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Formation and Stability of Food-Grade Water-in-Oil-in-Water Emulsions

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology

By

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Abstract

The inherent thermodynamic instability of water-oil-water (W/O/W) emulsions restricts their applications in food systems. The objective of this study was to develop food grade W/O/W emulsions with both high encapsulation efficiency (EE) of entrapped compounds and long term stability using minimal concentrations of emulsifiers. Emulsions were characterised by phase separation, confocal microscopy, droplet sizing and EEs of macromolecules such as poly R-478 dye (a water-soluble dye) and small molecules such as sorbitol, NaCl and sodium ascorbate (SA).

Emulsions were prepared using soybean oil, polyglycerol ester of polyricinoleic acid (PGPR) as the emulsifier for the primary water-in-oil (W/O) emulsions and sodium caseinate (NaCN) as the sole emulsifier for the secondary W/O/W emulsions, in both buffered (0.1 M sodium phosphate buffer) and non-buffered (distilled water) systems. The concentration of PGPR had a significant effect on the EE of W/O/W emulsions. In non-buffered systems, the PGPR concentration could be reduced to 2% (w/v) to obtain an EE > 90%, whereas, in buffered systems, 4% (w/v) PGPR was required to maintain a similar EE. The ionic environment created in buffered systems had an effect on the emulsifying ability of PGPR. However, the concentration of PGPR in the internal aqueous phase could be reduced to 2% (w/v) without affecting the EE and the stability of the W/O/W emulsion by partially replacing the PGPR with 0.5% (w/v) NaCN, added to the aqueous phase of the primary W/O emulsion. The results indicate that there may be a possible synergistic effect between PGPR and NaCN, thus allowing the formulation of double emulsions with reduced surfactant concentration.

A modified gum arabic (Acacia (sen) SUPER GUM™) was investigated as an emulsifier to aid in the stabilisation of W/O/W emulsions. SUPER GUM™ at a range of concentrations was added as an emulsifier to replace NaCN in the external aqueous phase of W/O/W emulsions. The addition of 10% (w/v) SUPER GUM™ to the external aqueous phase allowed the PGPR concentration to be further reduced to 0.5% (w/v) while maintaining an EE > 90%. W/O/W emulsions stabilised with SUPER GUM™ were also found to be stable over a wide pH range, in the internal or external

aqueous phase, compared with W/O/W emulsions stabilised with NaCN; therefore, they may be suitable for applications over a wide range of pH values, as may occur during ingestion or incorporation into different food systems.

The encapsulation of small molecular compounds (sorbitol, NaCl or SA) in the internal aqueous phase had a significant effect on the stability of W/O/W emulsions, because osmotic pressure gradients were created by the addition of these compounds. With the addition of 0.5 M sorbitol to the internal aqueous phase, the osmotic pressure induced a water flux from the external aqueous phase to the internal aqueous phase, resulting in swelling of the internal water droplets. The resulting enlarged water droplets were more susceptible to coalescence and were expelled to the external aqueous phase. The EE of poly R-478 dye decreased significantly, mainly because of the expelling of internal water droplets. In contrast, the pulsed field gradient nuclear magnetic resonance (PFG-NMR) technique showed that sorbitol diffused through the oil phase; however, there was no evidence that sorbitol had an adverse effect on the emulsifying ability of PGPR.

The encapsulation of 0.5 M NaCl not only created an osmotic pressure gradient between the internal and external aqueous phases, but also reduced the emulsifying ability of PGPR, as phase separation occurred in W/O emulsions. The release of NaCl from W/O/W emulsions took place quickly; this was mainly due to diffusion of NaCl through the oil phase, because it was observed that release via expelling of internal water droplets took much longer to occur. The release of NaCl from W/O/W emulsions could be reduced by increasing the PGPR concentration.

For identically formulated W/O/W emulsions, the release of encapsulated SA was slower than the release of NaCl. Both diffusion and expelling of internal water droplets contributed to the release, depending on the experimental conditions. The release of SA could be slowed by adding macromolecules such as 0.5% (w/v) NaCN to the internal aqueous phase, by increasing the viscosity of the internal aqueous phase by the addition of 2% (w/v) carboxy methyl cellulose or by using a rotor-stator homogeniser instead of a high-pressure homogeniser to prepare the W/O/W emulsions. In addition, the replacement of 0.5% (w/v) NaCN with 10% (w/v) SUPER

GUM™ improved the EE of SA, possibly by acting as a rigid barrier on the surface of the oil droplets and therefore blocking both the diffusion of SA to the external aqueous phase and the expelling of internal water droplets.

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Chapter 1: Introduction

An emulsion is prepared by dispersing one immiscible liquid in another, and then stabilising it using a third component, the emulsifier (Walstra, 1985). Multiple emulsions are more complex systems, in which the dispersed phase is itself an emulsion; these emulsions can be classified into two major types: water-oil-water (W/O/W) emulsions and oil-water-oil (O/W/O) emulsions. Multiple emulsions have been studied in attempts to find industrial applications for microencapsulation in various fields such as pharmaceuticals and cosmetics (Shimizu and Nakane, 1995; Couvreur, Blanco-Prieto, Puisieux, Roques and Fattal, 1997; Okochi and Nakano, 2000; Farahmand, Tajerzadeh and Farboud, 2006).

There are two main potential applications of multiple emulsions in the food industry: (1) multiple emulsions may provide protection for reactive food nutrients or volatile flavours and may also control their release through encapsulation (Dickinson and McClements, 1996); (2) the emerging interest in functional foods requires the technology to produce healthier foods. Compared with an oil-in-water (O/W) emulsion with the same volume fraction of the dispersed phase, a W/O/W emulsion contains a smaller oil fraction, potentially allowing less oil to be used and lowering costs (Dickinson and McClements, 1996; Garti and Aserin, 1996c). Despite their immense potential, the application of multiple emulsions in food systems has been limited by their inherent thermodynamic instability, which causes leakage of the encapsulant from the internal aqueous phase, flocculation of the droplets or phase separation during processing and storage (Hino, Shimabayashi, Tanaka, Nakano and Okochi, 2001; Benichou, Aserin and Garti, 2004).

The stability of multiple emulsions is determined by their composition, including the nature of the oil phase, the type of emulsifiers and the nature of entrapped materials or encapsulants, particularly if the encapsulant contains electrolytes. The techniques of formation and the conditions of storage also have a significant effect on the stability of multiple emulsions (Dickinson, Evison, Owusu and Williams, 1994b). Compared with single emulsions, the destabilisation processes experienced by multiple emulsion systems are much more varied (Florence and Whitehill, 1985). A better understanding

of the properties and characteristics of multiple emulsions under different conditions would increase the opportunity to improve their stability and to enhance their potential applications in the food industry.

The overall objective of this project was to study the properties and characteristics of W/O/W emulsions and to investigate the mechanisms behind the destabilisation processes. The study focused on W/O/W emulsions as they have more potential applications than O/W/O emulsions because most food systems are water based. The project included the selection of formation techniques and components to form a stable W/O/W emulsion. Once the model system was established, the properties and characteristics of the emulsions were studied, including their stabilities under different experimental conditions. The ability of designed W/O/W emulsions to encapsulate water soluble components was also explored.

Chapter 2: Literature review

2.1. Introduction

This literature review covers published information on the formation conditions and the composition of W/O/W emulsions. The physico-chemical properties that correlate with the stability of W/O/W emulsions are discussed. Potential applications of W/O/W emulsions in the food industry are also described.

2.2. Definition of a W/O/W emulsion

A W/O/W emulsion is an O/W emulsion in which the dispersed oil phase is a water-in-oil (W/O) emulsion. Three types of oil droplets can be found, as illustrated in Fig. 2.1. Type A contains only one internal water droplet, type B contains more than one but only a few internal water droplets and type C contains a large number of internal water droplets (Dickinson and McClements, 1996). The main factors that influence the characteristics and stabilities of W/O/W emulsions are their methods of production and their compositions (Garti and Aserin, 1996b; Einhorn-Stoll, Weiss and Kunzek, 2002).
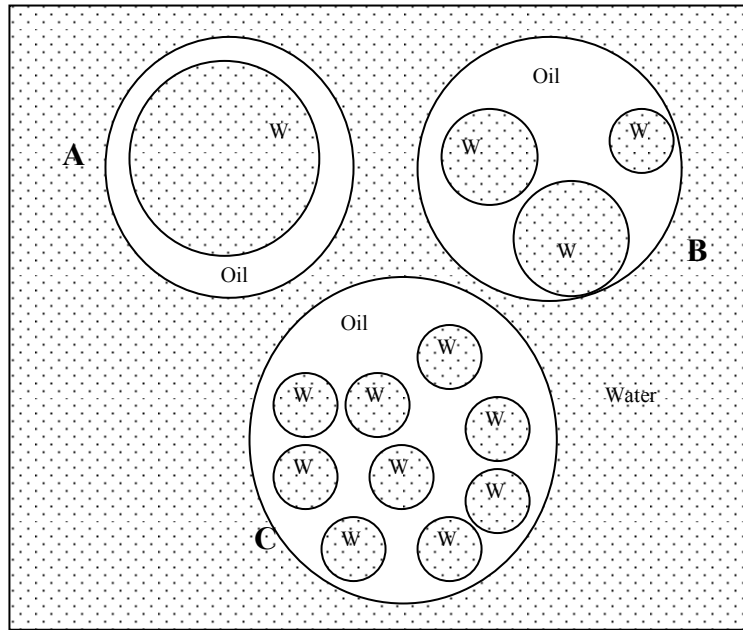
Fig. 2.1. Schematic diagram of a W/O/W emulsion, showing oil droplets, containing different distributions of water droplets, in the continuous water phase.

2.3.   Formation of W/O/W emulsions

W/O/W emulsions were first reported by Seifriz (1925). Various methods for preparing multiple emulsions are available; they are all start from the formation of a simple emulsion, which is generally achieved by applying mechanical energy (Garti and Aserin, 1996b). The interface between the two immiscible phases is deformed, forming a liquid film between the two liquids. Upon the continuous application of mechanical force, large droplets are formed, which are subsequently disrupted into smaller droplets. During the emulsification process, surfactant molecules are transported from the bulk solution to the interface and stabilise the formed droplets; the disruption of the large droplets to smaller droplets occurs only when the transport stage is faster than the droplet deformation stage (Garti and Aserin, 1996b; Benichou, Aserin and Garti, 2001).

There are two main methods for the formation of W/O/W emulsions: one-step emulsification and two-step emulsification (Benichou et al., 2001). One-step

emulsification methods include strong mechanical agitation and phase inversion. An aqueous solution of a hydrophilic emulsifier is vigorously blended with an oil phase containing a larger amount of hydrophobic emulsifier. A W/O emulsion is formed initially, but part of the emulsion inverts and forms a W/O/W emulsion (Eley, Hey and Symonds, 1988; Matsumoto and Kang, 1989).

The phase inversion method involves the gradual addition of an aqueous solution of hydrophilic emulsifier to a previously formed W/O emulsion. The W/O/W emulsion is formed as an intermediate stage before the system completely inverts into an O/W emulsion. Inversion is most likely when the dispersed aqueous phase is closely packed; ideally the fraction of aqueous phase should exceed 70% (Matsumoto, Koh and Michiura, 1985; Matsumoto and Kang, 1989). As in the mechanical agitation method, only a part of the W/O emulsion converts to a W/O/W emulsion and the extent of inversion appears to be sensitive to the chemical nature of the hydrophilic emulsifier and the temperature (Dickinson and McClements, 1996).

Two-step emulsification methods involve making a fine primary W/O emulsion and then dispersing the primary emulsion in a solution with a hydrophilic emulsifier (Fig. 2.2). A critical factor that determines the effectiveness of this method is the intrinsic stability of the internal W/O emulsion (Dickinson and McClements, 1996). It is important that the presence of hydrodynamic perturbations during the second emulsification stage does not lead to any significant breakdown of the primary emulsion (Dickinson and McClements, 1996; van der Graaf, Schroen and Boom, 2005).

Solution

Fraction %

Step 1

W/O
emulsion

Aqueous
solution of
hydrophilic
emulsifier

Step 2

W/O/W
emulsion

+

Fraction%

20 %

80 %

Oil Phase +
hydrophobic
emulsifier
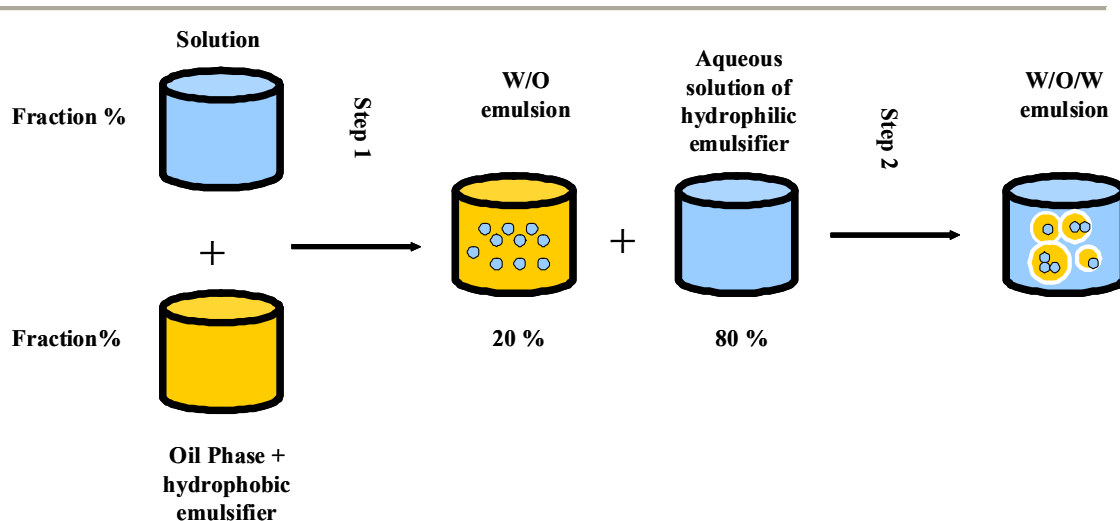
Fig. 2.2. Schematic illustration of a two-step method for the formation of a W/O/W emulsion.

The one-step methods are not widely used; they are difficult to reproduce because only part of the emulsion will form into a W/O/W emulsion. The two-step emulsification methods are more widely preferred (Dickinson and McClements, 1996). In contrast to the one-step methods, they can be used systematically to obtain stable multiple emulsions of well-defined composition and reproducible droplet size distribution (Florence and Whitehill, 1985; Matsumoto, 1985).

2.4. Process for the formation of W/O/W emulsions

Various techniques have been applied in two-step methods, including blender and mixer, high-pressure homogeniser, microfluidiser, membranes etc. (Fig. 2.3). Each method introduces energy into the system in a different manner, which results in different emulsion microstructures. The different techniques not only cause the reduction in droplet size but also influence the adsorption of the emulsifier (Liu and McGrath, 2005; Perrier-Cornet, Marie and Gervais, 2005). For a given formulation, the microstructure of the final emulsion is controlled by the formation process, which correspondingly contributes to the lifetime of the emulsion and the mechanism of destabilisation. Such control could be affected by the different techniques used or by a

change in the same technique used (Liu and McGrath, 2005; Scherze, Knoth and Muschiolik, 2006).

The most important conventional emulsification devices are stirrers, rotor-stator systems, high-pressure homogenisers and membranes (Schubert and Armbruster, 1992). With a stirrer, the dispersed phase is broken up by the shear stresses of the turbulence and the energy consumption is usually large. In rotor-stator systems, such as tooth-disc high-speed homogenisers and colloid mills, a high shear between a rotor and a stationary smooth, roughened or grooved surface is generated. Turbulence is the primary cause of fluid disruption, leading to the formation of droplets. These conventional methods have been used to form both W/O and secondary W/O/W emulsions. It is important not to use high shear stresses to form W/O/W emulsions, to prevent disruption of the internal emulsion and coalescence with the external aqueous phase (van der Graaf et al., 2005). However, as a result, only large droplets and wide size distributions are obtained under low shear conditions; these emulsions are susceptible to creaming (Perrier-Cornet et al., 2005). High-pressure homogenisers and membrane emulsification techniques are discussed below.
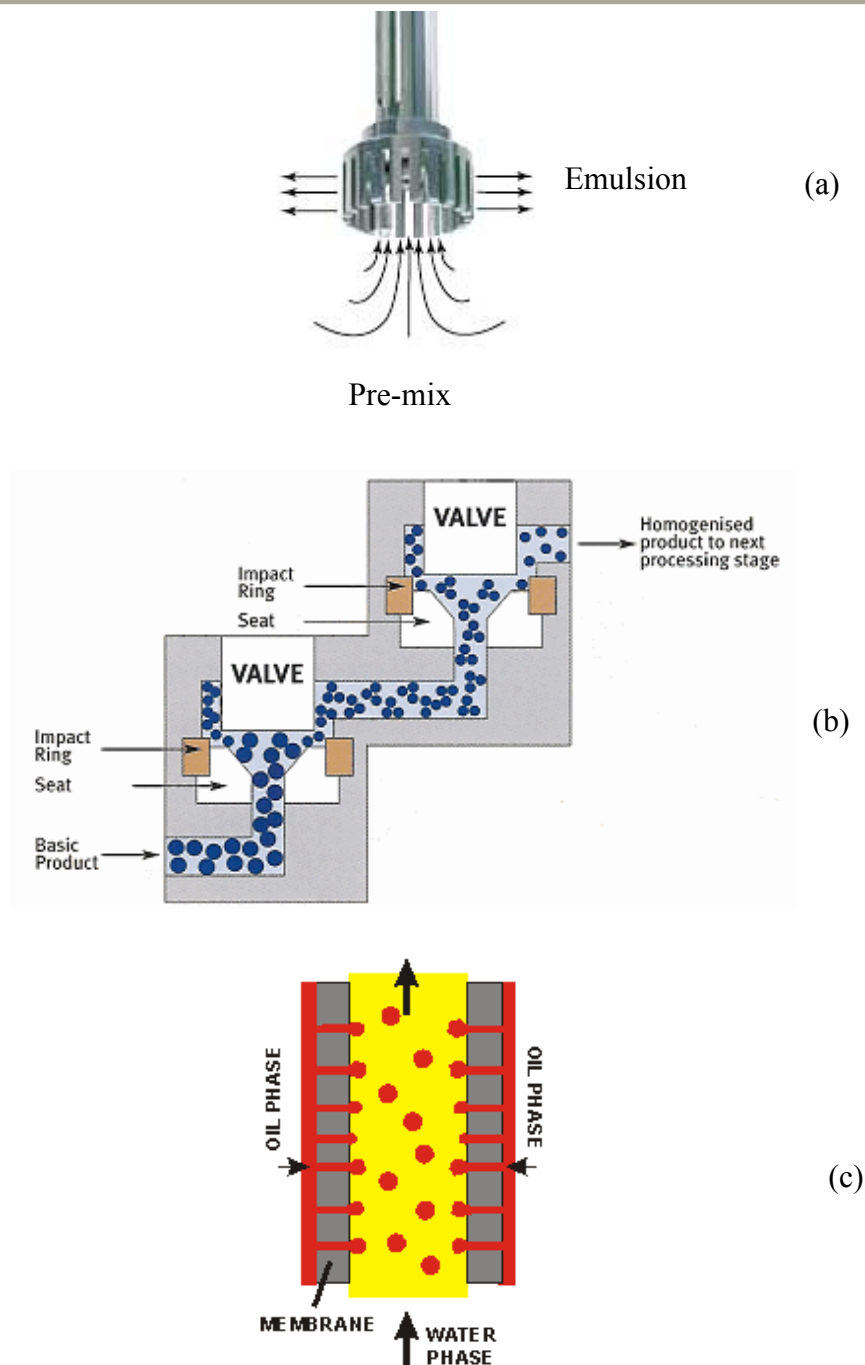
Fig. 2.3. Schematic illustration of commonly used emulsification techniques: (a) rotor-stator homogeniser; (b) two-stage high-pressure homogeniser; (c) membrane emulsification (from **www.homogeniser.com/principle.html**).

2.4.1. **High-pressure homogeniser**

In the high-pressure homogeniser systems, the emulsion mixture is passed through a narrow channel or orifice valve under pressure, deflected at right angles and forced to impact against either an impact ring (classical high-pressure homogeniser) or a stream from the opposite direction (microfluidiser); a sudden pressure drop occurs and the oil droplets in the aqueous phase are disrupted and emulsified (Burgaud, Dickinson and Nelson, 1990; Floury, Legrand and Desrumaux, 2004; Perrier-Cornet et al., 2005). The process may be assisted by the use of ultrasound or electrical fields. The energy dissipation is high and pressures in the range from 5×10^6 to 3.5×10^7 Pa are common. Emulsification is caused by turbulence, cavitation effects and high shear forces (Burgaud et al., 1990; Stang, Schuchmann and Schubert, 2001; Freudig, Tesch and Schubert, 2003; Floury et al., 2004; Perrier-Cornet et al., 2005).

High-pressure homogenisers can produce very fine droplets, which cannot be produced by mixers or rotor-stator homogenisers (Perrier-Cornet et al., 2005). The difference in droplet size is directly correlated to the energy density; the higher the energy density, the smaller are the droplets (Karbstein and Schubert, 1995; Schubert and Engel, 2004). The energy input is between 10^4 and 10^6 Wm^{-3} for the rotor-stator homogeniser (Karbstein and Schubert, 1995) and about 10^{12} Wm^{-3} for the high-pressure jet homogeniser (Marie and Perrier-Cornet, 2002).

In addition to pressure, other operating parameters that could also affect the efficiency of high-pressure homogenisers are temperature, number of passes, valve and impingement design and flow rate (Burgaud et al., 1990; Perrier-Cornet et al., 2005). In the early stage of the emulsification process, cavitation of the water droplets induces a reduction in the interfacial tension and new water-oil interfaces are quickly formed. Small and almost monodispersed droplets may be produced (Stang et al., 2001; Freudig et al., 2003; Schubert and Engel, 2004).

The mean droplet size of the emulsions after their production depends on both droplet disruption and droplet recoalescence (Burgaud et al., 1990; Karbstein and Schubert,

1995). The mean droplet size is the size of the droplets upon disruption but recoalescence of newly created droplets could occur shortly or even immediately after preparation. Above certain pressures, the beneficial effect of the homogenisation on the reduction in droplet size decreases. The amount of emulsifier initially present might not be sufficient to cover all the new interfaces created at the higher pressure, or structural changes in the emulsifier may occur because of the high pressure and the increasing temperature associated with the high pressure (Burgaud et al., 1990; Stang et al., 2001; Tesch and Schubert, 2002; Floury et al., 2004; Schubert and Engel, 2004; Perrier-Cornet et al., 2005). The adverse effect of high pressure on the emulsion droplet size may be reduced by changing the composition of the emulsion, such as increasing the emulsifier concentration, reducing the volume fraction of the dispersed phase or increasing the viscosity of the continuous phase (Burgaud et al., 1990; Einhorn-Stoll et al., 2002). Also, repeatedly passing the emulsion through the homogeniser reduces the droplet size until a limiting size is achieved (Perrier-Cornet et al., 2005).

2.4.2. *Membrane emulsification*

A more recent method that is used to form W/O/W emulsions is membrane emulsification (Fig. 2.3). Emulsion mixtures are forced under high pressure through a narrow gap of a few hundred microns in a microporous membrane. A combination of turbulence and intense laminar shear flow causes the disruption of the droplets; fine monodispersed emulsions with good stability towards creaming and flocculation are obtained (Kobayashi and Nakajima, 2002; Kobayashi, Lou, Mukataka and Nakajima, 2005). The technique is attractive because of the low energy consumption, better control of the droplet size and the droplet size distribution and, especially, the mildness of the process (Shima, Kobayashi, Fujii, Tanaka, Kimura, Adachi and Matsuno, 2004; Kobayashi et al., 2005). Membrane emulsification can produce emulsions with a minimum oil fraction of below 5% and can be used to prepare size-controlled monodispersed emulsions with droplet diameters of 3–90 μm (Mine, Shimizu and Nakashima, 1996; Kobayashi, Nakajima, Nabetani, Kikuchi, Shohno and Satoh, 2001; Sugiura, Nakajima and Seki, 2002). However, the main drawback of the

membrane emulsification method is that it is time consuming and has low productivity, which limits its use (Muschiolik, 2007).

2.5.   Compositions of W/O/W emulsions

After their formation, the stability of the disrupted droplets is determined by the correct selection of the composition of W/O/W emulsions (Taisne, Walstra and Cabane, 1996). The main constituents of a W/O/W emulsion are the internal aqueous phase, hydrophobic emulsifiers, the oil phase, hydrophilic emulsifiers and the external aqueous phase.

2.5.1. *Aqueous phase*

The aqueous phase is the dispersed phase in a W/O emulsion and the continuous phase in a W/O/W emulsion. Internal aqueous phases are often solutions of encapsulated compounds, such as sugar, salt and nutrients. (Garti and Aserin, 1996b). External aqueous phases are solutions of emulsifiers (e.g. proteins) and stabilisers (e.g. polysaccharides). The volume fraction of the aqueous phases and the addition of materials to the aqueous phases have a large effect on the stability of a W/O/W emulsion.

2.5.2. *Oil phase*

A stable W/O emulsion is essential to the stability of the secondary W/O/W emulsion, and it is strongly correlated to the hydrophobicity of the oil phase (Kanouni, Rosano and Naouli, 2002). The nature of the oil phase in W/O/W emulsions directly determines the encapsulation efficiency (EE), which is the internal aqueous phase retained in the final W/O/W emulsion, and the rate of transport of encapsulants through the oil phase (Omotosho, Whateley, Law and Florence, 1986b; Weiss, Scherze and Muschiolik, 2005).

To form a stable food-grade W/O/W emulsion, the oil phase should ideally have low viscosity and low water solubility (Garti, 1997a). However, commercially available vegetable oils usually have higher viscosity and higher water solubility than mineral oils (Hamilton, 1993). Emulsions made from vegetable oil require a higher energy input and the resulting emulsion is less stable to the migration of water in and out of the internal aqueous phase. Consequently, high hydrophobicity materials such as mineral oils or hydrocarbon solvents are commonly used as the oil phase in studies of W/O or W/O/W emulsions (Dickinson et al., 1994b; Dickinson and McClements, 1996; Benichou et al., 2001).

However, it is possible to use vegetable oils in W/O or W/O/W emulsions if suitable emulsifiers are used (Matsumoto et al., 1985; Dickinson et al., 1994b; Dickinson and McClements, 1996; Benichou et al., 2001; Kanouni et al., 2002). In a few studies, several vegetable oils such as soybean oil, corn oil and sunflower oil have been used to produce stable W/O/W emulsions using larger molecular weight emulsifiers. In these studies, larger molecules such as polyglycerol ester of polyricinoleic acid (PGPR), lecithin and proteins were used as hydrophobic and hydrophilic emulsifiers (Dickinson, Evison, Gramshaw and Schwope, 1994a; Olivieri, Seiller, Bromberg, Ron, Corvreur and Grossiord, 2001; Scherze, Knofel and Muschiolik, 2005; Dickinson, 2006).

2.5.3. *Volume fraction of dispersed phase*

To determine an optimum volume fraction of the dispersed phase in a W/O/W emulsion, both stability and EE need to be taken into consideration (Dickinson et al., 1994b). Researchers have shown that 20–30% of dispersed phase to continuous phase is the optimum volume fraction. At higher content of the dispersed phase, the emulsions were more condensed, the viscosity increased, the droplet sizes grew and the emulsions became unstable. When the content of the dispersed phase reached a certain level, the emulsions quickly inverted (Danner and Schubert, 2001). In contrast, an emulsion with a lower dispersed volume fraction will have low EE, which does not

have much commercial potential (Dickinson, Evison, Owusu and Zhu, 1993; Dickinson et al., 1994b).

2.5.4. Emulsifiers

Emulsifiers are amphiphilic compounds that possess two distinct groups in the same molecule: a hydrophobic group, which has an affinity for the oil phase, and a hydrophilic group, which has an affinity for water (Garti, 1997b; Kanouni et al., 2002). Emulsifiers lower the interfacial tension and facilitate droplet disruption, resulting in smaller droplets. An emulsifier determines which phase is the continuous phase and which is the dispersed phase; use of the wrong emulsifier could result in an inverted emulsion (Fennema, 1996; Benichou et al., 2001). Different emulsifiers are often classified by their hydrophilic/lipophilic balance, or HLB, ranging from zero to 20; this scale indicates an emulsifier's relative overall attraction to either oil or water. A low HLB indicates a strongly lipophilic emulsifier, whereas a high HLB indicates a strongly hydrophilic emulsifier. The HLB is also useful as a general indicator of an emulsifier's solubility in oil and water (Fennema, 1996).

Various ionic and non-ionic monomeric emulsifiers were used in early studies of W/O/W emulsions, in accordance with their performance and restrictions. Over time, researchers found that the classical W/O/W emulsions prepared with two sets of monomeric emulsifiers (hydrophobic and hydrophilic, e.g. Span 80 and Tween 80) could not provide long term stability to the W/O/W emulsion (Owusu and Zhu, 1996; Garti, 1997a; Hou and Papadopoulos, 1997; Pays, Giermanska-kahn, Pouligny, Bibette and Leal-Calderon, 2001), mainly because the monomeric emulsifiers served both as stabilising agents and as transport species. The W/O/W emulsions made with these types of emulsifiers usually had relatively large droplets and short-term stability, because of the migration of the emulsifiers from one interface to the other, and had altered interfacial emulsifier organisation (Kanouni et al., 2002). To date, monomeric surfactants have been progressively replaced by polymeric emulsifiers or macromolecular amphiphiles such as PGPR or milk protein, which can form strong and more rigid films at the interface (Benichou et al., 2004). Furthermore, a polymeric non-ionic emulsifier can form cross-linked interfaces after adsorption. These polymeric interfacial complexes are capable of withstanding extensive thinning

(caused by the osmotic-pressure-driven influx of water) and the resulting swelling of the internal water droplets (Garti and Aserin, 1996c; Benichou, Aserin and Garti, 2002a).

2.5.4.1. Commonly used polymeric hydrophobic emulsifiers in W/O/W emulsions

PGPR is derived from castor oil, is known to be one of the most efficient oligomeric emulsifiers for W/O emulsions and is extensively used by the food industry, alone or in combination with soy lecithin, to improve the flow properties of molten chocolate and to reduce the amount of cocoa butter required (up to 5 g/kg) (Wilson, Van Schie and Howes, 1998; Benichou et al., 2001). PGPR has an HLB value of 4, similar to that of Span 80, and a molecular weight of around 4400 g/mol, about 10 times that of Span 80 (Pays, Giermanska-kahn, Pouligny, Bibette and Leal-Calderon, 2002). PGPR generally provides the most stable W/O/W emulsions made with vegetable oil as the oil phase.

The functional component of lecithin is a mixture of phospholipids, which have a hydrophilic polar head and two fatty acid tails, the hydrophobic portion of the molecule. The various phospholipids can be separated to give specific functional properties to the lecithin. Phospholipids stabilise emulsions mainly by providing an electrostatic repulsion barrier to the emulsion droplets (Adachi, imaoka, Hasegawa and Matsuno, 2003; Muschiolik, Scherze, Preissler, Weiss, Knoth and Fechner, 2006). Hydrophobic lecithin with a reduced phosphatidyl choline content has been shown to provide good stability to W/O/W emulsions (Akhtar and Dickinson, 2001; Knoth, Scherze and Muschiolik, 2005).

2.5.4.2. Commonly used macromolecular hydrophilic emulsifiers in W/O/W emulsions

Macromolecular amphiphilic emulsifiers, both tailor-made synthetically and naturally occurring, are known to improve the interfacial coverage during emulsification, which provides better encapsulation and controlled release of the encapsulant (Cornec, Wilde, Gunning, Macckie, Husband, Parker and Clark, 1998; Rosano, Gandolfo and Hidrot, 1998). Typical examples are proteins and polysaccharides. The main

mechanisms of stabilisation by polymeric amphiphilic molecules appear to be electrostatic repulsions between two droplets carrying the same charge and steric stabilisation resulting from adsorbed biopolymers (Benichou et al., 2004).

The ability of sodium caseinate (NaCN), a milk protein, to act as an emulsifier and stabiliser at oil–water interfaces is well documented (Srinivasan, Singh and Munro, 2000; Ye and Singh, 2001). NaCN is a composite of four different proteins, α_{s1}-, α_{s2}-, β- and κ-caseins, in weight proportions of approximately 4:1:4:1. All caseins are amphiphilic proteins with a strong tendency to adsorb at oil–water interfaces during emulsion formation, reducing interfacial tension. This produces an adsorbed layer of protein around the oil droplets, which protects them against subsequent coalescence and flocculation (Srinivasan et al., 2000; Ye and Singh, 2001).

The high proline content of caseins is responsible for a disordered and flexible structure. NaCN is not monomeric but is aggregated to some extent in solution. The nature and the size of the aggregates are probably dependent on protein concentration, temperature, presence of ions and processing history (Dickinson, 1997). During the dynamic conditions of homogenisation, much of the protein material is transported to the oil–water interface by convection rather than by diffusion. The rate of adsorption of protein is determined by the size of the adsorbing proteins or aggregates and the immediately available binding sites on the molecule. NaCN is used at lower concentration, compared with monomeric surfactants, to avoid depletion flocculation of emulsions containing unabsorbed NaCN (Dickinson, 1997). Electrostatic interactions and steric stabilisation are the main mechanisms of stabilization in protein-stabilised emulsions.

Gum arabic is a versatile polysaccharide that is used in food systems because of its molecular flexibility (Islam, Phillips, Sljivo, Snowden and Williams, 1997). The functions of gum arabic include emulsification, encapsulation, stabilisation, water binding, adhesion and film forming (Dickinson and Galazka, 1991; McNamee, O'Riordan and O'Sullivan, 1998; Verbeken, Dierckx and Dewettinck, 2003).

Numerous physico-chemical studies of gum arabic have shown that it is heterogeneous, having either a variation in its monomer composition or a variation in the mode of linking and branching of the monomer units, in addition to a distribution in molecular mass (Williams, Phillips and Sephen, 1990; Dickinson and Galazka, 1991). Gum arabic consists of a major fraction of lower molecular mass (2.5×10^5 Da), which contains very little nitrogenous material, and arabinogalactan, a high-molecular-weight fraction ($1-2.5 \times 10^6$ Da) that represents about 10–30% of the total gum and that is associated with a small protein component (Randall, Phillips and Williams, 1988; Dickinson and Galazka, 1991; Dickinson, Galazka and Anderson, 1991b).

The amino acid composition in gum arabic has been studied in detail, as it has been demonstrated that it is the protein-containing high-molecular-weight fraction that adsorbs most strongly at the oil–water interface (Street and Anderson, 1983; Dickinson and Galazka, 1991; Dickinson et al., 1991b; Islam et al., 1997). However, it appears that the emulsifying behaviour of gum arabic samples depends not only on the nitrogen content but also on the molecular accessibility of the protein component for rapid adsorption and on the distribution of protein between the lower and the higher molecular-weight fractions (Dickinson, Murray, Stainsby and Anderson, 1988). It has been suggested that protein-associated high-molecular-weight fractions (arabinogalactan) are formed by the aggregation of smaller arabinogalactan units into larger units. The proportion of large and small arabinogalactan units in gum arabic has been reported to have a direct effect on the properties of the emulsifier, with a high proportion of larger units desired (Randall et al., 1988; Randall, Phillips and Williams, 1989; Williams et al., 1990). In emulsions stabilised by gum arabic, the stabilising mode of action is mainly steric repulsion (Chanamai and McClemennts, 2002; Benichou et al., 2004).

2.6.   Instability of W/O/W emulsions

Compared with simple emulsions, the destabilisation processes available to multiple emulsion systems are much more varied (Dickinson and McClements, 1996). Apart from the types of instability in simple emulsions that could affect both the primary W/O emulsion and the secondary W/O/W emulsion, there are other pathways of instability that are unique to W/O/W emulsions (Dickinson and McClements, 1996; Pays et al., 2002).

2.6.1. Destabilisation of simple emulsions

O/W emulsions can undergo several types of physical change, as illustrated in Fig. 2.4; the difference with W/O emulsions is that downward sedimentation rather than creaming would occur. Creaming, flocculation and coalescence can take place either in parallel or in sequence in each emulsion; all lead eventually to emulsion breakdown and phase separation. It is essential to distinguish between these processes and to learn to control them to some extent (Fennema, 1996).

2.6.1.1. Creaming

Creaming is the rise of dispersed droplets under the action of gravity, with the droplets remaining separated when they touch. Creaming takes place in any continuous aqueous phase if the dispersed phase is not exactly equal in density. Small droplets are more resistant to creaming (Dickinson, Evison and Owusu, 1991a). The rate of creaming can be lowered by decreasing the emulsion droplet size, equalising the densities of the two phases and increasing the viscosity of the continuous phase (Dickinson et al., 1991a; Garti and Aserin, 1996b).
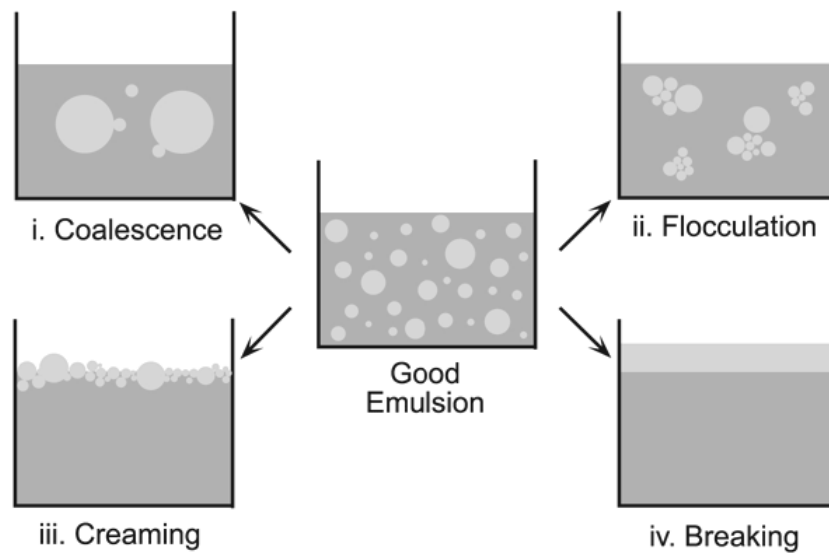
Fig. 2.4. Schematic representation of the instability processes occurring in O/W emulsions (Garti and Aserin, 1996b).

2.6.1.2. Flocculation

Flocculation is the act of the sticking together of droplets and the formation of three-dimensional clusters. It is a process in which an aggregation of droplets takes place as a result of collisions in combination with adhesive inter-droplet forces. Flocculation is a complex phenomenon that is caused by Brownian movement, gravity and shear motions of the droplets. In thermodynamically unstable emulsion systems, flocculation may be prevented if a large enough free energy barrier between the droplets exists; the free energy barrier is derived from the interfacial forces such as long-range van der Waals' forces and electrostatic forces. The net force of the two sets of interactions determines the behaviour of the emulsion droplet with respect to flocculation and further coalescence (Dickinson et al., 1991a; Garti and Aserin, 1996b). If there is no flow of liquid from one dispersed droplet to another, flocculation between droplets can be reversible (Dickinson et al., 1991a).

2.6.1.3. Coalescence

Coalescence is induced by rupture of the thin film (lamella) between closely approaching droplets. It reduces the interfacial tension and the surface area of droplets (Garti and Aserin, 1996b). The probability of coalescence is proportional to the time for which the droplets are close to each other. Therefore, it is especially likely to occur in a cream layer or in a flocculated system. It is a first-order rate process. The probability that rupture of a film occurs will be proportional to its contacting area. Coalescence is less likely to occur in smaller droplets or in droplets with a thick film, as these types of droplets have a smaller film area between the droplets or a stronger repulsive force between the droplets respectively. Coalescence leads to larger droplets, and eventually to phase separation (Fennema, 1996).

2.6.1.4. Ostwald ripening

In emulsions with polydispersed droplets, Ostwald ripening of the droplets could also destabilise the emulsion. Collisions between two droplets may lead to one bigger droplet and one smaller droplet. Eventually, the small droplets become very small and become solubilised in the continuous medium, and the polydispersity of the system decreases (Soma and Papadopoulos, 1996). Ostwald ripening occurs more often in W/O emulsions, when the aqueous phase has sufficient solubility in the oil phase. The main driving force for Ostwald ripening is generally agreed to be Laplace pressure, and it can be balanced by increasing the osmotic pressure by adding a suitable solute to the aqueous phase; as a result, the rate of Ostwald ripening can be delayed. With the addition of solute(s), as soon as a small droplet shrinks, its salt concentration and its osmotic pressure increase, thereby producing a driving force for water transport in the opposite direction. The net result is a stable droplet size distribution (Fennema, 1996; Rosano et al., 1998; Koroleva and Yurtov, 2003).

2.6.2. Coalescence between the internal and the external aqueous phases

Coalescence of the small internal water droplets within the oil droplets may lead to fewer water droplets in the oil droplets, but may not result in complete delivery of the small internal water droplets to the external aqueous phase. However, coalescence between the internal water droplets and the oil droplet interface can lead to complete delivery of the small internal water droplets to the external aqueous phase (Ficheux, Bonakdar, Leal-Calderon and Bibette, 1998).

Villa, Lawson, Li and Papadopoulos (2003) used capillary microscopy to study the coalescence between internal water droplets and the coalescence between the internal and external aqueous phases, in a single W/O/W emulsion droplet. It was found that the interaction between the internal water droplets in the oil droplet was similar to that between the water droplets in a W/O emulsion. In the absence of emulsifier and at low emulsifier concentration, the interaction energy between the internal water droplets exhibits a barrier, which becomes more pronounced as the emulsifier increases in concentration. A comparison between the energy diagrams reveals that the convex−convex surface interaction is much more stable than the convex−concave surface interaction. As a result, coalescence between the internal water droplets always occurs in conjunction with coalescence between the internal and external aqueous phases (Villa et al., 2003). When the oil droplets are destabilised, the internal water droplets may not coalesce among themselves, but rather may merge with the external aqueous phase. When the effective thickness of the adsorbed layer on the water droplets is larger than some critical value, the net interaction energy between the internal water droplets and the external aqueous phase becomes repulsive and constitutes the main contributor to stability (Hou and Papadopoulos, 1996).

The mechanisms that contribute to coalescence between the internal and external aqueous phases are yet to be fully understood. Mezzenga (2007) suggested that unbalanced Laplace pressures play an important role. In a pure W/O/W emulsion without osmotic pressure, the Laplace pressure associated with the small water droplets will lead to water diffusing from the internal phase to the external phase. The

Laplace pressure normally needs to be equilibrated by an opposite osmotic pressure. As mentioned, this could be achieved by dissolving compounds that are not soluble in the oil phase in the internal water droplet phase. Salt, sugar or even polysaccharides are able to decrease the chemical potential of water efficiently and thus induce an osmotic pressure opposing the Laplace pressure (Rosano et al., 1998; Koroleva and Yurtov, 2003; Mezzenga, Folmer and Hughes, 2004).

In contrast, the stability of a W/O/W emulsion can be significantly altered by an unbalanced osmotic pressure between the internal aqueous phase and the external aqueous phase. A large osmotic pressure gradient can lead to swelling or shrinkage of the internal water droplets as a result of diffusive passage of water molecules across the oil layer (Dickinson and McClements, 1996; Akhtar and Dickinson, 2001). It has been generally accepted that the rate of breakdown of the oil film or the loss of water droplets through coalescence between the internal water droplets and the external aqueous phase could be accelerated after swelling of the internal water droplets, which can be controlled by regulation of the osmotic pressure gradient (Raynal, Grossiord, Seiller and Clausse, 1993; Benichou et al., 2004).

2.6.3. *Transportation of water through the oil phase*

Coalescence of the water droplets in the internal aqueous phase and rupture of the oil droplets will release internal water droplets and encapsulant directly into the external aqueous phase. In contrast, diffusion of water and encapsulant across the oil phase has been detected, and could occur with or without significant alteration to the stability of the W/O/W emulsion (Benichou, Aserin and Garti, 2007).

Water transport through the oil phase in a W/O/W emulsion mainly occurs via three possible routes, as illustrated in Fig. 2.5. The bulk transport of water can be by diffusion of reverse micelles; reverse micelles are formed at one interface with water molecules entrapped from the aqueous phase and diffuse across the oil film to the other interface, where the entrapped water molecules are released into the other aqueous phase (Fig. 2.5 a). Another route of water transportation is through lamellar

thinning, in which water molecules pass through thin lamellae layers of surfactants, which partially form in the oil layer because of fluctuation of its thickness (Fig. 2.5 b). A hydrated surfactant transportation mechanism was proposed more recently (Fig. 2.5 c), in which the hydrophilic portion of the surfactant can hydrate on the water-oil interface and then diffuse to the oil-water interface, where it becomes dehydrated, thus transporting water from the internal water droplets to the external aqueous phase (Kita, Matsumoto and Yonezawa, 1977; Magdassi and Garti, 1984; Omotosho et al., 1986b; Garti, 1997a). The rate of water transportation has been related to the nature and concentration of surfactants, the type of oil used and the effect of the salt concentration gradient between two interfaces on the water transport rate (Wen and Papadopoulos, 2000a, b).

Previous studies of W/O/W emulsions stabilised by monomeric non-ionic emulsifiers (Span and Tween) have indicated that the osmotic pressure gradient between the two aqueous phases is a strong driving force for the mutual migration of water and encapsulants from one phase through the other (Magdassi and Garti, 1984, 1986). Garti (1997b) suggested that the transport of water is diffusion controlled, and that the diffusion rate through the oil phase can be altered by changing the nature of the oil, increasing its viscosity and adding various carriers. This suggests that the diffusion of the water or encapsulant through the oil is the rate-determining step, and that the internal water phase does not have an effect on the determination of the release rates.
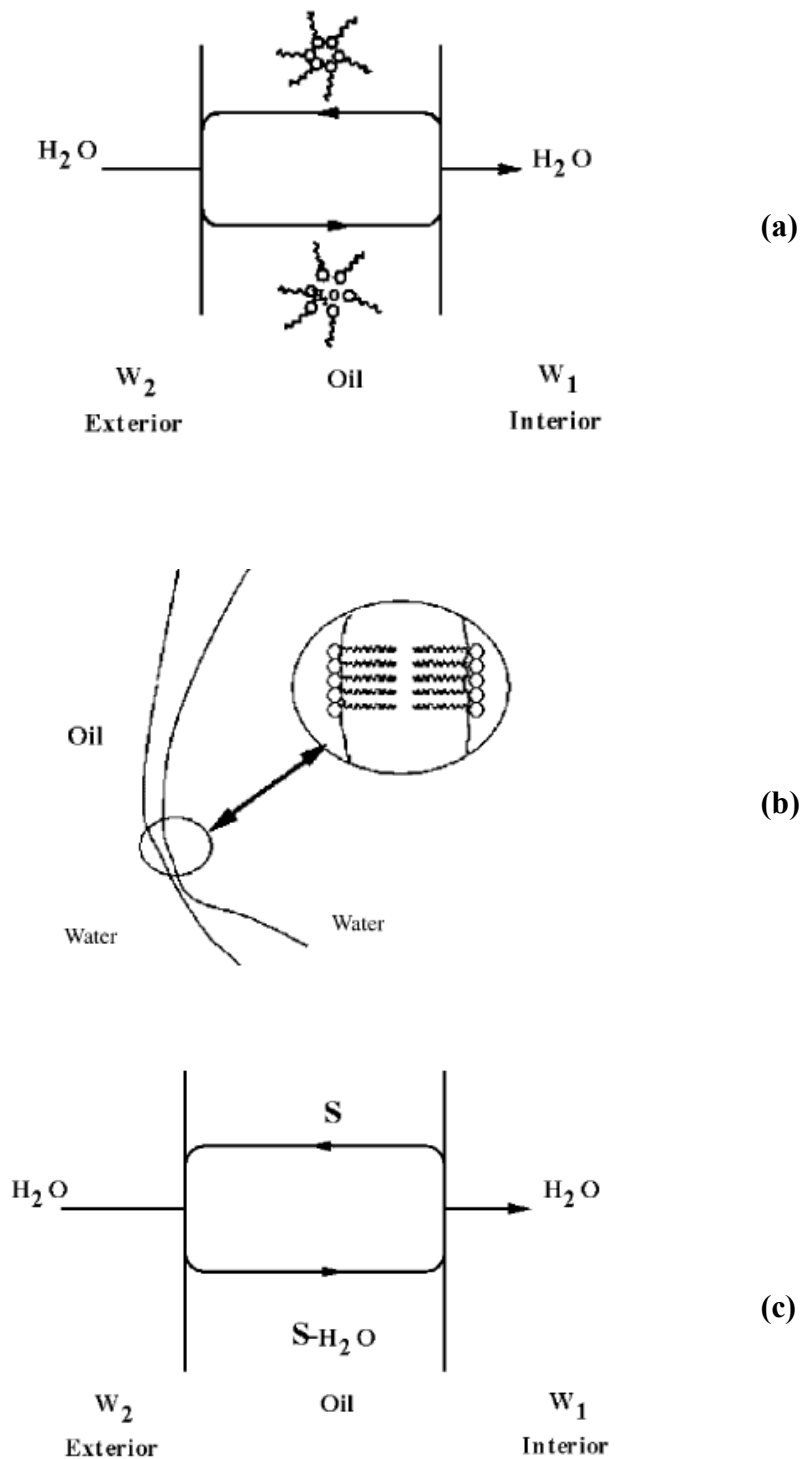
Fig. 2.5. Schematic illustration of three possible transport mechanisms in W/O/W emulsions: (a) reverse micellar transport; (b) lamellar thinning transport of marker from the internal aqueous phase to the continuous phase; (c) water transport via hydrated surfactant (emulsifier) (Benichou et al., 2004).

