Physicochemical properties of whey protein, lactoferrin and Tween 20 stabilised nanoemulsions: Effect of temperature, pH and salt

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\textbf{Abstract}

Oil-in-water nanoemulsions were prepared by emulsification and solvent evaporation using whey protein isolate (WPI), lactoferrin and Tween 20 as emulsifiers. Protein-stabilised nanoemulsions showed a decrease in particle size with increasing protein concentration from 0.25\% to 1\% (w/w) level with Z-average diameter between 70 and 90 nm. However, larger droplets were produced by Tween 20 (120–450 nm) especially at concentration above 0.75\% (w/w). The stability of nanoemulsions to temperature (30–90°C), pH (2–10) and ionic strength (0–500 mM NaCl or 0–90 mM CaCl\textsubscript{2}) was also tested. Tween 20 nanoemulsions were unstable to heat treatment at 90°C for 15 min. WPI-stabilised nanoemulsions exhibited droplet aggregation near the isoelectric point at pH 4.5 and 5 and they were also unstable at salt concentration above 30 mM CaCl\textsubscript{2}. These results indicated that stable nanoemulsions can be prepared by careful selection of emulsifiers.

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1. Introduction

Over the last two decades, considerable studies have been carried out to design novel emulsion systems with improved stability and delivery in the human gastro-intestinal tract. One of the possible delivery systems used for this purpose is in the form of nanoemulsions. Nanoemulsions can be defined as small emulsion droplets with mean particle diameter in the size range of 10–100 nm (Lee, Choi, Li, Decker, & McClements, 2011; Tadros, Izquierdo, Esquena, & Solans, 2004). In contrast, conventional emulsions have larger droplet size ranging from 0.1 to 100 \mu m in diameter (Lee et al., 2011; McClements, 2010). The relatively small droplet size of nanoemulsions compared to conventional emulsions means that nanoemulsions have different physicochemical properties with potential applications in food systems (McClements, 2010; Weiss, Gaysinsky, Davidson, & McClements, 2009). For instance, nanoemulsions tend to appear transparent or translucent due to the small particle size (smaller than the wavelengths of light) which results in lower reflectance of light (McClements, 1999). Therefore, nanoemulsions can be used to subtly incorporate bioactive compounds into transparent or translucent food systems without affecting their visual appearance. Nanoemulsions also have better physical stability against gravitational separation and droplet aggregation than conventional emulsions (McClements & Rao, 2011; Weiss et al., 2009).

In the present study, nanoemulsions were produced by a high pressure emulsification–solvent evaporation process (Lee & McClements, 2010; Lee et al., 2011). This method involves the use of high pressures to produce nanoemulsions but with the addition of solvent (e.g. ethyl acetate). The solvent added into the oil phase prior to high shear emulsification is subsequently removed from the system by evaporation, which results in the formation of oil droplets with smaller particle diameter (<100 nm). Several emulsifiers have been used to prepare nanoemulsions by this method, including whey protein isolate (WPI), Tween 20, sodium caseinate and soy protein isolate (Kim, Ha, Choi, & Ko, 2014; Lee et al., 2011; Troncoso, Aguilera, & McClements, 2012; Yi, Lam, Yokoyama, Cheng, & Zhong, 2014). It is fairly well established in conventional emulsions that emulsifiers play an important role to form and stabilise droplets by rapidly adsorbing at the droplet’s interface and conferring stability to emulsion through electrostatic
and/or steric repulsion (Degner et al., 2014; McClements, 1999). However, there is no systematic study on the physicochemical properties of nanoemulsions prepared by this method. In this work, we evaluated the role of different emulsifiers on the physical functionality of nanoemulsions prepared using emulsification and solvent evaporation. Three different emulsifiers, namely, whey protein isolate (WPI), lactoferrin and Tween 20 were selected and used based on their characteristics. In particular, WPI and lactoferrin are large protein emulsifiers with different electrical charges, e.g., negatively and positively charged, respectively, at neutral pH, while Tween 20 is a non-ionic small molecule surfactant. The effect of these emulsifiers on the formation and stabilisation of nanoemulsions were not well studied systematically.

