RESEARCH ARTICLE

Enhanced Delivery of Sesame Oil through Nanoformulation: Edible Protein Excipients and Nanotechnology as Protagonists

Pubali Dhar[1*], S. Pal[1], N Chatterjee[1], T Das[1], Dey S[2], A.Chakraborty[3]

[1]*Laboratory of Food Science and Technology, Food and Nutrition division, University of Calcutta, Alipore, Kolkata, West Bengal, India.*
[2]*Centre for Research in Nanoscience & Nanotechnology, University of Calcutta, Bidhannagar, Kolkata, West Bengal, India*
[3]*Department of Physiology, University of Calcutta, Rajabazar, Kolkata, West Bengal, India*

Received: 25th August, 2022; Revised: 03rd September, 2022; Accepted: 07th September, 2022; Available Online: 25th September, 2022

ABSTRACT

For decades, researchers have gained a lot of interest for lignans-rich sesame oil due to its potent antioxidative and anti-inflammatory properties. But its potential could not be fully achieved through traditional means of delivery due to the poor aqueous solubility of lipophilic bioactives. To eliminate the unsatisfactory outcome of conventional delivery strategies and to corroborate the perception of manufacturing the bio-compatible vehicle for therapeutic delivery, present-day researches are vigorously attempting to develop nanocarrier systems with food-grade excipients. In this current scenario, a primary objective of our venture is to explore soy protein for stable sesame oil nanoemulsion fabrication using high-energy ultrasonic devices. In the present study, the sesame oil nanoemulsion formulated with soy-protein isolate and tween 20 in 3:1 ratio has successfully minimized the hydrodynamic diameter to 105 nm and also improved the shelf-life stability remarkably during the 8-weeks storage period.

Furthermore, the evaluation of *in-vitro* digestibility of formulated nanoemulsion compared with the conventional emulsion seems crucial to facilitate the application of nanoemulsion as a delivery tool in the physiological system. The findings suggested that in the simulated gastrointestinal tract, the nanoemulsion achieved a better fatty acid release kinetics and thus an improved lipid digestion profile compared to the conventional one. The facts and facets obtained from this study would expect to elicit challenging openings as well as satisfactory possibilities in the frontier area of the food and pharmaceutical industries.

Keywords: Lipid digestibility**,** Nanoemulsion, Sesame oil, Soy-protein, Stability, Ultrasonication.

International Journal of Drug Delivery Technology (2022); DOI: 10.25258/ijddt.12.3.36

How to cite this article: Dhar P, Pal S, Chatterjee N, Das T, Dey S, Chakraborty A. Enhanced Delivery of Sesame Oil through Nanoformulation: Edible Protein Excipients and Nanotechnology as Protagonists. International Journal of Drug Delivery Technology. 2022;12(3):1139-1147.

Source of support: Indian Council of Medical Research (ICMR).

Conflict of interest: None

INTRODUCTION

Sesame oil enriched with poly-unsaturated fatty acid is already reported to offer various physiological and nutritional benefits.[1] Due to the fatty acid content, sesame oil plays a remarkable role in protecting the cardiac system, lowering cholesterol, preventing atherogenesis, and reducing inflammation[2] from PUFA, sesame oil contains several natural antioxidants such as sesamolin, sesamin, and tocopherol homologues[3] that also play a crucial role in maintaining redox homeostasis by scavenging free radicals and to arrest the oxidative damage and stress-mediated inflammation in the physiological system. Despite having numerous health benefits, the protective efficacy of sesame oil may not be fully achieved through traditional means of delivery due to rapid elimination through renal system, early clearance by the reticuloendothelial system (RES), difficulties in transport from the circulation to the target site and the harsh acidic environment of endo-lysosomes within the cell.[4] Furthermore, the therapeutic potency of these lipophilic bioactives have shown to be insufficient through traditional delivery strategies due to the reduction of digestion and absorption efficiency of impaired gastrointestinal system during severe systemic inflammation.[5] To surpass these disappointing outputs, nanoscale research has directed its focus in designing novel delivery devices to achieve target-specific transport, controlled release of the core and thus the maximum level of ameliorating efficacy of the bioactive components.[6] Nanoemulsion has been proposed as a novel carrier system suitable for delivering lipophilic bioactives

**Author for Correspondence: pubalighoshdhar@yahoo.co.in*

to bypass intermediate metabolic conversions and overcome physiological barriers.[7]

