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Formulation and stability assessment of ergocalciferol loaded oil-in-water nanoemulsions: Insights of emulsifiers effect on stabilization mechanism

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ABSTRACT

In the study, we investigated the effect of emulsifiers with different stabilizing mechanisms on the formulation and stability of ergocalciferol loaded oil-in-water (O/W) emulsions. O/W emulsion stabilized by modified lecithin (ML; electrostatic stabilization), sodium caseinate (SC; electrosteric stabilization) or decaglycerol monooleate (MO-7S; steric stabilization) were formulated using high-pressure homogenization. The Sauter mean diameter ($d_{3,2}$) of emulsions produced by ML, SC and MO-7S were 126 ± 1, 127 ± 4 and 138 ± 3 nm, respectively. The stability of resulting emulsions was evaluated when they exposed to different environmental stresses and during 30 days of storage at 25 and 55 °C. Results showed that the emulsions prepared by MO-7S or ML were stable against a wide range of pH (2–8), while SC-stabilized emulsions showed instability with extensive droplet aggregation at pH 4 or and 5. Only ML-stabilized emulsions showed droplet growth due to coalescence when treated at high NaCl concentration (300–500 mM). In the absence of glucose, SC-stabilized O/W emulsions showed better freeze-thaw stability, in comparison to those formed with ML or MO-7S emulsifiers. The emulsion produced by ML was found to be stable to droplet aggregation at heating temperatures (80–120 °C) for 1 h. All the O/W emulsions stored at 25 °C showed good physical and chemical stability. However, the chemical stability of ergocalciferol in emulsion system decreased in order of ML > MO-7S ≫ SC during storage at 55 °C for a period of 30 days. These findings provide valuable information for the development of nanoemulsion-based delivery system applied in food and beverage products.

1. Introduction

Recently, food and beverage products consisting of functional ingredients with additional health benefits attract an increasing attention in food industries (Piorkowski & McClements, 2014). Vitamin D is one of the most important nutraceutical compound gaining attention from researchers and food manufacturers due to its special role in maintaining the bone, teeth and cartilage development (Cranney, Weiler, O'Donnell, & Puil, 2008; Hark & Deen, 2005). In addition, vitamin D provides prevention against heart diseases, cancer and immune diseases (Haham et al., 2012; Holick, 2004). Vitamin D is a seco-steroid hormone that can be classified into two main different forms, namely vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is naturally present in mushrooms with low amount and commercially produced by UV irradiation of yeast, while vitamin D_3 is naturally synthesized in the skin of human and animal bodies via the exposure to sunlight (Khalid et al., 2015). However, an estimated one billion people worldwide have either vitamin D deficiency or insufficiency (Holick, 2007). The deficiency of vitamin D can be ascribed to the lack of exposure to sunlight, extensive use of UV protecting sun cream, or poor intake of food containing vitamin D (Haham et al., 2012; Holick, 2004; Tsiaras & Weinstock, 2011). Consequently, food and beverage incorporated this vitamin have raised considerable interest in practical applications. The utilization of vitamin D as nutraceutical ingredient in processed foods or beverages still represents a big challenge for several reasons: vitamin D is a highly hydrophobic compound that cannot be directly dispersed in aqueous phase; chemical degradation of vitamin D occurs when exposed to light, oxygen, or elevated temperatures, thus leading to the reduction of functionality and bioavailability (Guttoff, Saberi, & McClements, 2015; Tsiaras & Weinstock, 2011).

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In the past few decades, nanotechnology has received an increasing attention in foods, cosmetics and pharmaceutical applications, and it plays a very important role in encapsulating poor water-soluble bioactive compounds, i.e. polyunsaturated fatty acids, carotenoids, phytosterols, and many other ingredients. Several attempts were also carried out to improve the water solubility, stability and bioavailability of vitamin D using different nano-delivery systems including nanoparticles, solid lipid nanoparticles (SLN), nanoliposomes and so on (Abbasi, Djomeh, Mousavi, & Davoodi, 2014; Guttoff et al., 2015; Mohammadi, Ghanbarzadeh, & Hamishehkar, 2014; Ozturk, Argin, Ozilgen, & McClements, 2015; Patel & San Martin-Gonalez, 2012). O/W nanoemulsions is one of the most important nano-delivery systems for encapsulating lipophilic compounds. The nanoemulsions are the colloidal dispersions containing small lipid droplets (typically around 20–200 nm) dispersed with in aqueous phase (McClements, 2005; McClements, 2010; Mohammadi et al., 2014). Nanoemulsions have high optical clarity, increased oral bioavailability and great stability against gravitational separation and droplet coalescence (Guttoff et al., 2015; McClements, 2010; Thadros, Izquierdo, Esquena, & Solans, 2004; Zhao et al., 2013). Keeping these advantages in mind, the nanoemulsions are expected to be useful for encapsulating lipophilic compounds, such as oil-soluble vitamins and nutraceuticals, for application in food and beverage products.

