A and Figure 6.19B), with zeta-potential of about –22 mV, remained in suspension after 24 h. It was attributed to the electrostatic stabilisation provided by the ions in SMUF adsorbed onto the particles. It is interesting to note that the samples prepared with 0.5, 1 and 2% (w/w) (samples b, c, and d, respectively, in Figure 6.19A), corresponding to particles with about the same zeta-potential (about –19, –18 and –17 mV, respectively) but only partly coated with adsorbed caseins (Figure 6.15) appeared less turbid and were less stable against sedimentation, compared with either the particles fully coated by milk serum ions (sample a in Figure 6.20A) or the particles for which the surface was saturated with caseins (sample e in Figure 6.20A). As the surface protein concentration of SC increased from 0 to its saturation value (2.4 mg/m^2 reached for initial SC concentration of about 3%, w/w; see Figure 6.15), it appears that the stabilisation of the particles transitioned from a pure electrostatic stabilisation in SMUF (zeta-potential of –22 mV in SMUF, turbid suspension), to a steric stabilisation provided by the adsorbed layer of caseins at the surface of the particles (lower zeta-potential of –16 mV, but still turbid in SMUF). The intermediate samples, not fully coated by proteins but not either fully coated by milk ions, were less stable, probably because the charge was not enough to provide an electrostatic stabilisation, and there was not enough protein adsorbed on the HA surface to provide a steric stabilisation. For WPI-coated particles, there was no difference between the visual turbidity of the suspensions containing HA particles prepared at different WPI concentrations, and therefore coated by different amounts of whey proteins. It was suggested that considering the small amount of whey proteins adsorbing onto the particles in SMUF, and the very small change in the magnitude of the zeta-potential upon adsorption of whey proteins (from –22 mV to –19 mV; see Figure 6.19B) the adsorbed protein layer did not affect the suspension stability of the particles in SMUF. The

stabilisation was probably mainly due to the milk serum ions adsorbed onto the particles, rather than to the adsorbed whey proteins.

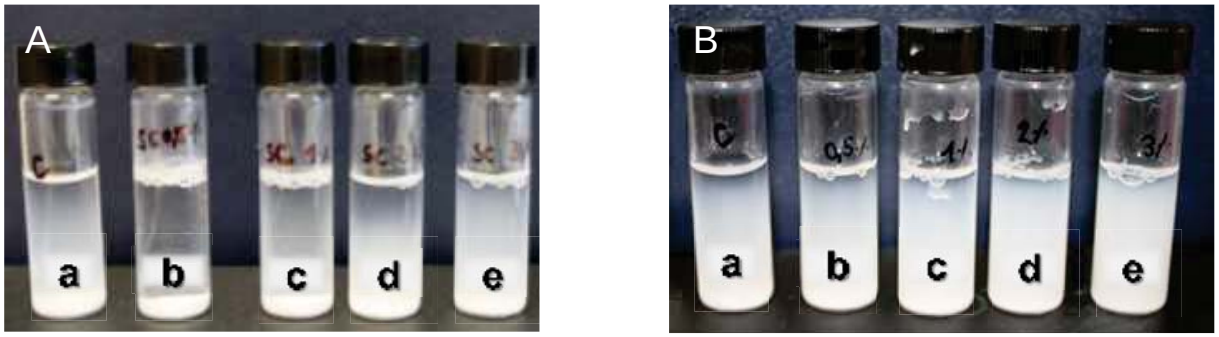

Figure 6.20: Suspension stability of (A) SC-coated HA particles and (B) WPI-coated particles, prepared in SMUF with increasing concentrations of protein. The particles were mixed with SC or WPI in SMUF, the HA pellets were centrifuged to obtain a pellet, the pellets were rinsed with SMUF and resuspended in SMUF. The initial SC or WPI concentrations (w/w) used to prepare the particles were: a, 0 (SMUF); b, 0.5%; c, 1%; d, 2%; e, 3%.

6.4 Conclusions

In conclusion, the results in this chapter showed that the amount of caseins from SC and whey proteins from WPI adsorbing onto HA particles varied depending on ionic strength, pH and mineral composition of the milk serum phase. Figure 6.21 summarises the effect of each variable on the electrostatic interactions between the binding sites on HA (C-sites and P-sites) and the binding groups on the milk proteins (carboxyl groups for whey proteins, phosphoserine groups for caseins) and on the characteristics of the adsorbed protein.

Ionic strength and pH were believed to affect the electrostatic interactions between HA and the proteins, as well as the structure of the proteins adsorbed onto HA. Calcium, citrate and phosphate ions were shown to specifically adsorb onto the C-sites and P-sites of HA particles. Therefore, in SMUF, there was probably a competition for adsorption between the phosphate and citrate contained in SMUF and the caseins and whey proteins. Consequently, both caseins and whey proteins adsorbed less onto HA in SMUF than in water, but caseins were less affected than whey protein, because of their stronger binding onto HA.

The observations on the suspension stability of SC and WPI-coated particles in different suspending conditions confirmed that caseins from SC may provide a steric stabilisation to the particles against aggregation; this mechanism was already suggested in Chapter 4. Conversely, the stabilisation of the particles provided by whey protein adsorption was only efficient in water, and not in salt solutions. This confirmed that whey proteins provide only an electrostatic stabilisation to the particles against aggregation.

HA particles as a calcium source are often added to calcium-fortified milk products that can have different pH, ionic strength or mineral composition characteristics. The results of this chapter characterised further the adsorption behaviour of caseins and whey proteins separately, under different physico-chemical conditions that could be used in milk products, using model systems (SC and WPI). The detailed understanding of the interactions between HA and model milk proteins acquired in this chapter will underpin the ability to understand interactions between milk proteins and HA particles in milk. The results in milk will be presented and discussed in Chapter 7 of this thesis.

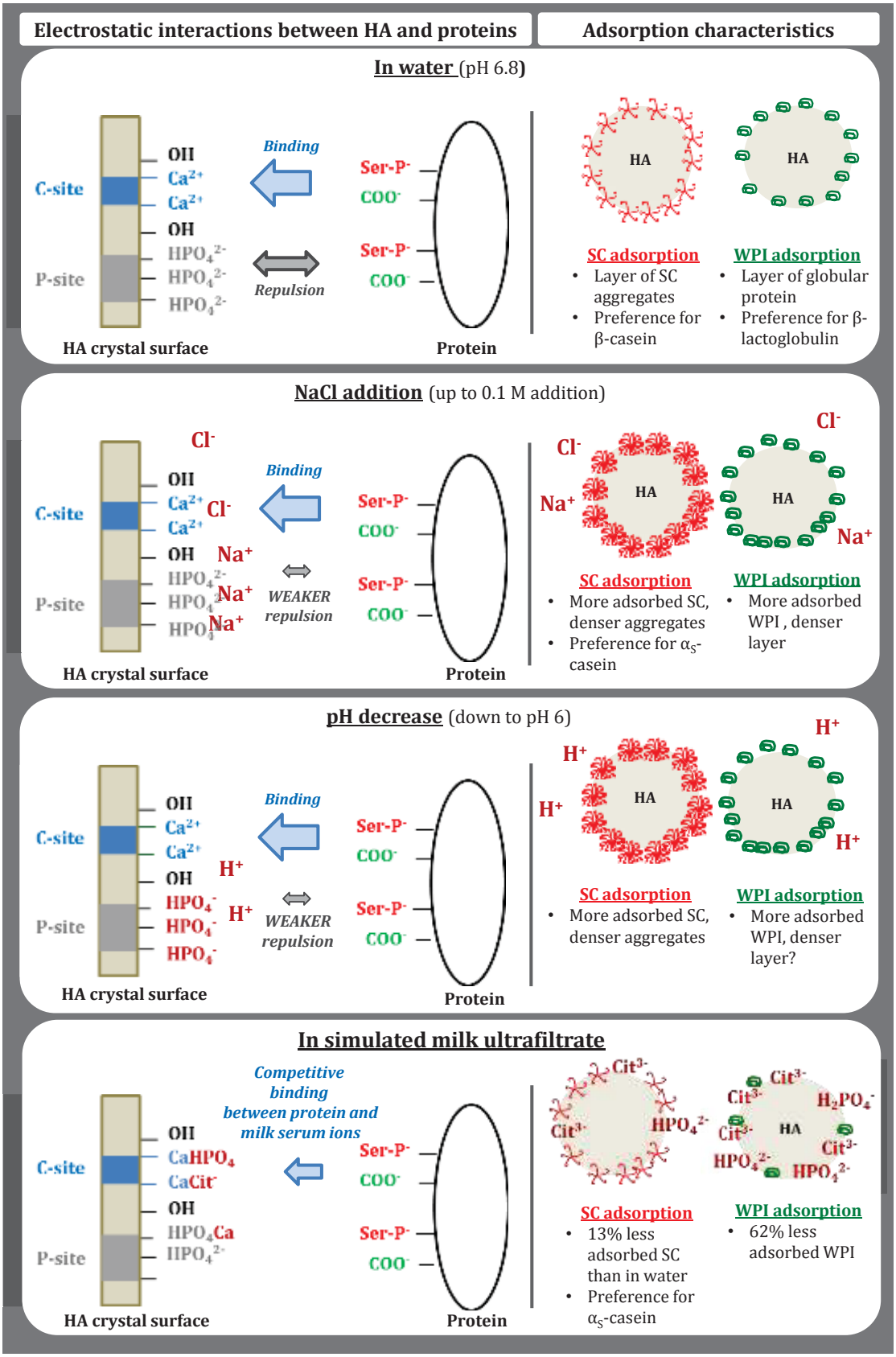

Figure 6.21: Graphical summary of the effect of ionic strength, pH, and SMUF on milk protein adsorption on HA. Ser-P$^-$, phosphoserine residue; COO$^-$, carboxyl residue.

CHAPTER 7 Interactions of casein micelles with hydroxyapatite particles *

7.1 Abstract

Hydroxyapatite (HA) particles are often added in calcium-fortified milks as they are considered to be chemically unreactive. However, this study showed that there was an interaction between the casein micelles in milk and HA particles. The caseins in milk were shown to bind to the HA particles, with the relative proportions of bound β casein, α_S-casein, and κ-casein different to the proportions of the individual caseins present in milk. Caseins from EDTA-dissociated casein micelles adsorbed to the same extent on HA particles as caseins from intact casein micelles, showing that the state of dissociation of the casein micelles did not have an effect on the amount of caseins that could bind onto HA. Transmission electron microscopy (TEM) showed no evidence of intact casein micelles on the surface of the HA particles, which suggested that the addition of HA particles caused the casein micelles to dissociate, either during or after binding of the proteins. This was confirmed by the fact that the unadsorbed casein micelles were partly dissociated. HA particles were shown to behave as ion chelators, with the ability to bind the ions contained in the milk serum phase. Consequently, the depletion of the serum minerals disrupted the milk mineral equilibrium, resulting in dissociation of the casein micelles in milk. It was believed that HA particles grew at the expense of the calcium phosphate nanoclusters of the casein micelles, with the nanoclusters being used as a reservoir of calcium and phosphate ions for HA growth.

---

* Part of the content presented in this chapter has been published as an original paper: Tercinier, L., Ye, A., Anema, S. G., Singh, A., & Singh, H. (2014b). *Journal of Agriculture and Food Chemistry, 62,* 5983–5992.
Part of the content was also presented as a conference presentation at the 2nd Mineral and Dairy Products Symposium, Auckland, New Zealand, February 26th-27th, 2014

7.2 Introduction

In the previous chapter, caseins and whey proteins from either pure milk protein solutions or sodium caseinate (SC) and whey protein isolate (WPI) were shown to bind onto hydroxyapatite (HA) particles. Whey proteins were believed to bind to HA particles through their carboxyl groups, whereas caseins were considered to bind more strongly onto HA particles through their phosphoserine groups. However, the caseins used in the previous experiment were not in a micellar form. For example, SC contains a mixture of the natural caseins of milk, but the caseins are soluble, rather than associated in the casein micelles (Wong et al., 1996). They can be associated randomly into small SC aggregates (Pitkowski, Durand, & Nicolai, 2008), but their phosphoserine groups are still available for binding to the calcium of HA particles. In milk, however, the phosphoserine groups of the caseins are involved in the casein micelle structure and are already bound to the colloidal calcium phosphate (CCP) (Dalgleish, 2011). Therefore, if exogenous HA particles are added to milk, it is not certain whether the caseins in the micelles will be able to bind to the HA particles. Also about 20% of the protein in milk is whey proteins, and these may also bind to HA particles.

The salt composition of milk is likely to affect the adsorption of proteins onto HA particles in milk, as the binding of proteins depends on the mineral composition of the suspending medium, as shown in Chapter 6, where the adsorption of both caseins and whey proteins decreased in simulated milk ultrafiltrate (SMUF). This was attributed to the binding of phosphate or citrate ions from the milk serum, which competed with the negatively charged groups of the proteins for adsorption. As milk contains free calcium, phosphate, and citrate ions in the milk serum phase, it is likely that these ions will adsorb onto HA particles, therefore competing with the proteins for binding. If the ions in milk bind to the HA surface, the mineral equilibrium of the milk serum phase could also be disrupted, potentially affecting the integrity of the casein micelles (Holt, 1985).

As HA particles are often added to milk for calcium fortification, it is important to understand the interactions that could take place between the milk proteins, the milk minerals, and the HA particles, and whether HA particles are likely to cause any instability in the products. Therefore, the objective of this chapter was to investigate whether or not the casein micelles and whey proteins adsorbed onto HA particles when HA particles were added to milk. The binding of casein micelles onto HA particles was investigated using both skim milk (SM) and whey-protein-depleted skim milk (WPD-SM). Transmission electron

microscopy (TEM) was used to check whether or not casein micelles could be observed at the surface of the HA particles. The binding of caseins from milks containing partially dissociated casein micelles was also investigated using EDTA-treated milks, to see whether the dissociation state of the casein micelles had an impact on the amount of proteins that adsorbed onto HA particles. To check whether the milk mineral equilibrium was affected by the addition of HA particles, increasing levels of HA particles were added to SMUF and SM and the soluble minerals were quantified. SM was also dialysed against SMUF or against SMUF containing HA particles to check whether the presence of HA particles caused the disruption of the casein micelles when casein micelles and HA particles were separated.

7.3 Material and methods

7.3.1 Adsorption experiments

Stock solutions of WPD-SM (See Section 3.7.1 for preparation) and SM were reconstituted from WPD-SMP and SMP in Milli-Q water (10%, w/w, total solids, TS, on a powder basis, corresponding to about 3.3%, w/w, total protein), as described in Section 3.2.1. HA powder (5%, w/w) was added to WPD-SM and SM solutions of different protein concentrations (0.15–2.4% w/w for WPD-SM and 0.15–3%, w/w, for SM), that were prepared by successive dilutions of the stock solutions in a SMUF solution (see Section 3.2.2.2 for SMUF preparation), to obtain different protein to HA ratios, as described in Section 3.2.3.1. Using SMUF for the successive dilutions of the WDP-SM and SM stock solutions enabled to obtain a suspending media for the HA particles that was different in protein concentration but similar in terms of pH, ionic strength, and mineral composition.

EDTA-treated SM were prepared at four levels of EDTA addition (0, 5, 10, and 20 mM), as described in Section 3.7.2, to obtain SM with different levels of casein micelle dissociation. Four different protein to HA ratios were obtained by adding different amounts of EDTA-treated SM sample to different amounts of HA particles, mixed to the following quantities of EDTA-treated SM to HA powder: 0.95 mg to 50 mg, 0.925 g to 75 mg, 0.9 g to 100 mg and 0.85 g to 150 mg.

After stirring for 2 h, the HA powder was separated from the SM, WDP-SM and EDTA-treated SM solutions and the unadsorbed protein concentration and composition was determined by analysing the supernatants using microchip fluidics- (MF-)electrophoresis

(Section 3.5.4) or traditional sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; Section 3.5.3). The amount and composition of adsorbed protein was determined by difference between the protein concentration and composition in the initial solution and in the supernatants (depletion method), as described in Section 3.2.4. The supernatants obtained from SM and WPD-SM, containing the unadsorbed protein, were also analysed for casein micelle size and non-micellar casein content, as described in Sections 3.4.1 and 3.4.2.

7.3.2 Transmission electron microscopy and light microscopy

Selected samples containing WDP-SM and HA particles were observed using TEM, and prepared according to the methods described in Section 3.3.3.2 (for samples containing HA particles) and Section 3.4.3 (for WDP-SM with no HA particles). Selected TEM sections were also observed using light microscopy, using toluidine blue to specifically stain protein material, as described in Section 3.4.4.

7.3.3 Dialysis experiments

A first dialysis experiment was carried out using a 10% (w/w, TS) SM solution (referred to as 10%-SM in this chapter) and a 5% (w/w, total solids) SM solution (referred to as 5%-SM in this chapter) prepared by diluting the 10%-SM in SMUF. Samples of the 5%-SM and 10%-SM (10 mL) were placed in dialysis bags (MEMBRA-CEL MC25-16×100 CLR) and dialysed overnight at room temperature against 120 mL SMUF (control) or SMUF containing 10% (w/w) HA particles. The SM samples were removed from the dialysis bags, their visual turbidity was assessed by taking photographs, and the samples were analysed for casein micelle size (see Section 3.4.1) and non-micellar casein content (see Section 3.4.2). The same dialysis experiment was then repeated with only 10%-SM, but with different concentrations of HA particles added in SMUF (0.1, 0.5, 1, 5 and 10%, w/w) and different dialysis times (1, 2 and 3 days).

7.3.4 Mineral quantification

Selected samples of SMUF and SM were analysed for total calcium (see Section 3.6.1), ionic calcium (see Section 3.6.2), inorganic phosphate (see Section 3.6.3) and citrate (see Section 3.6.4).

7.4 Results and discussion

7.4.1 Adsorption of caseins and whey proteins from WPD-SM and SM

7.4.1.1 Surface protein concentration

The adsorption studies were carried out by adding a constant amount of HA (5% w/w) to WPD-SM or SM solutions of different protein concentrations, in the range 0.15-2.4% for WPD-SM and 0.15-2.7% (w/w) for SM. The supernatants obtained after centrifugation of the HA particles were analysed for protein composition and concentration using MF-electrophoresis. The MF-electrophoresis gel patterns obtained for the initial protein solutions and the supernatants of the WDP-SM and SM adsorption experiments are shown in Figure 7.1A for WDP-SM and Figure 7.1B for SM, for five different initial protein concentrations (2.4, 2.1, 1.8, 1.5 and 1.2%, w/w). Each supernatant (lanes 2, 4, 6, 8, and 10) was loaded next to its corresponding initial solution (lanes 1, 3, 5, 7, and 9 on both figures), and diluted at the same dilution ratio in sample buffer. The intensities of the α_S-casein (α_{S1}- + α_{S2}) and β-casein bands decreased for all the samples between the initial solutions and the supernatant, showing that caseins adsorbed onto HA particles. However, the intensities of the κ-casein band in both WPD-SM and SM, and of the β-lactoglobulin and α-lactalbumin bands in SM did not seem to decrease much between the initial solutions and their respective supernatants, for all five initial concentrations, showing that κ-casein, β-lactoglobulin and α-lactalbumin probably only adsorbed to a negligible amount onto HA particles.

Figure 7.1 shows the unadsorbed protein concentration measured in the supernatants after adsorption and the amount of adsorbed proteins on the surface of the HA particles as a function of the initial protein concentration for both WPD-SM (Figure 7.2A) and SM (Figure 7.2B). For both WPD-SM and SM, at low initial protein concentration (<0.5%), the amount of

protein that remained in the supernatants (i.e., the unadsorbed protein) was very low. As the initial protein concentration increased above 0.5%, the amount of unadsorbed protein increased considerably, as unadsorbed protein started to accumulate in the supernatants. From this unadsorbed protein concentration values, the amount of total adsorbed proteins was calculated. For both systems, the amount of total adsorbed caseins increased gradually, until it reached a plateau value, corresponding to a maximum protein coverage of the surface of the HA particles. For HA particles mixed with WPD-SM, the surface casein concentration increased gradually with the initial protein concentration up to about 1.5% (w/w, total protein) and reached a maximum value of ~2.9 mg/m^2 (Figure 7.2A). For HA particles mixed with SM, the amount of adsorbed casein increased with the initial protein concentration up to about 2% (w/w, total protein) and reached a maximum value of ~2.8 mg/m^2 (Figure 7.2B).

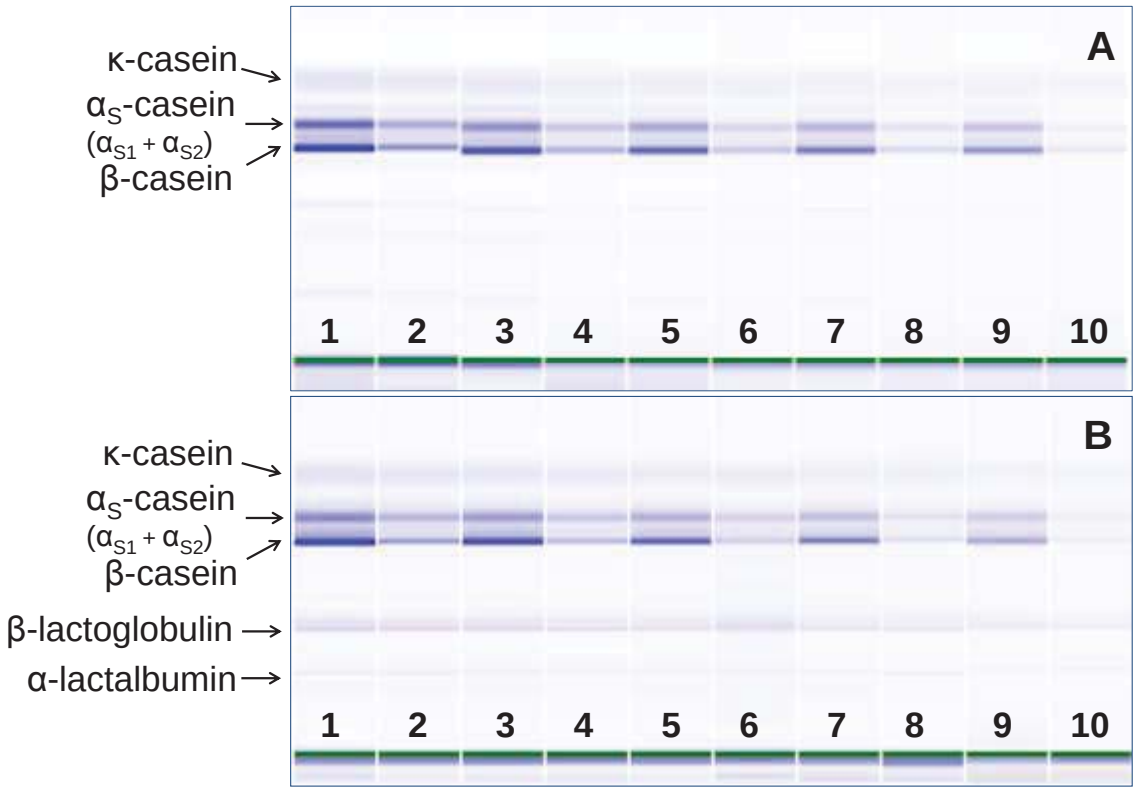

Figure 7.1: MF-electrophoresis gel pattern of initial protein solutions and supernatants obtained for adsorption experiments carried out with (A) WDP-SM and (B) SM solutions at different initial protein concentrations. Lanes 1 and 2, 2.4% (w/w); lanes 3 and 4, 2.1% (w/w); lanes 5 and 6, 1.8% (w/w); lanes 7 and 8, 1.5% (w/w); lanes 9 and 10, 1.2% (w/w); lanes 1, 3, 5, 7 and 9, control samples (i.e., initial solutions); lanes 2, 4, 6, 8 and 10, respective supernatant samples (i.e., containing the unadsorbed proteins).

These results clearly showed that the caseins from WPD-SM and SM adsorbed at the surface of HA particles, to similar maximum amounts of ~2.9 mg/m^2. In contrast, the maximum amount of whey proteins from SM that bound to HA particles was ~ 0.1 mg/m^2, which was negligible, as it represented only ~3% of the total protein bound (Figure 7.2B).

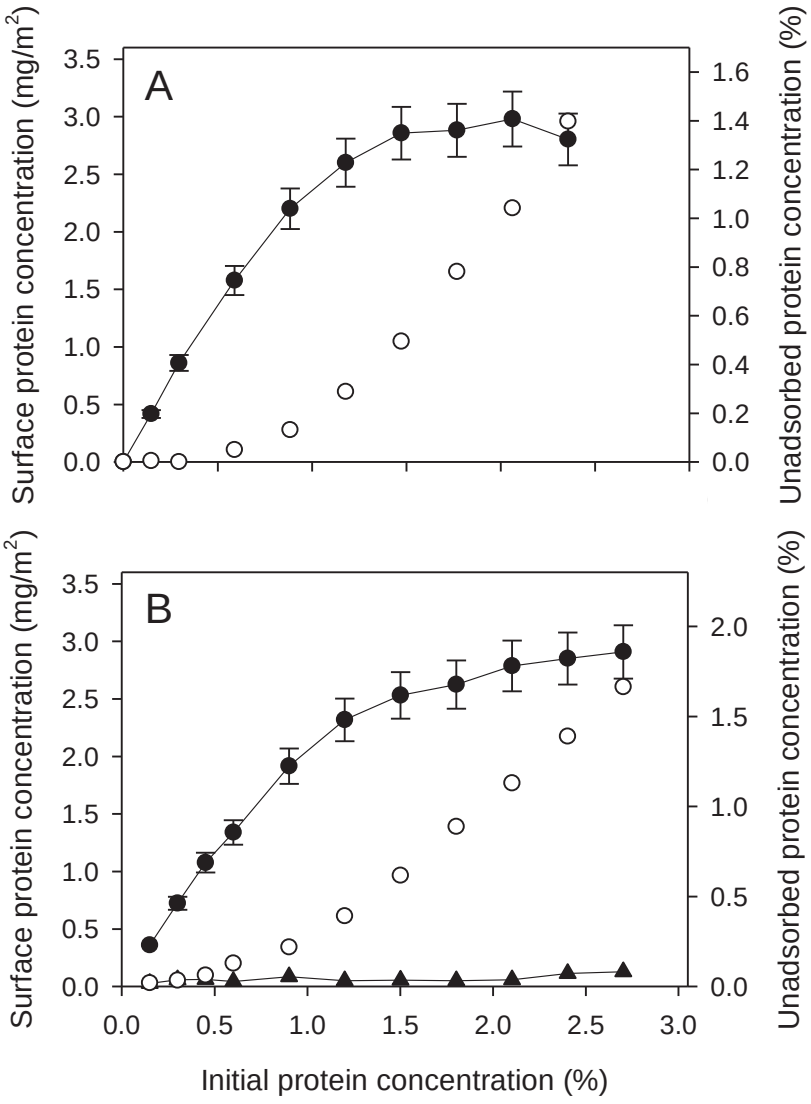

Figure 7.2: Surface protein concentration of (●) caseins and (▲) whey proteins adsorbed onto HA particles (mg/m^2) and (○) unadsorbed protein concentration (%, w/w) as a function of initial protein concentration: (A) WPD-SM; (B) SM. Results are means of duplicates or triplicates with error bars for the standard deviation.

