STABILIZED EMULSION SYSTEM FORMULATED WITH WHEY PROTEIN ISOLATE, HEXANOIC ACID, CASEIN HYDROLYSATE, CHITOSAN, LECITHIN, AND NACL

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Abstract
This study assessed emulsion system that creates several layers of compatible component and stabilizes the emulsion system with increased concentration of ionic interaction. Primary, secondary and tertiary emulsifiers are added consecutively to form several layers. A primary emulsion with whey protein isolates (WPI) and hexanoic acid was prepared, and chitosan (Ch) (0.01%, 0.02%, and 0.03%, w/v) was added to evaluate its impact on the particle size of the emulsion system. NaCl (0, 20, 40, and 80 mM) was added to stabilize the multilayer emulsion through increased ionic interactions with lecithin (0.5%, 1%, 2%, and 3%, w/v as primary emulsifier to produce secondary layer formation. Casein hydrolysate (CH) was used as secondary emulsifier to further stabilize the emulsion system without the use of NaCl for 28 d at 4°C. NaCl was also added to increase the ionic strength of the total emulsion system to potentially stabilize the system without disrupting layer formation for 28 d at 4°C. Addition of lecithin does not create any layer formation over the previously formed layers. No increase of particle size has been observed after addition of lecithin. The major objective of this study was to evaluate the formation of a multilayered emulsion system due to oppositely charged particles and also with increased strength of ionic interaction.

Keywords: Particle size, ionic interaction, amphiphilic proteins, multilayered emulsion

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1. INTRODUCTION

Oil-in-water emulsions are abundantly used to deliver bioactive compounds in food products. Emulsions are thermodynamically weak or unstable systems. Therefore, various emulsifiers are used to improve and create stable emulsion systems that are loaded with bioactive components to deliver into the respective applied field (Dickinson, 1994; McClements, 2004; McClements et al., 2007). Basically, emulsifiers form a thicker interfacial layer that enhances its stability of the emulsion system under different physiological conditions (Guzey and McClements, 2006). Dairy-based proteins are used as emulsifiers in emulsion systems because they can be adsorbed to the oil droplet interface, which creates a strong and non-aggregative protective film (Lee and McClements, 2010). Whey proteins are useful in food emulsions due to the amphiphilic properties that allow them to interact with hydrophobic and hydrophilic residues (Foegeding et al., 2002). Whey proteins can be transformed into small peptides by controlled enzymatic hydrolysis, which partially exposes hydrophobic residues through slightly denaturing secondary and tertiary structures (Christiansen et al., 2004; Gauthier and Pauliot, 2003; Tirok et al., 2001). This hydrolysis process enhances their ability to diffuse at a higher rate in the oil/water interface and increases their ability to disperse a larger area of the interface than the native protein (O'Regan and Mulvihill, 2010).

Chitosan, a linear polymer that consists of randomly distributed beta (1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is a cationic polysaccharide that is derived from the alkaline deacetylation of chitin. At a pH belowthepKa (6.5), this polymer's amino acids undergo protonation, which makes it a soluble cationic polyelectrolyte, while at a pH above its pKa it becomes insoluble (Kumar et al.,2000; Rinaudo, 2006; Cho et al., 2005). Chitosan can also form an interfacial complex with surface

active protein residues and/or anionic surfactants, which enhances the viscosity of the emulsion (Lapante et al., 2005; Mun et al., 2005; Klinkesorn, 2013). Chitosan also facilitates layer deposition due to its cationic nature. Lecithin is generally recognized as safe (GRAS) anionic emulsifier that has been used in many studies to stabilize the formation of a chitosan-lecithin layer through layer emulsion formation (Shchukina and Shchukina, 2012). The objective of this study was to form a multilayer emulsion (Figure 1) system to carry bioactive components through the use of whey protein, chitosan, and lecithin at varying NaCl levels.

2. MATERIALS AND METHODS

Materials

Whey protein isolate (WPI; Provon®292, Glanbia Inc., Lot# 0855701) was obtained from Glanbia Nutritionals (Fitchburg, WI). Disodium hydrogen phosphate, chitosan, sodium chloride, and chymotrypsin were purchased from Sigma Aldrich (St. Louis, MO). Hexanoic acid was purchased from FlukaPerformix (Company, location); lecithin (Performix®E) was obtained from Archers Daniel Midland (ADM, Decatur, IL).