However, there is evidence that water can be transported by other mechanisms, and factors other than osmotic pressure gradients and diffusion regulation could also affect the flow of water in W/O/W emulsions. Wen and Papadopoulos (2000a) studied water transportation between internal water droplets and the external aqueous phase in a single oil droplet using a capillary video-microscopy technique. The size of the internal water droplet and the distance between the water droplet and the external aqueous phase were monitored quantitatively (Fig. 2.6). It was found that water transportation was dominated by different mechanisms that depended on the distance between the internal water droplet and the external aqueous phase.
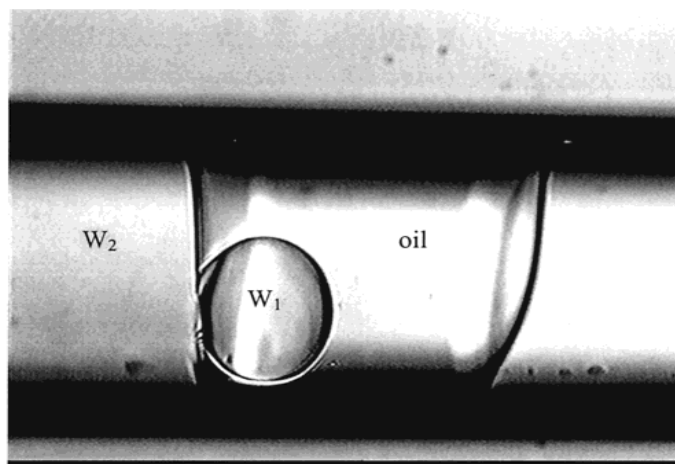
(a)
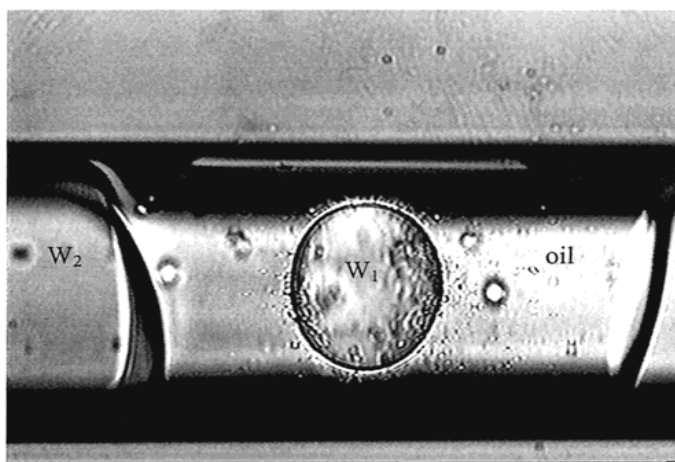
(b)

Fig. 2.6. Internal water droplet (W_1) and external aqueous phase (W_2) with (a) visual contact and (b) no visual contact (Wen and Papadopoulos, 2000a).

When the internal water droplets had visual contact (Fig. 2.6 a), water transportation was probably dominated by a hydrated surfactant transportation mechanism (Fig. 2.5 c); the transportation rate was dependent on the osmotic pressure gradient and diffusion regulation. The water transport rate was significantly lower when there was no contact between the internal water droplet and the external aqueous phase (Fig. 2.6 b) than when they were in visual contact. Water was most likely to be transported through a "reverse micelle transportation" mechanism (Fig. 2.5 a) and spontaneous emulsification, in which tiny droplets were formed spontaneously on the interface, then were detached and moved into the oil phase and eventually joined the external aqueous phase.

In the case of no visual contact (Fig. 2.6 b), it was found that the rate of water transportation was independent of the sizes of the water droplet and the oil droplet; it was also independent of the distance between the water droplet and the external aqueous phase and the osmotic pressure over a significant range (Wen and Papadopoulos, 2000a, 2001). In addition, the water transport rate was nearly constant. The results indicate that, under certain experimental conditions, water transportation could be controlled by the rates of interfacial processes, such as micelle formation or breakdown from the interface (Chen, Miller and Garrett, 1997), rather than diffusion regulation. Therefore, the effects of the osmotic pressure or diffusion regulation on water transport are expected to contribute to different extents under different conditions.

2.6.4. *Transportation of electrolyte through the oil phase*

Apart from water, ionised, ionisable and non-electrolyte water-soluble materials have been found to migrate through the oil phase without affecting the stability of W/O/W emulsions (Law, Florence and Wahateley, 1984; Magdassi and Garti, 1984, 1986). Although it has been proposed that water may carry electrolytes through the oil phase (Omotosho, Law and Whateley, 1986a), other observations suggest that the transportation of water and encapsulant could occur via different routes and

mechanisms (Owusu, Zhu and Dickinson, 1992; Wen and Papadopoulos, 2001; Cheng, Chen, Zhao, Luo, Wen and Papadopoulos, 2007).

The transportation of salts through the oil phase has been investigated by a number of researchers. Repeated observations under the microscope revealed a gradual decrease in the number of internal water droplets. At the same time, the remaining internal water droplets had similar size distribution over time. This suggests that larger water droplets coalesced and were expelled to the external aqueous phase, after a critical size had been reached. This indicates that the salt release was at least partially controlled by the coalescence of the internal water droplets on the oil droplet surface, and that the scenario of release may vary markedly as a function of the composition and the colloidal size of the W/O/W emulsion. It could occur by an entropy-driven diffusion and permeation of the salt across the oil droplet (Zak, Roberts and Gross, 1992; Goubault, Pays, Olea, Gorria, Bibette, Schmitt and Leal-Calderon, 2001).

Cheng et al. (2007) studied the transport of ions through the oil phase of a W/O/W emulsion. The direction of ion transportation was found to depend on the direction of the osmotic pressure gradient across the oil film; oil-soluble surfactants facilitated ion transport, and the ion transport was found to be independent of the thickness of the oil film. The results suggested that ion transportation depends mainly on the "reverse micelle" mechanism, in which ions are entrapped in reverse micelles with water and are transported across the oil phase, because ions exist in the hydrated state in the aqueous phase. Ions with smaller radii were transported more rapidly through the oil phase than ions with larger radii, probably because smaller molecules were more easily entrapped into the reverse micelles. In this and previous studies, there is no support for the molecular diffusion mechanism of ion transport through oils (Wen and Papadopoulos, 2001; Cheng et al., 2007). Other findings from ion transportation through the oil phase conclude that the diffusion rate of ions is dependent on the nature of the entrapped material, the emulsifiers used, the oil and the pH of the aqueous phase (Chang, Fuller, Frankenfeld and Rhodes, 1978; Dickinson et al., 1993; Garti, 1997a; Hai and Magdassi, 2004).

2.7.   Improvement of the stability of W/O/W emulsions

The main focus and efforts in more recent studies of multiple emulsions have been to improve the stability of W/O/W emulsions and to control the release of encapsulant and water in W/O/W emulsions. The compositions and processing techniques, which have direct influences on the properties of W/O/W emulsions, are subjected to various modifications to achieve improved stability (Garti and Aserin, 1996a; Benichou et al., 2004).

2.7.1. Control of the size of the internal water droplets

It has been proposed that the stability of W/O/W emulsions could be improved through stabilising the internal W/O emulsion by mechanically reducing its droplet size (forming microemulsions), or increasing the viscosity of the internal aqueous phase or forming microspheres (Garti and Aserin, 1996a). A smaller internal water droplet means that more water droplets will be encapsulated in the oil droplets. Dickinson and McClements (1996) suggested that oil droplets with substantial numbers of internal water droplets tend to exhibit the best stability and are generally regarded as being better controlled-release vehicles than droplets containing only a few water droplets, as internal water droplets are susceptible to flocculation (Dickinson and McClements, 1996).

More recently, Mezzenga et al. (2004) proposed that control of the size of the internal water droplets can be achieved by altering the chemical potential of the external and internal aqueous phases by dissolving neutral charged linear polysaccharides of suitable molecular weight. As a consequence of the different chemical potentials in the two aqueous phases, transport of water takes place, modifying the initial morphology of the W/O/W emulsion by altering the osmotic pressure balance between the two aqueous phases.

2.7.2. *Modification of the oil phase*

Modifying the oil phase of W/O/W emulsions can be a means of enhancing the stability and to slow down the release of encapsulant. Sela, Magdassi and Garti (1995) proposed that Pickering emulsions (emulsions stabilised with solid particles) could be shear resistant and that the release of encapsulant could be slowed. A later study by the same group used this concept. Fast freezing and slow thawing were applied to a W/O/W emulsion to accelerate micro-sized crystallisation of triglyceride in the oil phase, i.e. water droplets were covered by fat crystals rather than liquidised oil after the process. The release of encapsulated NaCl was near zero, indicating that high EEs can be obtained, and the emulsions were stable in terms of change in droplet size (Garti, Binyamin and Aserin, 1998; Garti, Aserin, Tiunova and Binyamin, 1999).

Weiss et al. (2005) used oil phases containing different concentrations and types of fat solid. The solidified oil phase was shown to play an important role in the release of encapsulant. The rate of release of encapsulant (L-tryptophan) was reduced by increasing the content of fat with higher melting point in the oil phase. Oil crystals were found to be formed in the lipid phase of the emulsion, but in an uncontrolled manner. These uncontrolled crystals had large sizes, which made the W/O/W emulsion susceptible to coalescence. It was also found that, after solidification of the oil phase, some of the characteristics of the W/O/W emulsions, such as viscosity, texture and resistance against shear, showed notable changes compared with the original emulsion (Guery, Bertrand, Rouzeau, Levitz, Weitz and Bibette, 2006). Nevertheless, the concept of Pickering emulsions might provide a good solution to the controlled release of encapsulants.

2.7.3. *Addition of macromolecules to the internal aqueous phase*

It has long been known in practice that the addition of naturally occurring macromolecular substances, such as gums or proteins, to the internal aqueous phase improves the stability of W/O and W/O/W emulsions, and slows down the release of

encapsulants (Garti and Aserin, 1996a; Garti, 1997b; Benichou et al., 2004; Lawson and Papadopoulos, 2004). Bovine serum albumin (BSA) is the macromoloecule that has been most extensively studied as a partial replacement for monomeric emulsifiers in the internal aqueous phase of multiple emulsions (Omotosho et al., 1986a; Dickinson et al., 1993; Garti, Aserin and Cohen, 1994; Evison, Dickinson, Owusu and Williams, 1995; Garti and Aserin, 1996a; Pays et al., 2002). All these studies showed that BSA improved the stability of W/O/W emulsions when it was added to the internal aqueous phase. Koberstein-Hajda and Dickinson (1996) incorporated unmodified faba protein into the internal aqueous phase of W/O/W emulsions using Span 80 as the hydrophobic emulsifier and NaCN as the hydrophilic emulsifier (external aqueous phase) with little change in the resulting EE values. Other proteins and polysaccharides that have been used as substitutes for monomeric emulsifiers are gelatine (Vaziri and Warburton, 1994), xanthan gum (Evison et al., 1995), human serum albumin (Koberstein-Hajda and Dickinson, 1996), whey proteins (Cornec et al., 1998) and cyclodextrins (Yu, Bochot, Cheron, Seiller, Grossiord, Le Bas and Duchene, 1999).

The possible formation of an interfacial complex between polymers added to the internal aqueous phase and the hydrophobic emulsifiers was considered to be the reason behind the significantly enhanced stability of W/O/W emulsions (Omotosho, Florence and Whateley, 1990; Dickinson and McClements, 1996). The addition of a macromolecule might slow down both the diffusion of encapsulant and the coalescence of internal water droplets and the external aqueous phase. Garti (1997b) suggested that polymers and hydrophobic emulsifiers could form an interfacial layer composed of loops and tails, well anchored into the oil and aqueous phases, and increase the repulsion force between droplets. It was also found that stable emulsions can be prepared by modifying their interfacial membranes by altering the electrostatic and steric interactions of natural biopolymers (Guzey, Kim and McClements, 2004; Wang, Tao and Hu, 2006).

The stabilising effect of polymers depends on the experimental conditions, such as the concentration of polymers and the type of emulsifiers used. For example, BSA at a low concentration (0.2% w/w) improved the EE of W/O/W emulsions using Span 80

as the hydrophobic emulsifier, but, at higher concentrations, the EE decreased and the emulsions showed flocculation (Benichou et al., 2004). Theories that can provide answers to these observations are yet to be developed.

2.7.4. Stabilisation of oil droplets in W/O/W emulsions

Fine oil droplets (< 1 μm) produced by high-pressure or membrane homogenisation show good stability. However, shear should be avoided in the second emulsification stage because it leads to the formation of relatively large W/O/W emulsion droplets with very limited thermodynamic stability, even when polymeric emulsifiers are used (Garti and Aserin, 1996b). The oil droplets obtained undergo destabilisation processes such as creaming, flocculation and coalescence. As mentioned, these types of instability could be reduced by reducing the oil droplet size using mild energy input (Goubault et al., 2001), using polymeric emulsifiers or increasing the viscosity of the external aqueous phase through the addition of polymeric stabilisers or thickeners to this phase. Some stabilisers or thickeners have no or poor emulsifying function, and have no interactions with other ingredients (Fennema, 1996; Kanouni et al., 2002; Tesch and Schubert, 2002; Benna-Zayani, Kbir-Ariguib, Trabelsi-Ayadi and Grossiord, 2008). Others could form complexes with hydrophilic emulsifiers and enhance the stability of emulsions through these interactions (Benichou et al., 2004; Kim, Decker and McClements, 2006).

More recent studies of the stability of W/O/W emulsions have paid more attention to the combination of hydrophilic emulsifiers and macromolecules (proteins or polysaccharides or phospholipids) in the external aqueous phase, aiming not only to reduce the creaming of the oil droplets but also to reduce the size of droplets and the release of water droplets or encapsulants (Muguet, Seiller, Barratt, Ozer, Marty and Grossiord, 2001; Lawson and Papadopoulos, 2004). Mixtures of monomeric emulsifiers/macromolecules and mixtures of macromolecules have mainly been investigated (Omotosho et al., 1986a; Garti and Aserin, 1996a; Rosano et al., 1998).

Proteins, polysaccharides and phospholipids are natural surface-active biopolymers. Under specific conditions, polysaccharides could interact with proteins in solution as well as at the interface, resulting in complex formation or immiscibility of the two biopolymers. The complexation alters the viscosity and the behaviour of the polymers at the interfaces, and enhances the functional properties compared with the proteins and polysaccharides alone (Yan, Zhang and Ni, 1992; Rosano et al., 1998; Benichou et al., 2004). Factors that influence these interactions are protein/polysaccharide ratio, pH, ionic strength, nature of the polymers, molecular weight, net charge, tertiary structure and flexibility of chains (Garti, 1997a; Musashino, Hasegawa, Imaoka, Adachi and Matsumo, 2001; Benichou et al., 2004).

For emulsion systems, electrostatic complexation between oppositely charged proteins and polysaccharides allows better anchoring of the newly formed macromolecular amphiphile on to oil-water interfaces (Benichou, Aserin and Garti, 2002b; Benichou et al., 2004). However, until now, very little on the use of protein-polysaccharide conjugates in W/O/W emulsions has been done. Caseinate-dextran conjugates and whey protein isolate (WPI)-xanthan gum complexes were reported to increase the coalescence stability and lower the encapsulant release, even at low pH, where protein cannot generally function well. In addition, although the hydrophobic emulsifier could not be replaced completely, its concentration could be reduced substantially (Benichou et al., 2007; Fechner, Knoth, Scherze and Muschiolik, 2007).

Compatible mixtures of biopolymers increase the viscosity of each of the phases, complex with the oil or the emulsifiers and form systems that will behave much like microcapsules, microspheres and mesospheric liquid crystals, which are more stable than emulsions (Kawakatsu, Tragardh and Tragardh, 2001; Benichou et al., 2004). They are an important basis for the discovery of new emulsifiers for preparing dispersed food systems with customised properties and for preparing double emulsions with different release behaviours. They could broaden the possibilities for the food applications of W/O/W emulsions (Fechner et al., 2007).

2.8. Characteristics of W/O/W emulsions

The complex nature of W/O/W emulsions has caused significant difficulties in the assessment of their characteristics and properties, which reflect the stability of the emulsions. For example, W/O/W emulsions are thermodynamically unstable and tend to coalesce; however, rupture, coalescence phenomena and change of interfacial tension within the oil droplets are difficult to detect (Dickinson and McClements, 1996; Garti, 1997a). Methods that provide indirect information often result in conflicting results as a subtle change in the experimental conditions or the compositions may have a significant impact on the sensitivity of the method used. More recently, a group of researchers has been able to monitor the change in water droplets inside a single oil droplet using a capillary video-microscopy technique (Wen and Papadopoulos, 2000a, b, 2001). However, it is worth noting that the system used in these studies was a single droplet and that the emulsion was formed without using mechanical force; what happens in a bulk system may be very different.

To date, the characteristics of double emulsions are generally defined using microscopic examination (photomicrography), counting the volume and the number of droplets (droplet size distribution) immediately after preparation and after prolonged storage, freeze-etching techniques, viscosity measurement and quantitative estimation of the encapsulant transported from the internal aqueous phase to the external aqueous phase (EE) and vice versa. In addition, interfacial tension can be measured, calculated and interpreted in terms of stability (Garti, 1997a; Kanouni et al., 2002).

2.8.1. Droplet size distribution

Droplet size distribution is one of the most important characteristics of an emulsion, as it will influence its rheology, stability, colour and taste (Danner and Schubert, 2001). The droplet size distribution of emulsions has generally been measured by light scattering techniques, electron microscopy and, in some cases, optical microscopy;

new techniques, such as pulsed field gradient nuclear magnetic resonance (PFG-NMR), to obtain more accurate data have also become available.

2.8.1.1. Multi-angle static laser light scattering (MasterSizer)

A MasterSizer, a static laser light scattering tool, is often used to probe the structure and dynamics of a dispersion system, based on the Mie theory (Evison et al., 1995). Particle size information is extracted from the intensity characteristics of the scattering pattern at various angles. The light scattering signal is due to the contrast of the refractive indices of different phases. In a simple emulsion, if the reflective indices of the oil phase and the aqueous phase are known, accurate particle size information can be obtained. The technique is rapid, a wide dynamic range of size can be measured (0.1–2000 μm), the entire sample is measured and the technique is suitable for very dilute systems (Evison et al., 1995). The most frequently used information from the MasterSizer is d_{43} (volume or mass moment mean) and d_{32} (surface area moment mean).

In a W/O/W emulsion, there is an index mismatch between the internal water droplets and the oil droplets, in addition to the mismatch between the oil droplets and the external aqueous phase. The structure and dynamics inside the oil droplet complicate the interpretation of laser light scattering; the results are obtained based on the assumption that the internal water droplets do not significantly change the refractive index of the oil droplets (Dickinson et al., 1994b; Wang et al., 2006).

2.8.1.2. Diffusing wave spectroscopy

Diffusing wave spectroscopy (DWS) is a non-intrusive dynamic light scattering technique that allows turbid colloidal samples to be studied (Hemar, Pinder, Hunter, Singh, Hebraud and Horne, 2003). The technique exploits the random multiple scattering of photons by the scattering particles present in the sample. Consequently, the variation in the scattering intensity with time can be directly related to the

mobility of particles in the sample. DWS can provide information about the local dynamics of colloidal particles from a few nanometres to several microns (Navabpour, Rega, Lioyd, Attwood, Lovell, Geraghty and Clarke, 2005). The DWS technique has recently been used to study the stability of macromolecular systems (renneting of milk), the mean droplet size of food emulsions and the characterisation of the viscoelastic properties of macromolecular networks (Michaut, Hebraud and Perrin, 2003).

Recent studies using DWS attempted to identify the droplet dynamics within multiple emulsions (Michaut et al., 2003; Wang et al., 2006); oil droplets from an O/W emulsion and those from a W/O/W emulsion showed significant differences in the decay of signals, indicating that the DWS technique has potential to be used to monitor the changes inside an oil droplet in W/O/W emulsions.

2.8.1.3. PFG-NMR techniques

PFG-NMR, which relies on the use of magnetic field gradients, has been used widely for the direct determination of self-diffusion coefficients (D) of molecules (Johns and Hollingsworth, 2007).

PFG-NMR has been used to determine the droplet size distribution in simple emulsions (Hollingsworth and Johns, 2006; Johns and Hollingsworth, 2007). The measurement of emulsion droplet size using PFG-NMR is based on the restriction of the self-diffusion of the molecules of the dispersed phase in an emulsion by the boundary between the phases. Advantages of this method include no sample dilution and discrimination of impurities (Hollingsworth and Johns, 2003; Hindmarsh, Hollingsworth, Wilson and Johns, 2004).

Only a few studies using this technique to determine the internal water droplet size of W/O/W emulsions can be found in the literature. The work conducted by Lonnqvist et al. (1997) used the well-documented PFG-NMR emulsion droplet sizing technique developed by Packer and Rees (1972) to monitor the size distribution of the internal

water droplets of a water-decane-water emulsion. The water signal was predominantly from the internal water droplets as the continuous water signal was relaxation weighted out (a spin-echo was used) and its contribution was further diminished by its significantly higher diffusion coefficient. Thus, the droplet size distribution was obtained (Lonnqvist, Hakansson, Balinov and Soderman, 1997). It was demonstrated that the technique could monitor the size distribution of the internal water droplets before and after formation into a W/O/W emulsion by tracing the diffusion characteristic of molecules inside the emulsion droplet, although the exchange of water between the internal and external aqueous phases was not studied. Mezzenga et al. (2004) considered the effect of osmotic pressure on water exchange between the internal aqueous phase and the external aqueous phase in a water-triglyceride-water multiple emulsion. Measurements from PFG-NMR of the diffusion of water in the internal droplets revealed an increase in the diffusion path, which was interpreted as being exchange of water molecules between droplets; laser light scattering measurements revealed enlargement of the oil droplets in response to water migration under dextran osmotic pressure gradients.

Typically, PFG-NMR experiments are time consuming, taking between 5 and 20 min to make a single droplet size distribution measurement. In a few recent studies, fast PFG-NMR methodologies have been developed and are based on application of the pulse sequence difftrain (diffusion train), in which restricted diffusion is made on a time scale of 3–10 s. The newly modified method opens up the possibility of probing faster transient processes in emulsion science (Hollingsworth, 2003; Hollingsworth, Sederman, Buckley, Gladden and Johns, 2004; Hindmarsh, Su, Flanagan and Singh, 2005).

2.8.2. *Confocal scanning light microscopy*

The basic principle of confocal scanning light microscopy (CSLM) is that a point in a sample is optimally illuminated and imaged in a detector pinhole. This leads to an increased resolution and a reduced depth of field because off-focus levels in the specimen contribute little to the image (Blonk and Van Aalst, 1993). CSLM is used in

the study of emulsions and foam stability; food components such as lipids and proteins can be stained selectively prior to processing of the product and also by diffusion of the stain into the product. This method has the advantage of avoiding destructive sampling. However, care has to be taken in checking the influence of the covalently linked marker on the structure of the macromolecule (Blonk and Van Aalst, 1993).

2.8.3. Encapsulation efficiency

The most important parameter characterising the success of a double emulsion preparation procedure is the EE (Owusu et al., 1992; Koberstein-Hajda and Dickinson, 1996). This is the fraction (% volume) of the aqueous phase of the primary emulsion or the encapsulant that is retained as the internal aqueous phase in the final W/O/W emulsion. It can be determined by measuring the release into the multiple emulsion continuous phase of an oil-impermeable solute marker present in the dispersed phase of the primary W/O emulsion (Dickinson et al., 1991a; Owusu et al., 1992). The EE can be based on Eq. 2.1.

$$EE = 100\left[1 - C^*V^*/C_wV_w\right] \qquad\qquad (2.1)$$

C^* the marker or encapsulant concentration in the external aqueous phase of the W/O/W emulsion;

V^* the volume of the external aqueous phase of the W/O/W emulsion;

C_w the known value of the concentration of marker or encapsulant solution initially encapsulated;

V_w the volume of the internal aqueous phase of the W/O emulsion.

The ideal 100% EE corresponds to a situation in which all the internal water droplets and encapsulant in the original primary emulsion remain intact during the subsequent processing and storage. The actual EE is affected by the severity of the second-stage emulsification process; in practice, as the primary W/O emulsion must break down to some extent under the influence of the hydrodynamic forces, the practical objective is

to minimise the extent of this breakdown as far as is possible (Dickinson and McClements, 1996). After the emulsions are formed, the change in EE is correlated with the stability of the emulsion.

2.9.   Potential applications of W/O/W emulsions

W/O/W emulsions have great potential to be used in many areas, such as in the pharmaceutical, cosmetic and food industries for the slow or controlled release of active encapsulants through the intermediate oil phase. The extent of release depends on several factors, such as the solubility of the encapsulant in the oil phase, the nature of the oil phase and the viscoelasticity of the adsorbed emulsifier layer at the oil phase (Matsumoto and Kang, 1989; Dickinson and McClements, 1996).

To date, the application of W/O/W emulsions is limited. The biggest hurdle is their inherent thermodynamic instability, and the difficulties in stabilising the system using safe, cost-effective ingredients and processing methods that are suitable for scaling up the production (Hino et al., 2001; Benichou et al., 2004). The presence of an additional component in the internal aqueous phase may itself compromise the stability of the system, by interfering with the stabilising properties of the hydrophobic emulsifier or by promoting an osmotic pressure gradient. The occurrence and the type of instability also depend on the chemical type of the encapsulated agent, the emulsion droplet size and the nature of the emulsifiers in the system (Matsumoto, 1985; Muschiolik, 2007).

To date, the relevant studies are mostly limited to pharmaceutical and cosmetic areas, as, in comparison with food systems, the systems applied in these areas are simpler and there is a broader choice of components. Foods contain multiple ingredients, all of which could have impacts on the stability of emulsions, and there is more regulation of food ingredients. The potential in pharmaceuticals includes: improved biological availability, prolonged drug delivery, sorbent reservoirs of drug over dosage treatments and immobilisation of enzymes (Omotosho et al., 1990; Garti and Aserin, 1996b; Couvreur et al., 1997; Garti, 1997a; Higashi, Tabata, Kondo, Maeda, Shimizu,

Nakashima and Setoguchi, 1999; Hino, Kawashima and Shimabayashi, 2000; Okochi and Nakano, 2000; Farahmand et al., 2006). Potential applications in cosmetics include the slow release of acidic compounds such as antioxidants (Tadros, 1992; Yazan, Seiller and Arslan, 1997; Gallarate, Carlotti, Trotta and Bovo, 1999; Ozer, Baloglu and Ertan, 2000; Lee, Kim, Han, Chang, Kang, Lee, Oh and Suh, 2004b).

In the food industry, there are two main potential applications of W/O/W emulsions. One is for the encapsulation of active compounds. The achievement of 100% EE of the internal aqueous phase during the emulsion preparation process is of particular interest, for example, to supplement products with sensitive micronutrients in the internal phase, to mask the unpleasant flavour of bioactive components or to achieve a controlled release of various substances, e.g. flavourants (Owusu et al., 1992; Dickinson and McClements, 1996; Muschiolik, 2007). Examples of this type of application are patented salted creams (encapsulation of salt) and aromatic mayonnaises (Asahi, 1987; Morinaga, 1994).

The other relates to the emerging interest in functional foods, which requires the technology to produce healthier foods, compared with the O/W emulsion of the same dispersed phase volume fraction. A W/O/W emulsion uses less oil phase, which indicates the potential for taking up less oil and lowering the cost (Dickinson and McClements, 1996; Garti and Aserin, 1996b). One promising application reported in this area is in the formulation of reduced-fat cheese-like products and dairy-like products. The development of dairy-like products containing less milk fat and a better saturated fat/unsaturated fat balance can contribute to improve the health of consumers (Lobato-Calleros, Rodrigues, Sandoval-Castilla, Vernon-Carter and Alvarez-Ramirz, 2006).

2.10. Conclusions

The characteristics and the stabilities of W/O/W emulsions have been studied over 30 years. Various theories about the mechanisms behind the stability of emulsions have been established and methods that could improve their stability have been developed.

However, the stability and the release patterns of complex double emulsion systems depend on various parameters that interact simultaneously. Some researchers tend to focus on certain mechanistic aspects whereas other researchers use very specific formulations and obtain specific results. A single or unique mechanism cannot explain all the pathways that occur in parallel in the system (Garti and Aserin, 1996b). To date, the mechanisms that contribute to the destabilisation and release of water and encapsulant from the internal aqueous phase to the external aqueous phase are not fully understood. A good control of any of the mechanisms has not yet been achieved (Benichou et al., 2004). The main goals remaining are to obtain submicron double emulsion droplets with long-term stability and to trigger and to control the release at will (Kent and Saunders, 2001).

The aim of this study was to investigate the characteristics and the stabilities of food-grade W/O/W emulsions. Most of the previous studies were conducted using compositions (e.g. hydrocarbon solvent) that are restricted for use in the food industry or applied techniques that have practical difficulties in the food industry. More studies using food grade compositions are necessary, from a simple system to more complicated systems, and from revealing the fundamental stability mechanisms to exploring the stability as a function of different processing and compositional variations. A better understanding of these aspects will enhance the potential for W/O/W emulsions to be used in the food industry.

Chapter 3: Formation of W/O/W emulsions: selection of materials and processing conditions

3.1. Introduction

The most important factors that could affect the properties of a W/O/W emulsion are its compositions and the processing conditions (Sourdet, Relkin, Fosseux and Aubry, 2002; Shima et al., 2004). This chapter presents preliminary experiments for selection of various compositions and processing conditions for the W/O/W emulsion. Although a wide range of materials have been used in studies investigating W/O/W emulsions, this study was to investigate the properties of a W/O/W emulsion that could be used in food systems, therefore only food grade materials were to be selected. Formation conditions for W/O/W emulsion are important as they will directly affect the particle size and coverage of emulsifier on the interface, which is vital for the stability of W/O/W emulsions (Srinivasan, Singh and Munro, 1996; Benichou et al., 2001). In this study, emulsions were prepared using a high pressure homogeniser and a rotor-stator style homogeniser. Properties of emulsions prepared under different pressures and speeds were compared and optimum processing conditions were determined. At the end of preliminary experiments, a W/O/W emulsion was formulated and processing conditions of formation were established.

3.2. Materials and Methods

3.2.1. Materials

Commercial soybean oil was purchased from AMCO Ltd., Auckland, New Zealand. Polyglycerol ester of polyricinoleic acid (PGPR 4150, Palsgaard Ltd., Denmark) and lecithin (Yelkingold) were purchased from Hawkins Watt Ltd, Auckland, New Zealand. Commercial sodium caseinate (NaCN) and whey protein isolate (WPI) were obtained from Fonterra Cooperative Ltd., Palmerston North, New Zealand. Violet dye poly R-478, Span 80 and Tween 80 were obtained from Sigma Ltd., St Louis, Missouri, USA, and Nile Blue and SDS were from BDH Ltd, Poole, UK.

3.2.2. Emulsion preparation

W/O/W emulsions were prepared by a two-step process. Phosphate buffer (0.1 M), pH 6.6 (containing 0.02% (w/v) sodium azide) was dispersed in soybean oil containing PGPR or other hydrophobic emulsifiers, and the mixtures were homogenised at 800/80 bar (first stage pressure/second stage pressure) using a two-stage valve homogeniser (APV 2000, Denmark). Samples were passed twice through the homogeniser to obtain the primary W/O emulsions. The second step involved the dispersion of the primary emulsions into solutions containing hydrophilic emulsifiers, prepared in 0.1 M phosphate buffer, pH 6.6, using the same homogeniser, at a pressure of 100/0 bar twice or using a rotor-stator style homogeniser (Heidolph DIAX600, Germany) at 8000 rpm for 5 mins.

3.2.3. Confocal laser microscopy

Confocal scanning laser microscopy (CSLM) was used to observe the primary emulsion and the multiple emulsion samples (Leica TCS 4D confocal microscope, Leica Lasertechnik GmbH, Heidelberg, Germany). Samples (1 mL) of emulsion were

stained with 0.1 mL of 1% (w/v) Nile Blue, and the stained samples were dropped on concave glass slides, covered by a cover slide and examined with a 100 × magnifications lens and an Ar/Kr laser with an excitation line of 488 nm; the resolution of this techniques is > 0.2 μm.

3.2.4. *Determination of average droplet size of primary W/O emulsions*

3.2.4.1. Diffusing wave spectroscopy (DWS) experimental set-up

The droplet sizes of W/O emulsions were measured using diffusing wave spectroscopy (DWS) measurements, which were performed on an experimental set-up similar to the one described by Hemar et al., (2003). The light source was a Spectra Physics 125A laser, operating at $\lambda = 632$ nm and delivering 35 mW. The laser beam was expanded to approximately 8 mm diameter at the sample cell. The diffuse light was collected by one of two single mode fibers (PI-3224-PC-5, Thorlabs Inc., Germany) fitted with Grin lenses (F230FC, Thorlabs Inc., Germany). One fiber was placed beyond the sample cell on the axis of the beam for the transmission geometry, a second fiber was placed at the front face of the cell for the back-scattering geometry. The optic fibers were connected to a Hamamatsu HC120-08 PMT photomultiplier tube module and a Malvern 7132 correlator was used to obtain the intensity autocorrelation function of the scattered light. The samples of primary W/O emulsions were placed in a 1 cm square cuvette at room temperature (23°C), and the autocorrelation functions were collected for 10 minutes. To obtain emulsion droplet size from the results of DWS technique, values of the kinematic viscosity of the samples were required and determined using a rheometer (Anton Parr Physica MCR 301, Austria).

3.2.4.2. DWS analysis

In an expanded beam mode DWS experiment, the field autocorrelation function $g_{(1)}(t)$, was obtained from the intensity autocorrelation function, using the Siegert relationship. In the back-scattering geometry, $g_{(1)}(t)$ is given by :

$$g_{(1)}(t) = \frac{\sinh\left[\sqrt{\frac{6t}{\tau}}\left(\frac{L}{l^*} - \frac{z_0}{l^*}\right)\right] + \frac{2}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\sqrt{\frac{6t}{\tau}}\left(\frac{L}{l^*} - \frac{z_0}{l^*}\right)\right]}{\left(1 + \frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{\frac{6t}{\tau}}\right] + \frac{4}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\left(\frac{L}{l^*}\right)\sqrt{\frac{6t}{\tau}}\right]} \qquad (3.1)$$

and in the transmission geometry, $g_{(1)}(t)$ is given by:

$$g_{(1)}(t) = \frac{\frac{L/l^*+4/3}{z_0/l^*+2/3}\left\{\sinh\left[\frac{z_0}{l^*}\sqrt{\frac{6t}{\tau}}\right] + \frac{2}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\frac{z_0}{l^*}\sqrt{\frac{6t}{\tau}}\right]\right\}}{\left(1 + \frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{\frac{6t}{\tau}}\right] + \frac{4}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\left(\frac{L}{l^*}\right)\sqrt{\frac{6t}{\tau}}\right]} \qquad (3.2)$$

were l^* is the scattering mean free path, z_0 is the penetration depth (in the present case we assume $z_0 \approx l^*$), and L is cell path length. For particles of radius a undergoing Brownian motion, the relaxation time, τ, is given by:

$$\tau = \left(D\,k_0^2\right)^{-1} \text{ and } \qquad D = \frac{k_B T}{6\pi\eta a} \qquad (3.3)$$

where $k_0 = 2\pi/\lambda$ is the incident wavevector, D the Stokes-Einstein diffusion coefficient, and η the viscosity of the continuous phase. Thus, knowing η the radius a can be obtained:

$$a = \frac{2\,k_B\,T}{3\,\pi\eta\,\lambda^2}\tau \qquad (3.4)$$

When DWS is carried out under conditions such that L/l^* is large, Eq. (3.1) for back-scattering reduces to:

$$g_{(1)}(t) = \exp\left[-\gamma\sqrt{\frac{6t}{\tau}}\right]$$ (3.5)

with γ=1.94 in this experiment, and the mean square displacement $\langle\Delta r^2(t)\rangle$ can be obtained from equation (3.1) (3.2) or (3.3) using:

$$\langle\Delta r^2(t)\rangle = 6t/\left(k_0^2\tau\right)$$ (3.6)

Using both the transmission and backscattering measurements and equation (3.1) and (3.2), the mean square displacements (MSD) of the W/O emulsion can be obtained. An l^* of 120 nm and a refractive indices of 1.45 for the W/O emulsion were used. In W/O emulsions, the Brownian motion is observed only at large-time scales ($t > $ 1ms). We believe that the deviation from the Brownian motion at very short time is due to absorption (Scheffold, 2003).

In order to quantify the particle size of W/O emulsions, backscattering field autocorrelation function $g_1(t)$ is plotted as a function the correlation time, and showed a linear relationship, indicating that equations (3.4) and (3.5) could be used to extract the apparent radius, using the viscosity of the oil (0.066 Pa.s) and water (0.001Pa.s). The current quantification method used here is valid in all cases for the primary emulsions.

3.2.5. Determination of average droplet size of W/O/W emulsions

The droplet sizes of W/O/W emulsions were measured by a Malvern MasterSizer MSE (Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). W/O/W emulsions were diluted in distilled water in the stirring cell. Volume average diameter (d_{43}) and surface average diameter (d_{32}) of W/O/W emulsions were determined. The lens range was set at 45 mm and the active beam length at 2.4 mm for emulsions prepared by high pressure homogeniser; and lens range set at 300mm and active beam length at 14.3 mm for emulsions prepared by rotor-stator homogeniser, as the later had much larger droplet size. The presentation code was 2NAD, the relative refractive index, i.e. the ratio of the refractive index of emulsion droplets to that of the dispersion medium was 1.095 and the absorbance value of the emulsion droplets was 0.001.

3.2.6. Determination of encapsulation efficiency

The encapsulation efficiency (EE) was defined as the fraction (% volume) of the aqueous phase of the primary emulsion which remained entrapped in the final W/O/W emulsion. The EE of W/O/W emulsions was determined by a modification to the method of Dickinson et al., (1994a) using a water soluble dye, Poly R-478. Poly R-478 is a polyporphyre (MW 5×10^4 Daltons) with a polyvinylamine sulphonate backbone and anthraquinone chromophore. It has been reported that there is no significant interaction between the poly R-478 dye and NaCN during the formation of multiple emulsions (Dickinson et al., 1991a; Dickinson et al., 1994b).

Poly R-478 was dissolved in sodium phosphate buffer (pH 6.6) to a final concentration of 1% ((w/v)). The solution was diluted to different concentrations and absorbance was measured at 522 nm. A standard curve was subsequently prepared ($r^2 = 0.9998$). Poly R-478 dye solution (1% w/v) was encapsulated in the inner aqueous phase of W/O/W emulsions. Samples of emulsions were diluted in phosphate buffer

(1:1) and centrifuged at 1×10^4 g for 20 minutes. An aliquot (1 mL) of the subnatant from centrifuged sample was obtained and the absorbance was recorded at 522 nm. The procedure was repeated on respective emulsions that had been prepared without dye to obtain blank values; these were subsequently subtracted from their counterparts with dye. The concentration of dye present in the subnatant was determined from the standard curve and the EE was expressed as a percentage of the initial concentration of dye encapsulated in the internal phase. The EE results were analysed using ANOVA and t-test for significance ($p < 0.05$) using the data analysis tool in Microsoft Excel.

3.3.   Results and Discussion

3.3.1. Internal and external aqueous phase

The internal and external aqueous phase selected are 0.1 M sodium phosphate buffer prepared with distilled water, as it was reported that the presence of electrolytes in aqueous phase slowed down Oswald ripening hence the stability of the emulsions is improved (Benichou et al., 2001). Both monosodium phosphate and disodium phosphate used to prepare phosphate buffer are common food additives (E 339 i and ii).

3.3.2. Oil phase

An oil phase that is stable at room temperature was desired as crystallisation of oil could destabilise W/O emulsions and W/O/W emulsions, causing breakdown of oil droplets and release of the encapsulant. Commercial grade vegetable oil, generally unsaturated long chain triglyceride, was suitable to be used as oil phase in this study. As these commercial vegetable oils have similar composition and properties (e.g. melting point, viscosity, etc.), only soy bean oil, the most commonly used vegetable oil, was tested (Ignacio, 1985; Rossell and J, 1991; Hamilton, 1993).

Volume fraction of water to oil was determined by adding different quantities of sodium phosphate buffer (10 – 50% v/v) to the soya oil prior to homogenization. The visual observations of primary W/O emulsions formed are presented in Fig. 3.1.

| 20:80 | 30:70 | 40:60 | 50:50 |
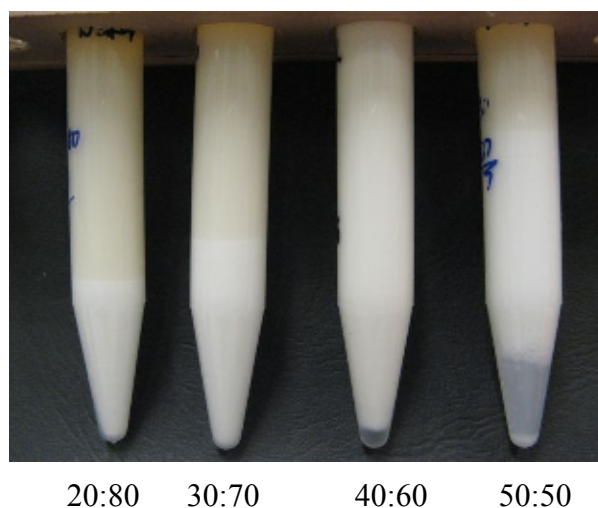
Fig. 3.1. Photograph of primary W/O emulsions with different volume ratios of water to oil. Emulsions were prepared with 2% (w/v) PGPR as the sole hydrophobic emulsifier and stored at 20 °C for 7 days.

It was found that emulsions with 20 -30% volume of water were stable without phase separation (the separation of water phase and remnant W/O emulsion) under present conditions. The increase of water phase induced greater instability, possibly due to increased incidence of coalescence and bridging at higher water volume, both of which led to reduction in total water droplet surface area (Benichou et al., 2001). The result was consistent with those obtained by other researchers that show that 20-30% of dispersed phase to continuous phase was optimum volume fraction for an emulsion if one took both stability and practical efficiency (e.g. EE) into consideration (Dickinson et al., 1993; Dickinson et al., 1994b). Based on this concept, the volume fraction of water in primary W/O emulsion, and oil (primary W/O emulsion) to the external aqueous phase of secondary W/O/W emulsion were both set at 20% dispersed phase: 80% continuous phase in all following experiments unless otherwise stated.

3.3.3. *Selection of hydrophobic emulsifier*

Hydrophobic emulsifiers are essential for the formation and stability of W/O emulsions. Several food grade hydrophobic emulsifiers, including nonionic hydrophobic emulsifiers sorbitan monooleate (span 80, HLB 4.7) (Sigma, St. Louise, Missouri, USA), a lecithin product (YelkinGold, HLB 4) (Hawkins Watt Ltd, Auckland, New Zealand) and polyglycerol ester of polyricinoleic acid (PGPR, HLB 4.3) (PGPR 4150, Palsgaard Ltd., Denmark) were tested in W/O emulsions using soy bean oil as the oil phase. All hydrophobic emulsifiers selected are commonly used in food products, such as margarine, ice cream and chocolate.

The visual observation of W/O emulsions prepared with different emulsifiers are shown in Fig. 3.2. W/O emulsions prepared with different emulsifiers had very different appearances. Samples with 10% (w/v) Span 80 showed phase separation immediately after homogenisation; samples with lecithin (10% w/v) resulted in a viscous paste-like sediment settling at the bottom of the flask; samples with PGPR (10% w/v) resulted in a uniform easy flowing emulsion without phase separation.

a   b   c
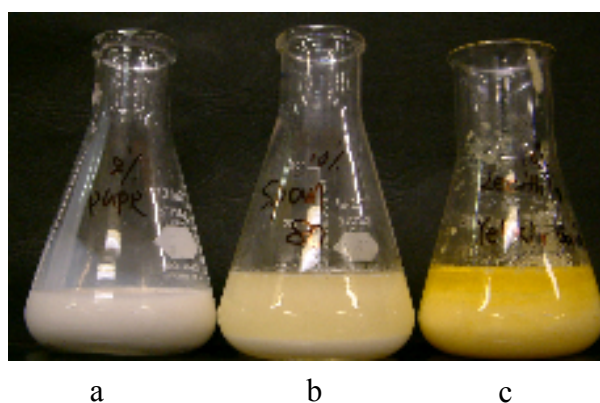
Fig. 3.2. Photograph of primary W/O emulsions with different hydrophobic emulsifiers (a): 10% (w/v) PGPR; (b): 10% (w/v) Span 80 or (c): 10% (w/v) lecithin immediately after homogenisation.

To further verify the ability of hydrophobic emulsifiers, the W/O emulsions prepared with different hydrophobic emulsifiers at various concentrations were incorporated into 0.5% NaCN solution in a ratio at 20: 80 to form W/O/W emulsions. Confocal images, droplet size determination and EE of W/O/W emulsion were obtained to determine the formation and practical efficiency of W/O/W emulsions. The results are shown in Figs. 3.3 - 3.5. Formation of W/O/W emulsions was observed in samples prepared with lecithin and PGPR, but not in samples prepared with Span 80 (Fig. 3.3). The droplet size distribution of samples with Span 80 closely resembled a simple O/W emulsion with a small mean droplet size (d_{43}=1.01μm), for lecithin it was also monmodal but mean droplet size had increased significantly (d_{43}=3.91μm) (Fig. 3.4). On the other hand, size distribution of sample with PGPR was bimodal. The first peak presented a population of smaller droplets (< 1μm) and second peak presented a population of larger droplets (>1μm). The smaller oil droplets with a d_{43} around 1 μm, i.e. similar to the droplet size of a simple emulsion, did not contain as many internal water droplets as in larger oil droplets (Fig. 3.3C). The oil droplet size distribution results indicated that W/O/W emulsion might be formed in samples with lecithin and PGPR, but not in samples with Span 80.

| A:10% (w/v) Span 80 | B:10% (w/v) lecithin | C:10% (w/v) PGPR |
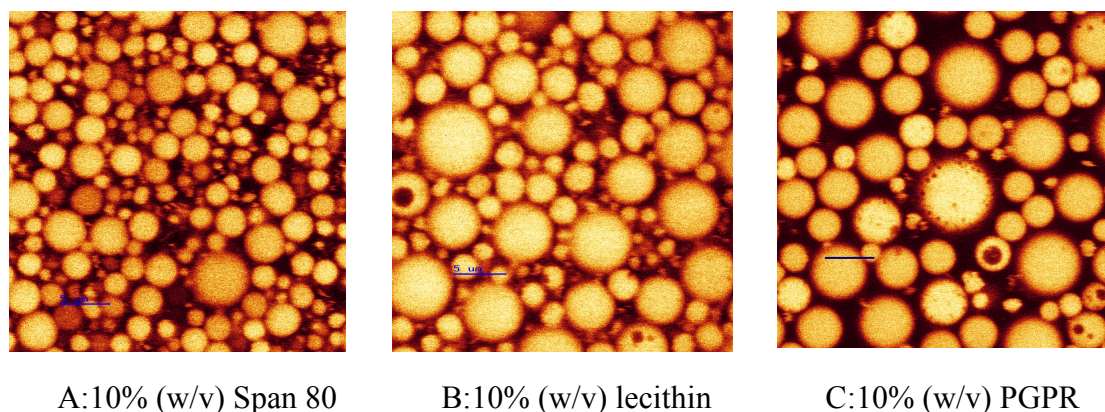
Fig. 3.3. Confocal microscopy images of freshly prepared W/O/W emulsions with different hydrophobic emulsifier. Scale bar represents 5 μm. 0.5% (w/v) NaCN in the external phase.
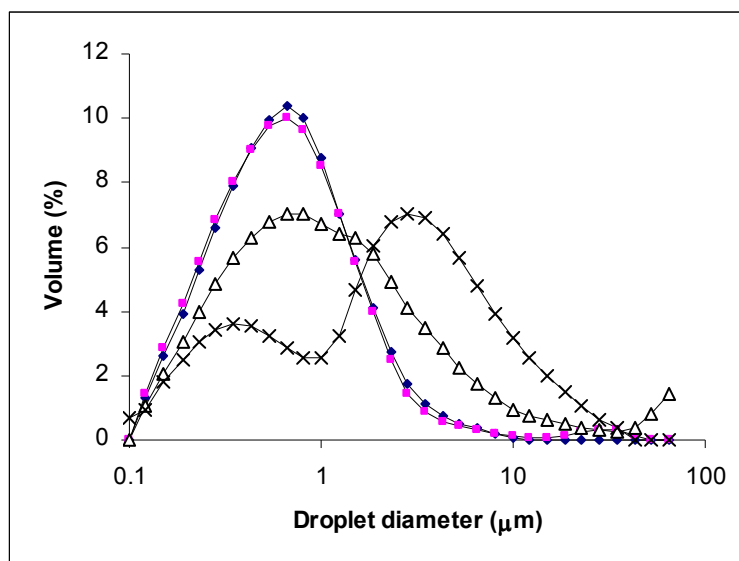
Fig. 3.4. Particle size distributions of W/O/W emulsions prepared with (◆) 10% (w/v) Span 80; (△) 10% (w/v) lecithin and (✕) 10% (w/v) PGPR in the oil phase. 0.5% (w/v) NaCN was in the external phase. The internal and external aqueous phase were made of 0.1 M phosphate buffer. Size distribution of a simple O/W emulsion made with 0.5% (w/v) NaCN (■) is also shown.

In EE determinations (Fig. 3.5), Span 80 appeared not to retain any dye (data not shown), whereas lecithin and PGPR retained various amounts of dye in the internal aqueous phase. Samples with PGPR had significantly higher EE than lecithin at all concentrations tested. The results again showed that PGPR is the best emulsifier among other emulsifiers under the present experimental conditions.
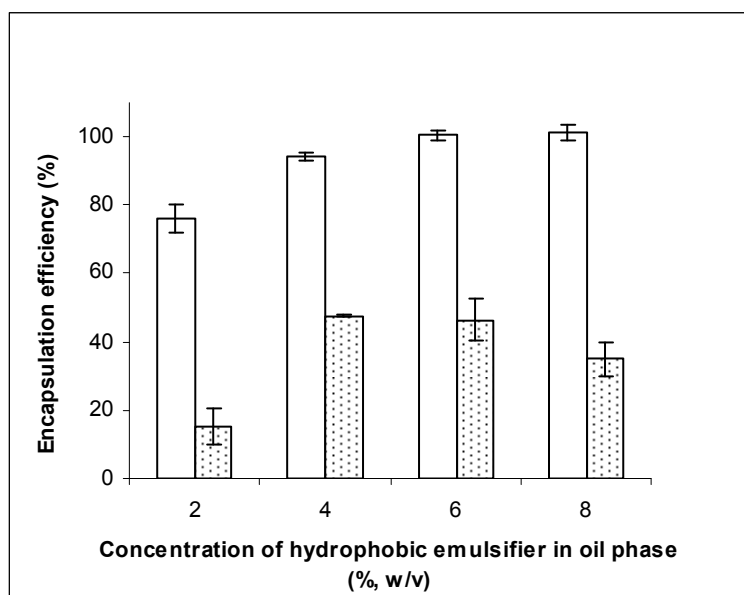
Fig. 3.5. Effect of hydrophobic emulsifier on encapsulation efficiency of W/O/W emulsion. Blank bar: PGPR, dotted bar: Lecithin. Error bars represent standard deviation. 0.5% (w/v) NaCN in the external aqueous phase.

Span 80, lecithin and PGPR have similar HLB value, theoretically they might possess similar emulsifying properties. In this study, however, results indicated that the emulsifying properties of emulsifiers were not solely dependent on the HLB value, other factors such as structures, chemical compositions and molecular weight was also important.