In the previous chapter, both caseins and whey proteins have been shown to bind onto HA at pH ~6.8, when adsorption studies were carried out in water, HEPES buffers and SMUF. However, because of the presence in their structure of phosphoserine residues grouped in clusters, the affinity of β-casein and α_S-casein for HA was stronger than that of whey proteins, and caseins were always able to bind more than whey proteins, under the different conditions studied. When mixed together with caseins, β-lactoglobulin and α-lactalbumin could hardly bind onto HA particles (Chapter 5). Therefore, when HA particles were added to SM, caseins were expected to bind onto HA preferentially to whey proteins. Only negligible whey protein binding was expected, and this is what was observed (Figure 7.2B). This is consistent with the results of Devold et al. (2006), who showed that, in milk at pH 6.7, only caseins were able to bind to HA in tooth enamel and there was no whey protein binding.

7.4.1.2 Langmuir modelling

The caseins in SC are in a non-micellar form and can be present as individual molecules or small aggregates (Pitkowski et al., 2008) whereas the caseins in WDP-SM and SM are associated together to form casein micelles (Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2006). The Langmuir model is often used in studies on the adsorption of protein onto HA, to compare the adsorption characteristics of different types of protein (Iafisco et al., 2010; Iafisco et al., 2011; Mohsen-Nia et al., 2012), and has been used in the previous chapters to characterise the adsorption of the different milk proteins onto HA. The Langmuir model was therefore fitted to the adsorption data obtained in this study for WPD-SM and SM and compared with the Langmuir model results obtained for SC in water (Chapter 4) and in SMUF (Chapter 6). The Langmuir isotherm curves for casein adsorption onto HA for the adsorption experiments carried out with WPD-SM and SM are given in Figure 7.3. Table 7.1 gives the calculated Langmuir constants for each of the isotherms and for the adsorption isotherms of SC onto HA, in water and in SMUF, obtained in Chapter 4 and Chapter 6. The maximum surface protein concentrations obtained for both WPD-SM and SM experiments (3.0 and 2.7 mg/m^2, respectively) were not different from the maximum adsorption value obtained for SC in water (2.9 mg/m^2, P <0.05) and were only slightly higher than the maximum adsorption value for SC in SMUF (2.4 mg/m^2) (Table 7.1).

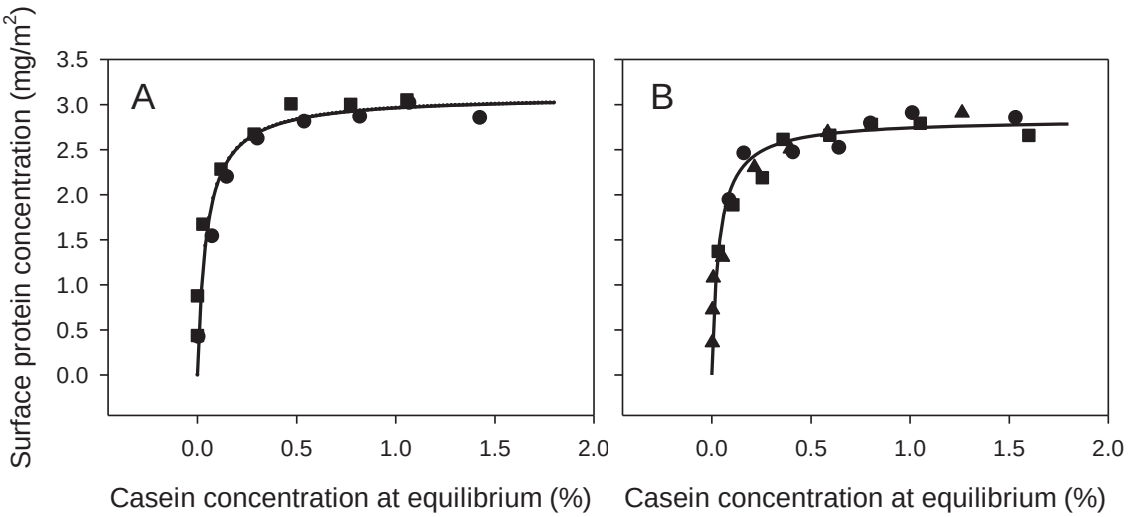

Figure 7.3: Adsorption isotherms of caseins onto HA particles: (A) WPD-SM; (B) SM
The symbols ●, ▲, and ■) are experimental data from different repeat experiments, the solid line gives the best-fit Langmuir curve.

Table 7.1: Parameters for the adsorption of SC and WPI onto HA particles in SMUF and in water, calculated according to the Langmuir model. [a]

Casein source	Affinity constant, K (100g/g)	Maximum surface coverage, q_m (mg/m^2)
WPD-SM	$24.5^{ab} \pm 9$	$3.0^{a} \pm 0.02$
SM	$31.5^{a} \pm 7.1$	$2.7^{ab} \pm 0.15$
SC in water	$13.7^{b} \pm 4.8$	$2.9^{a} \pm 0.2$
SC in SMUF	$31.5^{a} \pm 3.6$	$2.4^{b} \pm 0.01$

[a] Means in columns with different superscript letters differ significantly (P <0.05).

In food emulsion systems, casein micelles have been shown to bind as intact micelles onto oil droplets (Euston & Hirst, 2000; Ye, 2011) The surface protein concentration for casein-micelle-coated droplets has been reported to be much higher than that for SC-coated droplets (Singh & Ye, 2008; Ye, 2011) with usually two or three times more adsorbed protein for casein micelles than for SC. As casein micelles are larger than SC aggregates, they form a thicker layer at the surface of the oil droplets so that the protein surface load is higher. The fact that the maximum amount of casein adsorbed onto HA was similar for WPD-SM, SM and SC in SMUF (Table 7.1) could indicate that casein micelles do not bind as intact micelles onto HA particles.

The affinity constant of the Langmuir model characterises the initial slope of the adsorption isotherms and indicates a high affinity of protein for HA (Iafisco et al., 2011). There was a variability of the affinity constants in this study (Table 7.1), as a small variation in the determination of the unadsorbed protein concentration at low initial protein concentration (corresponding to the first points of the adsorption isotherms, see Figure 7.3) can lead to a high variation of the slope, and therefore of the affinity constant. However, it is interesting to note that the mean affinity constants were similar for the casein adsorption experiments carried out on SC in SMUF, WDP-SM, and SM binding to HA (Table 7.1). This suggests that the adsorption mechanisms and the affinity of HA for micellar and non-micellar forms of caseins in a milk environment are somewhat similar.

7.4.1.3 Transmission electron microscopy and light microscopy

TEM was carried out to examine the structure of the adsorbed caseins at the surface of the HA particles. Figure 7.4A and Figure 7.4B show TEM images from a solution of WPD-SM (10%, w/w, TS). The casein micelles in WPD-SM appear to be dark spherical aggregates, to be well dispersed, and to have diameters ranging from ~30 to 150 nm, consistent with the images of casein micelles observed in previous studies (Dalgleish, 2006; Schmidt & Buchheimi, 1992; Titapiccolo, Alexander, & Corredig, 2010). Figure 7.4C and Figure 7.4D show TEM images of the HA powder used in this study. It consists of particles of ~4 μm average size. However, these particles are made of elementary nanometer-sized rod-like particles (Figure 7.4D) that aggregate together into micron-sized particles, as observed previously in other studies (Sun & Lu, 2008; Xu et al., 2011). The presence of void space around the elementary small particles (Figure 7.4D) show that HA particles are porous, and illustrate the very high surface area of the particles, of about 65 m^2/g. Figure7.4E and Figure 7.4F show images of the HA particles suspended in WPD-SM (10%, w/w, total solids). As the particles had been stirred for 2 h in the WPD-SM before preparation for TEM, the caseins had adsorbed onto the HA particles. The sample corresponded to a stage of adsorption at which the maximum amount of adsorbed protein was reached, and some unadsorbed caseins were left in the supernatant. The HA particles had a similar appearance to the HA particles in the control (Figure 7.4C), but no casein micelles could be seen on the surfaces of the particles (Figure 7.4E and Figure 7.4F). It was therefore hypothesised that the caseins did not adsorb as intact micelles, but as small aggregates and/or individual molecules, which would be too small to be observed with TEM. The unadsorbed casein micelles could still be seen in the suspending phase around the particles, as shown by the

arrows in Figure 7.4F. These unadsorbed micelles had a hairy and disorganised appearance compared with the control (Figure 7.4B), which may have been because they were partly dissociated by the presence of HA particles.

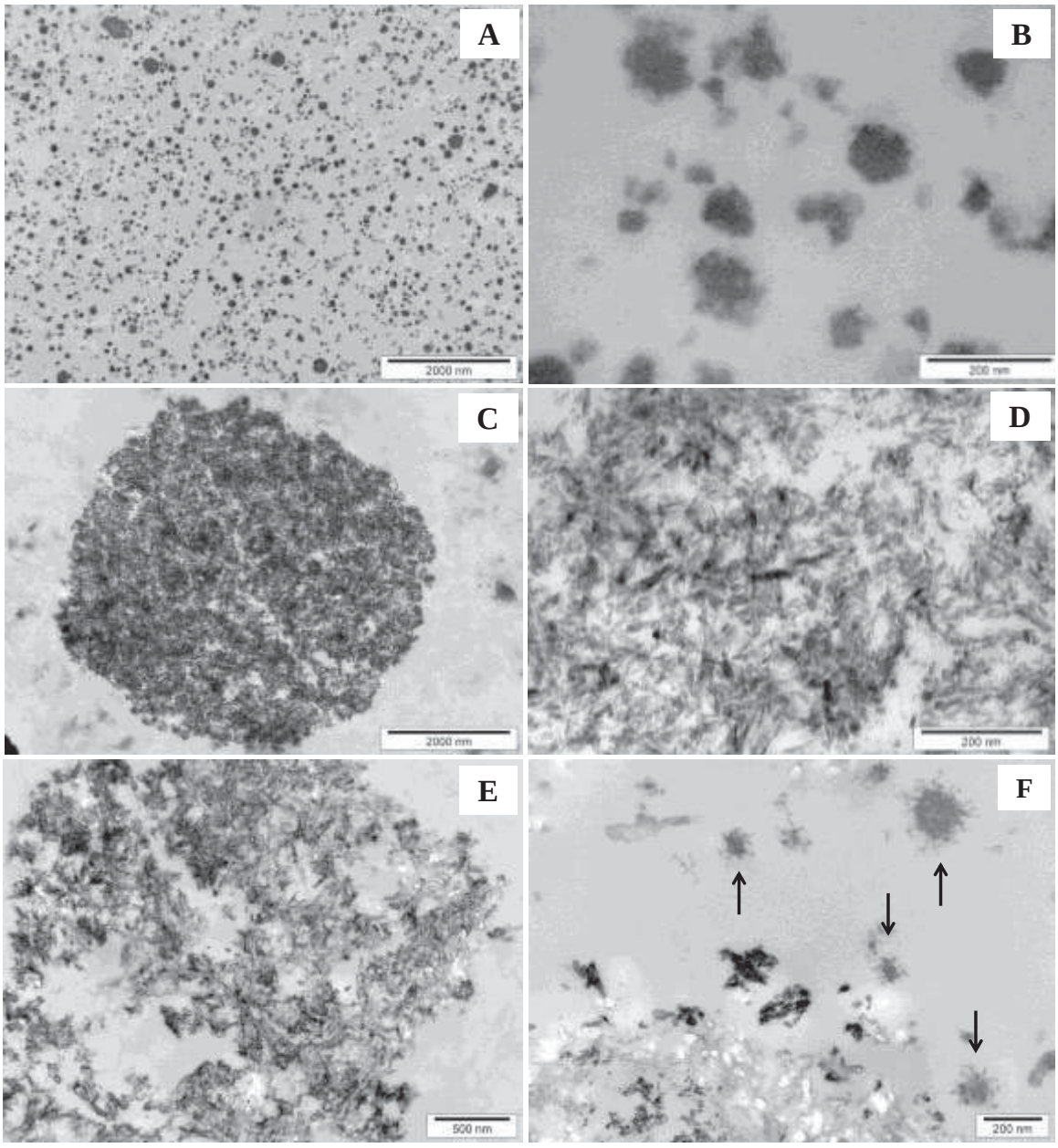

Figure 7.4: TEM micrographs obtained from: (A and B) WPD-SM solution reconstituted from WPD-SMP in water (10%, w/w); (C and D) suspension of HA particles in SMUF; (E and F) suspension of HA particles in WPD-SM solution, corresponding to a stage of adsorption at which the maximum amount of adsorbed protein was reached The arrows show the remaining unbound casein micelles in the suspending phase.

The TEM sections of the HA particles suspended in SMUF and in WPD-SM were also observed with a light microscope, using toluidine blue to specifically stain the protein material on the slide Figure 7.5). It can be seen that when suspended in SMUF, the HA particles appear like grey spherical particles, and they are not stained in blue, showing that no protein material is present.

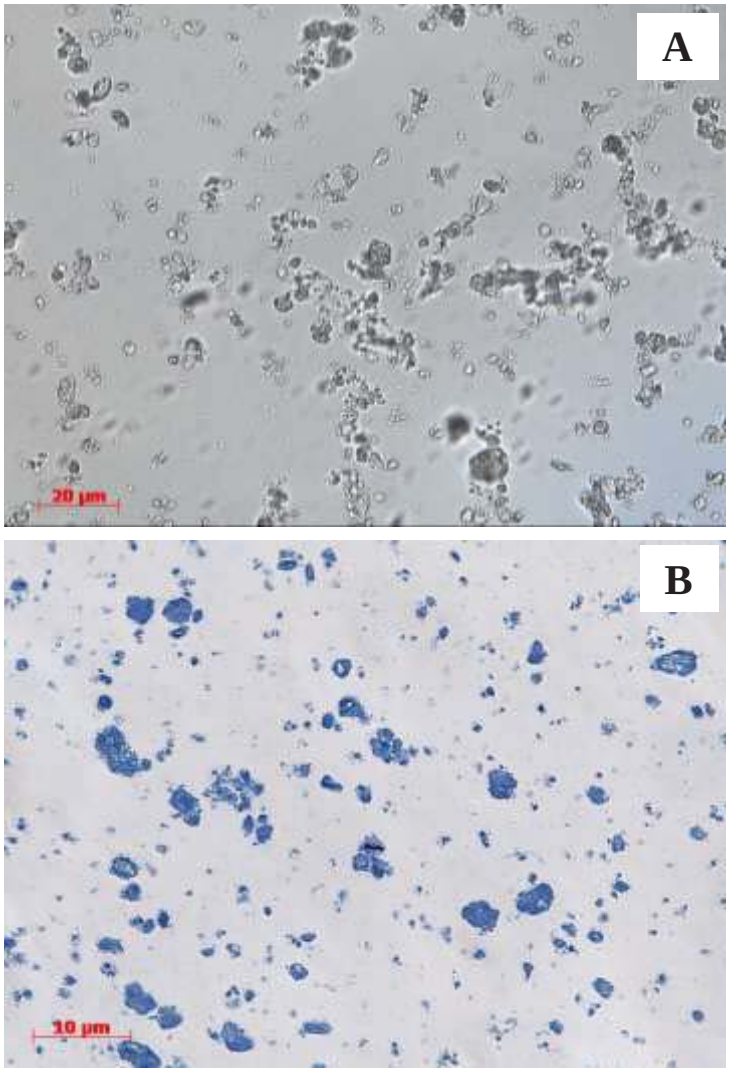

Figure 7.5: Light microscopy picture obtained from a suspension of HA particles in (A) SMUF and (B) WPD-SM solution. Toluidine blue specifically stained protein material.

The resolution of the light microscope was too small to see the inside structure of the particles. However, it is known from the TEM observations that each particle is porous and made of aggregated nano-size particles (Figure 7.4D). When suspended in WDP-SM, the HA particles are all stained in blue, including inside the particles. It means proteins were able to adsorb on all the HA surface, including on the elementary nano-size particles inside the

micron-sized particles. As the width of the porous channels inside the particles is very small, often under 200 nm (Figure 7.4D), the presence of protein inside the particles gives another indication that casein might not bind as intact micelles, but rather as small molecules that can penetrate inside the porous structure of the HA particles.

7.4.1.4 Analysis of unadsorbed casein

The initial adsorption measurements and TEM observations led to the hypothesis that casein micelles did not bind to HA particles as intact micelles, possibly because the micelles dissociated either before or shortly after binding. To verify this hypothesis, the supernatants obtained from the adsorption experiments carried out with WDP-SM and containing the unadsorbed proteins were analysed for non-micellar casein content, particle size, and protein composition.

For non-micellar content analysis, the supernatants obtained after adsorption, containing the total unadsorbed caseins, were centrifuged at 21,000 × *g* and 25 °C for 60 min; the pellet contained micellar caseins whereas the non-micellar caseins stayed in the serum phase. The supernatants (i.e., containing the total unadsorbed caseins) and their respective serum phase (i.e., containing only the non-micellar fraction of the total unadsorbed caseins), were analysed for protein content using MF-electrophoresis. Figure 7.6 shows the MF-electrophoresis computer-generated gel of the supernatants and their respective serum phase obtained in adsorption experiments carried out with WPD-SM at five different initial protein concentrations. The supernatants obtained at low initial protein concentrations of 0.6 and 0.9% (w/w; see lanes 1 and 3 in Figure 7.6) contained a very small amount of unadsorbed caseins, since most of the caseins were adsorbed onto HA. However, a high proportion of the unadsorbed caseins they contained was in a non-micellar form, as the intensities of the casein bands in their serum phase (see lanes 2 and 4 in Figure 7.6) were of respectively about 70% and 57% of the intensities of the casein bands in the respective controls (respectively lanes 1 and 3 inFigure 7.6). As the initial protein concentration increased to higher values of 1.2, 1.5 and 1.8% (w/w), the supernatants contained more and more unadsorbed proteins (see lanes 5, 7 and 9 in Figure 7.6), and a smaller portion of these unadsorbed proteins were in a non-micellar form, as shown by the very low intensities of the respective serum phases (see lanes 6, 8 and 10 in Figure 7.6).

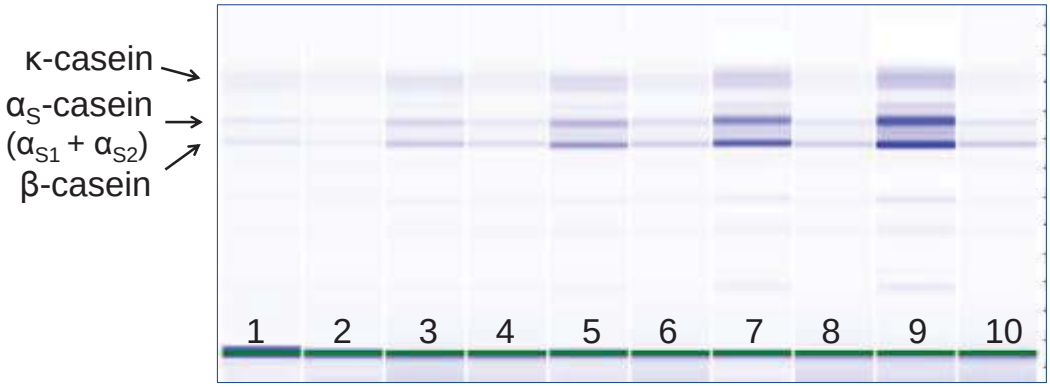

Figure 7.6: MF-electrophoresis gel pattern of supernatants containing the total unadsorbed protein and their respective serum phase containing the non-micellar unadsorbed caseins, obtained from adsorption experiments carried out with WDP-SM at different initial protein concentrations. Lanes 1 and 2, 0.6% (w/w); lanes 3 and 4, 0.9% (w/w), lanes 5 and 6, 1.2% (w/w), lanes 7 and 8, 1.5% (w/w), lanes 9 and 10, 1.8% (w/w); lanes 1, 3, 5, 7 and 9, control supernatant samples (i.e., containing the total unadsorbed caseins); lanes 2, 4, 6, 8 and 10, respective serum phase of the controls (i.e., containing the non-micellar unadsorbed caseins).

Figure 7.7 shows the distribution of the unadsorbed caseins between micellar and non-micellar forms (Figure 7.7A), calculated from the MF-electrophoresis results shown in Figure 7.6, and the casein micelle diameter of the unadsorbed casein (Figure 7.7B), as a function of the initial protein concentration used in the adsorption experiment.

At low initial protein concentration, more than half of the unadsorbed caseins were in a non-micellar form. As the initial protein concentration increased, the proportion of non-micellar casein in the unadsorbed protein decreased, reaching approximately 5% when HA particles were prepared with WPD-SM containing 2.4% protein (0.08%, w/w, of the caseins were in a non-micellar form out of 1.4%, w/w, of total unadsorbed protein), which was close to the level of non-micellar casein in the initial WPD-SM solutions (~3% non-micellar casein, data not shown). At low initial protein concentration, the average diameter of the unadsorbed casein particles was about ~122 nm (Figure 7.7B). As the initial protein concentration increased, the diameter increased, until it reached ~155 nm, which was close to the size of the casein micelle in the initial WPD-SM solutions (160 nm).

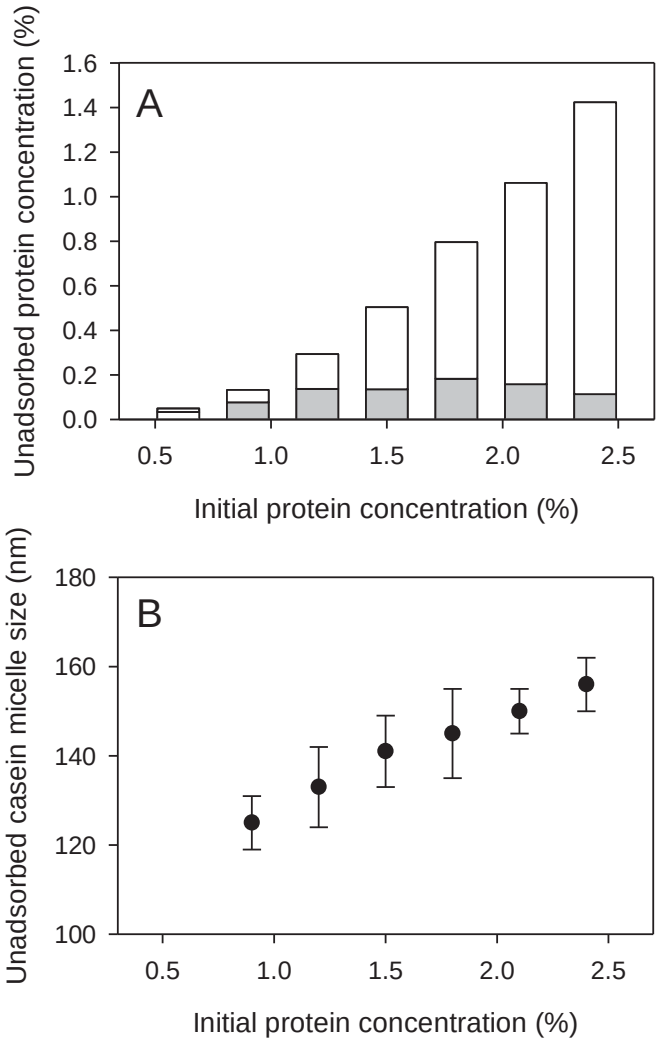

Figure 7.7: (A) Distribution of unadsorbed caseins between (white bar) micellar and (grey bar) non-micellar forms and (B) diameters of unadsorbed caseins. Error bars represent standard deviation.

Figure 7.8 shows the protein composition of the supernatants containing the unadsorbed proteins as a function of the initial protein concentration. For the adsorption experiments carried out with WPD-SM containing less than 1% (w/w) protein, the ratio of unadsorbed κ-casein to unbound α_S- and β-caseins in the supernatants was considerably greater than in the original WPD-SM (the composition of the original WPD-SM is indicated by the lines on Figure 5, and was of about 50% α_S-casein, 38% β-casein and 12% κ-casein). For example, when HA particles were mixed with WPD-SM containing 0.6% protein, ~60% of the unadsorbed protein was κ-casein, which is substantially higher than the proportion found in milk (12%). The ratio of unadsorbed α_S-casein to unadsorbed β-casein remained approximately constant. As the initial protein concentration in the SM increased, the relative

proportions of the three unadsorbed individual caseins gradually approached the initial WPD-SM composition as more and more unadsorbed protein accumulated in the supernatant.

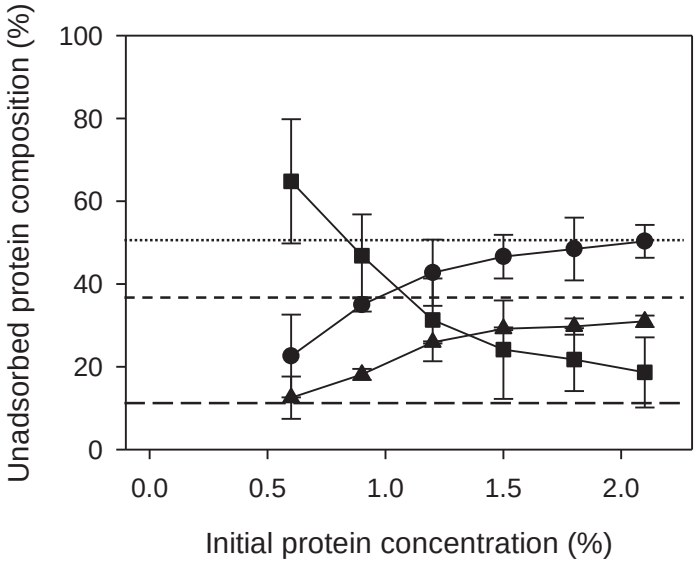

Figure 7.8: Composition of the supernatants containing the unadsorbed protein after adsorption of WPD-SM onto HA particles as a function of the initial protein concentration. ●, α_S-casein (α_{S1} + α_{S2}); ▲, β-casein; ■,κ-casein; the lines indicate the composition of the original WPD-SM in (dotted line) α_S-casein (α_{S1} + α_{S2}), (short dashed line) β-casein, and (long dashed line) κ-casein. Error bars represent standard deviation.

