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Heat-induced colloidal interactions of whey proteins, sodium caseinate and gum arabic in binary and ternary mixtures

Simon M. Loveday ^a * s.loveday@massey.ac.nz

Aiqian Ye ^a a.m.ye@massey.ac.nz

Skelte G. Anema ^b skelte.anema@fonterra.com

Harjinder Singh ^a h.singh@massey.ac.nz

a: Riddet Institute, c/o Massey University, Private Bag 11222, Palmerston North, New Zealand

b: Fonterra Research Centre, Dairy Farm Road, Private Bag 11029, Palmerston North, New Zealand

* corresponding author:

phone +64 6 356 9099 extension 81375 fax +64 6 350 5655

Abstract

Many food-grade proteins and polysaccharides will aggregate together when acidified or heated, due to electrostatic and hydrophobic interactions. At low concentrations, aggregates are soluble and colloidally stable, and they have potential applications as Pickering emulsifiers and nutrient carriers. Sodium caseinate (SC) and gum arabic (GA) at pH 7 will form colloidal aggregates when heated, but aggregation is largely reversed on cooling. Whey proteins (in the form of whey protein isolate, WPI) will aggregate irreversibly with GA when they are heated together, but aggregation is often so rapid and extensive that aggregates precipitate. Here we sought to overcome those limitations, and to develop an *in situ* method for quantifying heat-induced aggregation. Aggregation was measured using temperature-controlled dynamic light scattering equipment and transmission electron microscopy. Combinations of SC, WPI and GA were heated at either pH 7 or 3.5, and the weight ratio of protein to polysaccharide was held at 1:5 for simplicity. Heat-induced colloidally stable aggregates of SC + WPI + GA did not dissociate on cooling. Aggregation was measured *in situ*, both in temperature ramps and with isothermal experiments. *In situ* measurement allowed us to avoid potential artefacts stemming from the temperature changes and measurement delays associated with *ex situ* measurements. This work demonstrated how the size and heat-stability of colloidal protein-polysaccharide aggregates can be tailored by judicious selection of proteins, pH and heat treatment.

Keywords: Whey protein, casein, gum arabic, dynamic light scattering, colloidal aggregation, heat treatment

1 Introduction

Proteins and polysaccharides are common ingredients that are often used together in foods to manipulate viscosity, gelling, foaming, and emulsification behaviour. They can undergo segregative or associative interactions, resulting in phase separation, co-solubility or the formation of soluble or insoluble complexes (De Kruif, Weinbreck, & De Vries, 2004; Doublier, Garnier, Renard, & Sanchez, 2000; Ye, 2008). Here we focused on colloidal stable nano- and micro-particles, which may have applications as nutrient delivery vehicles (Zimet & Livney, 2009), fat mimetics (Paquin, 1999), antimicrobial agents (Chang, McLandsborough, & McClements, 2011a, 2011b) or Pickering emulsifiers (Dickinson, 2012).

Complexation between proteins and polysaccharides can be driven by hydrogen bonding, hydrophobic interactions or poor solvent conditions, but often the dominant driving force for complexation is electrostatic interactions (Doublier et al., 2000). Electrostatic complexing between proteins and polysaccharides in food systems is well-known, and applications of this phenomenon include fractionating mixtures of proteins and enhancing the stability of soluble proteins undergoing thermal treatments or changes to ionic conditions (Ye, 2008). Controlled acidification of protein-polysaccharide mixtures can produce colloidal nanoparticles by electrostatic association (Ye, Flanagan, & Singh, 2006). The main factors controlling association in this scenario are protein surface charge density, polysaccharide linear charge density, ionic strength and the ratio of protein to polysaccharide (Mattison, Wang, Grymonpre, & Dubin, 1999).

Unmodified protein-polysaccharide nanoparticles tend to disperse or aggregate when solution conditions change markedly, e.g. on dilution, pH change or the addition of salts. However nanoparticles can be stabilized with enzymatic cross-linking (Flanagan & Singh, 2006) or heat treatment (Jones & McClements, 2011).

Nanoparticles similar to those produced by acidification can be formed at neutral pH by heating dilute mixtures of protein and polysaccharide, which promotes reversible aggregation *via* hydrophobic interactions (Ye, Edwards, Gilliland, Jameson, & Singh, 2012). From a food processing point of view, heating at neutral pH is preferable to acidification because heating is a common unit operation in the food industry, and acidic solutions are corrosive to equipment. In the case of sodium caseinate (SC) and gum arabic (GA) (Ye et al., 2012), the transient nature of association is a barrier to the development of food ingredients from heat-induced protein-polysaccharide complexes.

Sodium caseinate is prepared by acidifying milk to pH \sim 4.6, separating and washing the precipitated curd, solubilising it by neutralizing to pH 6.6-6.8 with NaOH, then spray-drying (O'Regan & Mulvihill, 2011). SC is a mixture of the four major casein fractions (α_{s1} , α_{s2} , β and κ) aggregated into colloidal particles (Farrell, 2011), and is a common food ingredient in gel- and emulsion-based foods (O'Kennedy, 2011).

Whey protein isolate (WPI) contains >90% w/w protein, most of which is β -lactoglobulin (β -lg). β -lg is a globular protein comprising 162 amino acids, has an isoelectric pH of \sim 5.1 (Farrell Jr. et al., 2004), and undergoes covalent aggregation via disulphide bonds when heated at neutral pH (Schokker, Singh, Pinder, Norris, & Creamer, 1999). Electrostatic interactions between β -lg or WPI and various polysaccharides have been studied during acidification at 20-25°C (Sanchez, Mekhloufi, & Renard, 2006; Weinbreck, de Vries, Schrooyen, & de Kruif, 2003), or by heating at mildly acidic pH (Jones & McClements, 2011). WPI is substantially more expensive than SC, so cost is a barrier to its use in food manufacturing, but it is also highly reactive (Loveday, Hindmarsh, Creamer, & Singh, 2010), and susceptible to undesirable reactions such as Maillard browning.