Previous study have shown that Span 80 could form relatively stable W/O and W/O/W emulsion with organic solvent or mineral oil as the oil phase under certain conditions (Dickinson et al., 1993; Dickinson et al., 1994b), but in this study the emulsifying properties of Span 80 were poor. It has been found that Span 80 in the oil phase lowered O/W emulsion stability by travelling through the oil phase, involving disruption and displacement of adsorbed sodium caseinate (hydrophilic emulsifier); in addition, the Span 80 spontaneously formed W/O/W emulsions (Cornec et al., 1998; Michaut et al., 2003). These activities of Span 80 through the interface might be responsible for the destabilisation of W/O and W/O/W emulsions and consequential

total release of encapsulated dye in W/O/W emulsions with Span 80 as hydrophilic emulsifier.

The lecithin product used in these experiments was derived from soybean, and is commonly used as a hydrophobic emulsifier in food W/O emulsions, such as margarine and chocolate. However, in this study, lecithin functioned poorly in the present formulation and processing conditions. Separation of water droplets was detected in W/O emulsion and EE of dye was very low. Gaonkar (1992) found that the addition of electrolytes (e.g. sodium phosphate buffer in this study) in emulsions increased the concentration of surface-active impurities from commercial vegetable oil at the oil-water interface and reduced interfacial tension. Lecithin-stabilized W/O emulsions were found to be sensitive to the presence of surface-active impurities in the oil phase. The stability of W/O/W emulsions with lecithin as hydrophobic emulsifier was reduced and interfacial film breakdown and rapid coalescence of water droplets were observed (Knoth et al., 2005; Scherze et al., 2006). The detrimental effect of electrolytes on lecithin-stabilized W/O emulsions could also be attributed to electrostatic effects caused by the inherent structure of lecithin. In the presence of electrolytes, the polar heads of phospholipids in lecithin may repel each other by electrostatic repulsion, and their close association may be reduced, therefore reducing the emulsifying efficiency (Doig and Diks, 2003).

PGPR was the most suitable hydrophobic emulsifier in W/O/W emulsion using commercial vegetable oil in this study, in terms of stability of W/O emulsion and encapsulation efficiency.   However, 10% PGPR used in preliminary study is excessive, as the maximum level allowed to be used in food is up to 5 g/kg (Wilson et al., 1998). Further studies were conducted to reduce the amount of PGPR required for a stable W/O/W emulsion with high (> 90%) EE, and will be discussed in Chapter 4: Effect of PGPR and macromolecular materials on the formation and stability of W/O/W emulsions.

3.3.4. Selection of hydrophilic emulsifier

Various hydrophilic emulsifiers were investigated for their ability to form and stabilize W/O/W emulsions. These were sodium caseinate (NaCN) (96% protein content. Fonterra, Palmerston North, New Zealand), whey protein isolation (WPI)) (94% protein content. Fonterra, Palmerston North, New Zealand), a small molecular weight surfactant, polysorbate (Tween 80, HLB 14) (Sigma, St. Louise, Missouri, USA), a modified product of gum arabic (SUPER GUM^{TM}) (Sanwa-cho, Toyonaka, Japan) and an anionic surfactant, sodium dodecyl sulphate (SDS, HLB 40) (Sigma, St. Louise, Missouri, USA), which is one of the most competitive hydrophilic surfactants (Fairley, Krochta and German, 1997).

Initially, these emulsifiers were dissolved in sodium phosphate buffer. On dispersion of SUPER GUM™ in the sodium phosphate buffer system, precipitates were evident in the sample. It was unclear what these precipitates were, as protease digestion of SUPER GUM™ in the supernatant of these samples following centrifugation yielded identical molecular weight data to SUPER GUM™ in non-buffered systems (data not shown; S. Al-Assaf, personal communication). To avoid the presence of this precipitated material, all SUPER GUM™ samples in later study were prepared in distilled water (non-buffered) systems, also all emulsions involved in a later experiment were prepared in non-buffered systems to make the results comparable. It was then unexpectedly found that EE of W/O/W emulsion (0.5% w/v NaCN as hydrophilic emulsifier) in non-buffered systems had increased significantly compared to same formulation in buffered systems in previous experiments, indicating that the sodium phosphate buffer might alter the emulsifier properties of PGPR. The issue was further investigated and is discussed in Chapter 4: Effect of PGPR and macromolecular materials on W/O/W emulsions.

Figure 3.6 and Figure 3.7 shows the comparison of EE and droplet sizes of W/O/W emulsions prepared with different hydrophilic emulsifiers using high-pressure homogeniser. Comparing two milk proteins, samples with WPI had a narrow droplet size distribution and a smaller mean droplet size (d_{43} 1.44 vs. 4.79 μm of NaCN), but

were less stable; free oil was observed over time and its EE value was also lower compared to NaCN (Fig. 3.6), indicating that NaCN had better emulsifying ability than WPI. The results are consistent with studies that investigated the emulsifying properties of NaCN and whey protein in simple O/W emulsions (Srinivasan et al., 2000; Ye and Singh, 2001). The difference in emulsifying ability was consistent with the more flexible nature of caseins as opposed to the globular whey proteins. NaCN is composed of four different proteins, α_{s1}-, α_{s2}-, β- and κ- casein in weight proportions of approximately 4:1:4:1. All caseins are amphipathic proteins with a strong tendency to adsorb at oil-water interfaces during emulsion formation. Hydrophobic amino acids are heterogeneously distributed and form hydrophobic domains in caseins structure, sterically separated from more hydrophilic regions, resulting in a disordered structure (Fennema, 1996). Whey proteins contain mainly β-lactoglobulin ($\sim 65\%$), α-lactalbumin ($\sim 25\%$), and serum albumin ($\sim 8\%$), which are globular proteins. The major constituents of whey proteins show high levels of sulphur-containing amino acids, which are responsible for gelling properties through disulfide bond formation (Fennema, 1996).
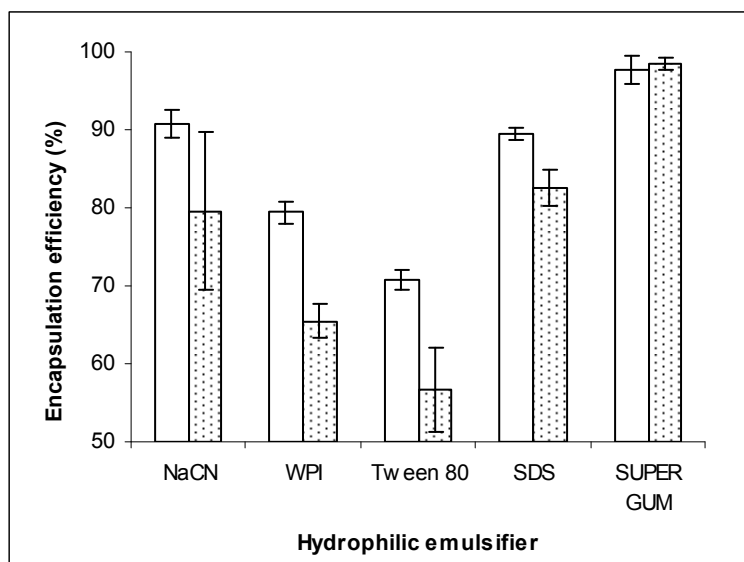
Fig. 3.6. Effect of different hydrophilic emulsifiers on encapsulation efficiency of W/O/W emulsion, blank bar: fresh sample, dotted bar: sample stored for 1 month at 20°C. W/O/W emulsions contained distilled water in the internal aqueous phase, 2% PGPR in the oil phase. The external phase contained 0.5% (w/v) NaCN, 2% (w/v) WPI, 2% (w/v) Tween 80, 0.1% (w/v) SDS or 10% (w/v) SUPER GUM^{TM}. Emulsions were prepared using high-pressure homogenizer.
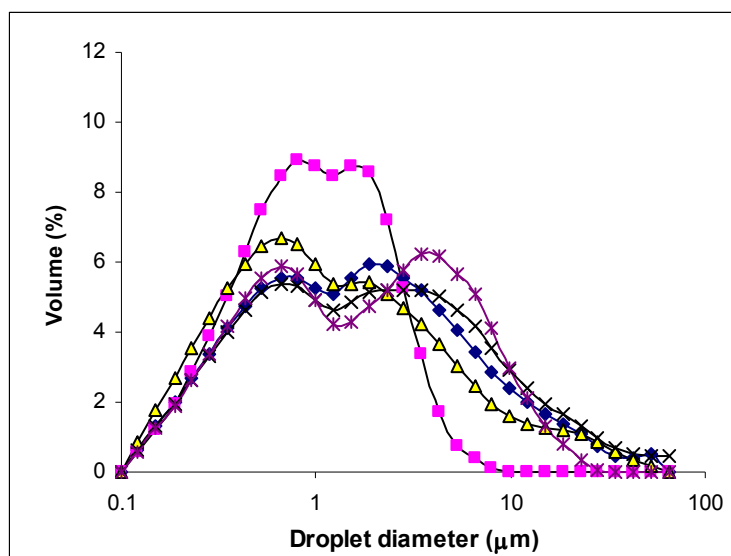
Fig. 3.7. Droplet size distribution of W/O/W emulsion with different hydrophilic emulsifier. W/O/W emulsions contained distilled water in the internal aqueous phase and 2% PGPR in the oil phase. The external phase contained (◆) 0.5% (w/v) NaCN, (■) 2% (w/v) WPI, (✕) 0.1% (w/v) SDS, (△) 2% (w/v) Tween 80 or (✳) 10% (w/v) SUPER GUM[TM]. Emulsions were prepared using high-pressure homogenizer.

Due to the structural differences the caseins are able to cover the interfacial area created during emulsification more effectively than the globular whey proteins. It was reported that NaCN has a minimum value for the surface concentration of 1.00 mg/m^2, compared with 1.5 mg/m^2 for WPI stabilized emulsions. In other words, at low protein concentrations caseinate can stabilize a large surface area than WPI (Hunt and Dalgleish, 1994).

Tween 80 resulted in largest droplet size and lowest EE, which is expected as it is well established that W/O/W emulsion are not stable when monomeric emulsifiers are used because of exchange of emulsifier through the oil phase (Michaut et al., 2003). W/O/W emulsion prepared with SDS as hydrophilic emulsifier were similar with

those with NaCN in terms of stability and EE, i.e., in this study, NaCN acted as efficiently as SDS.

W/O/W emulsion samples with SUPER GUM™ had the highest initial EE and were most stable over a period of month among all samples. The emulsifying ability of NaCN and SUPER GUM™ was further investigated in details in Chapter 5: Effect of hydrophilic emulsifiers on the formation and stability of W/O/W emulsions.

3.3.5. Processing techniques

The W/O emulsions were prepared using a two-stage high pressure homogeniser at various pressures to obtain smaller droplets, the secondary emulsification was prepared either with high pressure homogeniser using lower pressures, or with a rotor-stator homogeniser at 8000 rpm (revolution per minute), to avoid rupture of the internal droplets (van der Graaf et al., 2005).

3.3.5.1. Effect of homogenisation pressure on W/O emulsion

Reducing droplet size in W/O/W emulsions is of major importance, one of the routes to reduce particle size is to use high-pressure homogenization in which small and almost monodispersed droplets may be produced (Benichou et al., 2001; Stang et al., 2001; Schuchmann and Schubert, 2003). The high pressure homogeniser used in this study was a two-stage valve homogeniser (APV 2000, Denmark). A mixture of oil, emulsifier and water is forced through a narrow gap of a few hundred microns under high pressure. A combination of turbulence and intense laminar shear flow causes the disruption of the droplets, producing the final emulsion. The freshly disrupted small droplets have good stability toward creaming, flocculation and coalescence (Walstra and Smulders, 1997; Scherze et al., 2006). The operating parameters which would effect the efficiency of high-pressure homogenizers include pressure, temperature, number of passes, valve and impingement design and flow rate (Stang et al., 2001).

Effect of pressure on mean droplet diameter of W/O emulsion was studied and the mean size of W/O emulsion droplet measured by DWS is shown in Figure 3.8a. It was found that with increased pressure from 100 to 800 bars, the mean diameter of water droplet size did not decrease as expected, but increased significantly from 0.77 to 1.09 μm. The increase of water droplet size could be a result of coalescence. In studies of Karbstein & Schubert (1995) and Tesch & Schubert (2002), it was found that droplet size of an emulsion resulted from both droplet disruption and recoalescence after homogenisation. Coalescence frequency depends on the energy density, with increasing energy density the coalescence frequency rises first, reaches a maximum and then declines at higher energy densities.

W/O emulsions were also incorporated into sequential W/O/W emulsions and droplet size distribution of sequential W/O/W emulsions was measured by MasterSizer, and no significant difference were observed (Fig 3.8.b). The results indicate that larger water droplet size measured in W/O emulsion could be the result of flocculation of smaller water droplets, i.e., with sufficient amount of emulsifier (PGPR) at the surface, and higher viscosity in continuous phase (0.66 Pa.s of soybean oil vs. 0.001 Pa.s of water), coalescence time increased significantly, and water droplets flocculate rather than coalesce. As a result, the W/O emulsions prepared at higher pressure and the sequential W/O/W emulsions were as stable as their counterparts prepared at lower pressure.
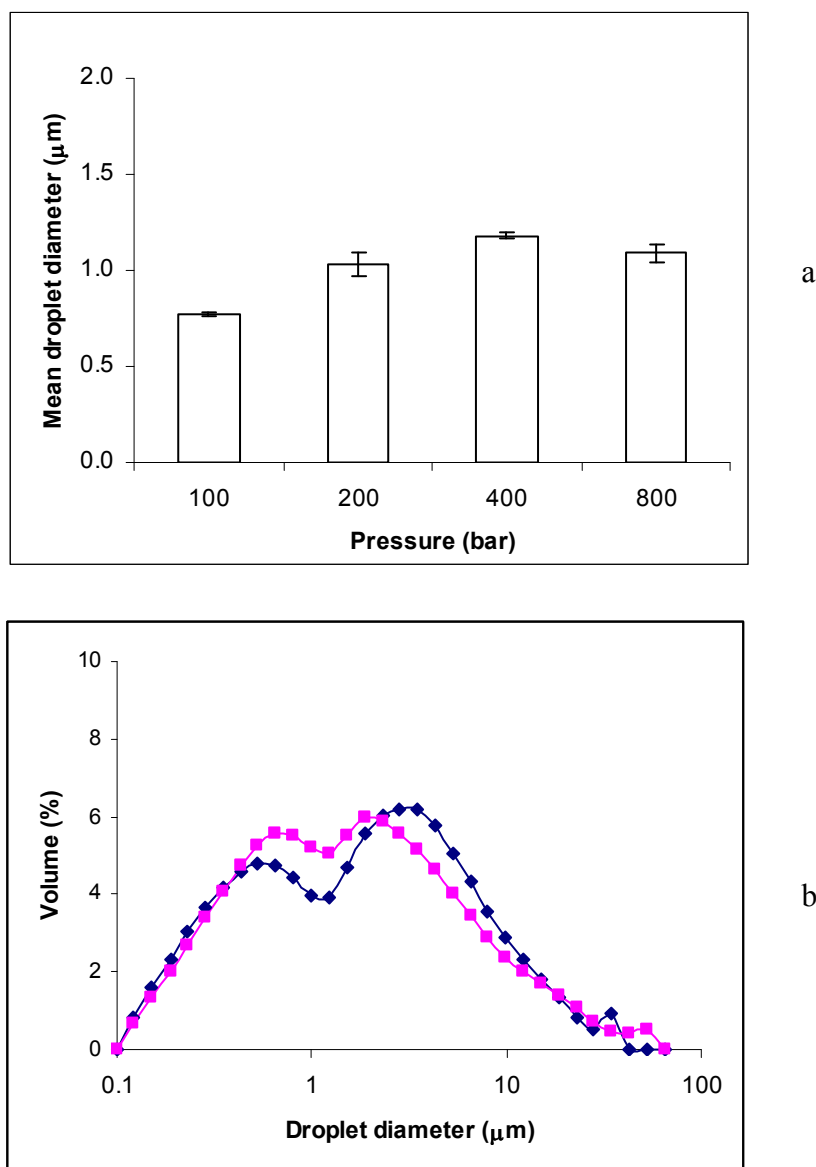
Fig. 3.8. Effect of homogenisation pressure on droplet size of (a) W/O emulsion measured by DWS technique, (b) sequential W/O/W emulsions measured by MasterSizer; (◆) 100 bar (■) 800 bar in formation of W/O emulsion. W/O/W emulsions contained distilled water in the internal aqueous phase and 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external phase.

To further verify the hypothesis, EE of sequential W/O/W emulsion was determined (Fig. 3.9). The EE of W/O/W emulsions formed with W/O emulsions prepared at

pressures above 400 bar increased significantly compared to W/O/W emulsions formed with W/O emulsions prepared at 100 and 200 bar, that was, from less than 65% to up to greater than 80%. The results showed that W/O emulsion prepared at higher pressure are more resistant to secondary homogenisation compared to the ones prepared at lower pressure. It provides further evidence that larger droplet sizes in W/O emulsions prepared at higher pressures might be due to the flocculation of smaller water droplets.
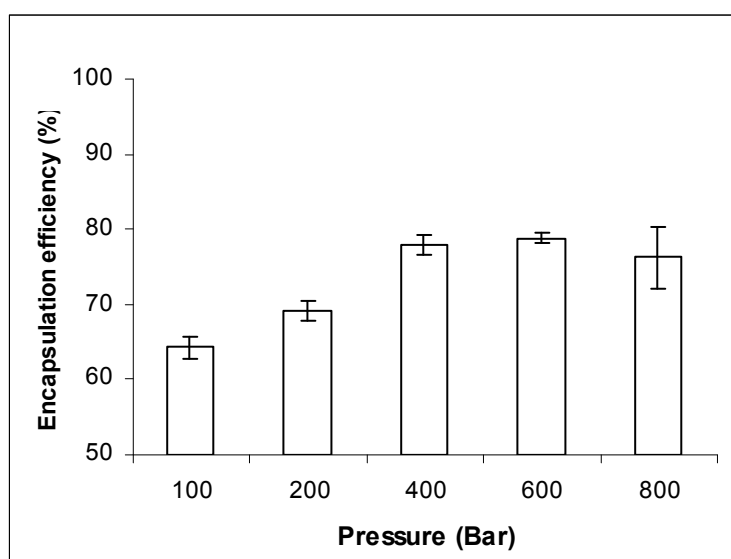
Fig. 3.9. Effect of homogenisation pressure for primary W/O emulsion on encapsulation efficiency of W/O/W emulsion. The W/O/W emulsions were all made at 100 bar using two-stage valve homogeniser. W/O/W emulsions contained distilled water in the internal aqueous phase and 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external phase.

Pressures from 400 to 800 bar were optimal for W/O emulsions formulated in this study, pressures above 800 bar were not used based on the finding of literature review and this study that higher pressure has an adverse effect on the formation and stability of emulsions, such as increase of coalescence frequency and rearrangement of emulsifier on the interface (refer to Chapter 2: Literature review).

3.3.5.2. Effect of homogenisation pressure on W/O/W emulsion

The droplet size distributions of W/O/W emulsions, prepared using different pressures and measured by MasterSizer are shown in Fig. 3.10a. W/O/W emulsions were prepared with a two-stage homogeniser at pressure of 20 to 400 bar. It was found that at all pressures, the droplet size of all samples showed a bimodal distribution. When the pressure was increased from 20 to 400 bar, the first peak increased whereas the second decreased, resulting in a d_{43} (mean volume diameter) reducing from 13.2 to 4.34 μm. The finding is in agreement with Dickinson et al., (1994a) and Stang et al. (2001) that when the energy density rises, the mean diameter of emulsion decreases.

The EE of emulsions in Fig. 3.10b showed that the EE decreased significantly when pressure increased. This result was expected, as water droplets that contained dye would undergo homogenising process again when W/O emulsions were incorporated into an aqueous solution to make W/O/W emulsions. At high pressure, energy density is very high and therefore more water droplets would be disrupted and more water-soluble dye would enter the aqueous solution. Therefore, the initial EE was reduced significantly.

Increasing homogenising pressure also increases the surface area of the oil droplets, and leads to increased spreading and rearrangement of adsorbed protein molecules at the interface. i.e. at smaller surface areas, multilayers of proteins might be formed at the interface, whereas at high homogenization pressure the layers of protein might be thinner and probably approaching a monolayer (Dickinson et al., 1994b). This could make easier for the dye to be transferred to the external aqueous phase.
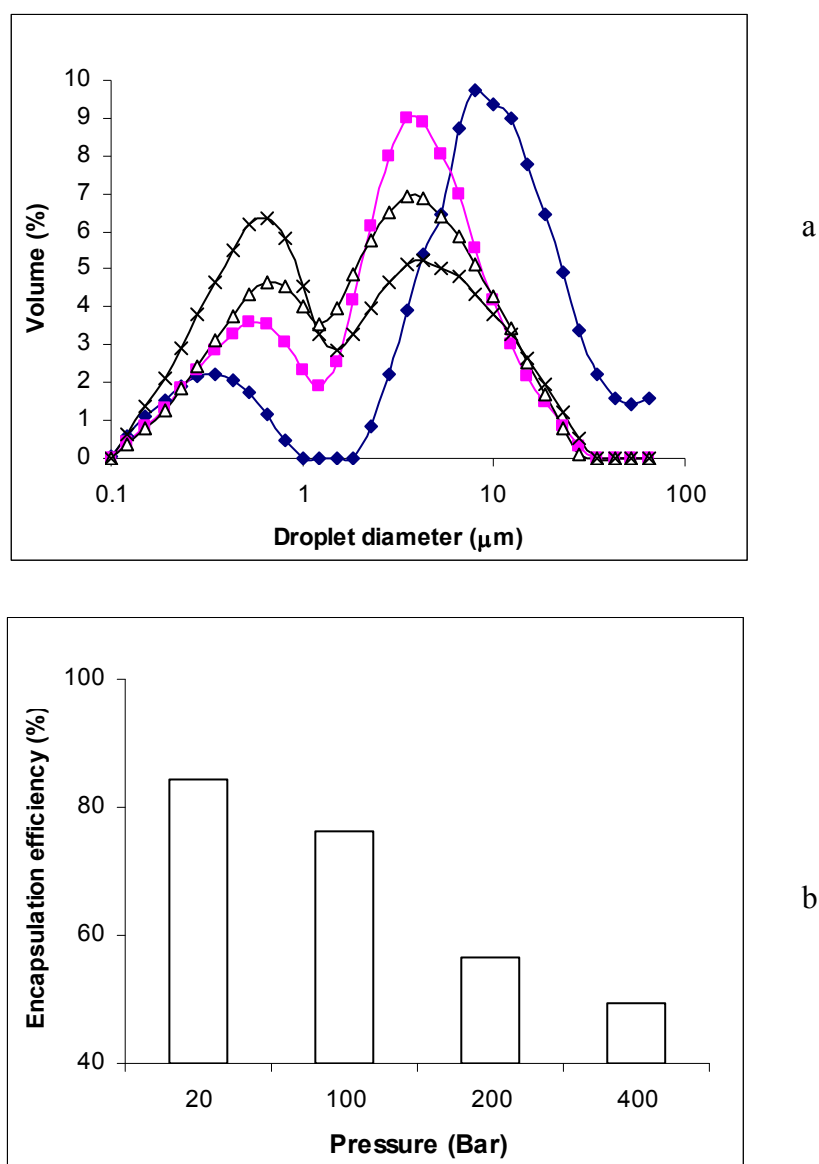
Fig. 3.10. (a): Droplet size of W/O/W emulsion prepared at (◆) 20 bar (■)100 bar (△) 200 bar (✕) 400 bar. (b): Effect of homogenization pressures on EE of poly R-478 dye of W/O/W emulsions. 2% (w/v) PGPR was used as the hydrophobic emulsifier and 0.5% (w/v) NaCN was used as hydrophilic emulsifier

3.3.5.3. Rotor-stator homogeniser

W/O/W emulsions reported in most previous studies were prepared with a rotor-stator style homogeniser (refer to Chapter 2: Literature review). The rotor-stator homogeniser used in this study was a Heidolph DIAX600. The sample is drawn up

into the apparatus by a rapidly rotating rotor positioned within a static head containing slots and holes. Because the rotor turns at a very high speed, the emulsion is rapidly reduced in size by a combination of extreme turbulence, cavitations and scissor-like mechanical shearing occurring within the narrow gap between the rotor and the stator. As this type of homogenizer has an open configuration, the product is repeatedly recirculated.

Comparisons between the droplet size distributions and EE of W/O/W emulsions prepared with high-pressure homogeniser and rotor-stator homogeniser are shown in Figs. 3.11 a and b, respectively. Emulsions prepared by rotor-stator are mono-model and are significantly larger in size compared to emulsion prepared with high-pressure homogeniser (d_{43} 89.86 µm vs. 2.29 µm). The result was expected as the energy density in rotor-stator homogeniser is lower (Perrier-Cornet et al., 2005). As expected, emulsions prepared with rotor-stator homogeniser underwent creaming soon after being prepared, the process was reversible up to one month as the d_{43} did not significantly change in this time frame (data not shown), i.e. the emulsion was stable against coalescence, which made it possible to compare samples prepared with high-pressure and rotor-stator homogeniser over time.

EE of emulsions prepared with rotor-stator homogeniser were found to be significantly higher than the EE of emulsions made with high-pressure homogeniser (Fig. 3.11b). Lower energy density in the rotor-stator homogeniser decreases the disruption rate of water droplet in W/O emulsion, increase emulsion droplet size and lead to higher coverage of emulsifier, all of which lead to decreased loss of dye into the external aqueous phase.
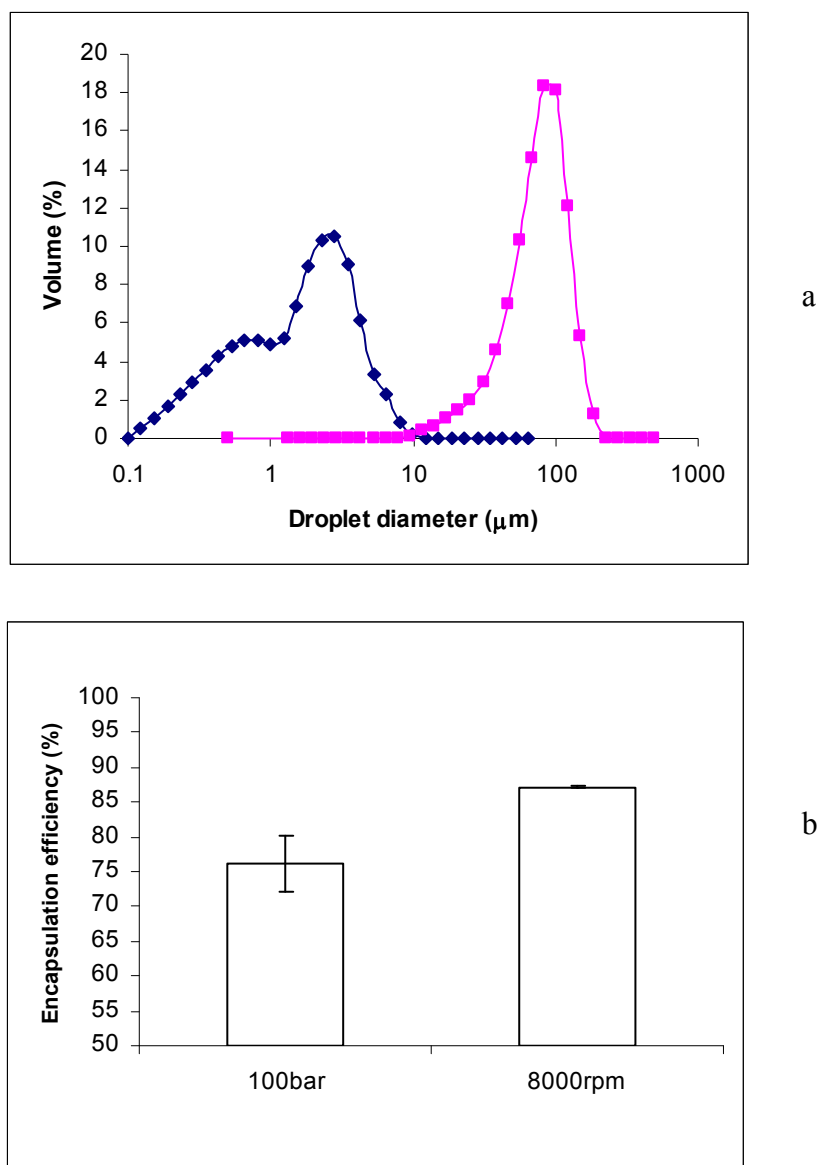
Fig. 3.11. (a): Comparison of droplet size distribution of W/O/W emulsion prepared with (◆) high-pressure homogeniser (100 bar) and (■) rotor-stator homogeniser (8000 rpm); (b): Encapsulation efficiency of W/O/W emulsion (2% (w/v) PGPR as hydrophobic emulsifier and 0.5% (w/v) NaCN as hydrophilic emulsifier) made with high-pressure homogeniser or rotor-stator homogeniser.

To determine the optimum speed of Heidolph DIAX600 that can produce W/O/W emulsions with desired droplet distribution and high EE, higher speeds from 8000 rpm to 24000 rpm were tested. Droplet size distribution and EE of W/O/W emulsion prepared with rotor-stator homogeniser at 8000 and 24000 rpm are shown in Fig.

3.12. The d_{43} of emulsion formed at 24000 rpm was about 50% lower than the samples produced at 8000 rpm (43.54 vs. 89.86 µm) (Fig 3.12 a). However, it was found that EE of samples formed at 24000 rpm was significantly lower than at 8000 rpm (Fig. 3.12 b). Therefore, 8000 rpm was adopted for preparing the W/O/W emulsion throughout the study using Heidolph DIAX600.
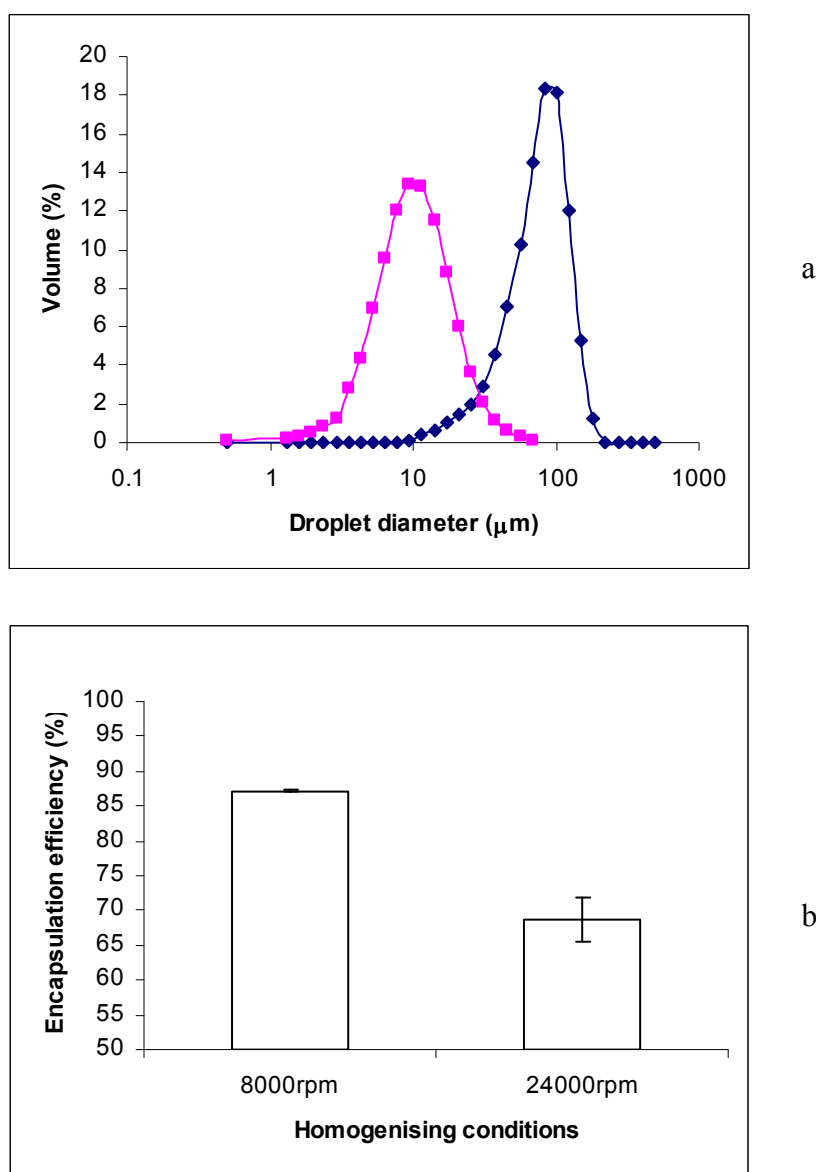
Fig. 3.12. (a): Comparison of droplet size distribution of W/O/W emulsion prepared with rotor-stator homogeniser at (◆) 8000 rpm and (■) 24000 rpm; (b): Encapsulation efficiency of W/O/W emulsion made using a rotor-stator homogeniser at 8000 or 24000 rpm. W/O/W emulsions contained 2% (w/v) PGPR as hydrophobic emulsifier and 0.5% (w/v) NaCN as hydrophilic emulsifier.

3.4.　　Summary

In this study, a model emulsion system was established after a selection of various types of oil, hydrophobic and hydrophilic emulsifiers. The formulation of the emulsion is described in Table 3.1.

Table 3.1. The composition of model W/O/W emulsion system

	Internal aqueous phase	**Oil phase + hydrophobic emulsifier**	**External aqueous phase + hydrophilic emulsifier**
Composition	sodium phosphate buffer (0.1 M) or distilled water	Commercial grade Soya oil + PGPR	Hydrophilic emulsifiers in 0.1 M sodium phosphate buffer or distilled water
Volume fraction (%, v/v)	20	80	-
	20		80

The emulsions were homogenised at 800/80 bars (two passes) for primary W/O emulsion and 100 bars (two passes) for secondary W/O/W emulsion when using two stage high-pressure homogeniser, and 8000 rpm for 5 mins when using rotor-stator homogeniser, unless stated otherwise.

Chapter 4: Effect of PGPR and macromolecular materials on the formation and stability of W/O/W emulsions

4.1. Introduction

A stable primary emulsion is essential for the overall stability of multiple emulsions. The stability of the primary W/O emulsion is directly determined by the type of components that forms the W/O emulsion, which includes the nature of oil, the type of emulsifiers and the nature of encapsulant in the internal aqueous phase, particularly if the encapsulant contains electrolytes (Dickinson et al., 1994b). Based on these concept, measures that could improve the stability of W/O emulsion include finding a suitable emulsifier, adding complexing agents to the oil to increase the viscosity of oil, or modifying the vegetable oil to be less water soluble and modifying the nature of the encapsulant by adding macromolecular materials (Omotosho et al., 1986b; Yan et al., 1992; Garti, 1997a).

This chapter focuses on investigating the conditions related to the emulsifying efficiency of PGPR as hydrophobic emulsifier; the effect of partially replacing PGPR with macromolecular materials, such as NaCN in the internal phase, on the formation and stability of the W/O/W emulsion.

4.2. Materials and Methods

All materials and methods were identical to those used previously (refer to chapter 3, section 3.2, Materials and Methods).

4.3.   Results and Discussion

4.3.1. Effect of PGPR concentration on W/O emulsions

The first step of the study of the emulsifying efficiency of PGPR on W/O/W emulsions was to investigate the effect of PGPR concentration on the stability of W/O emulsions, as a stable W/O emulsion is essential to a stable W/O/W emulsion. A varying range of concentrations of PGPR was tested.

Effects of varying PGPR concentration (0.5 -8% w/v) on the visual appearance of the W/O emulsions are shown in Fig. 4.1. For all the concentrations tested, no phase separation into discrete oil and water phases was observed in all samples for a long period. However, water droplet sedimentation occurred in all emulsions. Droplet sedimentation is a reversible process and results from Ostwald ripening and/or flocculation (Leal-Calderon, Cerhardi, Espert, Brossard, Alard, Tranchant, Stora and Bibette, 1996; Koroleva and Yurtov, 2003). Larger and heavier water droplets and cluster of water droplets were settled at the bottom of the tube and smaller and lighter water droplet made up the top layer. The results are in agreement with data reported by other studies regarding W/O emulsions (Aronson and Petko, 1993; Leal-Calderon et al., 1996; Saunders and Kent, 2001). The rate and extent of sedimentation (~20% of total volume) is similar in all W/O emulsions regardless of the concentration of PGPR studied. The results indicated that concentration as low as 0.5% of PGPR as sole hydrophobic emulsifier is sufficient to stabilise larger water droplets from coalescence.

a b c
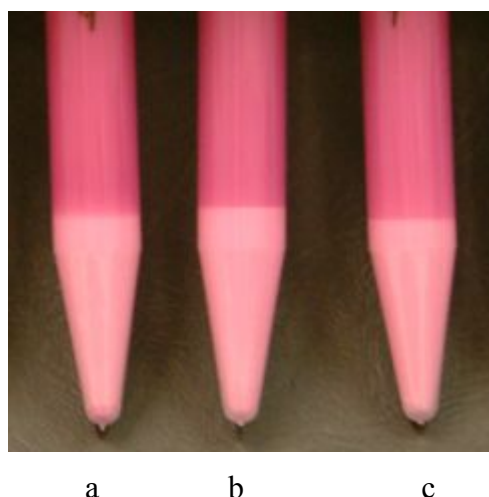
Fig. 4.1. Photographs of primary W/O emulsions prepared using (a): 0.5% (w/v), (b): 2% (w/v), (c): 8% (w/v) of PGPR after storage at 20°C for 7 days. W/O emulsions contained distilled water in the internal aqueous phase.

The mean water droplet size in W/O emulsion was determined using the DWS technique, and the results are shown in Fig. 4.2. The mean droplet size of W/O emulsions significantly increased as PGPR concentration decreased. As mentioned in Chapter 2: Literature Review, when emulsions are prepared under identical conditions, the initial size of water droplet should have been similar just after formation in the pressure homogeniser. However, after the emulsions are formed, the droplet size of an emulsion is the result of the combination of disruption of droplets, Ostwald ripening, flocculation and/or coalescence of droplets. Smaller mean water droplet size of W/O emulsion samples with higher concentration of PGPR can be interpreted as these samples being more stable to Ostwald ripening, coalescence and flocculation, due to sufficient coverage of PGPR on the water-oil interface and increased viscosity in oil phase (0.67 Pa.s for 0.5% PGPR and 1.01 Pa.s for 8% PGPR). Although it is difficult for the DWS technique to distinguish the single water droplet from a cluster of water droplets, we are still able to give evidence that W/O emulsion with higher concentration of PGPR resulted in smaller water droplets or smaller cluster of water droplets, which are likely to be more stable compared to larger water droplets in W/O emulsion with lower concentrations of PGPR.
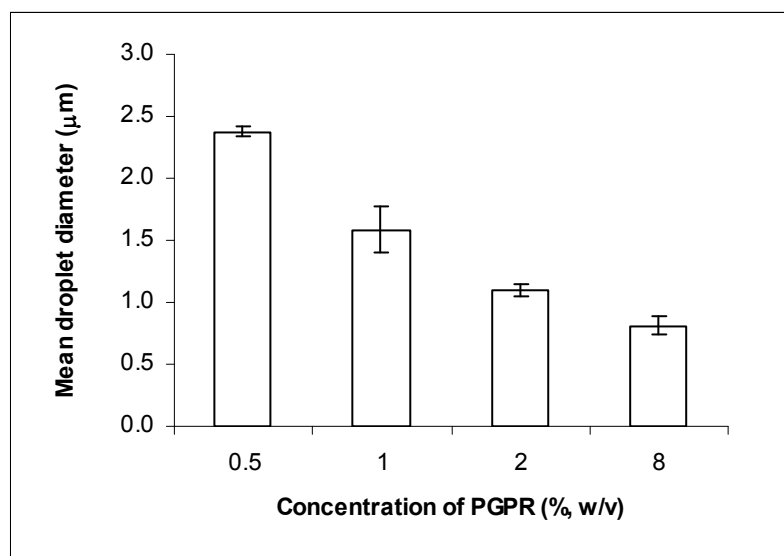
Fig. 4.2. Mean droplet size of W/O emulsions prepared with different concentration of PGPR in the oil phase. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation.

4.3.2. Effect of PGPR concentration on W/OW emulsions

The above W/O emulsions were added to 0.5% NaCN solution to form the sequential W/O/W emulsion. The properties of these W/O/W emulsions were studied to further determine the effect of PGPR concentration in the oil phase.

4.3.2.1. Droplet size distribution

The droplet size distribution of W/O/W emulsions prepared with varying range of concentrations of PGPR (between 1 and 8% w/v) and NaCN (0.5% w/v) in the external aqueous phase displayed a bimodal distribution (Fig. 4. 3). Generally, the size of emulsion droplets could be classified into two ranges: 1: diameter < 1.23 μm and 2: diameter > 1.23 μm and < 50 μm. PGPR concentration (between 1 and 8% w/v) had no significant effect on the droplet size distributions when 0.5% (w/v) NaCN was used in the external aqueous phase ($p < 0.05$).
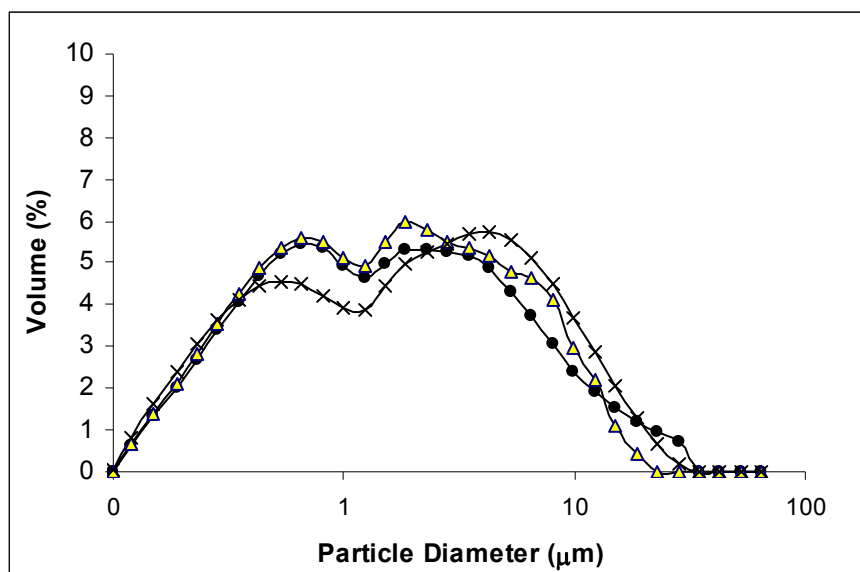
Fig. 4.3. Droplet size distributions of W/O/W emulsions prepared with distilled water in the internal aqueous phase, 0.5% (w/v) NaCN in the external phase and 1% (●), 2% (△) and 8% (✕) (w/v) PGPR in the oil phase. Emulsions were prepared using high-pressure homogenizer.

It appears that PGPR has an effect on the mean droplet size of W/O emulsions, but it does not have a significant effect on the droplet size distribution of W/O/W emulsions measured by the MasterSizer technique. As the oil droplets in W/O/W emulsion are significantly larger than water droplets inside them, changes to the oil droplets caused by entrapped water droplets migrating from inside the oil droplet to the aqueous continuous phase, disruption of the water droplets and coalescence inside the oil droplet may not cause detectable changes on the whole population of oil droplets as detected by the MasterSizer.

4.3.2.2. EE of W/O/W emulsion

The EE values of poly R- 478 dye (initially and after 4 weeks storage at 5 or 20 °C) of W/O/W emulsions prepared with different concentrations of PGPR (between 1 and 4% w/v) and NaCN (0.5% w/v) in the external phase are presented in Fig 4.4. The

initial EE value was greatly influenced by the PGPR concentration in the oil phase. An EE value of > 90% could be achieved at a concentration of 2% PGPR (w/v) in the oil phase. At PGPR concentrations of 4% (w/v), EEs of almost 100% were achieved. PGPR was not tested at higher concentrations as the results indicated that 4% (w/v) PGPR was sufficient to successfully encapsulate virtually all the poly R-478 dye. Reducing PGPR concentration from 4 to 0.5% (w/v) caused a significant decrease in EE values ($p<0.05$). For example, at 0.5% (w/v) PGPR concentration, an EE value of 38.3% was obtained, indicating that greater than 60% of the aqueous phase of the original primary emulsion was exposed to, and mixed with, the external aqueous phase during the second emulsification stage.

Although W/O emulsion prepared with 0.5% (w/v) PGPR do not show phase separation, the sequential W/O/W emulsions had very low EE. The intense mechanical stresses that W/O emulsions went through during the second homogenisation step have the potential for disrupting the W/O emulsion droplets (Surh, Vladisavljevic, Mun and McClements, 2007). PGPR at low concentrations (0.5% w/v) was not sufficient to resist the rupture force during the homogenization of the sequential W/O/W emulsions thereby significantly reducing the EE value of W/O/W emulsions. The results provide evidence that concentration of PGPR is critical to the EE and stability of W/O/W emulsions. The primary contribution of PGPR might be to allow the stabilisation of a stable interfacial area thereby resulting in reduced water droplet size during the formation of the primary W/O emulsion and hence a reduced rate of coalescence. In addition the increase in viscosity of the W/O emulsions as PGPR concentration increased could also have resulted in increased resistance to the effects of shearing during the secondary homogenisation stage and subsequently reduced the rate of coalescence of the water droplets.
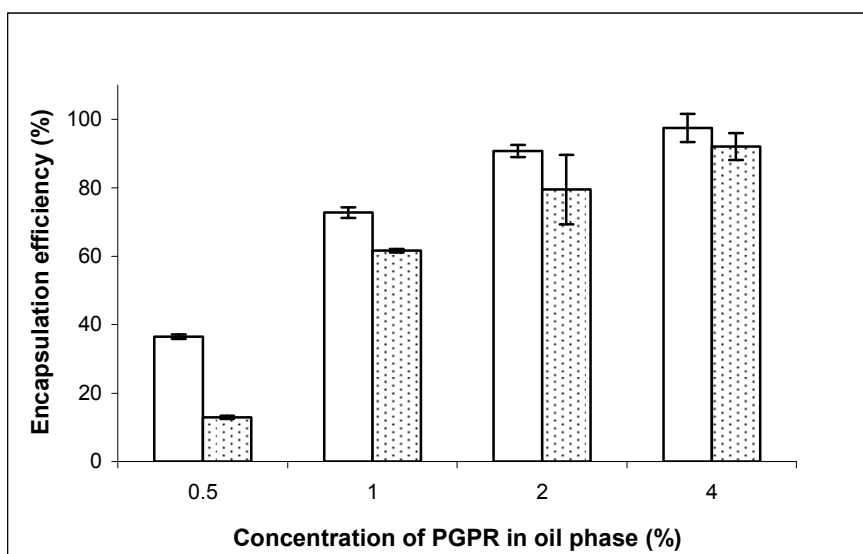
Fig. 4.4. Effect of different concentrations of PGPR in the oil phase on the encapsulation efficiencies of W/O/W emulsions. Blank bar: freshly prepared W/O/W emulsions; Dotted bar: same emulsions after storage for 4 weeks at 20 °C. External aqueous phase contained 0.5% (w/v) NaCN. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation. Emulsions were prepared in non-buffered system.

In addition, the EE of W/O/W emulsion decreased over time as a function of PGPR concentration (Fig. 4. 4.). At low PGPR concentrations (0.5 and 1%, w/v), storage of these emulsions for 4 weeks at 20 °C resulted in a significant ($p < 0.05$) decrease in EE values compared to the initial EE values. In contrast, the EE of W/O/W emulsion prepared with 2 and 4% (w/v) PGPR showed no significant change after 4 weeks storage at 20 °C, the result indicating that the dye remained entrapped within the internal aqueous phase during the storage period. Poly R-478 dye is a large molecular weight material (Mw 50,000 Da), it is unlikely that it could transport through the oil phase to the external aqueous phase. The loss of dye in emulsions with lower PGPR concentration is probably through the rupture or expelling of internal water droplets. This will be discussed in depth in Chapter 6.:Characteristics of W/O/W emulsions containing hydrophilic encapsulants in the internal aqueous phase.

4.3.3. Effect of ionic environment on emulsifying ability of PGPR

Visual observation of W/O emulsions in preliminary experiments showed that phase separation occurred in W/O emulsions with lower PGPR concentration (0.5-1% (w/v)) using phosphate buffer as the internal aqueous phase, whereas no phase separation occurred in emulsions using distilled water. It was suggested that sodium phosphate buffer (0.01M) might affect the emulsifying properties of PGPR. To investigate this effect in depth, two W/O/W emulsions systems were studied. One was a non-buffered system, using distilled water (pH~6.6) as the internal aqueous phase. The other was a buffered system, using phosphate buffer (0.1M, pH~6.6) as internal aqueous phase. The two systems were compared in visual observation of W/O emulsion, droplet size measurements of W/O and W/O/W emulsions, and EE of the sequential W/O/W emulsions.

4.3.3.1. Visual observations of W/O emulsions

The stabilities of W/O emulsions prepared with 0.5, 1 and 2% (w/v) PGPR using distilled water or 0.1 M sodium phosphate buffer at pH 6.6 (pH of distilled water) in the internal aqueous phase were compared and the results are shown in Table 4.1. W/O emulsions prepared in non-buffered systems with 0.5 - 2% (w/v) PGPR as the sole emulsifier did not show phase separation after storage at 20 °C for 7 days. On the other hand, W/O primary emulsions prepared in buffered systems, with 0.5 - 2% (w/v) PGPR as the sole emulsifier, showed phase separation soon after the emulsions were made.

Table 4.1: Effect of PGPR concentration and aqueous phase composition on phase separation in primary W/O emulsions.

