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# Characterisation of fish oil emulsions stabilised by sodium caseinate

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**Abstract**

Fish oil emulsions varying in sodium caseinate concentration (25% w/w oil and 0.1–1.0% w/w protein, giving oil-to-protein ratios of 250–25) were investigated in terms of their creaming stability, rheological properties, the mobility of oil droplets and the oil/protein interaction at the interface. The presence of excessive protein in an emulsion (i.e., at 1% w/w) caused the aggregation of oil droplets through depletion flocculation, resulting in low creaming stability and high low-shear viscosity. At a lower protein concentration (0.1% w/w), when protein was limited, the emulsion droplets were stabilised by bridging flocculation and showed good stability to creaming. Shear-thinning behaviour was observed for both flocculated emulsions. A reduction in the low-shear viscosity and a Newtonian flow was obtained for the emulsion containing an intermediate concentration of protein (0.25% w/w). At this concentration, there was relatively little excess unadsorbed protein in the continuous phase; thus the emulsion was most stable to creaming. NMR was used to characterise these emulsion systems without dilution. Shorter $T_2$ values (by low-field $^1$H NMR), for the emulsions containing both high (1% w/w) and low (0.1% w/w) amounts of protein, indicated increased restricted mobility of oils, caused by depletion or bridging flocculation. The line broadening in oil signals in the high-field NMR spectra ($^1$H, $^{13}$C) indicated increased interaction between oil molecules and proteins at the interface with increasing protein concentration in emulsions. In addition, $^{31}$P NMR spectra, which reflect the mobility of the casein component only, showed increased line broadening, with reduction in protein content due to the relatively higher proportion of the protein being adsorbed to the interface of the oil droplets, compared to that in the continuous phase (i.e., as the oil-to-protein ratio was increased). The $T_2$ values of resonances of the individual groups on oil molecules, obtained using high-field $^1$H NMR, reflected their different environments within the oil droplet.


*Keywords:* Emulsion; Particle size; Creaming; Flocculation; NMR

## 1. Introduction

Many traditional food products, such as milk, yoghurt, mayonnaise, cake batters, ice creams, whipped toppings and dairy creamers, are emulsion-based. The physicochemical properties of emulsions play an important role in food systems as they directly contribute to texture, sensory and nutritional properties of foods (Dalgleish, 2006; McClements, 2005). More recently, emulsion systems are being utilised for delivery of lipophilic health-active compounds, such as long chain polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), where they provide convenient and practical means for delivering these nutrients in human diets (Augustin & Sanguansri, 2003; Keogh et al., 2001; Kolanowski, Swiderski, & Berger, 1999).

Proteins, particularly dairy proteins, are often used as emulsifiers in foods. The amphiphilic nature of proteins

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means that they have the ability to adsorb to an interface, lower surface tension and provide a viscoelastic layer which prevents coalescence of the droplets (Dickinson, 1999, 2006). However, the ability of various milk proteins and their fractions to form and stabilise emulsions differs, depending on their structure, conformation and state of aggregation (Agboola & Dalgleish, 1995; Dickinson, Golding, & Povey, 1997; Euston & Hirst, 2000). Sodium caseinate is a well-used ingredient because of its good solubility and emulsifying properties and its stability during heating (Dickinson, 1999). It is known that the amount of protein required to stabilise oil-in-water emulsions depends, not only on the structure of the protein at the interface, and the average diameters of the emulsion droplets, but also on the types of oils, i.e., whether they are hydrocarbons such as *n*-tetradecane or triacylglycerides, such as soya oil (Dickinson et al., 1997; Fang & Dalgleish, 1993). A critical concentration ($c^*$) of protein in the aqueous phase is often needed to provide the full coverage of emulsion droplets. However, it has been reported that the presence of excessive caseinate in an emulsion containing a high concentration of protein ($c > c^*$) can induce depletion flocculation of emulsion droplets, resulting in creaming due to the strong tendency of the casein submicelles to form small protein particles (Dickinson et al., 1997). On the other hand, when the protein is limited in an emulsion containing a low concentration of caseinate ($c < c^*$), the oil interface may not be fully covered by the available protein, thus the adsorbed caseins are shared between two or more oil droplets to form bridging flocculation (Dickinson, Flint, & Hunt, 1989). Therefore, it is important to obtain optimum emulsion stability with an intermediate concentration of caseinate ($c = c^*$), which gives full coverage of the oil–water interface without any significant excess of protein remaining unadsorbed in the emulsion, (Dickinson, 1999).

The properties of both the interface and the continuous phase are important for understanding emulsion characteristics. Emulsions have been studied by numerous techniques, such as particle sizing, microscopy, rheology, and measurement of surface concentration, to characterise adsorbed protein at the interface and related physical properties of the emulsion (Agboola & Dalgleish, 1995; Dickinson et al., 1997; Euston & Hirst, 2000; Singh, Tamehana, Hemar, & Munro, 2003). All or most of these techniques involve some form of dilution. This dilution disrupts some structures that contribute to destabilisation, in particular, the structures of concentrated systems. The ability to study the stability of food emulsions in their undiluted forms may reveal subtle nuances about their stability.

The application of NMR spectroscopy for the analysis of liquid food systems has proved immensely valuable (Belton, Delgadillo, & Gil, 1998; Cornillon, 1998; Guillen & Ruiz, 2001). It may be used to measure the total fat content in fat-containing food products (Sorland, Larsen, Lundby, Rudi, & Guiheneuf, 2004; Veliyulin, van der Zwaag, Burk, & Erikson, 2005), coalescence of emulsions (Lee, McCarthy, & Dungan, 1998), the effect of fat crystals on the destabilisation of emulsions (Hodge & Rousseau, 2005), and the mobility of particles in fish oil-in-water emulsions (Shen, Udabage, Burgar, & Augustin, 2005). Over recent years, several NMR techniques have provided information on emulsion droplet measurement and the size distribution association in concentrated emulsions (Denkova et al., 2004; Kiokias, Reszka, & Bot, 2004; Lönnqvist, Khan, & Söderman, 1991). Both the dispersed and the continuous phase of emulsions can be characterised using NMR (Balinov, Mariette, & Söderman, 2004; Goudappel, van Duynhoven, & Mooren, 2001). Other researchers (Mine, 1997; ter Beek et al., 1996) have used $^{31}$P NMR to investigate the adsorption behaviour of caseins and lipid–protein interaction at an oil-in-water interface. The advantage of using NMR is that it can be applied to concentrated emulsions without pre-treatment or dilution of the sample. Acquisitions of the measurement data are usually fast and do not require excessive sample volumes. Therefore, NMR measurements could provide new insights into the destabilisation mechanisms of emulsions due to the ability to study concentrated emulsions.