Emulsion-based foods need to be stable against harsh condition (i.e., pH, ionic strength and temperature) during their processing, storage and transportation. It is well known that an emulsifier is one of the most important materials required to determine the stability of an emulsion-based food against the destabilization process. The selection of appropriate emulsifier is critical for producing emulsion-based products with high stability (Schubert & Engel, 2004). In the current study, three food-grade emulsifiers from different group, namely, modified lecithin (ML), sodium caseinate (SC) and decaglycerol monooleate (MO-7S) were used for evaluating their effect on the physicochemical stability of vitamin D-loaded nanoemulsions. The three emulsifiers were selected due to fact that they provide different stabilizing mechanisms to protect emulsion droplets from aggression. Limited systematic study was available on the comparison of influence of emulsifier with different stabilizing mechanisms on the stability of nanoemulsions loaded with vitamin D. ML used in our work is an enzymatically modified phospholipid derived from hydrolysis of soy lecithin. ML is a mixture of different phospholipids, in which lysophosphatidylcholine is the major compound and phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid are the secondary compounds (Sono, 2005). ML is a zwitter-ionic surfactant with small molecular weight, which stabilizes nanoemulsions by electrostatic repulsion. SC is a large molecular weight emulsifier derived from bovine milk, and it can provide a thick coating layer around the oil droplet to prevent droplet from growth mostly due to electrostatic repulsion. In addition, the long tail of disordered casein molecules adsorbed around the oil droplet provides partly steric stabilization effect (Raikos, 2010). MO-7S, a type of polyglycerol esters of fatty acids (PGEs), is synthetic non-ionic surfactant with small molecular size, which rapidly adsorbs onto oil droplet during homogenization and stabilizes the nanoemulsions by steric hindrance. The objective of this work was to provide a better understanding of the role of emulsifiers with different stabilizing mechanisms on the stability of oil-in-water nanoemulsions encapsulated ergocalciferol. The results obtained from this study provide useful information for the utilization of emulsifiers in the development of nanoemulsion-based delivery system for lipophilic bioactive compounds.

2. Materials and methods

2.1. Materials

Ergocalciferol, refined soybean oil, sodium caseinate, hydrochloric acid, sodium azide, ethanol (99.8%), sodium hydroxide, D (+)-Glucose, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Modified lecithin (ML, SLP White Lyso) was procured from Tsuji Oil Mills Co. Ltd. (Tokyo, Japan). Decaglycerol monooleate (MO-7S) was kindly donated by Sakamoto Yakuhin Kogyo Co. (Osaka, Japan). Milli-Q water with a resistivity of 18 MΩ cm was used for preparing all the solution in the current study. All other chemicals used in this study were of analytical grade and used as such during experimentation.

2.2. Preparation of ergocalciferol-loaded O/W nanoemulsions

The oil phase was prepared by dissolving 0.1% (w/w) ergocalciferol in refined soybean oil and water phase was prepared by dissolving 2.0% (w/w) emulsifier (either SC, MO-7S or ML) in 5 mM phosphate buffer (pH 7). Ergocalciferol nanoemulsions were formulated using two-step homogenization method described in our previous publication (Wang et al., 2012). Firstly, a coarse emulsion was prepared by mixing 10% (w/w) oil phase and 90% (w/w) water phase via a rotor-stator homogenizer (Polytron, PT-3000 Kinematica-AG, Littace, Switzerland) at 5000 rpm for 5 min. In the second step, the fine nanoemulsions were obtained by passing the coarse emulsions through a high pressure homogenizer (NanoVater, NV200, Yoshida Kikai, Japan) at 100 MPa for 4 passes. The resulting nanoemulsions were subjected to different treatments to investigate their stability under different conditions.

2.3. Emulsion stability testing

2.3.1. Effect of pH

The pH of ergocalciferol loaded nanoemulsions was adjusted within the range between 2 and 8 using 1 M HCl or 1 M NaOH. Samples were kept at room temperature for 24 h prior to droplet size and ζ-potential measurements.

2.3.2. Effect of ionic strength

5 mL of ergocalciferol loaded nanoemulsions were diluted by adding 5 mL NaCl solution (in phosphate buffer, pH 7) to adjust the NaCl concentration in samples (0–500 mM). The samples were stored at room temperature (25 ± 2 °C) for 24 h prior to analysis.

2.3.3. Effect of freeze-thawing

In this section, the influence of freeze-thaw cycles on the stability of ergocalciferol loaded nanoemulsions was evaluated in the absence and presence of glucose as cryoprotectant. Nanoemulsions with additives (emulsions: 10% glucose in phosphate buffer solution (pH 7) = 1:1 (v/v)) and without additive (emulsions and phosphate buffer = 1:1) were transferred to separate plastic test tubes. The test samples were placed at −20 °C for 22 h, and then left to thaw in a water bath at 30 °C for 2 h prior to droplet size measurement. For the freeze-thaw storage stability test, fresh emulsions with or without glucose were stored at −20 °C for 20 days followed by thawing in the water bath for 2 h prior to analysis.

2.3.4. Effect of high temperature treatment

10 mL nanoemulsions encapsulating ergocalciferol were shifted to a 15 mL glass test tube and well-sealed by a metallic cap. The samples were placed in oil bath or autoclaving device (KTS-2346, ALP Co., Ltd., Tokyo, Japan) at fixed temperature (80, 100 or 120 °C). After heating for 1 h, the samples were cooled at room temperature for 1 day prior to analysis.

2.3.5. Storage stability

The ergocalciferol loaded nanoemulsions with the addition of sodium azide (0.02% (w/w)) as antibacterial agent were incubated at 25 ± 2 °C and 55 ± 2 °C for 30 days under dark condition. The droplet

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size and ergocalciferol content retained in emulsion were measured under different storage intervals for a period of 30 days. Ergocalciferol encapsulation efficiency (EE) in samples was calculated with the following equation:

$$EE = \frac{C_t}{C_0} \times 100 \quad (1)$$

where C_t is the concentration of ergocalciferol at a specific time, while C_0 is the initial concentration of ergocalciferol loaded in the nanoemulsions.

2.4. Droplet size analysis

The measurement of droplet size was conducted using a laser diffraction particle size analyzer (LS 13320, Beckman Coulter, Brea, USA) which can measure the particle size in the range between 0.04 μm and 2000 μm. A suitable amount of emulsion droplets was dropped into the measuring cell containing Milli-Q water with the same pH as the samples being tested for determination of droplet size in the present study. The refractive index of the oil and water phases used in the measurement were 1.471 and 1.333, respectively. The particle size of the emulsions was expressed as Sauter mean diameter ($d_{3,2}$) or volume mean diameter ($d_{4,3}$). The measurement of each sample was repeated in triplicate.