Gum Arabic (GA) is exuded by trees of the *Acacia* spp., and is distinguished among food polysaccharides by containing a glycoprotein fraction with a structure thought to consist of a polypeptide backbone to which large carbohydrate 'blocks' are attached (Islam, Phillips, Slijvo, Snowden, & Williams, 1997; Mahendran, Williams, Phillips, Al-Assaf, & Baldwin, 2008). This gum, also known as acacia gum, is a good emulsifier due to its protein moiety, and it is widely used for encapsulating flavour oils in beverages.

Here we studied heat-induced complex formation between SC and GA, and between WPI and GA, both at pH 7 where negative charges predominate and at pH 3.5 where opposing charges will drive electrostatic attractions. We also explored the synergy between SC and WPI by making heat-induced nanoparticles in ternary solutions of WPI, SC and GA. We used a protein:polysaccharide weight ratio of 1:5 because it produced complexation in previous work (Ye et al., 2012), and we kept this ratio constant while modifying other parameters for the sake of simplicity. Our results show how the size and stability of colloidal protein-polysaccharide nanoparticles can be modulated by varying biopolymer concentrations, pH, temperature, and protein type.

2 Materials and Methods

2.1 Materials

SC (Alanate 185) and WPI (Alacen 895) were supplied by Fonterra Cooperative Ltd, Auckland, NZ. GA was supplied by Bronson and Jacobs Ltd., Auckland NZ. All chemicals were of analytical grade, and were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2 Preparation of WPI, SC and GA mixtures

SC, WPI and GA powders were dissolved in Milli-Q water separately under gentle stirring at room temperature for at least 2 h, and stock solutions were stored at 4 °C overnight to allow complete hydration. The pH of solutions was adjusted with NaOH and HCl at room temperature, and solutions were passed through 0.2 µm filters (Millipore, Billerica, Massachusetts, USA) to remove large aggregates. All concentrations are % w/v unless otherwise specified.

2.3 Notation

Mixtures of WPI, SC and GA are denoted using vertical bars ('|') to separate percentages w/v of each component, and the '%' sign is omitted for brevity. For example, a mixture of 0.25% WPI, 0.25% SC and 2.5% GA is denoted 0.25WPI|0.25SC|2.5GA.

2.4 Alkaline polyacrylamide gel electrophoresis

Native PAGE on alkaline polyacrylamide gels followed the method of Manderson *et al.* (1998), except that the running buffer was at pH 8.8 and the sample buffer comprised 2.5 mL of 0.5 M Tris-HCl, 8 mL glycerol, 0.4 mL of 0.5% w/v bromophenol blue and 9.1 mL Milli-Q water. Gels were scanned on a Molecular Imager Gel Doc XR system and integrated with Quantity One software (Bio-Rad Laboratories, Hercules, California, USA).

2.5 Particle size analysis

The hydrodynamic diameter of aggregates was measured by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern Instruments Ltd, Worcestershire, England), which measured scattering intensity at an angle of 173° and controlled the temperature of the measuring cell to an accuracy of ± 0.1°C. The refractive index of particles was assumed to be 1.45, and the

temperature-dependent viscosity of water was used in calculations. In heating ramp experiments, the temperature was varied between 25°C and 80°C in steps of 5°C, and two size measurements were taken at each temperature using automatic laser attenuation, measurement position and run number settings. The means of duplicate measurements are shown in figures, and vertical bars are the min and max of duplicates.

For isothermal experiments, the instrument was configured to seek the optimum laser attenuation setting for each new measurement, but the number of runs per measurement and the run time were fixed (6 runs of 10 s), and measurement position was fixed at a position chosen with prior experimentation. The zetasizer software calculated z-average diameter and polydispersity index (PDI) using cumulants analysis, and also did distribution analysis using a non-negative least squares fitting algorithm. The particle size distribution calculation was set to 'general purpose' mode unless otherwise specified.

2.6 Transmission electron microscopy

Negative staining and microscopy followed published methods (Loveday, Wang, Rao, Anema, & Singh, 2012), except that dispersions were not centrifuge-filtered or diluted.

3 Results

3.1 Single-component control experiments

Control experiments involving heating WPI, SC or GA alone were carried out at pH 7 and pH 3.5 (Table 1), and concentrations of 1% for proteins and 5% for GA were chosen so that any self-aggregation would be clearly observable. In 1% WPI solutions, the heating ramp appeared to cause a decrease in particle size at both pHs. However, the derived count rate (DCR) showed a 231% increase in scattering as a result of heating at pH 7, which indicates an increase in size and/or number of particles. Re-analysis of scattering data with the 'multiple narrow modes' function in zetasizer software gave peaks at 40-84 nm and 215-251 nm in unheated samples, and volumes of material in each population were approximately equal. Re-analysing data from heated samples gave peaks at 27-31 nm and 144-155 nm, with 90% of particle volume in the smaller peak. Results suggested that two phenomena occurred as a result of heating and cooling: a) breaking of large, loosely-associated aggregates into smaller components, and b) aggregation of molecules and very small aggregates whose scattering signals were swamped by those from larger particles in unheated samples.