The aim of this work was to examine whether NMR could provide further information that would complement that obtained by other conventional techniques used for characterising emulsions. The examination of emulsion systems involved the use of low and high-field $^{1}$H NMR and high-field $^{13}$C NMR and $^{31}$P NMR. $^{1}$H and $^{13}$C NMR were chosen to provide information about the oil components as it was expected that these spectra would be dominated by the oil molecules, which are more mobile than the colloidal protein components. $^{31}$P NMR measurements enabled the determination of the protein distribution in the system, i.e., in bulk aqueous vs. the interface as only the phosphoseryl residues on caseins would be observed. In this study, fish oil-in-water emulsions, prepared using varying amounts of sodium caseinate (25% w/w oil, 0.1–1.0% w/w protein; oil-to-protein ratio of 250–25) were first characterised in terms of their stability to creaming and their rheological properties in relation to the emulsion particle size and structuring behaviour, by light microscopy. NMR techniques were then employed to study the mobility of oil and protein components at the interface in the emulsions with distinctive stabilisation characteristics.

## 2. Materials and methods

### 2.1. Materials

Sodium caseinate (Alanate 180) was obtained from New Zealand Milk Products Pty Ltd. (Rowville, VIC, Australia). It contained 92.6% protein, 4.2% moisture, 1.17% sodium and 0.02% calcium. Fish oil (HiDHA R 25N FOOD – steam deodorised) was supplied by Clover Corporation Ltd. (Sydney, NSW, Australia). Deuterium oxide

was purchased from Novachem Pty Ltd. (Collingwood, VIC, Australia).

### 2.2. Preparation of emulsions

Sodium caseinate (6 g as supplied) was dissolved in deionised water to give a final concentration of 2% (w/w). The protein solution was mixed constantly at room temperature (about 21 °C) overnight prior to use. The protein solution was then diluted with the appropriate amount of deionised water to achieve final protein concentrations of 1, 0.75, 0.5, 0.25 and 0.1% (w/w). Pre-emulsions were prepared by homogenising a mixture of the aqueous phase (75 ml) and the oil phase (25 ml) with an Ultra-turrax T25 (Janke & Kunkel GmbH & Co., Staufen, Germany) homogeniser for 1.5 min at 20,500 rpm. The pre-emulsion was homogenized using a Foss Electric Milko Tester having four homogenizing stages equivalent to a treatment of approximately 14 MPa. Emulsions (oil-to-protein ratios of 25, 100 and 250) with protein concentrations of 1.0, 0.25 and 0.1% (w/w) were also prepared using deuterium oxide ($D_2O$) for the NMR studies.

### 2.3. Measurement of droplet size

The particle size distribution of the emulsion was measured using a laser diffractometer light scattering method with a Malvern Mastersizer 2000 instrument (Malvern Instruments Ltd, Worcestershire, UK) and standard optical parameters. The emulsion sample was dispersed to recirculating water in the Hydro SM measuring cell, at 2500 rpm until an obscuration rate of 10–20% was obtained. Background and sample were measured for 12 s. A differential refractive index of 1.092 (1.456 for oil/1.333 for water) and the absorption of 0.001 were used as the optical properties of the emulsion. Each emulsion sample was measured in triplicate.

### 2.4. Light microscopy

The emulsion structure was observed, on preparation, by smearing the emulsion sample onto a microscope slide and using a ×20 magnification on an Olympus BH-2 light microscope (Olympus Co., Tokyo, Japan).

### 2.5. Optical characterisation of emulsion creaming

The emulsion stability to creaming was measured by the optical characterisation of the liquid emulsion dispersion using a light scattering optical analyser Turbiscan MA2000 (Formulaction, France). The freshly prepared emulsion sample (6 ml) was placed in a transparent cylindrical glass measurement cell and completely scanned by a light source. The back scattering of light over the whole height of the sample was recorded every 40 μm along the sample tube. The optical scanning of the emulsion sample was carried out every minute over 1 h. A pattern of the light flux as a function of the sample height was obtained, giving a macroscopic fingerprint of the sample at a given time. The superimposed light scatting fingerprints characterised the stability or instability of the emulsion. The creaming volume was calculated according to the following equation (Roland, Peil, Delattre, & Evrard, 2003):

$$C = 100(V_t - V_s)/V_t,$$

where $C$ is the creaming volume percentage, $V_t$ (ml) is the total volume of the sample, and $V_s$ (ml) is the volume of the lower phase layer.

### 2.6. Emulsion rheology

The stress viscometry measurements were carried out using a Paar Physica M300 controlled-stress rheometer (PHYSICA Meßtechnik GmbH, Stuttgart, Germany) at 20 °C with a double-gap (DG26.7) geometrical arrangement. Measurements were made immediately after a 5 min equilibration in the rheometer.

### 2.7. $^1H$, $^{13}C$ and $^{31}P$ NMR measurements

Emulsions made with $D_2O$ were used for NMR studies in order to minimise the water resonance that would normally dominate the proton spectra. The $T_2$ values from these spectra provide useful information regarding oil and protein molecular dynamics. Only the emulsions prepared with the protein concentrations of 1.0, 0.25 and 0.1% (i.e., oil-to-protein ratios of 25, 100 and 250) were used for the NMR experiments.

The low-field proton NMR experiments were carried out on a Minispec-20 MHz spectrometer (Bruker Minispec PC120). The spin–spin relaxation time, $T_2$, for the proton signal was measured using the spin–echo pulse sequence (90°–180°–180°–180°, etc.) known as the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence. Analysis of each sample was completed on 50 data points, resulting from the amplitudes of 50 sequential spin–echoes, where the amplitude of each spin–echo signal was the average of 4 scans.