The amounts of the individual proteins adsorbed onto HA were calculated from the composition of the unadsorbed protein determined by MF-electrophoresis and the original protein composition of the WPD-SM (Figure 7.8), and are given in Figure 7.9. When the initial protein concentration was varied from 0 to 2.5%, the amounts of adsorbed α_S-casein, β-casein, and κ-casein increased gradually until they reached maxima of ~1.5, 1.3, and 0.15 mg/m^2, respectively (Figure 7.9). The maximum amount of bound κ-casein represented only ~5% of the total bound protein (0.15 mg/m^2 out of 2.9 mg/m^2), even though the original WPD-SM contained ~12% κ-casein, which is consistent with the fact that the supernatants containing the unadsorbed proteins were enriched in κ-casein compared to the initial WPD-SM solutions (Figure 7.8). This confirms that α_S-casein and β-casein are preferentially adsorbed over κ-casein, as already hypothesised from the MF-electrophoresis gels shown in Figure 7.1.

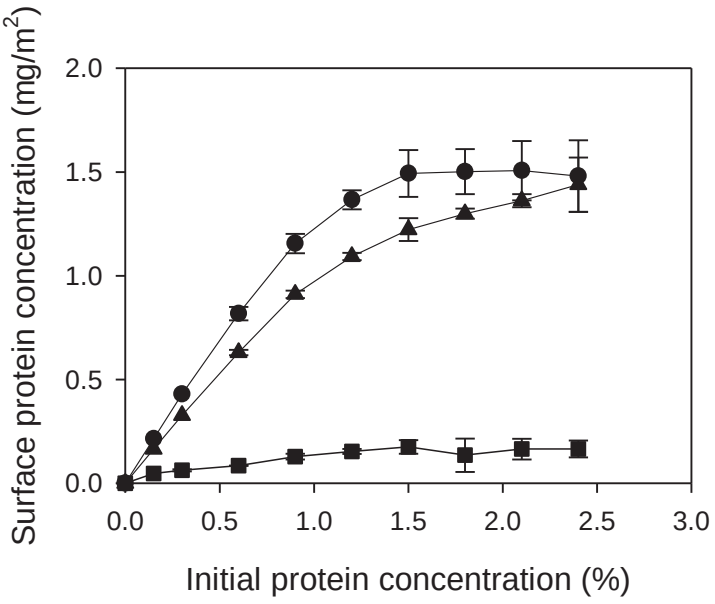

Figure 7.9: Estimated amount of adsorbed caseins (mg/m²) on HA particles as a function of initial protein concentration of WPD-SM. ○, α_S-casein (α_{S1}- + α_{S2}-); ▲, β-casein; ■, κ-casein. Error bars represent standard deviation.

Analysis of the unadsorbed proteins showed that the proportion of micellar casein in the unadsorbed casein fraction (Figure 7.7A), the casein micelle size (Figure 7.7B), and the protein composition (Figure 7.8) were not constant across the range of initial protein concentrations studied. The unadsorbed caseins obtained at low initial protein concentrations contained a higher proportion of κ-casein, contained a higher proportion of non-micellar casein, and were of a smaller size, compared with the unadsorbed caseins obtained at high initial protein concentration. This confirmed that the caseins were probably not bound to the HA particles as intact micelles, and that the casein micelle structure was disrupted by the addition of HA particles. If the caseins had bound as intact micelles, the proportion of adsorbed caseins on the HA surface would also have been expected to be the same as the proportion of the different caseins in WDP-SM, which was not the case (Figure 7.9). However, it was not possible to determine whether the casein micelles dissociated first and then the liberated caseins bound to HA or casein micelles adsorbed as intact micelles and then dissociated allowing only the strongly bound caseins to remain on the HA particle surface. However, if the casein micelles were binding as intact micelles first and were dissociating only in a second step, a difference in the proportions of the different caseins adsorbed onto the HA particles could possibly be observed as a function of adsorption time. A kinetic experiment was therefore carried out to check

whether the proportions of adsorbed caseins on the HA surface varied over time. 5% (w/w) HA particles were added in SM containing 1.25% (w/w) initial protein, and the amount and type of caseins adsorbed onto HA particles at different adsorption times, from 1 min to 3 h were measured using MF-electrophoresis. The results are given in Table 7.2. The total surface concentration on HA increased significantly between 1 and 120 minutes and then stabilised to a maximum value of about 2.6 mg/m^2, showing that after about 120 min, an equilibrium had been reached and no more protein adsorbed onto the HA particles (Table 7.2). There was no significant difference between the proportion of κ-casein adsorbed at the different adsorption times; all comprised between 6 and 8%, and lower than the proportion of κ-casein in the initial SM, equal to 12% (Table 7.2). This shows that from the start of the adsorption process, κ-casein adsorb less than α_S- and β-casein. It was therefore believed that the caseins might first dissociate from the casein micelles and then bound independently onto HA, rather than having the casein micelles binding first onto HA and then releasing the least preferred κ-casein. However, further experiments would be needed to confirm this hypothesis.

Table 7.2: Total surface protein concentration and relative proportions of individual caseins adsorbed onto HA particles after different adsorption times. [a]

Time (min)	Total surface casein concentration (mg/m^2)	Percentage (%) by type		
		α_S-casein	β -casein	κ-casein
1	0.48^a ± 0.18	47 ±4	45 ± 5	8 ±3
5	0.96^{ab} ± 0.22	46 ± 0.9	46 ± 0.4	8 ± 1.6
15	1.40^b ± 0.03	48 ± 0.2	44 ± 2.1	8 ± 2
30	1.68^c ± 0.01	48 ±0.2	45 ± 0.8	7 ± 0.6
60	2.11^d ± 0.1	51 ±0.2	43 ± 1.4	6 ± 1.3
90	2.44^e ± 0.12	51 ± 0.5	41 ± 1.2	7 ± 1.4
120	2.57^e ± 0.05	52 ±0.6	42 ± 0.6	6 ±0.8
180	2.64^e ± 0.04	53 ± 0.8	41 ± 0.7	6 ± 1
240	2.58^e ± 0.14	53 ± 0.3	41 ± 0.5	6 ± 0.6

[a] Means in columns with different superscript letters differ significantly (P <0.05).

7.4.1.5 Characterisation of the casein-coated particles

The aim of this chapter was mainly to understand the interactions between casein micelles and HA and the adsorption mechanism involved. However, before investigating further the adsorption mechanisms, the casein-coated HA particles prepared with WDP-SM were characterised for zeta-potential and suspension stability, as in previous chapters. The zeta-potential of the casein-coated HA particles prepared with different initial concentrations of WDP-SM was measured, and their suspension stability of the particles was assessed visually by taking a picture of the HA suspensions after 24 h left undisturbed on the bench, and these results were correlated with the amount of protein adsorbed onto the particles (Figure 7.10). The zeta-potential of the bare HA particles suspended in SMUF was of about –22 mV (Figure 7.10), as observed previously in Chapter 6, which was attributed to the adsorption of the citrate and phosphate ions contained in SMUF onto the particles. The adsorption of these negative ions was believed to provide an electrostatic stabilisation to the HA particles, which remained in suspension after 24 h (see tube a in Figure 7.10). As the initial protein concentration used to prepare the particles increased, a decrease of the magnitude of the zeta-potential was observed from –22 mV to a minimum plateau value of about –16 mV. This decrease of zeta-potential was probably due to the adsorption of the caseins onto HA particles, which modified the surface charge of the particles to less negative values, as observed for the adsorption of SC in SMUF in Chapter 6. However, it was interesting to notice that the zeta-potential reached the plateau value of about –16 mV for a low initial protein concentration of about 0.25 %(w/w), corresponding to low surface coverage of the HA particles by the caseins, of about 0.5 mg/m^2 (Figure 7.10). The particles corresponding to low surface coverage values of 0.5 mg/m^2 and 1 mg/m^2 (tubes b and c) were not as stable in suspension overtime as the bare particles in SMUF (tube a), and as the particles that were covered by a high amount of adsorbed caseins (tubes d to f), as shown by their lower visual turbidity after 24 h. These suspension stability results were similar to those observed when using SC-coated particles in SMUF, where it was attributed to a transition between a pure electrostatic stabilisation in SMUF (stabilisation by charge), to a steric stabilisation provided by the adsorbed layer of caseins at the surface of the particles, which was reached only when enough caseins were adsorbed onto the particles.

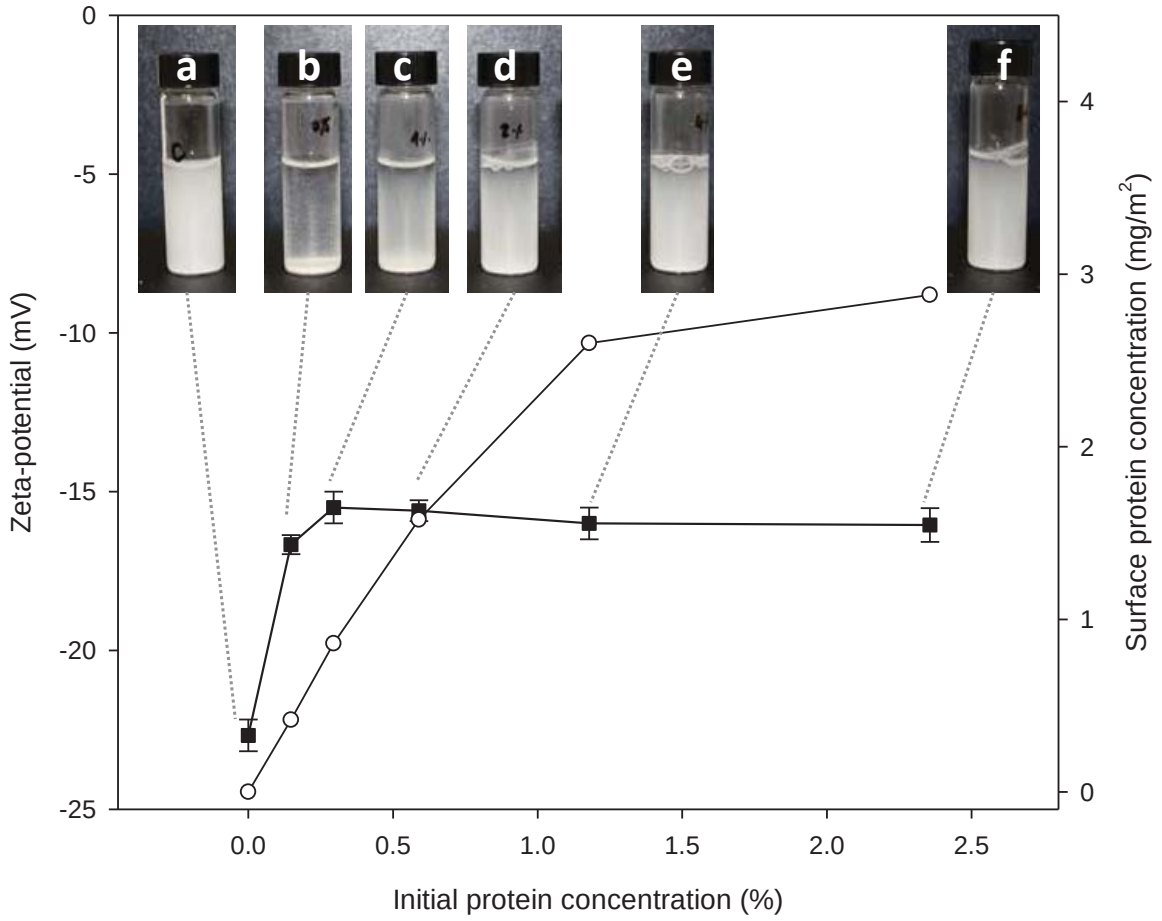

Figure 7.10: Zeta-potential, surface protein concentration and visual turbidity of HA particles prepared with WDP-SM at different initial protein concentrations. Particles were rinsed and re-suspended in SMUF for zeta-potential measurements and suspension stability observations; ■, zeta-potential, (○) surface protein concentration. Each tube is associated to one zeta-potential value and one surface protein concentration level and corresponds to one initial protein concentration (w/w) used to prepare the HA particles in suspension: a, 0 (SMUF); b, 0.15%; c, 0.30%; d, 0.60%; e, 1.2%, f, 2.4%.

7.4.2 Adsorption of caseins and whey proteins from EDTA-treated SM

7.4.2.1 Characterisation of EDTA-treated SM

The similarity of the maximum surface protein concentrations obtained with adsorption experiments carried out with SC and casein micelles from WDP-SM and SM (Table 7.1) suggested that the amount of proteins that can bind onto HA particles is the same, regardless of whether the caseins are in a micellar form, and therefore regardless of

whether the phosphoserine groups of the caseins are already bound to calcium phosphate in the calcium phosphate nanoclusters of the micelles. To verify this hypothesis, different levels of EDTA (0, 5, 10 and 20 mmol) were added to SM, to remove different amounts of CCP from the casein micelle and induce a partial dissociation of the casein micelles, using a method adapted from Griffin et al. (1988) and Ward et al. (1997). After EDTA addition, the milks were dialysed overnight (10 kilodalton cut-off) against the original SM, to remove the excess soluble calcium and phosphate and therefore restore the original pH and serum composition of the initial milk. The casein micelle diameter and the non-micellar casein content of the EDTA-treated milks were measured and reported in Table 7.3. Adding increasing levels of EDTA from 0 to 20 mM in SM induced a progressive decrease in casein micelle size from 245 nm when no EDTA was added (original SM) to 164 nm when 20 mM was added (Table 7.3). The proportion of non-micellar caseins increased from 2% in the original SM to 96% when 20 mM of EDTA was added (Table 7.3). This clearly shows that casein micelles progressively dissociated by the addition of EDTA and this was confirmed by the progressive decrease in the visual turbidity of the samples. As EDTA is a calcium chelator, its addition in milk causes the formation of complexes between EDTA and Ca^{2+} of the milk serum, therefore disrupting the milk mineral equilibrium. Consequently, CCP is released from the casein micelles, which causes the disruption and dissociation of the casein micelles (Griffin et al., 1988; Ward et al., 1997).

Table 7.3: Non-micellar casein content and casein micelle diameter of EDTA-treated skim milks.

EDTA concentration (mM)	Soluble casein content (% of initial)	Casein micelle diameter (nm)
0	2	245
5	25	240
10	44	235
20	96	164

7.4.2.2 Surface protein concentration of caseins from EDTA-treated SM on HA particles

The EDTA-treated SM samples all had the same protein composition as the original SM, but their casein micelles were dissociated to different levels. Therefore it was possible to investigate whether the level of dissociation of casein micelles in SM had an effect on the amount of caseins that could bind onto HA particles.

Various amounts of HA powder were added to various amount of each of the EDTA-added SM solutions (0, 5, 10, and 20 mM EDTA) to obtain four different SM to HA powder ratios: 50 mg of HA powder to 0.95 g of SM (very low HA to SM ratio), 75 mg of HA powder to 0.925 g of SM (low HA to SM ratio), 100 mg of HA powder to 0.900 g of SM (medium HA to SM ratio) and 150 mg of HA powder to 0.850 g of SM (high HA to SM ratio). These four ratios corresponded to different levels of protein surface coverage. The very low and low HA to SM ratio corresponded to levels where the adsorption plateau was reached (limited HA surface available, high surface coverage, a lot of protein in excess in the supernatants), whereas the medium and high HA to SM ratio corresponded to an incomplete surface coverage of the particle by the protein (excess of HA surface available, low surface coverage, low remaining amount of protein in the supernatants). The adsorption experiment was carried out for 2h, the HA particles were separated from the EDTA-added SM solutions by centrifugation and the initial solutions and the supernatants were analysed from protein content using SDS-PAGE (for qualitative results), and MF-electrophoresis (for quantitative results), as described before.

Figure 7.11 shows a SDS-PAGE gel obtained from the initial 0, 10 and 20 mM EDTA-added SM samples (controls, lanes 1 to 3) and the adsorption supernatants of the samples prepared with HA particles added to the initial 0, 10 and 20 mM EDTA-added SM, at three different HA to SM ratios. The intensity of the bands was similar for the three controls, showing that the three milks treated with different amounts of EDTA had the same protein content and composition. For each ratio (low HA to SM ratio, lanes 4 to 6, medium HA to SM ratio, lanes 7 to 9, and high HA to SM ratio, lanes 10 to 12), the intensity of the bands were similar for the samples prepared with SM prepared without EDTA (first lane of each group), SM prepared with 10 mM EDTA (second lane of each group), and SM prepared with 20 mM EDTA (third lane of each group).

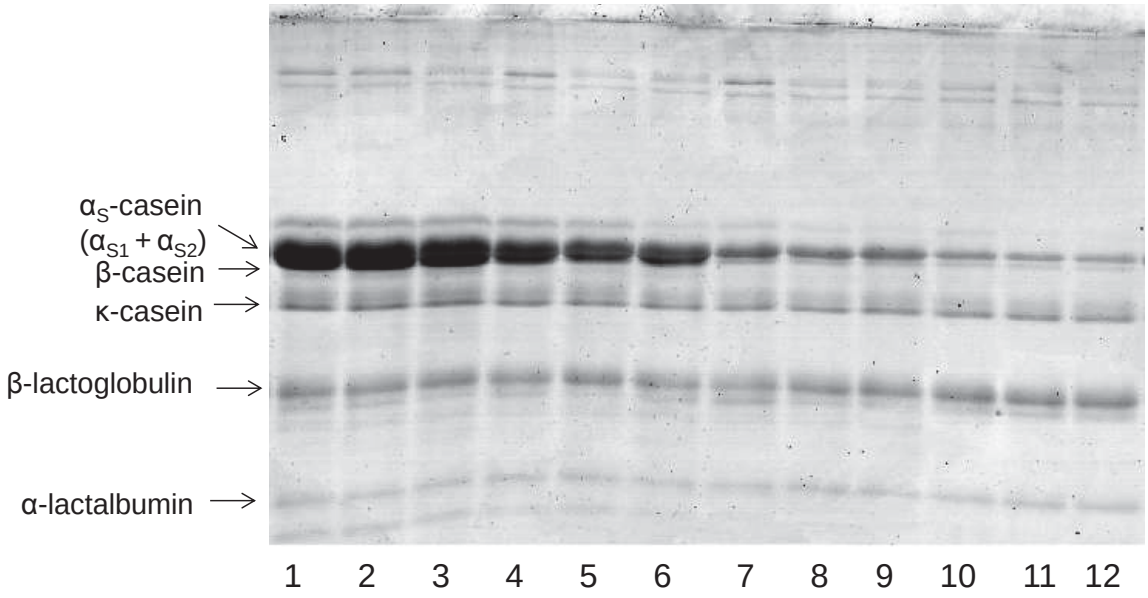

Figure 7.11: SDS-PAGE patterns of the initial solutions of EDTA-treated SM and the adsorption supernatants of the EDTA-treated SM and HA particles mixed to different ratios SM to HA powder. Lane 1-3: Controls 0, 10, 20 mM EDTA treated SM, no HA powder added; lane 4-6: supernatants of 50 mg of HA powder added to 0.95 mL of 0, 10, 20 mM EDTA-treated SM; lane 7-9: supernatants of 100 mg of HA powder added to 0.9 mL of 0, 10, 20 mM EDTA-treated SM; lane 10-12: supernatants of 150 mg of HA powder added to 0.85 mL of 0, 10, 20 mM EDTA-treated SM.

The amount of protein adsorbed onto HA for each HA to SM ratio and each EDTA-added SM sample was calculated based on three similar repeated experiments and the quantification of the protein remaining in the supernatants after adsorption was carried out using MF-electrophoresis. The adsorption results are shown in a Langmuir-type graph inFigure 7.12, with the surface casein concentration plotted as a function of the concentration of casein remaining in the supernatant (i.e., the casein concentration at equilibrium). For the four HA to SM ratios used in this experiment, there was no significant difference in the amount of caseins that adsorbed onto HA particles between the different EDTA-treated milks (Figure 7.12). The caseins adsorbed to values of about 2, 2.6, 2.9 and 3 mg/m^2, respectively, when HA particles were added to EDTA-treated SM at the following HA to SM ratios: 150 mg in 0.850 mL, 100 mg in 0.9 mL, 50 mg in 0.95 mL, and 25 mg in 0.975 mL. This means that the extent of dissociation of the casein micelles in SM had no effect on the amount of caseins that could bind onto HA particles. As EDTA-dissociated casein micelles had different sizes (Table 7.3), if the casein micelles had bound as whole micelles onto HA particles, the

adsorption of casein micelles from the different EDTA-treated milks would have led to different amounts of adsorbed caseins on the HA surface, with the samples containing the larger micelles binding more than the samples containing the smaller dissociated micelles. On the other hand, if the caseins in the casein micelles had been partially prevented from binding because their phosphoserine groups were already bound to CCP in the micelles and therefore could not detach from CCP to bind onto HA, less adsorbed caseins would have been observed from the sample containing the larger and non-dissociated micelles. As caseins micelles of different sizes and levels of dissociation in EDTA-added SM all bound to the same extent onto HA particles, this confirms again that casein micelles must not bind as intact micelles onto HA, but as individual caseins.

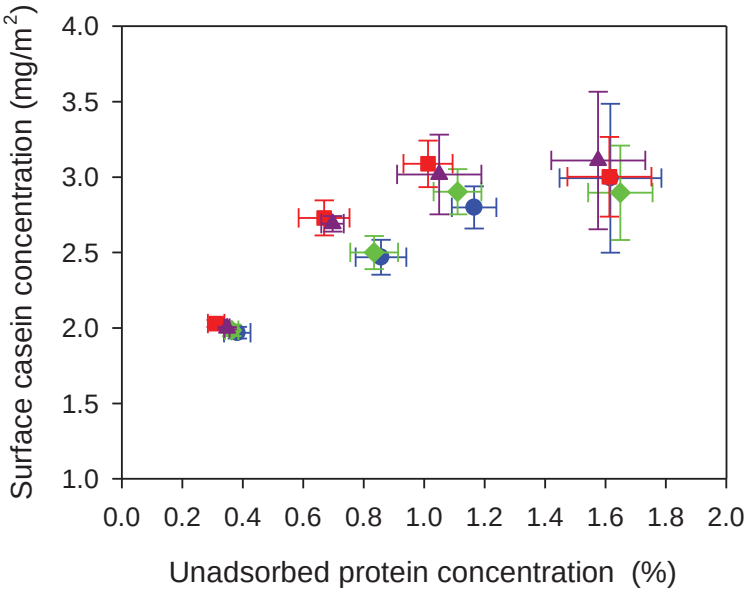

Figure 7.12: Langmuir-type representation of the surface protein concentration obtained in adsorption experiments carried out with HA particles added to different EDTA-treated SM. ●, SM control, no EDTA added; ◆, 5 mM EDTA-treated SM; ■, 10 mM EDTA-treated SM; ▲, 20 mM EDTA-treated SM; each group of point corresponds to one HA to SM ratio, from left to right: 150 mg in 0.95 mL, 100 mg in 0.9 mL, 50 mg in 0.95 mL and 25 mg in 0.975 mL. Error bars represent standard deviation.

In conclusion, the adsorption results obtained in this chapter for both WDP-SM, SM and EDTA-treated SM all showed that caseins of the casein micelles are able to bind onto HA to the same extent as blends of caseins in a non-micellar form, for example in SC or in EDTA-treated milks. The state of dissociation of the micelles does not seem to impact the amount

or the type of proteins that can bind onto HA. The preference for α_S- and β-caseins over κ-casein for binding onto HA that was observed in the previous chapters when using SC or purified caseins was also observed when using milk. This preference for α_S- and β-caseins can be attributed to the high content of phosphoserine residues in both α_S- and β-caseins, compared with κ-casein. As α_S- and β-caseins carry more residues that can bind to the C-sites on HA particles, they can bind onto HA to a greater extent. It has been shown previously that both α_S- and β-caseins contain specific phosphopeptides with a high ability to bind to calcium phosphate (Baum, Ebner, & Pischetsrieder, 2013; Cross et al., 2005; Reynolds et al., 1994). They contain similar calcium phosphate binding motifs, made of a sequence of amino acids containing three phosphoserine residues followed by two glutamic acid residues (FitzGerald, 1998). These casein-derived phosphopeptides (CPP) have been produced and purified by enzymatic digestion and have been bound to amorphous calcium phosphate (ACP) to form complexes (CPP–ACP) (Cross et al., 2007; Reynolds, 1998). It must be the same motifs of amino acids as those involved in the CPP–ACP complexes that are involved in the binding of α_{S1}-casein, α_{S2}-casein, and β-casein onto HA particles. Upon addition of HA particles in milk, the casein micelle must be disrupted and these motifs must be released from the calcium phosphate nanoclusters of the micelles, therefore becoming available to bind onto HA particles. However, it is not known why the addition of HA particles causes the dissociation of the casein micelles. It is possible that the presence of HA particles in milk disrupt the milk mineral equilibrium, since HA particles have been shown in Chapter 6 to be able to bind the milk serum ions. The rest of this chapter will therefore focus on investigating further these mechanisms.

7.4.3 Mechanism of casein micelle dissociation by HA particles

7.4.3.1 Dialysis experiment

A dialysis experiment was carried out using SM at 10% and 5% w/w total solids (respectively referred to as 10%-SM and 5%-SM). Both 10%-SM and 5%-SM were dialysed overnight against SMUF (controls) or SMUF containing HA particles to ascertain whether the presence of HA particles caused the dissociation of the casein micelles when not in direct contact. When removed from the dialysis bags, the 10%-SM and 5%-SM dialysed against SMUF containing HA particles (cuvettes B and D, respectively, in Figure 7.13) were less turbid than their respective controls dialysed against SMUF (cuvettes A and C, respectively, in Figure 7.13.

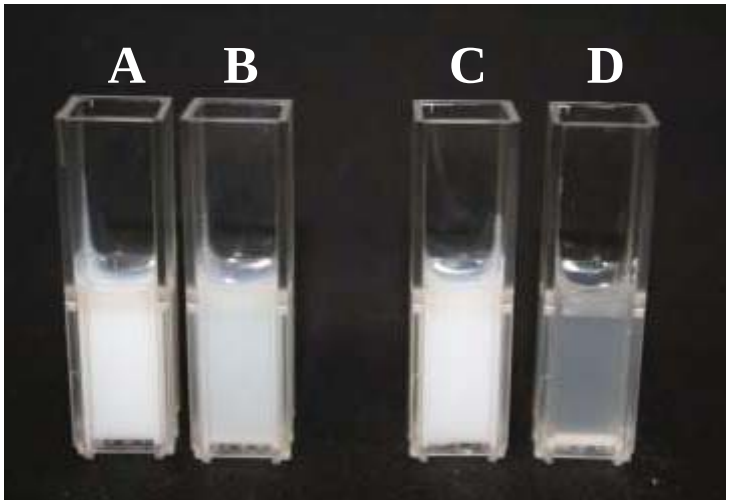

Figure 7.13: Visual turbidity of SM samples after dialysis against SMUF or against SMUF containing 10% (w/w) HA particles. Panels show: A, 10%-SM dialysed against SMUF; B, 10% SM dialysed against SMUF containing 10% (w/w) HA particles; C, 5%-SM dialysed against SMUF; D, 5%-SM dialysed against SMUF containing 10% (w/w) HA particles.