Whey proteins are a group of milk proteins classified as globular proteins consisting of mostly β-lactoglobulin and α-lactalbumin (Degner et al., 2014; Livney, 2010). Whey proteins can quickly adsorb to the droplet surface, reduce the interfacial tension and form a protective membrane around the oil droplets to prevent droplet aggregation (Kulmyrzaev, Chanamai, & McClements, 2000; McClements, 1999). One important feature of whey proteins is their good water solubility over a wide range of pH values (Hosseinpour, Izadi, Aminlari, Kamezani, & Tavana, 2011; Singh, 2011). They can remain soluble even at pH near the isoelectric point (around pH 4.9) if their native states have not been denatured. However, whey proteins are susceptible to heat denaturation at temperature above 70 °C (Anema, 2008). The denatured whey proteins unfold and expose reactive groups for protein aggregation.

Lactoferrin is an iron-binding glycoprotein with sugar moieties attached (Baker & Baker, 2005; Steijns & Van Hooijdonk, 2000). Due to the high levels of basic amino acids, lactoferrin has a high isoelectric point of around pH 9 with high concentrations of positive charges on the molecule’s surface (Steijns & Van Hooijdonk, 2000). Previous studies have also shown that lactoferrin is an excellent emulsifying agent as it adsorbs to the oil-water interface and produces a cationic emulsion (Tolke & McClements, 2011; Ye & Singh, 2006). Tween 20 (polyoxyethylene sorbitan monolaurate) is a non-ionic surfactant used to stabilise oil-in-water (O/W) emulsion. They can also rapidly adsorb to the surface of oil droplets and reduce interfacial tension to prevent droplet coalescence (Degner et al., 2014; Jo & Kwon, 2014).

The objectives of this work were to examine the influences of emulsifier types, concentrations, temperature, pH and ionic strength on the physicochemical properties of nanoemulsions.

2. Materials and methods

2.1. Materials

Whey protein isolate (Alacen™ 895) consisting of 93.9% protein, 0.3% fat, 4.7% moisture and 1.5% ash was supplied by Fonterra Co-operative Group Limited (New Zealand). Lactoferrin was obtained from Tatua Co-operative Dairy Company Limited (Morrinsville, New Zealand). Tween 20 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Corn oil was purchased from a local food supplier (Davies Trading Company, Palmerston North, New Zealand). Ultrapure water purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA) was used to prepare all the solutions used in the experimental works. Ethyl acetate (HPLC grade) was purchased from Fischer Scientific (New Jersey, USA). Hydrochloric acid, sodium hydroxide, sodium chloride, calcium chloride and sodium azide were analytical grade and purchased from Thermo Fisher Scientific (Victoria, Australia) or BDH Chemicals (Poole, England).

2.2. Preparation of nanoemulsions

Nanoemulsions were prepared by emulsification and solvent evaporation technique according to the method reported by Lee et al. (2011) but with some modifications. In this method, two different phases, denoted as organic phase and aqueous phase, were initially prepared separately and then mixed at an organic and aqueous phase ratio of 10:90. The aqueous phase consisted of an emulsifier solution (0.25–5% w/w) while the organic phase consisted of 90% (w/w) ethyl acetate and 10% (w/w) corn oil. The aqueous phase and organic phase were mixed together using a high shear mixer (Ultra Turrax® T25 Basic, IKA, Staufen, Germany) at 16,000 rpm for 2 min to form a coarse emulsion. The coarse emulsion was passed through a high-pressure homogeniser (M-110P, Microfluidics, Westwood, MA, USA) for 4 times at 12,000 psi (82.7 MPa) to produce a fine emulsion. The fine emulsion was subjected to evaporation in a round bottom flask to remove ethyl acetate using a rotary evaporator (Buchi Rotavapor R-215, Vacuum Controller V850 and Heating Bath B-491, BUCHI Labortechnik AG, Flawil, Switzerland) operated at 50 °C with a vacuum pressure of 153 mbar for 20–25 min. During evaporation, in addition to ethyl acetate, some water was also removed. The final oil concentration of nanoemulsions after evaporation was adjusted accordingly to 0.5% (w/w) by adding water. After adding 0.02% (w/w) sodium azide as an antimicrobial agent, the nanoemulsion samples prepared were stored for 1 day at room temperature (20 ± 1 °C) before analysis.