2.5. ζ-potential measurement

Determination of the ζ-potential of nanoemulsions droplet was performed by using a ζ-potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Before analysis, the emulsions at different pH mentioned in Section 2.3.1 were diluted (1:200) with Milli-Q water at the same pH as the initial samples being tested. The emulsions at different ionic strengths mentioned in Section 2.3.2 were diluted (1:100) with phosphate buffer (5 mM, pH 7). The diluted samples were then placed in a folded capillary cell and loaded in the instrument. The ζ-potential of samples were automatically measured using 10–100 runs per analysis after they were equilibrated for 120 s inside the instrument at 25 °C. The refractive indexes of the dispersed and continuous phases for calculation were set at 1.471 and 1.333, respectively, each measurement was conducted in triplicate.

2.6. Ergocalciferol quantification

The concentration of ergocalciferol in the O/W nanoemulsions was measured using High Performance Liquid Chromatography (HPLC). Ergocalciferol was extracted from the O/W emulsions prior to HPLC analysis using method previously described by Khalid et al. (2015). 0.2 mL of nanoemulsion was mixed with 3.8 mL ethanol and vortexed for 2 min, followed by ultra-sonicating for 20 min. The samples were placed at room temperature for 30 min and filtered by passing through nylon membrane with a pore size of 0.45 μm before analysis. For the samples with the measurement below the limit of detection, 2 mL of filtered ethanolic extract was transferred into amber vial and dried by asperging nitrogen. After concentrating the samples and re-dissolving in 0.2 mL of ethanol by vortexing and mixing for 2 min, they were filtered using nylon filter. The concentration of ergocalciferol in the emulsions was evaluated by using the HPLC system (JASCO International Co., Tokyo, Japan) equipped with a UV-970 UV–Vis spectrophotometric detector, a PU-980 pump system, and an AS-2055 autosampler. C-18 reversed phase column (4.6 × 250 mm; Shimpack VP-ODS, Japan) was used as stationary phase and the column temperature was set at 35 °C. The mobile phase was composed of acetonitrile and methanol with the isocratic mixture ratio at 75:25 (v/v) and the flow rate was set at 1 mL min^{-1}. The injection volume was 20 μL and the detection wavelength was set at 265 nm. The concentration of ergocalciferol in samples was calculated by the standard curve and all the measurements were repeated in duplicate.

2.7. Statistical analysis

All experiments were repeated at least in duplicate and standard deviations were calculated from these measurements. Analysis of variance (ANOVA) tests were used to analyze the characterization and stability data at a confidence level of 95%. Least significant difference (LSD) was used to compare the stability data with different emulsifiers. The LSD was calculated using Statistix 8.1 software (Tallahassee, USA) and according to the method described by Steel, Torrie, and Dickey (1997).

3. Results and discussion

3.1. Formulation of nanoemulsions encapsulating ergocalciferol

Nanoemulsions were formulated by homogenizing the oil and aqueous phase according to the procedure described in Section 2.2. Fig. 1 shows the $d_{3,2}$ and droplet size distribution of ergocalciferol loaded nanoemulsions stabilized by ML, SC or MO-7S. All the fresh emulsions exhibited mono-modal droplet size distribution (Fig. 1a) with $d_{3,2}$ ranging between 126 and 138 nm. The $d_{3,2}$ (Fig. 1b) of ML, SC and MO-7S stabilized nanoemulsions were 126 ± 1 nm, 127 ± 4 nm and 138 ± 3 nm, respectively. As previous study reported (Mao et al., 2009), small-molecule surfactants can absorb onto the droplet surface more rapidly

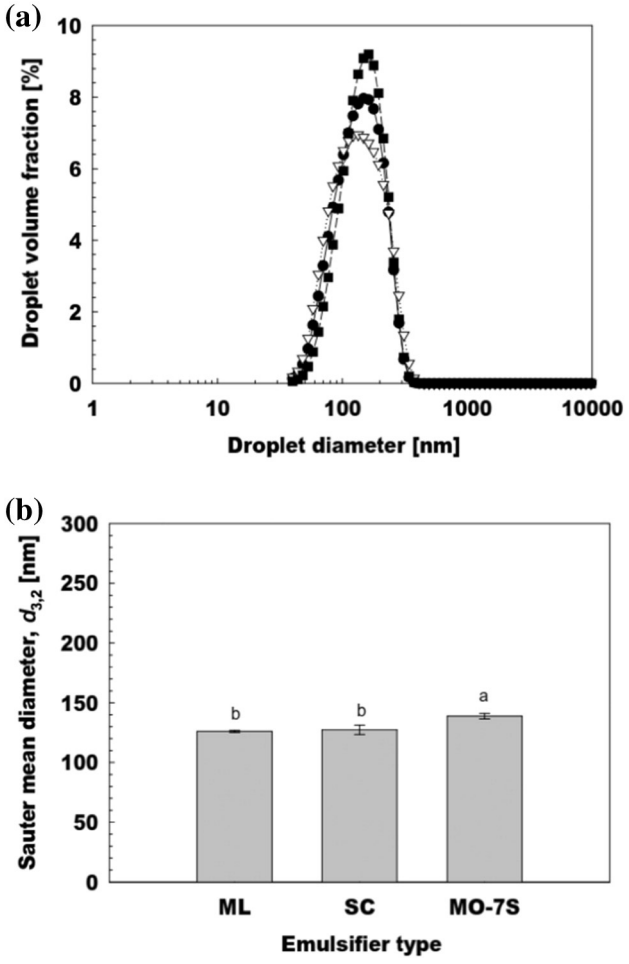

Fig. 1. Effect of emulsifier type on (a) droplet size distribution and (b) Sauter mean diameter $d_{3,2}$ of ergocalciferol-loaded nanoemulsions produced via high pressure homogenization. (–●–) denotes ML, (–▽–) denotes SC and (–■–) denotes MO-7S. The different letter shows significant difference at 95% probability level.