All high-field NMR spectra were acquired at room temperature on a 300 MHz Varian-UnityPlus NMR spectrometer. The proton spectra of the emulsions were measured at 300 MHz, with 4 scans accumulated for each spectrum. The spin–lattice, $T_1$, and spin–spin, $T_2$, relaxation times for proton signals were measured by employing the inversion recovery pulse sequence (180°–90°) and CPMG spin–echo sequence, respectively.

The standard $^{13}C$ spectra of the emulsions were obtained at 75.4 MHz. Each sample was acquired with 128 scans and a repetition time of 2 s. The $^{31}P$ spectra of the emulsions were obtained at 121.4 MHz. Each sample was acquired with 64 scans and a repetition time of 10 s. All the measurements were performed at room temperature (20 ± 1 °C).

## 3. Results and discussion

### 3.1. Emulsion droplet particle size

The particle size distribution of emulsions, as measured on the diluted emulsions by light scattering, changed from a unimodal distribution for the emulsion containing higher amounts of protein (i.e., 1% w/w) to bi- and multi-modal distributions with the deceasing of protein content to 0.25% and 0.1% w/w, respectively (Fig. 1). The average particle sizes ($D_{4,3}$) of emulsions containing protein at 0.25–1% w/w (i.e., oil-to-protein ratios between 25 and 100) were 1.90–2.42 μm (Table 1). Further decreasing of protein concentration in the emulsion to 0.1% w/w (i.e., the oil-to-protein ratio to 250) caused a significant increase in the average oil droplet particle size. A distinct tail in the size distribution with a small proportion of oil droplet size greater than $D_{4,3} \approx 10$ μm was observed (Fig. 1). At this protein concentration, the size of emulsion droplets is determined by the amount of protein. It is also possible that the flocculated oil droplets were held together by casein bridges which were moderately stable on dilution.

The trends in particle size obtained with varying caseinate concentrations in emulsions were similar to those obtained previously for caseinate-stabilised soy oil emulsions (Srinivasan, Singh, & Munro, 2002). Our results are also in general agreement with Hogan and co-workers who found that protein was limiting in 30% (total solids) emulsions (15% oil:15% Na caseinate-carbohydrate blend) containing a 1:69 ratio of protein-to-carbohydrate (i.e., at an oil-to-protein ratio of 70:1) (Hogan, McNamee, O'Riordan, & O'Sullivan, 2001). In their emulsions containing protein and carbohydrates, only the protein was surface active.

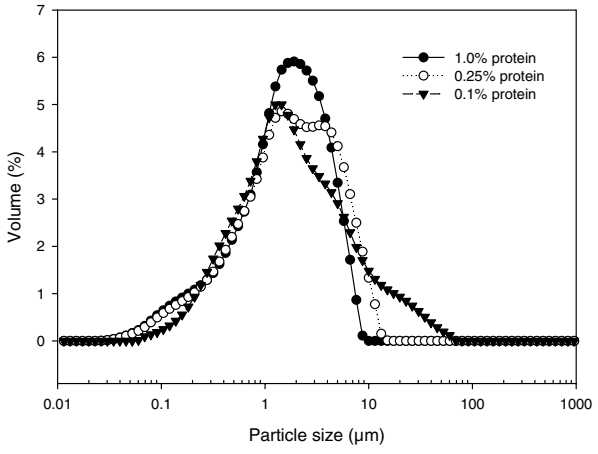


Fig. 1. Comparison of particle size distributions ($D_{4,3}$, volume%) of the emulsions containing 25% w/w oil with sodium caseinate concentrations of: ● 1.0% w/w (oil-to-protein ratio of 25); ○ 0.25% w/w (oil-to-protein ratio of 100); and ▼ 0.1% w/w (oil-to-protein ratio of 250).

In our work, the particle size was independent of protein content from 0.5% to 1.0% w/w, i.e., when the oil-to-protein ratio was between 25 and 50. Under these conditions, the particle size is governed primarily by the homogenisation pressure. There was a slight increase in particle size when the protein content was reduced to 0.25% w/w (i.e., at the oil-to-protein ratio of 100) and the appearance of bi-modal distribution (Fig. 1), indicating that the protein had started to become limiting for surface coverage. When protein is limited, there is no longer sufficient protein to fully stabilise the droplet interface, and therefore larger particles may be formed as a result of coalescence or bridging flocculation. With light scattering, it is not possible to differentiate between these two possibilities, although this can be determined using optical microscopy.

Microscopic examination showed that emulsions were (a) aggregated when the protein content was either at the high (1.0% w/w protein, oil-to-protein ratio of 25; Fig. 2A) or low (0.1% w/w protein, oil-to-protein ratio of 250; Fig. 2C) and (b) more homogeneous at an intermediate protein concentration (0.25% w/w protein, oil-to-protein ratio of 100; Fig. 2B). While the micrograph showed aggregates/flocculates in emulsions with high protein content, these were absent in the diluted emulsion used for the particle sizing by light scattering (Fig. 1). This may be explained by the occurrence of depletion flocculation in undiluted emulsions where there is sufficient protein for the coverage of oil droplet surface and excess protein in the aqueous phase. When the emulsion is diluted there is insufficient protein in the aqueous phase; therefore, no depletion flocculation occurs. Also the shear forces, exerted during the measurement of particle size in the Mastersizer, will be sufficient to break-up the weak reversible flocculation. The reversibility of depletion flocculation on dilution without coalescence has been previously observed in caseinate-stabilised oil-in-water emulsions (Dickinson et al., 1997). On the other hand, in cases where protein is limiting, the cause of aggregation could be coalescence or bridging flocculation. The data from the light scattering corroborate the information from the micrographs. At low protein concentration (0.1% w/w, oil-to-protein ratio 250; Fig. 2C), there is some evidence of coalescence and bridging flocculation.