Table 1. Particle size in control solutions of WPI, SC or GA alone at pH 7 or 3.5 before and after heating to 80°C in temperature ramp experiments. Data are the average of duplicates.

pH	solution components (% w/w)			particle diameter (nm)		Derived count rate (kilo counts per second)	
	WPI	SC	GA	unheated	heated	unheated	heated
7	1	0	0	62 & 233 ^a	29	7,861	26,051
7	0	1	0	241	245	25,140	31,013
7	0	0	5	6.0	6.4	21,291	21,084
3.5	1	0	0	217	161	14,751	13,533
3.5	0	1	0	26	40	51,468	124,868
3.5	0	0	5	8.6	7.8	27,531	26,761

a: approximately equal volumes of material in two particle populations

The 1% SC control at pH 7 contained a monomodal population of particles with diameter centred at 241 nm. This was somewhat larger than in earlier reports (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008), and probably reflected differences in materials and sample preparation protocols. Our earlier work (Ye et al., 2012) showed particles of comparable size to those seen here, as well as small concentration-dependent changes in particle size in SC solutions. SC showed substantial but completely reversible self-aggregation when heated at pH 7, probably due to hydrophobic associations, as seen in other studies (Ye et al., 2012). At pH 3.5, aggregates were much smaller than those obtained at pH 7.0, and appeared to become larger with heating and cooling. Heating increased the DCR by 143%. Distribution analysis clearly showed two peaks at 170-193 nm and 22-28 nm in unheated samples. The two populations scattered at approximately equal intensity, and on a volume basis 98% of material was in the smaller size range. Heating and cooling produced a monomodal distribution centred at 39-41 nm. Distribution analysis showed that typically >98% v/v of GA particles were 5-10 nm in diameter, and no change in particle size occurred with heating at either pH.

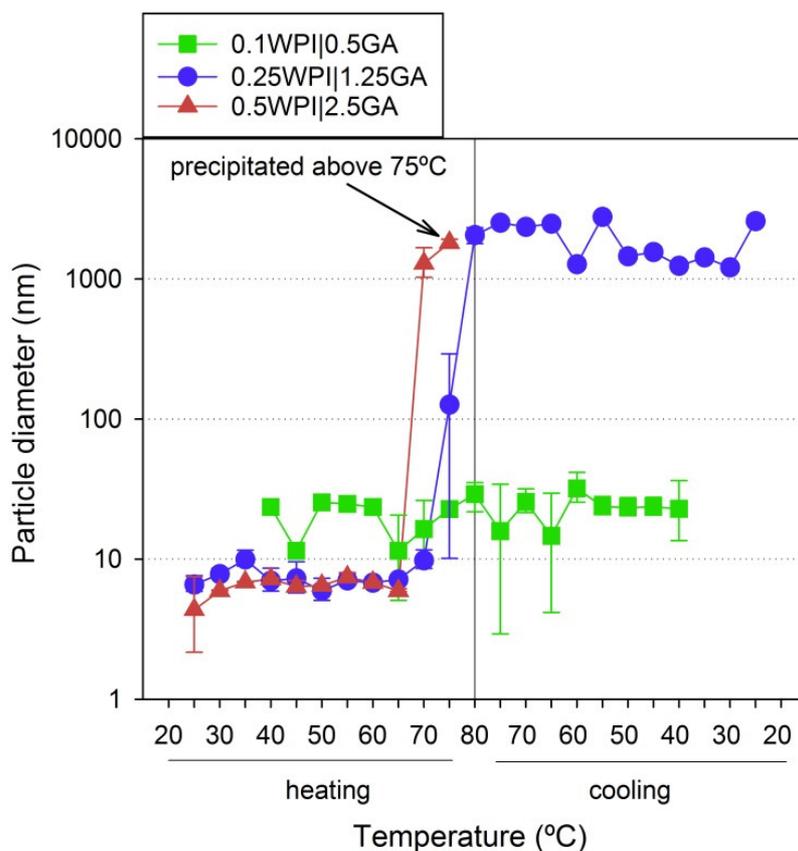
3.2 Binary Mixtures of SC and GA at pH 7

Heat-induced association in 0.1SC|0.5GA was examined in our previous work (Ye et al., 2012), although a slightly different measurement protocol was used. Very similar results were obtained with the measurement protocol used in the present work (see Figure S1 in Supplementary Material), and here we also tested higher concentrations at the same SC:GA ratio. SC|GA mixtures at all concentrations initially contained particles 6-11 nm in diameter, and aggregation occurred above a threshold temperature (35 - 50 °C), which decreased as concentration increased. Aggregation was mostly reversible on cooling; final particle size readings were up to 250 nm, but DCRs were similar to baseline levels before heating (Supplementary Material, Figure S2).

3.3 Binary Mixtures of WPI and GA at pH 7

A 20-30 nm complex was formed when 0.1% WPI and 0.5% GA were mixed at ambient temperature (particles this size were not seen in WPI or GA control solutions), and heating had no effect on particle size (Figure 1). However at higher concentrations, WPI and GA aggregated rapidly and irreversibly above 65 °C, and aggregation was so extensive in 0.5WPI|2.5GA that precipitation occurred. Heat-induced unfolding and aggregation of proteins (especially β -lactoglobulin) was the most likely factor triggering aggregation above 65 °C.