To corroborate the perception of manufacturing the biocompatible and edible nanoemulsion, recent researches are vigorously attempting to avoid commercial surfactants as well as to exploit biomaterials as emulsion stabilizers.[8] Though the formulation of safe and non-toxic nanoemulsion still remains an immense challenge, food-grade protein has gained considerable interest as formulation excipients owing to its inherent nutritional values as well as unique biocompatibility and biodegradability in physiological system.[9] Protein molecules amphipathic in nature have exhibited emulsifying and surface modifying a property. Different functional groups residing in proteins have been reported to bridge neighboring lipid molecules via chemical cross-linking.[10]

In this present scenario, soy protein is selected in our study as emulsifying agent owing to its non-toxic nature as well as cost-effective natural resource.[11] Soy-protein isolates consisting of 11S and 7S globulin is reported to significantly lower interfacial tension significantly[12] and possess distinctive functional properties such as emulsifying and gelling.[13] Interestingly, heat-induced aggregation of soy protein is found to facilitate the hydrophobic moieties enfolded in the native state to be exhibited towards the surface, increasing surface hydrophobicity.[14] In addition, denatured soy protein aggregates have been spotted to form stable emulsions as the emulsifier coating surrounding oil droplet comprises adsorbed aggregated proteins.[15] However, utilizing protein as a sole emulsifier at interface remains a major challenge for researchers. Protein-coated nanoemulsion is reported to be stabilized by electrostatic repulsion.[16] Therefore, it seems to be more prone to aggregation at isoelectric pH where the electrostatic repulsion fails to conquer attractive forces such as hydrophobic attractions and van der Waals interactions.

Moreover, at high ionic strength, i.e., the presence of higher salt concentration in the medium, is reported to neutralize the surface electrical charge and reduce electrostatic repulsion among the emulsified oil droplets owing to the electrostatic screening effect.[17] To overcome such undesirable output, in this study tween 20 was engaged with protein to fabricate a stable emulsion formulation. Tween 20, a small non-ionic surfactant with the polymeric head group was previously reported to partially displace soy-protein from the interface, interact with the residual proteins on an interface and impart steric stabilization enough to the emulsion formulation so that the droplets can overcome such aggregation.[18]

Successful fabrication of nanoemulsion can be made possible with several high and low-energy methods.[19] High-energy approaches utilize mechanical tools providing intense, disruptive forces to produce tiny oil droplets, whereas low-energy techniques exploit inherent interfacial properties of the excipients used in the system. To evade the requirement of the large amount of surfactant in low-energy techniques, till date, researchers mostly rely on high-energy approaches to formulate oil-in-water nanoemulsions using food-grade natural emulsifiers.[20] The present study primarily aims to fabricate soy-protein-based sesame oil nanoemulsions with the aid of ultrasound-assisted high-energy homogenization.

The final goal of this research is to investigate the free-fatty acid release kinetics and thus to evaluate the *in-vitro* lipid digestibility of sesame oil nanoemulsion compared with the conventional system. The endeavor is perhaps the first report exploring the stable sesame oil nanoemulsion with food-grade soy-protein excipients and evaluating the digestibility of formulated sesame oil in a simulated gastrointestinal environment.

MATERIALS AND METHOD

Materials

Refined sesame oil was procured from local supermarket of Kolkata, India. It was used without further purification. Soy protein isolate, Tween 20, and Nile red, were commercially obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents used in this work are of analytical grade.

Preparation of Nanoemulsion and Corresponding Conventional Emulsion

Oil-in-water nanoemulsions holding 2.5% (w/w) oil and 1.8% (w/w) emulsifier was prepared using a combined high-speed homogenization and ultrasonication approach as per previous report with required modification.[21] Soy protein isolate and tween 20 were mixed with in 3:1 ratio. 1.8% (w/w) emulsifier mixture was then dispersed in 10.0 mM sodium phosphate buffer solution (pH 7.0). 0.01% (w/w) Sodium azide was included in this solution to inhibit bacterial growth. After that 2.5% (w/w) sesame oil was added to this drop by drop followed by thorough mixing of oil and aqueous phase under continuous agitation at ambient temperature (25^{o}C) for 30 minutes. High-speed homogenization was then carried out with a T18 Ultra-Turrax homogenizer (IKA) at 15,000 rpm for 10 minutes to fabricate a conventional emulsion. Probe-ultrasonicator (Labman Pro-500, Probe diameter: 9.5 mm, India) operating at a frequency of 20 kHz with a power output of 300 W was successfully utilized for fabricating nanoemulsion utilizing ultrasonic waves. After high-speed homogenization, a certain amount of conventional emulsion was poured into a glass beaker immersed in an ice bath to minimize undesirable lipid oxidation during ultrasonication treatment. The following studies were performed with the freshly-prepared (O/W) conventional emulsion and nanoemulsion formulations.