	0.5% PGPR	1% PGPR	2% PGPR	4% PGPR
Distilled water	-	-	-	-
0.1 M sodium phosphate buffer	++	+	+	-

+ : Phase separation after 24 hours, ++ : phase separation immediately,

- : No phase separation after 7 days

The pH of solutions was 6.6

The results from the visual observations indicated that an ionic environment created by the sodium phosphate buffer might reduce the emulsifying ability of PGPR, probably through the influence of the ionic charge on the functional, charged groups at the water-oil interface. To verify this hypothesis, that an ionic environment had an effect on the emulsifying ability of PGPR, two experiments were conducted. In the first experiment, W/O emulsions were prepared with 0 to 0.5 M sodium phosphate buffer in the internal aqueous phase using 2% (w/v) PGPR as emulsifier. In the second experiment, 0.5 M sorbitol or NaCl was added to the internal aqueous phase of W/O emulsions prepared with 2% (w/v) PGPR, respectively. The experiments were conducted to determine whether the decrease of emulsifying ability of PGPR was directly related to the concentration of ions in the internal aqueous phase, and whether the loss of emulsifying ability of PGPR was specific to sodium phosphate buffer.

| i | ii | iii | iv | v | vi |
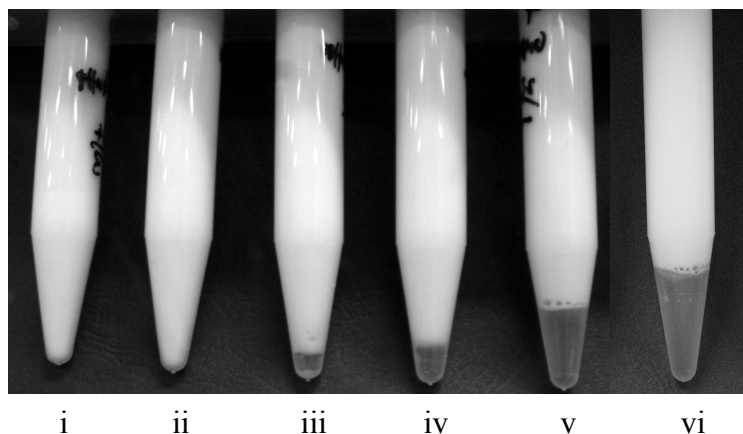
Fig. 4.5. Photographs of primary W/O emulsions prepared using different aqueous phase compositions after storage at 20°C for 1 month. (a): i: distilled water; ii-v: 0.05, 0.1 M, 0.25, 0.5 M sodium phosphate buffer, respectively; vi: 0.5 M NaCl. All emulsions contained 2% PGPR in the oil phase as hydrophobic emulsifier.

When increasing concentrations of sodium phosphate buffer were present in the internal aqueous phase of the W/O emulsions, it was found that the emulsions were increasingly destabilised, with phase separation proportionally related to the concentrations of buffer in the internal aqueous phase (Fig. 4.5). When 0.5 M NaCl was present in the internal aqueous phase, a similar effect to the 0.5 M sodium phosphate buffer was observed (Fig. 4.5). On the other hand, sorbitol (0.5 M), an organic compound that is often used as a marker in multiple emulsions, did not induce phase separation (data not shown) in W/O emulsions, indicating no damage to PGPR emulsifying ability. The results provide strong evidence that the ionic environment has an adverse effect on the emulsifying ability of PGPR.

4.3.3.2. Droplet size distribution of W/O emulsion

The mean droplet size of W/O emulsions in buffered and non-buffered system was measured using the DWS technique and is shown in Fig. 4.6. The addition of sodium phosphate buffer to the internal aqueous phase significantly reduced the water droplet

size in W/O emulsions. This is expected as addition of electrolytes with lower solubility in the dispersed phase is an efficient way to increase the emulsion stability. Previous studies have shown that the added electrolytes slow down the rate of Ostwald ripening, which occur due to diffusion of a substance from small to larger droplets in the internal aqueous phase, as the total pressure in smaller droplets is much higher than in the larger droplets (Aronson and Petko, 1993; Koroleva and Yurtov, 2003). As a result the small droplets will eventually disappear and the larger droplets grow bigger and have more tendencies to coalescence. Therefore, Oswald ripening tends to decrease polydispersity of a system and deteriorate its stability (Fennema, 1996).
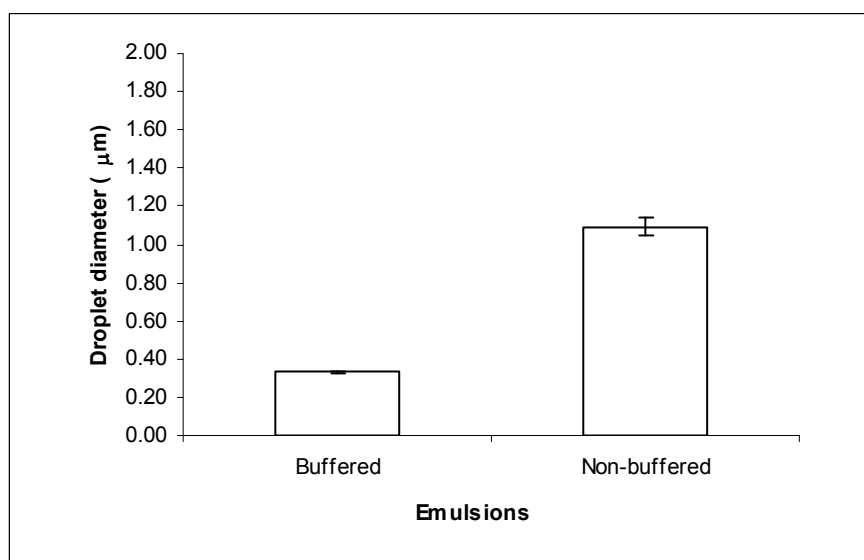
Fig. 4.6. Mean droplet size of W/O emulsions prepared in buffered or non-buffered system, with 2% (w/v) PGPR in the oil phase as sole hydrophobic emulsifier. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation.

The addition of sodium phosphate alters the diffusion rate and extent of water migration from smaller droplets to larger droplets. When the total pressure in smaller droplets decreased due to water diffusion to larger droplets, the concentration of electrolyte increased. In larger droplets, however, the concentration of electrolyte decreased and total pressure increased, resulting in a lower rate of diffusion of water from smaller droplets to larger droplets. As a result, the size of the water droplets

will be significantly stabilized, in particular small droplets (Kzling and kronberg, 1990; Aronson and Petko, 1993).

Although the addition of sodium phosphate in buffered system might reduce the rate of Ostwald ripening, phase separation observed in W/O emulsions in the buffered system (Figure 4.5) indicates that water droplets quickly went through a disruptive or a coalescence process.   The destabilisation is most likely due to the reduced emulsifying properties of PGPR caused by sodium phosphate, which resulted in insufficient coverage of PGPR at the oil-water interface and hence the instability of W/O emulsions.

4.3.3.3. Droplet size distribution of W/O/W emulsion

W/O emulsions in buffered or non-buffered system were incorporated in 0.5% NaCN solution in distilled water to prepare the W/O/W emulsions. Droplet size distributions of W/O/W emulsions in buffered and non-buffered system are shown in Fig. 4.7a. In buffered systems, the proportion of smaller droplets (<1.23 μm) decreased and the proportion of larger droplets (>1.23 μm) increased. As a result the d_{43} of emulsions of buffered systems was 5.99 μm, significantly higher than emulsions prepared in non-buffered system (3.22 μm).   This difference of droplet size distribution between buffered and non-buffered system was thought to be due to the diffusion of water from the external aqueous phase to the internal aqueous phase, with the driving force being osmotic pressure caused by a difference in the concentration of sodium phosphate buffer between the internal and external aqueous phase.

To verifying this hypothesis, W/O emulsions in a buffered system were incorporated into 0.5% NaCN solutions dissolved in 0.1 M sodium phosphate buffer, the same buffer concentration as the internal aqueous phase. In this formulation, the osmotic pressure difference between the internal and external aqueous phase was very small. The distribution of droplet size of the W/O/W emulsions was compared with W/O/W emulsions with buffered internal aqueous phase and non-buffered external phase (Fig. 4.7b). It was found that while both emulsions were polydisperse, the droplet size distribution shifted to the region of smaller droplets when buffered NaCN solution

was used as the external aqueous phase. This is expected as the osmotic pressure between the internal and external aqueous phase decreased when sodium phosphate buffer was present in both phases. Water diffusion from the external aqueous phase was reduced, and the swelling of the internal water droplet was less.
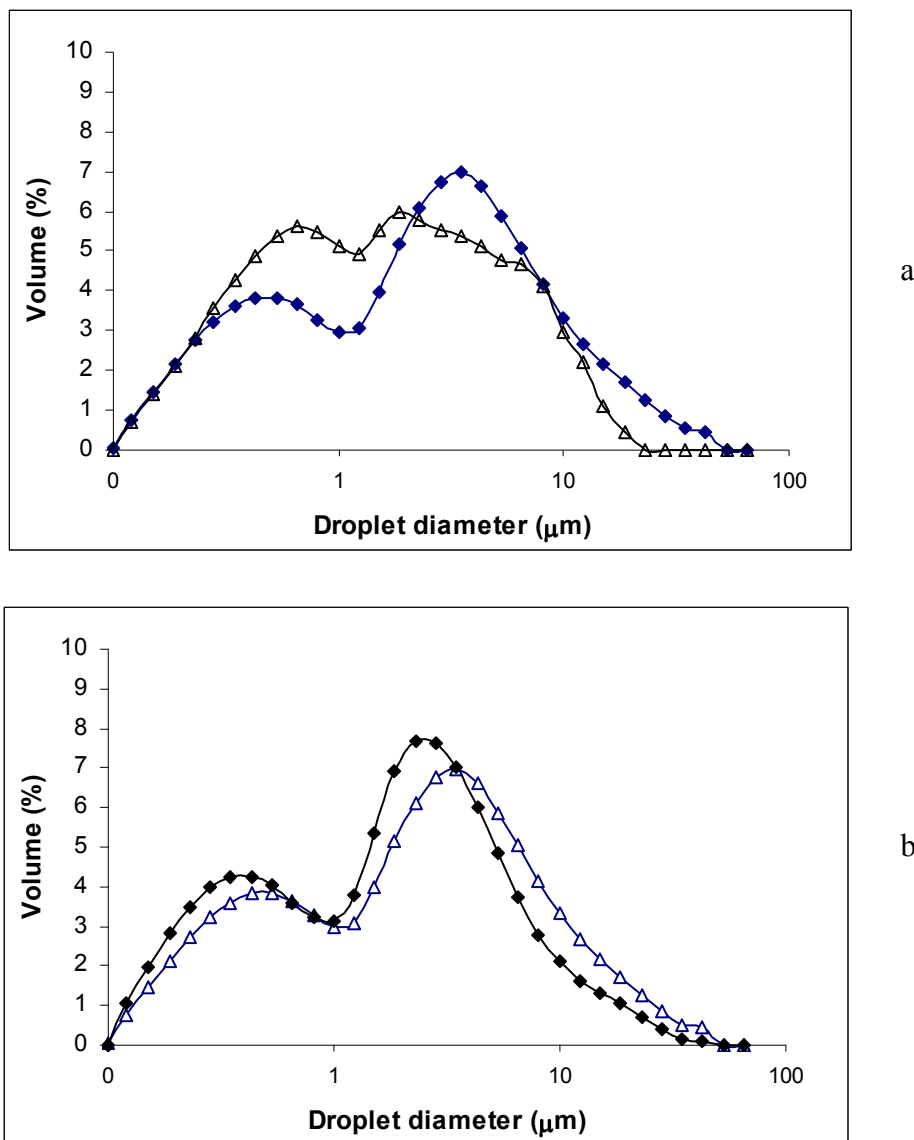
Fig. 4.7. (a): Droplet size distributions of W/O/W emulsions with internal aqueous phase composed of non-buffered (△) or buffered (◆) systems and with 0.5% (w/v) NaCN in distilled water as the external phase and 2% (w/v) PGPR in the oil phase. (b): Droplet size distributions of W/O/W emulsions with internal aqueous phase composed of a buffered system and with 0.5% (w/v) NaCN in anon- buffered (△) or buffered (◆) system as the external phase and 2% (w/v) PGPR in the oil phase.

4.3.3.4. EE of W/O/W emulsion

The EE of W/O/W emulsions prepared with varying PGPR concentrations in buffered and non-buffered systems in the oil phase are shown in Fig. 4.8. In both systems, when PGPR was used as the sole emulsifier, increased EE were obtained as PGPR concentration increased from 0.5 to 4% (w/v). However, the EE of W/O/W emulsions were significantly lower in buffered systems using 1 or 2% w/v PGPR compared to non-buffered systems at the same PGPR concentrations (p <0.05).

In non-buffered systems, EE of W/O/W emulsions increased significantly as PGPR in the emulsions was increased from 0.5 to 2.0% (w/v). W/O/W emulsions prepared with 2% (w/v) PGPR obtained an EE greater than 90%, and the EE increased to near 100% when 4% PGPR was added. It indicated that in non-buffered systems, 2-4% PGPR as a sole emulsifier is sufficient to obtain a W/O/W emulsion with high EE.

In buffered systems, both internal and external aqueous phase of W/O/W emulsions contained 0.1 M sodium phosphate buffer to reduce the effect of osmotic pressure as discussed previously. Between 0.5 and 4% (w/v) PGPR, the visual stabilities of W/O emulsions (Table 4.1) and the EE (Fig. 4.8) of the W/O/W emulsions are highly dependent on PGPR concentration. At 6 and 8% (w/v) PGPR, stable W/O emulsions which subsequently produce W/O/W emulsions with high EE in buffered system are produced.
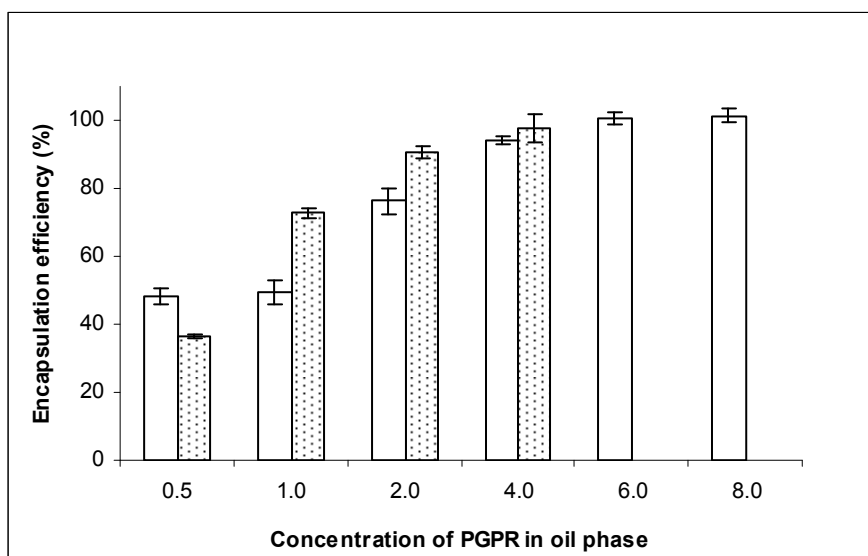
Fig. 4.8. Effect of different concentrations of PGPR in the oil phase on the encapsulation efficiencies of W/O/W emulsions. Blank bar: W/O/W emulsions in buffered systems; Dotted bar: W/O/W emulsions in non-buffered systems. External aqueous phase contained 0.5% (w/v) NaCN. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation.

These results are generally in accord with visual observation of W/O emulsions (Table 4.1), with the exception of the non-buffered systems containing 0.5% (w/v) PGPR which resulted in stable W/O emulsions, but displayed a very low EE (37%) when subsequent W/O/W emulsions were made. The low EE of W/O/W emulsions prepared with lower concentration of PGPR is considered due to insufficient coverage of PGPR on water droplet interface. Lower coverage of emulsifier resulted in the water droplets which tended to disrupt during second step of homogenisation and undergo coalescence/flocculation during storage. It has previously been shown that coalesced water droplets in a W/O/W emulsion were expelled from the oil droplet quicker than smaller intact water droplets (Gonzalez-Ochoa, Ibarra-Bracamontes and Arauz-Lara, 2003). This provides a further mechanism to explain the increased susceptibility of W/O emulsions prepared with low concentrations of PGPR to phase separate and the low EE in sequential W/O/W emulsions.

4.3.4. Effect of addition of macromolecular materials to the internal aqueous phase of W/O/W emulsions in buffered systems

The results from the previous section suggest that the addition of ions in the internal aqueous phase of W/O/W emulsions might alter the interfacial properties of PGPR, such as coverage of PGPR on surface of water droplets, the arrangement of PGPR molecules on the surface and the exposure of functional groups of PGPR molecules. As a result, decreases the encapsulation efficiency and stability of the emulsions. However, in real food emulsion systems, the presence of ions is unavoidable, and as a result more emulsifier (PGPR) will be required to stabilise the emulsions. It is desirable to reduce the amount of PGPR required for stable W/O/W emulsion with high EE. The addition of macromolecular materials could be a solution. Previous studies have successfully used various macromolecular materials such as proteins and/or polysaccharides to partially replace hydrophobic emulsifier(s) or to improve the EE and stability of W/O/W emulsions (Dickinson et al., 1993; Evison et al., 1995; Garti, 1997b; Benichou et al., 2004). In this study, several macromolecular materials were tested in the internal aqueous phase to investigate the effect on the properties of the W/O/W emulsions.

4.3.4.1. Effect of addition of NaCN to the internal aqueous phase of W/O/W emulsion in buffered systems

4.3.4.1.1. W/O emulsion

Although NaCN has been used as secondary hydrophilic emulsifier in the external aqueous phase in W/O/W emulsions, the effects of incorporating NaCN in the primary W/O emulsion does not appear to have been previously reported. As NaCN is a food grade, commercially available and relatively cost effective emulsifier, it may be possible to replace synthetic hydrophobic emulsifiers such as PGPR in the internal aqueous phase of W/O/W emulsions. NaCN (0.125 - 1%, w/v) was dissolved in 0.1 M sodium phosphate buffer and was incorporated as the internal aqueous phase to

investigate what effect it would have on the properties of W/O and W/O/W emulsions.

(A) (B) (C) (D) (E) (F) (G) (H)
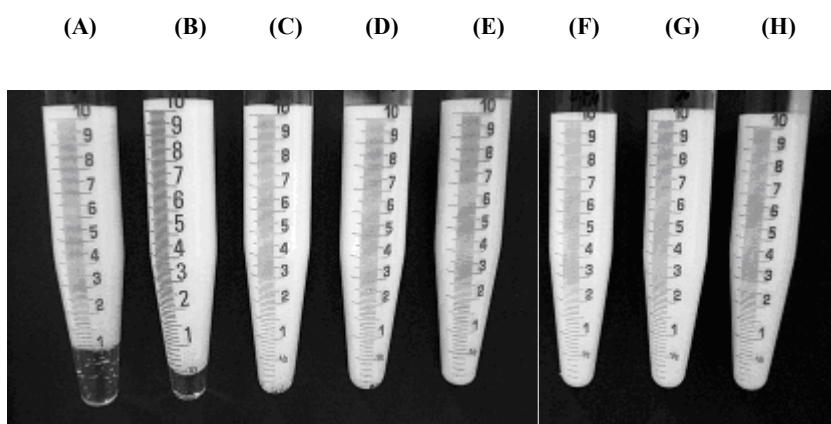
Fig. 4.9. Photograph of primary W/O emulsions stored at 20 °C for 7 days. Emulsions were prepared with (A): 0.5%; (B): 1%; (C): 2%; (D): 4%; (E): 6% (w/v) PGPR; and 0.5, 1 and 2% (w/v) PGPR in the oil phase with 0.5% (w/v) NaCN in the internal aqueous phase (F, G, H, respectively). Emulsions were prepared in buffered system.

The stabilities of W/O emulsions prepared with 0.5, 1 and 2% (w/v) PGPR in the oil phase with and without 0.5% (w/v) NaCN in the internal aqueous phase were compared visually (Fig. 4.9). W/O emulsions prepared with 0.5 and 1% (w/v) PGPR as the sole hydrophobic emulsifier showed phase separation after storage at 20 °C for 7 days (Fig. 4.9 A-B). However, when 0.5% (w/v) NaCN was added to the internal aqueous phase of these W/O emulsions, no phase separation was observed after storage at 20 °C for 7 days (Fig. 4.9, F-H). NaCN prevented or slowed down the rupture or coalescence of water droplet occurring in W/O emulsions when used in combination with PGPR in the buffered system.

4.3.4.1.2. Droplet size distribution of W/O/W emulsion

The droplet size distributions of W/O/W emulsions prepared with and without NaCN in the internal aqueous phase, and with 0.5 and 2% (w/v) PGPR are presented in Figure 4.10. Droplet size distributions exhibited a bimodal distribution typical of all

W/O/W emulsions prepared under the current set of conditions and did not change significantly (p>0.05) after the addition of 0.5% (w/v) NaCN in the internal aqueous phase at either 0.5 or 2% (w/v) PGPR (Fig. 4.10).
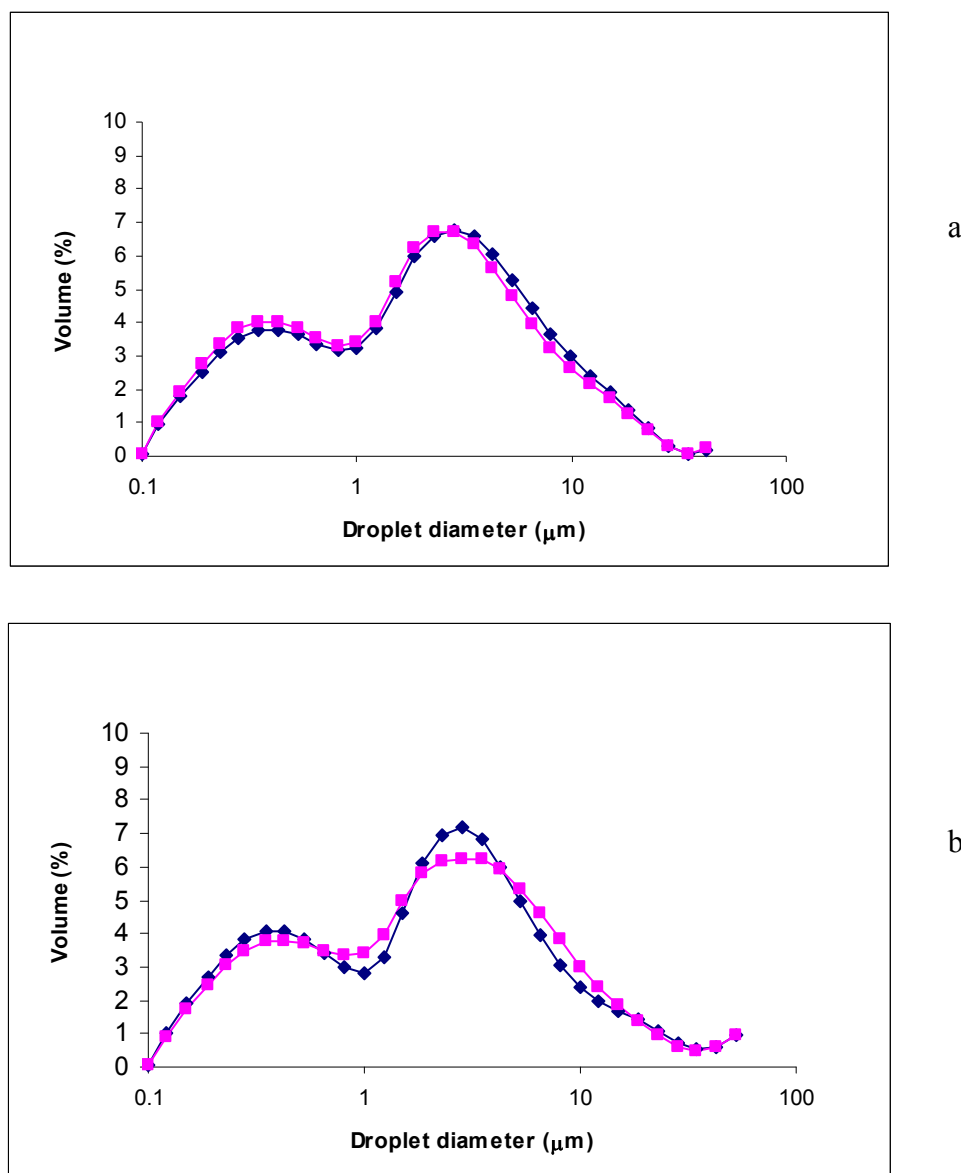
Fig. 4.10. Droplet size distributions of W/O/W emulsions prepared with 0.5% (w/v) NaCN in the external phase. (a): 0.5% (w/v) PGPR in the oil phase, (b): 2% (w/v) PGPR in the oil phase; with (■) and without (◆) 0.5% (w/v) NaCN in the internal aqueous phase. Emulsions were prepared in buffered system.

4.3.4.1.3. EE of W/O/W emulsions

The EE of W/O/W emulsions prepared with varying PGPR concentrations (0.5 and 2% w/v) in the oil phase, and a range of NaCN concentrations (0-1% w/v) in the internal aqueous phase were determined (Fig. 4.11a, b). In most cases, at identical NaCN concentrations, increased EE were observed as PGPR concentration increased (Fig. 4.11a, b). EE of W/O/W emulsions containing 0.5% (w/v) PGPR remained approximately constant on addition of low concentrations of NaCN to the internal aqueous phase (0.125 and 0.25% w/v), but subsequently increased at higher NaCN concentrations (0.5 and 1% w/v) (Fig. 4.11a). In general, the EE of W/O/W emulsions prepared with 2% (w/v) PGPR increased as NaCN concentration in the internal aqueous phase increased (Fig. 4.11b). On addition of 0.5% (w/v) NaCN to the internal aqueous phase, at 0.5 and 2% (w/v) PGPR, EE values increased from 48 ± 2.6 to 65 ± 11.9 and from 79 ± 6.1 to $92 \pm 2.2\%$, respectively, compared to the EE of emulsions prepared without NaCN in the internal aqueous phase, a significant increase ($p < 0.05$). When 1% (w/v) NaCN was added to the internal aqueous phase, higher EE were observed than emulsions without NaCN in the inner aqueous phase; although this increase was significant ($p<0.05$), it was not a significant improvement compared to the EE of W/O/W emulsions prepared with 0.5% NaCN (w/v) (Fig. 4.11a, b).

The EE of W/O/W emulsions prepared with 0.5 and 2% (w/v) PGPR in the oil phase, and a range of NaCN concentrations (0-1%, w/v) in the internal aqueous phase after storage at 5 and 20 °C for 4 weeks were also compared (Fig. 4.11a, b). Overall, the EE of emulsions decreased after storage at 5 or 20 °C for 4 weeks, regardless of the NaCN concentration in the internal aqueous phase. However, in the 2% (w/v) PGPR samples, EE did not decrease to the same extent after storage when increasing concentrations of NaCN (0.5 and 1% w/v) were added to the internal aqueous phase, compared to lower NaCN concentrations examined (Fig. 4.11b). In all cases, decreases in EE were greater after storage at 20 °C than 5 °C. The increased instability of W/O/W emulsions at higher temperatures may be explained as that at higher temperature, the Brownian motion of the droplets will be greater compared to

lower temperature, more interaction between droplets would occur which could accelerate the speed of dye released to the external aqueous phase.
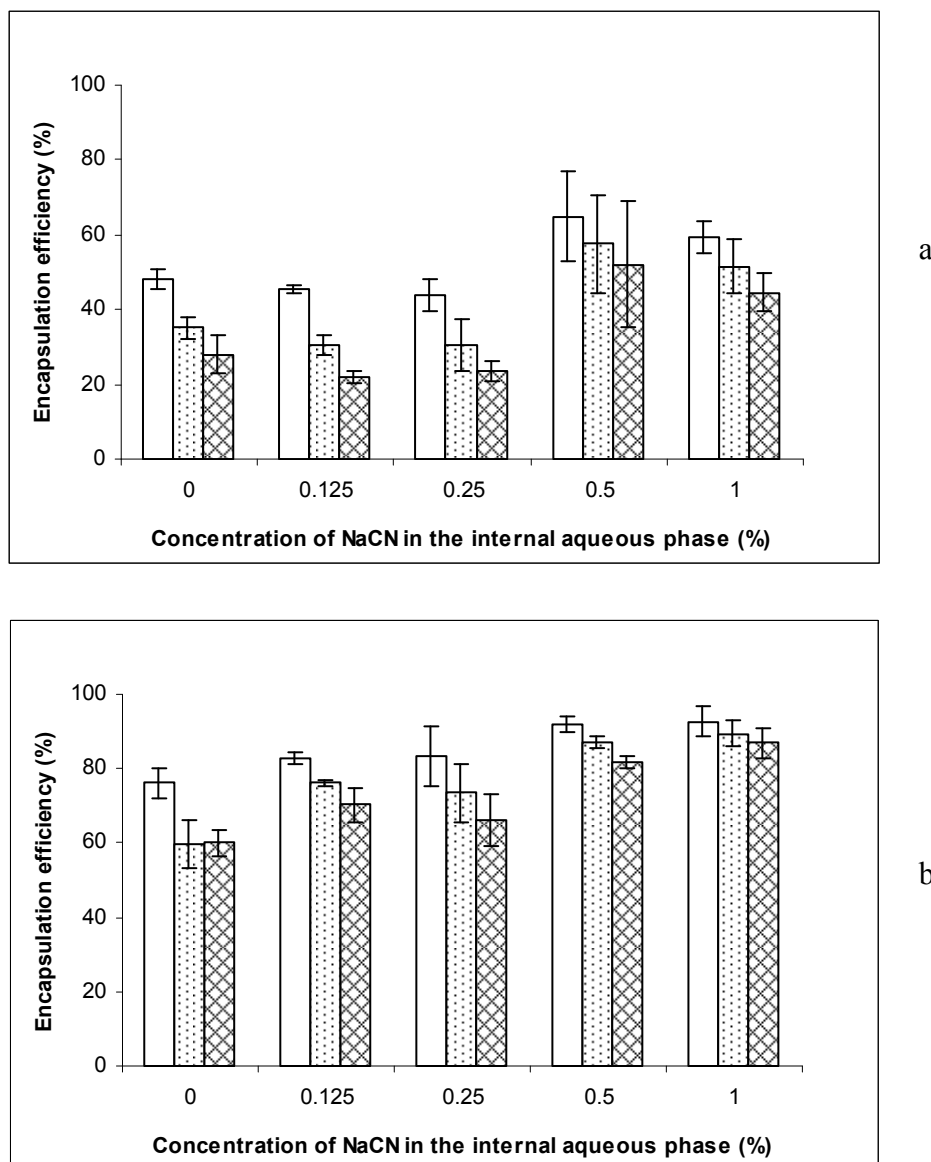
Fig. 4.11. Effect of NaCN concentration in the internal aqueous phase on the EEs of freshly prepared W/O/W emulsions (blank bar) and the same emulsions after 4 weeks storage at 5 °C (dotted bar) and 20 °C (netted bar). The emulsions were prepared with (a) 0.5% or (b) 2% (w/v) PGPR. External aqueous phase contained 0.5% (w/v) NaCN. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation. Emulsions were prepared in buffered system.

Although the initial EE increased after the addition of 0.5 and 1% (w/v) NaCN in the internal aqueous phase for W/O/W emulsions prepared with 0.5% (w/v) PGPR (Fig. 4.11a), the EE did not exceed 75%. The initial EE of W/O/W emulsions prepared with 2% (w/v) PGPR increased significantly to greater than 80% ($p < 0.05$) at all NaCN concentrations examined (Fig. 4.11b), and EE of greater than 90% was achieved at 0.5 and 1% (w/v) NaCN. Furthermore, at 2% (w/v) PGPR and 0.5 and 1% (w/v) NaCN in the internal phase, EE after storage at 5 and 20 °C for 4 weeks, did not decrease considerably compared to all other emulsions examined (Fig. 4.11a, b).

It is clear that the addition of NaCN to the internal aqueous phase improved the stability of W/O emulsions at 0.5 and 1% (w/v) PGPR (Fig. 4.9, F-H), and thereby the EE and stability of the W/O/W emulsion (Fig. 4.11a, b). W/O/W emulsions prepared with 2% (w/v) PGPR (Fig. 4.11 b) and containing 0.5 or 1% (w/v) NaCN showed similar initial EE (> 90%) of W/O/W emulsions prepared with 4% (w/v) PGPR without the addition of NaCN in the internal aqueous phase (Fig. 4.8). Therefore, the addition of 0.5% (w/v) NaCN in the internal aqueous phase could reduce the concentration of PGPR (from 4 to 2% w/v) while keeping the EE and stability at a satisfactory high level (> 90%).

4.3.4.2. Effect of addition of other macromolecular materials to the internal aqueous phase of W/O/W emulsion in buffered system

A few macromolecular materials, whey protein isolation (WPI), gelatine and carboxyl methyl cellulose (CMC), which have been used by other researchers in the internal aqueous phase to improve the stability of W/O/W emulsions were tested in this study to compare their effect to NaCN. The visual observation of W/O emulsions with addition of these macromolecular materials is summarised in Table 4.2. It was found that although these materials are different in nature, all the macromolecular materials tested prevented phase separation on W/O emulsions in buffered system, i.e. they slowdown the process of coalescence of water droplets.

Table 4.2: Effect of addition of macromolecular materials on phase separation in primary W/O emulsions prepared in buffered system, with 0.1 M sodium phosphate buffer in the internal aqueous phase and 0.5, 1 and 2% (w/v) PGPR in the oil phase.

	Control	0.5% NaCN	1% WPI	1% Gelatine	2% CMC
0.5% PGPR	++	-	-	-	-
1% PGPR	+	-	-	-	-
2% PGPR	+	-	-	-	-

Control: No addition of macromolecular in the internal aqueous phase

+ : Phase separation after 24 hours

- : No phase separation after 7 days

The pH of solutions was 6.6

The EE and droplet size distribution of sequential W/O/W emulsions were determined and are shown in Fig. 4.12 and Fig. 4.13, respectively. The EE of all W/O/W emulsions with addition of macromolecular materials in the internal aqueous phase increased, providing further evidence that these macromolecular materials could improve the EE and stability of W/O/W emulsion. On the other hand, the addition of macromolecular material did not show significant change in droplet size distribution, all W/O/W emulsions showed polydisperse distribution and had similar d_{43} and d_{32} values.
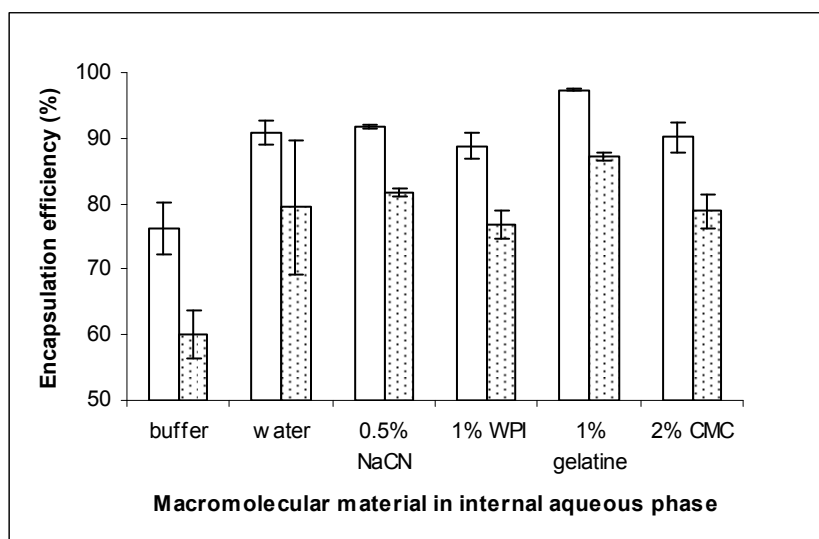
Fig. 4.12. Effect of macromolecular materials in the internal aqueous phase on the EEs of freshly prepared W/O/W emulsions (blank bar) and the same emulsions after 4 weeks storage at 20 °C (dotted bar). The emulsions were prepared with 2% (w/v) PGPR concentrations in the oil phase; External aqueous phase contained 0.5% (w/v) NaCN. Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation.

The mechanism(s) of stabilisation of W/O/W emulsions by macromolecule addition are not fully understood. However, it has been proposed that these macromolecules may form an extra film around the internal water droplets or form a "complex" with existing emulsifiers (Benichou et al., 2004). The improved EE, stability and unchanged droplet size of W/O/W emulsions in our study suggested that a possible synergistic effect existed. This synergistic effect appeared to stabilise the primary W/O emulsion interface against coalescence in the presence of both PGPR and macromolecular materials, such as NaCN, as hydrophobic emulsifiers. The combination of PGPR and macromolecular materials may produce a more viscoelastic adsorbed layer, thereby preventing the release of the dye from the internal aqueous phase to the external aqueous phase. Further investigations are necessary to determine how macromolecular materials acts synergistically with PGPR to stabilise the emulsions reported in the present study, and also as reported in previous studies (Omotosho et al., 1986b; Yan et al., 1992; Garti, 1997a).
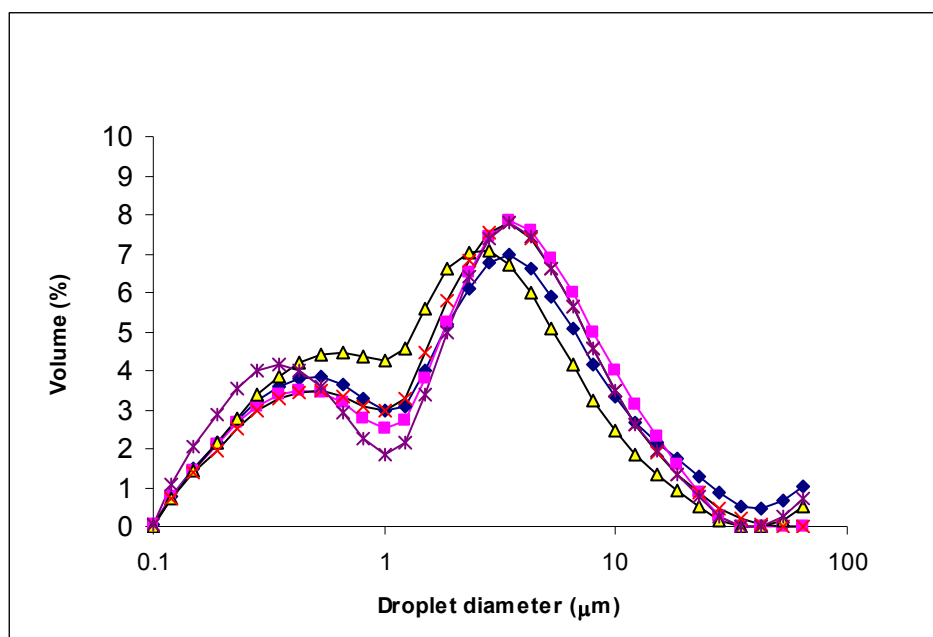
Fig. 4.13. Droplet size distributions of W/O/W emulsions with addition of 0.5% (w/v) NaCN (■), 1% (w/v) WPI (△), 1% (w/v) gelatine (✕), or 2% (w/v) CMC (✳), and W/O/W emulsion without addition in the internal aqueous phase (◆). 0.5% (w/v) NaCN was used in the external phase and 2% (w/v) PGPR was used in the oil phase.

For food applications, it is desirable to reduce the concentration of synthetic emulsifiers, such as PGPR, in W/O/W emulsions. The results presented herein clearly show that macromolecular materials such as NaCN may be used to reduce the concentration of PGPR used in multiple emulsions without affecting the EE and stability of the emulsions.

4.3.5. Effect of addition of macromolecular materials to the internal aqueous phase of W/O/W emulsions in non-buffered systems

Macromolecules were added to the internal aqueous phase of W/O/W emulsions in non-buffered systems to determine whether macromolecular materials have the same effect of improving the EE and stability of W/O/W emulsion as in buffered systems.

The effect of addition of macromolecular materials to the internal aqueous phase of the W/O/W emulsion of non-buffered system was studied to compare with buffered systems. The results are shown in Table 4.3. The addition of macromolecular materials destabilised the W/O emulsions in non-buffered systems, as phase separation were noticeable in W/O emulsions prepared with 0.5% (w/v) PGPR. While in buffered systems, phase separation in W/O emulsions prepared with 0.5 to 2% (w/v) were prevented. Although phase separations were not detected in W/O emulsion prepared with higher PGPR concentration (1 and 2% w/v), the particular destabilisation effect at lower PGPR concentration indicates that emulsifying properties of PGPR might be reduced by the addition of macromolecular materials.

Table 4.3: Effect of PGPR concentration and aqueous phase composition on phase separation in primary W/O emulsions in non-buffered system.

	0.5% PGPR	1% PGPR	2% PGPR
Distilled water	-	-	-
0.5% NaCN in distilled water	+	-	-
1% WPI in distilled water	+	-	-
1% Gelatine in distilled water	+	-	-
2% CMC in distilled water	+	-	-
10% SUPER GUM™ In distilled water	+	+	-

+ : Phase separation occurred after 24 hours

- : No phase separation after 7 days

The pH of the solutions was 6.6

EEs of sequential W/O/W emulsions are shown in Fig. 4.14. In accord with the observation of W/O emulsion, EE of W/O/W emulsion with 0.5% (w/v) PGPR as sole hydrophobic emulsifier decreased significantly with the addition of 0.5% (w/v) NaCN, indicating a destabilisation effect.
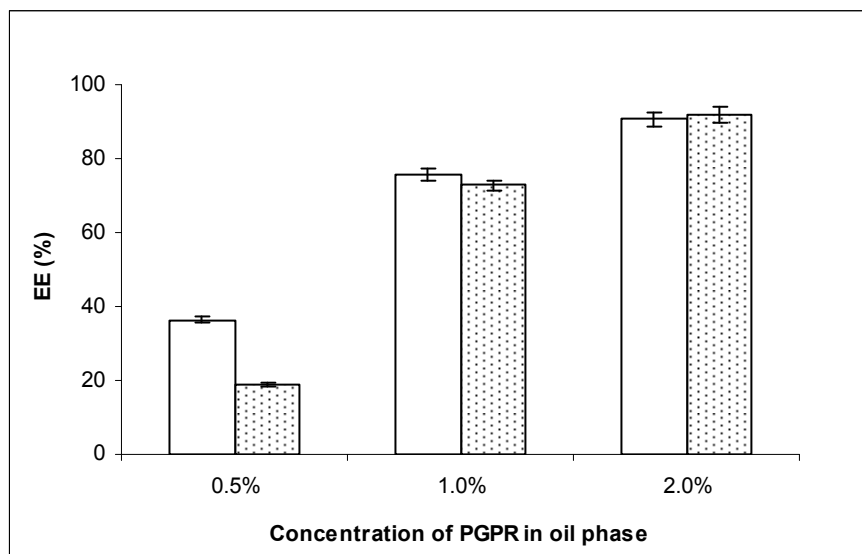
Fig. 4.14. Effect of different concentrations of PGPR in oil phase on the encapsulation efficiencies of W/O/W emulsions prepared using different aqueous phase compositions. Blank bar: non-buffered system; dotted bar: 0.5% (w/v) sodium caseinate solution in non-buffered system. Error bars show standard deviation of at least two measurements.

SUPER GUM™, a macromolecular material in the acacia gum family was also studied as possible source that may increase the stability of W/O emulsions. Due to the presence of precipitates when SUPER GUM™ was dissolved in the sodium phosphate buffer system, SUPER GUM™ was only studied in non-buffered system. SUPER GUM™ (10% w/v) was incorporated into the internal aqueous phase of W/O/W emulsions to investigate what effect it would have on primary W/O emulsion stability at 0.5 - 2% (w/v) PGPR. W/O emulsions were compared visually and the result are tabulated (Table 4.3).

While no phase separation was evident in W/O emulsions prepared with distilled water in the internal aqueous phase, W/O emulsions prepared with 0.5% and 1% (w/v) PGPR showed phase separation when 10% (w/v) SUPER GUM^{TM} was present in the internal aqueous phase, while W/O emulsions prepared with 2% (w/v) PGPR displayed no phase separation.

The EE values of W/O/W emulsions prepared with 2% (w/v) PGPR in the oil phase, and a range of SUPER GUM^{TM} concentrations (0-15% w/v) in the internal aqueous phase were determined (Fig. 4.15). The results showed that although W/O emulsions prepared with 2% (w/v) PGPR showed no phase separation after the addition of SUPER GUM^{TM} (Table 4.2), the EE values of subsequent W/O/W emulsions were significantly reduced compared to the W/O/W emulsion without SUPER GUM^{TM} ($p<0.05$). Significant decreases in EE values were observed after storage at 20 °C for one month for all W/O/W emulsions with or without SUPER GUM^{TM} in the internal aqueous phase ($p<0.05$). The addition of SUPER GUM™ to the internal aqueous phase did not cause observable changes to the appearance of W/O/W emulsion droplets as determined by confocal imaging and emulsion droplet size measurements (data not shown).
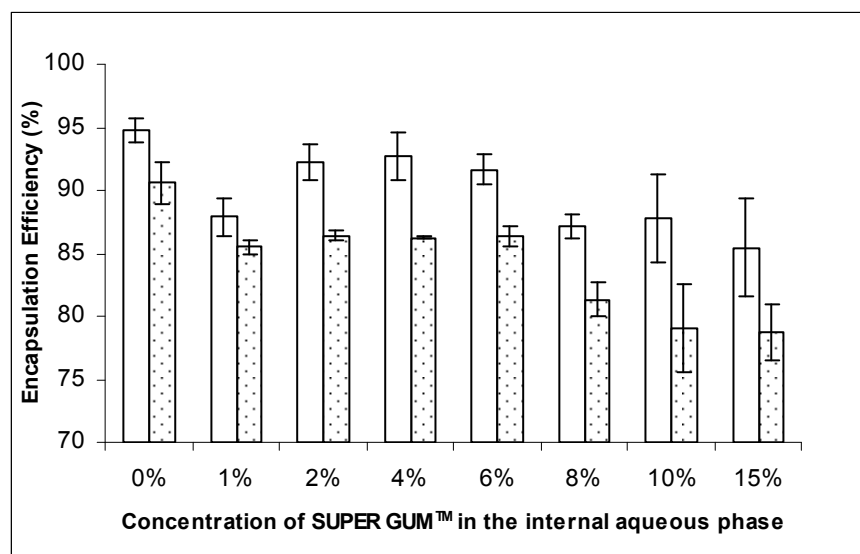
Fig. 4.15. Effect of SUPER GUM™ concentration in the internal aqueous phase on the encapsulation efficiencies of W/O/W emulsions (prepared with 2% (w/v) PGPR in oil phase and 0.5% (w/v) NaCN in the external aqueous phase). Blank bar: day 0, Dotted bar: one month storage at 20°C. Emulsions were prepared in non-buffered system. Error bars show standard deviation of at least two measurements.

The change of emulsifying properties of PGPR in the presence of ions (e.g. Na^+) may help explain the destabilisation of W/O emulsion in non-buffered systems after the addition of macromolecular materials. The phase separation of the primary W/O emulsions at lower PGPR concentrations (Table 4.3) and the decreased EE of W/O/W emulsions as the concentration of SUPER GUM^{TM} was increased (Fig 4.15), are likely due to the influence of the salts inherent in these macromolecular materials (for instance, SUPER GUM^{TM} contained approximately 1.7% salt) on the emulsifying ability of PGPR.

Some previously unexplained phenomena observed in W/O/W emulsion formation may also have an answer. Several studies have attempted to decrease the concentration of synthetic hydrophobic emulsifiers through the addition of macromolecular materials, such as proteins into the internal aqueous phase of W/O/W emulsions in the hope that the added emulsifier might occupy otherwise empty spaces

at the O/W or W/O interface (Evison et al., 1995; Koberstein-Hajda and Dickinson, 1996). While low concentrations of the added macromolecular materials were found to increase the stabilities of the W/O/W emulsions, using higher concentrations generally decreased the stabilities (Chapter 2: Literature review). This may be partly due to the inherent salts present in the macromolecular material used. Thus, there may be some mass balance effect between the beneficial effect of the emulsifier at stabilising the interface and the negative effect of the inherent salt content on the emulsifying ability of PGPR.

4.4.   Summary

A stable W/O emulsion is an essential basis for forming a W/O/W emulsion. The stability of W/O emulsions in this study was mainly determined by the concentration of PGPR, through stable interfacial area and increased viscosity of W/O emulsions. High concentration of PGPR (up to 8% (w/v)) produced fine W/O emulsion droplet (1 μm) and entrapped almost all internal water droplets in W/O/W emulsions (EE almost reach 100%). However, it was desired to reduce the amount of PGPR as it is a synthetic product. The minimum PGPR required to obtain a stable W/O emulsion without phase separation and a W/O/W emulsion with EE > 90% was 2% (w/v) in a non-buffered system. The minimum PGPR concentration increased to 4% (w/v) in a buffered system, as it was found that ions in buffered system changed the emulsifying properties of PGPR, resulted in phase separation in W/O emulsions and lower EE of W/O/W emulsions.

The addition of macromolecular materials, such as NaCN (0.5% w/v) in the internal aqueous phase of W/O/W emulsions could be a solution to reduce the PGPR in buffered system. The results in this study showed that addition of macromolecular materials could reduce the amount of PGPR from 4% to 2% (w/v) in a buffered sytem without changing the stability of W/O/W emulsions. It was postulated that macromolecules might have synergistic effect with PGPR, which appeared to enhance the stability of W/OW emulsions in the buffered systems. The effects of macromolecular materials were less pronounced in non-buffered systems.

Chapter 5: Effect of hydrophilic emulsifiers on the formation and stability of W/O/W emulsions

5.1. Introduction

The stabilisation of W/O/W emulsions not only depends on hydrophobic emulsifiers that stabilise the W/O emulsion, but also rely on hydrophilic emulsifier(s) to stabilise O/W emulsions. Several hydrophilic emulsifiers of different structures have been tested in a preliminary study (Chapter 3). It was found that W/O/W emulsions prepared with NaCN or SUPER GUM™, a modified gum arabic product, exhibit high EE and stability.

NaCN is widely used as an emulsifier in the food industry, as it contains both hydrophobic and hydrophilic regions, possesses a disordered structure and is stable at high temperatures (Britten and Giroux, 1991). NaCN was previously used as a hydrophilic emulsifier for W/O/W emulsions by other researchers, and stable W/O/W emulsions resulted (Dickinson et al., 1991a; Dickinson et al., 1994a; Dickinson et al., 1994b).

Gum arabic is a versatile ingredient used in food systems due to its molecular flexibility (Islam et al., 1997). Functions of gum arabic include emulsification, encapsulation, stabilisation, water binding, adhesion, film forming and dietary fibre (Dickinson and Galazka, 1991; McNamee et al., 1998; Verbeken et al., 2003). However, there have been no reports on the effects of gum arabic as an emulsifier on W/O/W emulsion systems.

The effect of a modified gum arabic product, referred to as SUPER GUM™, on the formation and stabilisation of W/O/W emulsion is investigated in the present study. Compared to conventional gum arabic, SUPER GUM™ has undergone an accelerated aggregation process, from which, the smaller arabinogalactan units join into larger molecular weight arabinogalactan protein aggregates. While the product is chemically and molecularly identical to the original gum, the amount of arabinogalactan protein

emulsifying component is up to more than double. This results in a significant increase in the interfacial surface properties and coverage of the oil droplet in O/W emulsions (Al-Assaf, Phillips, Aoki and Sasaki, 2007; Aoki, Al-Assaf, Katayama and Phillips, 2007).

The objective of this study is to investigate the effect of different concentrations of NaCN and SUPER GUM™ in the external aqueous phases on the formation and stabilisation of W/O/W emulsions at different conditions.

5.2.   Materials and Methods

All materials and methods were identical to those used previously, please refer to Chapter 3, section 3.2, materials and Methods.