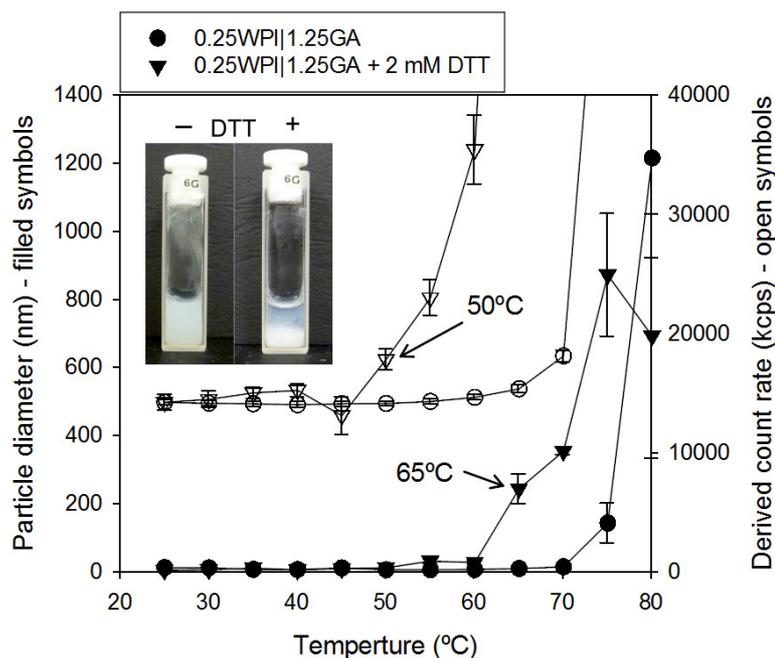
Figure 1. Heat-induced aggregation in heated WPI|GA mixtures at pH 7 and a range of concentrations.



The role of disulphide bonding was investigated by heating 0.25WPI|1.25GA with 2 mM dithiothreitol (DTT), which will reduce disulphide bonds and prevent the formation of new disulphide bonds. Aggregation began at 65°C with DTT (Figure 2), ten degrees lower than in non-reducing conditions, and the DCR showed early indications of aggregation beginning at 50°C with DTT, twenty degrees lower than the corresponding non-reduced sample. This suggested that intramolecular disulphide bonding inhibited aggregation, but also that disulphide bonding was not the only interaction driving aggregation. Even in mixtures with DTT, heating was necessary to induce aggregation, and hydrophobic interactions (Ye et al., 2012) were the most likely explanation for this.

The stepped temperature ramp protocol provided a broad picture of temperature-related effects on aggregation, but it had some disadvantages. In temperature ramp experiments, it took approximately 4 min to adjust and stabilise the temperature at each step. The time to complete a single size measurement, which depended on the number of runs selected by the software, varied in almost all cases from 110 s to 180 s (11-18 runs of 10 s each). The number of runs selected by the software depended on the size and polydispersity of particles, thus the holding time at each temperature varied slightly. This produced a variable temperature history, and a single temperature ramp experiment took up to 3.5 hours. Phenomena observed at a given temperature in a stepped heating ramp were a result of both the temperature *per se* and the thermal history of the sample.

Figure 2. Effect of a reducing agent, dithiothreitol (DTT), on aggregation of WPI and GA at pH 7 during heating. The inset shows the appearance of samples after cooling to 20 °C.



Isothermal holding experiments overcame some of these disadvantages. Samples were rapidly heated then held at a constant temperature, and particle size measurements were taken repeatedly over a period of time. The zetasizer was pre-heated to the required temperature before the sample cell was inserted, but it still took just over 2 min for the instrument to heat the sample and stabilise at the set temperature. Scattering data were collected during 6 runs of 10 s in order to capture the kinetics of aggregation. This produced noisier distribution analysis results than heating ramp experiments, which typically involved 12-14 data collection runs on the automatic setting. Polydispersity was low in the isothermal experiments, and cumulants analysis proved more resilient to data noise than distribution analysis.

Results from isothermal experiments in Figure 3 show that the rate of aggregation in mixtures of WPI and GA was highly sensitive to temperature in the range 72-78°C. Particle size increased most rapidly at the start of heating, and the rate of increase became less with time. Particle size distributions from an isothermal experiment at 75°C are shown in Figure 4. Although the '0 min' result is from a measurement taken several min after the sample was placed in the instrument (time taken for temperature to stabilise), the particle size distribution did not change materially during this time.

Figure 3. Aggregation in 0.25WPI|1.25GA mixtures during isothermal heating at different temperatures.

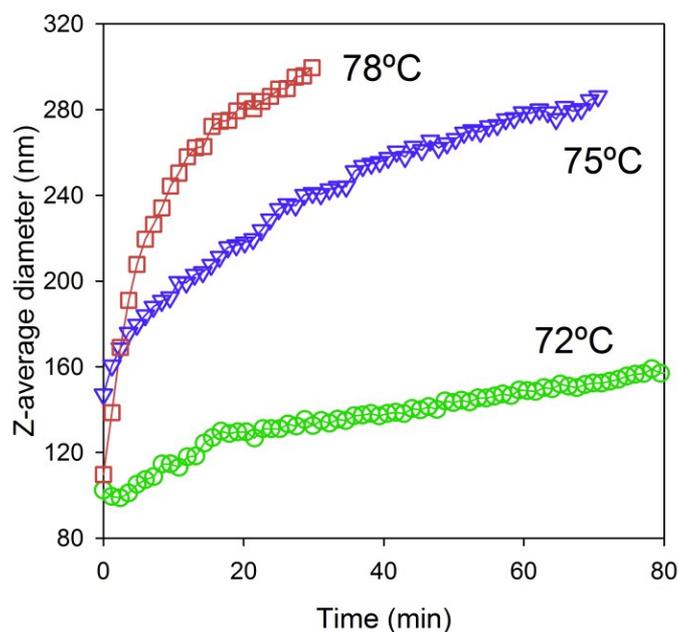
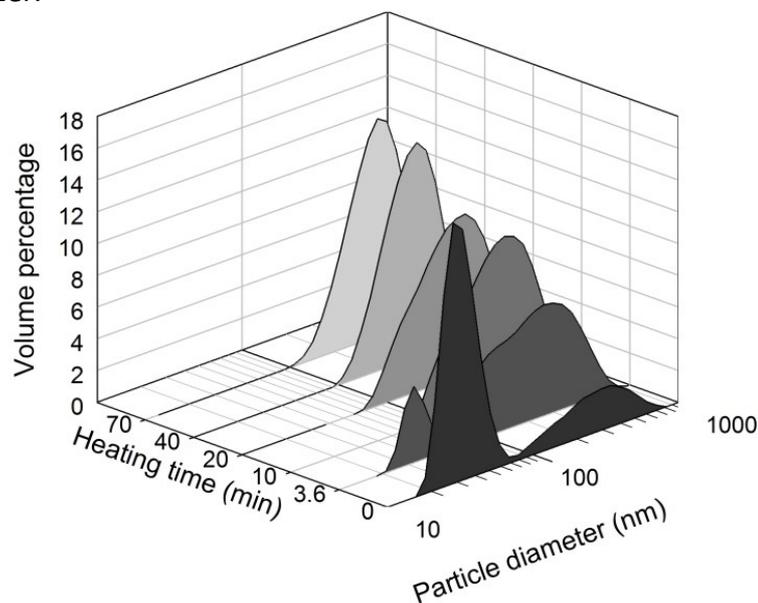