Detailed Droplet Characterization During Storage

Droplet Size Measurement During Storage

Nanoemulsions and conventional emulsions just after fabrication and after different time intervals during storage at 25°C were thoroughly characterized with respect to size and charge distribution of the droplets. The fabricated

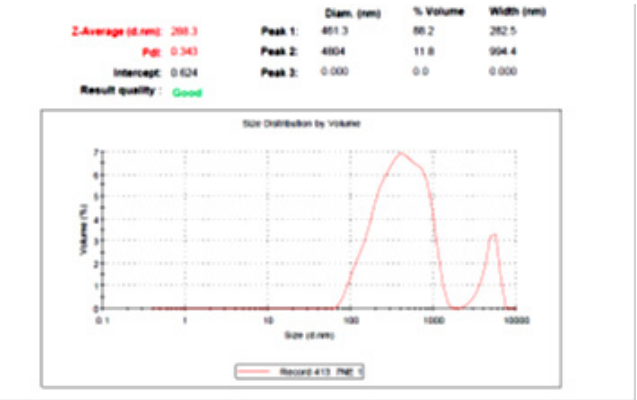

Figure 1a: Presenting initial hydrodynamic droplet diameter and poly dispersity index of conventional emulsion formulation

Figure 1b: Presenting initial hydrodynamic droplet diameter and poly dispersity index of nanoemulsion formulation

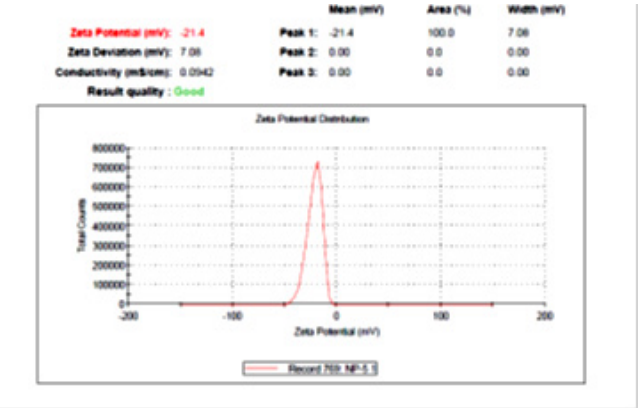

Figure 2a: Presenting initial zeta potential value of conventional emulsion formulation

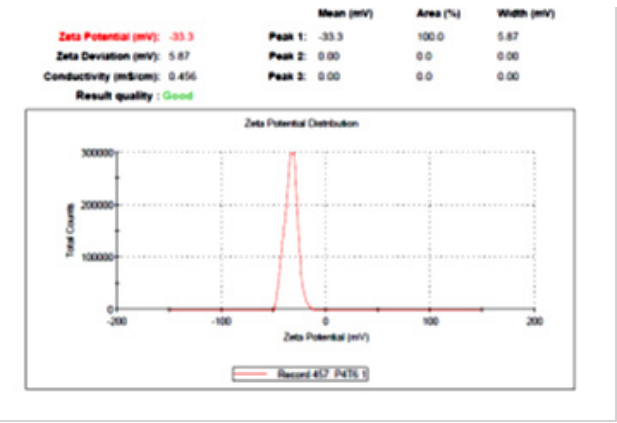

Figure 2b: Presenting initial zeta potential value of nanoemulsion formulation

Figure 3: Visual appearance of conventional emulsion and nanoemulsion during stipulated storage period

formulations' average droplet diameter and poly-dispersity index were measured with Malvern Nano ZS (Malvern Instruments, Zetasizer version 6.00, UK). The measurements were performed by diluting 100 times in triplicate.[22]

Droplet Electrical Charge Measurements During Storage

Surface charge or zeta potential (ζ-potential) of the conventional and nanoemulsion formulations were obtained from Malvern Nano ZS (Malvern Instruments, Zetasizer version 6.00, UK). Zeta potential values were determined in triplicate by using particle electrophoretic light scattering effect.[22]