5.3.   Results and Discussion

5.3.1. *Effect of NaCN concentration in the external aqueous phase on droplet sizes and EEs of W/O/W emulsions*

5.3.1.1. Buffered systems

W/O/W emulsions prepared with 2, 4 and 6% (w/v) PGPR in the internal phase (which form stable W/O emulsions) were used to investigate the change of emulsion droplet sizes as a function of NaCN concentration in the external aqueous phase (Fig. 5.1). Average droplet size (d_{43}) of W/O/W emulsion droplets decreased from 16.6 to 4.9 μm when NaCN concentration was increased from 0.03 – 0.125% (w/v) and 6% (w/v) PGPR was used as hydrophobic emulsifier. The decrease in average droplet size of W/O/W emulsions as NaCN concentration increased was also represented by changes in droplet size distribution. Increases in the volume of smaller droplets (< 1 μm) and decreases in the maximum diameter of larger droplets (from 2.8 to 4.3 μm) were observed as NaCN concentration increased from 0.03 to 2% (w/v). At 0.03%

(w/v) NaCN, some of the oil droplets were outside the range of the MasterSizer, indicating the presence of coalesced oil droplets of size greater than 50 μm.

Confocal microscopy showed the presence of several flocculated droplets in the W/O/W emulsion prepared at low concentrations of NaCN (Fig 5.1a), while flocculation was not observed in W/O/W emulsions prepared with ≥ 0.5% (w/v) NaCN in the external aqueous phase (Fig. 5.1b). The droplet size of all W/O/W emulsions showed no significant change (p>0.05) after 4 weeks storage at 20 ºC (data not shown).
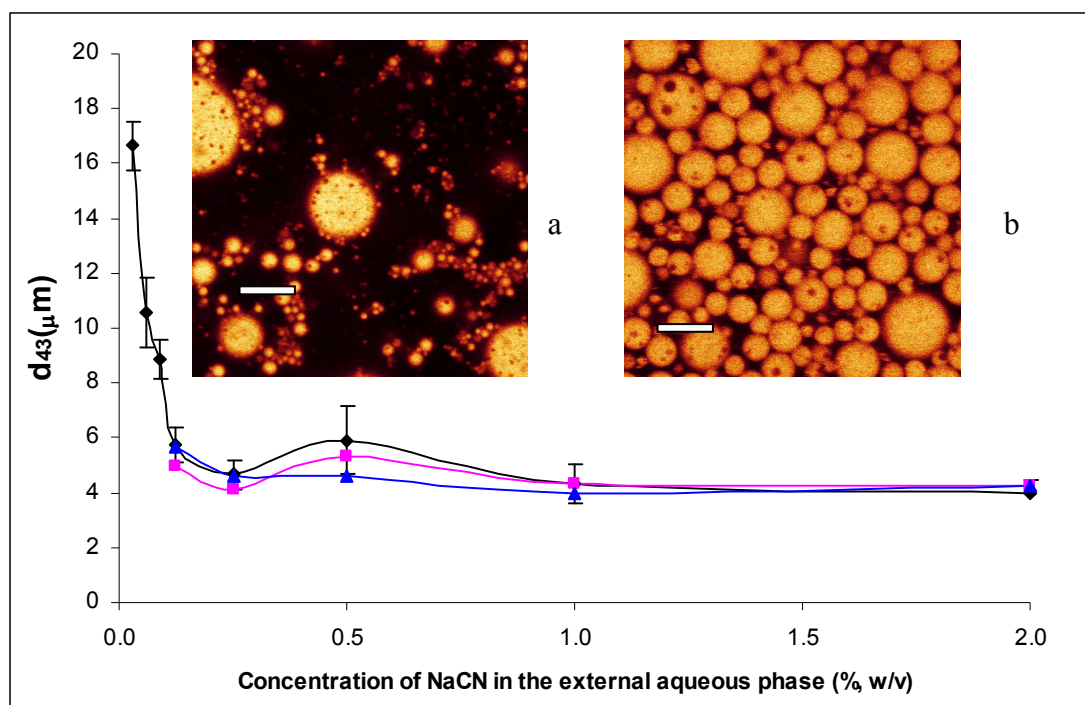
Fig. 5.1. Effect of NaCN concentration in external aqueous phase on droplet sizes (d_{43}) of W/O/W emulsions prepared with 2 (■), 4 (▲) and 6% (w/v) (◆) PGPR as hydrophobic emulsifier in oil phase. Error bars represent standard deviation. The inserted confocal microscopy images of freshly prepared emulsion represent the W/O/W emulsions at (a) 0.03% and (b) 2% (w/v) NaCN. Emulsions were prepared in buffered system. Scale bar represents 5 μm.

The EEs of W/O/W emulsions prepared with 1 and 2% (w/v) PGPR in the oil phase and varying NaCN concentrations (0.125 – 2% w/v) in the external aqueous phase are

shown in Figure 5.2. When NaCN concentration was increased from 0.125 to 0.25% (w/v), the EE values decreased slightly, but remained constant on further increases in NaCN concentration (Fig. 5.2a). A similar effect, i.e., a slight decrease in EE as NaCN concentration increased from 0.125 to 0.5% (w/v) followed by a plateau at higher NaCN concentrations, was also observed when 2% (w/v) PGPR was used as hydrophobic emulsifier (Fig. 5.2b). At high PGPR concentrations (4 and 6% w/v), greater than 93% EE was obtained at all concentrations of NaCN examined (Fig. 5.3).

The storage stabilities of the emulsions prepared with 1 and 2% (w/v) PGPR in the oil phase and varying NaCN concentrations (0.125 – 2% w/v) in the external aqueous phase were also determined (Fig. 5.2a & b). After 4 weeks storage, EEs decreased considerably more in emulsions which were stored at 20 ºC, compared to emulsions stored at 5 ºC. At higher PGPR concentrations (4 - 6% w/v), EE values remained high (> 90%) after storage for 4 weeks at either 5 or 20 ºC (data not shown). Increasing temperature will result in decreased viscosity of the oil phase, thereby increasing the rate of coalescence and expelling of the internal water droplets. In addition, the increase in Brownian motion on increasing temperature will increase the rate of flocculation leading to further instability in the multiple emulsions.
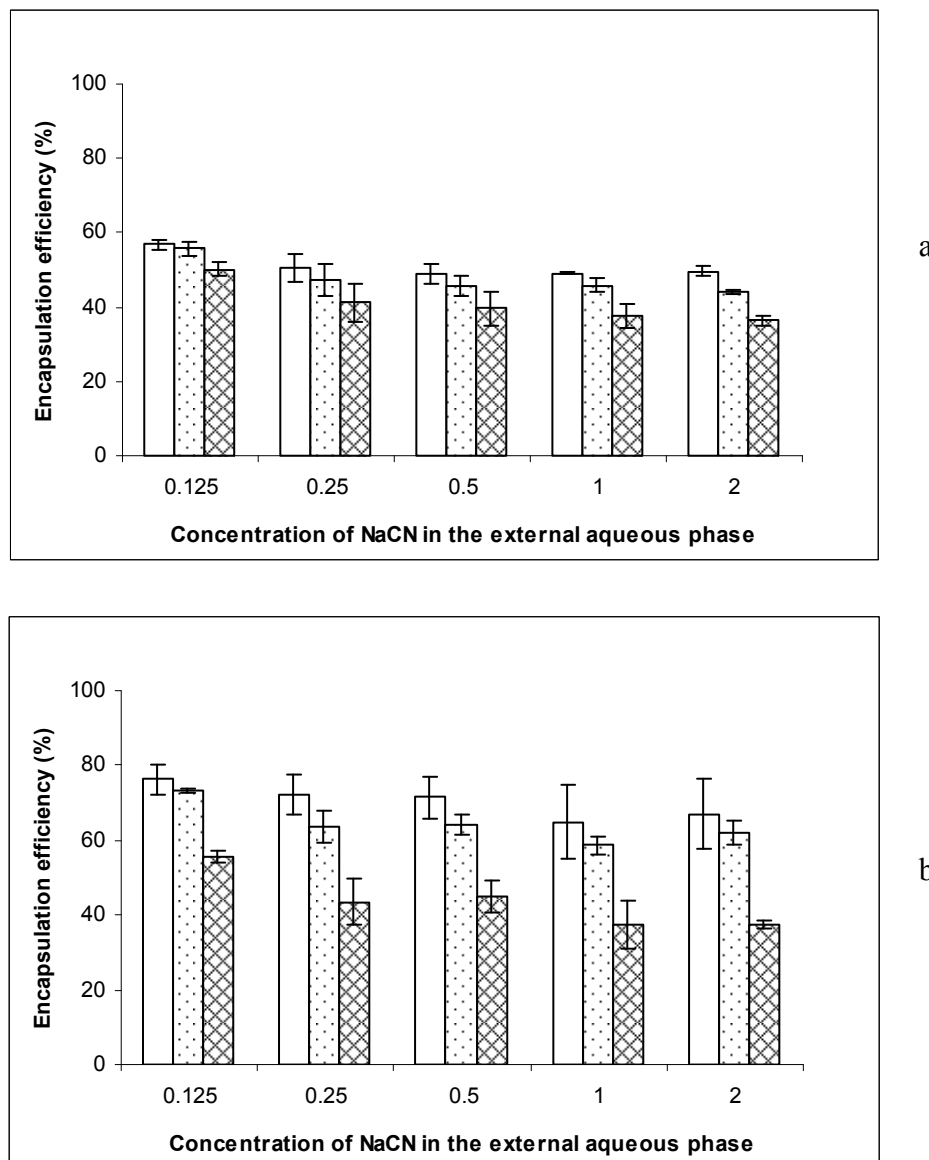
Fig. 5.2 Effect of NaCN concentration in the external aqueous phase on the EEs of freshly prepared W/O/W emulsions (blank bar), and the same emulsions after storage for 4 weeks at 5 (dotted bar) and 20 °C (netted bar). Oil phase contained (a) 1% and (b) 2% (w/v) PGPR. Emulsions were prepared in buffered system. Error bars show standard deviation of at least two measurements.

In a similar study, Dickinson et al. (1994b) prepared W/O/W emulsions using kerosene containing 8% Span 80 or soybean oil containing 8% PGPR for the oil phase and NaCN as the hydrophilic emulsifier. Their results showed that when NaCN concentration was increased from 0.125 to 1% (w/v) the droplet size (d_{43}) of the Span

80/kerosene system decreased from ~1.4 to ~0.85 μm, and resulted in a finer emulsion which was more stable against creaming. EE values also decreased from ~95 to ~75% with increasing NaCN concentrations in the Span 80/kerosene system, while in the PGPR/soybean oil system, EE values decreased from ~97 to ~90% on increasing NaCN concentration in the external aqueous phase from 0.125 to 0.5% (w/v) (Dickinson et al., 1994b). In this study, a reduction in d_{43} values was also observed (16.6 to 4.9 μm) on increasing NaCN concentration from 0.03 to 1% ((w/v)) when 6% PGPR was used in the oil phase (Fig. 5.1). On the other hand, EE values did not show significant change upon increasing NaCN concentration (Fig. 5.2).

Fig. 5.3 Effect of NaCN concentration in the external aqueous phase on the EEs of freshly prepared W/O/W emulsions prepared with 4% (w/v) PGPR (blank bar) and 6% (w/v) PGPR (dotted bar). Emulsions were prepared in buffered system. Error bars show standard deviation of at least two measurements.

5.3.1.2. Non-buffered systems

The effect of NaCN concentration in the external aqueous phase on the droplet sizes and EEs of W/O/W emulsions prepared in non-buffered system was also studied, and the results are shown in Fig. 5.4 and Fig. 5.5. It was found that as in buffered system, the increased concentration of NaCN from 0.125 to 0.5% (w/v) resulted in decreased average droplet size (d_{43}) from 10.1 to 3.2 µm (Fig. 5.4).   The increased NaCN concentration, at the same time, did not significantly change the EEs of W/O/W emulsions (Fig. 5.5). The effect of NaCN as hydrophilic emulsifier on the droplet size distribution and EE of W/O/W emulsions showed a similar pattern as W/O/W emulsions that were prepared in buffered systems.
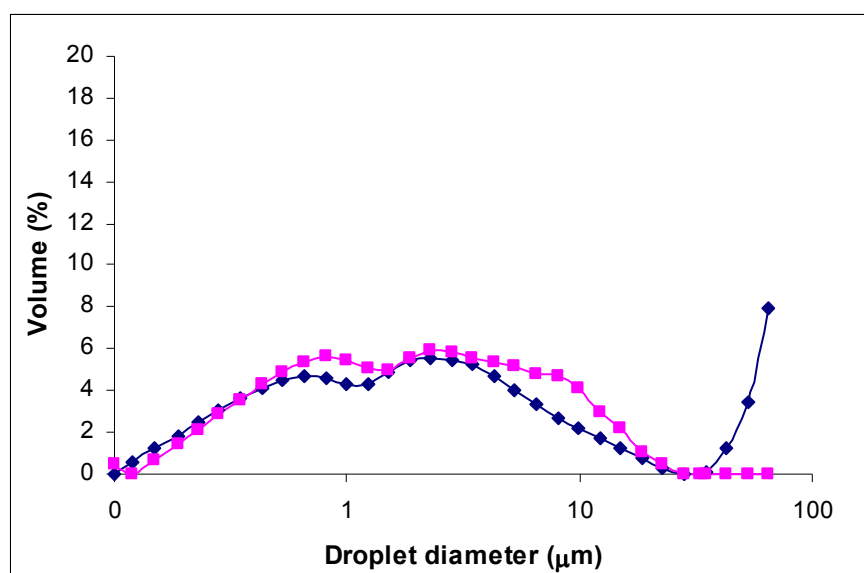
Fig. 5.4 Droplet size distributions of W/O/W emulsions prepared in non-buffered system with (◆) 0.125 and (■) 0.5% (w/v) NaCN in the external aqueous phase. 2% (w/v) PGPR in oil phase.
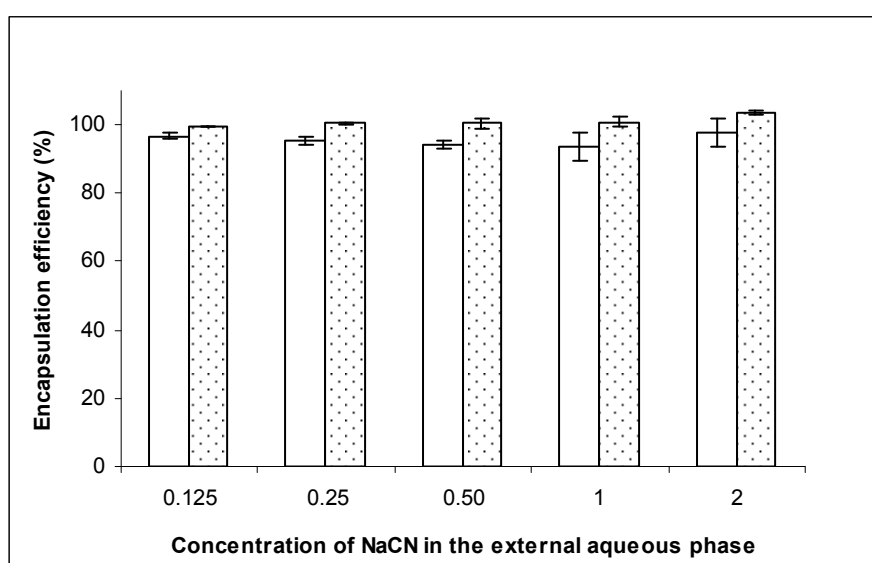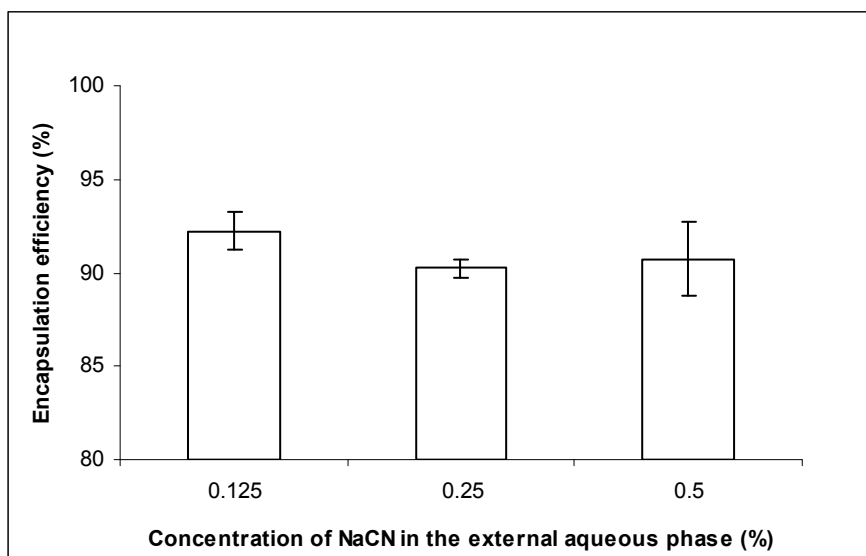
Fig. 5.5 Effect of NaCN concentration in the external aqueous phase on the EEs of freshly prepared W/O/W emulsions. Oil phase contained 2% (w/v) PGPR. Emulsions were prepared in non-buffered system. Error bars show standard deviation of at least two measurements.

5.3.2. Effect of SUPER GUM™ concentration in the external aqueous phase on droplet sizes and EEs of W/O/W emulsions

The EE of W/O/W emulsions prepared with 2% (w/v) PGPR in the oil phase, and a range of SUPER GUM^{TM} concentrations (2-10% w/v) in the external aqueous phase were determined (Fig. 5.5). The W/O/W emulsions were prepared in non-buffered systems, as in preliminary experiments SUPER GUM^{TM} precipitated in 0.1 M sodium phosphate buffer. Results showed that 2-10% (w/v) SUPER GUM^{TM} provided similarly high EE (> 90%), and the EE of W/O/W emulsions were all relatively stable over the one month storage period monitored (Fig. 5.5).
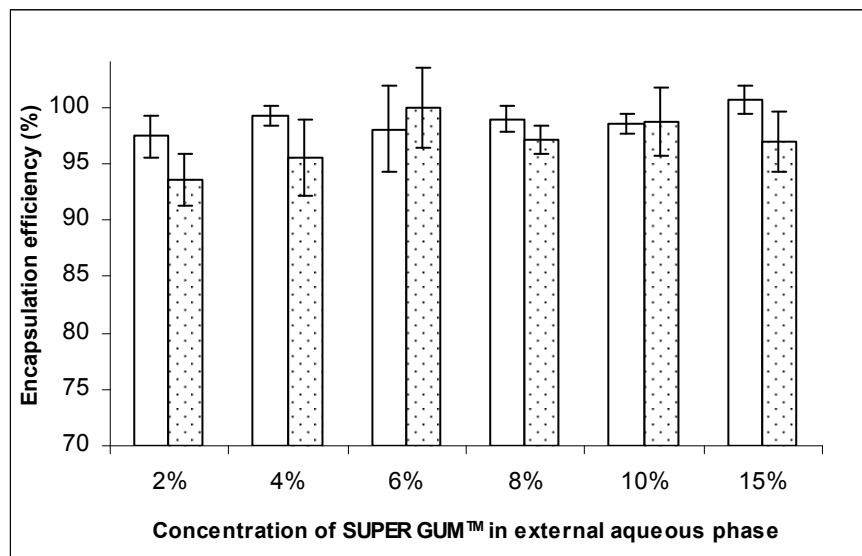
Fig. 5.6. Effect of SUPER GUM™ concentration in the external aqueous phase on the encapsulation efficiencies of W/O/W emulsions (prepared with 2% (w/v) PGPR in oil phase). Blank bar: day 0, Dotted bar: one month storage at 20°C. Emulsions were prepared in non-buffered system. Error bars show standard deviation of at least two measurements.

The droplet size distributions of W/O/W emulsions prepared using the same range of SUPER GUM^{TM} concentrations in the external aqueous phase are shown in Fig. 5.7. When measured immediately after emulsion formation, the droplet size distributions of all emulsions exhibited a bimodal distribution (Fig. 5.7a), typical of all W/O/W emulsions prepared under the same set of conditions. However, after one month at 20°C, large increases in the droplet size distributions were evident, indicative of droplet coalescence and/or flocculation in W/O/W emulsions prepared with 2-6% (w/v) SUPER GUM™ (Fig. 5.7b).
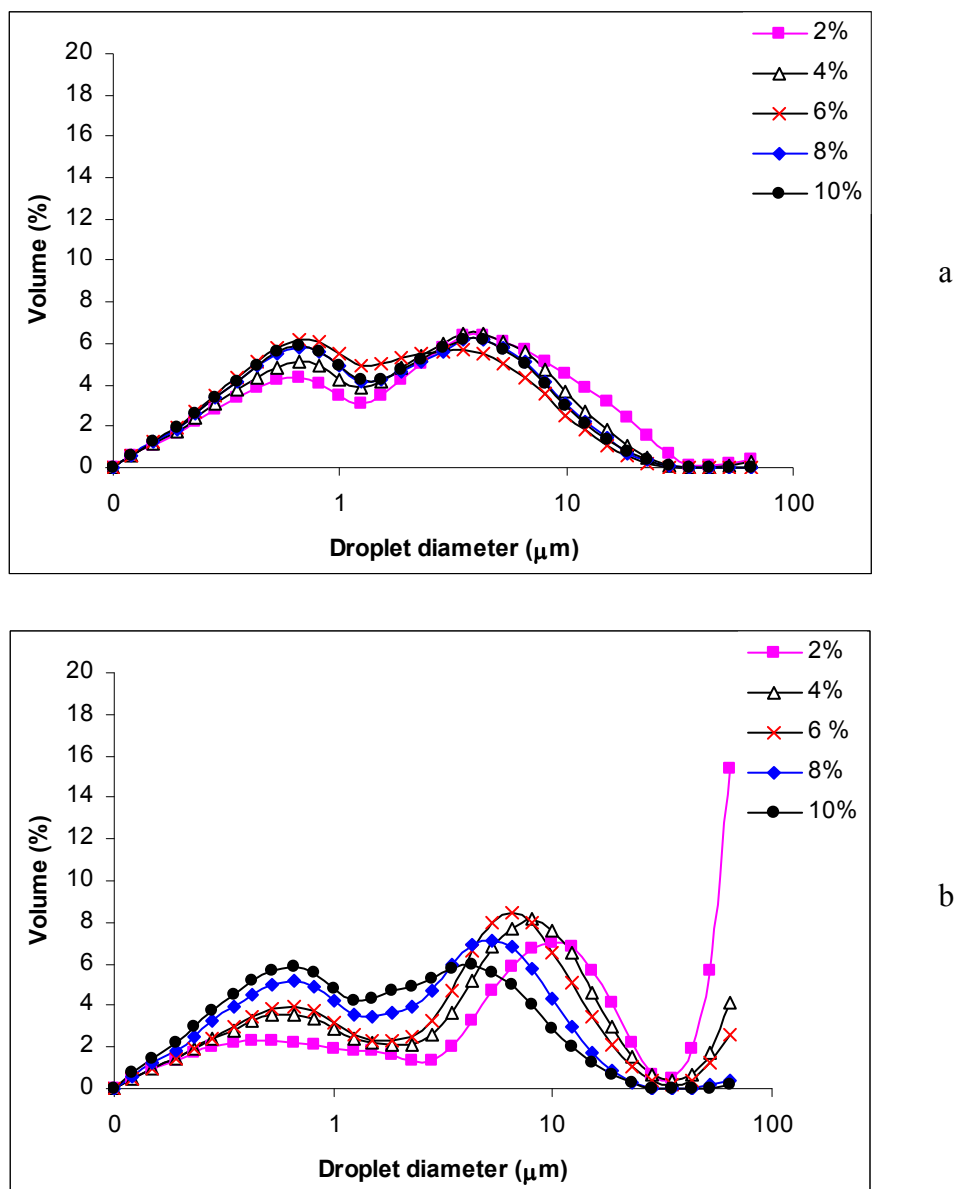
Fig. 5.7. Droplet size distributions of W/O/W emulsions prepared with 2% (w/v) PGPR in oil phase, distilled water in internal phase and varying (2-10% w/v) SUPER GUM™ concentrations in the external aqueous phase. (a): day 0; (b): 1 month at 20 °C.

Confocal microscopy (Fig. 5.8a, b) and droplet size distributions following dilution of W/O/W emulsions with 0.1% (w/v) Sodium lauryl sulphate (SDS) at low SUPER GUM™ concentrations confirmed that coalescence had occurred. As SDS, a highly effective anion surfactant, could replace SUPER GUM™ on oil droplets surface and would reverse the flocculation of oil droplets. Therefore, W/O/W emulsions prepared

with 2-6% (w/v) SUPER GUM™ were not stable over time in terms of droplet size. Coalescence was less evident in W/O/W emulsions prepared with 8-10% (w/v) SUPER GUM™ in the external aqueous phase (Fig. 5.8c). Sufficient SUPER GUM™ is required as emulsifier to prevent the particle size increasing dramatically during storage and it appears that at least 10% SUPER GUM™ is required to provide reasonable long-term stability.

It was noticed that high EE values were maintained even after extensive oil droplet coalescence had occurred in the emulsions formed at lower SUPER GUM™ concentrations. It was proposed by Gonzalez et al. (2003) that water droplets of different sizes escape from the oil droplets at different times after sample preparation. Contact between both interfaces, external (external aqueous phase and oil phase) and internal (oil phase and layer structured around the water droplets), is required for the droplet to exit, thus droplets close to the external interface are more likely to exit earlier than those closer to the centre of the oil droplet, which then might have the time to grow larger by internal coalescence. According to this theory and findings that larger water droplets are more likely to be expelled (Grossiord and Seiller, 2001; Villa, Lawson, Li and Papadopoulos, 2003; Cheng, Xu, Wen and Chen, 2005), it appears that changes in droplet size, caused by coalescence, may not influence EE values of SUPER GUM™ stabilised systems, provided the internal water droplet size remains constant.
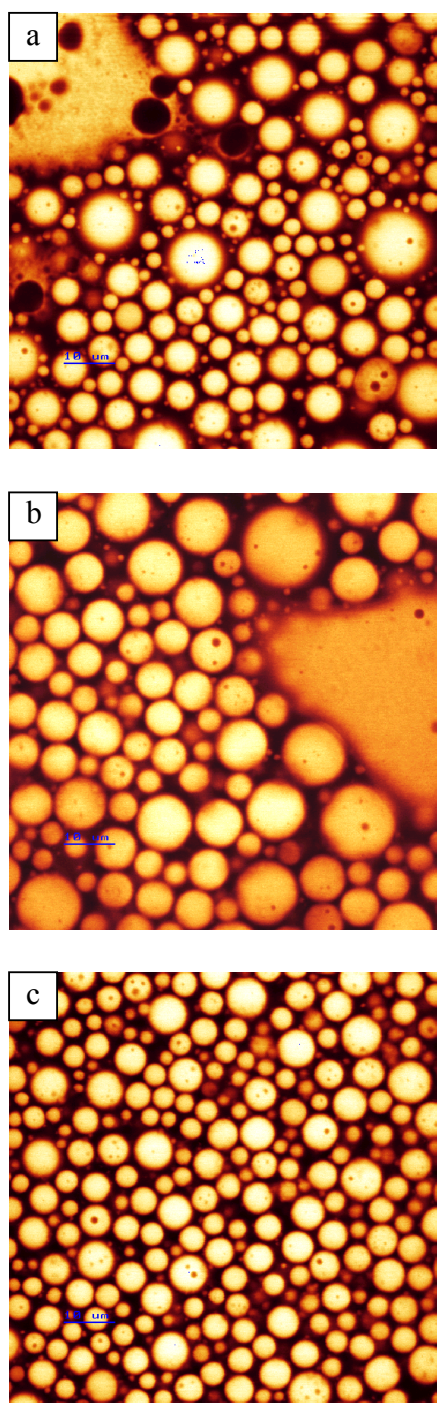
Fig. 5.8. **Confocal micrographs of W/O/W emulsion prepared with 2% (w/v) PGPR in oil phase, distilled water in internal phase and (a) 2% (w/v)' (b) 4% (w/v) and (c) 10% (w/v) SUPER GUM™ in the external aqueous phase. Size bar corresponds to 10 μm.**

5.3.3. *Comparison of NaCN and SUPER GUM™ as hydrophilic emulsifiers in non-buffered W/O/W emulsions*

As it has now been shown that 10% SUPER GUM™ is required to form stable W/O/W emulsions, a comparison was made to the previously reported system at which optimum stability and high EE was obtained using 2% (w/v) PGPR and 0.5% (w/v) NaCN (Chapter 4). The effects of PGPR concentration on the EE of W/O/W emulsions prepared using either 10% (w/v) SUPER GUM™ or 0.5% (w/v) NaCN as hydrophilic emulsifier in the external aqueous phase (both in non-buffered systems) are compared in Figure 5.9. When 0.5% (w/v) NaCN was used as emulsifier, increased EE was obtained as PGPR concentration increased and 2% PGPR was the minimum concentration required to obtain an EE over 95%.

The replacement of 0.5% (w/v) NaCN in the external aqueous phase with 10% (w/v) SUPER GUM™, however, significantly increased the EE from 35% and 67% to almost 100% in W/O/W emulsions prepared with 0.5 and 1% (w/v) PGPR, respectively (Fig. 5.9a), compared to the W/O/W emulsions with 0.5% NaCN as hydrophilic emulsifier. The W/O/W emulsions with SUPER GUM™ as hydrophilic emulsifier were also stable over time (Fig. 5.9b); after one month at 20°C, emulsions with 1% PGPR still retained an EE over 90%.
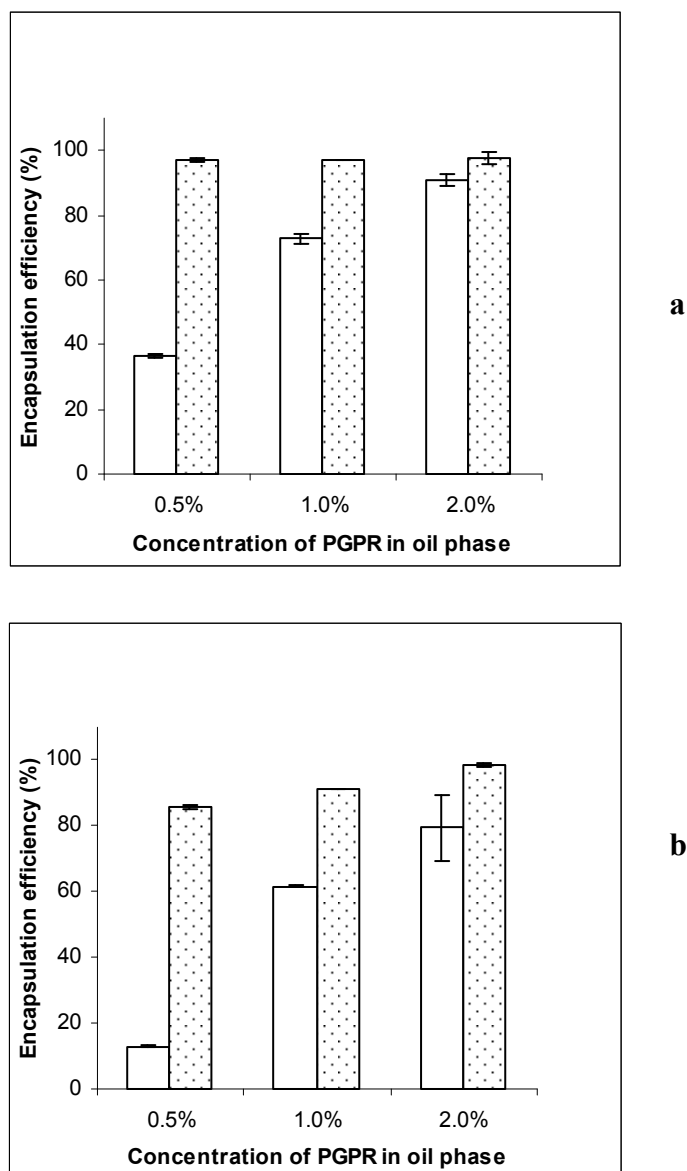
Fig. 5.9. Effect of PGPR concentration in the oil phase on the encapsulation efficiencies of W/O/W emulsions prepared using 0.5% (w/v) NaCN (blank bar) or 10% (w/v) SUPER GUM™ (dotted bar) as hydrophilic emulsifiers in the external aqueous phase. (a): day 0; (b): 1 month at 20 °C. Error bars show standard deviation of at least two measurements.

While the concentrations of SUPER GUM™ required to form a stable W/O/W emulsion are much higher than the concentration of NaCN, this is a function of the emulsifying properties of the materials themselves. It is necessary for sufficient protein fractions to adsorb onto the oil droplets during the emulsification to produce

the requisite stabilising emulsion. Proteinaceous emulsifiers such as NaCN are flexible in structure. When used to form emulsions, NaCN adsorbs to the oil-water interface in an unfolded way to gives a high surface coverage and large adsorbed layer dimension (Euston and Hirst, 2000). On the other hand, SUPER GUM™ has a protein content of ~2% (nitrogen content of 0.32%) (Al-Assaf et al., 2007; Aoki et al., 2007), in a form of aggregates of arabinogalactan protein, the effective number of protein "particles" is much lower than for free dissociated NaCN. Furthermore, the arabinogalactan protein, the functional group of emulsifying in SUPER GUM™ only comprises a small proportion (20%) of the SUPER GUM™ (Yadav, Igartuburu, Yan and Norhnagel, 2007). To compensate for this a high concentration of gum must be used (Dickinson et al., 1988; Dickinson, Elverson and Murray, 1989; Dickinson and Galazka, 1991; Dickinson et al., 1991b). Dickinson et al. (1991b) stated that roughly a 1: 1 ratio of gum arabic to oil compared with 1:10 protein to oil is necessary for a protein-stabilised emulsion.

As a result, SUPER GUM™ may cover the oil-water interface in a different way compared to NaCN. Dickinson et al. (1989 & 1991b) suggested that gum arabic forms a thick, sterically stabilising layer around emulsion droplets, which results in a low surface activity and thus results in a stable emulsion. A unique emulsifying property of this thick, sterically stabilising gum arabic layer around emulsion droplets is that the surface viscoelasticity is rather insensitive to dilution of the aqueous phase, which enables the emulsions to be stabilised both in a concentrated and diluted form (Dickinson et al., 1989).

The hypothesis was supported by other researchers. Shotton and Wibberly (1959) demonstrated that gum arabic was able to form thick viscoelastic films at the oil-water interface. Randall et al. (1989) showed that at least 12% gum arabic is required to obtain a stable 20% oil-in-water emulsion and it was the high-molecular-mass protein rich component that preferentially adsorbed at the oil-water interface. It has been proposed that the more hydrophobic polypeptide chain is adsorbed at the surface of the oil droplet, while the hydrophilic carbohydrate blocks attached to the chain protrude out into solution providing a strong steric barrier preventing droplet interaction (Snowden, Phillips and Williams, 1987). Based on the findings reported

herein, the increased emulsion stability of protein-containing polysaccharides may be attributed to its ability to form a thicker, more compact and viscoelastic interfacial layer compared to NaCN, and thus result in more stable W/O/W emulsions.

The use of multiple emulsions as a delivery system for food-derived bioactives has often been proposed, and a comparison of the behaviour of the NaCN and SUPER GUM™ stabilised W/O/W emulsions over a range of pH values, as may occur during ingestion or incorporation into different food systems, was therefore of interest. The droplet sizes of W/O/W emulsions prepared with SUPER GUM™ and NaCN in the external aqueous phase were monitored over a range of pH values, and the results are shown in Fig. 5.10.
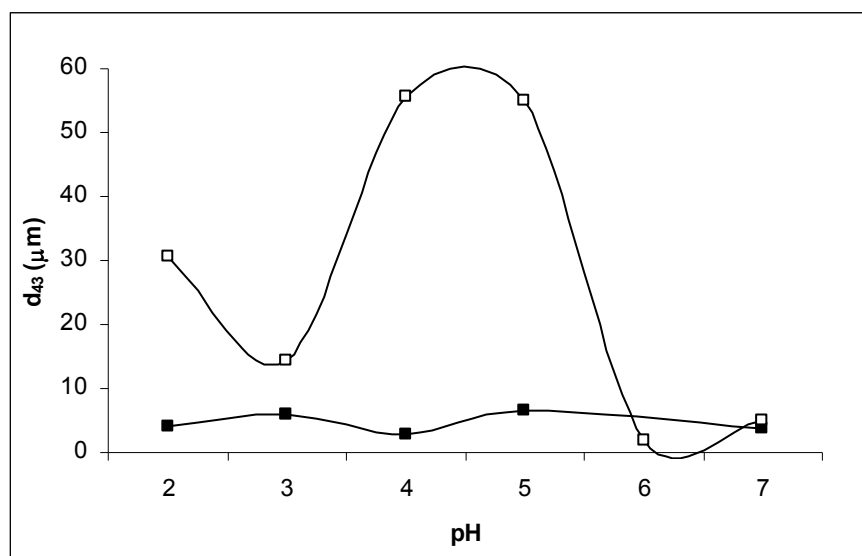
Fig. 5.10. Effect of pH on the mean volume droplet size (d_{43}) of W/O/W emulsions prepared with 2% (w/v) PGPR in the oil phase, distilled water in internal phase and either 10% (w/v) SUPER GUM™ (solid symbol) or 0.5% (w/v) NaCN (open symbol) in the external aqueous phase.

W/O/W emulsions prepared with 10% (w/v) SUPER GUM™ displayed a stable droplet size (d_{43}) from pH 7 to 2, whereas the particle sizes of W/O/W emulsions prepared with 0.5% (w/v) NaCN increased dramatically when the pH was reduced from pH 6 to 3. This indicates aggregation of NaCN coated droplets over this pH range, which is expected due to the self-associative tendencies of casein in their pI

region (pH 4.6). The finding suggested that W/O/W emulsions prepared with SUPER GUM™ may have advantages for use as a delivery system over a wide range of pH conditions.

5.4.   Summary

The effects of NaCN and SUPER GUM™ as hydrophilic emulsifier on the formation and stability of W/O/W emulsions were studied. Overall, the results suggest that EE of W/O/W emulsion was mostly dependent on the PGPR concentration, which determines the stabilisation of internal water droplets, rather than hydrophilic emulsifiers.

Hydrophilic emulsifiers contributed mainly on the stability of oil droplets, an appropriate amount of hydrophilic emulsifiers could prevent oil droplets from coalescence, therefore the droplet size distribution would not undergo significant change for a long period of time. Insufficient hydrophilic emulsifiers lead to larger oil droplets in emulsion as a result of coalescence. However, it was found that coalescence of oil droplets slightly increased EE of W/O/W emulsions.

W/O/W emulsions with 10% SUPER GUM™ as hydrophilic emulsifier had significantly higher EE compared to emulsions with NaCN. It could further reduce the minimum PGPR concentration to 0.5% (w/v) with an EE > 90%. It also showed advantages for use as a delivery system over a wide range of pH conditions.

Chapter 6: Characteristics of W/O/W emulsions containing hydrophilic encapsulants in the internal aqueous phase

6.1.   Introduction

During the formation of W/O/W emulsions, hydrophilic encapsulants may be incorporated into the internal aqueous phase. It may be possible to add sensitive food ingredients, such as enzymes or bioactive nutrients, into the internal aqueous phase of W/O/W emulsions, aiming to entrap them in the emulsion or release them in a controlled manner.

By incorporating hydrophilic encapsulants into the internal aqueous phase of W/O/W emulsions, a significant feature of multiple emulsions compared to ordinary emulsion arises, i.e. the osmotic gradient for the passage of water and hydrophilic encapsulants, such as electrolytes or sugars across the oil phase.   Swelling or shrinking of the internal water droplets results from the osmotic gradient between the internal and external aqueous phase and has a considerable influence on the stability of the W/O/W emulsions.

On the other hand, the hydrophilic compounds could change the viscosity and interfacial properties of W/O emulsions, which is essential to the stability of the sequential W/O/W emulsions (Dickinson and McClements, 1996). The effect of ionic environment in the internal aqueous phase on the properties of W/O/W emulsion has been discussed (Chapter 4.3.3). In this chapter, the effect of different types of small hydrophilic encapsulants on the properties of the W/O/W emulsions will be further investigated.

Sorbitol and NaCl were chosen as model hydrophilic encapsulants as they have been studied as osmotic gradient generators in W/O/W emulsions in previous studies (Hirasawa, Ashitani, Yoshikawa, Nagahisa, Furusawa, Katakura, Shimizu and Shioya,

2006), are cost effective and there are established methods for measuring their concentrations in solutions.

6.2. Materials and Methods

6.2.1. Raw materials

NaCl and sorbitol were obtained from Sigma Ltd., St Louis, Missouri, USA. Other materials were identical to those used previously; please refer to Chapter 3, Section 3.2, Materials and Methods.

6.2.2. Preparation of W/O/W emulsions

W/O/W emulsions were prepared by a two-step process. NaCl (0.1- 0.5 M) or sorbitol (0.1-0.5 M) solutions prepared in distilled water were dispersed in soybean oil containing PGPR, and the mixtures were homogenised at 800/80 bar (first-stage pressure /second stage pressure) using a two-stage high pressure homogeniser (APV 2000, Denmark). Samples were passed twice through the homogeniser to obtain the primary W/O emulsions. The second step involved the dispersion of the primary emulsions into solutions containing hydrophilic emulsifiers (e.g. NaCN), prepared in distilled water, using the same homogeniser, at a pressure of 100/0 bar twice.

6.2.3. Determination of encapsulation efficiency (EE)

The encapsulation efficiency (EE) is the fraction (% volume) of the encapsulants in the aqueous phase of the primary emulsion which remains entrapped in the final W/O/W emulsion. The EE of W/O/W emulsions encapsulated with sorbitol (0.5 M) was determined using poly R-478 dye as marker in the internal aqueous phase. For the details of measurement of poly R-478 dye please refer to Chapter 3.2.6: Determination of encapsulation efficiency.

The release of NaCl of W/O/W emulsions encapsulated with NaCl was determined using a Jenway Cl- ion-selective electrode combined to a reference electrode (DR359TX) (Barloworld ScientificLtd, London, England). To determine the concentration of NaCl released in the external aqueous phase of W/O/W emulsion, a series of standards within the expected sample concentration were measured in a blank W/O/W emulsion with distilled water in the internal aqueous phase. A subsequent standard curve was prepared (R^2=0.9981). The concentration of NaCl released from the internal to external aqueous phase was determined from the standard curve.

6.2.4. Determination of permeability of sorbitol through oil phase and the mean size of internal water droplets of W/O/W emulsions using the PFG-NMR technique.

6.2.4.1. Parameter setup

PFG-NMR diffusion measurements have been described by Hindmarsh, et al. (2005). The measurements were performed using a Bruker Avance 500 Mhz spectrometer featuring a 50 G cm^{-1} z-gradient. For each PFG-NMR experiment the gradient strength, g, ranged from 5 to 50 G cm^{-1} with 32 steps. For each experiment, 1 mL of solution was analysed at 298 K in a 5 mm NMR tube.

The diffusion coefficients for the sodium caseinate solutions with and without sorbitol were measured with a pulse width (δ) of 4 ms and a Δ of 50 ms (used as the free-diffusion coefficient in primary W/O emulsion droplet sizing). PFG-NMR emulsion droplet sizing of the two primary emulsions was undertaken with a δ of 5 ms and a Δ of 200 ms.

For the W/O/W emulsions, when tracing the diffusion of water, a δ of 5 ms was used for each sample and a number of measurements were taken where the value of Δ was varied between 20 to 100 ms by steps of 10 ms. When tracing the diffusion of sorbitol, a δ of 18 ms was used with a Δ of 400 ms.

6.2.4.2. Model fitting

6.2.4.2.1. W/O emulsion

Within the W/O emulsions, water molecules undergo self-diffusion, and they are only able to diffuse a fixed distance from their original positions. To calculate the droplet size of W/O emulsion, a probability distribution of radii, *P(R)* is used (eq 6.1), where the magnitude of the NMR signal (can be obtain using eq 6.3 and 6.4) is proportional to the volume of each droplet (Hollingsworth and Johns, 2003). *Ep* is the total signal attenuation corresponding to a population of droplets. The *P(R)* of primary W/O emulsions can be estimated with eq 6.2, where R_0 is volume weighted geometric median radius of the distribution and σ is variance (Packer and Rees, 1972; Hollingsworth and Johns, 2003).

$$Ep = \frac{\int_0^\infty R^3 P(R) E(q, \Delta, R) dR}{\int_0^\infty R^3 P(R) dR} \tag{6.1}$$

$$P(a) = \frac{1}{R\sigma(2\pi)^{1/2}} \exp\left[-\frac{(\ln R - \ln R_0)^2}{2\sigma^2} \right] \tag{6.2}$$

6.2.4.2.2. W/O/W emulsion

In W/O/W emulsions, the diffusion of water molecules in the external aqueous phase undergo free diffusion compared to water molecules in the internal water droplet, also, water molecules also exchange between the internal and external aqueous phase which have different diffusivities. For W/O/W emulsions samples prepared with sorbitol in the internal aqueous phase, the internal water droplet size and the permeability of water molecules were determined. The Pfeuffer et al (1998) model of

exchange between two aqueous phases with apparent diffusion in a sphere was fitted to the PFG-NMR experimental data using the non-linear least-squares regression analysis provided in Matlab (version 6.5 The Mathworks Inc), by doing so, both permeability and a distribution of internal water droplet size could be obtained (eq 6.3-6.6). The fitted parameters were p, D_2, τ_1, R_0 and σ. Where p_1 and p_2 were the relative volume fraction of the molecules in the internal and external aqueous phases. D the apparent diffusion coefficient of water, τ_1 is the internal aqueous phase exchange time.

$$D_{1,2}^{'} = \frac{1}{2}\left[D_1 + D_2 + \frac{1}{q^2}\left(\frac{1}{\tau_1} + \frac{1}{\tau_2}\right) \mp \sqrt{\left(D_2 - D_1 + \frac{1}{q^2}\left(\frac{1}{\tau_2} - \frac{1}{\tau_1}\right)\right)^2 + \frac{4}{q^4\tau_1\tau_2}} \right] \quad (6.3)$$

$$p_2^{'} = \frac{\left(p_1 D_1 + p_2 D_2 - D_1^{'}\right)}{D_2^{'} - D_1^{'}} \quad (6.4)$$

$$p_1^{'} = 1 - p_2^{'} \quad (6.5)$$

$$D_{app}(t_D) = -\frac{\partial \ln E(q,\Delta,R)}{\delta(q^2)} \cdot \frac{1}{t_D} \quad (6.6)$$

To achieve a more stable non-linear fitting of experimental data, the exchange models were first fitted with a single droplet radius for the internal aqueous phase (no distribution). The D_2 obtained was then used to fit the exchange model with a droplet size distribution but constrained to +/- 5%.

6.3. Results and Discussion

6.3.1. W/O/W emulsions with sorbitol

6.3.1.1. Visual observation of primary W/O emulsion with sorbitol (0.5 M) in the internal aqueous phase

The stabilities of W/O emulsions prepared with or without sorbitol (0.5 M) in the internal aqueous phase were compared visually. It appeared that after the addition of sorbitol into the internal aqueous phase (Fig. 6.1b), the stability of W/O emulsions improved, as they were more resistant to water droplet sedimentation compared to W/O emulsion without sorbitol in the internal aqueous phase (Fig 6.1). The addition of sorbitol at different concentrations (0.1 M and 1 M) was also tested and similar results were obtained.

line of sediment ⟶

     a   b
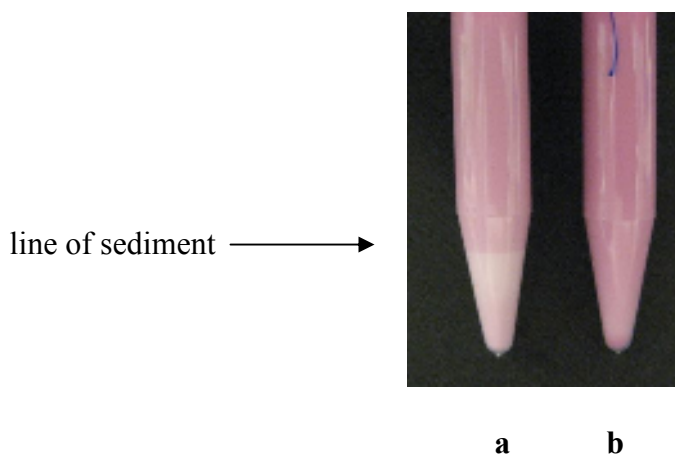
Fig. 6.1. Photographs of primary W/O emulsions prepared with (a) distilled water and (b) 0.5 M sorbitol solution in the internal aqueous phase. W/O/W emulsions were stored at 20°C for 1 month. Both emulsions contained 2% (w/v) PGPR in the oil phase as hydrophobic emulsifier.

Sorbitol did not show adverse effects on the emulsifying ability of PGPR in W/O emulsions, compared with sodium phosphate buffer and NaCl, which reduced the emulsifying ability of PGPR and caused phase separation. (Chapter 4.3.3.).

6.3.1.2. Mean droplet size of W/O emulsions with 0.5 M sorbitol in the internal aqueous phase

Mean droplet size of W/O emulsions containing 0.5 M sorbitol in the internal aqueous phase was determined using the DWS technique and results are shown in Fig. 6.2. Compared to control W/O emulsions, which were prepared with distilled water in the internal aqueous phase, W/O emulsions with 0.5 M sorbitol in the internal aqueous had significantly smaller mean water droplet diameter in fresh emulsions (1.0 μm vs. 0.33 μm). Over time, the mean droplet diameter of both emulsions increased compared to the freshly made emulsions due to flocculation/coalescence of water droplets, however, emulsion samples with sorbitol (0.5 M) in the internal aqueous phase always had significantly smaller droplet sizes than emulsions without sorbitol.
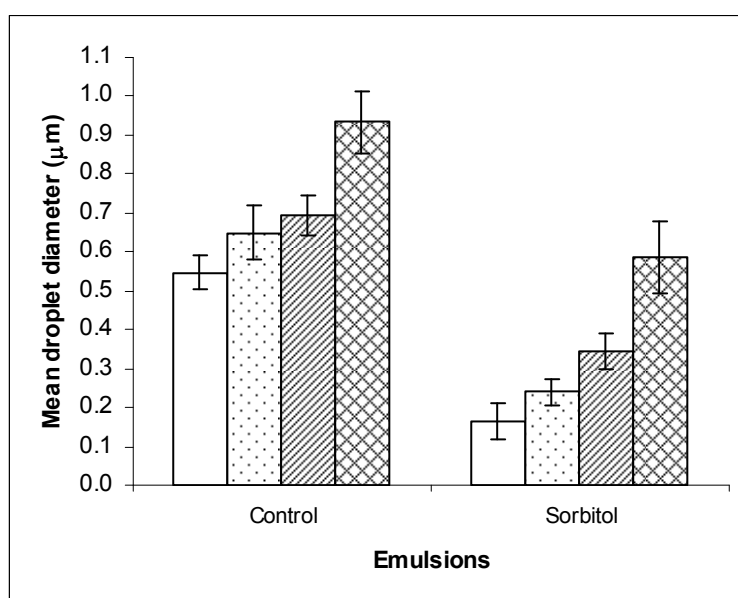
Fig. 6.2. Mean droplet size of W/O emulsions prepared with distilled water (control), or 0.5 M sorbitol solution in the internal aqueous phase. Samples were freshly prepared (blank bar), stored at 20°C for 1 day (dotted bar), 1 Week (striped bar) and 1 month (netted bar). Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation.