Figure 4. Particle size distributions measured during isothermal heating of 0.25WPI|1.25GA at 75°C in the zetasizer.



Initially there were two populations of particles – 75 % v/v of particles had diameter 20-30 nm and the remainder were 100-500 nm in diameter. The 20-30 nm particles disappeared after 5 min, and only 100-500 nm particles were seen at 5-20 min. With further heating, particle size became more homogeneous and slightly larger.

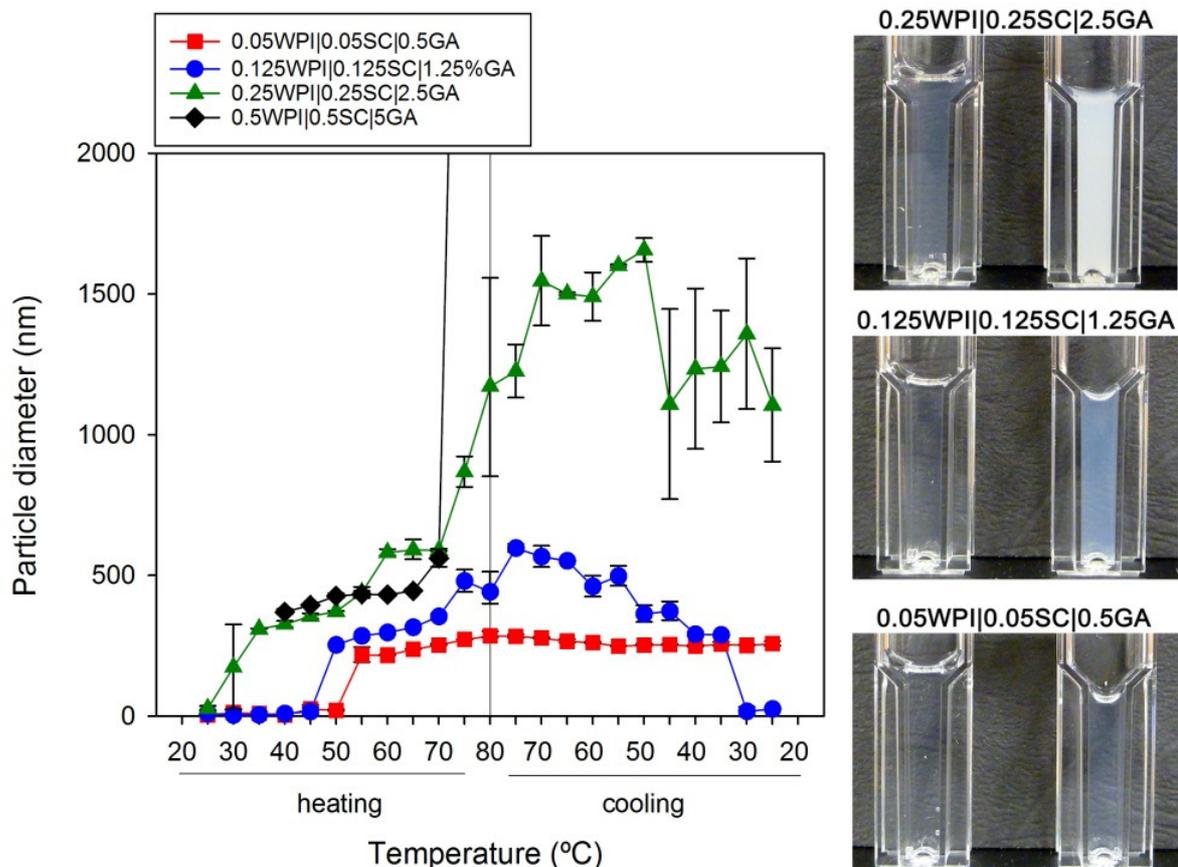
Samples heated at 75°C in the zetasizer for various times were examined with alkaline native PAGE (see Supplementary Material, figure S3). With an increase in heating time, the proportion of native proteins decreased, concomitant with an increase in the amount of large aggregates that wouldn't enter the gel. Densitometry showed that the decrease in the amount of the native β -lactoglobulin was approximately linear with time (data not shown).