Morphological Analysis

Transmission Electron Microscopy observed the droplet size and shape of the nanoemulsion formulation. Freshly prepared nanoemulsion and conventional emulsion samples were adsorbed on 300 mesh TEM copper grids for 1-minute. Then, the formulations were negatively stained by a drop of 1% (w/v) sodium-phospho-tungstate for 1-minute. Extra stain was removed by filter paper. The images of the samples were examined by TEM (JEM 1010, JEOL, Tokyo, Japan).[23]

Lipid Oxidation Measurements

Oxidative stability of conventional emulsion and nanoemulsion containing 2.5% (w/w) oil was expressed by quantifying the amount of TBARS (Thiobarbituric acid-reactive substance) in the emulsion system during storage.[24] 2 mL sample from each formulation was taken in two different capped test tubes and kept for 8 weeks in dark at 25°C. 15 g of TCA, 0.34 g of TBA, 1.76 mL of 12 M HCl, and 82.8 g of H_2O were vigorously mixed to prepare 100 mL TCA (trichloroacetic acid)-TBA (Thiobarbituric acid)-HCl solution. Further, this solution

Table 1: Presenting the stability study in terms of size, poly dispersity index and zeta potential for fabricated conventional emulsion and nanoemulsion throughout the 8-weeks storage period

	Conventional emulsion			*Nanoemulsion*		
	Size	*Pdi*	*Zeta potential*	*Size*	*PDI*	*Zeta potential*
0 Week	289.34 ± 10.12	0.34 ± 0.03	-21.4 ± 2.23	105.0 ± 7.47	0.24 ± 0.02	-33.3 ± 4.41
2 Weeks	385.9 ± 17.67	0.39 ± 0.01	-17.0 ± 3.74	109.1 ± 9.23	0.24 ± 0.03	-32.0 ± 2.89
4 Weeks	528.1 ± 16.23	0.41 ± 0.07	-9.1 ± 2.51	118.9 ± 4.89	0.23 ± 0.05	-30.9 ± 5.02
6 Weeks	786.7 ± 23.29	0.48 ± 0.09	-7.6 ± 1.03	137.0 ± 11.21	0.27 ± 0.01	-28.5 ± 3.46
8 Weeks	912.3 ± 28.59	0.45 ± 0.04	-3.7 ± 0.36	182.8 ± 8.51	0.36 ± 0.04	-26.1 ± 3.64

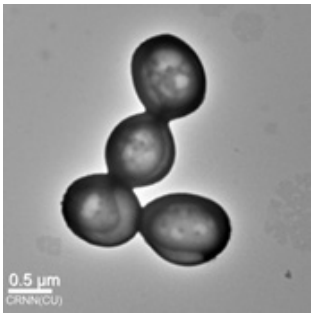

Figure 4a: Transmission electron microscopic images of conventional emulsion

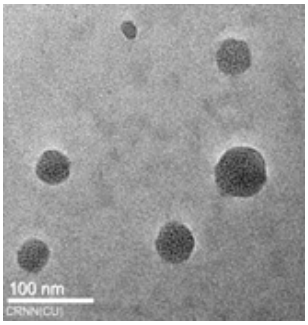

Figure 4b: Transmission electron microscopic images of nanoemulsion

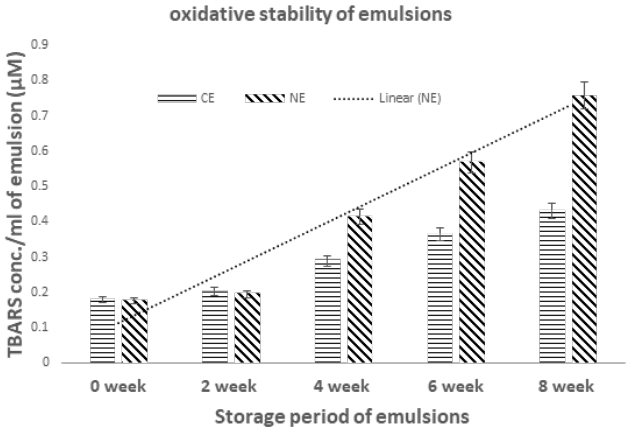

Figure 5: Evaluation of oxidative stability of conventional emulsion and nanoemulsion during stipulated storage period

was mixed with 3 mL of 2% (w/w) butylated hydroxytoluene in ethanol. Finally, 2 mL of emulsion sample was added to 2 mL of this solution. The mixture was heated at 80°C for 20 minutes and then cooled using running tap water for 10 minutes. Later the mixture was centrifuged at 2000 g for 5 min using a centrifuge (Remi R-24 Centrifuge). Finally, the absorbance was measured spectrophotometrically at 532 nm (Genesys 50 double beam UV/VIS Spectrophotometer, Thermo Fischer Scientific, USA).