Bridging and depletion flocculation in caseinate emulsions with a different non-polar core and composition (30–45 vol% *n*-tetradecane) have been previously observed (Dickinson et al., 1997). While the trends observed in our study were similar, it is difficult to make direct comparisons because proteins are adsorbed differently to hydrocarbon or triglyceride interfaces. The surface concentration of protein in the soy oil emulsion is approximately half of that in the tetradecane emulsion, due to the fact that the casein molecule adsorbed to a tetradecane–water interface has a secondary structure similar to that of the molecule in solution, while at a triglyceride–water interface it adopts a more open, stretched and fairly

Table 1
Formulations of emulsions containing 25% (w/w) oil with various amounts of sodium caseinate and particle size for each emulsion

| Protein content (%, w/w ingredient) | Tuna oil (%, w/w) | Oil-to-protein ratio | Emulsion total solids (% w/w) | Particle size | |
|---|---|---|---|---|---|
| | | | | $d_{32}$ (μm) | $d_{43}$ (μm) |
| 1.00 | 25.00 | 25 | 26.00 | 0.65 ± 0.01 | 1.90 ± 0.01 |
| 0.75 | 25.00 | 33 | 25.75 | 0.72 ± 0.04 | 1.91 ± 0.04 |
| 0.50 | 25.00 | 50 | 25.50 | 0.71 ± 0.04 | 1.97 ± 0.01 |
| 0.25 | 25.00 | 100 | 25.25 | 0.71 ± 0.04 | 2.42 ± 0.01 |
| 0.10 | 25.00 | 250 | 25.10 | 0.94 ± 0.03 | 13.3 ± 3.6 |

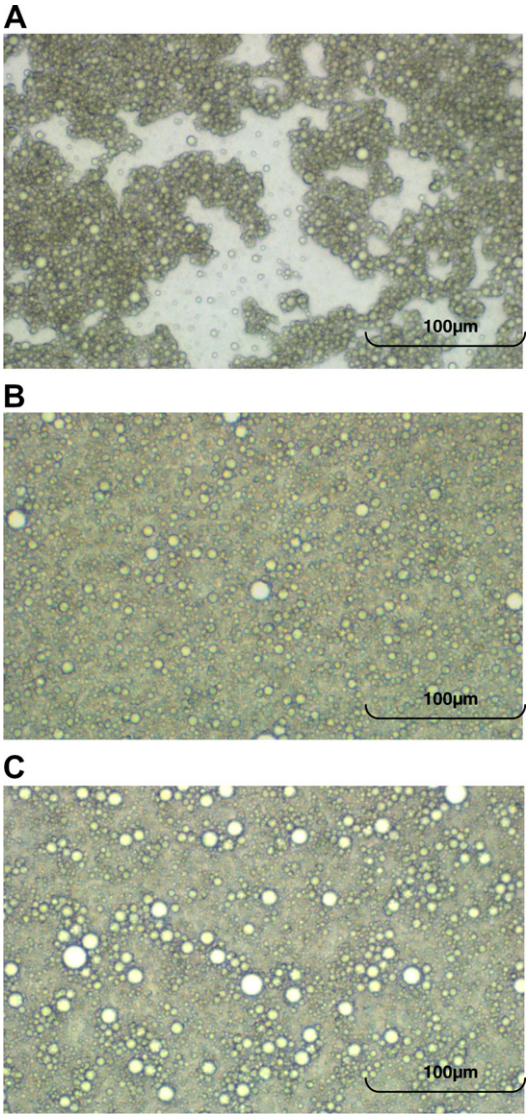


Fig. 2. Micrographs-emulsions containing 25% (w/w) fish oil at the protein concentrations of: (A), 1.0% w/w (oil-to-protein ratio of 25), (B), 0.25% w/w (oil-to-protein ratio of 100), and (C), 0.1% w/w (oil-to-protein ratio of 250).

flat conformation (Leaver & Dalgleish, 1992). Consequently, protein covers a larger surface area on the triglyceride than on the hydrocarbon oil. In other words, less protein is required to stabilise the triglyceride emulsion than the tetradecane emulsion. This work also showed that intermediate protein concentrations produce stable homogeneous emulsions, similar to that reported previously (Dickinson et al., 1997).

### 3.2. Emulsion stability and rheology

Emulsion stability was examined using the optical characterisation method with a TurbiScan. The backscattering patterns were measured as a function of the time and the emulsion height. The light scattering fingerprints of the emulsion samples, with protein concentrations of 1%, 0.25% and 0.1% w/w, respectively, are shown in Fig. 3. The emulsions were scanned over 1 h immediately after preparation and after 24 h of storage at room temperature. The absolute backscattering at a fixed level (i.e., 80%) was chosen to represent the phase change of emulsion (low layer) as a function of time. The creaming instability of the 1% w/w caseinate emulsion could be observed a few minutes after the preparation of the emulsion and significant changes in the backscattering during the first hour (Fig. 3A) clearly indicate the instability of this emulsion. The back scattering data obtained after 24 h also showed a further progress of emulsion instability, as indicated by the further creaming volume percentage reduction (Fig. 4). Similar observations were also found with the emulsion containing 0.75% and 0.5% w/w of protein (Fig. 4).

When the amount of protein used to stabilise fish oil-in-water emulsions was reduced to 0.25% and 0.1% w/w, the optical characteristics of these emulsions showed few changes in their backscattering patterns within the first hour after emulsion preparation (Fig. 3B and C, respectively), even though the particle size results and the microscopic examination indicate the presence of bridging flocculation in the 0.1% w/w caseinate emulsion. The emulsion showed that little or no creaming occurred, even after a 24 h storage (Fig. 4), suggesting that bridging flocculation may have a stabilising effect due to limited protein surface coverage (Dickinson et al., 1997). This is most likely due to the fact that, relative to depletion, bridging flocculation tends to progress much more slowly and will not necessarily affect the creaming rate during the early part of emulsion storage.