2.3. Effect of environmental conditions on nanoemulsions

The effects of heat treatment, pH and ionic strength on the stability of nanoemulsions prepared with different emulsifiers (WPI, lactoferrin or Tween 20) at 1% (w/w) emulsifier level were studied. The effect of heat treatment was carried out by heating the emulsion samples in water baths at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C) for 15 min. After heating, the samples were immediately cooled to room temperature by placing them in a chilled ice water bath. The pH stability of emulsions was determined by adjusting the pH of emulsions to different pH values (2, 3, 4, 5, 6, 7, 8, 9 and 10) using different concentrations of HCl or NaOH solutions. The addition of salt on the stability of emulsions was also determined by using sodium chloride (NaCl) or calcium chloride (CaCl₂). The emulsions were mixed with different concentrations of salt solutions and the final concentration of salt in the emulsions was 0, 50, 100, 200 and 500 mM NaCl or 0, 10, 30, 60 and 90 mM CaCl₂.

2.4. Characterisation of nanoemulsions

2.4.1. Particle size and size distribution

The particle size and size distribution of emulsions were measured by dynamic light scattering technique using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) equipped with a helium/neon laser at a wavelength of 633 nm and analysed at a backscattering of 173°. The emulsion samples were measured without further dilution during the size measurement. The particle size results were reported as the Z-average mean diameter. For those aggregated samples used in the environmental study, they were measured using a Mastersizer instrument (Mastersizer 2000 Hydro MU, Malvern Instruments, Worcestershire, UK). This applies to those WPI-stabilised nanoemulsions with pH adjusted to 4.5 and 5 and salt concentrations above 30 mM CaCl₂. Their particle size were reported as the volume mean diameter, \( D_{4,3} = \frac{\sum n_i d_i^3}{\sum n_i d_i} \), where \( n_i \) is the amount of droplets with diameter \( d_i \).
2.4.2. Zeta potential (\(\zeta\)-potential)

The \(\zeta\)-potentials of emulsions were determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) and disposable zeta potential cells (“Size & Zeta” folded capillary cell; DTS1070). The samples were used without further dilution during the measurement. The measurement was carried out after 30 s of equilibration at 25 °C and the \(\zeta\)-potential was calculated by the instrument software (Zetasizer Software, Version 7.10, Malvern Instruments, Worcestershire, UK) using the Smoluchowski model.

2.4.3. Transmission electron microscopy (TEM) analysis

The microstructure of emulsions was determined by transmission electron microscopy (TEM) and the emulsion samples were embedded in resin according to the method described by Gallier, Tate, and Singh (2013). The samples were injected into freshly made 3% agarose tubes (Hydragene Co. Ltd., Xiamen, China) and sealed at the end of each tube with remaining agarose. The embedded samples in agarose tubes were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (Merck, Darmstadt, Germany) at pH 7.2 and subjected to a second fixation with 1% osmium tetroxide (ProSciTech, Thuringowa, Australia) in the same buffer. The samples were dehydrated in a series of acetone washes consisting of 25% acetone (15 min), 50%, 75% and 90% acetone (30 min each) and 100% acetone (30 min for 3 times) (Merck, Darmstadt, Germany). This was embedded in fresh 100% resin (ProSciTech, Thuringowa, Australia) and polymerised at 63 °C for 48 h.