3.4 Ternary Mixtures of WPI, SC and GA at pH 7

The behaviour of ternary mixtures showed elements of both WPI|GA character and SC|GA character (Figure 5). With 0.05WPI|0.05SC|0.5GA and 0.125WPI|0.125SC|1.25GA, mixtures aggregated above 45-50 °C, and aggregation was partly reversible, as with SC|GA mixtures. The mixture of 0.5WPI|0.5SC|5GA aggregated dramatically and irreversibly above 70 °C, as with WPI|GA mixtures. However the intermediate concentration, 0.25WPI|0.25SC|2.5GA began aggregating above 25 °C, then the particle size plateaued between 35 and 70 °C, followed by a further large increase in particle size above 70 °C, which was irreversible on subsequent cooling.

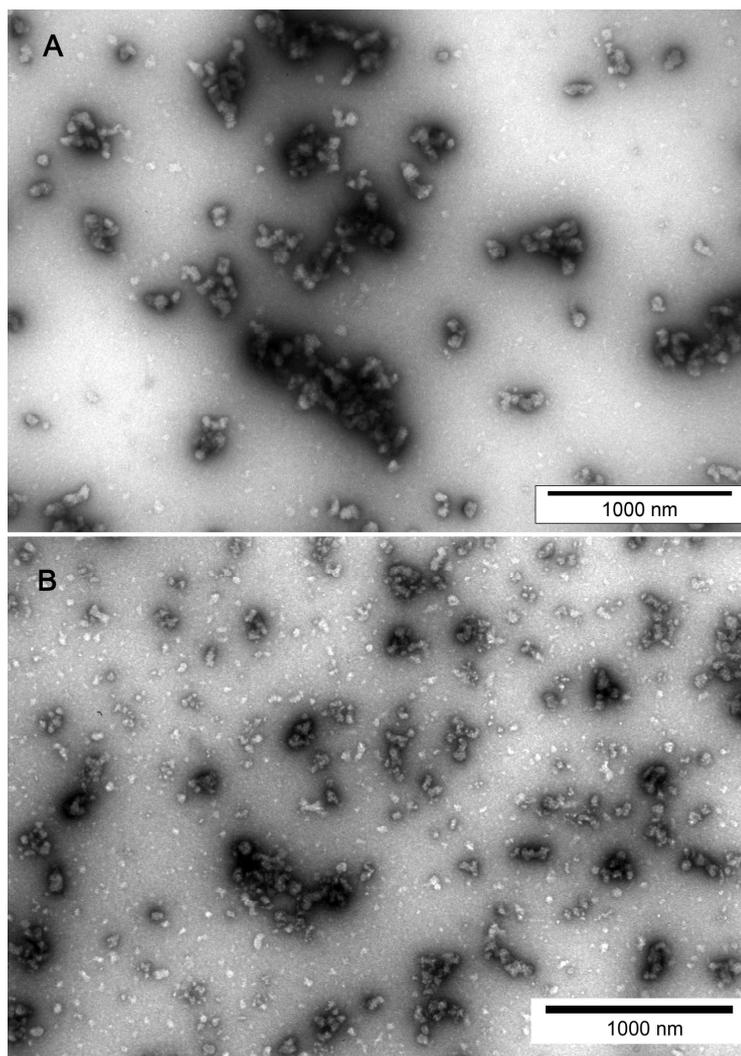
The appearance of heated mixtures was consistent with the particle size results (Figure 5), in that colloiddally stable dispersions were produced at the three lower concentrations, and dispersions were increasingly opaque with increasing concentration. The particle diameter for 0.125WPI|0.125SC|0.5GA appears to return to the original size in Figure 5, but this is because 55% v/v of the particles were ~30 nm in diameter and 45% v/v were ~265 nm. The DCR for 0.125WPI|0.125SC|0.5GA was an order of magnitude higher than for 0.05WPI|0.05SC|0.5GA, in agreement with the higher turbidity, indicating that aggregation was more extensive at the higher concentration.

Figure 5. Heat-induced aggregation in WPI|SC|GA solutions at pH 7 and different concentrations. Photos show mixtures before *in situ* heating (left cuvette) and after heating (right cuvette).



TEM images of heated 0.25WPI|0.25SC|2.5GA dispersions (Figure 6A) showed primary aggregates that were a few hundred nm in diameter, and they were grouped into much larger clusters with dimensions of up to several thousand nm. Primary aggregates were larger and more rounded than those in heated SC|GA dispersions (Figure 6B), and clustering was more common and extensive when both proteins were present.

Figure 6. Transmission electron micrograph of aggregates formed in mixtures of 0.25WPI|0.25SC|2.5GA (A) or 0.5SC|2.5GA (B) at pH 7 after *in situ* heating from 25 °C to 80 °C and cooling back to 25 °C.



3.5 Binary and Ternary Mixtures at pH 3.5

Heating ramp experiments were also done at pH 3.5 with the same concentrations and ratios in binary and ternary combinations of WPI, SC, and GA; results are shown in Table 2. Heating had little effect on particle size in WPI|GA mixtures at pH 3.5, although increased DCRs showed that the amount of aggregates increased. Aggregation in heated SC|GA mixtures was more sensitive to concentration at pH 3.5 than at pH 7; mixing 0.5%SC with 2.5%GA at ambient temperature immediately produced aggregates large enough to precipitate. In ternary mixtures, heating produced modest increases in particle size and DCR.