In-vitro Digestion and Determination of Lipid Digestibility

Two-stage technique for simulated *in-vitro* digestion was carried to evaluate the lipid digestibility of both conventional emulsion and nanoemulsion.[25] The ratio of emulsion formulation, simulated gastric fluid and simulated intestinal fluid was kept at 1:1.5:2 in this study. 10 mL conventional emulsion was added to 15 mL simulated gastric fluid (0.15 M sodium chloride and 3.2 mg/mL pepsin). pH was set to 2.0 using 1.0 M HCl and temperature was maintained at 37°C for two hours with magnetic stirring at 300 rpm. Then the sample taken from gastric phase was kept in a thermostatic water bath (37°C) and the pH was adjusted at 7.0 using 0.1 M sodium hydroxide solution. Then 20 mL of simulated intestinal fluid (24 mg/mL lipase, 20.0 mg/mL bile extract, 10 mM $CaCl_2$) at pH 7 was poured to the solution. The pH of the system was maintained at 7.0 with the addition of 0.1 M NaOH drop-by-drop for neutralizing the FFA produced during lipid digestion in the intestine. The temperature of the system was held constant at 37°C with a temperature-controlled bath under magnetic stirring at 300 rpm. The total volume of NaOH needed during intestinal digestion was noted.

Free Fatty Acid Release

The extent of free fatty acids (FFA) released from oil-in-water conventional emulsion and nanoemulsion were estimated using a titration method[26] with 0.1 mol·L^{-1} NaOH. The percentage of free fatty acids generated was determined using the following eqn.

$$\% \text{ FFA} = 100 \times ({}^{V}\text{NaOH} \times {}^{m}\text{NaOH} \times {}^{M}\text{Lipid}) / {}^{w}\text{lipid} \times 2$$

^{V}NaOH is the volume of sodium hydroxide utilized for neutralizing the FFA released (in L), ^{m}*NaOH* is the molarity of the sodium hydroxide (in mol·L^{-1}), ^{w}*lipid* is the total weight of sesame oil present initially in the system and ^{m}Lipid is the molecular weight of the sesame oil (the molecular weight of sesame oil was found to be 1103.8 g mol^{-1}).

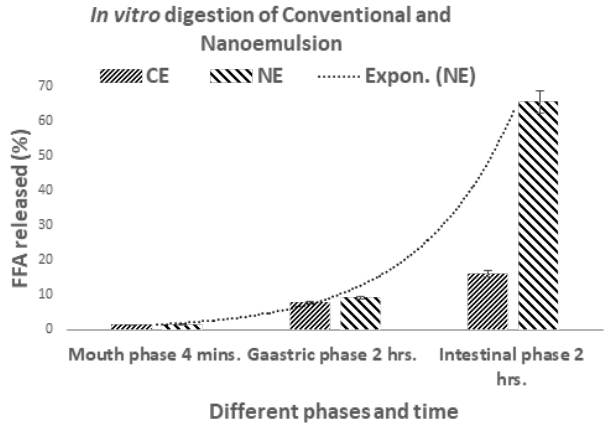

Figure 6a: Percentage of free fatty acid release (%) from conventional emulsion and nanoemulsion during different phases of digestion

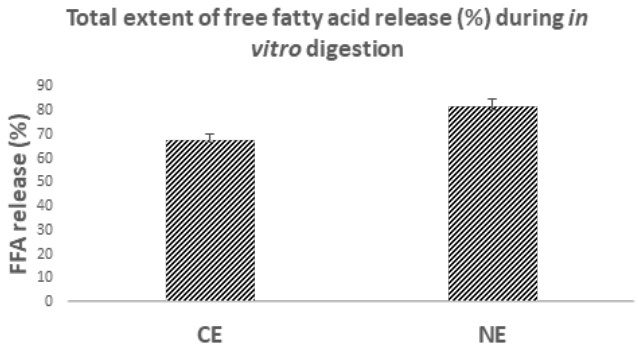

Figure 6b: Total extent of free fatty acid release (%) from conventional emulsion and nanoemulsion during stipulated period of simulated digestion