The embedded samples in resin blocks were cut using a glass knife on the ultramicrotome (Leica EM UC7, Heidelberg, Germany) and trimmed down to ultrathin sections using a diamond knife (Diatome, Hatfield, PA, USA). The thin sections of the embedded samples were placed on a copper grid using a Coat Quick “G” adhesive pen (Saiko, Japan) and stained with saturated uranyl acetate (BDH Chemicals, Poole, England) in 50% ethanol (Merck, Darmstadt, Germany) followed by 0.25% lead citrate (BDH Chemicals, Poole, England). This was mounted in a specimen holder and inserted into the microscope cooled by liquid nitrogen. The samples were viewed using a transmission electron microscope (FEI Tecnai™ G2 Spirit BioTWIN, Czech Republic) operated at 60 kV and equipped with a LaB6 filament. TEM images were captured with a 2 K × 2 K Veleta camera (14 bit) (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.5. Data analysis

All experimental works were carried out in replicates on new, freshly prepared samples and the results were reported as averages and standard deviations of the measurements. The data were analysed statistically by Analysis of Variance (Microsoft Excel 2010) at P-value ≤ 0.05.

3. Results and discussion

3.1. Influences of emulsifier types and concentrations on the physicochemical properties of nanoemulsions

The use of different types and concentrations of emulsifiers had a noticeable influence on the characteristics of nanoemulsions. The protein-stabilised nanoemulsions containing WPI or lactoferrin appeared more translucent than Tween 20 nanoemulsions and the particle size of nanoemulsions was significantly different (\(P < 0.05\)) (Fig. 1). WPI or lactoferrin nanoemulsions were smaller than those containing Tween 20 at concentrations above 0.75% (w/w). This result is interesting as many previous studies showed that smaller droplets were formed by small molecule surfactants rather than proteins (Jo & Kwon, 2014; Qian & McClements, 2011). This is because small molecule surfactants can adsorb more rapidly onto the droplet surface during emulsification as they are more flexible and smaller in size than large globular proteins (Jafari, He, & Bhandari, 2007; Jo & Kwon, 2014; McClements, 1999).

The particle size of WPI or lactoferrin nanoemulsions was around 100 nm at 0.25% (w/w) protein which was decreased to 70 and 90 nm, respectively as the protein concentration increased but remained steady at above 1% (w/w). In the case of Tween 20, the mean particle diameter decreased slightly from 115 to 94.3 nm when Tween 20 concentration was increased from 0.25% to 0.5% (w/w). However, Tween 20 produced nanoemulsions with increasingly larger droplet size (120–450 nm) when the concentration was increased from 0.75% to 5% (w/w). Jafari et al. (2007) also observed an increase in the droplet size of emulsions stabilised with Tween 20 from 183 to 505 nm (\(D_{3,2}\)) at high surfactant concentrations above 2.5% (w/w). It was suggested that the unadsorbed Tween 20 when used at high levels formed surfactant micelles which caused the oil droplets to flocculate due to depletion effect. The presence of surfactant micelles in the aqueous phase can result in the exclusion of surfactant molecules between the oil droplets when they are at a distance less than the diameter of the surfactant micelles due to osmotic pressure and eventually cause the droplets to flocculate (Jafari et al., 2007; McClements, 1999). Interestingly, the \(\zeta\)-potential of Tween 20 emulsions was negative (around –10 mV) (Fig. 2) even though Tween 20 is a non-ionic surfactant. It is thought that the negative surface charge was due to adsorption of anionic species derived from the materials used to make the nanoemulsions, such as free fatty acids (\(\text{COO}^-\)) in oils or hydroxyl ions (\(\text{OH}^-\)) present in sodium hydroxide solution when adjusting the pH of emulsion (Jo & Kwon, 2014).