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than emulsifier with large molecule such as protein, hence leading to form smaller droplet size. Interesting, bigger $d_{3,2}$ of SC-stabilized nanoemulsions comparing those stabilized by ML and MO-7S was not observed in our study. This phenomenon can be explained by relatively high concentration of SC used in our study can provide sufficient emulsifier molecular to cover the newly formed droplets to prevent them from re-aggregation with each other during high pressure homogenization process, hence be capable of producing droplets with small size (Kanafusa, Chu, & Nakajima, 2007). Nanoemulsion delivery systems had great stability due to their small droplet sizes. However, different environmental factors and storage condition may affect the stability and droplet sizes of nanoemulsions. The effect of these parameters on ergocalciferol loaded nanoemulsions were described in sections below.

3.2. Effect of pH

Food products and beverage typically exhibit broad range of pH, hence it is important to understand the influence of pH on the stability of ergocalciferol loaded nanoemulsions for future commercial applications. Fig. 2a and Fig. S1 showed the change in $d_{3,2}$ and $d_{4,3}$ of ML, SC and MO-7S-stablized nanoemulsions as a function of pH, respectively. The obtained results showed the $d_{3,2}$ and $d_{4,3}$ of nanoemulsions stabilized by ML and MO-7S remained constant over a wide range of pH. On the other hand, the $d_{3,2}$ and $d_{4,3}$ of emulsions stabilized by SC increased sharply at pH 4 and 5 and these samples became highly unstable with an observed serum layer at the bottom and a creamed layer on the top of the tested tube (date not shown). We also examined the effect of pH on electrical characteristics (ζ-potential) of oil droplets (Fig. 2b). As reported previously, emulsions with ζ-potential >30 mV (absolute value) were considered to be stable to oil droplet aggregation (Jacobs, Kayser, & Muller, 2000). For SC-stabilized emulsions, the absolute values of ζ-potential were <30 mV at pH 4 and 5. These pH values were closed to the isoelectric point (pI) of SC, which was reported between pH 4.1 and 4.6 (Ching, Bhandari, Webb, & Bansal, 2015; Zhang, Zhang, Decker, & McClements, 2015). Thus, the electrosteric repulsion of SC-coated droplets is not strong enough to keep the emulsion stable, at pH near to pI, since the attractive force between particles dominates electrosteric repulsion, thereby causing droplet aggregation. The high stability of SC-stabilized emulsions at other pH values were related to highly positive or negative charge of droplet at pH below or above the pI, respectively. Theoretically, MO-7S as a non-ionic emulsifier was expected to have no charge. Nevertheless, we observed a negative charge on the MO-7S-stabilized oil droplet at higher pH, and a considerable increase in ζ-potential as the pH was reduced to 2. The charge on the droplet was attributed to the presence of ionized impurities in MO-7S, such as free fatty acids (Wang et al., 2012). Although, less charge (absolute value < 30 mV) was observed on the droplet and there was no increase in droplet size when MO-7S emulsions were adjusted to low pH. Unlike SC, the MO-7S provides steric hindrance between droplets to stabilize the emulsions, and this steric repulsion is fairly insensitive to pH and ionic strength (McClements, 2005). In the case of ML-stabilized emulsions, the high stability can be explained by the negative charge on the droplets across the entire pH range, which prevents emulsion droplets from aggregating.

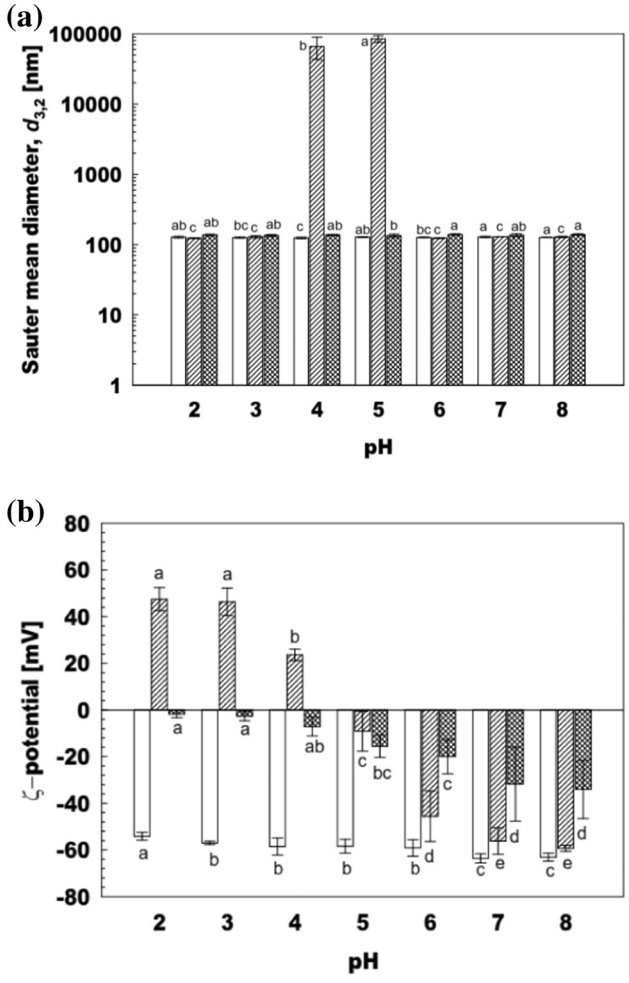

Fig. 2. Effect of pH on the stability of O/W nanoemulsions. (a) $d_{3,2}$ and (b) ζ-potential of ergocalciferol loaded nanoemulsions stabilized by different emulsifiers. (▭) denotes ML, (▨) denotes SC and (▩) denotes MO-7S. The different letter shows significant difference at 95% probability level.