Dialysis of WPI

Forty grams of WPI was weighed and dispersed in 400 ml of de-ionized (DI) water followed by gentle stirring for 2 h at 20°C. The dispersed WPI solution was poured into dialysis tubing (50 ml per tubing (8 dialysis tubes). The four dialysis tubes were placed in a container and dialyzed against 4 L of DI water for one day by changing the water every 4 hours WPI solutions were collected, freeze-dried in a FreeZone® Triad™ Freeze Dry System (LabconcoCorp., Kansas City, MO) and stored in airtight containers -20°C.

Preparation of enzymatic hydrolysate of casein

Skimmed milk was adjusted to pH 4.5-4.6 by adding 0.1 N HCl, and the mixture was kept unstirred at 22°C for 30 min. The curd (casein) was drained and re-dispersed by vortexing in 0.1 N NaOH (final pH 7.8) to obtain 1% (w/v) dispersion. Chymotrypsin was used to digest freshly prepared acidic casein (Haque et al., 1993) at chymotrypsin: casein ratio of 1:60. The digestion mixture was adjusted to a pH of 7.8 using 100 mMTris-HCl, and was stored at 37°C for 1 h. Digestion was terminated by adjusting the pH to 2.0 using 0.1 M HCl. After digestion, the mixture was centrifuged (speed and time) and the pellet was freeze-dried and stored in an airtight container -20°C.

Emulsion preparation

Oil-in-water emulsions were prepared with dialyzed WPI (1% and 5%, w/v), hexanoic acid (disperse phase fraction, Φ=0.05), chitosan (1%, 2%, and 3%, w/v), and NaCl (0, 10, 20, 40, 80 and 160 mM) through gentle stirring for 1 h with a magnetic stirrer. These emulsions were then homogenized in a blender for 3 min on the slow speed setting and subjected to ultrasonic treatment for 30 s for 3 times with an interval of 5 minutes.This primary emulsion (tetra-system) was stabilized in different concentrations of NaCl and added to lecithin at different concentrations (1%, 2%, 3%, and 4%, w/v) to formulate a layer-by-layer (LBL) emulsion (Penta-system). Lecithin was first dispersed in 50 ml of buffer (Mcllvaine's isotonic buffer, pH 6.0) and homogenized by blending for 2 min and sonicated for 30 s. Lecithin solutions were added to the previously described tetra-system followed by gentle stirring for 1 h. The final pH of all emulsions was adjusted to 6.0 with McIlvaine's buffer.

Particle size measurements

The mean globular size of the emulsion, expressed as dispersed phase volume-surface average diameter (d_{vs}), was measured on a TrilaserMicrotrac S3500 Particle Size Analyzer (Nikkiso, Tokyo, Japan) based on the expression

$$\sum V_i / \sum \left(\frac{V_i}{d_i}\right)$$

where V_i is denoted as the volume of globules in a size class of mean diameter d_i in micrometer(μm).

Statistical analysis

There were six experiments conducted in this study, all within completely randomized designs with 3 replications. The first three experiments were 2 × 5 factorial designs with treatment effects of chitosan and sodium chloride concentration. The fourth experiment was a 3 × 5 factorial design with 3 different concentrations of chitosan and 5 different NaCl concentrations. The fifth experiment was a 4 × 5 factorial design with 4 concentrations of lecithin and 5 concentrations of sodium chloride. The sixth experiment included was a 2 ×10 factorial design with two treatments of with or without chitosan and 10 different sodium chloride concentrations in a completely randomized design (Table 1). The data analysis was performed using SAS 9.3 (SAS Institute Inc., Cary, NC USA). Means were separated within each treatment factor using p diff ($P<0.05$) when significant differences existed among treatments ($P<0.05$).