Of the two protein emulsifiers, WPI produced nanoemulsions with smaller particle sizes (70–110 nm) at all concentrations (0.25–5% w/w) than those stabilised by lactoferrin (90–100 nm) (Fig. 1). The mean particle diameter of nanoemulsions stabilised by WPI decreased from 110 to 70 nm when the protein concentration was increased from 0.25% to 0.75% (w/w) but remained fairly constant thereafter. In a study reported by Cornacchia and Roos (2011), the decrease in droplet size from 1500 to 600 nm (\(D_{3,2}\)) with increasing WPI concentrations up to 0.8% (w/w) was also reported for emulsions with 10% (w/w) sunflower oil or hydrogenated palm kernel oil. No further decrease in droplet size was observed at higher WPI concentrations. The authors also reported that excessive proteins could result in the formation of multi-layer proteins around the droplets as the surface protein coverage was found to increase from 1.5 to 4.8 mg/m² with increasing protein concentration. It was suggested that multiple interfacial layers may be formed in WPI-stabilised emulsions as the globular whey proteins become partially denatured during homogenisation. Denatured protein molecules expose amino acids containing reactive groups, such as non-polar and sulphhydril groups and promote protein–protein interactions via hydrophobic interactions and disulphide bonds. In this study, at WPI concentration above 0.75% (w/w), the excess WPI molecules could have been adsorbed on the droplet surface and form multi-layer coatings. This explains the lower concentrations of non-adsorbed proteins in the continuous phase which is favorable in preventing depletion flocculation. Presumably, the excess proteins form thicker multi-layer coatings around the droplets which can provide steric repulsion apart from electrostatic repulsion between the negatively charged droplets. This may explain why depletion flocculation did not occur in WPI-stabilised nanoemulsions at higher concentrations. As expected, all the WPI-stabilised nanoemulsions were negatively charged at pH 7 (Fig. 2). The net negative charge was decreased with increasing protein concentration especially from 0.2% to 1% but still high enough to confer electrostatic repulsive force between the droplets.
There was also a decrease in the particle size of lactoferrin-stabilised nanoemulsions but it did not decrease further at higher concentrations above 0.5% (w/w) (Fig. 1). In a previous study done on the adsorption behaviour of lactoferrin in oil-in-water emulsions, Ye and Singh (2006) reported that the droplet size of emulsion decreased with increasing lactoferrin concentration from 0.3% up to 1% (w/w) level. They also found the surface protein coverage increased from 1 to 4.7 mg/m² when lactoferrin concentration was increased from 0.3% to 3% (w/w), indicating the formation of lactoferrin multi-layers at the droplet surface. In our case, the unadsorbed lactoferrin molecules can be thought to form multi-layer coatings at higher concentrations (0.5% w/w) and stabilise the emulsions through electrostatic and steric hindrance. The higher molecular weight lactoferrin (approximately 84 kDa) with carbohydrate groups attached to the polypeptide backbone provides a strong steric repulsion in emulsions (Steijns & van Hooijdonk, 2000; Tokle, Decker, & McClements, 2012). The \( \zeta \)-potential measurements of emulsions showed that the lactoferrin nanoemulsions were positively charged (Fig. 2) as lactoferrin contains a high proportion of basic amino acids with a high isoelectric point of around 9 (Baker & Baker, 2005; Steijns & van Hooijdonk, 2000).

Fig. 1. Mean particle diameter and photographs of nanoemulsions prepared with different types and concentration of emulsifiers at an organic to aqueous phase ratio of 10:90 and adjusted to 0.5% (w/w) oil.