Table 2. Particle size in mixed solutions of WPI, SC or GA at 3.5 before and after heating to 80°C in temperature ramp experiments. Data are the average of duplicates.

solution components (% w/w)			particle diameter (nm)		Derived count rate (kilo counts per second)	
WPI	SC	GA	before heating	after heating	before heating	after heating
0.1	0	0.5	15.9	16.4	17,118	37,741
0.25	0	1.25	13.3	13.3	25,707	90,805
0.5	0	2.5	13.7	12.1	40,959	85,476
0	0.1	0.5	16.2	117	103,974	227,419
0	0.25	1.25	146	244	324,584	1,522,431
0	0.5	2.5	- ^a	-		
0.05	0.05	0.5	104	168	119,303	158,855
0.125	0.125	1.25	17	259	104,134	191,828
0.25	0.25	2.5	395	479	159,964	438,481

a: mixing 0.5% SC with 2.5% GA produced particles large enough to precipitate within minutes.

4 Discussion

Cumulants analysis fits a single exponential function to autocorrelation decay data, and assumes a monomodal particle size with a Gaussian distribution. The z-average size that it calculates is intensity-weighted, and therefore highly biased towards large particles, because scattering intensity at a given wavelength scales with the sixth power of particle size for Rayleigh scatterers. The alternative 'distribution analysis' fits correlation decay data with a multi-exponential function, and uses Mie theory to calculate a volume distribution, thereby mitigating the bias towards large particles.

The zetasizer software uses a non-negative least squares algorithm with quadratic intensity weighting to fit autocorrelation decay with multi-exponential functions and produce particle size distributions. The software contains a 'multiple narrow modes' function based on a proprietary algorithm, for use when *a priori* knowledge suggests that multiple populations of particles may be present. Jamting *et al.* (2011) found that this mode improved discrimination to some extent in bimodal samples, though not as much as an alternative analytical approach proposed by Frisken (2001).

Rasteiro *et al.* (2008) highlighted the perils of applying inversion algorithms to multi-modal light scattering data in order to deconvolute the autocorrelation function into a particle size distribution. They concluded that the CONTIN algorithm was more robust than the non-negative least squares calculation for bimodal samples. Unfortunately our software license did not permit access to the full implementation of CONTIN, and the software listed only peak locations derived from a CONTIN analysis with unknown parameters. However these were generally in agreement with the results of 'multiple narrow modes' distribution analyses. Solutions of SC or WPI contained some colloidal aggregates <200 nm in diameter even before heating or mixing with other biopolymers. This was not unexpected and has been reported before (Schmitt, Sanchez, Thomas, & Hardy, 1999; Ye *et al.*, 2012). Heating single-component solutions produced relatively minor changes in particle size (Table 1), except in the case of SC

at pH 7, which aggregated at high temperature then dissociated in a fully reversible way, as previously reported (Ye et al., 2012).

Heating produced little change in size for mixtures of protein and polysaccharide at pH 3.5 (Table 2), where they bear opposite net charges. Opposing charges may cause GA to coat the outside of existing protein aggregates as soon as the two come in contact on mixing at ambient temperature. The GA coating would then inhibit further protein aggregation during heating.

To our knowledge, heat-induced colloidal aggregation of WPI and GA has not been previously investigated. This aggregation in heated WPI|GA mixtures was most likely due to disulphide bridging between β -lactoglobulin molecules, but breaking disulphide bonds with DTT did not inhibit aggregation (Figure 2), indicating that the hydrophobic forces seen in SC|GA aggregation (Ye et al., 2012) also operated with whey proteins. Reducing disulphide bonds substantially lowered the temperature at which WPI and GA started aggregating, which suggested that the two disulphide bonds stabilizing the native tertiary structure of β -lactoglobulin (Cys66-Cys160 and Cys106-119) inhibited aggregation with GA. This was borne out by the strong temperature-sensitivity of aggregation kinetics between 72°C and 78°C (Figure 3), where β -lactoglobulin denatures most rapidly at neutral pH (Boye, Ma, Ismail, Harwalkar, & Kalab, 1997; Wada, Fujita, & Kitabatake, 2006).

This is apparently the first time that colloidal aggregation has been examined in ternary combinations of two proteins and a polysaccharide. At the two lowest concentrations, the SC character dominated the behaviour of the ternary combination, perhaps because the concentration of whey proteins was too low to form a covalently cross-linked network that binds proteins together within particles. At the highest concentration, this networking ability of whey proteins produced extensive aggregation and very large particles. This may have been due to heat-induced sulfhydryl-disulphide interchange reactions between β -lactoglobulin (in WPI) and κ -casein (in SC), which are known to reduce the extent of whey protein aggregation in heated milk (Anema & Li, 2003). Thus, a combination of SC and WPI overcame the shortcomings of both, i.e. reversibility of SC|GA aggregation and rapid, extensive aggregation in WPI|GA.

Investigations of the kinetics of protein-polysaccharide aggregation in dilute systems are relatively rare, and the *in situ* evolution of particle size during heating has apparently not been studied before. *Ex situ* studies on dilute acidic mixtures of β -lactoglobulin and pectin (Jones & McClements, 2010; Kazmierski, Wicker, & Corredig, 2003) have shown little effect of temperature (65-90°C) on the rate of aggregation or the size of aggregates, in agreement with our findings at pH 3.5 (Table 2).

The *in situ* method offers significant advantages in terms of analysis time and avoiding experimental artefacts associated with cooling, storage and dilution of samples heated in a water/oil bath or heating block. Although *in situ* measurements were not instantaneous (6 runs of 10 s in this case), they did allow us to follow aggregation at high temperature as it happened.