3. RESULTS AND DISCUSSION

Mean the particle size of the primary emulsion

When 5% WPI was used in the primary emulsion system, no differences existed in particle size between emulsions with and without chitosan at 1 h after blending with 0 and 20 mMNaCl solutions (Experiment 1, Figure 2A). However, adding chitosan at 0.01% increased the particle size of the emulsion at 10, 40, and 80 mMNaCl. This increase in particle size was not practically important since the size of the emulsions were between 8 and 12 μm, which indicated that droplets were too large for any of these treatments to form stable emulsions. Similar results occurred 24 h after blending, with the exception that the chitosan treatments had greater particle size after 24 h of blending ($P<0.05$) for the 0, 40 mM and 80mM concentrations of NaCl (Experiment 2, Figure 2B). Since all emulsion treatments were unstable when 5% WPI was included in these first 2 experiments and 3 through 6 were formulated with 1% WPI. It is likely that the emulsion became unstable because of high pressure greater than 50 MPa is required to stabilize emulsions with high concentrations of protein.

When 1% WPI was used in the emulsion formulation, the 0 and 10 mMNaCl treatments (Experiment 3) had greater particle size at 24 h after blending when chitosan was added to the formulation ($P<0.05$) (Figure 3). When NaCl concentration ranged from 20 to 80 mM, the addition of chitosan did not affect ($P>0.05$) the particle size. However, all emulsions were more stable since the particle size was approximately 3 - 4 μm for each treatment, which was less than the emulsions that were formulated with 5% WPI. The lack of increase in particle size indicates that the chitosan may have interacted with the whey protein to help form a stable emulsion but did not form a multilayer since this would have increased the size of the globules. To verify this, imaging analysis would need to be conducted. Since smaller globular sizes were achieved after the addition of chitosan, a fourth experiment was conducted to evaluate the effect of chitosan percentage on the mean globular diameter of the emulsion. At all salt concentrations tested (0 to 160 mM), the mean globular diameter was greater at 0.03% chitosan when compared to the 0.01 and 0.02% chitosan treatments (Experiment 4, Figure 4). In addition, the only treatments that had increased particle size were the 40 mM and 160 mM NaCl treatments with 0.03% chitosan. Based on these results, 0.02% chitosan was used in the formulation for experiments 5 and 6 to produce the secondary emulsion.

Mean particle size of the tertiary emulsion

Addition of casein hydrolysate (2% of WPI, w/w) with the secondary emulsion completed the formation of the tertiary emulsion. In the last experiment, casein hydrolysate (CH) was added to increase ionic reactions in the emulsion system. Results indicated that the

emulsion remained stable after the addition of CH, which is important for future research on delivering curcumin in emulsion systems (Experiment 6, Figure 6). Even though these ingredients were successfully used in the emulsion systems, it was evident that the emulsions were unstable over storage time. Therefore, future research will be conducted on nanoemulsions to determine if they will remain stable over storage time.

Table 1: Summary of all the experiments with their respective statistical factorial designing

Experiments	Statistical Design	Ingredients and their number of concentrations
First	2 × 5 factorial design	Chitosan (with and without) and NaCl (5)
Second	2 × 5 factorial design	Chitosan (with and without) and NaCl (5)
Third	2 × 5 factorial design	Chitosan (with and without) and NaCl (5)
Fourth	3 × 5 factorial design	Chitosan (3) and NaCl (5)
Fifth	4 × 5 factorial design	Lecithin (4) and NaCl (5)
Sixth	2 × 10 factorial design	Casein Hydrolysate (with and without) and NaCl (10)

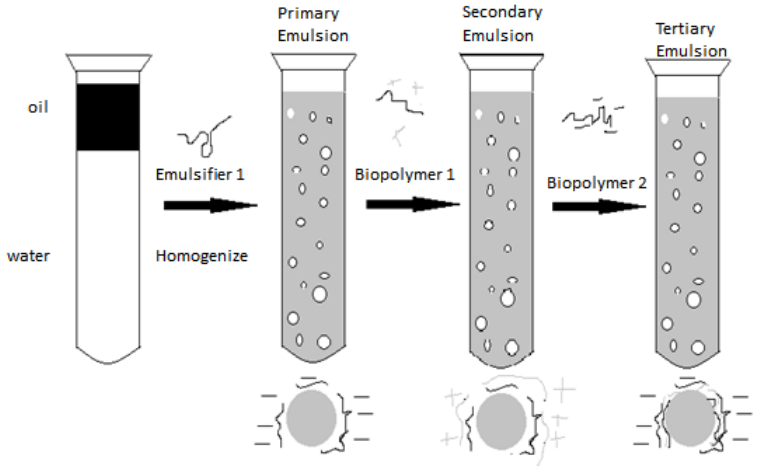
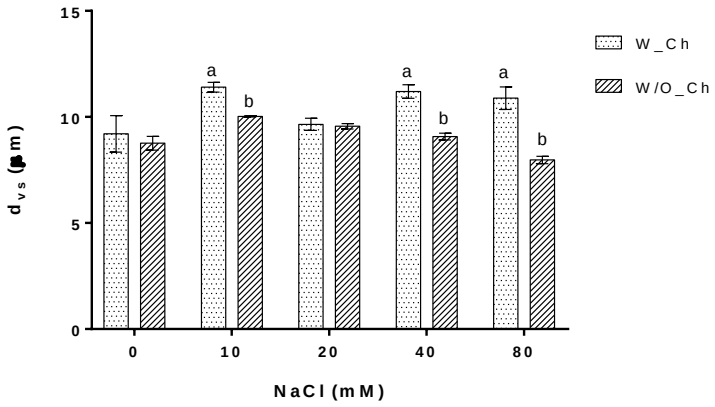