Fig. 2. Mean \( \zeta \)-potential of nanoemulsions prepared with different types and concentrations of emulsifiers at an organic to aqueous phase ratio of 10:90 and adjusted to 0.5% w/w oil.
The microstructure of selected nanoemulsions stabilised by WPI, lactoferrin and Tween at 1% (w/w) emulsifier level was observed by transmission electron microscope (TEM) as shown in Fig. 3. The TEM images showed that the oil droplets formed by the three different emulsifiers were uniformly spherical and the droplet size corresponded well to the particle size measurements. Overall, the results showed that the three types of emulsifiers can be used to prepare nanoemulsions with an optimal concentration to form small droplets. WPI and lactoferrin produced small droplet sizes (<100 nm) at all different protein concentrations (0.25–5%, w/w) used in this study while Tween 20 formed larger droplets at concentration higher than 0.75% (w/w), possibly due to coalescence caused by depletion flocculation. The ζ-potentials of nanoemulsions were different due to the molecular characteristics of different emulsifiers used. The stability of nanoemulsions formed by three different emulsifiers was further evaluated under varying environmental conditions which is discussed in subsequent sections.

3.2. Effect of heat treatment on the stability of nanoemulsions

The effect of temperature on the stability of nanoemulsions prepared at 1% (w/w) emulsifier level was examined. Nanoemulsions were heated in water baths at different temperatures ranging from 30 to 90 °C for 15 min and rapidly cooled to room temperature. The particle size and ζ-potential of the heat-treated nanoemulsions were measured after 1 day of storage at room temperature. The results showed that all the nanoemulsions except Tween 20 heated at 90 °C were stable against droplet aggregation as there was no significant change in the appearance and particle size over the temperature range used (P < 0.05) (Fig. 4a).

Oil droplet aggregation which could result from heat treatment in protein-stabilised emulsions was not observed in this study. Similar results were shown in a previous study by Lee et al. (2011) that whey protein-stabilised nanoemulsions were stable to heat treatment against droplet aggregation due to strong electrostatic repulsion between them. On the other hand, it has been reported that large particles were formed in protein-stabilised emulsions (Fig. 4b). This may be related to the droplet coalescence occurring at temperature near the phase inversion temperature (PIT) of Tween 20. The phase inversion of an emulsion would occur when the temperature is near the cloud point of non-ionic surfactants (Shinoda & Ari, 1964). The cloud point is defined as the temperature at which the surfactant solution becomes insoluble and undergoes phase separation due to dehydration of the head group of surfactant molecules at higher temperature (Mahajan, Chawla, & Bakshi, 2004). According to the manufacturer, the cloud point of Tween 20 is around 76 °C and therefore some droplet coalescence may occur at higher temperatures as indicated by an appreciable increase in the mean particle size of Tween 20 nanoemulsions, especially after heat treatment at 90 °C (Fig. 4a).

These results suggested that the nanoemulsions stabilised by WPI or lactoferrin have better thermal stabilities than those stabilised by Tween 20. This has important implications in the selection of suitable surface active molecules to stabilise nanoemulsions that can withstand thermal processing.

3.3. Effect of pH on the stability of nanoemulsions

The pH stability of nanoemulsions was investigated for their potential applications in foods. The pH of nanoemulsions stabilised by WPI, lactoferrin or Tween 20 was adjusted to different pH values ranging from 2 to 10. Visual observations showed that WPI-stabilised nanoemulsions were stable at all pH values except pH

![Fig. 3. TEM images of nanoemulsions prepared with different types of emulsifiers; (a) WPI, (b) lactoferrin and (c) Tween 20, at the emulsifier concentration of 1% (w/w). Nanoemulsions were prepared at an organic to aqueous phase ratio of 10:90 and adjusted to 0.5% (w/w) oil.](image-url)
4.5 and 5 which are near to the isoelectric point (pl) of WPI around 4.9 (Fig. 5a). The particle size measurements showed that the oil droplets remained small at pH below or above the pl but there was a marked increase in the particle size at pH values near to the pl of WPI (Fig. 5b). This is because WPI-stabilised nanoemulsions are stabilised by electrostatic repulsion but they tend to form aggregates via hydrophobic attractions and van der Waals interactions when the electrostatic repulsion is not strong enough to overcome these attractive forces at pH close to the pl (Harnsilawat, Pongsawatmanit, & McClements, 2006; McClements, 1999). This was confirmed by the \( \zeta \)-potential measurements of the emulsions as the electric net charges of droplets were small at the pl but became highly positive or negative at pH below or above the pl, respectively (Fig. 5c).