The *in situ* aggregation data in Figure 4 showed an increasing amount of 100-500 nm particles at the expense of the 20-30 nm particles, rather than a progressive increase in the size of smaller particles. Similarly, alkaline native PAGE (Figure S3 in Supplementary Material) showed no β -lactoglobulin oligomers larger than dimers, but rather a progressive disappearance of native β -lactoglobulin and progressive accumulation of high molecular mass material that did not enter the gel. These data suggest a nucleation and growth mechanism, in which small protein aggregates attach to existing larger nuclei in preference to aggregating

with other small aggregates. Indeed, such a mechanism has been seen before in acid-induced complex coacervation of β -lactoglobulin and acacia gum (Sanchez et al., 2006). Schmitt *et al.* (2000) showed that pre-existing aggregates of β -lactoglobulin (much larger than we observed here) had a strong influence on the surface properties, size and structure of colloidal coacervates with acacia gum, and they suggested that food ingredient functionality in such a system could be tailored by controlling the initial size and amount of protein aggregates.

5 Conclusions

Here we have further explored the use of heating as an alternative to acidification for inducing colloidal aggregation in protein-polysaccharide mixtures. Earlier work with SC and GA at pH 7 was extended to mixtures of WPI and GA, and heat-induced aggregation at pH 3.5 was examined. We have shown how WPI and SC can be used in combination to make protein-polysaccharide nanoparticles in a way that exploits the desirable characteristics of each protein – the cross-linking ability of WPI and the cost-effectiveness of SC. We have developed a protocol for tracking aggregation while heating *in situ*. The *in situ* method avoids potential artefacts stemming from temperature changes and measurement delays, which are intrinsic to *ex situ* measurements.

The protein to polysaccharide mass ratio was kept constant for the sake of clarity here, and we are currently investigating the effects of varying this ratio. We will also examine how salts affect the formation and stability of colloidal protein-polysaccharide aggregates.

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SUPPLEMENTARY MATERIAL

Figure S1. Aggregation in mixtures of SC and GA at a range of concentrations.

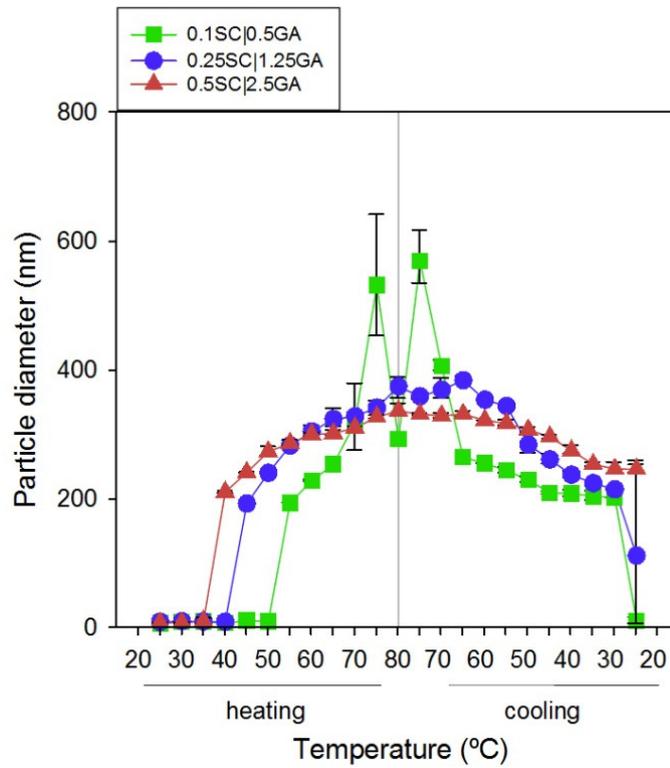


Figure S2. Derived count rate (kilo counts per second) in heated mixtures of SC and GA.

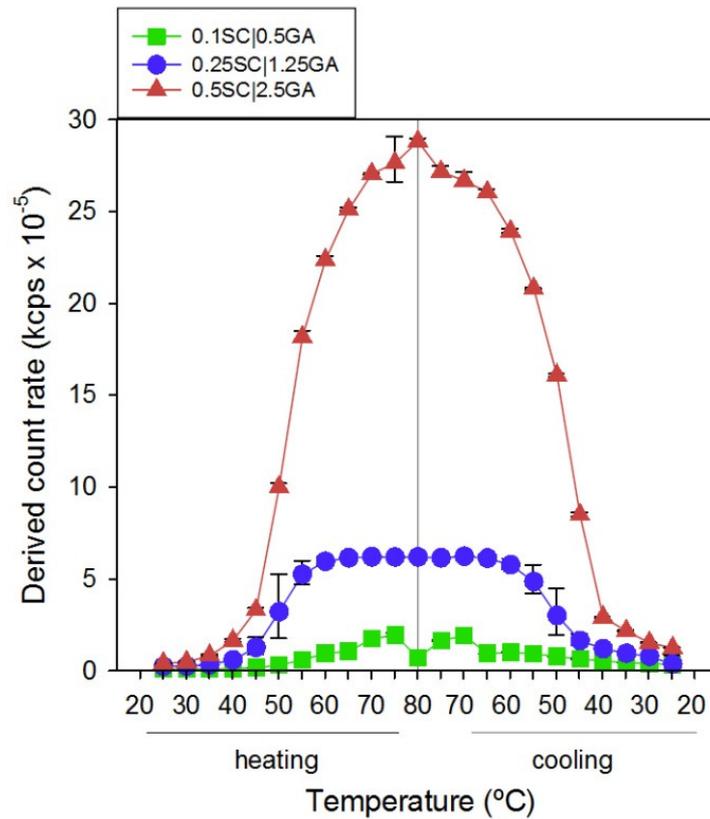


Figure S3. Alkaline native PAGE of 0.25WPI|1.25GA during isothermal heating at 75°C for various times.