Fig. 1.Schematic representation of layer-by-layer emulsion system (Adopted from Ogawa et al., 2004)

A



B

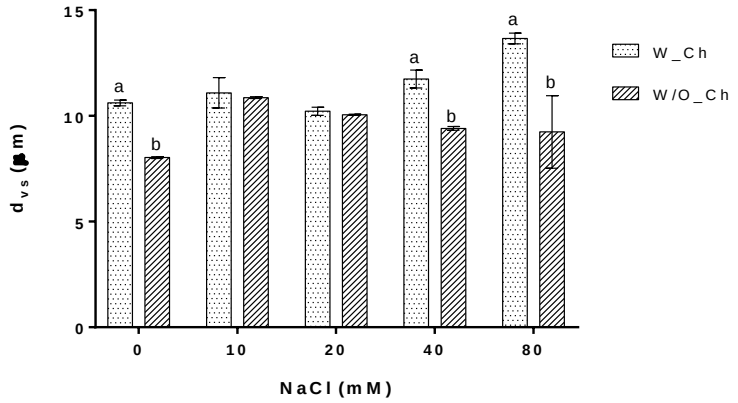


Fig.2. Mean globular diameter of a WPI (5%, w/v) and hexanoicacid (Φ=0.05) based emulsion with (W_Ch) or without (W/O_Ch) chitosan after1 h (A) and 24 h(B) of blending. Significant differences between the W_Ch and W/O_Ch treatments at each NaCl concentration are indicated by different letters (P<0.05). Data are presented as the mean ± standard deviation.

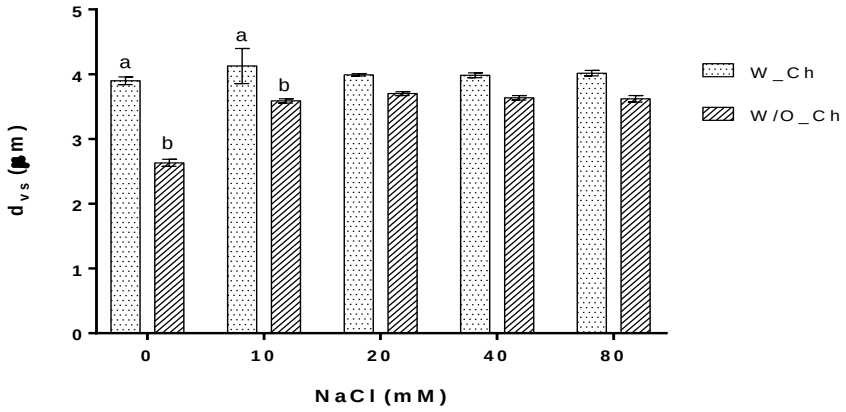


Fig.3. Mean globular diameter of a WPI (1%, w/v) and hexanoicacid (Φ=0.05) based emulsion with (W_Ch) or without (W/O_Ch) chitosan after 24 h of blending. Significant differences between the W_Ch and W/O_Ch treatments at each NaCl concentration are indicated by different letters (P<0.05). Data are presented as the mean ± standard deviation.