Confocal Microscopy Before and After Digestion

The comparative analysis of morphology of the oil droplets in the conventional and nanoemulsion before and after digestion was carried out with a confocal laser scanning microscope (EVOS M5000 Imaging system, Thermo Fischer Scientific, USA) with a 20X magnifying lens.[27] Nile Red dissolved in dimethyl sulfoxide was mixed with emulsion samples in 1:10 ratio to stain and visualize oil droplets. A minute amount of the stained emulsion formulations throughout the digestion procedure was placed in a glass slide and covered with a glass coverslip to finally prepare the slide for confocal microscopy.

Statistical Analysis

All experiments were carried out in triplicate using fabricated formulations and the data were represented as mean ± standard error of the mean (SE). For statistical evaluation of the results and determination of level of significance level, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was executed thoroughly. The statistical differences between the values were considered to be significant at $p < 0.05$ significance level.

RESULTS AND DISCUSSION

Detailed Droplet Characterization

Characterization of formulated nanoemulsion and conventional emulsion with Physico-chemical properties, *i.e.,* hydrodynamic diameter polydispersity index and surface electrical charge for a stipulated storage period, was presented in Table 1.

In the presence of soy protein and tween 20 as an emulsifier, lignans-rich sesame oil was primarily emulsified into an

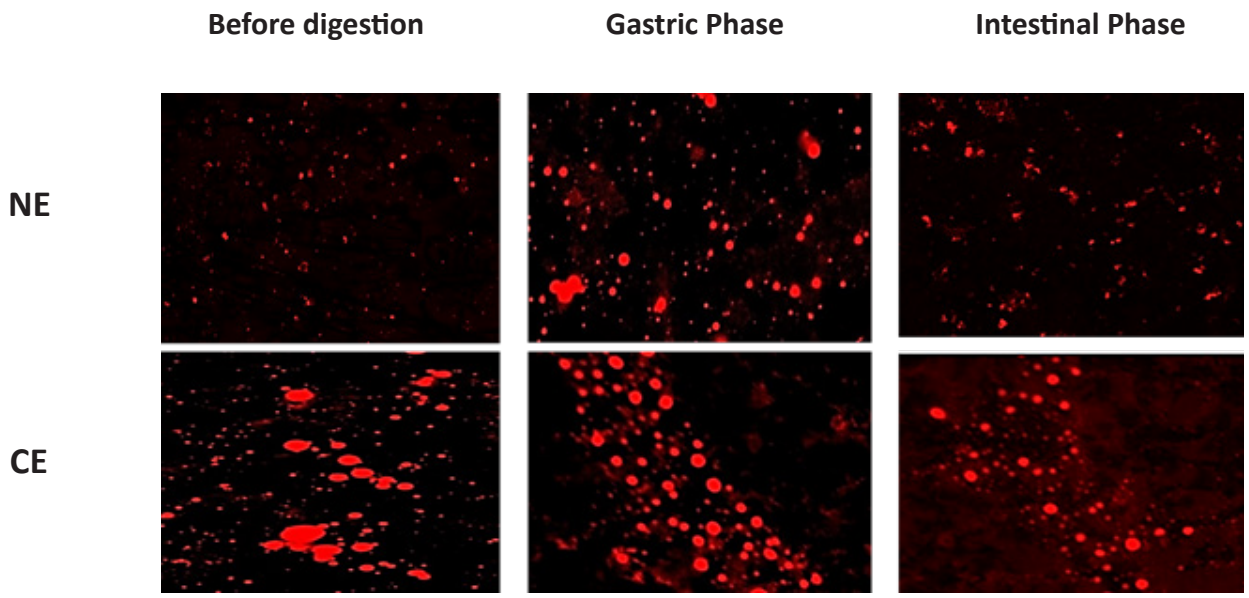

Figure 7: Confocal microscopy images revealing the fate of the fabricated formulations, *i.e.,* oil-in-water conventional emulsion and nanoemulsion in different phases of simulated digestion

aqueous phase through high-speed homogenization. On the other hand, nanoemulsion was formulated by a high-speed homogenization process followed by ultrasonication. The type and concentration of oil and emulsifier and the fabrication technique were reported to immensely affect the bulk physico-chemical characteristics and stability of the formulation. Therefore, in the current study all the process parameters, excluding the fabrication technology, were kept constant for fabricating nanoemulsion and conventional emulsion. The average droplet sizes, as well as polydispersity index (PDI) were found to decrease significantly after the application of ultrasonication.