The rheology behaviour of these fish oil emulsions was studied by the measurement of their steady-state shear

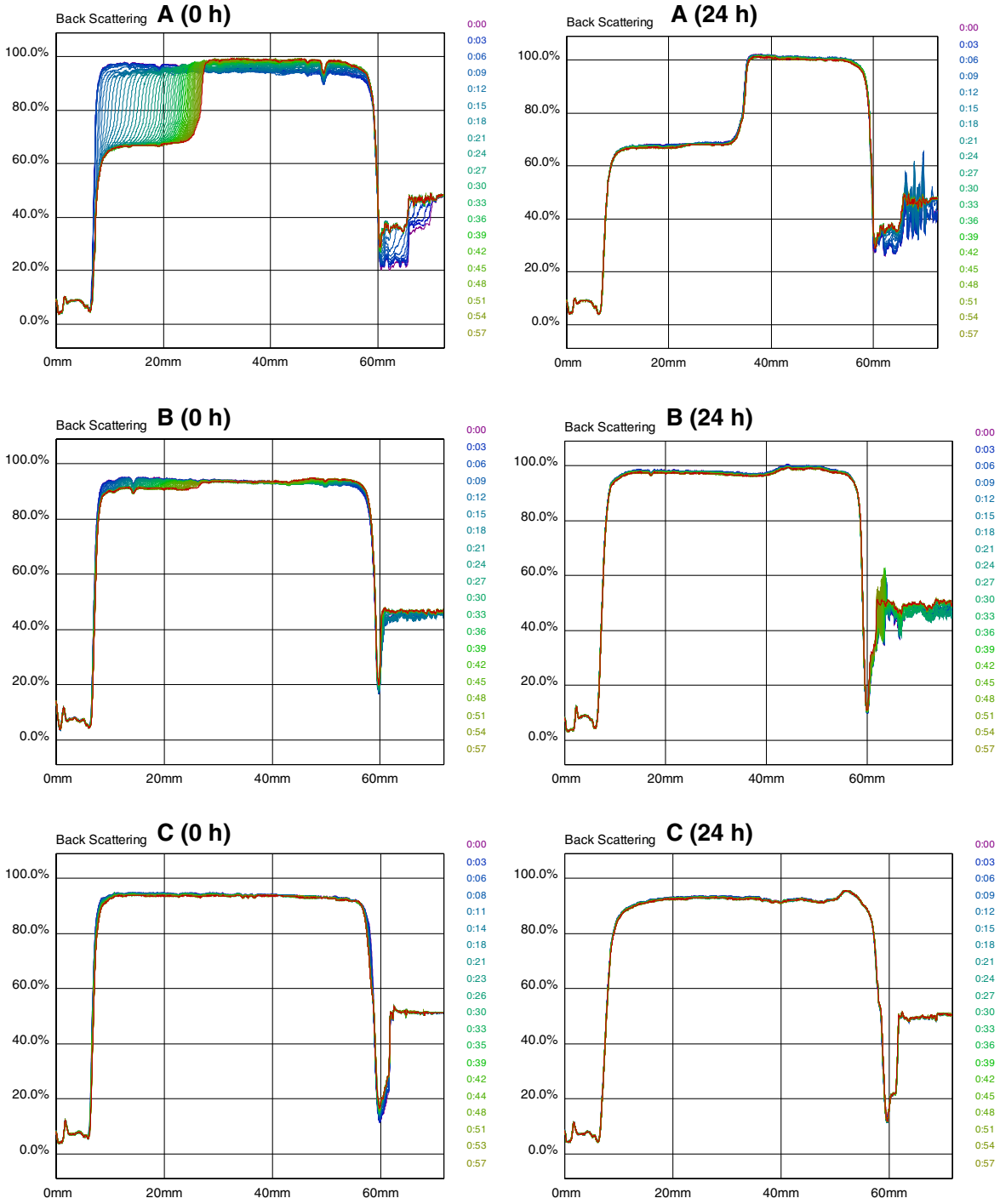


Fig. 3. Back scattering patterns of emulsions containing 25% (w/w) oil at the protein concentrations of: (A), 1.0% w/w (oil-to-protein ratio of 25), (B), 0.25% w/w (oil-to-protein ratio of 100), and (C), 0.1% w/w (oil-to-protein ratio of 250); recorded for 1 h immediately after emulsion preparation and after a 24 h storage using TurbiScan.

viscometry. The apparent viscosities as a function of shear stress, for emulsion samples containing 1, 0.25 and 0.1% w/w of protein, are shown in Fig. 5. The fish oil emulsion containing 0.25% w/w caseinate showed a constant viscosity, independent of shear stress, i.e., a stable Newtonian liquid, while the emulsions prepared with high (1.0% w/w) and low (0.1% w/w) protein contents showed a shear-thinning behaviour. However, the low-shear viscosity of the 1% w/w caseinate emulsion was much higher than that of the 0.1% w/w caseinate emulsion, and both were higher than that of the 0.25% w/w caseinate emulsion.

The results showed emulsion rheological behaviour similar to the *n*-tetradecane-in-water emulsion systems (35 vol% *n*-tetradecane, 1–6% wt% caseinate) studied by

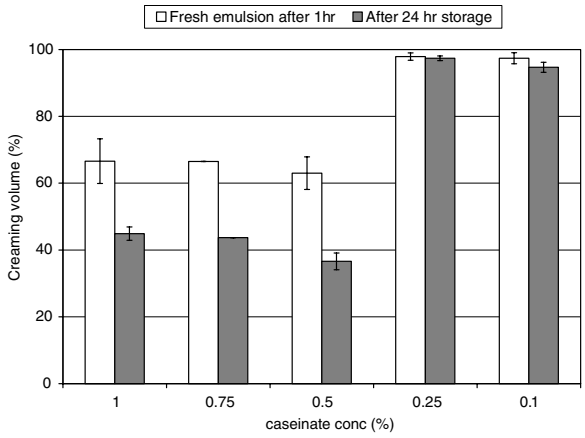


Fig. 4. Creaming stability of emulsions containing 25% (w/w) oil stabilised by sodium caseinate at various concentrations after 1 h and 24 h, determined by the creaming volume percentage from the back scatting data using TurbiScan. A large value of creaming volume percentage is an indication of a more stable emulsion (Roland et al., 2003).

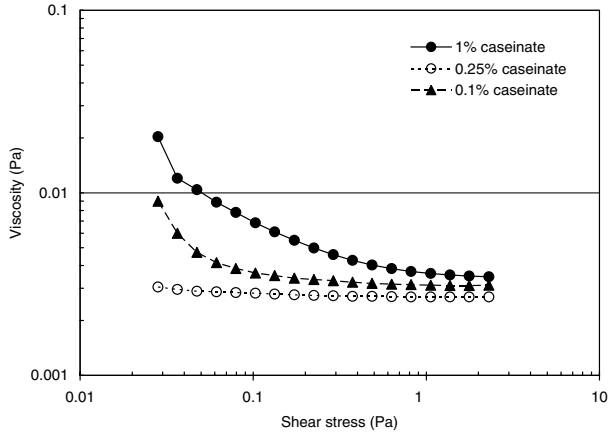


Fig. 5. Steady-state shear viscometry of emulsions containing 25% (w/w) oil stabilised by sodium caseinate at the concentration of ● 1.0% w/w (oil-to-protein ratio of 25), ○ 0.25% w/w (oil-to-protein ratio of 100), and ▲ 0.1% w/w (oil-to-protein ratio of 250). Apparent viscosity is plotted against applied stress.