3.3. Effect of ionic strength

It is imperative to see the stability of emulsified foods and beverages against ionic strength, as the emulsion-based products may be added with different amounts of mineral ions during product development. We therefore, investigated the influence of ionic strength on the physical stability of ergocalciferol loaded nanoemulsions. The addition of NaCl from 0 to 500 mM, resulted in no apparent changes in $d_{3,2}$ and $d_{4,3}$ of nanoemulsions stabilized by MO-7S and SC (Fig. 3a and Fig. S2). MO-7S stabilized nanoemulsions exhibited good stability in the presence of NaCl and was attributed to the non-ionic nature that forms steric repulsion between oil droplets and was fairly insensitive to the change in ionic strength as previously described in Section 3.2. The addition of salt to SC-coated nanoemulsions resulted in destabilization of emulsions and was attributed to electrostatic screening effects (Israelachvili, 2011). However, little increase in $d_{3,2}$ of SC-stabilized nanoemulsions was observed at highest concentration of NaCl (500 mM) in our study. This effect was probably due to the shielding effect of the salt on the electrostatic repulsion between SC-coated oil droplets was not strong enough (Fig. 3b). On the other hand, the casein molecules formed partial steric interaction between the droplets, which may be sufficiently large enough to prevent the droplet from growth. In contrast, a thin cream layer was occurred on the top of the ML-stabilized emulsions at ≥400 mM NaCl (data not shown), and there was moderate increase in $d_{3,2}$ and $d_{4,3}$ of the samples when NaCl concentration exceeded 300 mM. Similar, results have been reported by other researchers (McClements, Decker, & Choi, 2014). Previous studies indicated that the electrostatic screening effect of electrolytes might cause a significant reduction in electrostatic repulsion between droplets coated by ionic emulsifier at high salt concentration, which leads to the emulsion instability (Ozturk, Argin, Ozilgen, & McClements, 2014; Tan et al., 2016). However, no apparent reduction in the magnitude of the ζ-potential was observed in ML-stabilized emulsions with increasing NaCl concentration. Therefore, the shielding effect due salt ions on the electrostatic repulsion between droplets could not explain the instability of ML-stabilized emulsions in our study. We predict that the

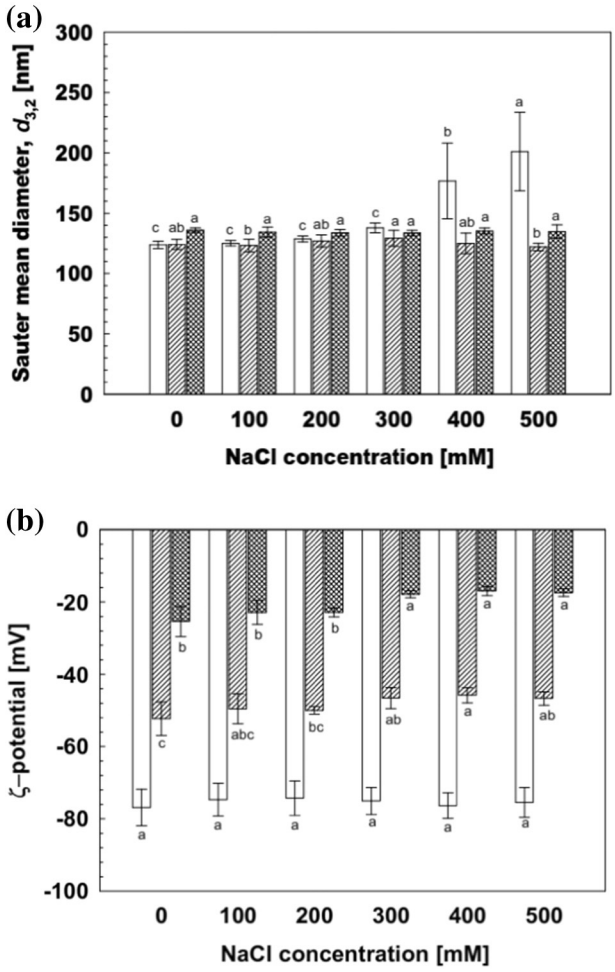

Fig. 3. Effect of NaCl concentration on (a) $d_{3,2}$ and (b) ζ-potential of ergocalciferol loaded nanoemulsions. (▭) denotes ML, (▨) denotes SC and (▩) denotes MO-7S. The different letter shows significant difference at 95% probability level.

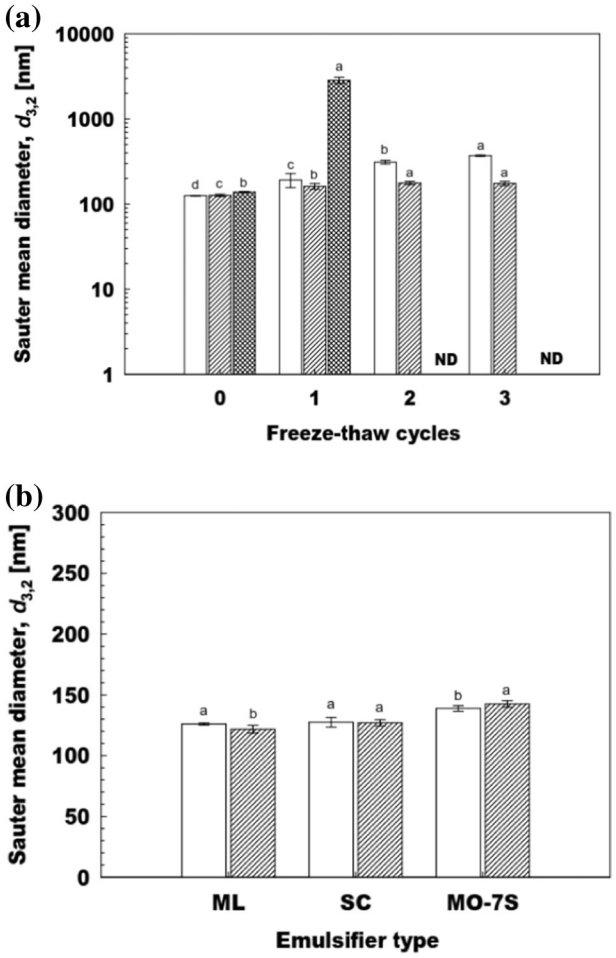

Fig. 4. Effect of freeze-thawing on the stability of nanoemulsions loaded with ergocalciferol formulated with different emulsifier types. (a) The change in $d_{3,2}$ of nanoemulsions without glucose. (▭) denotes ML, (▨) denotes SC and (▩) denotes MO-7S. (b) The change in $d_{3,2}$ of nanoemulsions with glucose. (▭) denotes cycle 0 and (▨) denotes cycle 3. ND means no determination of $d_{3,2}$, due to oiling off. The different letter shows significant difference at 95% probability level.