For the lower concentration of SM (5%-SM), the sample dialysed against SMUF containing HA particles was almost transparent, indicating that almost all the casein micelles had dissociated. This shows that the casein micelles in the milks dialysed against HA particles dissociated during the dialysis process, even though they were not in direct contact with the particles. This was confirmed by measurement of the non-micellar casein content and the particle size of the dialysed milks. The casein micelle diameter of the control SM was ~230 nm for both 10%-SM and 5%-SM and both milks contained less than 5% non-micellar caseins, which was similar to the original non-dialysed SM (Table 7.4). This shows that the milks dialysed against SMUF were stable during dialysis. The 10%-SM and 5%-SM dialysed against SMUF containing HA particles contained 73 and 94% non-micellar caseins, respectively. The casein micelle size of 10%-SM dialysed against SMUF containing HA particles was 165 nm; that of 5%-SM treated identically could not be measured by the Zetasizer because the sample did not show the required turbidity.

Table 7.4: Non-micellar casein content and casein micelle size of the dialysed milks

Sample	Non-micellar caseins (%)	Casein micelle diameter (nm)
10%-SM		
Dialysed against SMUF	3	230
Dialysed against HA in SMUF	73	165
5%-SM		
Dialysed against SMUF	5	227
Dialysed against HA in SMUF	94	N/D[a]

[a] N/A: not determined

7.4.3.2 Mineral quantification

The observations that HA caused the casein micelles to dissociate even when not in direct contact (Figure 7.13) leads to the hypothesis that HA particles were able to draw out some of the minerals contained in SMUF, which would cause the casein micelles to dissociate. It has been shown in Chapter 6 that calcium, phosphate, and citrate ions could all adsorb onto HA. If the ions contained in milk serum adsorb onto HA particles to a significant amount, it might disrupt the salt balance in milk serum, causing the casein micelles to dissociate. This hypothesis was tested by adding different amounts of HA particles (from 0 to 18%, w/w) into SMUF and SM and then measuring ionic calcium, total calcium, and inorganic phosphate.

Figure 7.14 shows the variation in the ionic calcium concentration when HA particles at different concentrations, from 0 to 18% (w/w), were added to SMUF or to SM. The SM and SMUF contained ~1.7 and ~1.45 mM ionic calcium, respectively, which was in accordance with the values reported in the literature (Gao et al., 2009; Lewis, 2011; Lin et al., 2006). As more HA particles were added to both SMUF and SM, the ionic calcium concentration decreased, with the decrease being more pronounced in SMUF. The addition of 10% (w/w) HA particles led to ionic calcium concentrations of ~0.1 mM in SMUF and ~0.55 mM in SM, which represented losses of 93 and 68% of the initial ionic calcium, respectively. The decrease in ionic calcium on the addition of HA particles indicates that ionic calcium binds to these particles. As ionic calcium binds to HA particles, CCP must be released from the casein micelles to compensate for the loss of ionic calcium in the serum, thereby explaining

the smaller decrease in ionic calcium for SM than for SMUF. The binding of ionic calcium onto the HA particles was a relatively fast process; the ionic calcium concentration reached a stable level in less than 5 min.

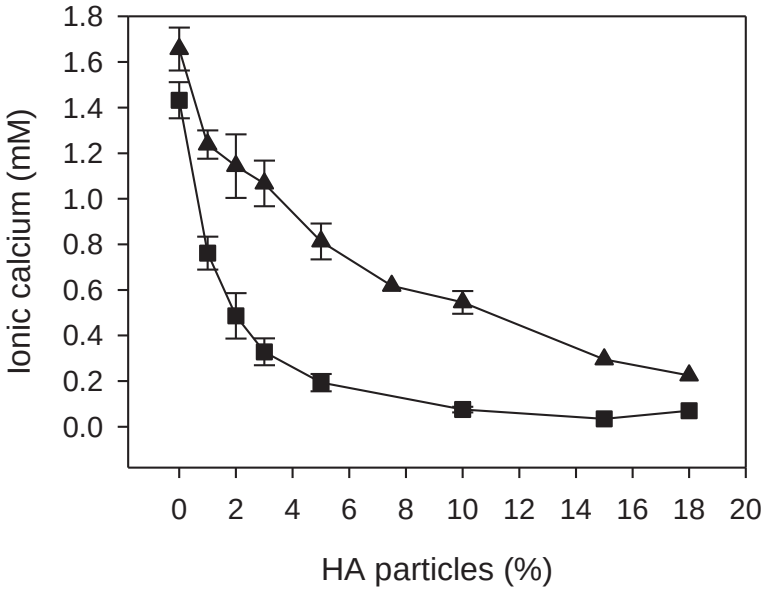

Figure 7.14: Effect of addition of HA particles in (■) SMUF and in (▲) SM on ionic calcium concentration. Error bars represent standard deviation.

A mineral analysis was also carried out on SMUF and SM samples after the HA particles had been removed from the solution by centrifugation. As HA particles were added to SM at increasing concentration from 0 to 18% (w/w), the concentrations of total calcium, citrate, and inorganic phosphate decreased (Figure 5.15A). The initial SM contained ~21.5 mmol/kg of inorganic phosphate, ~28.5 mmol/kg of calcium, and ~11 mmol/kg of citrate, which is consistent with the values reported previously for SM (Holt, 1985). On the addition of 18% HA particles to SM, the calcium concentration in the SM decreased by ~93%, from 28.5 to 2.2 mmol/kg, the inorganic phosphate concentration decreased by ~68%, from 21.6 to 6.8 mmol/kg, and the citrate concentration decreased by ~74%, from 11 to 2.85 mmol/kg. Part of the decrease was probably because the calcium, phosphate, and citrate that were contained in the casein micelles before they dissociated and adsorbed on the HA particles sedimented out with the HA particles during centrifugation. However, CCP contains only ~50% of the total inorganic phosphate, ~70% of the total calcium, and ~20% of the total citrate contained in milk (Gaucheron, 2005). As the percentage decreases in calcium, phosphate, and citrate on the addition of 18% HA particles to SM were higher than the

percentage of the corresponding mineral in CCP, calcium, phosphate, and citrate ions must have bound to HA particles, depleting the milk serum in these ions.

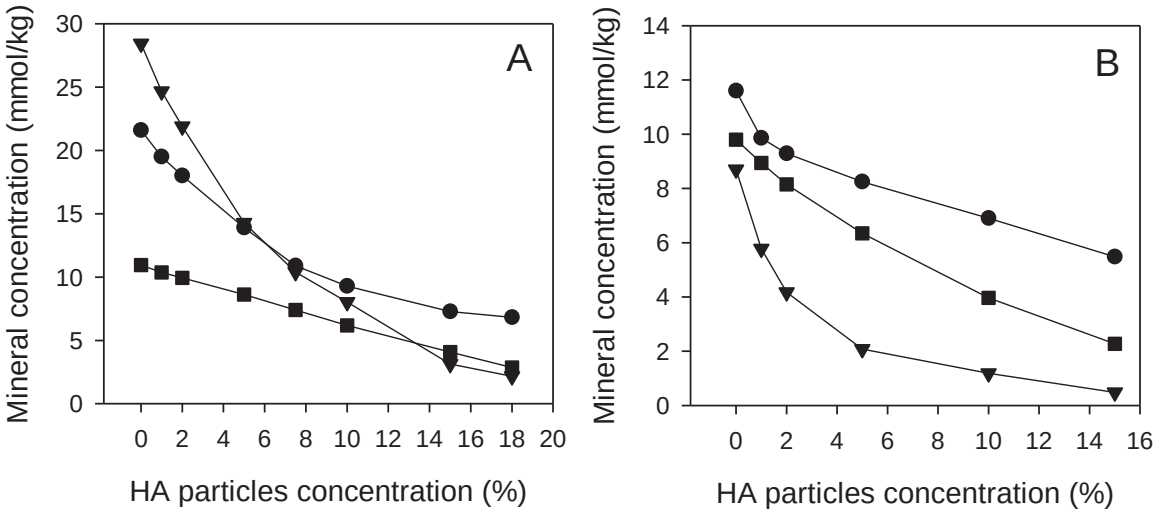

Figure 7.15: Effect of addition of HA particles in (A) SM and (B) SMUF on (▼) total calcium, (■) total citrate, and (●) inorganic phosphate concentrations. HA particles were removed from the solution by centrifugation before the measurements.

This was confirmed by the mineral analysis results when HA particles were added to SMUF (Figure 7.15B). On the addition of 15% HA particles to SMUF, the total calcium concentration decreased by ~95%, from 8.7 to 0.5 mmol/kg, the inorganic phosphate concentration decreased by ~53%, from 11.6 to 5.5 mmol/kg, and the citrate concentration decreased by ~77%, from 9.8 to 2.3 mmol/kg. The decrease in calcium was faster and more pronounced than the decreases in phosphate and citrate, probably because phosphate and citrate have to compete for the same binding sites on the HA surface (positive sites, C-sites), whereas only calcium ions can bind to the negative sites (P-sites), and maybe also because the number of P-sites available for calcium adsorption is higher than that of C-sites for phosphate and citrate adsorption at natural milk pH, since it has been shown that more P-sites than C-sites are expressed on the surface of HA at pH ~6.8 (Kandori et al., 2005, 2009).

The disruption effect of HA on the integrity of the casein micelle and on the milk mineral equilibrium can also be explained by taking into account the thermodynamic aspects of calcium phosphate growth and sequestration. In milk, calcium and phosphate are

sequestered in the form of calcium phosphate nanoclusters inside the casein micelle (Little & Holt, 2004). The phosphoserine groups of the caseins are bound to the calcium phosphate nanoclusters inside the micelles, keeping the calcium phosphate in a thermodynamically metastable state in which its precipitation and further growth are prevented (Holt, Wahlgren, & Drakenberg, 1996a; Holt et al., 1998). However, when HA particles are added to milk, it is possible that the most stable form of calcium phosphate (HA) grows at the expense of the least stable form (calcium phosphate sequestered in the casein micelles) (Holt, Lenton, Nylander, Sørensen, & Teixeira, 2014). The calcium phosphate nanoclusters sequestered inside the casein micelle can therefore be seen as a reservoir of calcium and phosphate ions that contributes to the crystal growth of HA. As the integrity of the casein micelle structure is maintained by the calcium phosphate nanoclusters, the release of the ions from the nanoclusters must disrupt the structure of the casein micelles, leading to their dissociation (De Kruif et al., 2012; Holt et al., 2013). The process of calcium phosphate growth is generally very slow. However, the HA particles used in this study may have provided a very large surface of initially crystallised precursor HA that would allow a fast crystallisation of calcium phosphate from the minerals that were drawn out of the milk serum and of CCP onto the HA particles (Nancollas & Tomazic, 1974). The phenomena observed in this study might almost be seen as a Ostwald ripening-like phenomena, with the large HA particles growing further by adsorbing the small calcium phosphate crystals sequestered in the micelles (Cazalbou, Combes, Eichert, & Rey, 2004; Nonoyama et al., 2011).

7.4.3.3 Kinetics of casein micelle dissociation by HA particles

In Section 7.4.3.1, SM samples were dialysed against SMUF containing 10% (w/w) HA particles. This HA concentration was purposely chosen to be high, as the aim was to maximise the HA to protein ratio to check whether the presence of HA particles caused the casein micelles in the dialysis bag to dissociate. However, if as hypothesised, the HA particles outside the dialysis bag are able to draw the minerals out of the milk serum and grow at the expense of the calcium phosphate of milk, smaller concentrations of HA particles in SMUF should lead to the same effect of casein micelle dissociation, but it might take longer for the dissociation to happen. To check this hypothesis, 10%-SM was dialysed against four different concentrations of HA particles in SMUF (0, 0.1, 0.5, and 5%, w/w). The dissociation state of the caseins was determined for each concentration after days 1, 2, 3 and 7, by MF-electrophoresis.

Figure 7.16 shows the MF-electrophoresis computer-generated gels of the dialysed milks and their respective serum phase (obtained after centrifugation at 14,000 × g for an hour and containing the non-micellar fraction of the caseins) for each dialysis time and each HA concentration. The percentages of non-micellar caseins contained in the dialysed SM, calculated from the MF-electrophoresis data, are reported in Table 7.5. At each time point, it was observed that the higher the concentration of HA particles, the greater degree of casein micelle dissociation, as shown by the increasing intensities of the casein bands in the milk serums (vertical comparison between samples in lines A, B, C and D of the same columns in Figure 7.16). For example, at Day 1, SM dialysed against SMUF with no HA particles (control) only contained 2% of non-micellar caseins, whereas SM dialysed against 0.1, 0.5 and 5% (w/w) HA particles respectively contained 5, 30 and 97% of non-micellar caseins (Table 7.5). In addition, at each concentration of HA particles, there was an increase in casein micelle dissociation with time (horizontal comparison of the band intensities of the serum phase of samples in columns 1, 2, 3 and 4 of the same lines in Figure 7.16), until the casein micelles were fully dissociated. For example, SM dialysed against 0.5% (w/w) HA particles contained respectively 30, 87 and 99% dissociated caseins after Day 1, 2 and 3 (Table 7.5). Even the control sample of SM dialysed against SMUF showed an increase in casein micelle dissociation overtime, from 2% at Day 1 to 27% at Day 7, but to a much lesser extent than the samples dialysed against HA particles. It was attributed to some dissociation of the casein micelles due to plasmin activity in the milks left at room temperature for a few days, since bands corresponding to γ-casein and proteose-peptones appeared under the α-lactalbumin band for γ-casein and above the β-lactoglobulin band for proteose-peptones in the gels at Day 3 and 7 (very visible for samples A3, A4, B3 and B4 in Figure 7.16), and γ-casein and proteose-peptones are known to be hydrolysis products of β-CN by plasmin enzymes (Andrews & Alichanidis, 1983; Anema, 2009a). These dialysis results show that the dissociation of casein micelles caused by HA particles is concentration-dependent and time-dependent. Higher concentrations of HA particles offer more initial available HA surface for the milk serum ions to adsorb, which probably allows a faster depletion of the milk serum in minerals, and therefore a faster dissociation of the casein micelles. However, even low concentrations of HA particles seem to cause the casein micelles to dissociate overtime, probably by progressive growth of the initial HA particles at the expense of the micellar calcium phosphate. In theory, it looks like only a very small initial amount of added HA particles could be enough to form an initial crystallised calcium phosphate seed precursor, from which calcium phosphate would grow further at the solid/liquid interface by progressive withdrawing of the minerals from the milk serum and from CCP (Nancollas & Tomazic, 1974; Nancollas, Amjad, & Koutsoukos, 1979).

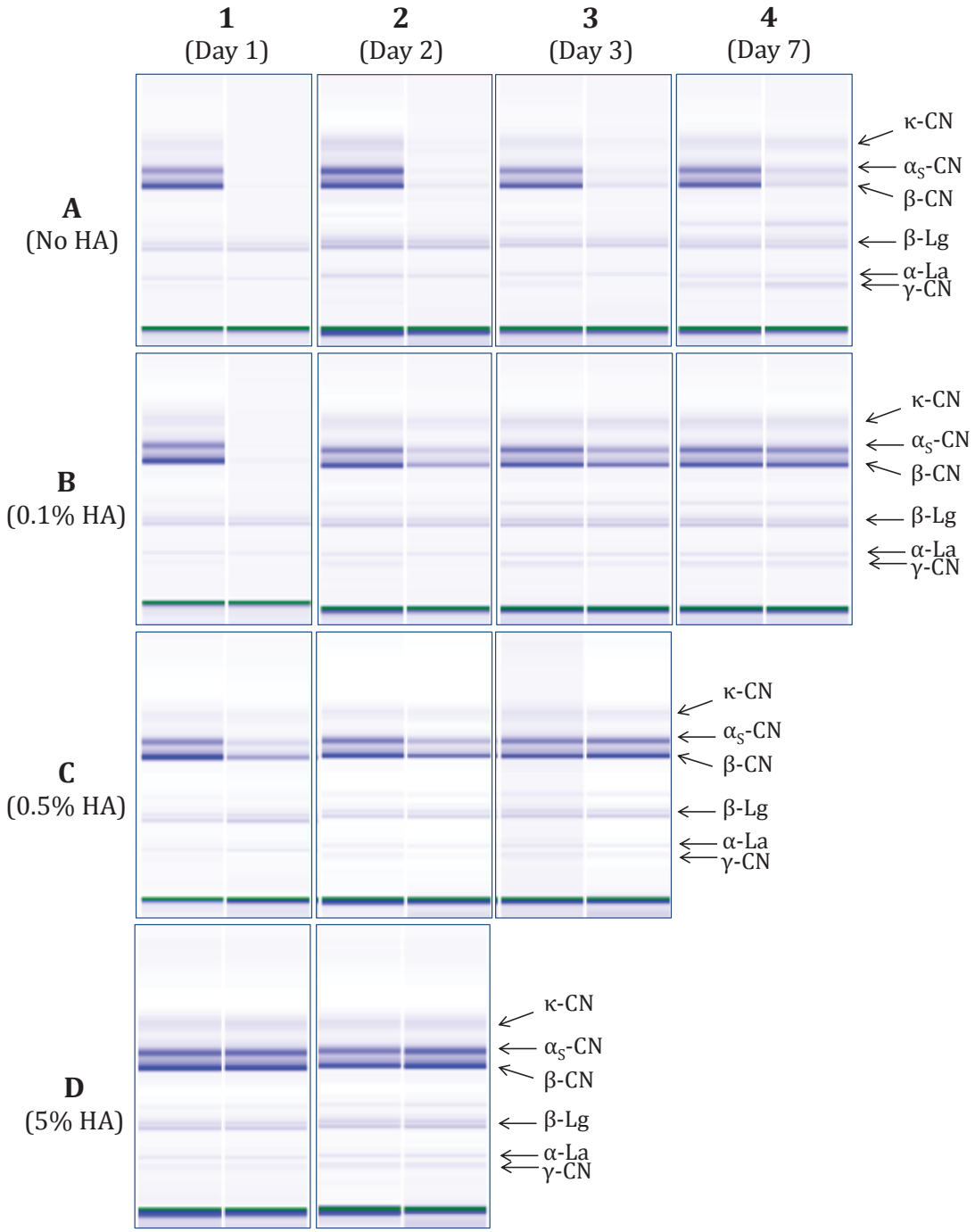

Figure 7.16: MF-gel electrophoresis showing the dialysed SM samples and their respective serum phase (i.e., containing the dissociated casein micelles), for SM samples dialysed against different concentrations of HA particles (0, 0.1, 0.5 and 5%, w/w, lanes A to D) and for different dialysis times (1, 2, 3 and 7 days, columns 1 to 4). For each pair of lanes, the left sample is the dialysed SM and the right sample is the respective serum phase. Abbreviations are: CN, casein; Lg, lactoglobulin; La, lactalbumin.

In the dialysis experiment, the caseins were separated from the HA particles. However, when HA particles are added in milk, the presence of caseins will probably inhibit the growth of the HA particles. The inhibitory action of caseins on HA crystal growth was previously demonstrated by Van Kemenade and de Bruyn (1989), and attributed to the strong binding of the phosphoserine groups of α_{S1}-casein and β-casein to the growing calcium phosphate phase. Caseins are also known to inhibit calcium phosphate dissolution in tooth enamel, because their strong adsorption onto HA stabilise the HA crystal surface (Barbour et al., 2008; Reynolds, 1987; Weiss & Bibby, 1966). Other phosphorylated calcium-binding proteins such as osteonectin and osteopontin have also been shown to bind onto HA in the bone matrix and inhibit its crystal growth, because the adsorbed phosphopeptides of the proteins block the growth sites (George & Veis, 2008; Holt et al., 2014; Hunter, 2013).

Table 7.5: Percentage of non-micellar caseins in SM, after dialysis of SM against SMUF containing different concentrations of HA particles, for different times.

HA concentration (%)	Non-micellar caseins (%)			
	Day 1	Day 2	Day 3	Day 7
0	2	7	13	27
0.1	5	41	66	75
0.5	30	87	99	-
5	97	98	-	-

7.5 Conclusions

In conclusion, the results in this chapter showed the existence of interactions between casein micelles and HA particles, as summarised in Figure 7.17. The addition of HA particles to SM causes the dissociation of the casein micelles and the binding of the individual caseins onto HA. This dissociation of the casein micelles is a consequence of the milk serum ions (calcium, phosphate and citrate at least) binding onto the surface of the HA particles, and contributing to the further growth of the HA particles. Consequently, the CCP in the casein micelles is drawn out of the micelles and the structure of the casein micelles is disrupted. Therefore, the phosphoserine groups of α_S- and β-caseins become available to bind to HA

particles. Both α_S- and β-caseins preferentially bind onto HA compared with κ-casein, because of their higher content in phosphoserine residues.

The results in this Chapter show that the addition of HA particles in milk may compromise the stability of the milk by causing the adsorption of a fraction of the milk proteins onto the HA particles, by disrupting the milk mineral equilibrium and by causing the dissociating of some of the casein micelles. The implications of these findings for the stability of calcium-fortified milks are detailed in the next chapter of this thesis (Chapter 8).

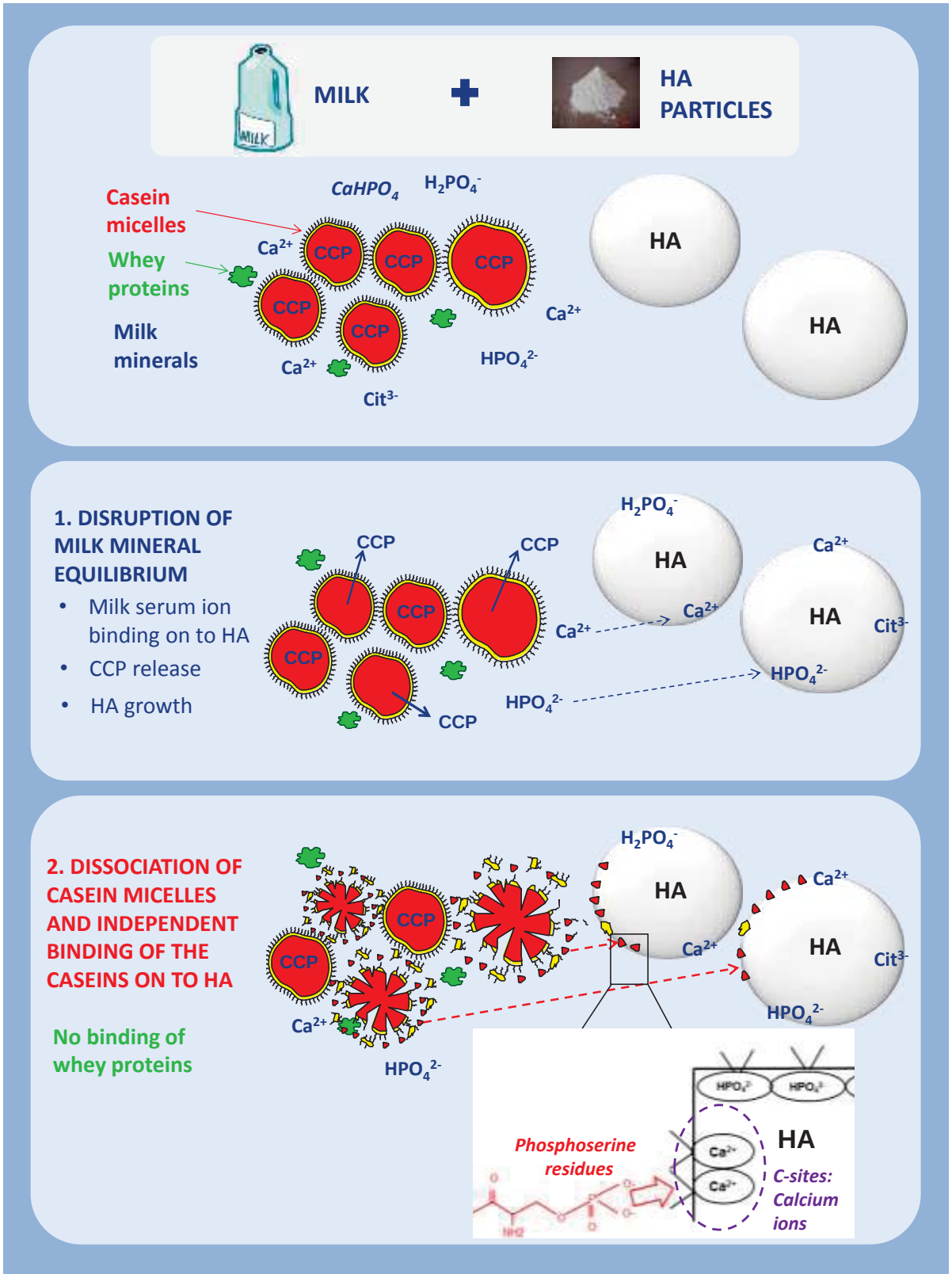

Figure 7.17: Proposed mechanism explaining the dissociation of casein micelles upon addition of HA particles in milk and the subsequent binding of caseins to the particle

CHAPTER 8 Overall summary and recommendations

Whilst calcium fortification of milk products is extremely beneficial for consumer health, particularly in terms of bone health, the ability to do this successfully is limited by a number of technical issues. In ultra-high temperature treated (UHT) milks in particular, the application of heat treatment exacerbates these issues. Soluble calcium salts disrupt the milk mineral equilibrium, shielding the charge of the casein micelles in milk and reducing the electrostatic repulsion between the micelles, leading to their aggregation upon heat treatment. Because of this, insoluble calcium salts are usually used for commercial heat-treated calcium-fortified milks, since they do not dissolve and are regarded as chemically unreactive, and therefore do not cause heat instability issues. A hydrocolloid or stabiliser is then required to keep these insoluble calcium salts in suspension during shelf-life, and it is also recommended that the calcium salts have a small particle size. However, even with the use of stabilisers, shelf-life instability phenomena such as phase separation, gelation and sedimentation are often observed with insoluble calcium salts.