Dickinson and Golding (1997). It was concluded that emulsions having full protein surface coverage, but relatively little excess unadsorbed protein in the continuous phase, were stable Newtonian liquids. Taking into account the particle size, microscopic and creaming stability results, it is clear that, in our system, the fish oil (25% w/w) emulsion stabilised by 0.25% w/w sodium caseinate was the most homogeneous stable emulsion with full protein surface coverage and relatively little excess protein in the aqueous phase.

At the highest protein concentration, i.e., 1% w/w caseinate, the aggregated structure from the depletion-flocculated emulsion droplets, due to the existence of a substantial excess amount of unadsorbed protein in the aqueous phase, leads to an increase in the effective volume fraction of hydrodynamically interacting particles, thereby resulting in a much higher apparent viscosity than for the homogeneous emulsion. As a result, the break-up of the aggregates under the high local stress would lead to a reduction in the effective dispersed phase volume fraction, and hence a reduction in the apparent viscosity as a function of increased shear stress (Dickinson & Golding, 1997).

At the lowest protein concentration, i.e., 0.1% w/w caseinate, increased particle size was observed (Figs. 1 and 2); however, increasing average droplet size is not normally associated with an increase in emulsion viscosity. The increase in emulsion particle size and low-shear viscosity is likely due to very slow bridging flocculation at the relatively low protein surface coverage (Dickinson & Golding, 1997). The associated shear-thinning behaviour was related to the breaking of the protein bridges between the droplets under the applied shear stress.

### 3.3. NMR

Low and high-field $^{1}H$ NMR and high-field $^{13}C$ and $^{31}P$ NMR were used to examine the emulsion systems with different stability characteristics that had been characterised above, namely, (a) the emulsion containing excessive protein (1% w/w) with low stability to creaming due to depletion flocculation, (b) the emulsion with medium protein content (0.25% w/w) with full surface coverage of oil droplets and stable to creaming, and (c) the emulsion containing a limited amount of protein (0.1% w/w) with primarily bridging flocculation. These emulsions were prepared in $D_2O$ in order to eliminate strong proton signals from water in the NMR spectra. The oil signals are expected to dominate the $^{1}H$ NMR and $^{13}C$ NMR spectra due to the higher mobilities of the oil molecules in comparison to the protein at the interface and in the bulk aqueous phase. Both $^{1}H$ NMR and $^{13}C$ NMR can provide information about oil content and its mobility. Since phosphorus is only present in the protein component of the emulsion, specifically the phosphoserine groups of caseins, $^{31}P$ NMR spectra can reveal the restricted mobility of the casein at the interface and differentiate it from protein in the bulk aqueous phase.

In evaluating the proton NMR, it is instructive to first consider the different mobilities of the oil molecules. As a first approximation, the motion of the oil molecules within the oil droplet can be assumed to be comprised of the motion of the oil molecules in the interior of the oil droplet and those near the protein interface. It can be further expected that the oil molecules in the interior of the droplet will have higher mobility than those that are more immobilised at the interface due to their increased interaction with the protein. In addition to the motion of the oil within the droplets, there will also be some contribution from translational diffusion of oil droplets within the emulsion. In the homogeneous emulsion systems, where oil droplets exist as discrete entities for most of the time, the diffusion is unrestricted; however, in emulsions, when there is flocculation, the translational movement of the oil droplets will be restricted. The

proton signal is therefore expected to be a multi-exponential function due to the different proton environments and various segmental dynamics of the glyceride chains. The relative contributions of the motion of the oil molecules to the signals obtained in low-field and high-field NMR were the combination of the observed total signal.

The analysis of the decay from the low-field proton NMR signals of the emulsions (1.0, 0.25 and 0.1% w/w caseinate; oil-to-protein ratios of 25, 100 and 250), using a double exponential function, yielded upper and lower limiting values of $T_2$. The short and long spin–spin relaxation times and their amplitudes are presented in Table 2. The total spin–echo signal amplitudes were similar for all three emulsions, thus confirming that the oil component (25% w/w same amount in all emulsions) dominated the signal. This is understandable, as the CPMG spin–echo sequence was applied at a pulse spacing of 1 ms, so that the proton signal from the mobile molecules (oil) dominated the relaxation time $T_2$. The protein components that stabilise the interface or exist as colloidal particles in the aqueous phase make no or only a negligible contribution to the NMR signal, as their $T_2$ values are shorter than the pulse spacing in the CPMG sequence. Thus the $T_2$ values from low-field NMR provide information about the mobility of the oil, with the shorter values indicating more restricted motion. While the mobility of the oil can be comprised of contributions from the diffusion of the oil droplets and the mobility of the individual oil molecules within the oil droplet, the $T_2$ values from low-field NMR appeared to be dominated by the structure of the bulk emulsion. The $T_2$ values from low-field NMR were shorter in the flocculated emulsions containing either excessive (1% w/w) or limited amounts of protein (0.1% w/w) and longest for the homogeneous emulsion with 0.25% w/w protein where the diffusion of the whole oil droplet was less restricted (Table 2). The flocculation, irrespective of whether it is depletion or bridging flocculation, restricted the mobility of the oil molecules and this was clearly reflected by their shorter $T_2$ values in the low-field NMR signal, in comparison to the $T_2$ value obtained for the homogeneous emulsion with the protein content of 0.25% w/w.

The high-field $^1$H NMR spectra of the three emulsions had the same resonance pattern but varying line-width (Fig. 6). Decreasing line-width with decreasing protein content was evidence of the increasing mobility of the oil components. This may be attributed to the decreased interaction between the oil and protein in the emulsion with the decrease in overall protein content in the system. The assignment of the peaks from the $^1$H NMR spectra and the relaxation times of the proton resonances are given in Table 3. The mobility of the individual groups of oil resonances varied (Table 3). The residual water (non-deuterated) was assigned at 4.8 ppm and used as an internal reference proton peak. The relaxation time, $T_2$, of the residual water increased as the protein concentration in the emulsions decreased. This was expected because the protein concentration in the bulk decreases relative to the concentration of protein adsorbed at the oil–water interface. Therefore, in comparison to the emulsion with higher protein content, there would be less hydrated protein molecules in the aqueous phase and consequently an increased mobility of water molecules in the emulsion with lower protein content.