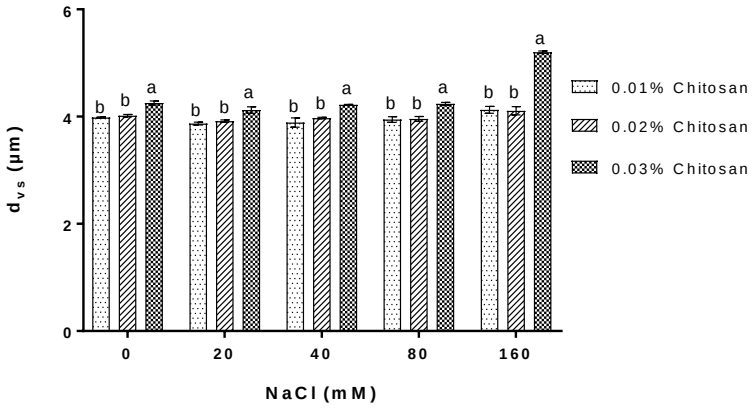


Fig. 4.Mean globular diameter of a WPI (1%, w/v) and hexanoicacid (Φ=0.05) based emulsion containing chitosan (0.01%, 0.02% and 0.03%) after 1 h of blending. Significant differences between the chitosan treatments at each NaCl concentration are indicated by different letters (P<0.05). Data are presented as the mean ± standard deviation.

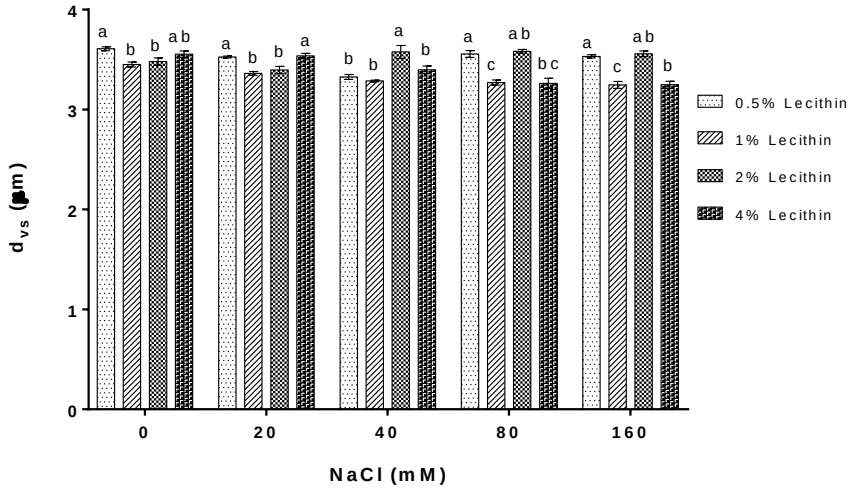


Fig. 5. Mean globular diameter of a WPI (1%, w/v) and hexanoicacid (Φ=0.05) based emulsion containing chitosan (0.02%) and lecithin (0.5%, 1%, 2%, 4%) after24 h of storage. Significant differences between the lecithin treatments at each NaCl concentration are indicated by different letters (P<0.05). Data are presented as the mean ± standard deviation.

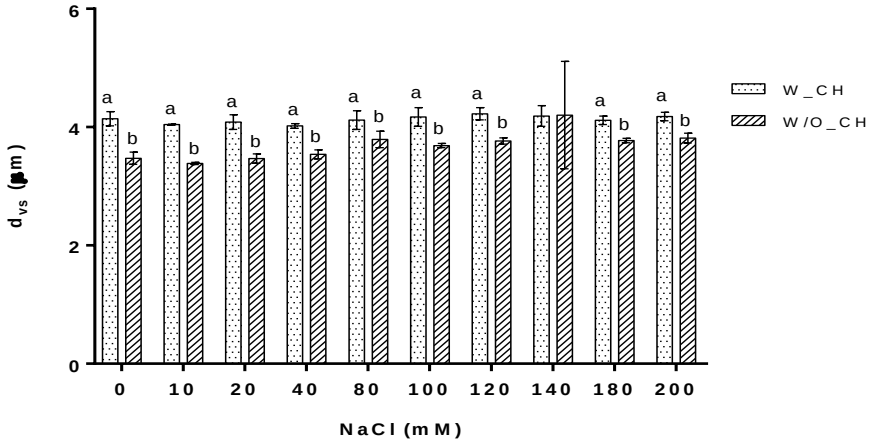


Fig. 6. Mean globular diameter of a WPI (1%, w/v), hexanoicacid (Φ = 0.05), chitosan (0.02%) and lecithin (2%) based emulsion containing casein hydrolysate (2% of WPI, w/w) (W_CH) and without casein hydrolysate (W/O_CH) after 28 days of storage. Significant differences between the W_CH and W/O_CH treatments at each NaCl concentration are indicated by different letters (P<0.05). Data are presented as the mean ± standard deviation.