Figure 1(a) depicted that the initial droplet size of the conventional emulsion was 288.3 nm with higher Poly Dispersity Index (PDI) of 0.34 while the droplet size and PDI were reduced to 105 nm and 0.24, respectively in nanoemulsion illustrated in Figure 1(b). A lower value of PDI was shown uniform distribution of the emulsified oil droplet size indicating improved stability of emulsion formulation.[28]

The application of an intense mechanical energy is quite essential for the generation of tiny oil droplets from the larger. This disruptive shear force comes from the power amplitude and the sonication time during ultrasonication.[29] In ultrasonication, the pressure amplitude of the sound wave raises with increasing the power amplitude, which will further expand the extent of cavitation regarding the growth and collapse intensity of the bubbles. With increasing the cavitation, the extent of the interfacial destabilization rises and thus the droplet number increases as a consequence of liquid thread break-up.[30] The dispersed phase droplet size has also shown to decrease gradually with an expansion in the sonication time. With the increase in power amplitude and the sonication time, the energy dissipation rate within the system is supposed to be enhanced and thus the rate of increase in temperature. An increase in temperature leads to raise the vapor pressure of the cavitating medium. This in turn might cause a rise in the number of nuclei giving rise to cavitation. With an elevation in the number and intensity of cavitational events, the disruption of oil phase to generate fine oil droplets is perceived to be amplifying in ultrasonic emulsification. Furthermore, with a rise in temperature, the viscosity and interfacial tension have decreased considerably, decreasing the cavitation threshold and increasing interfacial instability facilitating oil phase disruption. From the findings obtained from our study, ultrasonication generating compressive and tensile stresses was suggested to be the most crucial factor for the significant reduction in droplet size during emulsification.

The surface electrical charge of the emulsified oil droplets in nanoemulsion was quantified by measuring the zeta potential of droplets. Figure 2b indicated that the nanoemulsion was initially found to possess higher negative zeta potential (-33.3 mV) compared to conventional emulsion (-21.4 mV) shown in Figure 2a.

Significantly high zeta potential values (positive or negative) indicated strong electrostatic repulsion among the dispersed oil droplets in the continuous aqueous medium leading to good electrical stabilization. In contrast, low zeta potential value tends to coagulate or flocculate leading to physical destabilization. Despite the non-ionic nature of Tween 20, the droplet coated with tween 20 was shown to impart negative charge possibly caused by surface-active anionic impurities (*e.g.,* free fatty acids) in the oil phase or adsorption of hydroxyl ions (OH^- ions) from water.[31] When the aqueous medium's pH is higher than the isoelectric point (pI) of the protein, protein molecules are reported to impart negative charges since at that point carboxyl groups possess negative charge and amino groups remain neutral.[32] Hence, soy protein was found to impart negative charges in the system as the isoelectric point (pI) of soy protein (pH 4.0-5.0) > pH of the medium (pH 7.0). In the present study, higher negative zeta potential values were obtained in a nanoemulsion compared to a conventional emulsion. The findings were in agreement with the previous literature, indicating an inevitable increase in surface potential with decreasing size of the droplets in the system owing to its Brownian motion and polydisperse distribution of the droplets in nanoformulation.[33]

During the 8-weeks storage period conventional emulsion was found to be visually opaque and susceptible to creaming and subsequent phase-separation, indicating droplet aggregation but the nanoemulsions appeared to be stable without any observable coalescence or flocculation throughout the storage (Figure 3).

The droplet diameter of the nanoemulsion formulation was almost remained same for the period and the electrical potential of the formulation decreased marginally at the end of storage duration for 8 weeks. In previous reports, flocculation was observed when a number of droplets came close and aggregated keeping intact the initial sizes of the droplets.[34] Coalescence was reported to occur when several droplets merged together after they came close, producing a larger droplet. Droplets were found to be resistant to aggregation when the electrostatic or steric repulsive forces dominated but unstable when the van der Waals' interactions and hydrophobic attractions became more effective. The susceptibility for flocculation and coalescence was reported to generally decline with reducing droplet size[35.] Thus, nanoemulsions are more stable and resistant to droplet aggregation in comparison with conventional emulsions. Additionally, gravitational separation might be maximally inhibited in kinetically stable nanoemulsions as the Brownian motion of very fine droplets in the system overpowered the gravitational forces.[36] In our approach for nanoemulsion fabrication, primary oil phase was shattered and disrupted by the high-speed homogenization and ultrasonication, respectively, leading to decrease in the droplet diameter and poly-dispersity index, increasing the surface potential and thus to improve the stability of nanoemulsion.