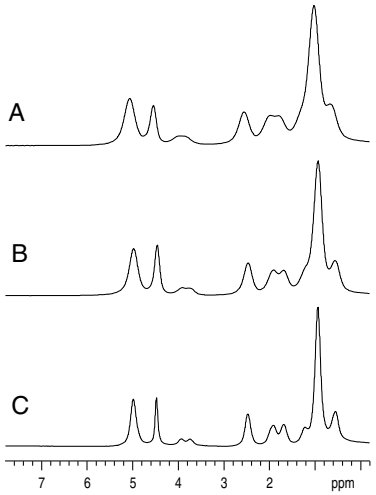


Fig. 6. The high-resolution proton NMR spectra of the emulsions in $D_2O$ containing 25% (w/w) oil with protein contents of: (A), 1.0% w/w (oil-to-protein ratio of 25), (B), 0.25% w/w (oil-to-protein ratio of 100), and (C), 0.1% w/w (oil-to-protein ratio of 250).

Table 2
The short and long $T_2$ values, as determined by a double exponential fit of spin–echo signal decay, from low-field proton NMR spectra of the deuterated emulsions containing 25% (w/w) fish oil

| Protein content (%) | $T_2$[a] (ms)$^{short}$ | $T_2$[a] (ms)$^{long}$ | Amplitude[b] (%)$^{Short}$ | Amplitude[b] (%)$^{long}$ |
|---|---|---|---|---|
| 1.00 | 5.3 | 93.4 | 16 | 84 |
| 0.25 | 12.4 | 106.5 | 13 | 87 |
| 0.10 | 6.0 | 91.2 | 13 | 85 |

[a] Standard error ± 3%.
[b] Standard error ± 3%.

Table 3
Assignment of resonances obtained in high-field $^1$H NMR for the groups on the oil molecule (Guillen & Ruiz, 2003), and the short and long $T_2$ values, as determined by a double exponential fit of spin–echo signal decay (standard error ± 3%)

| Protein content in emulsion (% w/w) | | $T_2$ (ms) | | |
|---|---|---|---|---|
| Resonance $\delta$ (ppm) | Group position | 1.00 | 0.25 | 0.10 |
| 5.36 | =C*H*– | 104 | 117 | 122 |
| 4.8 | Water | 200 | 292 | 302 |
| 4.28 and 4.11 | –O–C*H*$_2$–C | 24 | 27 | 20 |
| 2.85 | =CH–C*H*$_2$–CH= | 84 | 93 | 97 |
| 2.27 | =CH–C*H*$_2$–CH$_2$–CH=O | | 49 | 48 |
| 2.04 | =CH–CH$_2$–C*H*$_2$–CH=O | 81 | 65 | 71 |
| 1.60 | =CH–C*H*$_2$–CH$_2$–C | | | |
| 1.30 | C–C*H*$_2$–C | 67 | 70 | 71 |
| 0.97 | –C*H*$_3$ | 161 | 155 | 184 |

The $T_2$ values from the high-field NMR varied between the different groups of the triacylglycerol molecule (Table 3). There are many factors that may affect the mobility of these groups, such as the size of the droplet, the positions of the groups within the droplet and the state of aggregation of the droplets. However, the data did not appear to be dominated by the bulk properties of the emulsions as there is no consistent trend for the $T_2$ values in flocculated emulsions (i.e., with 0.10 and 1.0% w/w protein) or the homogeneous emulsion (0.25% w/w protein). This could be because the signal was predominantly related to the spatial positions of the groups of the triacylglycerol moiety within an individual oil droplet. In this case, the mobility of the oil molecules is dominated by its interactions with other oil molecules inside the droplet. There will be a contribution from the interactions between the oil molecules and the proteins at the interface but this will be small in comparison to the interactions among oil molecules. This is because there are a smaller number of all oil molecules at the interfaces than within a single oil droplet. This explains why the observed high-field proton NMR signal of the same groups on the triacylglycerol within the oil droplets exhibit similar dynamics, depending on their position within the droplet. The greater mobility of the $-CH_3$ group ($\delta = 0.97$ ppm) compared to other resonances of the triacylglycerol molecule suggests that it is further away from the interface than the other groups.

The $^{13}C$ NMR spectra of emulsions are shown in Fig. 7. The oil signal dominates as it is the most mobile species in the emulsions. The line-width of the oil peaks decreased with the decrease of protein content in the emulsion (i.e., as the oil-to-protein ratio increased). These trends are similar to those observed in the high-field $^1H$ NMR spectra, confirming the increased mobility of the oil molecules at the lower protein concentration (i.e., higher oil-to-protein ratio). As there is no interference from proton peaks in $^{13}C$ NMR, it is possible to use these $^{13}C$ NMR resonance lines to confirm the oil contents of emulsions. Quantification of the oil content requires collection of the carbon spectra under the same spectroscopic conditions and at the same signal scale calibration. The first major condition that needs to be satisfied is to have sufficient delay between the scans ($>5 \times$ longest $T_1$) to detect the proportional amount of each molecule (i.e., no saturation) and the second major condition is that the observed sample volume is homogeneous (i.e., no phase separation) during the time of data collection. The carbon spectra obtained under such conditions confirmed that the oil content was nearly the same in all samples, within 3% experimental error, as it should be according to the composition. The higher peaks in the spectrum coincide with a narrower line-width but with nearly the same integral intensity.