The context for this thesis was therefore to develop an understanding of the challenges faced in the calcium supplementation of milk. In this regard, hydroxyapatite (HA) was chosen as the model insoluble calcium salt since it is often used in the calcium fortification of heat-treated milks. However, the findings of this thesis on HA may also be applied to other insoluble calcium salts, such as calcium carbonate or calcium citrate, since they present similar characteristics.

HA particles (and other particles of insoluble calcium salts) are considered to be chemically unreactive when added to milk at neutral pH, since they do not release ionic calcium in the milk, and therefore do not cause casein micelle aggregation and heat instability when the milk is heated. However, HA particles are surface active and carry charged surface sites that can potentially be adsorbing sites for many charged molecules, including proteins. The ability of proteins to bind onto HA particles is well known and used in biomaterial applications. Surprisingly, however, despite their industrial relevance, the adsorption interactions between milk proteins and HA have never been studied properly in food systems.

The objectives of this thesis were, therefore, to characterise the adsorption of milk proteins onto HA using both model systems and milk, and to study the consequences of the adsorption on the colloidal stability of the particles and the stability of the milk protein fraction. The key findings of this research are highlighted and discussed in this chapter, and recommendations for further research are made.

8.1 Key summary points

In this thesis, the adsorption of milk proteins onto HA particles was studied using:

(i) pure protein solutions of the five main milk proteins, β-casein, α_S-casein (this term being used to cover the combination of α_{S1}-casein and α_{S2}-casein), κ-casein, β-lactoglobulin and α-lactalbumin (Chapter 5);
(ii) commercial milk protein ingredients containing a blend of caseins or whey proteins, i.e., sodium caseinate (SC) and whey protein isolate (WPI) (Chapters 4 and 6); and
(iii) whey-protein depleted skim milk (WDP-SM) and skim milk (SM) (Chapter 7).

In addition, the adsorption of the milk proteins was studied under different physico-chemical conditions of pH (Chapter 6), ionic strength (Chapters 5 and 6) and mineral composition (Chapter 6, using simulated milk ultrafiltrate, SMUF, to mimic the composition of the milk serum and SMUF containing added citrate). This provided the basis from which to ultimately understand how the compositional characteristics of the milk (pH of ~ 6.8, ionic strength of ~ 0.1 M and presence of milk serum ions) affect the adsorption of the different milk proteins, when HA particles are added (Chapter 7).

- **The adsorption of milk proteins onto HA is of a competitive nature between the different milk proteins and varies depending on the physico-chemical characteristics of the solution, i.e., pH, ionic strength and mineral composition**

Individually, α_S- and β-casein have higher affinities for HA than κ-casein, β-lactoglobulin or α-lactalbumin and both adsorb to higher maximum levels.

When studied individually, β-casein, α_S-casein, κ-casein β-lactoglobulin and α-lactalbumin were all able to bind onto HA particles, but the amount of proteins that could adsorb and the affinity for HA were different for each protein (Chapter 6). In HEPES buffer, pH 6.8, at the

two levels of ionic strength studied (7 mM and 100 mM), α_S- and β-casein had higher affinities for HA and were able to adsorb to higher maximum levels (of ~ 3 mg/m^2) than κ-casein, β-lactoglobulin or α-lactalbumin (maximum adsorption values of only ~ 1.5 mg/m^2).

The stronger binding of α_S- and β-casein to HA is due to phosphoserine clusters; self-association probably adds to the level of maximum binding.

The higher affinities of α_S- and β-casein for HA and their higher surface loads were attributed to a stronger binding of α_S- and β-casein onto HA, due to the presence in their sequences of clusters of phosphoserine residues that formed many anchor points capable of binding very strongly onto the HA calcium sites (C-sites) (Gorbunoff & Timasheff, 1984a; Johnsson et al., 1993). Comparatively, the binding of κ-casein, β-lactoglobulin and α-lactalbumin probably occurred only through their side carboxyl groups binding onto the HA C-sites, which was believed to explain their lower affinities and their lower surface loads (Kawasaki, 1991). It was also suggested that α_S- and β-casein may have been able to self-associate or associate together in the adsorbed layer, therefore forming thicker layers of small aggregates on the surface of HA particles, which could also explain their higher surface loads, compared with the other proteins.

In mixtures of the milk proteins, κ-casein, β-lactoglobulin and α-lactalbumin only bind to HA at significant levels when all the α_S-casein and β-casein in the mix has been adsorbed.

A preferential adsorption of α_S- and β-casein over the other milk proteins (κ-casein, β-lactoglobulin and α-lactalbumin) was observed in all the systems containing a blend of the different proteins studied in this thesis, regardless of the initial protein source (pure protein solutions, SC, WPI or milk) and of whether the caseins were in a soluble form (in pure protein solutions or SC) or a micellar form (in milk). When the different milk proteins were mixed together, α_S-casein and β-casein adsorbed almost exclusively onto HA. It was only when there was no more α_S-casein and β-casein available for adsorption that the other proteins could bind in significant amounts, in the preferential order κ-casein > β-lactoglobulin > α-lactalbumin. This could be explained by the stronger nature of the binding of α_S- and β-casein compared with that of κ-casein, β-lactoglobulin and α-lactalbumin.

Decreased pH and increased ionic strength lead to an increase in the amount of adsorption of milk proteins onto HA due to a decrease in the extent of electrostatic repulsions.

Both pH and ionic strength were shown to have an effect on the maximum amounts of proteins that could adsorb onto HA particles. In general, when the absolute value of the zeta-potential decreased because of a decrease in pH or an increase in ionic strength, more

protein was found to adsorb onto the HA particles. This was attributed to a decrease in the electrostatic repulsions between the HA particles and the protein species, which favoured protein adsorption. With ionic strength increasing or pH decreasing, the electrostatic repulsions within the protein molecules would also have decreased, causing the protein to form a denser layer around the HA particles, and also contributing to a higher surface load.

Milk serum ions can specifically bind onto HA particles and therefore compete for adsorption with milk proteins

It was also shown that milk serum ions could specifically bind onto the HA particles, therefore affecting the binding of the milk proteins onto HA. It was suggested that a competitive adsorption existed between milk serum ions and milk proteins. For example, caseins and whey proteins adsorbed onto HA in lower maximum amounts in SMUF than in water (Chapter 6), but the caseins were less affected (smaller decrease), probably because they could compete better than whey proteins with the milk serum ions for adsorption (Chapter 6), due to their phosphorylated nature and stronger binding. Also, when citrate was added in SMUF, the maximum amount of caseins that could bind onto HA decreased as the citrate concentration increased, which confirmed that citrate ions competed with the caseins for adsorption. Binding of milk serum ions onto HA was also observed when HA particles were added to milk in Chapter 7. Calcium, phosphate and citrate ions from the milk all adsorbed onto HA particles, depleting the milk serum in ions.

It is individual casein molecules or small casein aggregates that bind onto the HA particles, not intact micelles

As the milk mineral equilibrium was disrupted, CCP was released from the casein micelles leading to dissociation of the casein micelles. Therefore, the casein micelles were considered not to bind as intact micelles onto the HA particles but as individual molecules or small aggregates. This hypothesis was consistent with the fact that the caseins adsorbed to the same maximum amounts regardless of the state of dissociation of the casein micelles. Whether the initial solution contained caseins from SC caseinate in SMUF (Chapter 6), partly dissociated casein micelles (in EDTA-added milk in Chapter 7) or intact casein micelles (in WDP-SM and SM), the maximum amount of adsorbed caseins was usually of ~ 3 mg/m^2, with the adsorbed layer made up mostly of α_S- and β-casein, because of their stronger affinities for HA compared with κ-casein.

- **The adsorption of milk proteins onto HA particles affects the colloidal properties of the HA particles in suspension**

The results described in the various chapters on the suspension stability of HA particles showed that when HA particles were added to solutions containing milk proteins, the zeta-potential of the HA particles depended on both the physico-characteristics of the suspending media (pH, ionic strength and mineral composition) and on the amount of protein adsorbed onto the HA surface.

The adsorption of both caseins and whey proteins to HA particles results in the particles becoming negatively charged, thus improving suspension stability; casein adsorption also provides steric stabilisation.

The adsorption of both caseins and whey proteins caused a decrease in the magnitude of the zeta-potential of the HA particles in water (Chapter 4). The surface of the particles became negatively charged, and their suspension stability was improved. It is believed that the whey proteins provide electrostatic stabilisation to the HA particles by charging the surface of the particles negatively, therefore increasing the repulsion between the particles, and slowing down their aggregation and sedimentation rates. Caseins, however, are considered to provide an electrosteric (electrostatic and steric) stabilisation to the particles by charging the HA surface negatively, and forming a hairy layer on the surface of the particles, thus preventing their aggregation and sedimentation. The different nature of the stabilisation between caseins and whey proteins (charge stabilisation only for whey proteins, charge plus steric stabilisation for caseins) was confirmed by the fact that the stabilisation provided by the whey proteins was not sustained when the magnitude of the zeta-potential of the whey protein-coated particles was lower than –20 mV (for example at salt concentrations ≥0.05 M), whereas the stabilisation provided by the caseins was maintained regardless of the magnitude of the zeta-potential of the casein-coated particles. For example, in salt concentrations ≥0.1 or in SMUF, despite the fact that the magnitudes of the zeta-potential of the casein-coated particles were ≤–16 mV, the suspension stability of the HA particles was maintained.

A hypothesis was generated that milk protein-coated nano-sized HA particles could be small enough to eliminate the need for additional stabilisers in milk, but it proved too difficult to make such particles at bench-scale and therefore the hypothesis could not be confirmed.

At the level of addition of HA particles to milk commercially, the ratio of HA particles to casein is in most cases sufficiently low to allow full coverage of the HA particles by the

caseins. The adsorbed caseins will therefore provide steric stabilisation against particle aggregation, so the particles will not aggregate and sediment as quickly as when suspended in water. However, as the diameter of the particles is of a few microns, despite the casein layer providing partial stabilisation to the particles against aggregation, the particles only remained suspended for a short period in milk. Over the product shelf-life, which can be anywhere up to six to nine months for UHT milk, in the absence of another type of stabilisation, the HA particles added into milk will end up sedimenting to the bottom. Therefore, hydrocolloids, such as carrageenan, microcrystalline cellulose or gellan gum, usually need to be added to commercial calcium-fortified milks to provide extra stabilisation. Hydrocolloids form weak gel networks with the milk proteins, entrapping the insoluble HA particles and keeping them in suspension over shelf-life (Blakemore & Harpell, 2010; Tuason et al., 2009).

One theory resulting from this work was that it may be possible to produce nano-sized HA particles that are surface-functionalised by a layer of adsorbed proteins. If the HA particles had an initial small size and were coated and stabilised by caseins or whey proteins, they could potentially form stable nano-suspensions that would not aggregate and sediment overtime. Such nano-particles could potentially be added to calcium-fortified product, without the need of adding hydrocolloids. This hypothesis was briefly tested, and the preliminary results are reported in Appendix 1, where two different methods were used to try to produce milk protein coated-nanosized HA. Some particles were obtained (probably not HA particles, but rather some particles of amorphous calcium phosphate), but their size was in the micrometric range, so even though their surface was charged negatively (due to the adsorption of the milk proteins on their surface), the particles were too big to form a stable suspension. This suggested that the milk proteins were not able to bind onto the calcium particles when initially formed, and therefore were not able to prevent the further growth and aggregation of the particles. It was believed that the pH at which the particles were initially formed (pH 5) was too close to the isoelectric point of the milk proteins, so the phosphoserine and carboxyl groups of the milk proteins were not charged enough to bind onto the particles. Even though this technique did not produce stable suspensions of protein-coated HA particles, it may be worth investigating further whether it is possible to make food-grade surface-functionalised HA particles. Many methods have been reported in the literature for the making of stable suspensions of nano-sized HA particles, but they usually use non-food grade polymers to bind onto the HA surface (Borum-Nicholas et al, 2003; Yu et al, 2007). However, many food-grade polymers have been shown to adsorb onto HA particles, e.g., xanthan gum and carboxymethylcellulose (Barbour et al., 2005), bovine

serum albumin (BSA) (Brandes et al., 2006), gelatin (Chang et al. 2003), chitosan (Wilson & Hull, 2008) or gum arabic (Roque & Wilson, 2008) . These polymers may function better than caseins and whey proteins for surface modification or encapsulation of small HA particles, and may have the potential to produce novel calcium fortifiers.

- **The interactions between casein micelles and HA particles in milk may have consequences on the stability of calcium-fortified milks**

At the normal levels of fortification with HA particles, casein micelle dissociation should not be an issue, but at higher concentrations of HA particles, micellar disruption will destabilise the milk.

It was shown in Chapter 7 that the addition of HA particles to milk disrupted the milk mineral equilibrium, causing the dissociation and adsorption onto HA of a fraction of the milk casein micelles. The consequences of these phenomena on the stability of the milk will most likely depend on the extent of disruption of milk mineral equilibrium and of the casein micelle adsorption and dissociation, and this will be determined by the ratio of protein to HA particles in the milk. In Chapter 7, HA particles were added at a range of concentrations in milk, from 0.1 to 20% (w/w). However, the normal concentration of HA particles added to high-calcium commercial milks can be up to approximately 1% (w/w). It has been estimated that upon addition of 1% (w/w) HA particles to milk, the ionic calcium concentration of the milk decreases by ~ 20%, and approximately 10% of the initial casein will adsorb onto the HA particles, and only a negligible fraction of the unadsorbed casein micelles will be dissociated. At this level, these changes are not expected to compromise significantly the stability and sensory properties of the milk. A lower concentration of ionic calcium in milk containing HA particles will not affect the heat stability of the milk, as it is usually an increase in ionic calcium that causes the protein in milk to aggregate upon heat treatment (Philippe et al., 2003; Sievanen et al., 2008). As HA particles act as calcium chelators, the addition of HA particles to milk may even improve the heat stability of the milk (de Kort et al., 2012; Kaliappan & Lucey, 2011).

The ratio of HA particles to milk protein has to be taken into account when formulating high-calcium milks. Using much higher concentrations of HA particles or increasing the ratio of HA particles to protein (for example in milk-based beverages usually formulated with lower protein contents than milk) would also increase the extent of casein micelle

adsorption and dissociation, possibly to levels that may affect the stability or sensory characteristics of the products.

Particle size, surface area, crystallinity and nature of the calcium phosphate phase need to be carefully considered when choosing calcium fortificants.

In the general context of calcium fortification, the formulation of calcium-fortified milks using particles of HA (or other insoluble calcium salts) should always be approached with awareness of the adsorption phenomena demonstrated in this thesis. For example, the characteristics of the particles such as particle size, surface area, crystallinity and nature of the calcium phosphate phase should be considered carefully in the choice of calcium ingredients. It is usually believed that smaller particles would be preferable for use in calcium-fortified milks, since their suspension stability is better and they are less detectable in the mouth (Augustin & Williams, 2002). Different processing methods used to manufacture HA particles can give HA powders with very different surface areas, ranging from a few square metres per gram of powder to about 100 mg/m^2 (Budenheim, personal communication). If HA particles of smaller size or higher surface area than those used in this study were added to calcium-fortified milks, the adsorption of milk ions and milk proteins onto the particles could reach levels that may have consequences for the stability of the products. For example, phase separation may occur in the milk, resulting in a clear milk phase depleted in casein micelles and minerals, with the casein-covered HA particles sedimenting to the bottom over time. If the extent of casein micelle dissociation due to HA addition becomes important, the presence of soluble caseins in the serum phase of the milk may enhance age gelation over shelf-life (Datta & Deeth, 2001).

8.2 Recommendations for future work

The results of this thesis provide a good step forward in understanding the interactions that occur in calcium-fortified milks. The adsorption phenomena occurring between the milk proteins and HA particles were characterised in different systems, and the consequences on the stability of the milk were highlighted. Even though they are often considered chemically inert when added to milk, HA particles were shown to interact with the milk protein fraction, which could potentially compromise the stability of the milk during processing and shelf-life. The interactions and adsorption phenomena described in this thesis were only characterised at room temperature, for only one type of HA ingredient, and for reaction times of only a few hours. This study could be extended to a wider range of commercial

insoluble calcium salts and fortification levels, and to a wider range of reaction times and temperatures as detailed below.

- **Adsorption studies with other calcium salts and further characterisation of the adsorption mechanisms between milk proteins and calcium salts**

HA ingredients with different characteristics, such as different particle sizes, different surface areas or densities, or different degrees of crystallinity could be used for adsorption studies to determine whether these factors affect the rate and extent of the adsorption reactions. Other calcium salts of a different chemical nature, such as calcium carbonate and calcium citrate, could be used to determine whether similar adsorption phenomena would occur. It should be noted that many food-grade insoluble calcium salts are not characterised fully by the suppliers. Their nomenclature for example, is often incorrect, with the commonly tricalcium phosphate (TCP) being in fact hydroxyapatite. Therefore, if the chemical nature, or the characteristics of surface area or crystallinity are not provided by the supplier, additional testing should be carried out on insoluble calcium salts. For example, testing methods, such as Fourier-Transform Infra-Red Spectrometry, X-ray diffraction analysis and determination of the specific surface area by the Brunauer–Emmett–Teller (BET) method, if available, should be carried out prior to the use of insoluble calcium salts in commercial products. The adsorption of other minor milk proteins such as lactoferrin and bovine serum albumin could also be studied, for example. As lactoferrin and bovine serum albumin are neutral proteins (pI > 7), they are likely to adsorb onto HA particles following a different mechanism and affecting the colloidal properties of HA particles differently, compared to the proteins studied in this thesis (Zhu et al., 2007; Iafisco et al., 2011).

- **Development of surface-functionalised food-grade HA particles**

The development of a method to produce nano-sized HA particles stabilised by a layer of adsorbed polymer should be investigated further. It should be possible to produce stable suspensions of nano-sized hydroxyapatite, using food-grade polymers such as proteins or hydrocolloids as adsorbants. This research path may have potential to produce novel calcium fortifiers, as discussed previously.

- **Effect of processing and shelf-life on the adsorption phenomena**

Since calcium-fortified milks are heat-treated at high temperatures, it would also be interesting to characterise the adsorption phenomena and the disruption of the milk mineral equilibrium after heat treatment, and compare them with the results obtained in this thesis carried out at room temperature. However, it is believed that heat treatment should not have a major effect on the maximum coverage values or affinity of the milk proteins for HA (Goobes et al., 2007), since the adsorption mechanisms are mostly driven by electrostatic interactions and are therefore not temperature-dependent.

Since calcium-fortified milks can have a shelf-life up to 9 months, the adsorption phenomena should also be followed during shelf-life. For example, the amount of adsorbed protein and adsorbed minerals, the ionic calcium content of the milk and the fraction of soluble casein in the milk serum could be determined at different time intervals, as it is possible that the growth of HA particles may continue during shelf-life, as further binding of the milk serum ions onto HA particles could theoretically occur over time. However, results for other protein sources in the literature showed that proteins can inhibit the growth of HA, by binding onto the adsorption sites, rendering them inaccessible for the calcium and phosphate ions (Romberg et al., 1986; Van Kemenade & de Bruyn, 1989). Therefore it is believed that when HA particles are added to milk, the binding of the caseins would probably inhibit HA crystal growth, and prevent further binding of the milk serum ions onto HA. In this case, only a limited amount of the milk ions would be withdrawn from the milk serum and adsorb onto the HA particles in the first hours of contact between the particles and the milk, and therefore, the adsorption of the milk serum ions and the dissociation of the casein micelles may not continue further over-shelf life. However, this hypothesis has not been verified in this thesis and further research is required to confirm whether or not HA growth continues during shelf-life, as it could potentially deplete the milk further in caseins and milk ions, causing dissociation of a high proportion of the casein micelles and affecting the product stability over shelf-life.

- **Identification of stability boundaries in calcium-fortfied milks**

The results of this thesis showed that the extent of the adsorption reactions was dependent on the HA surface to protein ratios in the product. Further experiments using different protein to HA surface ratios would extend the knowledge of these ratio-dependent phenomena, potentially enabling the determination of regions of stability and instability

when HA particles are added to products. The quantification of maximum levels of fortification, above which the adsorption phenomena start to have some consequences on the milk stability, could be extremely useful for the formulation of calcium-fortified milks.

- **Adsorption studies in real food systems, for example in the presence of other ingredients**

Interactions of HA particles and proteins with other ingredients should also be considered in systems more typical of commercial products. Calcium-fortified milks not only contain milk proteins and HA particles, but also a broader range of ingredients, including fat, stabilisers and flavours. These ingredients may affect the interactions between milk proteins and HA particles. The presence of fat in the product could possibly lead to a three-way interaction between fat droplets, milk proteins and HA particles, for example if the casein-covered HA particles interact with the fat droplets through their protein layers and adsorb on their surface. Also, the addition of various stabilisers, such as carrageenans, microcrystalline cellulose or gellan gums may affect the adsorption interactions between protein and HA particles, for example, by competing with the proteins for adsorption onto HA, or by competing with HA for interaction with the proteins.

Finally, further studies combining the compositional factors (type of calcium salts, level of fortification, and presence of other ingredients) with the processing variables would yield useful information for the development of calcium-fortified milks with optimal stability over shelf-life.

References

Adamson, N., Riley, P. F., & Reynolds, E. C. (1993). The analysis of multiple phosphoseryl-containing casein peptides using capillary zone electrophoresis. *Journal of Chromatography A, 646*, 391-396.

Addeo, F., Chobert, J.-M., & Ribadeau-Dumas, B. (1977). Fractionation of whole casein on hydroxyapatite. Application to a study of buffalo κ-casein. *Journal of Dairy Research, 44*, 63-68.

Andersen, D. L., Keller, D. J., & Streiff, P. J. (1989). *Aseptically packaged calcium enriched milk*. US Patent 4851243 A

Andrews, A. T., & Alichanidis, E. (1983). Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal of Dairy Research, 50*, 275-290.

Anema, S. G. (2009a). The use of "lab-on-a-chip" microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. *International Dairy Journal, 19*, 198-204.

Anema, S. G. (2009b). Stability of milk-derived calcium phosphate suspensions. *Dairy Science and Technology, 89*, 269-282.

Anema, S. G., & Klostermeyer, H. (1996). [zeta]-Potentials of casein micelles from reconstituted skim milk heated at 120 °C. *International Dairy Journal, 6*, 673-687.

AOAC. (1984). *Official methods of analysis*. Washington DC, USA: Association of Official and Analytical Chemists.

Augustin, M. A., & Williams, R. P. W. (2002). Technological aspects of calcium fortification of milk and dairy products. *Food Australia, 54*, 131-133.

Bankston, T. E., Dattolo, L., & Carta, G. (2010). pH Transients in hydroxyapatite chromatography columns—Experimental evidence and phenomenological modeling. *Journal of Chromatography A, 1217*, 2123-2131.

Barbour, M. E., Shellis, R. P., Parker, D. M., Allen, G. C., & Addy, M. (2005). An investigation of some food-approved polymers as agents to inhibit hydroxyapatite dissolution. *European Journal of Oral Sciences, 113*, 457-461.

Barbour, M. E., Shellis, R. P., Parker, D. M., Allen, G. C., & Addy, M. (2008). Inhibition of hydroxyapatite dissolution by whole casein: the effects of pH, protein concentration, calcium, and ionic strength. *European Journal of Oral Sciences, 116*, 473-478.

Barroug, A., Lemaitre, A., & Rouxhet, P. (1989). Lysozyme on apatites: a model of protein adsorption controlled by electrostatic interactions. *Colloids and Surfaces, 37*, 339-355.

Baum, F., Ebner, J., & Pischetsrieder, M. (2013). Identification of multiphosphorylated peptides in milk. *Journal of Agricultural and Food Chemistry, 61*, 9110-9117.

Baumy, J. J., & Brule, G. (1988a). Effect of pH and ionic strength on the binding of bivalent cations to β-casein. *Lait, 68*, 409-417.

Baumy, J. J., & Brule, G. (1988b). Binding of bivalent cations to α-lactalbumin and β-lactoglobulin: effect of pH and ionic strength. *Lait, 68*, 33-48.

Baumy, J. J., Guenot, P., Sinbandhit, S., & Brulé, G. (1989). Study of calcium binding to phosphoserine residues of β-casein and its phosphopeptide (1–25) by 31P NMR. *Journal of Dairy Research, 56*, 403-409.

Bell, A. E. (1993). Rheology of liquids. In R. Macrae, R. K. Robinson, & M. J. Sadler (Eds.) *Encyclopaedia of food science, food technology and nutrition* (pp. 4979-4982). London, UK: Academic Press.

Bell, L., Posner, A., & Quirk, J. (1972). Surface charge characteristics of hydroxyapatite and fluorapatite. *Nature, 239*, 515-517.

Bell, L. C., Posner, A. M., & Quirk, J. P. (1973). The point of zero charge of hydroxyapatite and fluorapatite in aqueous solutions. *Journal of Colloid and Interface Science, 42*, 250-261.

Benaziz, L., Barroug, A., Legrouri, A., Rey, C., & Lebugle, A. (2001). Adsorption of O-phospho-L-serine and L-serine onto poorly crystalline apatite. *Journal of Colloid and Interface Science, 238*, 48-53.

Bernardi, G., & Kawasaki, T. (1968). Chromatography of polypeptides and proteins on hydroxyapatite columns. *Biochimica et Biophysica Acta (BBA) - Protein Structure, 160*, 301-310.

Bernardi, G., Giro, M.-G., & Gaillard, C. (1972). Chromatography of polypeptides and proteins on hydroxyapatite columns: Some new developments. *Biochimica et Biophysica Acta (BBA) - Protein Structure, 278*, 409-420.

Blakemore, W. L., & Harpell, A. R. (2010). Carrageenan. In A. Imeson (Ed.), *Food stabilisers, thickeners and gelling agents* (pp. 73-94). Oxford, UK: Wiley-Blackwell.

Borum, L., & Wilson, O. C., Jr. (2003). Surface modification of hydroxyapatite. Part II. Silica. *Biomaterials, 24*, 3681-3688.

Borum-Nicholas, L., & Wilson, O. C., Jr. (2003). Surface modification of hydroxyapatite. Part I. Dodecyl alcohol. *Biomaterials, 24*, 3671-3679.

Brandes, N., Welzel, P. B., Werner, C., & Kroh, L. W. (2006). Adsorption-induced conformational changes of proteins onto ceramic particles: Differential scanning calorimetry and FTIR analysis. *Journal of Colloid and Interface Science, 299*, 56-69.

Brew, K. (2003). α-Lactalbumin. In *Advanced dairy chemistry. Vol. 1 Proteins* (pp. 387-419). London, UK: Springer-Verlag.