electrolytes might change the hydrophilic–lipophilic balance and hydration conditions of the emulsifiers by depletion of the hydration shell around the polar head groups of emulsifier molecule (McClements et al., 2014), which induces to the instability of ML-stabilized emulsions when exposed to high concentrations of NaCl.

3.4. Effect of freeze-thaw treatment

Many emulsion based food products like ice creams are exposed to fully or partially frozen state during their formulation, storage and utilization. The stability of these products during freeze-thawing is still a major challenge to food scientists and food industries (McClements, 2005). Hence, in this section, we investigated the influence of freeze-thaw cycles on the stability of ergocalciferol-loaded nanoemulsions produced using different types of emulsifier was in the presence or absence of glucose.

Fig. 4a presented the formulation results without glucose in the continuous phase of O/W nanoemulsions. The results indicated that the ML-stabilized nanoemulsions showed an appreciable increase in $d_{3,2}$ from 126 to 371 nm after 3 cycles of freeze-thaw treatment. For the SC-coated nanoemulsions, the droplet size presented a slight increase in $d_{3,2}$ from 127 nm (initial) to 175 nm (3 cycles). However, the $d_{3,2}$ increased drastically to 2816 nm even after 1 cycle of freeze-thawing for MO-7S-stabilized nanoemulsions. A similar trend was also observed, when data was plotted in terms of $d_{4,3}$ (Fig. S3). Further freeze-thawing of MO-7S-stabilized nanoemulsions showed extensive coalescence, leading to phase separation and oiling off (data not shown). These findings demonstrated that the stability of ergocalciferol loaded nanoemulsions towards freeze-thawing depends on the emulsifier type and follows the order of SC > ML > MO-7S.

Crystallization of water takes place during freeze-thawing, as a result the emulsion droplets were forced to come closer to each other in the remaining non-frozen aqueous phase (Ghosh & Coupland, 2008; Ogawa, Decker, & McClements, 2003). Thus, the emulsifier used should have the capability of protecting the emulsion droplet from coming closer and to stop the aggregation process. It is proven that the protein-stabilized emulsion had better freeze-thawing stability in comparison to those stabilized by the surfactants with smaller molecular weight. The proteins formed a thicker interfacial film around the droplets and inhibit the droplets from coalescence towards the freeze-thawing conditions (Cramp, Docking, Ghosh, & Coupland, 2004; McClements, 2005; Palanuwech & Coupland, 2003). In our study protein based emulsifier (SC) protects the nanoemulsions better against freeze-thawing, in comparison to synthetic emulsifier (MO-7S), which agrees well with the previous literatures. Tangsuphoom and Coupland (2009) found that SDS-stabilized coconut milk emulsion provides better freeze-thaw stability than those with Tween 20-coated samples. They reported that the association with SDS provided substantial electrostatic repulsion around lipid droplet and prevented the particles from coalescence, in comparison to Tween 20 during freeze-thawing.

In our study, ML was used as a small zwitter-ionic emulsifier to formulate the O/W nanoemulsions, similarly to SDS, it provides an

electrostatic repulsion between lipid particles and prevents the droplet coalescence to some extent. Comparing to the nonionic surfactant (MO-7S), this repulsion provided by ML is relatively stronger to slow down particle size growth when exposed to freeze-thaw treatment. In contrast, steric repulsion formed by MO-7S is not strong enough to inhibit lipid droplet coalescence after freeze–thawing, ultimately resulting in creaming and oil phase separation as the freeze-thawing process progressed. On the other hand, no increase in $d_{3,2}$ or phase separation was observed in any of the samples, when glucose was added to the continuous phase (Fig. 4b). The preceding results indicated that nanoemulsions containing glucose had good freeze-thawing stability after 3 cycles, regardless of type of emulsifier used for stabilizing nanoemulsions. It is well known that the addition of sugars in O/W emulsions can effectively improve their freeze-thawing stability by reducing the amount of formed ice and prevent the droplets from coalescence (Ghosh, Cramp, & Coupland, 2006).

3.5. Effect of high temperature treatment

Thermal stability of emulsion-based delivery systems is important during processing and storage, since temperature always fluctuate specially during hot filling, pasteurization and autoclaving. We examined the effect of high temperature (80–120 °C) on the stability of ergocalciferol-loaded O/W nanoemulsions for a period of 1 h at specific temperature. For MO-7S stabilized nanoemulsions (Fig. 5), the $d_{3,2}$ remains stable only at 80 °C, with further increase in temperature to 100 or 120 °C, a clear layer of oil phase was observed at the top of sample bottles (data not shown), which suggested that higher temperature induced the emulsions to droplet coalescence. As previously reported, dehydration of surfactant hydrophilic head group occurred when heating the nonionic emulsifier-stabilized O/W emulsion close to or above the phase inversion temperature (PIT), which led to the change in the optimum curvature of the protective monolayer (Israelachvili, 2011; McClements, 2005). This progressive dehydration of emulsifier at the oil-water interface would lead to an ultralow interfacial tension, therefore facilitate the droplet coalescence and phase separation (Kabalnov & Wennerstrom, 1996; McClements, 2005). Presumably, this destabilization of MO-7S-coated oil droplet due to the higher temperature (100 and 120 °C) used in this study is far above the PIT, which might have caused phase separation in emulsion system.