The phosphorus spectra of these emulsions are presented in Fig. 8. When the concentration of protein decreased, there was a decreased phosphorus signal in the $^{31}P$ NMR spectra. In addition, with decreasing protein concentration (i.e., when the ratio of oil-to-protein increased), the resonance line became broader. This broadening of the peak is consistent with increased spreading of the protein at the interface. At a lower protein concentration (0.1% w/w protein), there is less protein to cover oil surfaces and therefore a higher proportion of the protein immobilised at the interface compared to that in the bulk. It has been reported that increasing the oil concentration whilst maintaining a constant protein level, leads to a reduced surface concentration of protein, thus suggesting the spreading of protein at an interface to form a thinner layer (Srinivasan, Singh, & Munro, 1996). Another contributing factor to the broadening peak observed at the low protein concentration (0.1% w/w) could be the bridging flocculation in these emulsions. However, it appears that protein–protein interactions have less of an effect on protein mobility than has the immobilisation caused by adsorption to an interface. This explains the narrowing of the peak obtained in depletion-flocculated emulsions

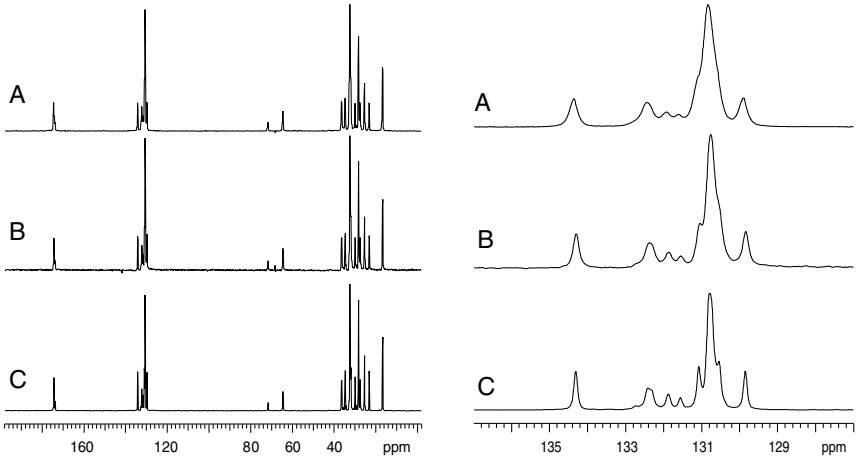


Fig. 7. The high-resolution carbon spectra of the emulsions in $D_2O$ containing 25% (w/w) oil with protein contents of A, 1.0% w/w (oil-to-protein ratio of 25), B, 0.25% w/w (oil-to-protein ratio of 100), and C, 0.1% w/w (oil-to-protein ratio of 250). Double bond regions are shown on right.

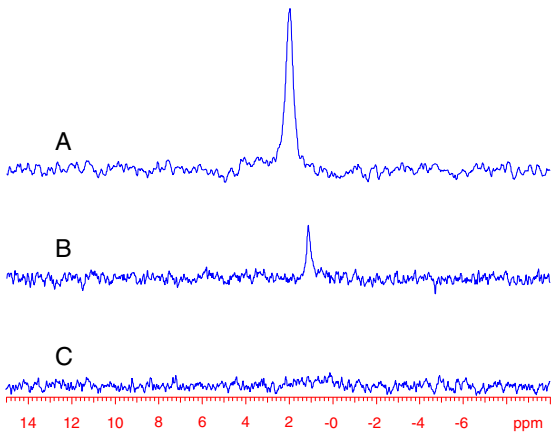


Fig. 8. The $^{31}P$ NMR solution spectra of the emulsions in $D_2O$ containing 25% (w/w) oil with protein contents of: (A), 1.0% w/w (oil-to-protein ratio of 25), (B), 0.25% w/w (oil-to-protein ratio of 100), and (C), 0.1% w/w (oil-to-protein ratio of 250).

with a high protein concentration (1.0% w/w) where there was protein–protein interactions in the bulk but also sufficient protein to cover the oil droplets, so that less spreading of the protein was necessary. Work by Mine (1997) has indicated that the adsorption of caseins to the oil–water interface causes broadening of phosphorus NMR resonances which are associated with a change in conformation of the protein on adsorption.

## 4. Conclusions

Fish oil emulsions, stabilized using sodium caseinate (25% w/w oil and 0.1–1.0% w/w sodium caseinate giving oil-to-protein ratios of 250–25), were either homogeneous or exhibited depletion or bridging flocculation, depending on the concentration of protein in the formulation. These were characterised by a suite of techniques (light scattering, microscopy, Turbiscan and rheology) and NMR ($^1H$, $^{13}C$ and $^{31}P$ NMR).

Reversible depletion flocculation was evident in the emulsion containing excessive amount of caseinate (1% w/w) by the microscopic examination, its high low-shear viscosity and shear-thinning behaviour. The emulsion prepared with 0.25% w/w protein showed a homogeneous distribution of oil droplets with good stability to creaming and viscosity independent of shear stress (i.e., a Newtonian liquid), confirming that the oil droplets having full protein surface coverage and relatively little excess unadsorbed protein were in the continuous phase. Emulsion particle sizes were relatively constant until the amount of protein used to stabilise the emulsion was reduced to 0.1% (w/w). Although some oil droplet coalescence might have occurred in the 0.1% w/w caseinate emulsion, as indicated by the microscopy, the predominant origin of the increased particle size is likely to be contributed by the slow aggregation procession of bridging flocculation, as demonstrated by its rheological shear-thinning behaviour and relatively high stability in creaming.

The examination of these emulsions (in $D_2O$) by low- and high-field NMR provided further information in terms of oil motion in the emulsion and oil/protein interaction at the interface. The $T_2$ values obtained from low-field $^1H$ NMR measurements were sensitive to the structure of the bulk emulsion, with shorter $T_2$ values being obtained in flocculated emulsions. The high-field NMR spectra ($^1H$, $^{13}C$), which primarily detect the oil molecules, had increased line broadening with the increase in protein content, due to increased interaction between oil molecules and proteins at the interface. In addition, $^{31}P$ NMR spectra, which reflect the mobility of the casein component only, showed increased line broadening at lower protein content, due to the increasing proportion of the protein immobilised at the interface of the oil droplets compared to that in the continuous phase at the higher oil-to-protein ratio. The $T_2$ resonance values of the individual groups of oil molecules, obtained using high-field $^1H$ NMR, were influenced by the mobility of individual groups on the triacylglycerol molecule, reflecting the different environments within the oil droplet and indicating that $-CH_3$ was the most mobile and furthest away from the oil/protein interface. The techniques used provide complementary information and new insights into the characteristics of the emulsion.

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