Brownlow, S., Cabral, J. H. M., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., et al. (1997). Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure, 5*, 481-495.

Byler, D. M., & Farrell, H. M., Jr. (1989). Infrared spectroscopic evidence for calcium ion interaction with carboxylate groups of casein. *Journal of Dairy Science, 72*, 1719-1723.

Calvo, M. S., Eyre, D. R., & Gundberg, C. M. (1996). Molecular basis and clinical application of biological markers of bone turnover. *Endocrine Reviews, 17*, 333-368.

Canabady-Rochelle, L. S., Sanchez, C., Mellema, M., Bot, A., Desobry, S., & Banon, S. (2007). Influence of calcium salt supplementation on calcium equilibrium in skim milk during pH cycle. *Journal of Dairy Science, 90*, 2155-2162.

Cazalbou, S., Combes, C., Eichert, D., & Rey, C. (2004). Adaptative physico-chemistry of bio-related calcium phosphates. *Journal of Materials Chemistry, 14*, 2148-2153.

Chandrapala, J., Augustin, M. A., McKinnon, I., & Udabage, P. (2010). Effects of pH, calcium-complexing agents and milk solids concentration on formation of soluble protein aggregates in heated reconstituted skim milk. *International Dairy Journal, 20*, 777-784.

Chang, M. C., Ko, C.-C., & Douglas, W. H. (2003). Conformational change of hydroxyapatite/gelatin nanocomposite by glutaraldehyde. *Biomaterials, 24*, 3087-3094.

Chaplin, L. C. (1984). Studies on micellar calcium phosphate: composition and apparent solubility product in milk over a wide pH range. *Journal of Dairy Research, 51*, 251-257.

Cheng, J., & Xie, P. (2001). Study on the choice of calcium and the stability of neutral high calcium milk. *Science and Technology of Food Industry, 22,* 27-28.

Chu, B. S., Ichikawa, S., Kanafusa, S., & Nakajima, M. (2008). Stability of protein-stabilised beta-carotene nanodispersions against heating, salts and pH. *Journal of the Science of Food and Agriculture, 88*, 1764-1769.

Chu, B., Zhou, Z., Wu, G., & Farrell, H. M. (1995). Laser Light Scattering of Model Casein Solutions: Effects of High Temperature. *Journal of Colloid and Interface Science, 170*, 102-112.

Correia, R., Magalhaes, M., Marques, P., & Senos, A. (1996). Wet synthesis and characterization of modified hydroxyapatite powders. *Journal of Materials Science: Materials in Medicine, 7*, 501-505.

Cross, K. J., Huq, N. L., Palamara, J. E., Perich, J. W., & Reynolds, E. C. (2005). Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *Journal of Biological Chemistry, 280*, 15362-15369.

Cross, K. J., Huq, N. L., & Reynolds, E. C. (2007). Casein phosphopeptides in oral health - Chemistry and clinical applications. *Current Pharmaceutical Design, 13*, 793-800.

Dalgleish, D. G. (2006). Food emulsions—their structures and structure-forming properties. *Food Hydrocolloids, 20*, 415-422.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles-review and possible improvements. *Soft Matter, 7*, 2265-2272.

Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology, 3*, 449-467.

Dalgleish, D. G., & Morris, E. R. (1988). Interactions between carrageenans and casein micelles: electrophoretic and hydrodynamic properties of the particles. *Food Hydrocolloids, 2*, 311-320.

Dalgleish, D. G., & Parker, T. G. (1980). Binding of calcium ions to bovine αsl-casein and precipitability of the protein–calcium ion complexes. *Journal of Dairy Research, 47*, 113-122.

Das, K., & Kinsella, J. (1989). pH dependent emulsifying properties of b-lactoglobulin. *Journal of Dispersion Science and Technology, 10*, 77-102.

Datta, N., & Deeth, H. (2001). Age gelation of UHT milk—a review. *Food and Bioproducts Processing, 79*, 197-210.

Dattolo, L., Keller, E. L., & Carta, G. (2010). pH transients in hydroxyapatite chromatography columns—Effects of operating conditions and media properties. *Journal of Chromatography A, 1217*, 7573-7578.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986). *Data for biochemical research* (3rd edN., pp. 148-149). New York, NY, USA: Oxford University Press.

de Kort, E., Minor, M., Snoeren, T., Van Hooijdonk, T., & Van Der Linden, E. (2009). Calcium-binding capacity of organic and inorganic ortho-and polyphosphates. *Dairy Science and Technology, 89*, 283-299.

de Kort, E., Minor, M., Snoeren, T., van Hooijdonk, T., & van der Linden, E. (2011). Effect of calcium chelators on physical changes in casein micelles in concentrated micellar casein solutions. *International Dairy Journal, 21*, 907-913.

de Kort, E., Minor, M., Snoeren, T., van Hooijdonk, T., & van der Linden, E. (2012). Effect of calcium chelators on heat coagulation and heat-induced changes of concentrated

micellar casein solutions: The role of calcium-ion activity and micellar integrity. *International Dairy Journal, 26*, 112-119.

De Kruif, C. G., & Grinberg, V. Y. (2002). Micellisation of β-casein. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 210*, 183-190.

De Kruif, C. G., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Vol. 1. Proteins* (3rd edn., pp. 233-276): London, UK: Springer-Verlag.

De Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. *Advances in Colloid and Interface Science, 171-172*, 36-52.

De la Fuente, M. A. (1998). Changes in the mineral balance of milk submitted to technological treatments. *Trends in Food Science and Technology, 9*, 281-288.

De la Fuente, M. A., Belloque, J., & Juarez, M. (2004). Mineral contents and distribution between the soluble and the micellar phases in calcium-enriched UHT milks. *Journal of the Science of Food and Agriculture, 84*, 1708-1714.

Desmet, J., Hanssens, I., & van Cauwelaert, F. (1987). Comparison of the binding of Na+ and Ca2+ to bovine alpha-lactalbumin. *Biochimica Biophysica Acta, 912*, 211-219.

Devold, T. G., Rykke, M., Isabey, D., Sørensen, E. S., Christensen, B., Langsrud, T., et al. (2006). In vitro studies of adsorption of milk proteins onto tooth enamel. *International Dairy Journal, 16*, 1013-1017.

Dickinson, E. (1998). Stability and rheological implications of electrostatic milk protein-polysaccharide interactions. *Trends in Food Science and Technology, 9*, 347-354.

Dickinson, E. (1999). Caseins in emulsions: interfacial properties and interactions. *International Dairy Journal, 9*, 305-312.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces, 20*, 197-210.

Dickinson, E. (2011). Mixed biopolymers at interfaces: Competitive adsorption and multilayer structures. *Food Hydrocolloids, 25*, 1966-1983.

Dogan, M., Toker, O. S., & Goksel, M. (2011). Rheological behaviour of instant hot chocolate beverage: Part 1. Optimization of the effect of different starches and gums. *Food Biophysics, 6*, 512-518.

Donnelly, W. (1977). Chromatography of milk proteins on hydroxyapatite. *Journal of Dairy Research, 44*, 621-625.

Draget, K. I. (2009). Alginates. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of hydrocolloids* (pp. 807-828). London, UK: Woodhead Publishing Limited & CRC Press LLC.

Edwards, P. B., Creamer, L. K., & Jameson, G. B. (2009). Structure and stability of whey proteins. In A. Thompson, M. Boland & H. Singh (Eds.), *Milk proteins: From expression to food* (pp. 163-203). Oxford, UK: Academic Press/Elsevier.

Elofsson, U. M., Paulsson, M. A., & Arnebrant, T. (1997). Adsorption of β-lactoglobulin A and B in relation to self-association: Effect of concentration and pH. *Langmuir, 13*, 1695-1700.

Euston, S. R., & Hirst, R. L. (2000). The emulsifying properties of commercial milk protein products in simple oil-in-water emulsions and in a model food system. *Journal of Food Science, 65*, 934-940.

Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., et al. (2004). Nomenclature of the proteins of cows' milk - sixth revision. *Journal of Dairy Science, 87*, 1641-1674.

FitzGerald, R. J. (1998). Potential uses of caseinophosphopeptides. *International Dairy Journal, 8*, 451-457.

Fox, P. F. (2003). *Advanced dairy chemistry.* London, UK: Kluwer Academic/Plenum.

Fox, P. F. (2009). Milk: an overview. In A. Thompson, M. Boland & H. Singh (Eds.), *Milk proteins: From expression to food* (pp. 1-54). Oxford, UK: Academic Press/Elsevier.

Fox, P. F., & McSweeney, P. L. H. (1998). Milk proteins. In *Dairy chemistry and biochemistry* (pp. 146-238). London, UK: Blackie Academic and Professional.

Gagnon, P. (1998). An enigma unmasked: How hydroxyapatite works, and how to make it work for you. In *Validated Biosystems quarterly resource guide for downstream processing*. Singapore: Validated Biosystems, Inc.

Gao, R. (2010). *Ion speciation in milk-like systems*. Wageningen Netherlands: Wageningen Universiteit (Wageningen University).

Gao, R., Temminghoff, E. J. M., van Leeuwen, H. P., van Valenberg, H. J. F., Eisner, M. D., & van Boekel, M. A. J. S. (2009). Simultaneous determination of free calcium, magnesium, sodium and potassium ion concentrations in simulated milk ultrafiltrate and reconstituted skim milk using the Donnan Membrane Technique. *International Dairy Journal, 19*, 431-436.

Gaucheron, F. (2000). Iron fortification in dairy industry. *Trends in Food Science and Technology, 11*, 403-409.

Gaucheron, F. (2003). Enrichissement en calcium et en fer des produits laitiers. In, *Mineraux et produits laitiers* (pp. 415-451). Paris, France: Lavoisier.

Gaucheron, F. (2004). *Mineraux et produits laitiers*. Paris, France:Lavoisier.

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development, 45*, 473-483.

Gaucheron, F., Le Graet, Y., Boyaval, E., & Piot, M. (1997). Binding of cations to sodium caseinate: importance of physicochemical conditions. *Milchwissenschaft, 52*, 322-327.

George, A., & Veis, A. (2008). Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. *Chemical Reviews, 108*, 4670-4693.

Gerstner, G. (2005). Feasibility of calcium fortification in dairy and soy drinks. *Marketing and Technology, 18*, 10-13.

Goobes, G., Goobes, R., Shaw, W. J., Gibson, J. M., Long, J. R., Raghunathan, V., et al. (2007). The structure, dynamics, and energetics of protein adsorption—lessons learned from adsorption of statherin to hydroxyapatite. *Magnetic Resonance in Chemistry, 45*, S32-S47.

Gorbunoff, M. J. (1984). The interaction of proteins with hydroxyapatite: II. Role of acidic and basic groups. *Analytical Biochemistry, 136*, 433-439.

Gorbunoff, M. J., & Timasheff, S. N. (1984a). The interaction of proteins with hydroxyapatite: III. Mechanism. *Analytical Biochemistry, 136*, 440-445.

Gorbunoff, M. J., & Timasheff, S. N. (1984b). The interaction of proteins with hydroxyapatite: I. Role of protein charge and structure. *Analytical Biochemistry, 136*, 425-432.

Grenby, T. H., Andrews, A. T., Mistry, M., & Williams, R. J. H. (2001). Dental caries-protective agents in milk and milk products: investigations in vitro. *Journal of Dentistry, 29*, 83-92.

Griffin, M. C. A., Lyster, R. L. J., & Price, J. C. (1988). The disaggregation of calcium-depleted casein micelles. *European Journal of Biochemistry, 174*, 339-343.

Guéguen, L., & Pointillart, A. (2000). The bioavailability of dietary calcium. *Journal of the American College of Nutrition, 19*, 119S-136S.

Gurr, M. (1999). *Calcium in nutrition.* Brussels, Belgium: International Life Sciences Institute Europe.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids, 22*, 1460-1466.

Han, Y., Wang, X., & Li, S. (2009). A simple route to prepare stable hydroxyapatite nanoparticles suspension. *Journal of Nanoparticle Research, 11*, 1235-1240.

Harding, I. S., Rashid, N., & Hing, K. A. (2005). Surface charge and the effect of excess calcium ions on the hydroxyapatite surface. *Biomaterials, 26*, 6818-6826.

Hauschka, P. V., & Carr, S. A. (1982). Calcium-dependent .alpha.-helical structure in osteocalcin. *Biochemistry, 21*, 2538-2547.

He, H. W., Li, G. D., Li, B., & Chen, Z. Q. (2008). Effects of surface microstructure of hydroxyapatite on protein adsorption and biological performance of osteoblasts. *Applied Surface Science, 255*, 565-567.

Heaney, R. P. (2000). Calcium, dairy products and osteoporosis. *Journal of the American College of Nutrition, 19*, 83S-99S.

Heaney, R. P., Recker, R. R., & Weaver, C. M. (1990). Absorbability of calcium sources: The limited role of solubility. *Calcified Tissue International, 46*, 300-304.

Heaney, R. P., Dowell, M. S., & Barger-Lux, M. J. (1999). Absorption of calcium as the carbonate and citrate salts, with some observations on method. *Osteoporosis International, 9*, 19-23.

Hemar, Y., Hall, C. E., Munro, P. A., & Singh, H. (2002). Small and large deformation rheology and microstructure of kappa-carrageenan gels containing commercial milk protein products. *International Dairy Journal, 12*, 371-381.

Hlady, V., & Füredi-Milhofer, H. (1979). Adsorption of human serum albumin on precipitated hydroxyapatite. *Journal of Colloid and Interface Science, 69*, 460-468.

Holt, C. (1985). The milk salts: Their secretion, concentrations and physical chemistry. In P. F. Fox (Ed.), *Developments in dairy chemistry* (Vol. 3., pp. 143-181). Amsterdam, The Netherlands: Springer Netherlands.

Holt, C. (1992). Structure and stability of bovine casein micelles. *Advances in Protein Chemistry, 43*, 63-151.

Holt, C. (2004). An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *European Biophysics Journal, 33*, 421-434.

Holt, C., & Horne, D. (1996). The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Nederlands melk en Zuiveltijdschrift, 50*, 85-111.

Holt, C., & Timmins, P. A. (1997). Modelling milk: the structure of calcium phosphate nanoclusters. *Rapport annuel de l'Institut Laue-Langevin, Grenoble, France*.

Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Caseins and the casein micelle: Their biological functions, structures, and behavior in foods1. *Journal of Dairy Science, 96*, 6127-6146.

Holt, C., Dalgleish, D. G., & Jenness, R. (1981). Calculation of the ion equilibria in milk diffusate and comparison with experiment. *Analytical Biochemistry, 113*, 154-163.

Holt, C., De Kruif, C. G., Tuinier, R., & Timmins, P. A. (2003). Substructure of bovine casein micelles by small-angle X-ray and neutron scattering. *Colloids and Surfaces a-Physicochemical and Engineering Aspects, 213*, 275-284.

Holt, C., Lenton, S., Nylander, T., Sørensen, E. S., & Teixeira, S. (2014). Mineralisation of soft and hard tissues and the stability of biofluids. *Journal of Structural Biology, 185*, 383-396.

Holt, C., Parker, T. G., & Dalgleish, D. G. (1975). The thermochemistry of reactions between alpha-s1 casein and calcium chloride. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 379*, 638-644.

Holt, C., Sørensen, E. S., & Clegg, R. A. (2009). Role of calcium phosphate nanoclusters in the control of calcification. *FEBS Journal, 276*, 2308-2323.

Holt, C., Timmins, P. A., Errington, N., & Leaver, J. (1998). A core-shell model of calcium phosphate nanoclusters stabilized by β-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. *European Journal of Biochemistry, 252*, 73-78.

Holt, C., van Kemenade, M. J., Nelson, L. S., Sawyer, L., Harries, J. E., Bailey, R. T., et al. (1989). Composition and structure of micellar calcium phosphate. *Journal of Dairy Research, 56*, 411-416.

Holt, C., Wahlgren, N., & Drakenberg, T. (1996a). Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochemical Journal, 314*, 1035-1039.

Holt, C., Wahlgren, N. M., & Drakenberg, T. (1996b). Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochemical Journal, 314*, 1035-1039.

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal, 8*, 171-177.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current Opinion in Colloid and Interface Science, 11*, 148-153.

Horne, D. S. (2009). Casein micelle structure and stability. In A. Thompson, M. Boland & H. Singh (Eds.), *Milk proteins: From expression to food* (pp. 133-162). Oxford, UK: Academic Press/Elsevier.

Huang, F., Shen, Y., Xie, A., Zhu, J., Zhang, C., Li, S., et al. (2007). Study on synthesis and properties of hydroxyapatite nanorods and its complex containing biopolymer. *Journal of Materials Science, 42*, 8599-8605.

Hunt, J. A., & Dalgleish, D. G. (1994a). Adsorption behaviour of whey-protein isolate and caseinate in soya-oil-in-water emulsions. *Food Hydrocolloids, 8*, 175-187.

Hunt, J. A., & Dalgleish, D. G. (1994b). Effect of pH on the stability and surface composition of emulsions made with whey protein isolate. *Journal of Agricultural and Food Chemistry, 42*, 2131-2135.

Hunt, J. A., & Dalgleish, D. G. (1995). Heat stability of oil-in-water emulsions containing milk proteins: Effect of ionic strength and pH. *Journal of Food Science, 60*, 1120-1123.

Hunter, G. K. (2013). Role of osteopontin in modulation of hydroxyapatite formation. *Calcified Tissue International, 93*, 348-354.

Iafisco, M., Di Foggia, M., Bonora, S., Prat, M., & Roveri, N. (2011). Adsorption and spectroscopic characterization of lactoferrin on hydroxyapatite nanocrystals. *Dalton Transactions, 40*, 820-827.

Iafisco, M., Sabatino, P., Lesci, I. G., Prat, M., Rimondini, L., & Roveri, N. (2010). Conformational modifications of serum albumins adsorbed on different kinds of biomimetic hydroxyapatite nanocrystals. *Colloids and Surfaces B: Biointerfaces, 81*, 274-284.

Jenness, R., & Koops, J. (1962). Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk and Dairy Journal, 16*, 153-164.

Johnsson, M., Levine, M., & Nancollas, G. (1993). Hydroxyapatite binding domains in salivary proteins. *Critical Reviews in Oral Biology and Medicine, 4*, 371-378.

Johnsson, M., Richardson, C., Sallis, J., & Nancollas, G. (1991). Adsorption and mineralization effects of citrate and phosphocitrate on hydroxyapatite. *Calcified Tissue International, 49*, 134-137.

Jones, K. L., & O'Melia, C. R. (2000). Protein and humic acid adsorption onto hydrophilic membrane surfaces: effects of pH and ionic strength. *Journal of Membrane Science, 165*, 31-46.

Juriaanse, A., Arends, J., & Ten Bosch, J. (1980). The adsorption of acidic and basic homopolypeptides to whole bovine dental enamel. *Journal of Colloid and Interface Science, 76*, 212-219.

Juriaanse, A. C., Booij, M., Arends, J., & Bosch, J. J. T. (1981). The adsorption in vitro of purified salivary proteins on bovine dental enamel. *Archives of Oral Biology, 26*, 91-96.

Kaliappan, S., & Lucey, J. (2011). Influence of mixtures of calcium-chelating salts on the physicochemical properties of casein micelles. *Journal of Dairy Science, 94*, 4255-4263.

Kandori, K., Kuroda, T., Togashi, S., & Katayama, E. (2011). Preparation of calcium hydroxyapatite nanoparticles using microreactor and their characteristics of protein adsorption. *Journal of Physical Chemistry B, 115*, 653-659.

Kandori, K., Masunari, A., & Ishikawa, T. (2005). Study on adsorption mechanism of proteins onto synthetic calcium hydroxyapatites through ionic concentration measurements. *Calcified Tissue International, 76*, 194-206.

Kandori, K., Miyagawa, K., & Ishikawa, T. (2004). Adsorption of immunogamma globulin onto various synthetic calcium hydroxyapatite particles. *Journal of Colloid and Interface Science, 273*, 406-413.

Kandori, K., Mukai, M., Yasukawa, A., & Ishikawa, T. (2000). Competitive and cooperative Adsorptions of bovine serum albumin and lysozyme to synthetic calcium hydroxyapatites. *Langmuir, 16*, 2301-2305.

Kandori, K., Oda, S., Fukusumi, M., & Morisada, Y. (2009). Synthesis of positively charged calcium hydroxyapatite nano-crystals and their adsorption behavior of proteins. *Colloids and Surfaces B: Biointerfaces, 73*, 140-145.

Kandori, K., Oda, S., & Tsuyama, S. (2008). Effects of pyrophosphate ions on protein adsorption onto calcium hydroxyapatite. *Journal of Physical Chemistry B, 112*, 2542-2547.

Kandori, K., Tsuyama, S., Tanaka, H., & Ishikawa, T. (2007). Protein adsorption characteristics of calcium hydroxyapatites modified with pyrophosphoric acids. *Colloids and Surfaces B: Biointerfaces, 58*, 98-104.

Karman, A., & Van Boekel, M. (1986). Evaluation of the Kjeldahl factor for conversion of the nitrogen content of milk and milk products to protein content. *Netherlands Milk and Dairy Journal 40,* 315-336.

Kawasaki, T. (1991). Hydroxyapatite as a liquid chromatographic packing. *Journal of Chromatography A, 544*, 147-184.

Kawasaki, T., Takahashi, S., & Ideda, K. (1985). Hydroxyapatite high-performance liquid chromatography: column performance for proteins. *European Journal of Biochemistry, 152*, 361-371.

Kinsella, J. E., & Morr, C. V. (1984). Milk proteins: physicochemical and functional properties. *Critical Reviews in Food Science and Nutrition, 21*, 197-262.

Kirby, B. J., & Hasselbrink, E. F. (2004). Zeta potential of microfluidic substrates: 1. Theory, experimental techniques, and effects on separations. *Electrophoresis, 25*, 187-202.

Kligerman, A. E. (1987). Calcium-fortified milk prod - obtd. by dispersing calcium phosphate in cows milk with stabilising and suspending agent e.g. carrageenan. In: LACTAID INC (LACT-Non-standard).

Knights, R. J., & Kjelsberg, L. M. (1998). *Method of suspending insoluble calcium in protein composition*. US Patent 5766330 A

Kontopidis, G., Holt, C., & Sawyer, L. (2004). β-Lactoglobulin: Binding properties, structure, and function. *Journal of Dairy Science, 87*, 785-796.

Koutsopoulos, S. (2002). Synthesis and characterization of hydroxyapatite crystals: A review study on the analytical methods. *Journal of Biomedical Materials Research, 62*, 600-612.

Kraulis, P. J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *Journal of Applied Crystallography, 24*, 946-950.

Krawczyk, G., Venables, A., & Tuason, D. (2009). Microcrystalline cellulose. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of hydrocolloids* (2nd Edn., pp. 741-759). Oxford, UK: Woodhead Publishing Limited & CRC Press LLC.

Kronman, M. J., & Andreotti, R. E. (1964). Inter-and intramolecular interactions of α-lactalbumin. I. The apparent heterogeneity at acid pH. *Biochemistry, 3*, 1145-1151.

Kronman, M., Andreotti, R., & Vitols, R. (1964). Inter-and intramolecular interactions of α-lactalbumin. II. Aggregation reactions at acid pH. *Biochemistry, 3*, 1152-1160.

Kronman, M., Holmes, L., & Robbins, F. (1967). Inter-and intramolecular interactions of α-lactalbumin. VIII. The alkaline conformational change. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 133*, 46-55.

Kull, T., Nylander, T., Tiberg, F., & Wahlgren, N. M. (1997). Effect of surface properties and added electrolyte on the structure of β-casein layers adsorbed at the solid/aqueous interface. *Langmuir, 13*, 5141-5147.

Kuwajima, K. (1996). The molten globule state of alpha-lactalbumin. *FASEB journal, 10*, 102-109.

Lamkin, M. S., Arancillo, A. A., & Oppenheim, E. G. (1996). Temporal and compositional characteristics of salivary protein adsorption to hydroxyapatite. *Journal of Dental Research, 75*, 803-808.

Langendorff, V., Cuvelier, G., Michon, C., Launay, B., Parker, A., & De kruif, C. G. (2000). Effects of carrageenan type on the behaviour of carrageenan/milk mixtures. *Food Hydrocolloids, 14*, 273-280.

Leach, S. (1960). Electrophoresis of synthetic hydroxyapatite. *Archives of Oral Biology, 3*, 48-56.

Lee, H. J., Choi, H. W., Kim, K. J., & Lee, S. C. (2006). Modification of hydroxyapatite nanosurfaces for enhanced dolloidal stability and improved interfacial adhesion in nanocomposites. *Chemistry of Materials, 18*, 5111-5118.

Lee, S. C., Choi, H. W., Lee, H. J., Kim, K. J., Chang, J. H., Kim, S. Y., et al. (2007). In-situ synthesis of reactive hydroxyapatite nano-crystals for a novel approach of surface grafting polymerization. *Journal of Materials Chemistry, 17*, 174-180.

Lee, W.-H., Loo, C.-Y., Van, K. L., Zavgorodniy, A. V., & Rohanizadeh, R. (2011). Modulating protein adsorption onto hydroxyapatite particles using different amino acid treatments. *Journal of the Royal Society Interface*.

Le Graet, Y., & Brule, G. (1993). Effects of pH and ionic strength on mineral balance of milk. *Lait, 73,* 51-60.

Le Graet, Y., & Gaucheron, F. (1999). pH-induced solubilization of minerals from casein micelles: influence of casein concentration and ionic strength. *Journal of Dairy Research, 66*, 215-224.

Le Ray, C., Maubois, J. L., Gaucheron, F., Brule, G., Pronnier, P., & Garnier, F. (1998). Heat stability of reconstituted casein micelle dispersions: changes induced by salt addition. *Lait, 78*, 375-390.

Levenson, D. I., & Bockman, R. S. (1994). A review of calcium preparations. *Nutrition Reviews, 52*, 221-232.

Lewis, M. J. (2011). The measurement and significance of ionic calcium in milk - A review. *International Journal of Dairy Technology, 64*, 1-13.

Lewis, M., Grandison, A., Lin, M. J., & Tsioulpas, A. (2011). Ionic calcium and pH as predictors of stability of milk to UHT processing. *Milchwissenschaft-Milk Science International, 66*, 197-200.

Li, C., Zhao, L., Han, J., Wang, R., Xiong, C., & Xie, X. (2011). Synthesis of citrate-stabilized hydrocolloids of hydroxyapatite through a novel two-stage method: A possible aggregates–breakdown mechanism of colloid formation. *Journal of Colloid and Interface Science, 360*, 341-349.

Lin, J., Raghavan, S., & Fuerstenau, D. (1981). The adsorption of fluoride ions by hydroxyapatite from aqueous solution. *Colloids and Surfaces, 3*, 357-370.