It is interesting to note that the phase separation of nanoemulsions did not form a cream layer on top of the emulsion but instead they formed white precipitation at the bottom of the tube at pH values of 4.5 and 5 (Fig. 5a). This phenomenon was similar to a previous study done on the pH stability of WPI-stabilised nanoemulsions and conventional emulsions (Lee et al., 2011). It was shown that a cream layer was formed on top of the conventional emulsions but a suspension of white particulates was found at the bottom of the tube for nanoemulsions. The observed difference in the phase separation behaviour of the two emulsion systems was explained by the overall particle density difference between the oil and water phases. The oil droplets in the conventional emulsions were relatively large in size and surrounded by a thin layer of protein and the particle density was thus less than the surrounding liquid for the droplets to exhibit creaming. However, the droplets in the nanoemulsions were smaller and coated by a thicker layer of protein which caused the particle density to be higher than the surrounding liquid for sedimentation to occur. This could explain why the particle characteristics between nanoemulsions and conventional emulsions are different.

The pH stability of lactoferrin-stabilised emulsions (5% w/w corn oil and 0.5% w/w lactoferrin) has been reported to be stable in the pH range from 2 to 6 with \( z \)-average diameter less than 250 nm (Tokle & McClements, 2011). However, droplet aggregation was observed at higher pH values from 7 to 9 which are close to the pl of lactoferrin (Fig. 5a). Interestingly, the latter was not observed in this study as lactoferrin-stabilised nanoemulsions did not form large aggregates at higher pH values (Fig. 5b) and remained positively charged (Fig. 5c). It is thought that the stability of nanoemulsions coated with lactoferrin can be maintained by both electrostatic and steric repulsive forces as mentioned above. This is because lactoferrin is a glycoprotein with carbohydrate moieties that stabilises emulsions against aggregation by a combination of electrostatic and steric repulsion (Mao, Dubot, Xiao, &
However, the decrease in positive charges of lactoferrin nanoemulsions with increasing pH (Fig. 5c) suggests that the higher pH stability of nanoemulsions observed in this study could be largely attributed to steric repulsion.

The nanoemulsions stabilised by Tween 20 were also stable to pH changes as the particle size did not increase across the pH range studied (Fig. 5b) and the \(\zeta\)-potential of the emulsions remained the same (Fig. 5c). This is so because Tween 20 is a non-ionic surfactant and thus the stability of emulsions was not affected by pH changes.

Based on the results, all the nanoemulsions were stable across the pH range found within most food products except those stabilised by WPI at pH 4.5 and 5. The WPI-stabilised nanoemulsions were unstable to droplet aggregation at pH near the pI and this could pose a problem for WPI-stabilised nanoemulsions in food systems with pH values around 4.5 and 5.

### 3.4. Effect of salt addition on the stability of nanoemulsions

The stability of nanoemulsions in the presence of NaCl (0–500 mM) or CaCl\(_2\) (0–90 mM) at different concentrations was examined. All the nanoemulsions exhibited good stability to NaCl with little change in particle size at all NaCl concentrations up to 500 mM (data not shown). However, WPI-stabilised nanoemulsions became opaque and exhibited phase separation at salt concentrations above 10 mM CaCl\(_2\) while those stabilised by...

**Fig. 5.** Influence of pH changes on (a) the visual appearance and stability, (b) mean particle diameter and (c) \(\zeta\)-potential of nanoemulsions stabilised by different types of emulsifiers.
lactoferrin or Tween 20 remained physically stable at all salt concentrations studied (Fig. 6a). As shown in Fig. 6b, the mean particle size of WPI-stabilised nanoemulsions increased slightly when the CaCl₂ concentration was increased from 0 to 10 mM but they formed larger aggregates and phase separated at higher concentrations. The phase separation behaviour of nanoemulsions which resulted in sedimentation instead of creaming can be similarly explained by the pH-induced aggregation of nanoemulsions.