The results of mean water droplet size measured by the DWS technique was in accordance with the observation of W/O emulsions with or without addition of sorbitol in the internal aqueous phase (Fig. 6.1). Larger water droplets in control emulsion were more unstable in terms droplets flocculation, which led to sedimentation of water droplets (Aronson and Petko, 1993; Kent and Saunders, 2001).

Benichou et al (2001) found that after the addition of polyols, such as propylene glycol or glycerol, in the internal aqueous phase of W/O emulsions, the interfacial tension between the aqueous phase and the oil phase is drastically reduced and the ease of formation improves. This is due to the polyols lowering the attractive forces between the internal water droplets by enhancing the miscibility of the aqueous phase and the oil phase, and equalisation of the refractive indices between the oil and aqueous phase (Kzling and kronberg, 1990; Benichou et al., 2001). Under pressure, polyols act as co-surfactant in the system and play an important role in the stabilization of the W/O emulsions (Gregory, 1991). On the other hand, Benichou et al. (2001) and Dickinson and Golding (1998) found that the addition of polyols in the aqueous phase of W/O emulsion facilitated Ostwald ripening, which could affect the long-term stability of W/O emulsion as the number of larger water droplets increased over time. However, in this study, water droplet sedimentation or phase separation was not observed for up to one month in W/O emulsions with addition of 0.5 M sorbitol in the internal aqueous phase, thus it was assumed that the positive effect of sorbitol far outweighed the adverse effect on stability of W/O emulsions.

6.3.1.3. Confocal microscopy of W/O/W emulsions

W/O emulsions with sorbitol (0.5 M) in the internal aqueous phase were dispersed in 0.5% (w/v) NaCN solutions to form W/O/W emulsions, and the internal water droplets in the W/O/W emulsion were observed by confocal microscopy (Fig. 6.3 b). W/O/W emulsions with sorbitol (0.5 M) added in the external aqueous phase (Fig. 6.3 c) or in both the internal and external phase (Fig. 6.3 d) were also prepared to create osmotic pressure gradients from different directions or balanced osmotic pressure, respectively.

Fig. 6.3b shows that after the formation of W/O/W emulsions, osmotic pressure was created from the internal to the external aqueous phase, and water transferred from the external to internal aqueous phase. As a result the internal water droplets swelled and increased in size, and flocculation/coalescence of water droplets occurred, as suggested by previous researchers (Grossiord and Seiller, 2001; Villa et al., 2003; Cheng et al., 2005). Fig. 6.3 c shows much less visible water droplets inside the oil droplets, as the osmotic pressure gradient was from the external to internal aqueous phase, the internal oil droplets were expected to shrink over time due to water loss from internal aqueous phase. However, compared to Fig 6.3 a, the blank emulsion without addition of sorbitol, the oil droplets did not show apparent change in size (Fig. 6.3 c). Fig. 6.3 d shows W/O/W emulsion with 0.5 M sorbitol in both internal and external aqueous phases, the osmotic pressure being balanced. However, flocculation/coalescence of large water droplets still occurred, but they were less visible compared to emulsions with sorbitol in the internal aqueous phase (Fig. 6.3 b).
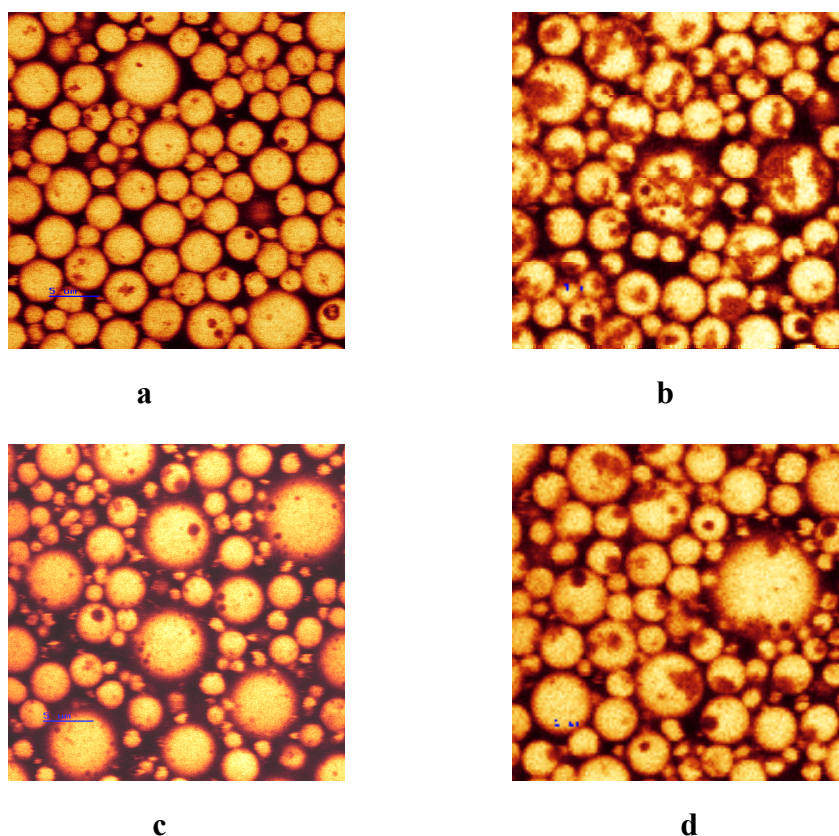
Fig. 6.3. Confocal microscopy images representing W/O/W emulsions. (a) emulsion with no addition of sorbitol, (b) emulsion with osmotic gradient from the internal to external aqueous phase (0.5 M sorbitol in internal aqueous phase only), (c) emulsion with osmotic gradient from external to internal aqueous phase (0.5 M sorbitol in external aqueous phase only), (d) emulsion with 0.5 M sorbitol in both internal and aqueous phase. Scale bar represents 5 μm.

6.3.1.4. PFG-NMR analysis of water diffusion and internal droplet size distribution of W/O/W emulsions with 0.5 M sorbitol in the internal aqueous phase

PFG-NMR analysis of the emulsions was undertaken 24 hours after formation, as after this time it was estimated from the results of past studies that the emulsions would have stabilised and any transport processes occurring would be under equilibrium conditions (Matsumoto, Inoue, Kohda and Ikura, 1980; Garti, Magdassi and Whitehill, 1985; Yan and Pal, 2001). PFG-NMR analysis and modeling were

carried out by Dr. Jason Hindmarsh, and the results have been reported (Hindmarsh et al., 2005).

Fig 6.4 shows representative curves (*ln {E(q,Δ)]* versus q^2) predicted by Pfeuffer et al's model for a sequence of values of Δ (20-100 ms). In simulating the plots using Eq. 6.3-6.6, the diffusion coefficient of water at 25ºC ($D = 2.3 \times 10^{-9}$ m^2/s), a droplet distribution with a mean radius R_0 of 5×10^{-7} m and a standard deviation σ of 0.8, and an internal aqueous phase exchange time τ_1 of 50 ms were used. The curves are characterised by two slopes; the first larger slope at low values of q^2 is dominantly the attenuation due to the free-diffusion of water in the external aqueous phase (S_{outer}) and the second is the restricted diffusion in the internal aqueous phases of water droplets (S_{inner}). The transition from the free to restricted diffusion attenuation becomes less gradual for increasing Δ, due to increased population of molecules transporting from a low diffusivity environment (internal aqueous phase) to a higher one (external aqueous phase) during the time period Δ, as the signal from the larger droplets attenuates faster, as predicted by Eq. 6.2. This also leads to less residual signal from the internal aqueous phases at increasing values of the observation time Δ, as seen by the stepwise decrease in the signal level at higher values of q^2. The level of attenuation increases due to increased population of molecules transporting from a low diffusivity environment (the internal aqueous phase) to a higher one (the external aqueous phase) during the time period Δ. The change in the curve intercept at high values of q^2 is determined by the internal aqueous phase exchange time. The models demonstrate that there is indeed internal aqueous phase exchange of water between the internal and the external aqueous phase for the W/O/W emulsions.
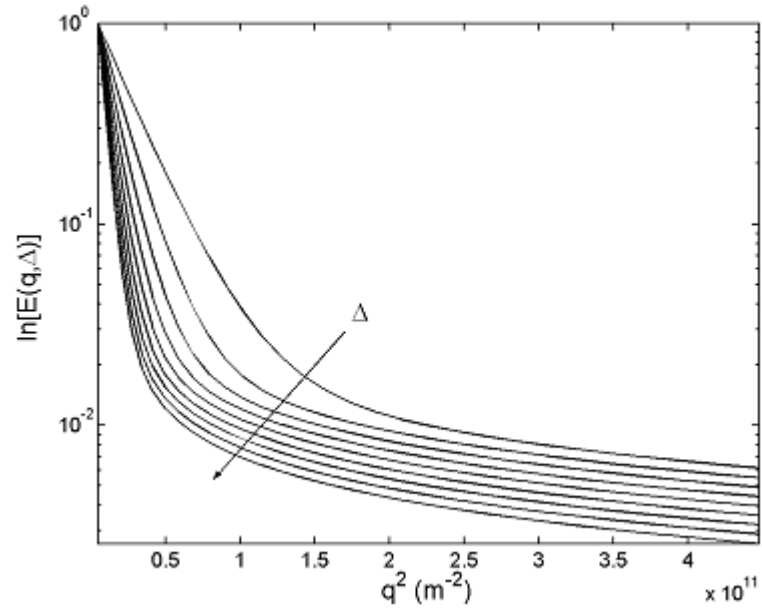
Fig. 6.4. Representative curves of the echo attenuation in a two compartment model with free and restricted diffusion including inter-compartment exchange (Pfeuffer et al and Price et al models) at different observation times Δ. Parameters δ= 5 ms, p_1=0.05, R_0=5×10^{-7} m, σ=0.8, $D_{1,2}$=2.3×10^{-9} m^2/s, τ_1= 50 ms and Δ varied between 20 to 100 ms by steps of 10 ms (arrow direction of increasing Δ) (Hindmarsh et al., 2005).

Fig 6.5 shows the results of fitting the Pfeuffer et al model to the experimental echo attenuation data of each W/O/W emulsion at an observation time Δ of 100 ms. The fitted parameters are shown in Table 6.1. Also shown is the mean permeation coefficient P of the oil phase for water as calculated from Schoberth et al. (2000).

$$P = \frac{1}{\tau_1}\frac{R_0}{3} \tag{6.7}$$
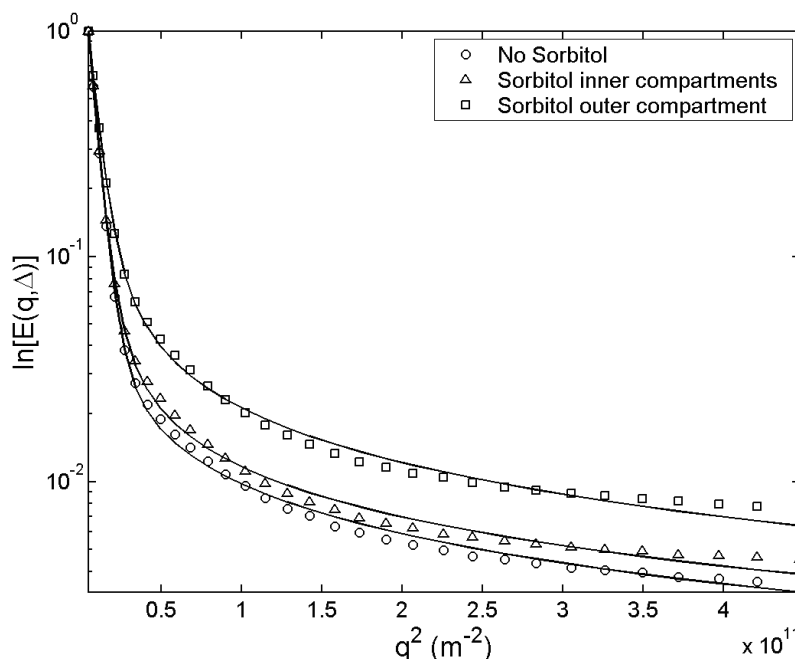
Fig. 6.5. Pfeuffer et al model curve fits for experimental data of the echo attenuation of water in W/O/W emulsions; no sorbitol present, sorbitol added to internal aqueous phases and sorbitol added to external aqueous phase. Experimental parameters; δ = 5 ms and Δ= 100 ms (Hindmarsh et al., 2005). Summary of model fitted parameters are shown in Table 6.1.

The permeability values obtained (Table 6.1) are in reasonable agreement with those reported in previous studies of W/O/W emulsions. Using optical microscopy, Matsumoto et al (1980) found the permeability of water to range between 2×10^{-6} to 8×10^{-6} m/s for a series of water/hydrocarbon/water emulsions. They also found that the permeability was influenced by the concentration of emulsifying agent, oil composition and osmotic pressure gradient between the aqueous phases. Ueda and Matsumoto (1991) studied the influence of a series of sugars on the water permeability and found that for all the sugars investigated, there was an increase in the permeation coefficient when a sugar was added to either the internal or external aqueous phases. This was due to the presence of an osmotic pressure gradient. This was also evident in this study, as shown in Table 6.1.

The results in Table 6.1 indicate that for each W/O/W emulsion the predicted internal aqueous phase water volume fraction (p_1) was greater than the theoretical maximum initial volume fraction. Assuming complete emulsification of the primary emulsion, p_1 would be 0.05 for each W/O/W emulsion. The value of 0.06 for the W/O/W with no sorbitol present can be neglected as it is likely to fall within the uncertainty of the data, but the change in the volume fraction is significant for both W/O/W emulsions containing sorbitol, in the internal or external aqueous phases. This suggests that the internal water droplets have undergone swelling. This is supported by the oil droplet sizing results obtained from MasterSizer (Fig. 6.6), which shows that with the addition of sorbitol there is an increase in the size of the oil droplet distributions. The size increase predominately occurs in larger sized distribution of the binominal distributions ($d_{43} > 1.23$ μm), leading to a greater separation of the two peaks. The changes in the mean droplet diameter of the larger size distributions are 135% and 150% for the addition of sorbitol to the internal aqueous phases and the external aqueous phases, respectively. These are reasonably consistent with the observed changes in internal aqueous phase volumes.

Swelling of the internal water droplets leads to swelling of the oil droplets. This is consistent with the observation of the water exchange and is the basis of the optical microscope technique of measuring the oil membrane permeability (Matsumoto et al., 1980; Ueda and Matsumoto, 1991; Yan and Pal, 2001; Mezzenga et al., 2004). The swelling levels are comparable to those reported by Yan and Pal (2001), who observed that a W/O/W emulsion could remain stable with levels of swelling up to 200% of their initial size.

Table 6.1: Summary of fitted parameters of Pfeuffer et al model to experimental data. For water diffusion in W/O/W emulsions at an observation time Δ of 100 ms. Parameter characterise water exchange and the internal aqueous phase droplet size distribution (Hindmarsh et al., 2005).

W/O/W Emulsion	External aqueous phase diffusivity D_2	Mean droplet radius R_0	Standard Deviation σ	Internal aqueous phase volume fraction	Internal aqueous phase exchange time τ_1	Mean permeation coefficient P
	$(\times 10^{-7}$ $m^2/s)$	$(\times 10^{-7}$ m)	-	-	(ms)	$(\times 10^{-6}$ m/s)
No Sorbitol	2.2	3.30	0.82	0.06	0.08	1.43
Sorbitol Internal	2.2	4.03	0.78	0.09	0.05	2.55
Sorbitol External	2.0	5.75	0.74	0.13	0.06	3.25
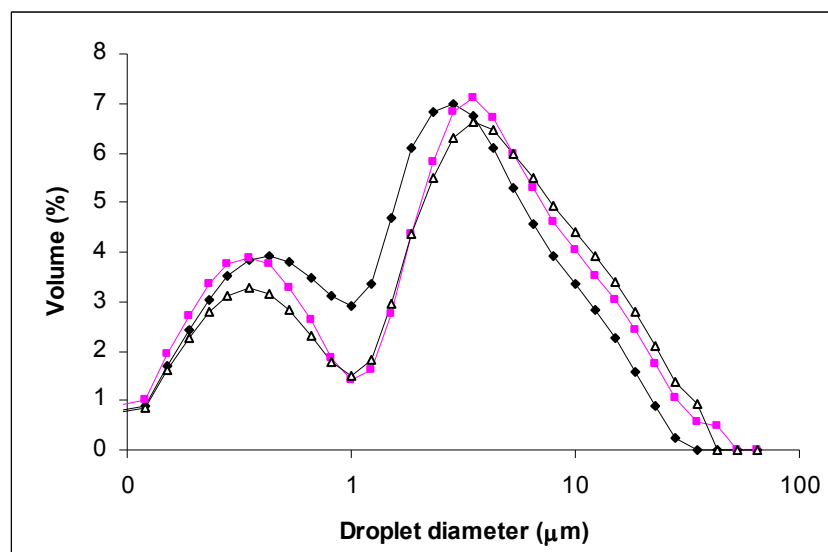
Fig. 6.6. Droplet size distributions (determined using MasterSizer) of W/O/W emulsions with no sorbitol (◆), with 0.5 M sorbitol in the internal aqueous phase only (■), and with 0.5 M sorbitol in the external aqueous phase only (△).

Monitoring the oil droplet size distribution will detect swelling and a certain level of rupture of the internal aqueous phase water droplet, monitoring of the internal aqueous phase droplet size distribution provides a more valuable and direct indication of the stability of the W/O/W emulsion. Fig. 6.7 a and b shows the comparison of the droplet size distributions for the primary W/O emulsions and the internal aqueous phases of the W/O/W emulsions.

The mean internal water droplet diameter of W/O emulsion measured using PFG-NMR technique (Fig. 6.7 a and b) was verified by results obtained using DWS technique (Fig. 6.2), the results from two different methods showed similar trends. The water droplet size decreased significantly after the addition of 0.5 M sorbitol in the internal aqueous phase in W/O (0.93 μm vs. 0.50 μm by PFG-NMR; 1.0 μm vs. 0.33 μm by DWS). The similarity of results provides evidence that the addition of sorbitol in the internal aqueous phase might have improved the stability of W/O emulsion by reducing the interfacial tension between the aqueous phase and the oil phase.

After W/O emulsions were incorporated into W/O/W emulsions, internal droplet size distribution showed increased mean diameter and spread (standard deviation) of their distributions from the primary emulsion from which they were formed. The increased mean diameter can be mainly due to swelling of the droplets but the increased spread of the distribution is presumably the product of coalescence and rupture of the water droplets during the secondary emulsification and aging of the W/O/W emulsion. The presence of coalescence and rupturing indicates a certain level of instability of the W/O/W emulsions (Hindmarsh et al., 2005).

It was unexpected that swelling of water droplets would have occurred in the W/O/W emulsion with sorbitol in the external aqueous phase (Fig. 6.7 a), as it was predicted that the osmotic gradient formed would be from the internal to the external aqueous phase therefore shrinkage of the internal aqueous phase droplets would have been expected. Confocal image (Fig.6.3 c) did not show apparent swelling of the internal water droplets. However, only the very large water droplets attached to the surface of oil droplets can be observed in the confocal image. An explanation could be that the presence of sorbitol in or around the oil/water interface in both the internal and external aqueous phase affected the properties of interfacial layer. Sorbitol is well known to affect the hydration capacity of the hydrophilic head groups of surfactants (Lawrence and Rees, 2000). Any change in the interfacial tension of the water droplets will lead to a change in its equilibrium size. Therefore, the final droplet size is dependent on both the osmotic swelling and the interfacial properties of the surfactants (Hindmarsh et al., 2005).
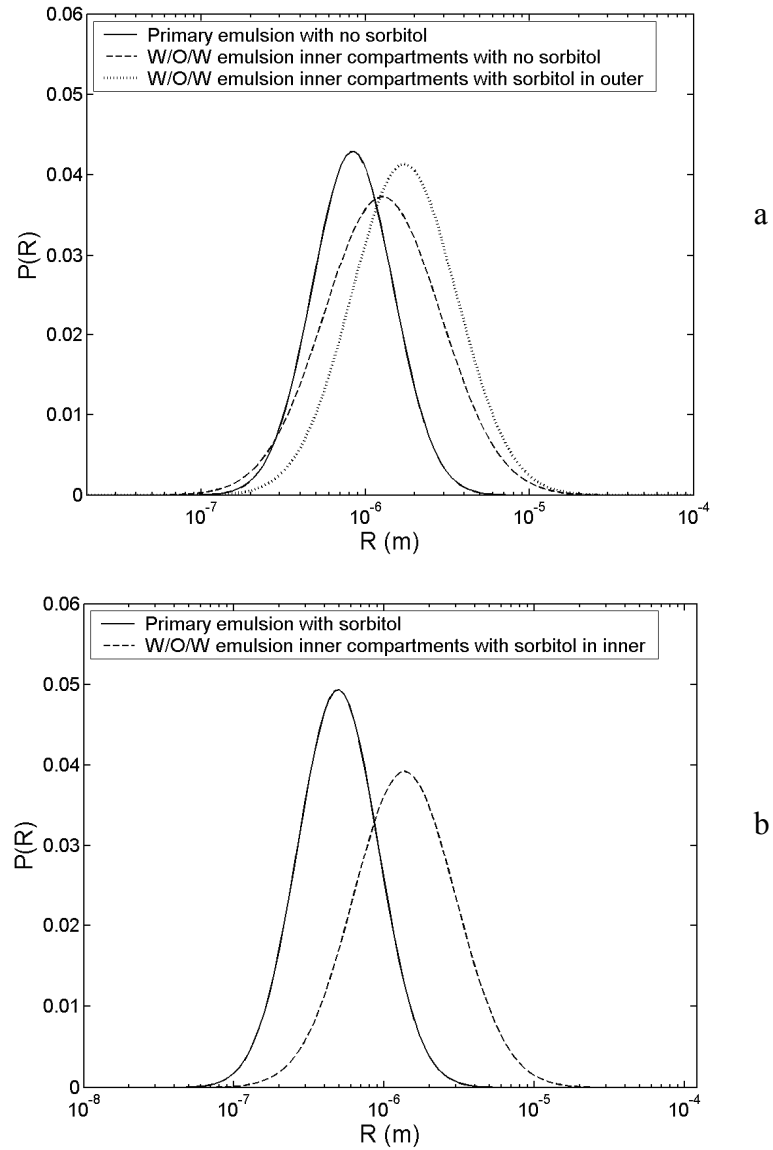
Fig. 6.7. Comparisons of water droplet size distributions for W/O emulsion (primary emulsion) and W/O/W emulsion internal aqueous phases. W/O emulsion droplet distributions predicted from fitting PFG-NMR attenuation data with Eqns. (3) and (5). W/O/W emulsion internal aqueous phases distribution predicted from Pfeuffer et al model curve fits. Normalised distributions plotted (all values of P(R) scaled so that maximum is equal to 1). (a): Water droplet size distributions of emulsions with no sorbitol added to W/O emulsion; (b): Water droplet size distributions of emulsions with sorbitol added to W/O emulsion (Hindmarsh et al., 2005).

6.3.1.5. PFG-NMR analysis of sorbitol permeation through oil phase

The presence of sorbitol in the internal or external aqueous phase for both W/O/W emulsions (emulsion with sorbitol added in the internal aqueous phase only or emulsion with sorbitol added in the external aqueous phase only) can be observed from the PFG-NMR echo attenuation of the sorbitol in each emulsion (Fig. 6.8). Both *ln[E(q,Δ)]* versus q^2 curves are indicative of self-diffusion within two domains with different diffusivities. Two distinctive attenuation slopes are present in *ln[E(q,Δ)]* versus q^2 curve. A large initial slope indicating free diffusion in the external aqueous phase is followed by a much smaller slope to the attenuation due to the restricted diffusion in the internal aqueous phase water droplets. A proportion of the release of sorbitol into the external aqueous phase will be the product of droplet rupture during the secondary emulsification of the primary emulsion containing sorbitol. But it is less probable that there would be any significant entrapment of sorbitol into internal aqueous phases during the emulsification of the primary emulsion containing no sorbitol into a solution containing sorbitol. Studies showed that a range of sugars other than sorbitol do permeate the oil membrane of W/O/W emulsions (Ueda and Matsumoto, 1991; Dickinson et al., 1993). It is likely that the sorbitol permeates the oil phase to equalise the osmotic pressure between the internal and external aqueous phase. In the case where sorbitol is initially in the external aqueous phase, it is possible that once it permeates into the internal aqueous phases it changes the interfacial properties and osmotic gradient which leads to a swelling of the internal water droplet size. Fig. 6.8 demonstrates that PFG-NMR is able to analyse the exchange of multiple species whereas other methods such as optical microscopy are limited to one species that can be traced.
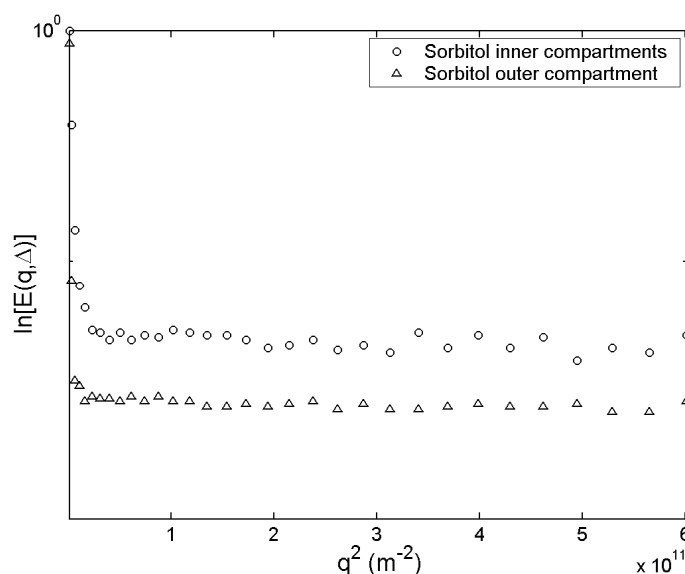
Fig. 6.8. PFG-NMR echo attenuation of sorbitol in W/O/W emulsions; sorbitol added to the internal aqueous phase and sorbitol added to the external aqueous phase. Experimental parameters; δ = 18 ms, Δ = 200 ms (Hindmarsh et al., 2005).

6.3.1.6. EE of W/O/W emulsions with 0.5 M sorbitol in the internal aqueous phase

Poly R-478 dye was used as a marker to determine the EE of W/O/W emulsions with addition of sorbitol in the internal aqueous phase (Fig. 6.9). The adsorption spectra of the dye did not change after mixing with 0.5 M sorbitol. Four sets of W/O/W emulsions were evaluated for EE: emulsions with no added sorbitol, emulsions with addition of sorbitol in the internal phase only, emulsions with addition of sorbitol in the external phase and emulsions with addition of sorbitol in both phases.

The results presented in Fig. 6.9 show that emulsions with sorbitol (0.5 M) present in the internal aqueous phase had markedly lower EE (59.9 ± 8.8%) compared to emulsions without sorbitol in the internal aqueous phase. EE of control emulsions with no added sorbitol had similar EE (90.7 ± 1.8%) to emulsions with sorbitol in the external aqueous phase only (92.2 ± 2.9%). However, EE of emulsion with sorbitol in both phases was significantly lower (85.7 ± 0.1%) than EE of these emulsions.
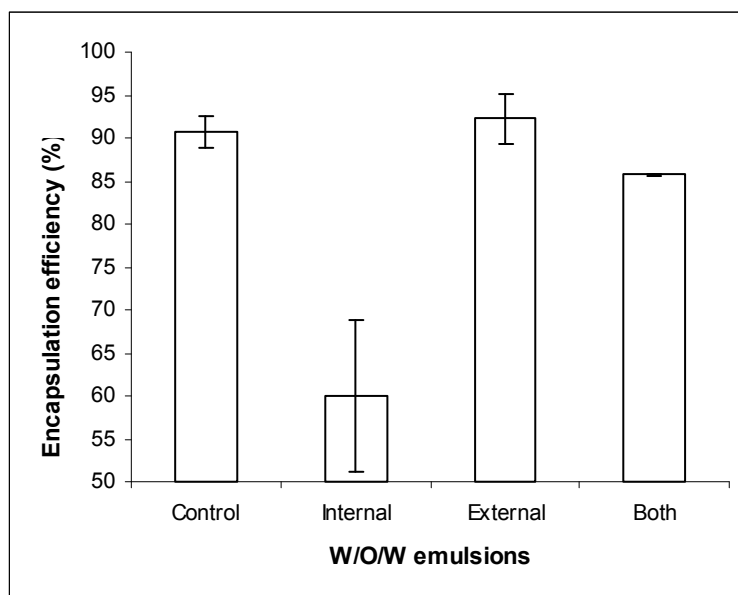
Fig. 6.9. Effect of addition of 0.5 M sorbitol on the EEs of W/O/W emulsions prepared with 0.5% (w/v) NaCN as hydrophilic emulsifier. Control: no sorbitol in W/O/W emulsion. Internal: 0.5 M sorbitol in the internal aqueous phase only, External: 0.5 M sorbitol in external aqueous phase only, Both: 0.5 M sorbitol in both internal and external aqueous phases. Measurements were conducted within 24 hours of formation of W/O/W emulsions.

Addition of sorbitol in the internal aqueous phase also decreased EE of W/O/W emulsions prepared with 10% (w/v) SUPER GUM^{TM} as hydrophilic emulsifier (Fig 6.10). SUPER GUM^{TM} was found results in higher EE compared to NaCN in the previous study (Chapter 5). The addition of sorbitol to the internal aqueous phase did not result in a significant decrease in the initial EE compared to the control emulsion, but over time, the EE of W/O/W emulsions with 0.5 M sorbitol added to the internal aqueous phase reduced significantly compared to the control emulsions. The instability of the W/O/W emulsions was likely resulted from the addition of 0.5 M sorbitol, and was independent of the hydrophilic emulsifier used to stabilise the W/O/W emulsions.
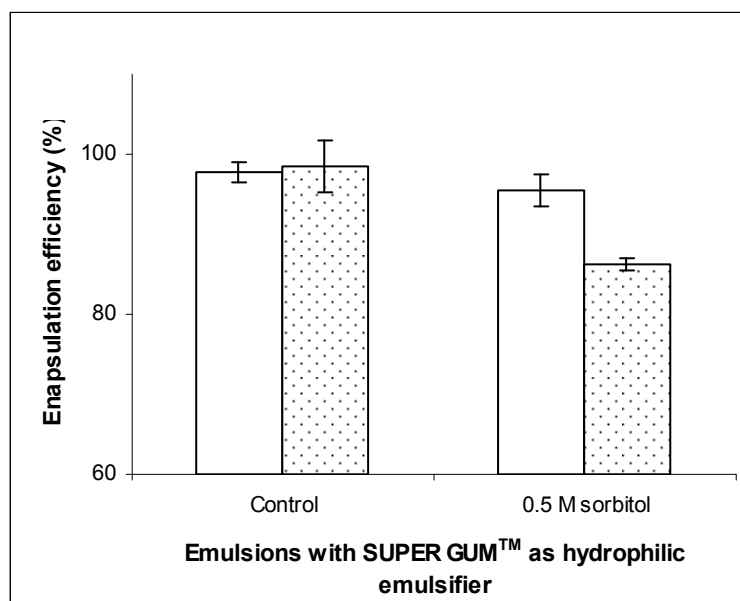
Fig.6.10. Effect of 0.5 M sorbitol in the internal aqueous phase on the EEs of W/O/W emulsions with 10% (w/v) SUPER GUM[TM] as hydrophilic emulsifier. control: no sorbitol in W/O/W emulsion, sorbitol: 0.5 M sorbitol in the internal aqueous phase.   Blank bar: day 0, Dotted bar: one month storage at 20°C.

Laplace pressure is believed to be the main forces that destabilises W/OW emulsions through Ostwald ripening that cause the water droplets to be attracted and expelled into the external aqueous phase (Davis, Round and Purewal, 1981; Aronson and Petko, 1993; Rosano et al., 1998; Kanouni et al., 2002). It is well accepted that added 0.1-0.5 M NaCl or sorbitol create osmotic pressure that balances the Laplace pressure and stabilises the internal water droplets. The addition could also increase the resistance of the oil film to coalescence by increasing adsorption density and lowering the interfacial tension of the interfacial film (Aronson and Petko, 1993; Garti and Aserin, 1996b).

However, in this study, it was found that the Laplace pressure did not bring about a significant effect on the stability of W/O/W emulsion without osmotic gradient. The EE of blank W/O/W emulsion decreased slowly over a time period of months

(Chapter 4, Fig.4.4). The osmotic gradient generated by addition of sorbitol had more significant effects on W/O/W emulsions.

Apart from water droplets expelling, there are other possible paths for releasing of water/encapsulant from the internal to the external aqueous phase, such as rupture of the water droplets during homogenising, direct-diffusion of water/encapsulant through the oil phase, reverse micelle diffusion and changes in interfacial properties of W/O/W emulsions (Wen and Papadopoulos, 2001).

However, the high initial EE of control emulsions (Figs 6.9 and 6.10) showed that there was only a small amount of dye released during homogenising as a result of oil droplet rupture. Previous researchers have studied the diffusion of small molecular weight substances through oil phases and found there was little evidence to support the theory of direct diffusion; the diffusion of small molecular substances was mainly dependent on reverse micelle diffusion, and the larger the molecule, the less and slower the diffusion (Ueda and Matsumoto, 1991; Cheng et al., 2007). As mentioned in Chapter 4 earlier, the poly-R-478 dye has a large molecular weight (50,000Da), thus dye molecules might be too large to be trapped in reverse micelles and to be transferred. Based on the finding of this study and published work, under the present experimental conditions, the contribution of the rupture of the oil droplets during homogenising, direct-diffusion or reverse micelle diffusion on the release of poly R-478 dye was believed to be negligible.

It is most likely that dye was released mainly through expelling of water droplets through the contact of internal water droplets and the oil droplet interface as observed in other studies (Leal-Calderon et al., 1996; Wen and Papadopoulos, 2000b, 2001; Gonzalez-Ochoa et al., 2003). In W/O/W emulsion without osmotic gradient, the EE of the poly R-478 represents the EE of water incorporated initially into the emulsions, as the volume fraction of the internal aqueous phase did not show any significant change.

However, after sorbitol was added to the internal aqueous phase in W/O/W emulsion, osmotic pressure was created from the internal to the external aqueous phase and water transferred from the external to internal aqueous phase in order to reduce the osmotic gradient, at the same time, the internal volume fraction increased. The EE of the poly R-478 represents the EE of increased internal volume fraction.

Larger internal water droplets were more unstable, and more likely to be expelled from oil droplets compared to smaller ones, resulting in significantly reduced EE of W/O/W emulsions (Leal-Calderon et al., 1996; Benichou et al., 2001; Kent and Saunders, 2001; Mezzenga, 2007). When osmotic pressure was created from the internal aqueous phase to the external aqueous phase, the size of water droplets close to the surface of oil droplets grew in size and were expelled promptly from the oil droplets; those water droplets in the middle of oil droplets may undergo swelling and coalescence, which also resulted in larger water droplets. These larger water droplets were easier to be expelled from the oil droplets. As a result, the EE of W/O/W emulsion decreased significantly (Fig.6.9 and Fig 6.10).

W/O/W emulsion with 0.5 M sorbitol in both internal and external aqueous phase would have a balanced osmotic pressure (Fig. 6.9). Although these emulsions had a significantly lower EE compared to emulsions with no added sorbitol, their EE was significantly higher than W/O/W emulsions with 0.5 M sorbitol in the internal aqueous phase only. Combined with the results from PFG-NMR, it can be assumed that changes to interfacial properties caused by diffusion of sorbitol into the oil phase may also play a part in decreasing the stability of W/O/W emulsions.

6.3.2. W/O/W emulsions with NaCl in the internal aqueous phase

6.3.2.1. Visual observations of primary W/O emulsions

It was found in earlier studies that the ionic environment created by NaCl reduced the emulsifying ability of PGPR in W/O/W emulsion at (2% PGPR (w/v)). As a result,

phase separation (resulting from coalescence of water droplets) occurred after the formation of the W/O emulsions (Chapter 4.3.3.1). In this study, W/O/W emulsions containing different PGPR concentrations were examined. NaCl (0.1-0.5 M) was added to the internal aqueous phase of W/O/W emulsions along with 2-8% (w/v) PGPR. Table. 6.2 shows the occurrence of phase separation as a function of different concentrations of PGPR in the oil phase of W/O emulsions.

The results showed that the adverse effect of NaCl was particularly visible in W/O emulsions with lower PGPR concentrations (2-4% w/v) where NaCl concentration as low as 0.1 M caused phase separation. However at higher concentrations of PGPR (6-8% (w/v)), the W/O emulsions were more resistant to phase separation at additions of NaCl up to 0.5 M. At a given concentration of PGPR, the level of phase separation was proportional to the concentration of NaCl added. The results suggest that NaCl may change the emulsifying properties of PGPR, and this can be overcome by increasing the concentration of PGPR in the oil phase. NaCl may change the emulsifying properties of PGPR through the influence of the ionic charge on the functional, charged groups of the surfactant molecule at the water-oil interface as discussed before (Chapter 4.3.3.1).

Table 6.2: Effect of PGPR concentration and NaCl addition in the aqueous phase on phase separation in primary W/O emulsions.

	2% PGPR	4% PGPR	6% PGPR	8% PGPR
Distilled water	-	-	-	-
0.1 M NaCl	+	+	-	-
0.5 M NaCl	++	+	+	-

+ : Phase separation after 24 hours, ++ : phase separation immediately,

- : No phase separation after 7 days

The pH of solutions was 6.6

6.3.2.2. Mean droplet size of W/O emulsions

The W/O emulsion droplets size was measured using DWS technique. The addition of NaCl reduces the mean water droplet diameter significantly in both emulsions prepared with 2 and 8% (w/v)(Fig. 6.11).
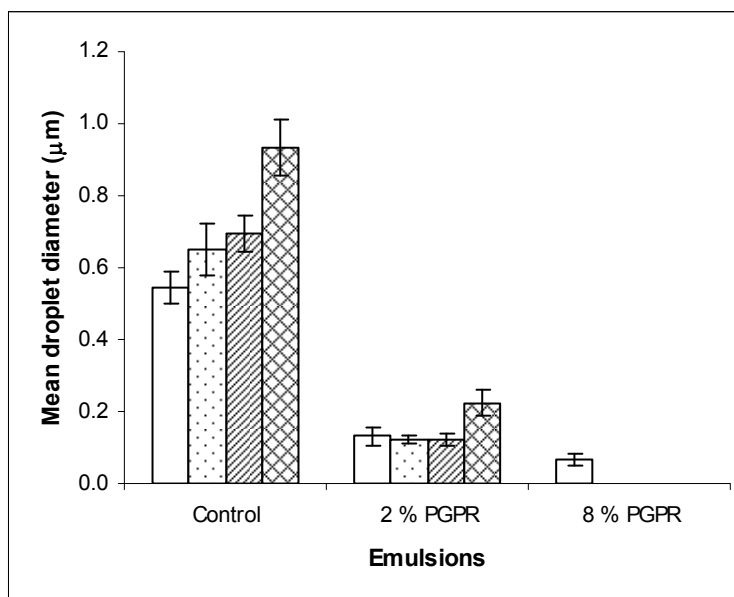
Fig. 6.11. Mean droplet size of W/O emulsions prepared with distilled water (control), or 0.5 M NaCl solution in the aqueous phase and with either 2% (w/v) PGPR or 8% (w/v) PGPR in the oil phase. Samples were freshly made (blank bar), stored at 20 °C for 1 (dotted bar), 7 (stripped bar) and 30 days (netted bar). Measurements were performed at least twice on duplicate emulsions, error bars represent standard deviation. The measurements were obtained using DWS technique.

For W/O emulsions with lower PGPR concentrations (e.g. 2% w/v), due to the occurrence of phase separation within 24 hours, it was assumed that as the emulsifying properties of PGPR was reduced by NaCl, inadequate emulsifier would result in coalescence between water droplets which eventually would lead to phase separation. Significant increases in mean water droplet sizes within 24 hours were expected if excessive coalescence between water droplets occurred. W/O emulsions were measured for mean water droplet size using DWS technique within 24 hours

after the preparation of emulsions (Fig. 6.11). However, the mean droplets size did not show any significant increase within 24 hours after the formation of emulsions and neither over a period of a week, significant increase of water droplet size was detected only after a month of storage. The result suggests that phase separation in W/O emulsions with lower concentrations of PGPR mainly result from the rupture of single water droplets or coalescence of small cluster of water droplets, rather than excessive coalescence between water droplets. Water was released from ruptured droplets and moved to the bottom of the container due to gravity, whereas the remaining stable water droplets remain. The mechanism behind the finding is unclear; NaCl might dehydrate the PGPR molecules, causing the interface to become more rigid. The energy barrier between water droplets would then increase and result in more repulsive force between water droplets, which could halt coalescence between water droplets (Hou and Papadopoulos, 1996).

The effect of NaCl on stability of W/O might be considered in two ways. Though NaCl may change the emulsifying properties of PGPR, and cause phase separation of W/O emulsions. In contrast, as discussed before, NaCl may slow down the Ostwald ripening between water droplets, therefore resulting in smaller mean droplet sizes and hence the W/O emulsion is more resistant to flocculation/coalescence (Hino et al., 2001; Pays et al., 2001).

For W/O emulsions with higher concentration of PGPR (e.g. 8% w/v), water droplets did not rupture or show phase separation as there was an excess amount of PGPR in the oil phase. This excess of PGPR could overcome the structure-altered PGPR by NaCl or binding with NaCl, and the water droplets were effectively protected from rupture and coalescence.

6.3.2.3. Confocal microscopy for W/O/W emulsions

NaCl solution (0.5 M) was incorporated into the internal aqueous phase or external aqueous phase of W/O/W emulsions to create osmotic pressure gradients in different directions. NaCl solution was also added to both aqueous phases to make double

emulsion with balanced osmotic pressure. Double emulsion without addition of NaCl was used as a control sample. The presence of W/O/W emulsion was observed by confocal microscopy (Fig. 6.12a, b & c). Similar to W/O/W emulsions with the addition of sorbitol, a greater number of larger water droplets were clearly visible within the oil droplets when the osmotic gradient was from the internal to external aqueous phase (Fig. 6.12a; NaCl only present in the internal aqueous phase). The water transferred into the internal water droplet from the external aqueous phase immediately after the W/O/W emulsion was prepared. Fewer and smaller water droplets were visible in the oil droplets in other two type emulsions (Fig. 6.12 b & c), due to opposite osmotic gradient direction and balanced osmotic pressure, respectively.

(a)   (b)   (c)
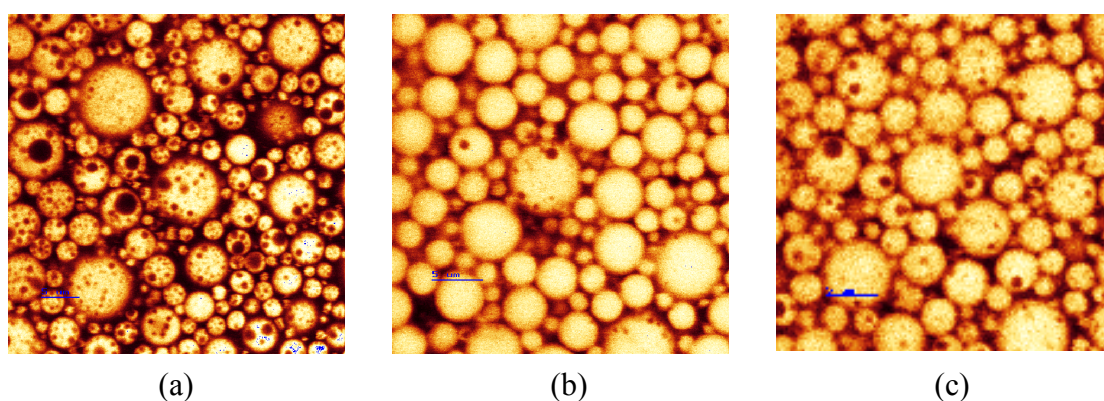
Fig. 6.12. Confocal microscopy images representing W/O/W emulsions. (a) emulsion with osmotic gradient from the internal to external aqueous phase (0.5 M NaCl in internal aqueous phase only), (b) emulsion with osmotic gradient from external to internal aqueous phase (0.5 M NaCl in external aqueous phase only), (c) emulsion with 0.5 M NaCl in both internal and aqueous phase. Scale bar represents 5 μm.

As discussed earlier for W/O/W emulsions containing sorbitol, it was believed that mass transfer in W/O/W emulsions, that have an osmotic gradient, includes the migration of water and/or NaCl through the oil phase and this contributes to the physical properties of the W/O/W emulsions under the current experimental conditions.

The distributions of oil droplet size of W/O/W emulsion were determined by the MasterSizer (Fig. 6.13) and the results were in agreement with the confocal images. The W/O/W emulsions with addition of 0.5 M NaCl in the internal aqueous phase had significantly larger average size of oil droplets compared to all other emulsions tested. Emulsions with addition of 0.5 M NaCl in the external aqueous phase and emulsions with 0.5 M NaCl in both internal and external aqueous phases did not show significant changes in oil droplet size compared with the blank W/O/W emulsions (control).
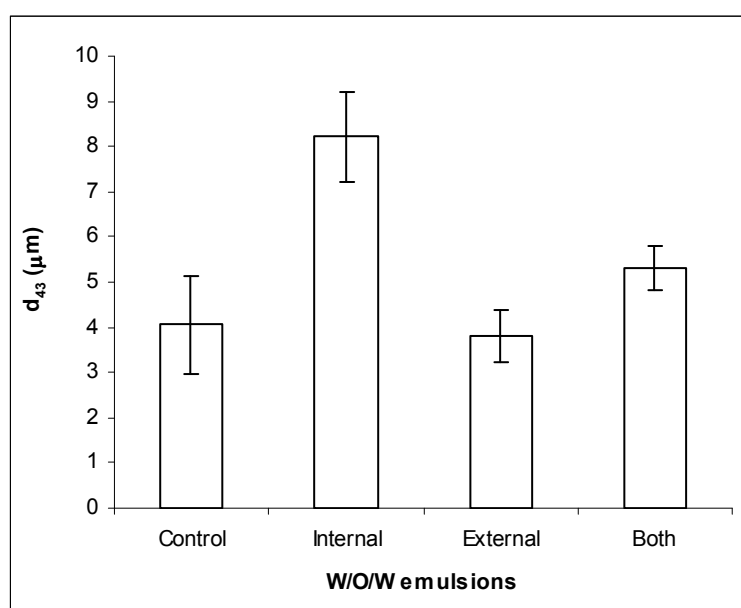
Fig. 6.13. Average oil droplet size of W/O/W emulsions prepared without addition of NaCl (Control), with 0.5 M NaCl solution in the internal aqueous phase only (internal), with 0.5 M NaCl solution in the external aqueous phase only (external) and 0.5 M NaCl in both aqueous phase (both). All emulsions had 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase. Measurements were carried out using the MasterSizer.

It was unexpected that W/O/W emulsions with addition of NaCl in the external phase would have a similar oil droplet size compared to the control emulsions, as it was assumed that water would diffuse from the internal droplets to the external aqueous phase, driven by the osmotic gradients. Average size of the oil droplets in the W/O/W emulsion was expected to decrease due to the shrinkage of the internal water droplets.

Two phenomena might explain this observation. The external phase inclusion (a small fraction of external aqueous phase was incorporated into the internal aqueous phase) is a possible phenomenon during the two-stage homogenising process. The external phase inclusion would increase the concentration of NaCl in the internal water droplets of the W/O/W emulsion, which could reduce the water flux from the external aqueous phase (Shima et al., 2004). At the same time, like sorbitol, NaCl might diffuse through the oil phase to the internal aqueous phase and balance the osmotic gradient between the internal and external aqueous phases and also reduce the water flux from the internal aqueous phase. It is not clear, in this study, which of these two effects were dominant.

6.3.2.4. PFG-NMR analysis of water diffusion in W/O/W emulsions

6.3.2.4.1. Analysis method

Attempts have been made to investigate the distribution of internal water droplet sizes for W/O/W emulsion with NaCl in the internal aqueous phase using the PFG-NMR technique. However, addition of NaCl significantly changes the relaxation properties of the internal aqueous phase, and as a consequence, the Pfeuffer exchange model used for W/O/W emulsions with sorbitol as a pressure gradient generator is invalid and could not be used to extract properties of the W/O/W emulsions with NaCl as a pressure gradient generator. Alternatively, the properties of emulsion were then represented by the diffusivity coefficient distributions.

Experimental data were analysed by non-negative least squares method (Prospa version 2.1, Magritek, Wellington New Zealand). The fitting procedure for the transformation is such that the measured attenuation data is mapped as a summation of single exponential decays. The method takes x-y data and produces a spectrum of exponentials providing a best fit to the original data in a least squares sense. The distribution and intensity of water diffusivity can be determined and compared.