Lin, M. J., Lewis, M. J., & Grandison, A. S. (2006). Measurement of ionic calcium in milk. *International Journal of Dairy Technology, 59*, 192-199.

Lin, S. H. C., Leong, S. L., Dewan, R. K., Bloomfield, V. A., & Morr, C. V. (1972). Effect of calcium ion on the structure of native bovine casein micelles. *Biochemistry, 11*, 1818-1821.

Little, E. M., & Holt, C. (2004). An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein phosphopeptides. *European Biophysics Journal, 33*, 435-447.

Liu, Y., & Nancollas, G. H. (1997). Crystallization and colloidal stability of calcium phosphate phases. *Journal of Physical Chemistry B, 101*, 3464-3468.

Livney, Y. D. (2010). Milk proteins as vehicles for bioactives. *Current Opinion in Colloid and Interface Science, 15*, 73-83.

López-Huertas, E., Teucher, B., Boza, J. J., Martínez-Férez, A., Majsak-Newman, G., Baró, L., et al. (2006). Absorption of calcium from milks enriched with fructo-oligosaccharides, caseinophosphopeptides, tricalcium phosphate, and milk solids. *American Journal of Clinical Nutrition, 83*, 310-316.

López-Macipe, A., Gómez-Morales, J., & Rodriguez-Clemente, R. (1998). The role of pH in the adsorption of citrate ions on hydroxyapatite. *Journal of Colloid and Interface Science, 200*, 114-120.

Lucey, J. A., & Horne, D. S. (2009). Milk salts: Technological significance. In P. L. H. McSweeney & P. F. Fox (Eds.), *Advanced dairy chemistry, Vol. 3: Lactose, water, salts and minor constituents* (3rd edn., pp. 351-390). London, UK: Springer-Verlag.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Agricultural and Food Chemistry, 48*, 1610-1616.

Luey, J.-K., McGuire, J., & Sproull, R. D. (1991). The effect of pH and NaCl concentration on adsorption of β-lactoglobulin at hydrophilic and hydrophobic silicon surfaces. *Journal of Colloid and Interface Science, 143*, 489-500.

Luo, Q., & Andrade, J. D. (1998). Cooperative adsorption of proteins onto hydroxyapatite. *Journal of Colloid and Interface Science, 200*, 104-113.

Lynch, S. R. (2005). The impact of iron fortification on nutritional anaemia. *Best Practice and Research Clinical Haematology, 18*, 333-346.

Lyster, R. L. J. (1981). Calculation by computer of individual concentrations in a simulated milk salt solution. II. An extension to the previous model. *Journal of Dairy Research, 48*, 85-89.

Marinova, K. G., Basheva, E. S., Nenova, B., Temelska, M., Mirarefi, A. Y., Campbell, B., et al. (2009). Physico-chemical factors controlling the foamability and foam stability of milk proteins: Sodium caseinate and whey protein concentrates. *Food Hydrocolloids, 23*, 1864-1876.

Martins, M. A., Santos, C., Almeida, M. M., & Costa, M. E. V. (2008). Hydroxyapatite micro-and nanoparticles: nucleation and growth mechanisms in the presence of citrate species. *Journal of Colloid and Interface Science, 318*, 210-216.

Mavropoulos, E., Costa, A. M., Costa, L. T., Achete, C. A., Mello, A., Granjeiro, J. M., et al. (2011). Adsorption and bioactivity studies of albumin onto hydroxyapatite surface. *Colloids and Surfaces B: Biointerfaces, 83*, 1-9.

McDougall, W. (1977). Effect of milk on enamel demineralization and remineralization in vitro. *Caries research, 11*, 166-172.

McGann, T. C., Kearney, R. D., & Donnelly, W. J. (1979). Developments in column chromatography for the separation and characterization of casein micelles. *Journal of Dairy Research, 46*, 307-311.

McKenna, A. B., Lloyd, R. J., Munro, P. A., & Singh, H. (1999). Microstructure of whole milk powder and of insolubles detected by powder functional testing. *Scanning, 21*, 305-315.

McMahon, D. J. (1996). Age-gelation of UHT milk: changes that occur during storage, their effect on shelf life and the mechanism by which age-gelation occurs. In *Heat treatments and alternative methods. IDF Symposium, Vienna (Austria), 6-8 Sep 1995.* Geneva, Switzerland: International Dairy Federation.

McMahon, D. J., & McManus, W. R. (1998). Rethinking casein micelle structure using electron microscopy. *Journal of Dairy Science, 81*, 2985-2993.

McNamee, B. F., O'Riordan, E. D., & O'Sullivan, M. (1998). Emulsification and microencapsulation properties of gum arabic. *Journal of Agricultural and Food Chemistry, 46*, 4551-4555.

Mekmene, O. (2010). *Comportement de la fraction minérale laitière en fonction des conditions physicochimiques: de la précipitation des sels de phosphate de calcium à la prediction des équilibres minéraux du lait*. PhD Thesis. Agrocampus Ouest, Rennes, France.

Mekmene, O., & Gaucheron, F. (2011). Determination of calcium-binding constants of caseins, phosphoserine, citrate and pyrophosphate: A modelling approach using free calcium measurement. *Food Chemistry, 127*, 676-682.

Mekmene, O., Le Graet, Y., & Gaucheron, F. (2009). A model for predicting salt equilibria in milk and mineral-enriched milks. *Food Chemistry, 116*, 233-239.

Mekmene, O., Le Graët, Y., & Gaucheron, F. (2010). Theoretical model for calculating ionic equilibria in milk as a function of pH: Comparison to experiment. *Journal of Agricultural and Food Chemistry, 58*, 4440-4447.

Mizuno, R., & Lucey, J. A. (2005). Effects of emulsifying salts on the turbidity and calcium-phosphate-protein interactions in casein micelles. *Journal of Dairy Science, 88*, 3070-3078.

Mohsen-Nia, M., Bidgoli, M. M., Behrashi, M., & Nia, A. M. (2012). Human serum protein adsorption onto synthesis nano-hydroxyapatite. *Protein Journal, 31*, 150-157.

Moreno, E. C., Kresak, M., & Hay, D. I. (1984). Adsorption of molecules of biological interest onto hydroxyapatite. *Calcified Tissue International, 36*, 48-59.

Morr, C. V. (1967). Effect of oxalate and urea upon ultracentrifugation properties of raw and heated skimmilk casein micelles. *Journal of Dairy Science, 50,* 1744-1751.

Morr, C. (1982). Functional properties of milk proteins and their use as food ingredients. *Developments in dairy chemistry, 1*, 375-399.

Morris, G. A. (2002). The self-assembly and structure of caseins in solution. *Biotechnology and Genetic Engineering Reviews, 19*, 357-376.

Mostafa, N. Y., & Brown, P. W. (2007). Computer simulation of stoichiometric hydroxyapatite: structure and substitutions. *Journal of Physics and Chemistry of Solids, 68*, 431-437.

Müller-Mai, C., Voigt, C., & Gross, U. (1990). Incorporation and degradation of hydroxyapatite implants of different surface roughness and surface structure in bone. *Scanning Microscopy, 4*, 613-622.

Mulvihill, D., & Murphy, P. (1991). Surface active and emulsifying properties of caseins/caseinates as influenced by state of aggregation. *International Dairy Journal, 1*, 13-37.

Mura-Galelli, M. J., Voegel, J. C., Behr, S., Bres, E. F., & Schaaf, P. (1991). Adsorption/desorption of human serum albumin on hydroxyapatite: a critical analysis of the Langmuir model. *Proceedings of the National Academy of Sciences U S A, 88*, 5557-5561.

Nakanishi, K., Sakiyama, T., & Imamura, K. (2001). On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *Journal of Bioscience and Bioengineering, 91*, 233-244.

Nancollas, G., & Tomazic, B. (1974). Growth of calcium phosphate on hydroxyapatite crystals. Effect of supersaturation and ionic medium. *Journal of Physical Chemistry, 78*, 2218-2225.

Nancollas G, H., Amjad, Z., & Koutsoukos, P. (1979). Calcium phosphates speciation, solubility, and kinetic considerations. In E. A. Jenne (Ed.) *Chemical modeling in aqueous systems: American Chemical Society Symposium Series* (Vol. 93, pp. 475-498). Washington, DC, USA: American Chemical Society.

Nelson, C. E., Crowley, D. E., & Weatherford, W. M. (1987). *Calcium fortified milk*. US Patent 4701329 A.

Neuman, W., & Neuman, M. (1953). The nature of the mineral phase of bone. *Chemical Reviews, 53*, 1-45.

Noisuwan, A., Hemar, Y., Wilkinson, B., & Bronlund, J. E. (2011). Adsorption of milk proteins onto rice starch granules. *Carbohydrate Polymers, 84*, 247-254.

Nonoyama, T., Kinoshita, T., Higuchi, M., Nagata, K., Tanaka, M., Sato, K., et al. (2011). Multistep growth mechanism of calcium phosphate in the earliest stage of morphology-controlled biomineralization. *Langmuir, 27*, 7077-7083.

Norton, J., Malik, K. R., Darr, J. A., & Rehman, I. (2006). Recent developments in processing and surface modification of hydroxyapatite. *Advances in Applied Ceramics, 105*, 27.

O'Connell, J., Grinberg, V. Y., & De Kruif, C. (2003). Association behavior of β-casein. *Journal of Colloid and Interface Science, 258*, 33-39.

Ohshima, H. (2007). Electrokinetics of soft particles. *Colloid and Polymer Science, 285*, 1411-1421.

Omoarukhe, E. D., On-Nom, N., Grandison, A. S., & Lewis, M. J. (2010). Effects of different calcium salts on properties of milk related to heat stability. *International Journal of Dairy Technology, 63*, 504-511.

Ono, T., Odagiri, S., Kaminogawa, S., & Yamauchi, K. (1976). A study on the binding of calcium ions to alpha (S-1)-casein. *Agricultural and biological chemistry*.

Ono, T., Yahagi, M., & Odagiri, S. (1980). The binding of calcium to kappa-casein and para kappa-casein. *Agricultural and Biological Chemistry, 44*, 1499-1503.

Pak, C. C., & Avioli, L. (1988). Factors affecting absorbability of calcium from calcium salts and food. *Calcified Tissue International, 43*, 55-60.

Patrick, L. (1999). Comparative absorption of calcium sources and calcium citrate malate for the prevention of osteoporosis. *Alternative Medicine Review, 4*, 74-85.

Payens, T. A. J., & Van Markwijk, B. W. (1963). Some features of the association of β-casein. *Biochimica et Biophysica Acta, 71,* 517-530.

Philippe, M., Gaucheron, F., Le Graet, Y., Michel, F., & Garem, A. (2003). Physicochemical characterization of calcium-supplemented skim milk. *Lait, 83*, 45-59.

Philippe, M., Le Graet, Y., & Gaucheron, F. (2005). The effects of different cations on the physicochemical characteristics of casein micelles. *Food Chemistry, 90*, 673-683.

Pitkowski, A., Durand, D., & Nicolai, T. (2008). Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science, 326*, 96-102.

Prakash, S., Huppertz, T., Karvchuk, O., & Deeth, H. (2010). Ultra-high-temperature processing of chocolate flavoured milk. *Journal of Food Engineering, 96*, 179-184.

Prince, R. L., Smith, M., Dick, I. M., Price, R. I., Webb, P. G., Henderson, N. K., et al. (1991). Prevention of postmenopausal osteoporosis: a comparative study of exercise, calcium supplementation, and hormone-replacement therapy. *New England Journal of Medicine, 325*, 1189-1195.

Ramírez-Sucre, M. O., & Vélez-Ruiz, J. F. (2010). Formulation and characterization of a flavored dairy beverage. In (Vol. 7, pp. 5894-5909). Pittsburgh, PA.

Rehman, I., & Bonfield, W. (1997). Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy. *Journal of Materials Science: Materials in Medicine, 8*, 1-4.

Ren, F., Ding, Y., & Leng, Y. (2014). Infrared spectroscopic characterization of carbonated apatite: A combined experimental and computational study. *Journal of Biomedical Materials Research Part A, 102*, 496-505.

Reynolds, E. (1987). The prevention of sub-surface demineralization of bovine enamel and change in plaque composition by casein in an intra-oral model. *Journal of Dental Research, 66*, 1120-1127.

Reynolds, E. C. (1997). Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *Journal of Dental Research, 76*, 1587-1595.

Reynolds, E. C. (1998). Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: A review. *Special Care in Dentistry, 18*, 8-16.

Reynolds, E., & Del Rio, A. (1984). Effect of casein and whey-protein solutions on caries experience and feeding patterns of the rat. *Archives of Oral Biology, 29*, 927-933.

Reynolds, E. C., & Wong, A. (1983). Effect of adsorbed protein on hydroxyapatite zeta potential and *Streptococcus mutans* adherence. *Infection and Immunity, 39*, 1285-1290.

Reynolds, E. C., Riley, P. F., & Adamson, N. J. (1994). A selective precipitation purification procedure for multiple phosphoseryl-containing peptides and methods for their identification. *Analytical Biochemistry, 217*, 277-284.

Rölla, G. (1971). Adsorption of dextran to saliva-treated hydroxyapatite. *Archives of Oral Biology, 16*, 527-533.

Rollema, H. S., & Brinkhuis, J. A. (1989). A 1H-NMR study of bovine casein micelles; influence of pH, temperature and calcium ions on micellar structure. *Journal of Dairy Research, 56*, 417-425.

Roque, A., & Wilson, O. C. Jr. (2008). Adsorption of gum Arabic on bioceramic nanoparticles. *Materials Science and Engineering: C, 28*, 443-447.

Rouahi, M., Champion, E., Gallet, O., Jada, A., & Anselme, K. (2006). Physico-chemical characteristics and protein adsorption potential of hydroxyapatite particles: Influence on in vitro biocompatibility of ceramics after sintering. *Colloids and Surfaces B: Biointerfaces, 47*, 10-19.

Sadeghian, Z., Heinrich, J. G., & Moztarzadeh, F. (2006). Influence of powder pre-treatments and milling on dispersion ability of aqueous hydroxyapatite-based suspensions. *Ceramics International, 32*, 331-337.

Sawyer, L. (2003). β-Lactoglobulin. In P. L. H. McSweeney, & P. F. Fox (Eds.) *Advanced dairy chemistry. Vol. 1. Proteins* (pp. 319-386). London, UK: Springer-Verlag.

Schmidt, D. G. (1970). The association of αs1-casein-B at pH 6.6. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 207*, 130-138.

Schmidt, D. G. (1982). Association of caseins and casein micelle structure. *Developments in Dairy Chemistry, 1*, 61-86.

Schmidt, D. G., & Buchheimi, W. (1992). The application of electron microscopy in dairy research. *Journal of Microscopy, 167*, 105-121.

Schmidt, D. G., & Van Markwijk, B. (1968). Further studies on the associating subunit of αs1-casein. *Biochimica et Biophysica Acta (BBA)-Protein Structure, 154*, 613-614.

Schüpbach, P., Neeser, J., Golliard, M., Rouvet, M., & Guggenheim, B. (1996). Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. *Journal of Dental Research, 75*, 1779-1788.

Sedlmeyer, F., & Kulozik, U. (2006). Impact of process conditions on the rheological detectable structure of UHT treated milk protein-carrageenan systems. *Journal of Food Engineering, 77*, 943-950.

Sharpe, J. R., Sammons, R. L., & Marquis, P. M. (1997). Effect of pH on protein adsorption to hydroxyapatite and tricalcium phosphate ceramics. *Biomaterials, 18*, 471-476.

Shimabayashi, S., Tanaka, H., & Nakagaki, M. (1986). Adsorption of dodecyl sulfate ion on hydroxyapatite and concurrent release of phosphate and calcium ions from the surface of hydroxyapatite *Chemical and Pharmaceutical Bulletin, 34*, 4474-4478.

Sievanen, K., Huppertz, T., Kelly, A. L., & Fox, P. F. (2008). Influence of added calcium chloride on the heat stability of unconcentrated and concentrated bovine milk. *International Journal of Dairy Technology, 61*, 151-155.

Singh, G., Arora, S., Sharma, G. S., Sindhu, J. S., Kansal, V. K., & Sangwan, R. B. (2007). Heat stability and calcium bioavailability of calcium-fortified milk. *LWT-Food Science and Technology, 40*, 625-631.

Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology, 57*, 111-119.

Singh, H., & Creamer, L. K. (1991). Aggregation and dissociation of milk protein complexes in heated reconstituted concentrated skim milks. *Journal of Food Science, 56*, 238-246.

Singh, H., & Fox, P. F. (1987). Heat stability of milk: role of β-lactoglobulin in the pH-dependent dissociation of micellar κ-casein. *Journal of Dairy Research, 54*, 509-521.

Singh, H., & Ye, A. (2008). Interactions and functionality of milk proteins in food emulsions. In A. Thompson, M. Boland & H. Singh (Eds.), *Milk proteins* (pp. 321-345). San Diego, CA, USA: Academic Press.

Skwarek, E., Janusz, W., & Sternik, D. (2014). Adsorption of citrate ions on hydroxyapatite synthetized by various methods. *Journal of Radioanalytical and Nuclear Chemistry, 299*, 2027-2036.

Snoeren, T. H. M., Both, P., & Schmidt, D. G. (1976). An electron-microscopic study of carrageenan and its interaction with kappa-casein. *Netherlands Milk and Dairy Journal, 30*, 132-141.

Spinelli, M., Brudevold, F., & Moreno, E. (1971). Mechanism of fluoride uptake by hydroxyapatite. *Archives of Oral Biology, 16*, 187-203.

Srinivasan, M., Singh, H., & Munro, P. A. (1996). Sodium caseinate-stabilized emulsions: Factors affecting coverage and composition of surface proteins. *Journal of Agricultural and Food Chemistry, 44*, 3807-3811.

Srinivasan, M., Singh, H., & A. Munro, P. (1999). Adsorption behaviour of sodium and calcium caseinates in oil-in-water emulsions. *International Dairy Journal, 9*, 337-341.

Srinivasan, M., Singh, H., & Munro, P. A. (2000). The effect of sodium chloride on the formation and stability of sodium caseinate emulsions. *Food Hydrocolloids, 14*, 497-507.

Stanciu, G., Sandulescu, I., Savu, B., Stanciu, S., Paraskevopoulos, K., Chatzistavrou, X., et al. (2007). Investigation of the hydroxyapatite growth on bioactive glass surface. *Journal of Biomedical and Pharmaceutical Engineering, 1*, 34-39.

Stayton, P. S., Drobny, G. P., Shaw, W. J., Long, J. R., & Gilbert, M. (2003). Molecular recognition at the protein-hydroxyapatite interface. *Critical Reviews in Oral Biology and Medicine, 14*, 370-376.

Sugiarto, M., Ye, A., & Singh, H. (2009). Characterisation of binding of iron to sodium caseinate and whey protein isolate. *Food Chemistry, 114*, 1007-1013.

Sun, R.-X., & Lu, Y.-P. (2008). Fabrication and characterization of porous hydroxyapatite microspheres by spray-drying method. *Frontiers of Materials Science in China, 2*, 95-98.

Swaisgood, H. E. (1982). Chemistry of milk proteins. In P. F. Fox (Ed.), *Developments in dairy chemistry* (pp. 1-60). Barking, UK: Elsevier Applied Science Publishers Ltd.

Swaisgood, H. (2003). Chemistry of the caseins. In P. L. H. McSweeney, & P. F. Fox (Eds.) *Advanced dairy chemistry. Vol. 1. Proteins* (pp. 139-201). London, UK: Springer-Verlag.

Syrbe, A., Bauer, W. J., & Klostermeyer, N. (1998). Polymer science concepts in dairy systems - An overview of milk protein and food hydrocolloid interaction. *International Dairy Journal, 8*, 179-193.

Tan, J., Chen, M., & Xia, J. (2009). Water-dispersible hydroxyapatite nanorods synthesized by a facile method. *Applied Surface Science, 255*, 8774-8779.

Tercinier, L., Ye, A., Anema, S., Singh, A., & Singh, H. (2013). Adsorption of milk proteins on to calcium phosphate particles. *Journal of Colloid Interface Science, 394*, 458-466.

Tercinier, L., Ye, A., Anema, S. G., Singh, A., & Singh, H. (2014a). Effects of ionic strength, pH and milk serum composition on adsorption of milk proteins on to hydroxyapatite particles. *Food Biophysics*, DOI: 10.1007/s11483-014-9360-5

Tercinier, L., Ye, A., Anema, S. G., Singh, A., & Singh, H. (2014b). Interactions of casein micelles with calcium phosphate particles. *Journal of Agriculture and Food Chemistry, 62,* 5983–5992.

Thaiudom, S., & Goff, H. D. (2003). Effect of κ-carrageenan on milk protein polysaccharide mixtures. *International Dairy Journal, 13*, 763-771.

Tijssen, R. L. M., Canabady-Rochelle, L. S., & Mellema, M. (2007). Gelation upon long storage of milk drinks with carrageenan. *Journal of Dairy Science, 90*, 2604-2611.

Titapiccolo, G., Alexander, M., & Corredig, M. (2010). Rennet-induced aggregation of homogenized milk: Impact of the presence of fat globules on the structure of casein gels. *Dairy Science and Technology, 90*, 623-639.

Townend, R., & Timasheff, S. N. (1960). Molecular interactions in β-lactoglobulin. III. Light scattering investigation of the stoichiometry of the association between pH 3.7 and 5.22. *Journal of the American Chemical Society, 82*, 3168-3174.

Townend, R., Weinberger, L., & Timasheff, S. N. (1960). Molecular interactions in β-lactoglobulin. IV. The dissociation of β-lactoglobulin below pH 3.52. *Journal of the American Chemical Society, 82*, 3175-3179.

Tsioulpas, A., Koliandris, A., Grandison, A. S., & Lewis, M. J. (2010). Effects of stabiliser addition and in-container sterilisation on selected properties of milk related to casein micelle stability. *Food Chemistry, 122*, 1027-1034.

Tuason, D. C., Krawczyk, G. R., & Buliga, G. (2009). Microcrystalline cellulose. In A. Imeson (Ed.), *Food stabilisers, thickeners and gelling agents* (pp. 218). London, UK: Wiley-Blackwell.

Udabage, P., McKinnon, I. R., & Augustin, M. A. (2000). Mineral and casein equilibria in milk: effects of added salts and calcium-chelating agents. *Journal of Dairy Research, 67*, 361-370.

Uskoković, V., & Uskoković, D. P. (2010). Nanosized hydroxyapatite and other calcium phosphates: Chemistry of formation and application as drug and gene delivery agents. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 96B*, 152-191.

Van Kemenade, M. J. J. M., & de Bruyn, P. L. (1989). The influence of casein on the kinetics of hydroxyapatite precipitation. *Journal of Colloid and Interface Science, 129*, 1-14.

Varela, P., & Fiszman, S. (2013). Exploring consumers' knowledge and perceptions of hydrocolloids used as food additives and ingredients. *Food Hydrocolloids, 30*, 477-484.

Verheul, M., Pedersen, J. S., Roefs, S. P. F. M., & de Kruif, K. G. (1999). Association behavior of native β-lactoglobulin. *Biopolymers, 49*, 11-20.

Vreeman, H., Brinkhuis, J., & Van der Spek, C. (1981). Some association properties of bovine SH-κ-casein. *Biophysical Chemistry, 14*, 185-193.

Vyas, H. K., & Tong, P. S. (2004). Impact of source and level of calcium fortification on the heat stability of reconstituted skim milk powder. *Journal of Dairy Science, 87*, 1177-1180.

Wahlgren, M., & Arnebrant, T. (1991). Protein adsorption to solid surfaces. *Trends in Biotechnology, 9*, 201-208.

Wahlgren, M. C., Arnebrant, T., & Paulsson, M. A. (1993). The adsorption from solutions of β-lactoglobulin mixed with lactoferrin or lysozyme onto silica and methylated silica surfaces. *Journal of Colloid and Interface Science, 158*, 46-53.

Walstra, P. (1999). Casein sub-micelles: do they exist? *International Dairy Journal, 9*, 189-192.

Walstra, P., & Jenness, R. (1984). *Dairy chemistry and physics*. New York, NY, USA: John Wiley and Sons.

Wang, A., Yin, H., Liu, D., Wu, H., Wada, Y., Ren, M., Xu, Y., Jiang, T., & Cheng, X. (2007). Effects of organic modifiers on the size-controlled synthesis of hydroxyapatite nanorods. *Applied Surface Science, 253*, 3311-3316.

Wang, J., Zhang, H., Zhu, X., Fan, H., Fan, Y., & Zhang, X. (2013). Dynamic competitive adsorption of bone-related proteins on calcium phosphate ceramic particles with different phase composition and microstructure. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 101B*, 1069-1077.

Wang, K., Zhou, C., Hong, Y., & Zhang, X. (2012). A review of protein adsorption on bioceramics. *Interface Focus*.

Ward, B. R., Goddard, S. J., Augustin, M.-A., & McKinnon, I. R. (1997). EDTA-induced dissociation of casein micelles and its effect on foaming properties of milk. *Journal of Dairy Research, 64*, 495-504.

Wassell, D. T. H., Hall, R. C., & Embery, G. (1995). Adsorption of bovine serum albumin onto hydroxyapatite. *Biomaterials, 16*, 697-702.

Weaver, C. M. (1998). Calcium in food fortification strategies. *International Dairy Journal, 8*, 443-449.

Weaver, C. M., & Heaney, R. P. (2005). *Calcium in human health*. Totawa, NJ, USA: Humana Press.

Weiss, M., & Bibby, B. (1966). Effects of milk on enamel solubility. *Archives of Oral Biology, 11*, 49-57.

WHO. (2004). Calcium. In *Vitamin and mineral requirements in human nutrition* (pp. 59-93). Geneva, Switzerland: World Health Organization.

Wilson, O. C., Jr. (2009). Surface modification of hydroxyapatite: a review. *Advances in Biomedical and Biomimetic Materials: Ceramic Transactions, 96*, 171.

Wilson, O. C., Jr., & Hull, J. (2008). Surface modification of nanophase hydroxyapatite with chitosan. *Materials Science and Engineering: C, 28*, 434-437.

Wong, D. W. S., Camirand, W. M., Pavlath, A. E., Parris, N., & Friedman, M. (1996). Structures and functionalities of milk proteins. *Critical Reviews in Food Science and Nutrition, 36*, 807-844.

Wood, G. B., Reid, D. S., & Elvin, R. (1981). Calculation by computer of individual concentrations in a simulated milk salt solution. I. *Journal of Dairy Research, 48*, 77-83.