SC has been proven to be a good protein emulsifier against high temperature due to its disordered structure. The emulsions stabilized by SC remained stable during 30 min of heating at 90 °C or 15 min of heating at 121 °C (Hunt & Dalgleish, 1995; Srinivasan, Singh, & Munro, 2002). As indicated in Fig. 5 and Fig. S4, an appreciably increase in mean droplet diameter was observed in our study only when SC-stabilized emulsion was heated at 120 °C for 1 h, which suggested that particle aggregation occurred due to heating. Our results somewhat agreed with a previous study reported by Chu, Ichikawa, Kanafusa, and Nakajima (2008), in which 1 wt% SC-stabilized β-carotene nanodispersions showed an appreciably increase in particle size after heating them at 60 °C for 1 h, and a rapid increase in size after heating for 4 h. The difference in results might be due to the longer heating time of 1 h in our study, rather than 15 min in the previous studies. The release of phosphorus from caseinate would take place when placing them at high temperature and dephosphorization lead to the reduction of negative charge on the caseinate and further promotes caseinate-caseinate interactions that is dependent on heating time and temperature (Chu et al., 2008; Guo, Fox, Flynn, & Mohammad, 1989; Howat & Wright, 1934). The oil droplets growth was not observed in ML-stabilized emulsions during the entire temperature range, which suggested that the electrostatic repulsion between ML-coated droplets was stronger enough to overcome the attractive interactions and prevent droplets aggregation at elevated temperatures.

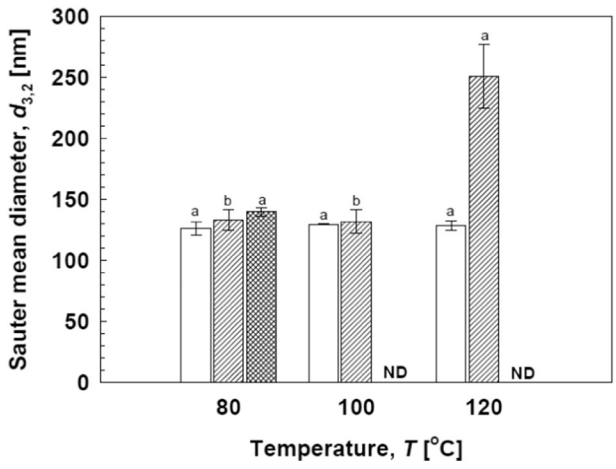

Fig. 5. Effect of heating temperature on $d_{3,2}$ and stability of ergocalciferol loaded nanoemulsions. (▭) denotes ML, (▨) denotes SC and (▩) denotes MO-7S. ND means no determination of $d_{3,2}$, due to oiling off. The different letter shows significant difference at 95% probability level.

3.6. Long-term storage stability

Long term storage stability of ergocalciferol loaded nanoemulsions was investigated with different emulsifiers (ML, SC or MO-7S) during storage at two different temperatures (25 or 55 °C) for a period of 30 days. This experiment was performed at neutral pH without salt addition.

3.6.1. Physical stability

Fig. 6 depicts the change in $d_{3,2}$ of ergocalciferol loaded nanoemulsions during 30 days of storage at 25 °C and 55 °C, respectively. All nanoemulsions stored at 25 °C exhibited good physical stability against oil droplet growth and phase separation and no change in their visible appearance and $d_{3,2}$ was observed during 30 days of storage (Fig. 6a). It is well accepted that nanoemulsions containing small droplet size are fairly stable due to the fact that Brownian motion of the nano-sized droplets can resist gravitational separation, flocculation and coalescence (Thadros et al., 2004). On the other hand, soybean oil was used as dispersed phase in present study, due to long chain triglyceride structure, soybean oil is quite water-insoluble, hence limiting the effect of the Ostwald ripening (McClements & Rao, 2011). The three different emulsifiers used in our study provided strong coated layer around the oil droplets and refrained from their growth during storage period of 30 days.

An accelerated test was performed to investigate the long-term physical stability of nanoemulsions loaded with ergocalciferol by placing them at an elevated temperature (55 °C) for 30 days (Fig. 6b). In general, there is no distinct change in $d_{3,2}$ of nanoemulsions formed with ML and MO-7S, suggesting that the emulsions stabilized by ML and MO-7S were physically stable during 30 days of storage at 55 °C. In contrast, SC-stabilized nanoemulsions showed a slight increase in mean droplet size at the end of storage time, indicating that these nanoemulsions were slightly destabilized due to the long-term storage at an elevated temperature. The nanoemulsions prepared by SC were unstable to long-term storage at 55 °C, presumably attributed to caseinate-caseinate interactions, which were discussed in Section 3.5.

3.6.2. Chemical stability

The long-term chemical stability of nanoemulsions stored at room temperature (25 °C) and at elevated temperature (55 °C) was evaluated by determining the change in retention of ergocalciferol during 30 days of storage. The fresh O/W nanoemulsions stabilized by ML, SC and MO-7S contained 89.5 ± 3.1, 88.3 ± 3.2 and 91.7 ± 2.6 mg/L ergocalciferol, respectively. The resulting retention of ergocalciferol was regarded as 100% at day 0. In terms of ergocalciferol retention (Fig. 7a), all the

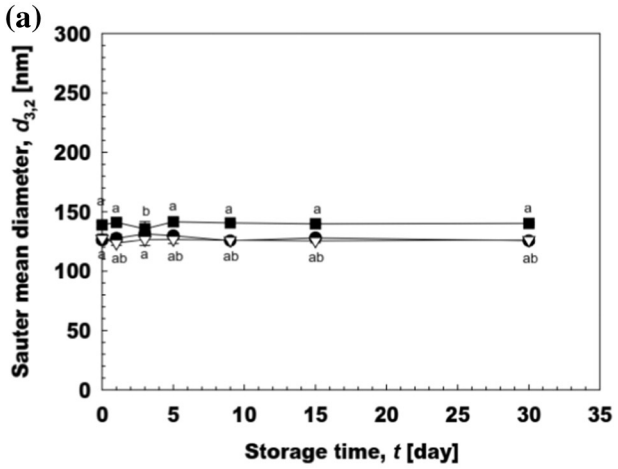

Fig. 6. Effect of storage temperature on long-term physical stability of ergocalciferol loaded nanoemulsions stabilized by different emulsifiers. (a) 25 °C and (b) 55 °C. (–●–) denotes ML, (–▽–) denotes SC and (–■–) denotes MO-7S. The different letter shows significant difference at 95% probability level.