Discussion

Particle size reduction is the primary objective of the emulsion formulation. A smaller particle exposes hydrophilic side chains that solubilize the protein structure (Mayers et al., 1995). Also, as droplet size decreases, the interfacial area increases which confer better stability by reducing protein loads (Hunt and Dalgleish, 1994). It has been theorized that this increased stability can promote the creation of monolayer coverage of the nascent droplet. In protein-based emulsion systems, competitive absorption must be considered. Competition is only observed when there is excess protein in the system that is not needed in the emulsion system. An absorptive layer can be formed when WPI concentration exceeds 0.9% (McClements, 2005). Since it was assumed that

a higher concentration of WPI would provide more protein for absorption, 5% WPI was used to formulate the primary emulsion. However, the use of 5% WPI caused a larger particle size and emulsion instability. In addition, proteins can be included to formulate emulsion droplets for subsequent layer deposition, especially at pHs away from the isoelectric point (pH 6.0 for our system) (Gu et al., 2005). The ionic strength of the solution and the intra and inter-molecular electrostatic interaction leads to the formation and stabilization of a multilayered film (Decher et al., 1992; Poptoshev et al., 2004). The presence of salt during multilayer deposition may increase the thickness of the deposited layer (Decher et al., 1992). In the absence of salt, polyelectrolytes form comparatively thin layers with flattened chains that absorb against the surface. This results in electrostatic repulsion, which restricts further post absorption molecular rearrangement with other molecules. In the presence of salt, weaker intra-molecular repulsion in the adsorption layer allows the molecular rearrangement of charged electrolytes and surface groups (Steitz et al., 2000). Therefore, the addition of salt in an emulsion allows molecular interaction on the interfacial surface of the droplet. Our first and followed experiment(after the primary emulsion formation) supports this view. The anionic lipid lecithin was used as a stabilizer and has been previously used as a second deposition layer of the cationic polysaccharide chitosan (Ogawa et al., 2003; Klinkeson et al., 2009). Decreased particle size in the secondary emulsion does not indicate that deposition of lecithin has occurred on the exterior surface of the droplet. Our studies confirmed the deposition of lecithin over chitosan. However, increased concentrations of lecithin established that deposition may occur, but higher concentrations may contribute to emulsion instability, since 2% lecithin lead to larger emulsion droplets than emulsions with 1% lecithin.

Addition of casein hydrolysate enhanced emulsion stability for all treatments and decreased the importance of NaCl to emulsion stability. Lack of significant differences establishes that no molecular interaction occurs, which is possibly due to ionic saturation (Huang et al., 2017). Notenough protein surface was available for further deposition of lecithin over chitosan. Addition of CH exposed the protein surface, which contributed to further molecular rearrangement and stabilization of the emulsion, even without salt. A continuum of electrolytes or oppositely charged polyelectrolytes may have stabilized the emulsion. Antioxidant properties of CH presumably results from the redox activity of thiol groups in the peptides (Brandes et al., 2009). These groups are particularly redox-sensitive when they are in the proximity of neighboring positively charged or aromatic residues (e.g., phenylalanine, tyrosine) (Antelmann and Helmann, 2011; Phelan et al., 2008). This in turn potentially increases the ionic interaction or electrostatic repulsion of the emulsion system.

4. CONCLUSIONS

Addition of lecithin did not reduce the particle size of the emulsion droplets. There was no evidence of the deposition of oppositely charged polyelectrolytes. A continuum of electrolytes or oppositely charged polyelectrolytes may have stabilized the emulsion. Further research is needed to confirm the deposition of oppositely charged particles since results from this study were not indicative of the formation of a multilayer emulsion system. Since a multilayer emulsion system was not formed with our ingredients of choice, it was proposed that a nanoemulsion system would potentially be a better delivery system for insoluble bioactive than a multilayer emulsion system.

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