A representative distribution of water diffusivity obtained from W/O/W emulsions with 0.5 M NaCl in the internal aqueous phase is shown in Fig. 6.14. The large peak at 1.3×10^{-9} m^2s^{-1} (peak 1) represents the diffusivity of the free water in the external aqueous phase, which is unrestricted by emulsion walls. This diffusion peak is a stable contribution in the distribution of diffusivity from the beginning of all samples, indicating that this corresponds to a critical volume fraction relationship between the continuous and disperse phases (Egger and McGrath, 2006). As the distribution of diffusivity of the free water is stable and similar for all the emulsions investigated, and as it dominates the distribution graph, in the following experimental data analysis it was eliminated from the graph to give a clear picture of peak 2 and 3, which is of interest.
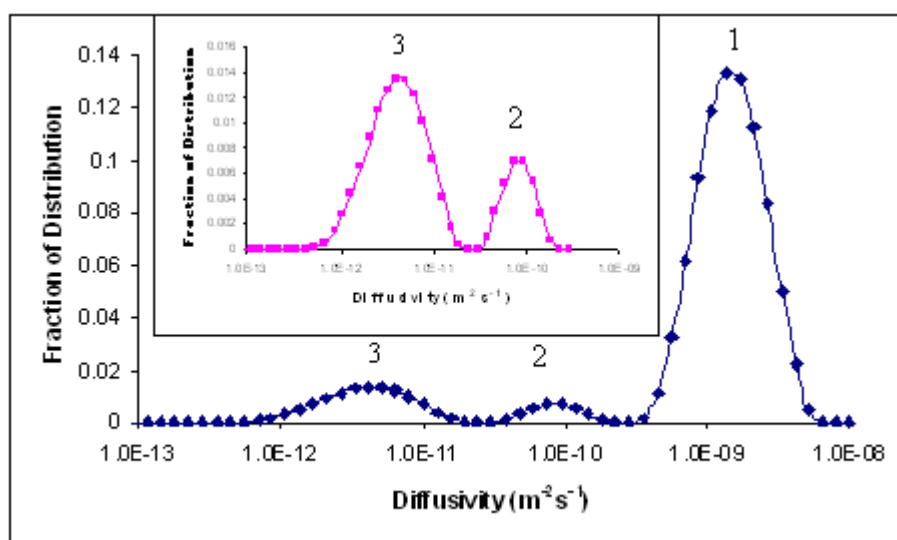
Fig. 6.14. Distribution of water diffusion coefficient obtained from W/O/W emulsions. The insert shows the distribution of two slower water diffusion coefficients (peak 2 and 3) in the same sample. W/O/W emulsions were prepared with 0.5 M NaCl in the internal aqueous phase, 8% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase.

The peak at 9.3×10^{-11} m^2s^{-1} (peak 2) is noted as the water exchanging between the internal and the external phase. It occurred in the oil phase and was the transition stage for water diffusion between the internal aqueous phase and external phase. The

diffusion coefficient of this portion of water is slower than free water in the external aqueous phase, due to the higher viscosity of oil compared to water, and it was faster than water in the internal droplets as there is no restricted boundary. The peak at 4.1×10^{-12} m^{2}s^{-1}(peak 3) represents the restricted diffusion of water in the internal aqueous phase of W/O/W emulsion, as the total net displacement of the water molecules is restricted by the walls of the internal water droplets.

6.3.2.4.2. The trend of water diffusion

Fig. 6.15 shows distributions of diffusivity coefficient of water molecules for W/O/W sample over a time period of 12 hours. During the period the samples were observed to undergo the changes in terms of the distribution of diffusivity coefficients of water molecules. It was noted that over the time of observation, the faster diffusion coefficient (peaks at 6.6 $\times 10^{-11}$ m^{2}s^{-1}), which is seen to be the diffusion of water between the internal and external phase, were observed with decreasing intensity and also shifted to slower diffusion rates, indicating decrease of water exchange between the internal and external phase over time. After about 10 hours, the form and position of the diffusion coefficient were not altered indicating that equilibrium has been reached.
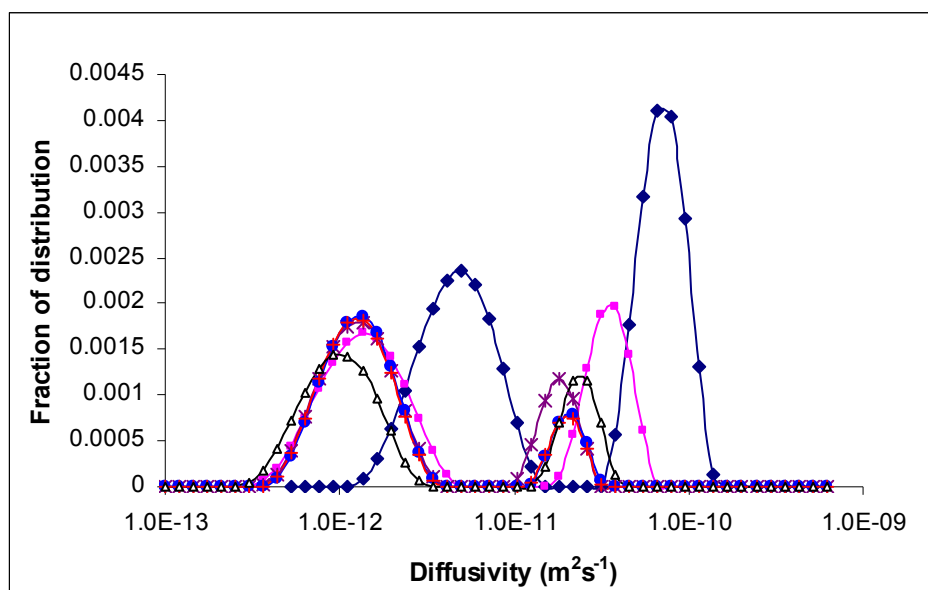
Fig. 6.15. Distribution of water diffusion coefficient obtained from W/O/W emulsion over 12 hours. W/O/W emulsions were prepared with 0.5 M NaCl in the internal aqueous phase, 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase. (◆) 1 hour, (■) 2 hours, (△) 3 hours, (ж) 7 hours, (●) 10 hour and (+) 12 hours.

Diffusion of water in the internal water droplet showed a slightly different pattern. After the appearance of a fast diffusion peak at 1 hour (4.9×10^{-12} m^2s^{-1}), the diffusion coefficient shifted to the slower position (1.2×10^{-12} m^2s^{-1}) and the intensity of the diffusion coefficient decreased, after that, the intensity of the diffusion coefficient remained unchanged over the time of observation. The results indicated that the transfer of water from the external aqueous phase to the internal aqueous phase due to osmotic gradient was a rapid process, initial fast swelling of internal water droplet results in large internal water droplets, which resulted in a faster diffusivity peak and higher intensity. However, water droplets that became too large may have ruptured or merged to the external phase, and the volume fraction of water in the internal phase subsequently decreased. At some critical volume fraction the growth of the internal water droplets is matched by the rate of expelling of water, when the two transformations reach equilibrium, a constant diffusion coefficient resulted. It is difficult to be more quantitative on this point due to technique limitation. However,

we can qualitatively determine the expected trend of the restricted diffusion of the water as a function of time, as NMR results agree well with the values obtained from the light scattering (MasterSizer) and confocal image experiments.

6.3.2.4.3. Effect of addition of NaCl in the internal aqueous phase on water diffusivity

The comparison of distribution of water diffusivity of W/O/W emulsions with or without 0.5 M NaCl in the internal aqueous phase is shown in Fig 6.16 a. It appeared that W/O/W emulsion with 0.5 M NaCl in the internal phase had much higher internal volume fraction, and diffusion coefficient of water in oil phase and internal water droplet were distinctly separated, compared to the W/O/W emulsion without NaCl, which has much less volume fraction of internal water. The result indicated that firstly, water flux from the external aqueous phase to the internal aqueous phase increased due to osmotic gradient created by NaCl in the internal aqueous phase (peak 2). Secondly, the water droplet increased in size, the volume fraction of water in the internal aqueous phase increased (peak 3).

The variation in position and intensity of water diffusion coefficient obtained from W/O/W emulsions with or without addition of NaCl in the internal aqueous phase fits well with the data obtained from confocal imaging (Fig. 6.12) and MasterSizer (Fig. 6.16 b). The confocal images showed an increased number and size of internal water droplets when 0.5 M NaCl was added to the internal aqueous phase of W/O/W emulsions. The MasterSizer data showed that the average size of oil droplets in W/O/W emulsion with NaCl in the internal aqueous phase increased (d_{43} 6.3 ± 1.1 μm) compared to oil droplets of W/O/W emulsions without NaCl (d_{43} 4.0 ± 1.1 μm), as the internal water droplets grew in size, the size of the oil droplet also increased.
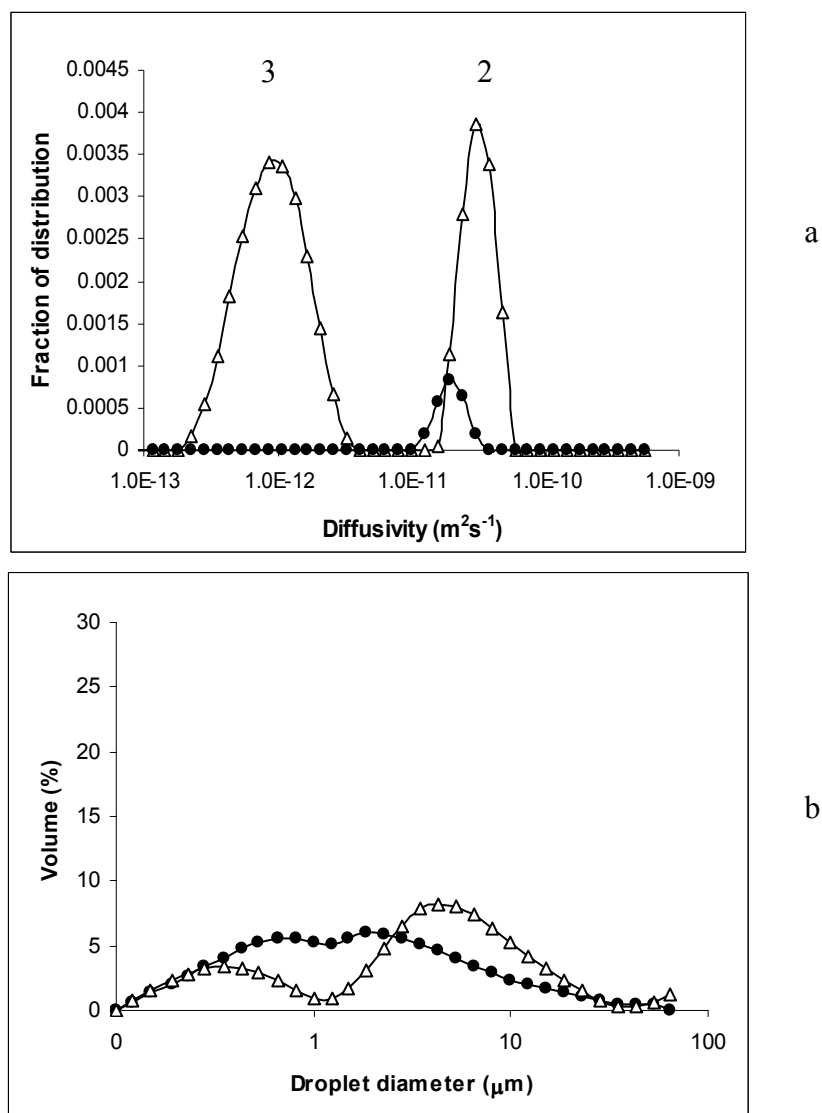
Fig. 6.16. Effect of addition of NaCl in the internal aqueous phase on (a) distribution of water diffusivity and (b) distribution of oil droplet size of W/O/W emulsion with distilled water (●) or 0.5 M NaCl (△) in the internal aqueous phase. All emulsions had 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase. Particle size was measured by the MasterSizer.

6.3.2.4.4. Effect of PGPR concentration on water diffusivity

The effect of PGPR concentration on the distribution of water diffusivity of W/O/W emulsions was investigated (Fig. 6.17 a).   It appears that W/O/W emulsions with 8% PGPR had much higher diffusion intensity at both peaks, and both peaks of diffusion coefficients of water droplets were faster than that of W/O/W emulsion with 2% PGPR.   The higher diffusion intensity indicated that W/O/W emulsions with 8% PGPR had more stable and larger internal water droplets after water transferred to the internal water droplets from the external aqueous phase, and therefore a higher internal water volume fraction. The larger amount of water on the other hand resulted in a fast diffusion coefficient.   The variation in position and intensity of water diffusion coefficients obtained from W/O/W emulsions with 2 or 8% (w/v) PGPR fits well with the results from the particle sizing data obtained from the MasterSizer (Fig. 6.17b), which showed the size distribution of sequential oil droplets in W/O/W emulsion.   The enlargement of internal water droplets increased the size of oil droplets from a d_{43} of 6.3 ± 1.1 µm to a d_{43} of 15.2 ± 2.1 µm for W/O/W emulsions with increase in PGPR concentration from 2 to 8% (w/v). It is possible that sufficient PGPR in the oil phase is essential to stabilize enlarged internal water droplets from droplet rupture or from merging with the external aqueous phase.

The change of internal water droplets of W/O/W with the addition of NaCl to the internal aqueous phase was studied, and it was found that the transfer of water from the external aqueous phase to the internal aqueous phase is a speedy process reaching equilibrium within 24 hours.   It was also aimed to study the EE of internal water volume fraction over time, using poly R-478 dye, as what was done for W/O/W emulsions with addition of sorbitol.   However, we were not able to monitor the EE Poly R-478 under the conditions used in this experiment as the dye did not dissolve in solutions containing salt.
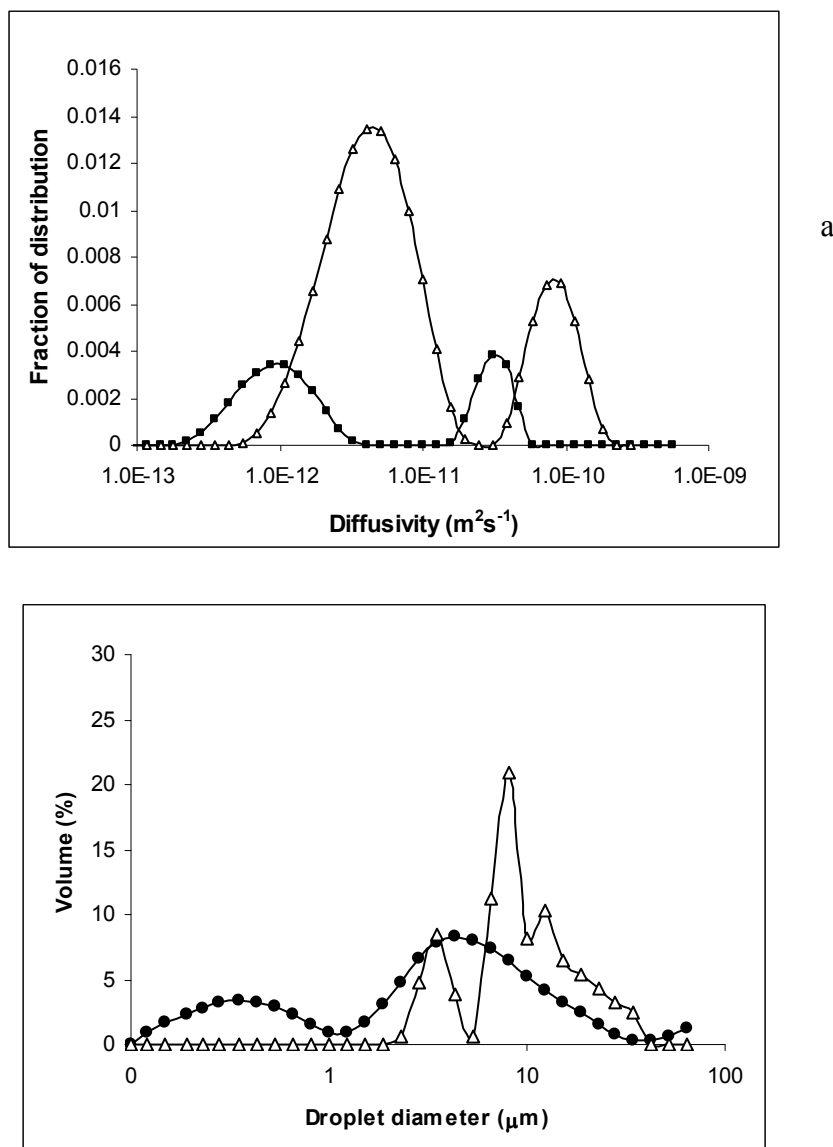
Fig. 6.17. Effect of the concentration of PGPR in the oil phase on (a) distribution of water diffusivity and (b) distribution of oil droplet size of W/O/W emulsions with 0.5 M NaCl in the internal aqueous phase and 2% (w/v) PGPR (●) and 8% (w/v) PGPR (△) in the oil phase. All emulsions had 0.5% (w/v) NaCN in the external aqueous phase.

As an alternative, we monitored the size change of oil droplets over a period of 1 month, as an indirect signs of loss of internal water droplets and the results are presented in Fig. 6.18. W/O/W emulsions prepared with 2% (w/v) PGPR and with 0.5 M NaCl in the internal aqueous phase exhibited decreased average size of oil droplets, from 6.3 ± 1.1 to 5.4 ± 0.7 μm, over one month storage (Fig. 6.18 a). W/O/W emulsions prepared with 8% (w/v) PGPR showed a similar pattern, the average droplets size were reduced from 15.2 ± 2.1 to 10.1 ± 1.4 μm (Fig. 6.18b). It appears that the decrease of the internal aqueous phase volume fraction (loss of internal water droplets) is a much slower process compared to the increase of internal aqueous phase volume fraction.

The increase in internal volume fraction is a speedy process, mainly caused by water diffusion from the external aqueous phase to the internal aqueous phase driven by osmotic gradient (Wen and Papadopoulos, 2000a; Gonzalez-Ochoa et al., 2003). Decrease of the internal volume fraction, as discussed earlier (Chapter 6.3.1), is mainly caused by destabilisation of water droplets, including water droplets rupture, coalescence between water droplets and the external aqueous phase. It could take months or longer to complete (Hino et al., 2001; Pays et al., 2001). By understanding the mechanism behind the changes of physical properties of W/O/W emulsions, it is possible to control the increase and decrease of the internal aqueous volume fraction at a desirable rate, which have the potential for food application (Hino et al., 2001).
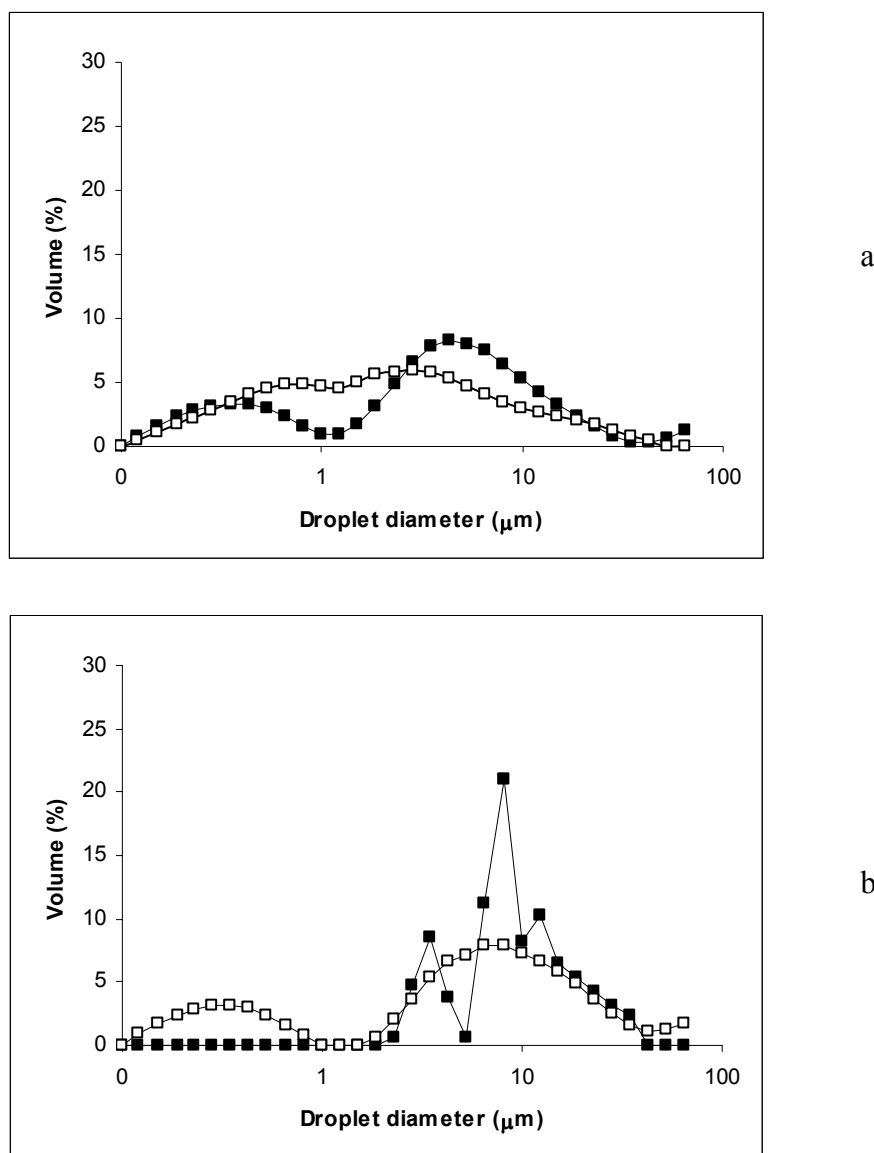
Fig. 6.18. Distribution of oil droplet sizes of W/O/W emulsions prepared with (a) 2% (w/v) PGPR and (b) 8% (w/v) PGPR after they were freshly made (■) or stored at 20 °C for 1 month (□). Emulsions were prepared with 0.5 M NaCl in the internal aqueous phase and had 0.5% NaCN in the external aqueous phase.

6.3.2.5. The release of NaCl from the internal aqueous phase of W/O/W emulsions

6.3.2.5.1. Effect of hydrophobic emulsifier on the release of NaCl from W/O/W
            emulsions

In addition to the study of water diffusion in W/O/W emulsions with an osmotic gradient, we also studied the transportation of NaCl from the internal aqueous phase to the external aqueous phase. It was expressed as the release of NaCl from the internal to external aqueous phase, and was studied by measuring the Cl^- ion released into the external aqueous phase. The concentration of Cl^- in the external aqueous phase of W/O/W emulsion was determined immediately after the formation of emulsions and over 24 hours. Fig. 6.19 shows the effect of PGPR concentration on the release of Cl^- from the internal aqueous phase of W/O/W emulsions.

Decreased release of NaCl was observed as PGPR concentration increased from 2 to 15% (w/v) in W/O/W emulsions (Fig. 6.19a and b). It was notable that lower PGPR concentrations resulted in a higher initial loss of NaCl, indicating that NaCl was released immediately after the formation of the W/O/W emulsion. This was expected as the visual observation of W/O emulsions showed that W/O emulsions with 2% (w/v) PGPR had more water separated from the emulsion than W/O emulsions with higher PGPR concentrations (Table 6.2). Internal water droplets in this emulsion appear to have an insufficient amount of PGPR and would be more susceptible to damage by mechanical forces during the second emulsification step.
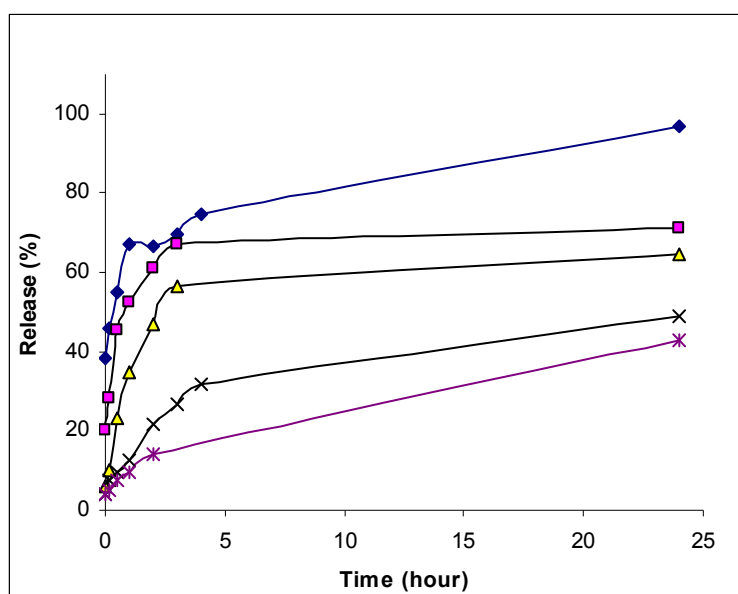
Fig. 6.19. Effect of time on the release of NaCl from the internal to external aqueous phase in W/O/W emulsions. W/O/W emulsions contained 0.5 M NaCl in the internal aqueous phase and 2 (◆), 4 (■), 6 (△), 8 (✕) and 15% (✱) (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase.

For W/O/W emulsions with lower concentrations of PGPR (2-6% w/v), the initial loss of NaCl was up to 40%, but less initial loss of NaCl was observed at higher PGPR concentration (8 and 15% w/v). The release of NaCl in W/O/W emulsions with lower concentration of PGPR was very rapid and reached between 50-80% within the first 5 hours, and between 60-100% within 24 hours. W/O/W emulsions with higher concentration of PGPR (8 and15% w/v) released NaCl in a much slower and constant manner.

It is well accepted that ion release from W/O/W emulsions is caused by three possible pathways as discussed in Chapter 2: Literature review: ion diffusion through the oil phase from the internal aqueous phase to the external phase (by reverse micelles mechanism), expelling of internal water droplets to external aqueous phase caused by coalescence and droplet rupture (Raynal et al., 1993; Ficheux et al., 1998). All pathways could be dominant, depending on the experimental conditions, such as the concentration and type of emulsifier, ionic strength and encapsulants added. In this

study, it was difficult to determine which pathway dominated the release of the Cl⁻ ion. The release of NaCl from W/O/W emulsions was a relatively fast process, especially for W/O/W emulsion with lower PGPR concentrations, and the complete release of Cl⁻ could occur within a few hours. If the release of NaCl was entirely due to the rupture or expelling of internal water droplets, most of the internal water droplets would disappear from the oil droplets after the release of Cl⁻ reaches 100%. The results obtained from NMR technique and confocal image showed that this was not the case, indicating that NaCl was also released from W/O/W emulsion through diffusion. For W/O/W emulsion with lower PGPR concentration, the rupture/expelling mechanism might dominate as the internal water droplets are not stable due to insufficient PGPR coverage; the visual observation of W/O emulsion support this view (Table 6.2). For W/O/W emulsions with higher PGPR concentration, the contribution of rupture/expelling mechanism to the release of Cl⁻ might be reduced, as the increased PGPR concentration increase the viscosity in the oil phase, which might form a stronger membrane, and slow down water permeation coefficients, and the internal water droplets are more resistant to rupture or coalescence. In addition, high concentrations of hydrophobic emulsifier was shown to have a negative influence on the formation or the rupture of reverse micelles, which are the carrier of ion at the interfaces and hence slow down ion migration (Wen and Papadopoulos, 2001; Cheng et al., 2007).

6.3.2.5.2. Effect of hydrophilic emulsifiers on release of NaCl from W/O/W emulsions

The effects of hydrophilic emulsifiers on the release of NaCl from W/O/W emulsions were investigated as they play an essential role in the stability of oil droplets (Wen and Papadopoulos, 2000a). The release of NaCl as function of concentration or type of hydrophilic emulsifier was examined and the results are shown in Fig. 6.20 and Fig. 6.21, respectively.

Increased concentrations of NaCN (0.5 to 5% w/v) in the external aqueous phase slightly increased the release of NaCl (Fig. 6.20). It was observed that creaming of

W/O/W emulsions with 5% (w/v) NaCN occurred immediately after the formation of the emulsion, faster compared to emulsions with lower concentrations of NaCN. However, there were no significant changes in terms of oil droplet size measured by the MasterSizer at the range of concentration tested.
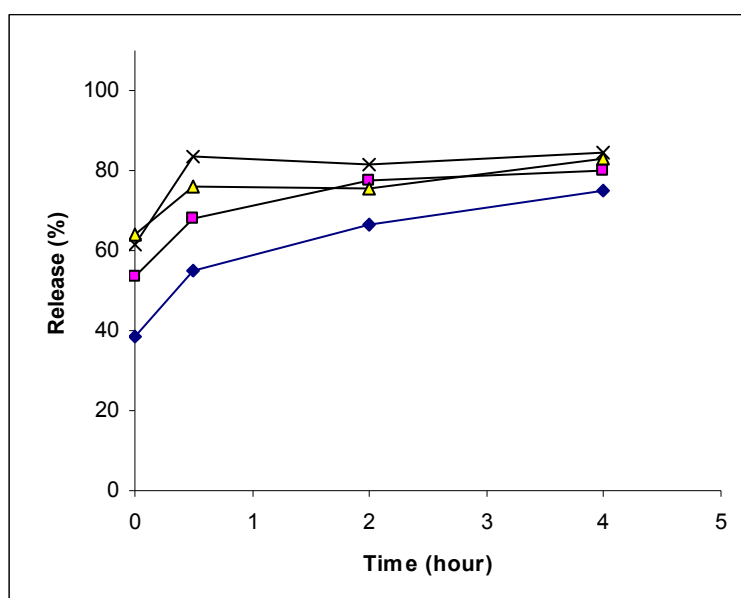
Fig. 6.20. Effect of storage time on the release of NaCl from the internal to external aqueous phase in W/O/W emulsions. W/O/W emulsions contained 0.5 M NaCl in the internal aqueous phase and 2% (w/v) PGPR in the oil phase, and 0.5% (◆), 1% (■), 2% (△) and 5% (×) (w/v) NaCN in the external aqueous phase.

Unlike hydrophobic emulsifiers, which may act as carriers in transportation of water or ions through the oil phase, hydrophilic emulsifiers are documented to have little or even a negative effect on coalescence between internal water droplets and the external aqueous phase in W/O/W emulsions. Wen et al. (2000a,b) used Span 80 and Tween 80 as hydrophobic and hydrophilic emulsifiers, respectively in W/O/W emulsion, and it was found that the presence of Tween 80 in the external aqueous phase accelerated coalescence between the internal water droplets, therefore increasing the transportation of water and ions. Cheng et al. (2007) also found that excessive concentration of hydrophilic emulsifier (SDS) could destabilise the emulsion by

travelling to the interface of internal water droplets, i.e. reduce EE of W/O/W emulsions. In this study, as NaCN is a macromolecule, it is unlikely to move from the external aqueous phase to the internal aqueous phase to destabilise the W/O emulsion. Therefore the reason for the increase of release of NaCl in W/O/W with high concentration of NaCN is likely due to an excess amount of NaCN which destabilised the O/W emulsions (Dickinson et al., 1991a).

NaCN (0.5% w/v) was replaced with 10% (w/v) SUPER GUM™ as hydrophilic emulsifier in W/O/W emulsion (Fig. 6.21). In an earlier study, it was found that W/O/W emulsion with SUPER GUM™ as hydrophilic emulsifier always had higher EE of poly R-478 dye, which represents the EE of internal water droplets in W/O/W emulsions without the addition of sorbitol or NaCl in the internal aqueous phase.
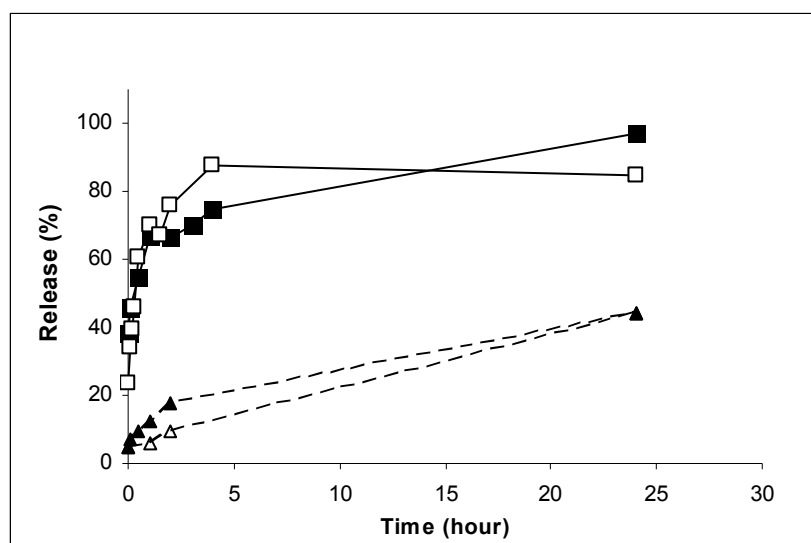
Fig. 6.21. Effect of storage time on the release of NaCl from the internal to external aqueous phase in W/O/W emulsions. W/O/W emulsions contained 0.5 M NaCl in the internal aqueous phase and 2% (—) and 8% PGPR (---) in the oil phase, and 0.5% (w/v) NaCN (solid symbol) and 10% (w/v) SUPER GUM™ (open symbol) in the external aqueous phase.

However, results in Fig. 6.21 showed that SUPER GUM™ did not significantly change the release of Cl⁻ in both W/O/W emulsions prepared with 2 or 8% (w/v) PGPR. The explanation of this scenario might be that with the presence of osmotic

gradient from the internal to external aqueous phase, the size of internal water droplets significantly increased, the balance of repulsion and attraction forces between the internal water droplets and between the internal water droplets and the external aqueous phase was altered. Hence, the effect of SUPER GUM™ on delaying the coalescence between internal water droplets and external aqueous phase was overcome.

6.4.   Summary

In this chapter, the effects of addition of a sugar (sobitol) and a salt (NaCl) in the internal aqueous phase on the stability of W/O/W emulsion were investigated. The transportation of water induced by the osmotic gradient was studied, along with the study of transportation of sorbitol and NaCl in W/O/W emulsion. It appeared that water diffused from the external to the internal aqueous phase in a rapid manner due to osmotic gradient created by sorbitol or NaCl and the diffusion of water quickly reached equilibrium within 24 hours due to diffusion of sorbitol or NaCl in oil phase. The loss of water droplets, on the other hand, was a much slower process mainly through the rupture or expelling of water droplets.

The release of NaCl from the internal aqueous phase to the external aqueous phase was found to be significantly faster compared to the release of poly R-478, which represented the loss of water droplets. The complete release of NaCl took a few hours whereas the release of water droplets could take months.   The observation indicated that the release of NaCl is not only through rupture or expelling of water droplets from oil droplet, but also by diffusion through the oil phase. The release of NaCl had a significant inverse relationship with the concentration of hydrophobic emulsifier PGPR, but was independent of the two hydrophilic emulsifiers examined.

Chapter 7: Encapsulation of sodium ascorbate in W/O/W Emulsion

7.1. Introduction

Ascorbic acid (AA) is of great importance in biochemical reactions as a reducing agent. It is also a powerful antioxidant in foods, preventing colour changes and alterations of aroma and flavour and extending the shelf life of food products. The strong reducing power of ascorbic acid is due to the two-step oxidation that gives hydrated dehydroascorbic acid (DHAA) by way of the intermediate ascorbate free radical monodehydro ascorbic acid (MDHA) (Davies, Austin and Partridge, 1991; Szymula, Szczypa and Friberg, 2002). Further degradation may produce various five carbon compounds (Kimoto, Tanaka, Ohmoto and Choami, 1993). High reducing power of AA, on the other hand, makes it susceptible to degradation in homogenous or heterogeneous systems, as AA is freely soluble in water. The stabilization of this antioxidant in water-based formulations is a prime requirement to fulfil its role in the final applications (Szymula et al., 2002). Efforts have been made to apply W/O/W and O/W/O emulsion technologies to stabilize AA in pharmaceutical and cosmetic products (Kim, Kang, Suh and Oh, 2003; Lee, Ahn, Lee and Kwak, 2004a; Farahmand et al., 2006).

The aim of this study is to evaluate the stabilization of AA in W/O/W emulsions and the effect of encapsulating AA on the properties of W/O/W emulsions. To maintain the internal aqueous phase at pH 6.6-7, sodium ascorbate (SA), a salt form of ascorbic acid, was incorporated into the W/O/W emulsion. SA has identical function groups compared to AA, except that as a sodium salt of ascorbic acid, every 1000 mg SA contains 889 mg AA (Davies et al., 1991). The pH of a SA solution (2% w/v) is 6.7 compared to 2.4 of the same concentration of AA.

7.2.   Materials and Methods

Sodium ascorbate (SA) was obtained from Sigma Ltd., St Louis, Missouri, USA. Other materials were identical to those used previously; please refer to Chapter 3, Section 3.2, Materials and Methods.

To determine the amount of SA released into the external aqueous phase of W/O/W emulsion, a high performance liquid chromatography (HPLC) method was applied. W/O/W emulsions were centrifuged at 1×10^4 g for 2 hours for samples prepared with high pressure homogenizer and 5 minutes for samples prepared with a rotor-stator homogenizer, respectively. An aliquot (1 mL) of the subnatant from the centrifuged sample was mixed with 1 mL of meta-phosphoric acid (MPA, 1.5% w/v), which is a extractant and a stabiliser, and the mixture was passed through a 0.45 μm filter prior to analysis (Graham and Annette, 1992).

HPLC was conducted using a HP1100 series equipped with a UV detector and an autosampler. The chromatographic separation was achieved using an Aminex HPX-87H column (300×7.8mm, Bio-Rad, CA, USA), the column temperature was maintained at 25 °C. The mobile phase consisted of 0.005 M sulfuric acid solution (Graham and Annette, 1992). The flow rate was set at 1.0 mL/min. The solution was diluted to different concentrations and absorbance determined at 245 nm. A standard curve was subsequently prepared ($r^2 = 0.9998$) and the concentration of sodium ascorbate present in the subnatant was determined from this standard curve.

7.3.   Results and Discussion

7.3.1. SA in the aqueous phase of W/O/W emulsions with 0.5% (w/v) NaCN as hydrophilic emulsifier

7.3.1.1. W/O emulsions with SA in the aqueous phase

SA (2% w/v) was incorperated in the aqueous phase of W/O emulsions. The mean droplet size of water droplets was determined using the DWS technique and the results are shown in Fig. 7.1. The addition of 2% SA to the internal aqueous phase significantly reduced mean droplet size of water droplets, similar to that of sorbitol and NaCl, as discussed previously in Chapter 6 (Fig. 6.2 and Fig. 6.11). No phase separation was detected in W/O emulsions with 2% SA (0.1 M) upon storage for up to 7 days. The results indicated that SA might have a different effect on the emulsifying ability of PGPR compared to NaCl, as the latter caused phase separation soon after the W/O emulsions were formed (Table 6.2).
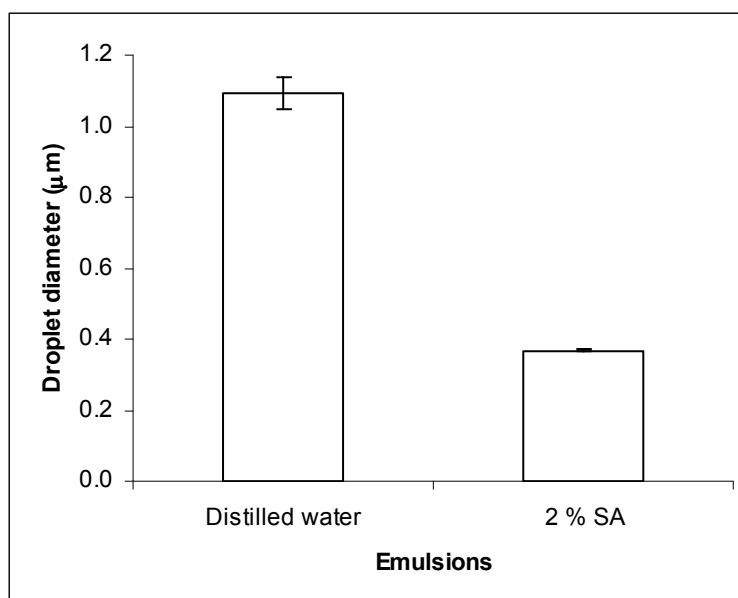
Fig. 7.1. Mean droplet size of W/O emulsions prepared with distilled water or 2% SA solution in the internal aqueous phase. Both W/O emulsions were prepared with 2% (w/v) PGPR. Measurements were performed at least twice on duplicate emulsions; error bars represent standard deviation. The measurements were conducted using DWS.

7.3.1.2. Properties of W/O/W emulsion with SA in the internal aqueous phase

The confocal image (Fig. 7.2 b) of W/O/W emulsion with SA in the internal phase showed less enlarged water droplets compared to emulsions with addition of 0.5 M sorbitol (Fig. 6.3 b) or 0.5 M NaCl (Fig. 6.12 a). The result was expected, because 2% (w/v) SA (0.1M) would generate less osmotic gradient compared to 0.5 M sorbitol or 0.5 M NaCl. As a result, less water would be transferred from the external to the internal aqueous phase.

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a   b

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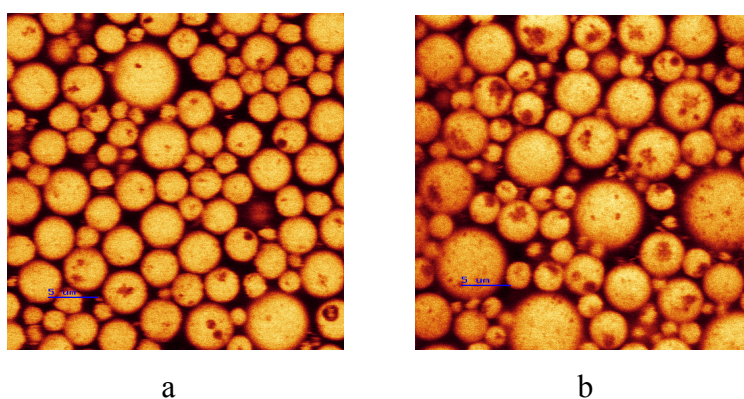
Fig. 7.2. Confocal microscopy images representing W/O/W emulsions with (a) distilled water and (b) 2% SA in the internal aqueous phase. Emulsions contained 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase. Scale bar represents 5 µm.

The droplet size distribution of oil droplets in the W/O/W emulsions changed after the addition of 2% SA (Fig. 7.3 a). The volume of droplets in the larger size region (>1.23 µm) increased and the volume of droplets in the smaller size region (<1.23 µm) decreased, increasing d_{43} of emulsion droplets from 3.4 ± 0.2 µm to 4.7 ± 1.3 µm. The change of droplet size distribution was more pronounced when 8% (w/v) PGPR was used. The volume of smaller sized droplets decreased, in addition, the peak of smaller sized droplet shifted to smaller size region. At the same time, the volume of larger sized droplets increased, and the peak of larger droplets shifted to larger size region. Overall, the d_{43} of the emulsion significantly increased to 8.7 ± 0.6 µm.
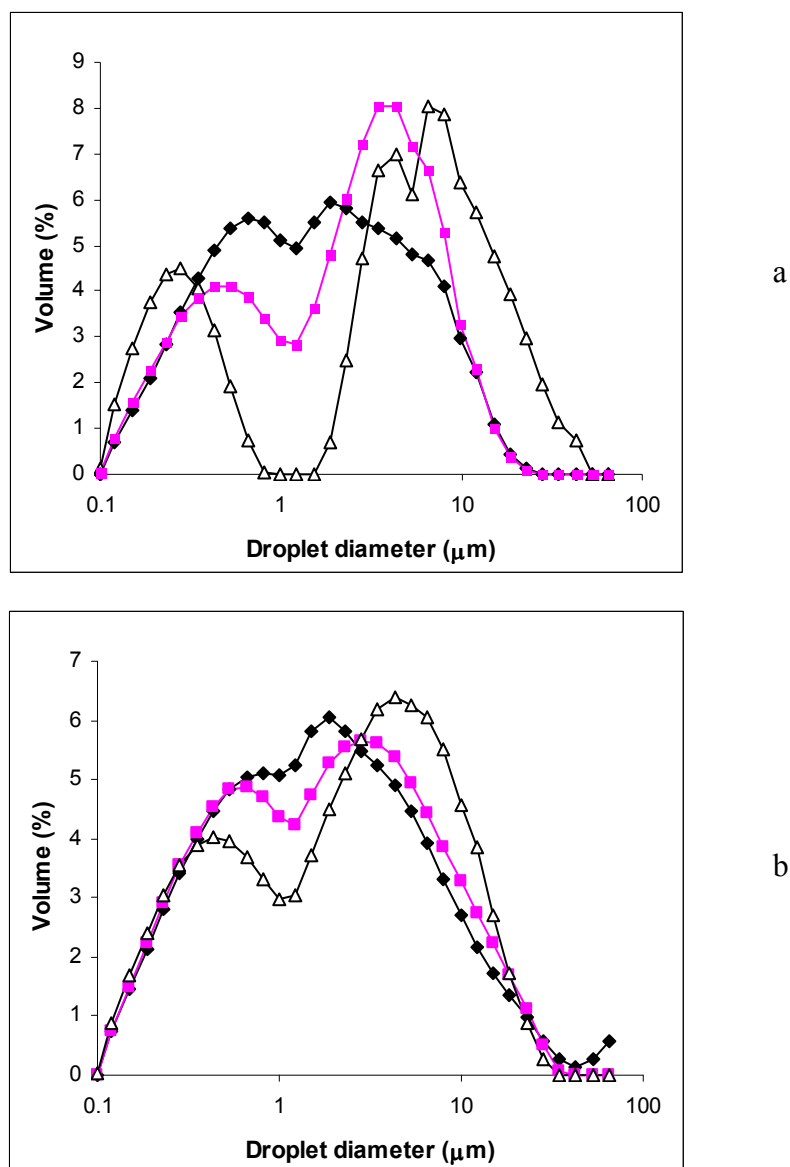
Fig. 7.3. Droplet size distributions of W/O/W emulsions (a) freshly made or (b) after one month storage at 20°C. Emulsions were prepared with distilled water in the internal aqueous phase and 2% PGPR in the oil phase (♦), 2% (w/v) SA in the internal aqueous phase and 2% (w/v) PGPR in the oil phase (■), and 2% (w/v) SA in the internal aqueous phase and 8% (w/v) PGPR in the oil phase (△).

This change of pattern in W/OW emulsions with 8% PGPR indicated that the W/O/W emulsions contained oil droplets with and without water droplets entrapped. Some small oil droplets only contained one or a few water droplets, and had a similar size as

single oil droplets without water droplet entrapped, and the size distribution of these two different types of oil droplets became overlapped. With the addition of SA, the volume fraction and mean size of oil droplets containing water droplets increased considerably, whereas the mean size of single oil droplets remained unchanged and the volume fraction of these droplets decreased (Fig. 7.3 a). Two groups of oil droplets could be distinguished in terms of size distribution.

Over time, the osmotic gradient reached equilibrium and the larger oil droplets decreased in size due to the expelling of water droplets to the external aqueous phase. The size distribution of two groups of oil droplets became overlapped again, like blank W/O/W emulsions (Fig. 7.3 b). The pattern of distribution of oil droplets for W/O/W emulsions with 2% (w/v) SA is similar to W/O/W emulsions with 0.5 M NaCl (Fig. 6.17b). The increase of size of oil droplets after the addition of SA is less compared to NaCl, which is in agreement with the observations from the confocal images (Fig 7.2).

7.3.1.3. Release of encapsulated SA from W/O/W emulsions

The release pattern of SA encapsulated in W/O/W emulsions prepared with a high-pressure (two-stage valve) homogeniser was determined using HPLC analysis (Fig 7.4a). The release was a fast process, nearly 80% SA was released within first 24 hours, after that there was a lag phase lasting about 3 days. Increasing the concentration of PGPR from 2 to 8% (w/v) slightly reduced the release of SA. It is believed that diffusion of SA through the oil phase was the dominant mechanism behind the early stage release of SA (day 0-1). As 80% of SA released during the first 24 hours, over this time frame, enlarged water droplets and oil droplets were still well detected from confocal images and distribution of oil droplet size. The type of diffusion of SA was not further investigated as it was beyond the scope of this study.

It was noted that SA underwent significant loss after release to the emulsion external aqueous phase of emulsions at 20°C over a period of one month. This was due to SA degradation, the rate of degradation was faster compared to the degradation rate of SA

in pure solution. The observation was expected, as the degradation of SA during prolonged sample storage is well recognized, and it is well known that the antioxidant activity of SA in homogenous solutions may not be the same as that in heterogeneous media. In the external aqueous phase of W/O/W emulsion, SA is likely to be oxidised by singlet oxygen, oxide group from the oil droplet surface and emulsifiers which accelerate the degradation process (Szymula et al., 2002; Szymula and Narkiewicz-Michalek, 2003; Lee et al., 2004b; Farahmand et al., 2006).

W/O/W emulsions prepared with the rotor-stator homogeniser showed significantly slower release rate of SA compared to emulsions prepared with the high-pressure homogeniser (Fig. 7.4 b). The release of SA reached a peak at 40% at about 3 days. The release was further reduced significantly when higher concentration of PGPR (8%) was applied. As expected, the oil droplets formed were significantly larger compared to their counterparts in the emulsions prepared with high-pressure homogeniser. This probably halted both the diffusion of SA and the expelling of water droplets. As a result the release of SA in the external aqueous phase decreased. High concentrations of PGPR (8%) further reduced both diffusion and water droplet expelling and hence the release of SA was significantly slower.
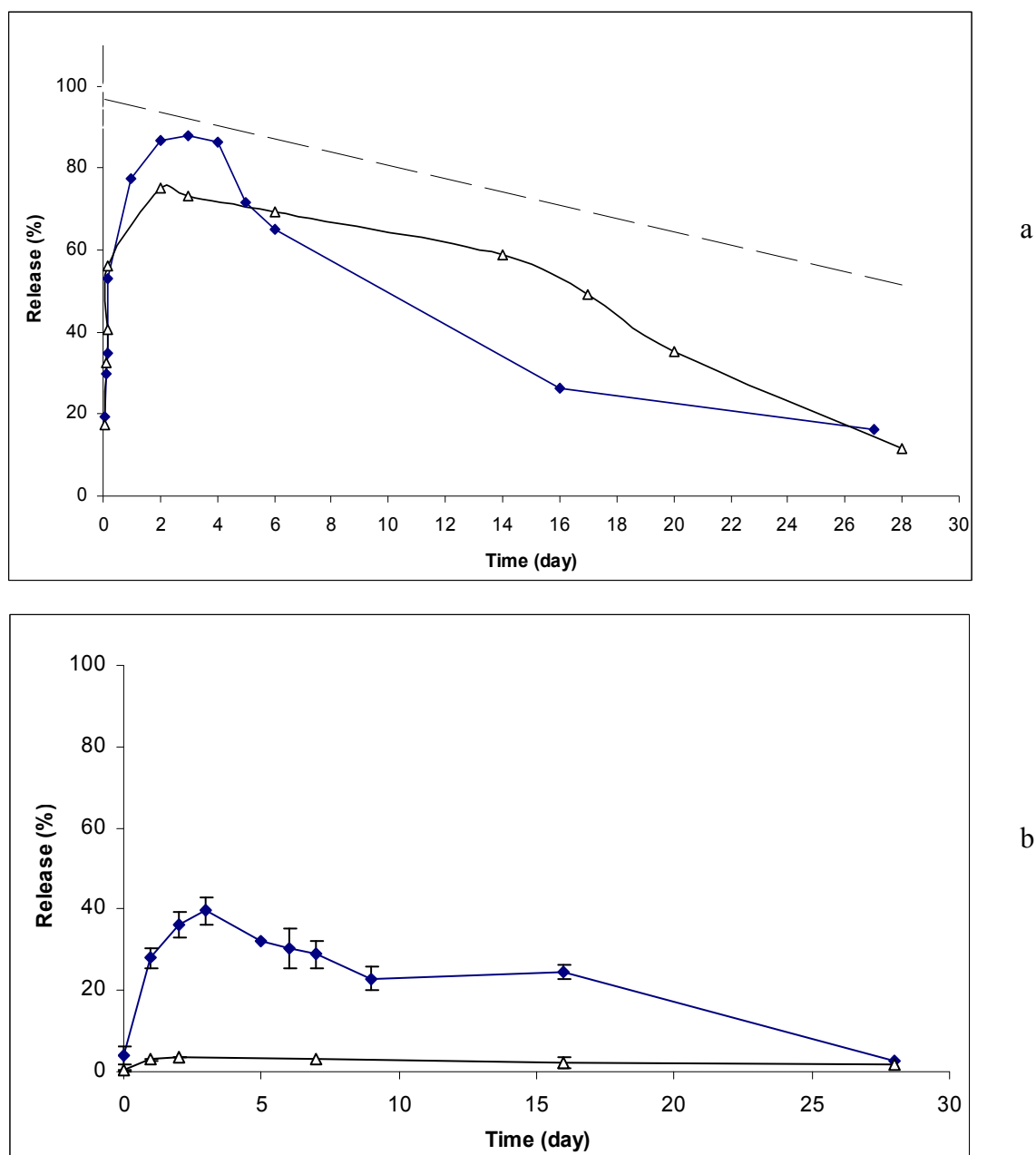
Fig. 7.4. Release of SA (%,fraction of SA measured in the external aqueous phase) from the internal to the external aqueous phase as a function of time in W/O/W emulsions prepared with (a) high-pressure homogeniser and (b) rotor-stator homogeniser. W/O/W emulsions contained 2% (w/v) SA in the internal aqueous phase and (◆) 2 and (△) 8% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase. Measurements were mean of duplicate emulsions. Dashed line represents the degradation rate of 2% (w/v) SA in its pure solution.