Xu, W., Ganz, C., Weber, U., Adam, M., Holzhuter, G., Wolter, D., et al. (2011). Evaluation of injectable silica-embedded nanohydroxyapatite bone substitute in a rat tibia defect model. *International Journal of Nanomedicine, 6*, 1543-1552.

Yanes, M., Duran, L., & Costell, E. (2002). Effect of hydrocolloid type and concentration on flow behaviour and sensory properties of milk beverages model systems. *Food Hydrocolloids, 16*, 605-611.

Ye, A. (2011). Functional properties of milk protein concentrates: Emulsifying properties, adsorption and stability of emulsions. *International Dairy Journal, 21*, 14-20.

Ye, A., & Singh, H. (2006). Heat stability of oil-in-water emulsions formed with intact or hydrolysed whey proteins: Influence of polysaccharides. *Food Hydrocolloids, 20*, 269-276.

Yin, G., Liu, Z., Zhan, J., Ding, F., & Yuan, N. (2002). Impacts of the surface charge property on protein adsorption on hydroxyapatite. *Chemical Engineering Journal, 87*, 181-186.

Yin Hsu, F., Chueh, S.-C., & Jiin Wang, Y. (1999). Microspheres of hydroxyapatite/reconstituted collagen as supports for osteoblast cell growth. *Biomaterials, 20*, 1931-1936.

Yu, W. W., Chang, E., Falkner, J. C., Zhang, J., Al-Somali, A. M., Sayes, C. M., et al. (2007). Forming biocompatible and nonaggregated nanocrystals in water using amphiphilic polymers. *Journal of the American Chemical Society, 129*, 2871-2879.

Zhang, Z., Dalgleish, D. G., & Goff, H. D. (2004). Effect of pH and ionic strength on competitive protein adsorption to air/water interfaces in aqueous foams made with mixed milk proteins. *Colloids and Surfaces B: Biointerfaces, 34*, 113-121.

Zhu, H., & Damodaran, S. (1994). Effects of calcium and magnesium ions on aggregation of whey protein isolate and its effect on foaming properties. *Journal of Agricultural and Food Chemistry, 42*, 856-862.

Zhu, X. D., Fan, H. S., Li, D. X., Xiao, Y. M., & Zhang, X. D. (2007). Protein adsorption and zeta potentials of a biphasic calcium phosphate ceramic under various conditions. *Journal of Biomedical Materials Research Part B-Applied Biomaterials, 82B*, 65-73.

Zittle, C., DellaMonica, E., Rudd, R., & Custer, J. (1958). Binding of calcium to casein: influence of pH and calcium and phosphate concentrations. *Archives of Biochemistry and Biophysics, 76*, 342-353.

APPENDIX 1 Synthesis of HA particles in the presence of milk proteins: a preliminary study

Abstract

In a preliminary work, two different methods were tested to prepare HA particles in the presence of sodium caseinate (SC) or whey protein isolate (WPI). The synthesis of HA particles in the presence of SC was not possible, as caseins precipitated at pH values around their isoelectric point, even at low temperature. In the presence of WPI, stable suspensions of particles could be obtained. Whey proteins probably adsorbed to the surface of the particles, as shown by the negative zeta-potential values, and resulted in particles that were in the micrometre range, possibly because the proteins were not effective at preventing the growth of the particles, or because the shearing and mixing conditions were not strong enough. Further work would be needed to investigate other methods to form surface-functionalised nano HA particles.

Introduction

In Chapter 4, it was shown that the adsorption of caseins and whey proteins onto hydroxyapatite (HA) particles improved the suspension stability of the particles in water. The protein layer around the particles was believed to provide an electrostatic (for whey proteins) and an electrosteric (for caseins) stabilisation to the particles. By charging the surface of the particles negatively (for both whey proteins and caseins), and possibly by forming a protruding layer at the surface of the HA particles (for caseins only), the protein layer increased the repulsion between the particles, rendering them less prone to aggregation and sedimentation over time. However, the HA particles still sedimented to the bottom of the solution within a few days.

These results led to the idea that it might be possible to make nano-sized calcium phosphate particles that are surface-functionalised by a layer of adsorbed proteins, and that could therefore stay in suspension for a long time. Many studies have looked at modifying the surface properties of HA particles to reduce particle aggregation in bioceramic applications, to solve the problems caused by the spontaneous aggregation of HA particles in the processing and use of bioceramic bone implants (Norton et al., 2006). Large chain polymers such as dodecyl alcohol (Borum-Nicholas & Wilson, 2003), silica (Borum & Wilson, 2003), polyethylene glycol (Yu et al., 2007) and dextran (Rölla, 1971) have been used successfully to improve the colloidal stability of HA particles and create stable nano-dispersions, but not in food applications. Polymers commonly used in food products such as gum arabic (Roque & Wilson, 2008) and xanthan gum (Barbour et al., 2005) have also been shown to synthesise HA particles with improved colloidal stability. However, even though many proteins have been shown to bind to already-made HA particles and improve their colloidal properties, for example collagen (Yin Hsu et al., 1999), gelatine (Chang et al., 2003), bovine serum albumin and lysozyme (Brandes et al., 2006) and lactoferrin (Iafisco et al., 2011), none of the work reported in the literature looked at synthesising HA particles in the presence of proteins.

Many methods to prepare stable suspensions of HA nanoparticles have been reported in the literature (Han, Wang, & Li, 2009; Huang et al., 2007; Kandori et al., 2009; Koutsopoulos, 2002; Lee, Choi, Kim, & Lee, 2006; Lee et al., 2011). The most common methods for HA synthesis involves a wet precipitation of HA crystals from supersaturated aqueous solutions containing calcium and phosphate ions in a ratio of calcium to phosphate ions equal to 1.67 (corresponding to the ratio of the respective ions in HA crystals). Various polymers are usually added in the aqueous solution to control the sizes of the particles and obtain a stable

dispersion of nanoparticles, as they prevent the further growth and aggregation of the particles by adsorbing onto the crystal surface. The synthesis of HA nanoparticles also involves a maturation step, which is necessary to obtain a pure crystallised HA solid. The HA suspensions are usually aged at high temperature in dry ovens under a controlled CO_2-free atmosphere. However, the reported maturation times varied a lot between different studies, from a few hours (Kandori et al., 2009; Lee et al., 2011) to a few months (Koutsopoulos, 2002). In the absence of maturation steps, a calcium phosphate precipitate is still obtained, but is usually not pure HA as it contains transient precursor calcium phosphate phases such as β-tricalcium phosphate (β-TCP) or α-TCP, and exhibits only a low crystallinity (Koutsopoulos, 2002; Uskoković & Uskoković, 2010).

The work in this thesis showed that caseins and whey proteins adsorbed to already-made HA particles, and improved the suspension stability of the particles in water. A study by Van Kemenade and de Bruyn (1989) also showed that HA formation was retarded in the presence of caseins, showing that casein bound onto the HA crystals during synthesis and inhibited the growth and maturation of the HA crystals. It may therefore be possible to use caseins from sodium caseinate (SC) or whey proteins from whey protein isolate (WPI) for the synthesis of surface-functionalised HA nanoparticles with milk proteins. The aim of this chapter was therefore to investigate whether it was possible to synthesise HA particles in the presence of SC and WPI, and whether the colloidal properties of the particles (particle size, zeta-potential and suspension stability) were improved, compared with the HA particles synthesised without SC or WPI.

Material and methods

Preparation of HA particles

Two simple methods were used to precipitate HA from aqueous solutions containing calcium and phosphate ions. The goal was not to make HA particles of precise characteristics, but only to precipitate a calcium phosphate phase (likely to be HA), with or without the presence of milk proteins.

Dissolution and pH re-adjustment of suspensions of commercial HA particles

Commercial HA particles (TCP 53-83, see Section 3.1.2.2 for characterisation) were suspended in water at a concentration of 1% (w/w). The pH of the HA suspension was ~7.2. The pH was adjusted to pH 2 using 6 M HCl and 1 M HCl solutions added drop by drop, so the HA particles were fully dissolved. SC or WPI at different concentrations (0 to 1%) were added in 50 mL of the acidic solution containing the dissolved HA, using stock solutions of SC or WPI at pH 2. A solid white precipitate was formed in solution and the suspension was kept aside for analysis.

Chemical wet synthesis of HA particles

HA particles were prepared from calcium hydroxide $Ca(OH)_2$ and calcium phosphate monobasic $Ca(H_2PO_4)_2$, using a method adapted from Han et al. (2009).

Preparation of saturated calcium hydroxide solution

A saturated calcium hydroxide solution was prepared by adding 5 g of $Ca(OH)_2$ in a 1 L Schott bottle filled up to the top with Milli-Q water. The solution was stirred for three days in the fridge. The solution was then poured through a Buchner funnel equipped with a filter paper and filtered under vacuum and stored in an air tight container.

Preparation of calcium phosphate monobasic monohydrate solution

A stock solution of calcium phosphate monobasic monohydrate $Ca(H_2PO_4)_2$ was prepared by dissolving 10 g of $Ca(H_2PO_4)_2$ in 1 L of water. The pH was ~3.8. It was adjusted to pH 2.7 to allow complete dissolution of the powder.

Preparation of HA

20 mL of $Ca(H_2PO_4)_2$ were stirred in a 100 mL plastic container using a magnetic stirrer. WPI or SC were added to the $Ca(H_2PO_4)_2$ solution at different concentrations (0 to 1%) using stock solutions and stirred until complete dilution. Under constant stirring, 80 mL of saturated calcium hydroxide solution was added. A white precipitate was formed in suspension and kept aside for analysis.

Characterisation of HA particles

The HA particles obtained with the two different methods were analysed for particle size by laser diffraction (See Section 3.3.1), photon correlation spectroscopy using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd.) and for zeta-potential (See Section 3.3.2). Photographs of the HA suspensions were taken after leaving the suspensions undisturbed on the bench for 24 h.

Real-time absorbance monitoring of HA particles precipitation

The synthesis of HA particles described in Section 0 was carried out at a smaller scale and using a smaller concentration of initial HA particles in a Jasco V-560 spectrophotometer (Japan Spectroscopic Co.), to monitor in real-time the precipitation of HA particles. HA particles (0.25%, w/w) were dissolved by dropping the pH to pH 2 with 6 M HCl. Two milliltres of the solution were placed in a plastic cuvette inside the spectrophotometer and 1 M NaOH was added to the solution in the cuvette in 10 μL aliquots, under constant stirring. The pH was left to equilibrate before adding the next aliquot of NaOH, and was recorded every minute. Absorbance values at a wavelength of 900 nm were recorded every ten seconds, as absorbance values indicated the change of turbidity caused by the formation of the HA precipitate.

Results

HA particle characterisation

Particles synthesised by dissolution and pH re-adjustment of suspensions of commercial HA particles, in the presence of WPI at different concentrations

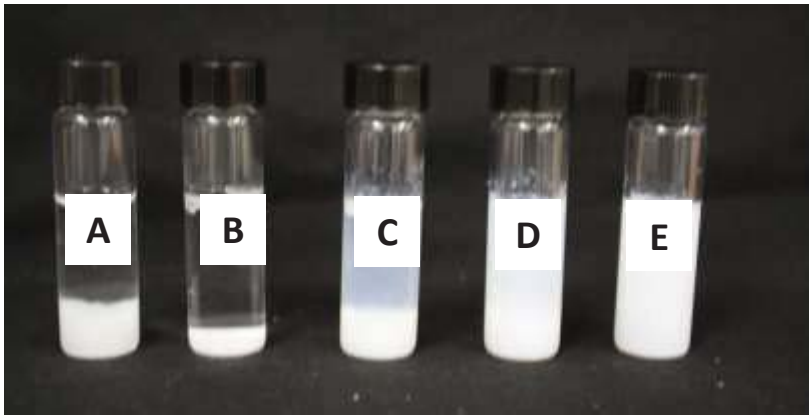

Figure A1: Suspension of particles prepared by dissolution and pH re-adjustment (to pH 7.2) of suspensions of HA particles, containing different concentrations of WPI. A, control, no WPI; B, 0.1 % WPI; C, 0.25% WPI; D, 0.5% WPI; E, 1% WPI.

Table A1: Characteristics of WPI-coated particles made by progressive increase of pH of an acidic solution of dissolved 0.25% (w/w) HA particles.

Sample in Figure 8.1	WPI concentration (%)	Final pH	Particle size (μm)	Zeta-potential (mV)
A	0	7.05	23.2	-4
B	0.1	7.1	13.7	-10
C	0.25	6.98	15.6	-13.3
D	0.5	7.03	9.9	-15.4
E	1	7.13	8.5	-16

- Readjusting the pH of a solution of dissolved HA particles to pH 7.2 using aliquots of NaOH led to the formation of particles. The nature of the precipitate is probably not HA, but an amorphous form of calcium phosphate (George and Veis, 2008).

- As WPI concentration increased, the particle size of the particles decreased and the zeta-potential of the particles became more negative (Table A1), showing that whey proteins probably adsorbed to the particles during their precipitation.

- However, the obtained particles were of micrometre size, even in the presence of WPI, showing that whey proteins did not prevent the growth of the particles and therefore did not allow the formation of nano-metre particles. The binding of the whey proteins onto the particles in formation may not be effective at preventing the growth and aggregation of the particles, or the conditions of mixing and shearing may not be strong enough to form nanosized-particles.

- Making calcium phosphate particles with this method in the presence of SC (at different concentrations from 0 to 1%, w/w) did not work, as the caseins precipitated at the same time as calcium phosphate particles precipitated. The precipitate obtained in suspension sedimented almost immediately and was believed to be made of a mixture of large calcium phosphate particles and large casein aggregates. The experiment was also carried out at low temperature (4 °C) to try to avoid casein precipitation but the caseins still precipitated.

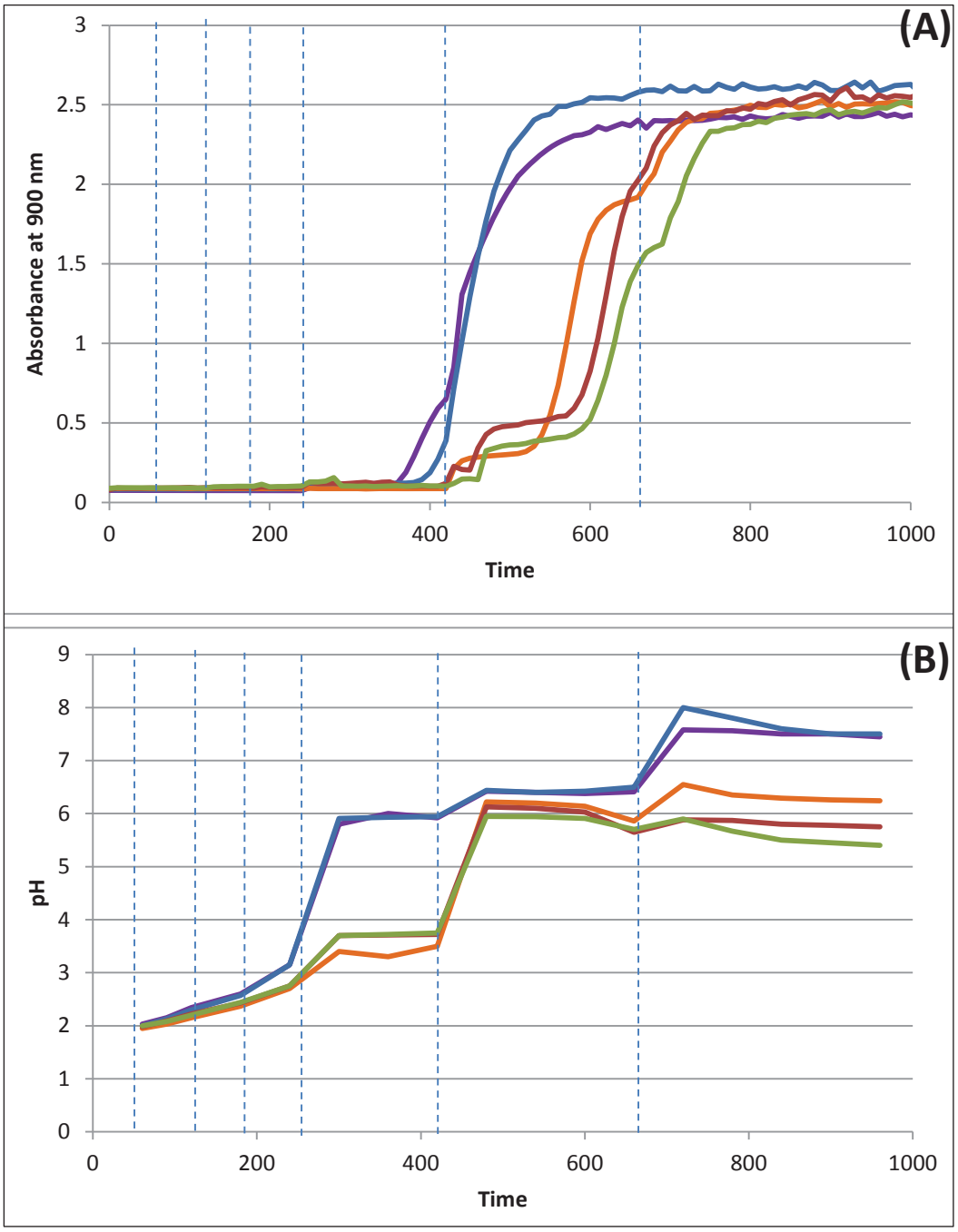

Figure A2: Real-time absorbance (Panel A) and pH (Panel B) monitoring during particles precipitation, when particles were prepared by dissolution and pH re-adjustment of suspensions of HA particles with different WPI concentrations. Purple line, no WPI; blue line, 0.1% (w/w) WPI; orange line, 0.25% (w/w) WPI; red line, 0.5% (w/w) WPI; green line, 1% (w/w) WPI. The dashed lines correspond to the addition of one 10 µL aliquot of NaOH in the spectrophotometer cuvette.

- pH and absorbance were recorded over time during the precipitation of the particles. The presence of WPI seemed to delay the formation of the particles. For any given amount of NaOH added, the pH increased less for the samples containing whey protein than for samples without whey protein. Also, the particles started to precipitate after a longer time (after about 400 s) for solutions containing 0.25%, 0.5% and 1% WPI compared to solutions containing no WPI or 0.1% WPI (after about 250 s). This may be due to the buffering capacity of the whey proteins, and a method would be needed to adjust the pH to always the same pH value for the samples containing different amounts of whey proteins, and measure the absorbance at equal pH values.

- The precipitation of the particles occurred mostly in the pH range 4 to 6, close to the pI of the whey proteins. This suggests that the whey proteins are not sufficiently charged to prevent the precipitation of the particles.

Particles synthesised by chemical wet synthesis

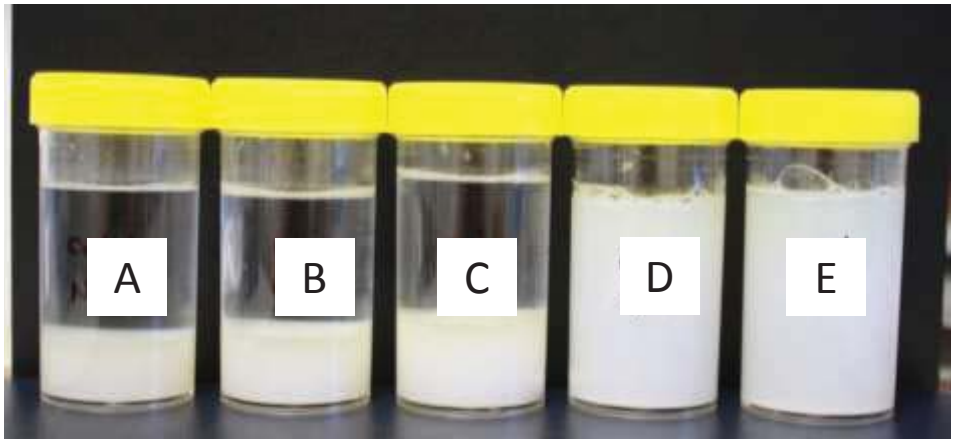

Figure A3: Suspension of particles prepared by chemical wet synthesis from calcium hydroxide $Ca(OH)_2$ and calcium phosphate monobasic $Ca(H_2PO_4)_2$ and different concentrations of WPI. A, control, no WPI; B, 0.05%; C, 0.1%; D, 0.5%; E, 1%.

Table A2: Characteristics of WPI-coated particles made by wet chemical synthesis

Sample in Figure 8.3	WPI concentration (%)	Final pH	Particle size (μm)	Zeta-potential (mV)
A	0	5.4	22.9	-2.85
B	0.05	5.5	20.6	-14.5
C	0.1	5.58	18.5	-17.9
D	0.5	5.64	10.9	-22.9
E	1	5.60	8.7	-21.3

- Mixing calcium hydroxide $Ca(OH)_2$ and calcium phosphate monobasic $Ca(H_2PO_4)_2$ in the presence of WPI (0 to 1%, w/w) also led to the formation of a white precipitate, probably again an amorphous calcium phosphate phase (George and Veis, 2008). As was the case with the dissolution and pH re-adjustment method, as the concentration of WPI increased, the particles became stable in suspension. The particles prepared with 0.5% and 1% WPI were stable for a few days, with no sedimentation observed in the bottles (Figure A3).

- As the WPI concentration increased, the particles had smaller particle size and more negative zeta-potential values, showing that the whey proteins must have bound to the particles, and the improvement of suspension stability of the particles must be correlated to the adsorption of the whey proteins. However, the particles obtained were of micrometre size, and it appears that, as was the case with the dissolution and pH re-adjustment method, making nano-size particles of calcium phosphate in the presence of whey proteins is unlikely to work with this method. The mixing and shearing conditions were probably not strong enough to allow nanosized particles to form.

- This method did not work in the presence of caseins; as was the case with the dissolution and pH re-adjustment method, casein precipitated and a “slushy” white precipitate was obtained that sedimented to the bottom of the containers within an hour.

APPENDIX 2 Poster

The poster was presented at the São Paulo School of Advanced Science Advances in Molecular Structuring of Food Materials, Pirassununga, April 1st – 5th, 2013.

Binding of milk proteins onto calcium phosphate particles to improve their suspension stability

L. Tercinier [1,2], A. Ye[1], S.G. Anema[2], A. Singh[2], H. Singh[1]
[1]Riddet Institute, Massey University, New Zealand; [2]Fonterra Co-operative Group Limited, New Zealand
lucile.tercinier@fonterra.com

Introduction

- Insoluble calcium phosphate salts are often used for calcium fortification of milk. **Proteins are known to adsorb to calcium phosphate materials**, for example on hydroxyapatite (HA) chromatography columns or on bone substitute materials [1].
- The aim of this study was to investigate **whether or not milk proteins interact with insoluble food-grade HA particles**. Adsorption studies were carried out and the effect of protein binding on the particles stability was examined.

Materials & Methods

Results

Adsorption isotherms and modeling

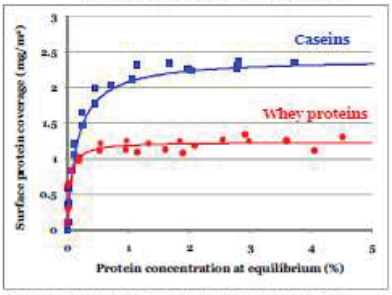

Figure 1. Isotherms of milk proteins adsorbed on to HA. The points are from experimental data. The lines are the best-fit Langmuir model curves.

Surface composition

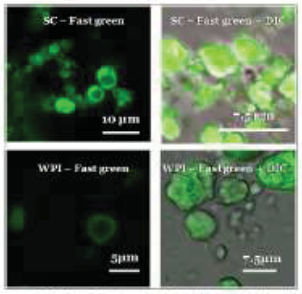

Figure 2. Confocal microscopy of protein-coated particles. Fast Green channels and overlapping of Fast Green and DIC (differential interference contrast) channel.

Surface charge

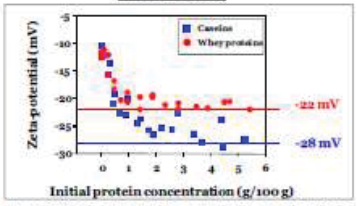

Figure 3. Effect of initial protein concentration on zeta-potential of HA particles made with SC or WPI solutions of different concentrations. Particles were centrifuged, rinsed with water and and re-suspended in water..

Suspension stability

Figure 4. Suspension stability of particles made with increasing concentrations of SC. Particles were rinsed with water and left to sediment for 24h.

Preferential adsorption

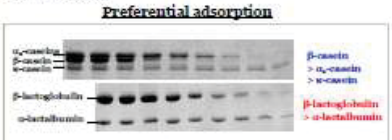

Figure 5. SDS-PAGE gel obtained from the supernatants of samples made with constant SC concentration and increasing HA concentrations

- Both caseins and whey proteins bound to the surface of HA particles. However, caseins had a higher maximum surface coverage, as shown by the Langmuir adsorption isotherms (Figure 1) and the brightness and thickness of the Fast Green protein layer on the microcopy images (Figure 2). β-casein and β-lactoglobulin were the two preferred proteins for adsorption (Figure 5).
- The surface charge of the particles increased after adsorption of caseins or whey proteins. SC-coated particles were more negatively charged than WPI-coated particles (Figure 3). The suspension stability of protein-coated particles was improved in water (Figure 4), due to steric and electrostatic stabilisation.

Discussion

- The adsorption of milk proteins onto HA particles was driven by **electrostatic interactions** [2] (Figure 6). It was explained by a **mixed ion pairing between the calcium-sites of HA and the carboxyl groups of the milk proteins** and, to a lesser extent, between the phosphate-sites of HA and the amino groups of the milk proteins.
- A **specific interaction between negative phosphoserine clusters in casein molecules and the calcium-site of HA** explained the higher affinity of caseins for HA [3].

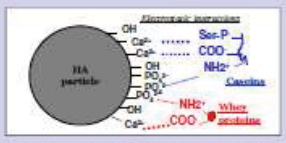

Figure 6 . Proposed mechanism to explain the surface interactions between HA and milk proteins.

Conclusion

The suspension stability of calcium phosphate particles was enhanced by coating the particles with milk proteins. This work demonstrates the possibility of surface modification of food-grade nano or micro-sized particles, that could be potentially of interest in the design of food products with improved stability and functional properties.

References

[1] Q. Luo, J.D. Andrade, J. Colloid Interface Sci. 200 (1998) 104
[2] L. Tercinier, A. Ye, S.G. Anema, A. Singh, H. Singh, J. Colloid Interface Sci. 394 (2013) 448.
[3] T. Kawasaki, J. Chromatogr. A 544 (1991) 147

Riddet Institute
FOOD | INNOVATION | HEALTH

Fonterra
Dairy for life