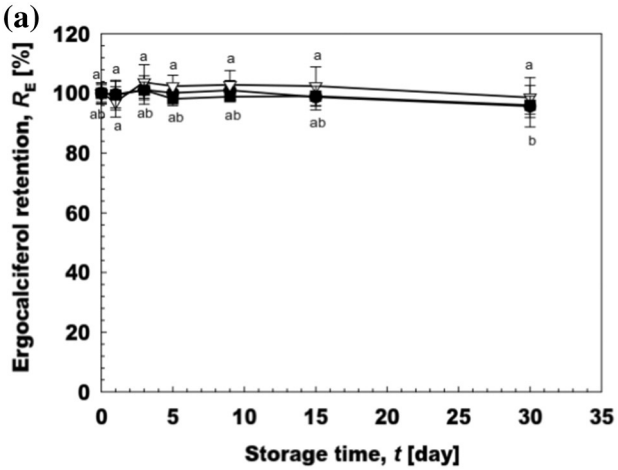

Fig. 7. Effect of storage temperature on long-term chemical stability of ergocalciferol loaded nanoemulsions stabilized by different emulsifiers. (a) 25 °C and (b) 55 °C. (–●–) denotes ML, (–▽–) denotes SC and (–■–) denotes MO-7S. The different letter shows significant difference at 95% probability level.

samples showed high chemical stability after storage at 25 °C for 30 days, with a slightly loss of 4.3%, 1.4% and 4.0% in ML, SC and MO-7S-stabilized nanoemulsions, respectively. Oxidation of vitamin D in emulsion-based products would occur easier when they are exposed to light, in which light acts as catalyst (Banville, Vuillemard, & Lacroix, 2002), as well as vitamin D is highly sensitive to high temperature. Thus, the high chemical stability of ergocalciferol in our study can be ascribed to the dark storage with mild storage temperature (25 °C).

Although, all nanoemulsions stored at 25 °C showed high chemical stability with almost no loss of ergocalciferol during storage period, the effect of emulsifier on the degradation of ergocalciferol at elevated temperature (55 °C) was rather significant. Fig. 7b illustrates the influence of emulsifier type on ergocalciferol stability in nanoemulsions stored at 55 °C. After 30 days of storage, 54.4%, 4.5% and 49.0% of ergocalciferol retained in the nanoemulsions stabilized by ML, SC and MO-7S, respectively. The data obtained from our study suggested that emulsifier type plays a vital role in maintaining the retention of ergocalciferol in nanoemulsions, and the loss of ergocalciferol decreased in the following order: SC ≫ MO-7S > ML. In the literature, it was reported that protein emulsifiers, such as whey protein isolate (WPI) and SC, provide better chemical stability for encapsulated compounds, compared to emulsifiers with low molecular weight, due to the thick protective layers and anti-oxidative properties (Tan et al., 2016). Also it was reported that WPI exhibited better β-carotene chemical stability in nanoemulsions stored at 55 °C, comparing to those stabilized by small molecule surfactants such as Tween 20 and decaglycerol monolaurate (Mao et al., 2009). The obtained results in our study disagreed with those reported by Mao et al. (2009). The reasons for these opposite results are still unclear. However, it was thought to be related to the difference in protein emulsifier tested. The adsorbed SC molecules on the surface of oil droplet are less close to each other than WPI molecule, due to the higher flexibility of casein than globular whey protein isolates (Dickinson, 2005). Thus, the most severe loss of vitamin D in SC-stabilized nanoemulsions, comparing to those coated with compacted emulsifier layer (small emulsifier or WPI) presumably attributed to huger gap between SC molecules induced ergocalciferol to be exposed to oxidation and degradation more quickly at an elevated temperature. Another reason that could explain SC-stabilized nanoemulsions exhibit lowest chemical stability in our study is that SC might undergo thermal degradation (Boland, Singh, & Thompson, 2014), resulting in weakening the membrane layer on the surface of oil droplet. Ergocalciferol in ML-stabilized nanoemulsions was least sensitive to thermal degradation among the samples and was attributed to phospholipids that play a role as anti-oxidizing agent, and play a vital role in encapsulation by reducing permeation of free radicals across the emulsion interface (Pan, Tikekar, & Nitin, 2013).

4. Conclusion

The current study showed that ergocalciferol loaded nanoemulsions was successfully formulated via the high pressure homogenization using three different emulsifiers (ML, SC or MO-7S). In addition, some

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of the major factors affecting the stability of resulting nanoemulsions were systematically evaluated. The stability of nanoemulsions to different environmental stresses were highly dependent on the emulsifier type, whereas no particular emulsifier tested was found to provide absolute stability to the nanoemulsions when exposed to pH, ionic strength, freeze-thaw cycles and high temperature treatment. Independent of emulsifier type, the nanoemulsions exhibited good physical and chemical stability during storage at 25 °C up to 30 days. In contrast, the long-term stability of nanoemulsions was highly dependent on the type of emulsifier tested at elevated temperature. The results of our study could provide useful information on the selection of appropriate emulsifier for producing stable nanoemulsions encapsulating functional bioactive compounds for commercial usage.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.foodres.2016.10.021.

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