To further study the mechanisms of the release of SA, 0.1 M NaCl was added to the external aqueous phase of W/O/W emulsion to balance the osmotic gradient between the internal and the external aqueous phase (Fig. 7.5). For emulsions prepared with the high-pressure homogeniser, the addition of NaCl in the external aqueous phase increased the release of SA to almost 100%, however, the peak of release was delayed slightly from day 2-4 to day 3-4 (Fig. 7.5 a). For W/O/W emulsions prepared with the rotor-stator homogeniser, the peak of release of SA was delayed from day 3 to day 14, indicating a different dominant release mechanism in these two types of emulsions (Fig.7.5 b).
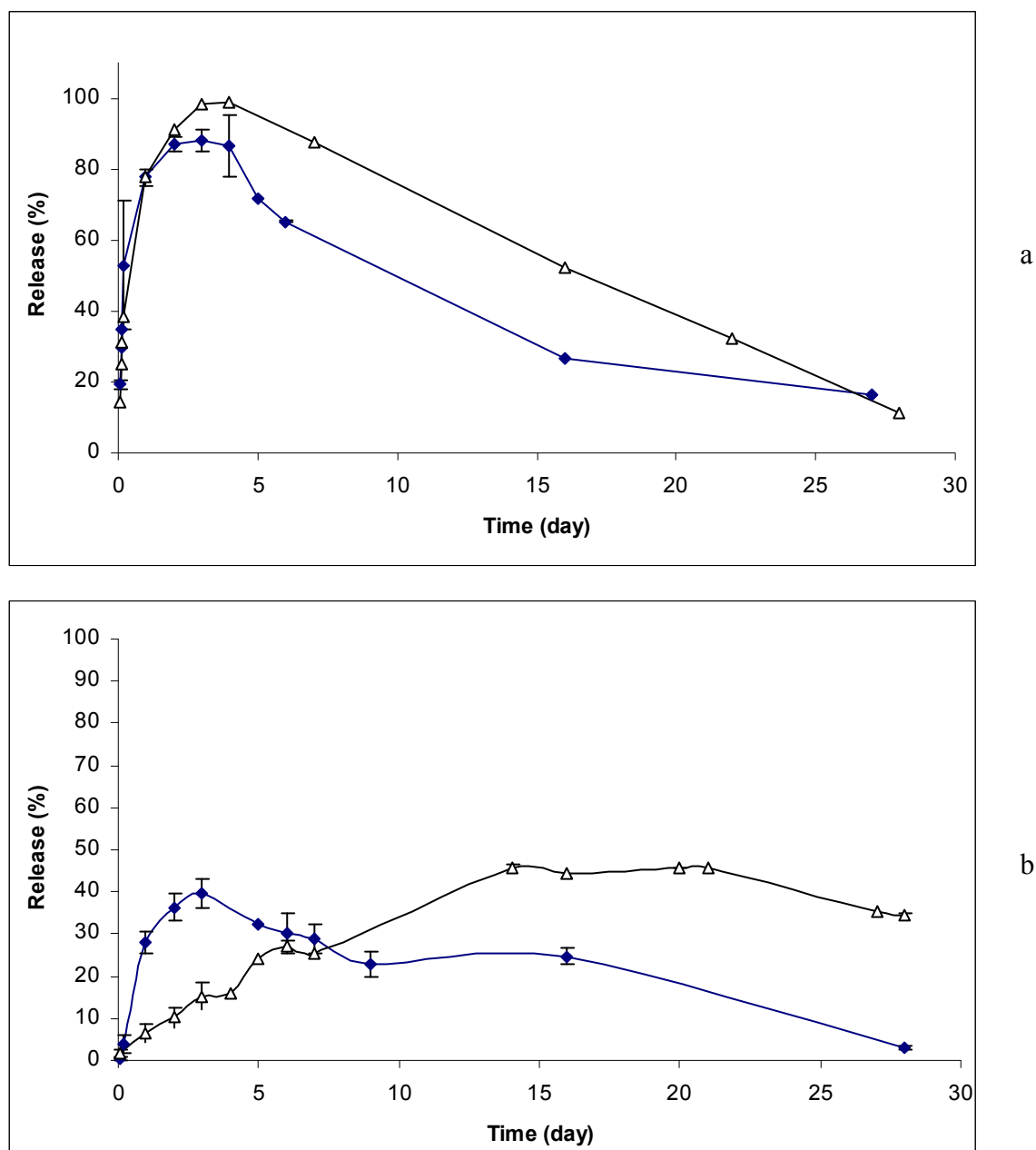
Fig. 7.5. Release of SA (%, fraction of SA measured in the external aqueous phase) from the internal to external aqueous phase as a function of time in W/O/W emulsions prepared with (a) high-pressure homogeniser and (b) rotor-stator homogeniser. W/O/W emulsions presented are emulsions containing 2% (w/v) SA in the internal aqueous phase (◆) and emulsions containing 2% (w/v) SA in the internal aqueous phase and 0.1 M NaCl in the external aqueous phase (△). All emulsions contained 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase.

For W/O/W emulsions prepared with the high-pressure homogeniser, due to the addition of 0.1 M NaCl in the external aqueous phase, the osmotic gradient was balanced. Water droplets swelled less, due to reduced water flow from the external aqueous phase, which could be indirectly observed by changes in the oil droplets sizes (Fig. 7.6 a). As discussed in Chapter 5, the smaller water droplets were less susceptible to expelling from the oil droplets. In addition, theoretically, NaCl might diffuse to the internal water droplet, and change the properties of interface of water droplets, such as dehydrating the emulsifier. This could result in a rigid interface that slows down the coalescence between the water droplets and the external aqueous phase and also contribute to reduction in the expelling of water droplets (Wen and Papadopoulos, 2000a, 2001). With less contribution from water expelling, the release peak of SA was delayed. On the other hand, the diffusion of SA through oil phase might be accelerated due to higher SA concentration in less-swollen water droplets. The hypothesis was supported by the results that nearly 80% SA was still released at first 24 hours with the addition of 0.1 M NaCl in the external aqueous phase. Diffusion of SA through the oil phase is likely the dominant mechanism of release at the early stages in W/O/W emulsions prepared with high-pressure homogeniser.

For emulsions prepared with rotor-stator homogeniser, the addition of NaCl in the external aqueous phase significantly delayed the release of SA. As mentioned above, the addition of NaCl mainly balanced the osmotic gradient and the direct result of it was less swelling of water droplets. The less swelling of water droplets could be determined indirectly by measuring the size distribution of the oil droplets (Fig. 7.9b). Less water droplets were expected to be expelled from the oil droplets. However, in contrast to emulsions prepared with the high-pressure homogeniser, the effect of reduced water droplet expelling largely overcame the effect of increased diffusion of SA, the amount and rate of SA release were both significantly reduced. The observation indicates that water droplet expelling was likely the dominant release mechanism in W/O/W emulsions prepared with the rotor-stator homogeniser.
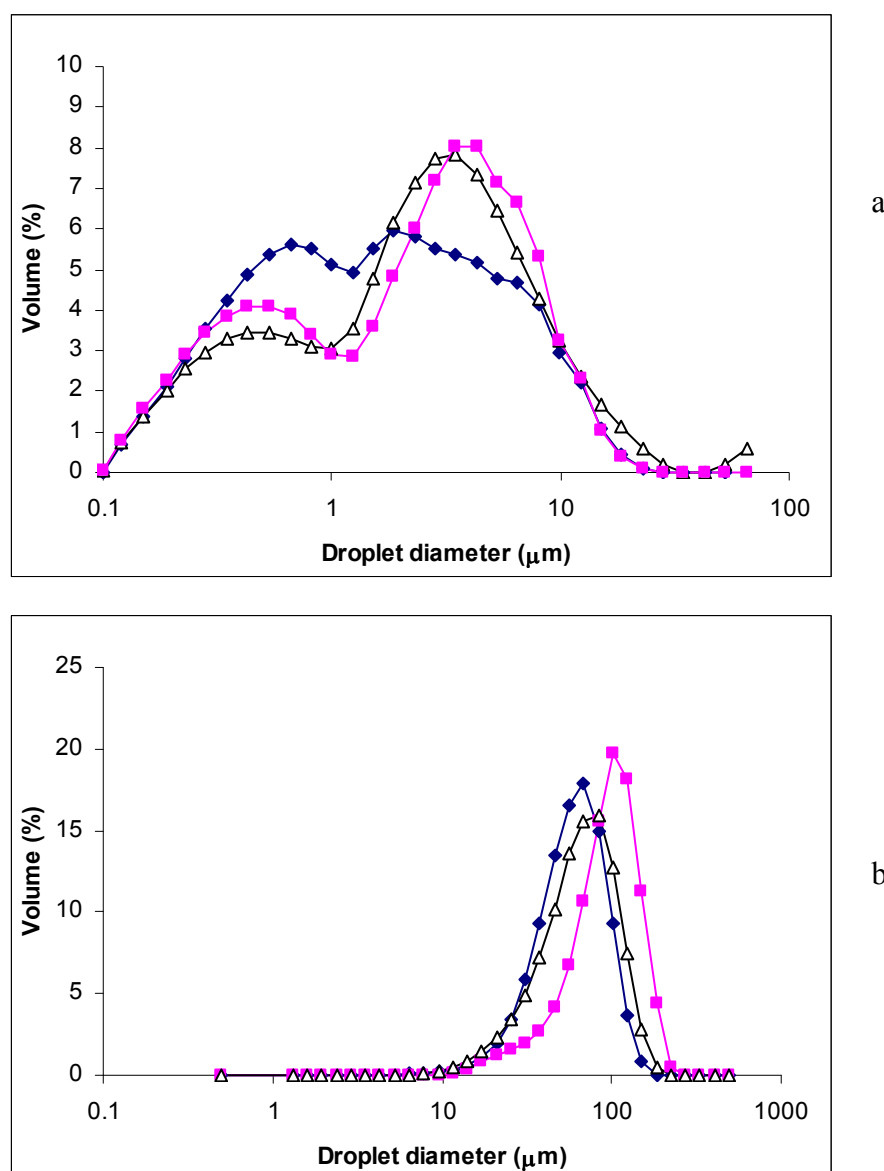
Fig. 7.6. Droplet size distributions of W/O/W emulsions prepared with (a) high-pressure homogeniser and (b) rotor-stator homogenisers. Emulsions presented are blank emulsions (◆), emulsions with addition of 2% (w/v) SA in the internal aqueous phase (■) and emulsions with addition of 2% (w/v) SA in the internal aqueous phase and 0.1 M NaCl in the external aqueous phase (△). All emulsions contained 2% (w/v) PGPR in oil phase and 0.5% (w/v) NaCN in the external aqueous phase.

7.3.1.4. Encapsulated SA in W/O/W emulsion

It is desirable to break down the emulsion and release SA entrapped in the W/O/W emulsion to compare the degradation rate of released and entrapped SA, which would show whether the stability of SA could be enhanced by encapsulation. Methanol extraction (demulsifying) combined with ultrasonic destabilisation and freeze-thaw methods were applied according to the protocols set up by Lee et al. (2004a) and Rojas and Papadopoulos (2007). The destabilization technique was used for W/O/W emulsions prepared with the rotor-stator homogeniser, as substantial amount of SA remained in the emulsions compared to their counterparts prepared with the high-pressure homogeniser.

W/O/W emulsions prepared under the current conditions could not be destabilised completely by either of the methods. The repeated freezing and thawing method released more SA compared to methanol extraction method, and the results of W/O/W emulsions before and after destabilisation by freezing and thawing are shown in Fig. 7.7 a and b. Overall, there was always some SA released after the destabilisation, especially for emulsions with 8% PGPR. Though it is difficult to recover all SA remaining in the W/O/W emulsion, it still appears that W/O/W emulsions provide some protection of SA from degradation.
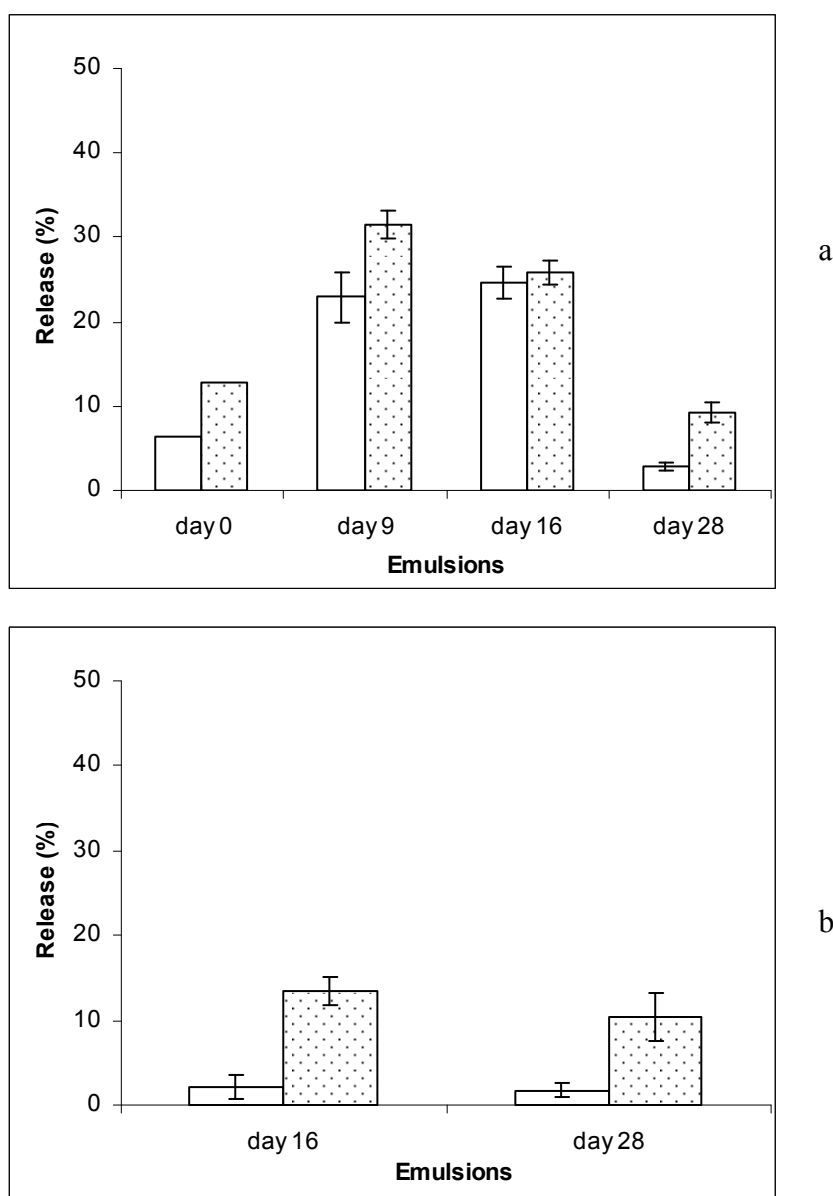
Fig. 7.7. **Effect of freeze and thaw destabilization on the release of SA (%, fraction of SA measured in the external aqueous phase) in W/O/W emulsions stored at 20 °C for 0-28 days. Emulsions contain (a) 2% (w/v) and (b) 8% (w/v) PGPR, and 0.5% (w/v) NaCN in the external aqueous phase. Blank bar: original Sample, dotted bar: destabilised samples by the freeze-thaw method.**

Rojas and Papadopoulos (2007) investigated the effect of repeated freeze and thaw cycle on the properties of W/O/W emulsions. In their study, n-hexadecane, Span 80 and Tween 80 were used as oil phase, hydrophobic and hydrophilic emulsifiers,

respectively. It was found that the freezing and thaw method could destabilize W/O/W emulsions, and the size of water droplets was vital in the process of destabilisation. The critical ratio of internal water droplets relative to the effective diameter of the oil droplet was around 0.3, above which external coalescence occurred upon oil thawing. The hypothesis was that external coalescence occurs when the internal water droplet becomes large enough to attraction over the electrostatic repulsion, due to their larger volume and the significant presence of water-soluble surfactant. In addition, large water droplets are inherently more unstable than their smaller counterparts and are more susceptible to destabilizing effects of external stimuli such as oil thawing.

In this study, d_{43} of oil droplets in W/O/W emulsions (with 2% SA) prepared with rotor-stator homogeniser was 70-110 μm, and the mean diameter of internal water droplets was less than 0.8 μm (the internal water droplet size of W/O/W emulsion with addition of 2% SA was not measured, the mean internal water droplet size of W/O/W emulsion with addition of 0.5 M sorbitol determined by PFG-NMR was used as a reference). The ratio of water droplets and oil droplet size was far less than 0.3. According to the hypothesis of Rojas and Papadopoulos (2007), the W/O/W emulsions were expected to be stable over repeated freeze and thaw cycles.

7.3.1.5. Effect of macromolecules on the release of SA from W/O/W emulsions

Previous studies found that the addition of proteins (NaCN and WPI) or increased viscosity of the internal aqueous phase could improve the stability of W/O/W emulsions and delay the release of encapsulants. In this study, NaCN and CMC were added to the internal aqueous phase to investigate the effect of addition of protein and increased viscosity in the internal aqueous phase on the release of SA from W/O/W emulsions (Fig. 7.8).
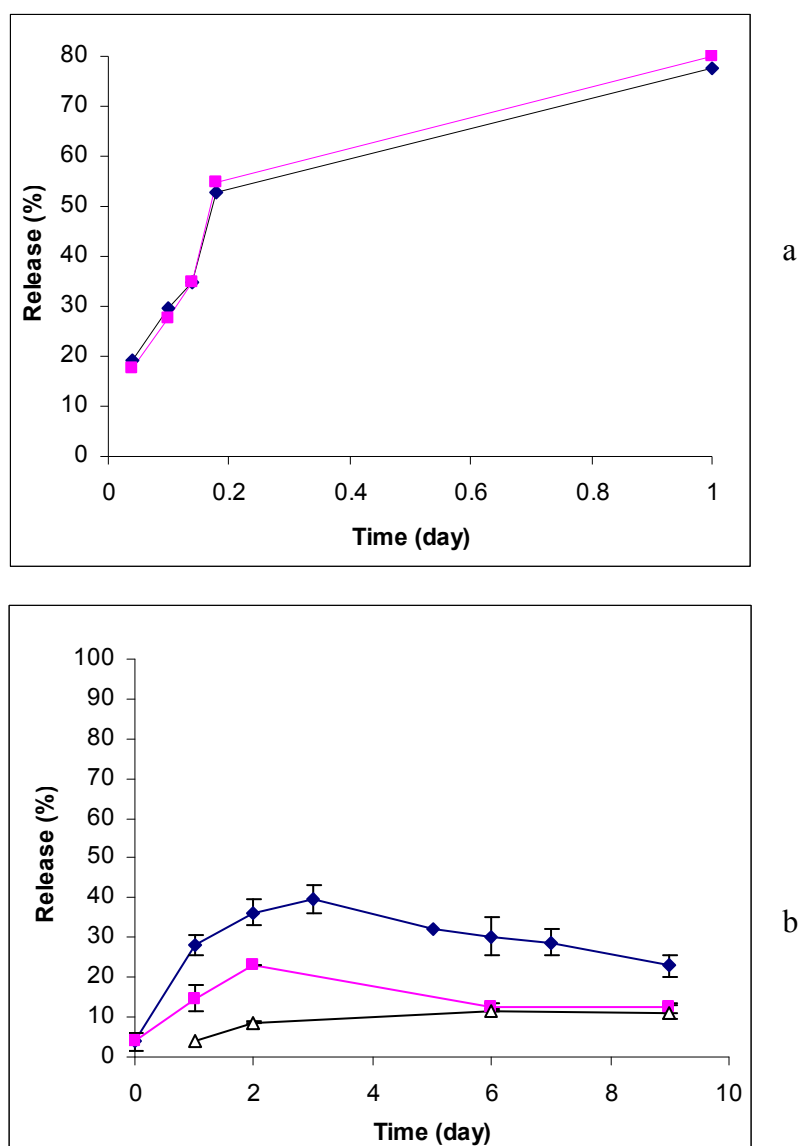
Fig. 7.8. Effect of time on release of SA (%, fraction of SA measured in the external aqueous phase)to the external aqueous phase of W/O/W emulsions prepared with (a) high-pressure homogeniser and (b) rotor-stator homogeniser. W/O/W emulsions were prepared with 2% (w/v) SA in the internal aqueous phase (◆), 2% SA and 0.5% NaCN in the internal aqueous phase (■) and 2% (w/v) SA and 2% (w/v) CMC (△) in the internal aqueous phase. W/O/W emulsions contained 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN in the external aqueous phase.

Fig. 7.8 shows that the addition of 0.5% NaCN in the internal aqueous phase of W/O/W emulsion prepared with the high pressure homogenizer did not have a

significant effect on the release of SA. The effect of addition of NaCN and CMC, however, were obvious in W/O/W emulsions prepared with the rotor-stator homogenizer. Emulsions with NaCN showed a similar pattern to the control emulsion, with the peak of SA release at day 2, and then the release declined, probably due to the degradation of SA. Emulsions with CMC showed slower but more gradual release of SA. CMC (2% w/v) may improve the stability better than NaCN (0.5% w/v), probably due to the increased viscosity of the internal aqueous phase which may reduce the rate of diffusion of ions and coalescence between water droplets and the external aqueous phase.

7.3.2. Release of SA in W/O/W emulsions with 0.5% (w/v) NaCN or 10% (w/v) SUPER GUM™ as hydrophilic emulsifier

In this study, the release profile of SA was studied by replacing NaCN (0.5% w/v) with SUPER GUM™ (10% w/v) in the external aqueous phase of W/O/W emulsions as hydrophilic emulsifier (Fig. 7.9). The results showed that for W/O/W emulsions prepared with the high-pressure homogeniser, emulsions with SUPER GUM™ as hydrophilic emulsifier resulted in significantly less release of SA. The pattern of release, however, is similar to emulsions with NaCN as emulsifier. It suggested that SUPER GUM™ might provide a strong barrier to either coalescence between water droplets and the external aqueous phase and/or diffusion of SA.
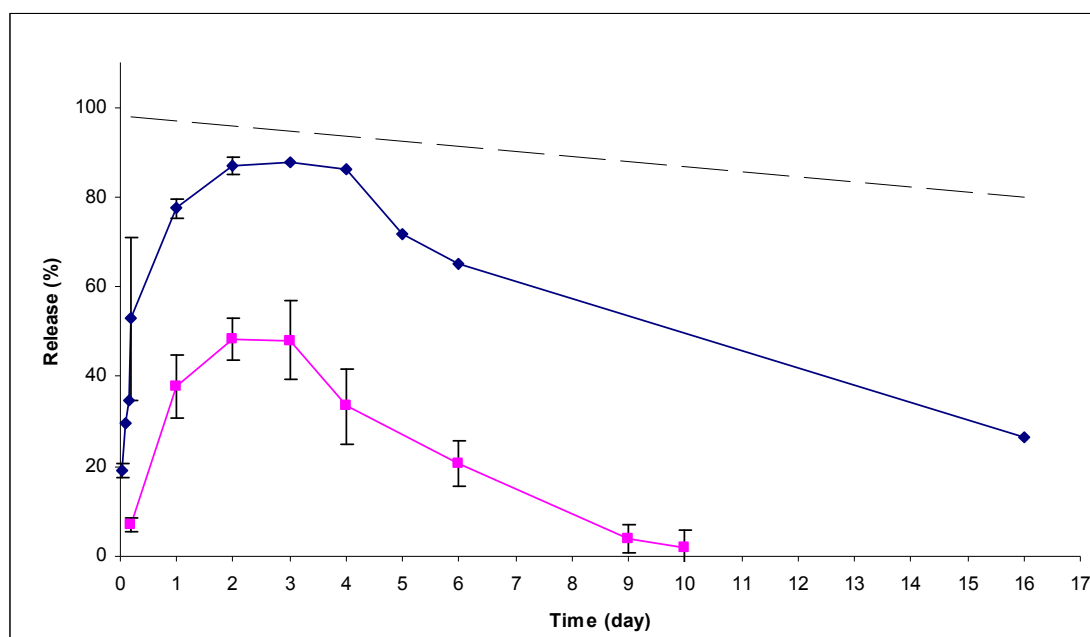
Fig. 7.9. Release of SA (%, fraction of SA measured in the external aqueous phase)from the internal to external aqueous phase as a function of time in W/O/W emulsions prepared with high-pressure homogeniser. W/O/W emulsions contained 2% (w/v) SA in the internal aqueous phase, 2% (w/v) PGPR in the oil phase and 0.5% (w/v) NaCN (◆) or 10% (w/v) SUPER GUM^{TM}(■) in the external aqueous phase. Dashed line represents the degradation rate of 2% (w/v) SA in pure solution.

It is well established that protein can be desorbed from the oil droplet surface by competitive adsorption of small surfactants, such as SDS (Heertje, van Aalst, Blonk, Don, Nederlof and Lucassen-Reynders, 1996; Bos, Nylander, Arnebrant and Clark, 1997; Dickinson, 1998). To verify the effect of SUPER GUM™ on the release of SA, an experiment using SDS to replace SUPER GUM™ as the hydrophilic emulsifier was conducted in this study and results are shown in Fig. 7.10.
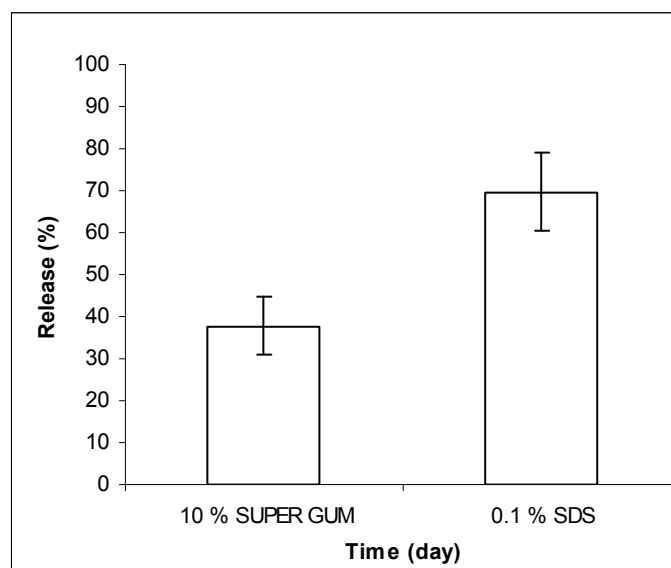
Fig. 7.10. Effect of addition of 0.1% SDS in the external aqueous phase on the release of SA (%, fraction of SA measured in the external aqueous phase) from the internal to the external phase of W/O/W emulsions prepared freshly using 10% (w/v) SUPER GUM™ as hydrophilic emulsifiers in the external aqueous phase on day 1 after the formation of W/O/W emulsion.

It was found that the release of SA significantly increased from 37.7 to 69.6% instantly after 0.1% SDS was added to the external aqueous phase. The results indicates that enlarged water droplets or SA might be accumulated around the interface of oil droplets, blocked by the thick layer formed by SUPER GUM™ from coalescence with the external phase or to diffuse through. Because SDS at least partially replaced SUPER GUM™ on the surface of oil droplets, the barrier that was formed by SUPER GUM™ was partially ruptured, immediately releasing SA to the external aqueous phase.

The W/O/W emulsion with SUPER GUM™ as hydrophilic emulsifier also showed reduced release of SA when prepared with the rotor-stator homogeniser. Results (Fig. 7.11) showed that in the SUPER GUM™ system, much less PGPR (0.5% w/v) was required to achieve similar release of SA (~ 40%) compared to NaCN systems (2% w/v PGPR) (Fig. 7.8 b), whereas the mean droplet size is considerably smaller (66.9 vs. 107.2 μm).
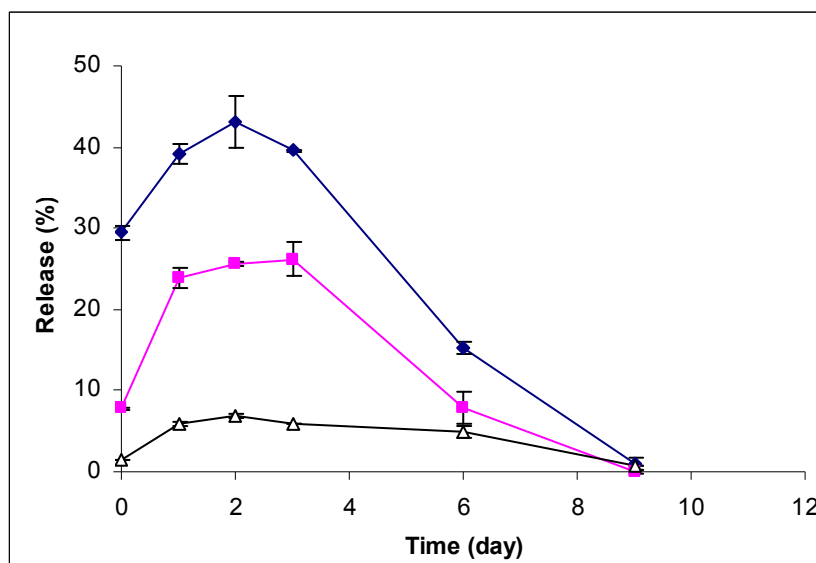
Fig. 7.11. Effect of time on the release of SA (%, fraction of SA measured in the external aqueous phase) in W/O/W emulsions prepared with rotor-stator homogeniser as function of time. W/O/W emulsions contained 2% SA in the internal aqueous phase and (◆) 0.5, (■) 1 and (△) 2% (w/v) PGPR in the oil phase and 10% (w/v) SUPER GUM™ in the external aqueous phase.

After the peak of SA release (at day 2-3), both systems underwent degradation processes; complete degradation of SA in NaCN system occurred at day 28 (Fig. 7.5 b), whereas it took its counterpart only 10 days in SUPER GUM™ system (Fig. 7.11). It is suspected that SUPER GUM™ may accelerate the degradation of SA.

The degradation processes of SA are very complex and contain a number of oxidation/reduction and intermolecular rearrangement reactions (Davies et al., 1991). Dehydroascobic acid (DHAA), the oxidised form of ascorbic acid, has the same bioactive function as ascorbic acid but is dark yellow in colour which is undesirable in food products. It is highly unstable and may be converted to a variety of degradation products, such as 2-furoic acid, 3-hydroxy-2-pyrone, 5-methyl-3,4-dihydroxytetrne, furfural, etc., depending upon the conditions of the degradation reaction (Davies et al., 1991; Graham and Annette, 1992; Yuan and Chen, 1998). DHAA can be reduced

rapidly and completely to ascorbic acid by homocysteine at pH 7.0, and this method is fast and specific, and has been applied since Hughes's (1956) finding to estimate ascorbic acid in the presence of homocysteine without interference from the latter oxidation compounds (Graham and Annette, 1992).

To reduce DHAA in the external aqueous phase, homocysteine (3:1) was added to W/O/W emulsions with NaCN or SUPER GUM™ as hydrophilic emulsifier at day 6 and day 9 to determine the SA presented in external phase. The reduction took 30 minutes at room temperature (Kennedy, White, Warner, Lloyd and Rivera, 1989; Graham and Annette, 1992). The results of SA in the external aqueous phase before and after the addition of homocysteine are shown in Fig. 7.12. As homocysteine can only specifically reduce DHAA to ascorbic acid, but not other oxidation products, the ascorbic acid would not be fully recovered. The result represents a combination of ascorbic acid and DHAA in the external phase at the time of test.

The results showed that before and after the addition of homocysteine, the concentration of ascorbic acid was always higher in the NaCN system, and the concentration of DHAA (the difference between release of SA before and after the addition of homocysteine) was also higher in NaCN system. The results indicate that both SA and DHAA were oxidised slower in NaCN system compared to the SUPER GUM™ system. There are two possibilities for this observation. SUPER GUM™ has a more complex composition, which could be the reason that it oxidised SA faster than did NaCN, and/or the SUPER GUM™ system had smaller oil droplets, thus more total surfaces were exposed to release SA.
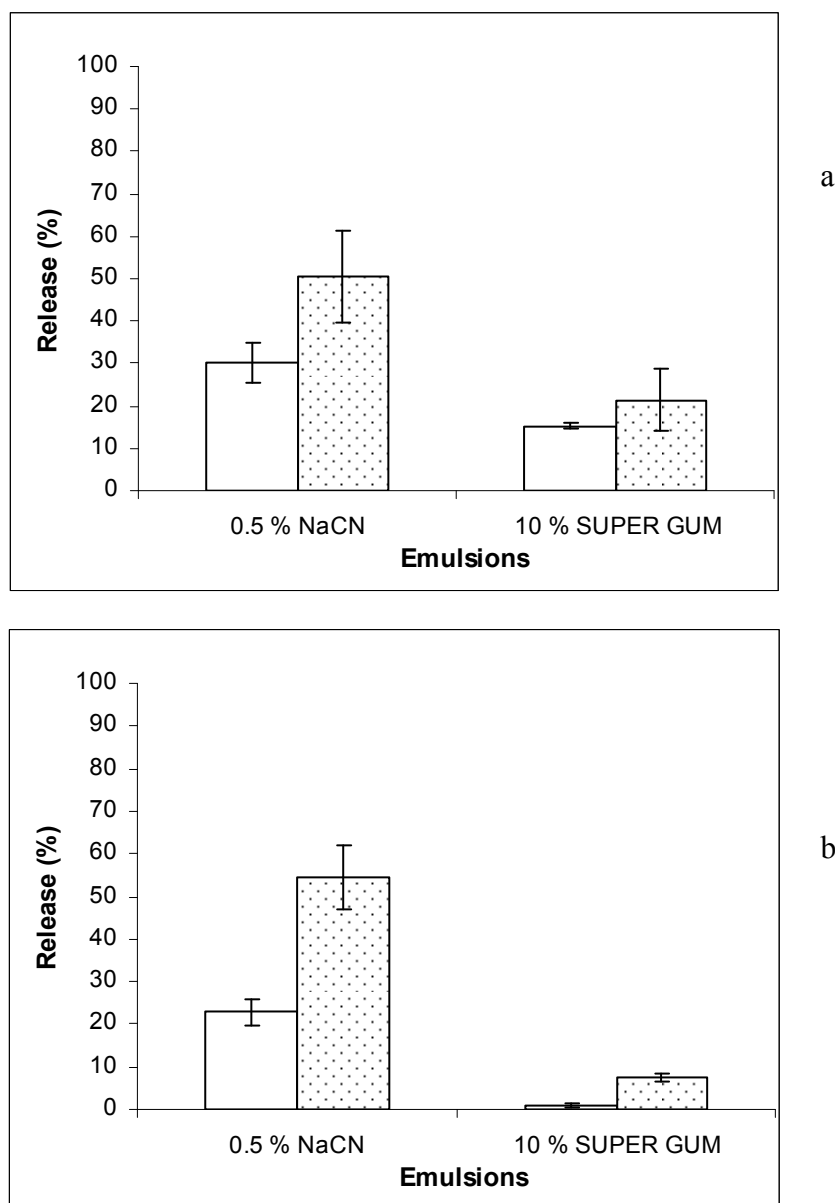
Fig. 7.12. Concentration of SA in the external aqueous phase of W/O/W emulsions before (blank bar) or after (dotted bar) the addition of reducing agent homocysteine at (a) day 6 and (b) day 9 after prepared with rotor-stator homogeniser. W/O/W emulsions were prepared with 2% (w/v) SA in the internal aqueous phase, 2% PGPR (for NaCN system) and 0.5% (w/v) PGPR (for SUPER GUM™ system) in the oil phase, and 0.5% (w/v) NaCN or 10% (w/v) SUPER GUM™ in the external aqueous phase, respectively.

7.4.   Summary

The encapsulation of sodium ascorbate (SA), a salt form of ascorbic acid, in W/OW emulsions was studied. It was found that the release of SA was greatly affected by the composition and formation techniques of W/O/W emulsions. Increasing the concentration of PGPR, adding macromolecules in the internal aqueous phase or using rotor-stator homogeniser could all slow down the release of SA. It was noted that replacement of NaCN by SUPER GUM™ as hydrophilic emulsifier could also significantly reduce the release of SA from W/O/W emulsions. However, on the other hand, oxidation rate of SA was higher in W/O/W emulsions use SUPER GUM™ as hydrophilic emulsifier.

Chapter 8: Conclusions and Recommendations

The overall objective of this study was to establish a stable W/O/W emulsion model system that would have the potential to be used in various food systems. The properties and characteristics of the emulsions were studied, the stability of the emulsions under different experimental conditions was investigated and the potential applications of the designed W/O/W emulsions were explored. The findings from this study are summarised as follows.

8.1. Formation of stable W/O/W emulsions

Compositions and techniques were compared and selected to form stable food-grade W/O/W emulsion systems. Stable W/O/W emulsions could be formed using soybean oil as the oil phase, PGPR as the hydrophobic emulsifier and NaCN or SUPER GUM™ as the sole hydrophilic emulsifier. The ratios of dispersed phase to continuous phase were 1:4 for both the primary W/O emulsion and the secondary W/O/W emulsion, based on the experimental results or the literature. W/O/W emulsions were prepared using the two-step emulsification method.

8.1.1. The effect of the formation technique on W/O/W emulsions

Optimum pressures to obtain a stable W/O/W emulsion with a fine oil droplet size ($d_{43} \approx 2-5$ μm) and a high EE (> 90%) were determined using a high-pressure homogeniser. Stable W/O emulsions with a mean water droplet size of about $0.8-1.0$ μm could be formed at 800/80 bar, whereas higher pressures did not produce significantly smaller water droplets. The secondary W/O/W emulsions were formed at 100 bar (using a high-pressure homogeniser) or 8000 rpm (using a rotor-stator homogeniser). Although higher pressures could reduce the oil droplet size, the EEs of the W/O/W emulsions were significantly lower, because the higher mechanical force resulted in more surface exposure of the water droplets to the external aqueous phase.

8.1.2. *The effect of hydrophobic emulsifiers on the stability of W/O/W emulsions*

A stable W/O emulsion is essential for a stable secondary W/O/W emulsion. W/O emulsions prepared with PGPR as the hydrophobic emulsifier were significantly more stable than emulsions prepared with Span 80 or lecithin. The concentration of PGPR greatly affected the stability of the W/O emulsions. W/O emulsions had smaller mean water droplet sizes and were more stable towards phase separation when higher concentrations of PGPR were used. Higher PGPR concentrations increased the viscosity of the oil phase and the coverage of PGPR on the water droplet surface. This in turn increased the repulsion forces between water droplets and reduced the extent of flocculation/coalescence. However, as PGPR is a synthetic emulsifier and its use in food products is restricted, low PGPR concentrations in emulsions are preferred. The minimum concentration of PGPR to form a stable W/O/W emulsion (EE > 90%) was 2% (w/v) in a non-buffered system using NaCN (0.5%) as the hydrophilic emulsifier, and could be further reduced to 0.5% (w/v) when SUPER GUM™ was used as the hydrophilic emulsifier.

In buffered W/O/W emulsions, the minimum concentration of PGPR required to obtain an EE of 90% was 4% (w/v). The reason for the reduced EE was because the ionic environment created by the phosphate buffer had an adverse effect on the emulsifying ability of the PGPR. The addition of macromolecules, such as NaCN (0.5%), improved the EE and reduced the minimum concentration of PGPR to 2% (w/v). It was postulated that proteins and PGPR may have a synergistic effect on the interface to stabilise the internal water droplets. The improvement in EE on the addition of macromolecules was more pronounced in buffered systems than in non-buffered systems, probably because of the inherent ionic content naturally present in the macromolecules.

8.1.3. *The effect of hydrophilic emulsifiers on the stability of W/O/W emulsions*

The effectiveness of NaCN or SUPER GUM™ as the hydrophilic emulsifier was evaluated and compared. W/O/W emulsions prepared with 10% (w/v) SUPER GUM™ had significantly higher EE than emulsions prepared with 0.5% (w/v) NaCN. SUPER GUM™ may build thick and rigid layers on the oil droplet surface, which retard the expelling of internal water droplets to the external aqueous phase. SUPER GUM™ was also more resistant to pH change (2–7) in the external aqueous phase and it may have advantages for use as a delivery system over a wide range of pH conditions.

Change in the concentration of the hydrophilic emulsifier had an effect on the oil droplet size distribution. A low concentration of hydrophilic emulsifier caused coalescence between the oil droplets, but the EE of the W/O/W emulsion did not change significantly because the W/O emulsion was not destabilised by the hydrophilic emulsifier.

8.1.4. *The effect of encapsulated compounds on W/O/W emulsions*

Sorbitol and NaCl were added to the internal aqueous phase of W/O/W emulsions. By adding hydrophilic encapsulants into the internal aqueous phase of W/O/W emulsions, an osmotic pressure gradient was generated for the passage of water and hydrophilic encapsulants such as electrolytes or sugars across the oil phase.

8.1.4.1. Addition of sorbitol

The addition of 0.5 M sorbitol to the aqueous phase of W/O emulsions significantly reduced the mean water droplet size; as a result, the emulsion was more resistant to water sedimentation. The addition of sorbitol might reduce the attractive force between water droplets by lowering the interfacial tension between the aqueous and

oil phases. For the secondary W/O/W emulsions, the osmotic pressure created by sorbitol in the internal aqueous phase induced rapid water transportation from the external aqueous phase to the internal aqueous phase. As a result, the internal water volume fraction and the mean size of the water droplets increased. The increase in the mean size of the water droplets was measured quantitatively using the PFG-NMR technique, a relatively new technique for non-invasively determining the droplet size in W/O/W emulsions, and that provides a promising means of detecting changes to the droplet size distribution of the internal water of W/O/W emulsions.

The EEs of W/O/W emulsions after the addition of sorbitol were also determined quantitatively, using poly R-478 as a marker. It was found that the EE decreased significantly after the addition of sorbitol, because of swelling of the water droplets, which were unstable and were expelled from the oil droplets more rapidly.

The transportation of sorbitol through the oil phase was detected using the PFG-NMR technique. Transportation occurred from higher concentration to lower concentration. A diluted concentration of sorbitol in the swelled water droplets and transfer of sorbitol across the oil phase may contribute to the osmotic pressure gradient balance between the internal and external aqueous phases being restored.

8.1.4.2. Addition of NaCl

NaCl is commonly added to the internal aqueous phase in W/O/W emulsion studies, to reduce Ostwald ripening of the water droplets by balancing the Laplace pressure and hence stabilising the emulsion. However, in this study, it was found that as low as 0.1 M NaCl caused phase separation in W/O emulsions. The destabilisation was caused by a reduction in the emulsifying ability of PGPR. This could be improved by the addition of macromolecules to the internal aqueous phase.

The transportation of water in W/O/W emulsions with NaCl added to the internal aqueous phase was studied using the PFG-NMR technique. It appeared that water was transported from the external aqueous phase to the internal aqueous phase rapidly

because of the osmotic pressure gradient created by the NaCl. The swelling of the water droplets reached equilibrium within 24 h. Loss of water from the internal aqueous phase, observed from indirect evidence, such as the oil droplet size distribution of the W/O/W emulsion, was a significantly slower process mainly because of the rupture and expelling of water droplets to the external aqueous phase. Osmotic pressure gradients generated by NaCl and sorbitol had a similar effect on water diffusion.

The release of NaCl across the oil phase to the external aqueous phase was also studied. The release of NaCl was found to be significantly faster than the release of poly R-478, which represented the loss of water droplets. There are three possible pathways of NaCl release: diffusion of NaCl across the oil phase, expelling of internal water droplets to the external aqueous phase and rupture of water droplets. Under the current experimental conditions, it appeared that all three pathways occurred, but which mechanism was dominant was not clear. The release of NaCl was found to be dependent on the concentration of PGPR, the hydrophobic emulsifier of the W/O/W emulsions. PGPR may reduce the release of NaCl by increasing the coverage of the water droplets and by increasing the viscosity of the oil phase. The effect of the hydrophilic emulsifier, NaCN or SUPER GUM™, on the release of NaCl did not appear to be significant compared with the effect of PGPR.

8.2. Potential applications of W/O/W emulsions for encapsulation

Sodium ascorbate (SA), the salt form of ascorbic acid, was added to the internal aqueous phase (2% w/v) of W/O/W emulsions. The release of SA was determined quantitatively using HPLC analysis. SA showed similar release characteristics to NaCl. The release of SA was also believed to be a combination of all three pathways mentioned above, and could be slowed by increasing the concentration of PGPR, by adding macromolecules or by using the rotor-stator homogeniser to prepare the W/O/W emulsion.

However, in all formulations, the release of SA was significantly slower than the release of NaCl. It was also worth noting that SUPER GUM™ significantly slowed the release of SA from W/O/W emulsions, which did not occur for the release of NaCl. This finding indicates that W/O/W emulsions could be a feasible means of encapsulating SA and of achieving controlled release, by modifying the formation conditions and the formulations. The inherent structure, composition and surface charges of W/O/W emulsions may contribute to the release properties observed for SA.

8.3. Recommendations for future work

The destabilisation of W/O/W emulsions after the addition of small molecular hydrophilic encapsulants and the fast release of these small molecular encapsulants from W/O/W emulsions were the major problems encountered in this study. The results showed that addition of protein or polysaccharide to the internal aqueous phase improved the stability of W/O/W emulsions, indicating that modifying the properties of the components of W/O/W emulsions may hold a key, in future study, to improving the stability of W/O/W emulsions and to slowing the release of encapsulants.

8.3.1. *Internal aqueous phase*

In this study, carboxy methyl cellulose (CMC), a polysaccharide, was used to increase the viscosity of the internal aqueous phase, and the stabilities of the W/O/W emulsions were improved by this addition. The viscosity of the internal aqueous phase might be further enhanced, through gelation, to form an S/O/W emulsion; the solidified internal aqueous phase will resemble microcapsules, which will be more resistant to coalescence and may have better EE than water droplets.

8.3.2. Oil phase

It was accepted that the diffusion of small molecules in the oil phase is a rate-determining step for transportation of the hydrophilic encapsulant. A change in the properties of the oil phase in W/O/W emulsions will have a marked influence on the release pattern of encapsulants. Pickering emulsions, described in Chapter 2, can be explored further, because they were proven to provide a W/O/W emulsion that had a significantly low release of encapsulant. Further work in this area may be focused on producing a shear-stable W/O/W emulsion in which the internal water droplets can be stabilised by the fat crystals in the oil phase. The oil phase will ideally be crystallised in a controlled manner in terms of size, type and shape of crystals.

8.3.3. External aqueous phase

Gum arabic is a mixture of saccharides and glycoproteins. The use of a modified gum arabic, SUPER GUM™, as the hydrophilic emulsifier in W/O/W emulsions to reduce the release of encapsulants showed the possibility of using a mixture of polysaccharides and proteins as a co-emulsifier to further increase the stability of W/O/W emulsions by forming thick and rigid interfacial layer(s) on the oil droplet surface, or to change the viscosity of the external aqueous phase. A compatible polymeric mixture will contribute to a stable W/O/W emulsion and the controlled release of encapsulants. It may also be useful for the creation of food emulsions with improved physico-chemical properties or for the development of novel delivery systems.

8.3.4. Evaluation of interfacial properties

This study investigated the properties of W/O/W emulsions mainly by their physical properties such as droplet size and concentration of encapsulant release from the internal aqueous phase. A synergistic effect between the hydrophobic emulsifier and

added proteins was postulated. However, because of technical limitation, it was not clear how the synergistic effect was formed and what properties of the interfaces it changed.

To create emulsions with regulated stability and tailored release or encapsulation behaviour, it will be important to better describe the interfacial properties of W/O/W emulsions, such as the role of the interfacial thickness and density in the release of encapsulants with different polarity and molecule size. The interfacial rheological behaviours against different shear forces should also be characterised. New techniques and methods need to be developed to provide more information on the interfacial properties of W/O/W emulsions.

8.3.5. *Advanced model system*

The results obtained from this study can serve as a starting point for study in a more complicated model that mimics a real food system. Food systems containing multiple types of ingredients may undergo various processing conditions such as heating, shearing and turbulent flow. It will be important to describe the stability of such systems under different processing conditions and to create new methods for releasing encapsulants from these systems without significant changes to their stability. Factors contributing to the stability of W/O/W emulsions, such as energy input, interactions between ingredients and ion diffusion, all need to be studied and understood before tailoring the droplet formation, selecting encapsulants and defining the release kinetics, so as to give the emulsions sufficient shelf life for real food applications.

8.4.   Conclusions

This study showed that a stable system of a food-grade W/O/W emulsion with a simple formulation and that uses sole polymeric emulsifiers in both the W/O and O/W interfaces can be established. It provides basic information on the instability and interactions between components of the W/O/W emulsion, and can be used as a

starting point for further study in more complicated systems. The study experienced a common problem in this field; that is, the methods are based on the physical properties of emulsions and the concentration of ions in the external aqueous phase. There are gaps in our knowledge of which instability mechanisms are important, how they occur and how they can be exploited. More studies of W/O/W emulsions will provide information on predicting the behaviour of a W/O/W emulsion in real food systems and on better selecting ingredients or formation techniques to achieve long-term stability, high EE and controlled release of encapsulants from W/O/W emulsions.

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Appendix