Initially, WPI-coated emulsion droplets were stabilised by electrostatic repulsive forces due to their negative charges. However, the presence of the dissociated Ca²⁺ cations screened the electrical charges and reduced the electrostatic repulsion between the droplets for aggregation to occur (Degner et al., 2014; Ye, Lo, & Singh, 2012). This was confirmed by the measurement of electrical charges on the emulsions. The ζ-potential of WPI-stabilised nanoemulsions decreased with the addition of CaCl₂ and eventually reached almost neutrality at higher concentrations (Fig. 6c). In addition to the screening of negative charges, the divalent Ca²⁺ ions could also form calcium bridges between the adsorbed proteins on the droplets to promote extensive aggregation. This is similar to a previous study on the destabilisation of protein-stabilised emulsions caused by adding CaCl₂ in conventional emulsions (Ye et al., 2012).

Fig. 6. Influence of CaCl₂ concentrations on (a) the visual appearance and stability, (b) mean particle diameter and (c) ζ-potential of nanoemulsions stabilised by different types of emulsifiers.
In the case of nanoemulsions stabilised by lactoferrin or Tween 20, they were stable against droplet aggregation in the presence of NaCl or CaCl₂. Their particle size remained the same without any sign of phase separation at different salt concentrations (Fig. 6b). However, the ζ-potential of the nanoemulsions decreased as compared to their original value in nanoemulsions without the addition of salt. The emulsions stabilised by lactoferrin became less positively charged (from +25.5 to +2.19 mV) while those of Tween 20 became less negatively charged (from -16.0 to -2.84 mV) with increasing CaCl₂ (Fig. 6c). The change in ζ-potential measurements suggested that there was some charge screening by the Cl⁻ ions in the case of lactoferrin and Ca²⁺ ions in the case of Tween 20. Nevertheless, the nanoemulsions stabilised by lactoferrin or Tween 20 remained stable as the ion binding effect was not sufficient to induce droplet aggregations to overcome their steric repulsion. Similarly, Tokle and McClements (2011) did not find any droplet aggregations in 0.5% (w/w) lactoferrin-stabilised conventional emulsions (containing 5% corn oil) up to 100 mM CaCl₂ but some aggregation was found at higher concentrations above 150 mM CaCl₂. In another study, Tween-stabilised conventional emulsions (10% MCT oil) were also reported to be stable up to 500 mM NaCl (Yang, Leser, Sher, & McClements, 2013). The good salt stability of lactoferrin or Tween 20-stabilised nanoemulsions suggested that their stability was largely dominated by steric repulsion rather than electrostatic repulsion.

4. Conclusions

This study showed that nanoemulsions with small particle size can be produced by emulsification and solvent evaporation using different emulsifiers. The protein emulsifiers produced smaller droplets than those prepared with Tween 20. Protein-stabilised nanoemulsions were stabilised by a combination of electrostatic and steric repulsion whereas Tween 20-stabilised nanoemulsions was stabilised by steric repulsion which could explain why their nanoemulsions were more susceptible to depletion flocculation and formed large droplets at high surfactant concentrations. The study also found that WPI-stabilised nanoemulsions were unstable to pH near to the pl of whey proteins as compared to nanoemulsions stabilised by lactoferrin or Tween 20. Similar phenomenon was observation in WPI nanoemulsions in the presence of CaCl₂ addition when the concentration went above 30 mM. Salt destabilised WPI nanoemulsions via screening of the negative charge and formed calcium bridges which then led to droplet aggregation. However, the presence of CaCl₂ up to 90 mM did not affect the stability of lactoferrin or Tween 20-stabilised nanoemulsions. These results indicated that the selection of emulsifier type used is important to producing stable nanoemulsions in different environmental conditions.

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References