Spherical Morphology of Nanoemulsion

Morphological analysis indicated the droplets of nanoemulsion to be spherical in shape and unimodal in distribution (Figure 4a). The data pertaining to mean droplet size obtained

by transmission electron microscopy (TEM) image can be correlated with the range of hydrodynamic diameter acquired by DLS analysis. Agglomeration of significantly larger droplets of conventional emulsion were also observed by TEM (Figure 4b) to confirm the appreciable difference between conventional and nanoemulsion in terms of in size and appearance.

Stability Against Lipid Oxidation

In this study, our focus was to assess the stability of sesame oil conventional and nanoemulsions against lipid oxidation during storage. For both the conventional emulsion and nanoemulsion systems, a noticeable surge in TBARS was observed for the storage period. This rise indicated that a substantial portion of lipid got oxidized in both formulations. Although the typical tendency of oxidation was indistinguishable, the amount of TBARS generated in the nanoemulsions was considerably elevated compared with the conventional emulsions at the same storage period. In our study, the TBARS level increased from 0.18 to 0.431 μM/mL of conventional emulsions and from 0.177 to 0.758 μM/mL of nanoemulsion (Figure 5).

As per previous reports, quite a few feasible justifications may be suggested to explain the less oxidative stability of nanoemulsions in comparison with the conventional emulsions at same storage period[16]: (i) Nanoemulsion offered a larger surface area owing to their smaller droplet size compared to that of conventional emulsion; (ii) Nanoemulsion permitted more light penetration into them owing to their weaker light scattering effect. In the future, comprehensive research based on the correlation between the particle size and oxidative stability in a protein-stabilized formulation is needed for vivid understanding of the effect.

Lipid Digestibility Analysis of the Conventional Emulsion and Nanoemulsion *in-vitro*

Better Lipid Digestibility of Nanoemulsion in Terms of FFA Release

As the fabricated formulations will be used as delivery vehicles for natural bioactive compounds, it is essential to check their *in-vitro* digestibility in simulated human gastrointestinal tract. No significant difference in free fatty acid release extent was observed for nanoemulsion and conventional emulsion after digestion in the simulated mouth and gastric phase. Small intestine is the specific area of the gastrointestinal system where major lipid digestion of humans takes place in the presence of the pancreatic enzyme lipase. In the present study, the extent of lipolysis for conventional emulsion was found 1.5, 7.8 and 16.2% in mouth, gastric, and intestinal phases, respectively (Figure 5a). Similarly, for nanoemulsion, the percentage of lipolysis was shown 1.6, 9.2 and 65.7% in mouth phase, gastric phase and intestinal phase, respectively (Figure 6a). After the completion of *in-vitro* digestion, the percentage of free fatty acid release for nanoemulsion was 81.47 ± 3.48, suggesting better lipid digestion than conventional emulsion, where the percentage of lipolysis was 67.66 ± 2.56 (Figure 6b).

To summarize, our findings reported that the amount of lipolysis and free fatty acid production after intestinal digestion was significantly higher in the nanoemulsions compared to conventional emulsions. Nanoemulsions would be expected to be digested rapidly in small intestine due their significantly small droplet size. Previous reports also indicated the inverse relationship between the lipid digestion rate and the oil droplet size in the system.[37] Moreover, for the nanoemulsion with a larger surface area to volume ratio, there was an enhanced chance to accumulate lipase on the oil-water interface more profoundly than in conventional formulation.

Comparative Study and Comprehension of Lipid Digestibility by Confocal Microscopy

From confocal microscopy images (Figure 7), the nanoemulsion prior to digestion was found to possess uniformly dispersed droplets with a relatively small mean diameter, whereas the conventional emulsion was found to contain relatively larger-sized droplets distributed in the medium. In the gastric stage of simulated digestion, the size of droplets in both nanoemulsion and conventional emulsion exhibited a significant increase in droplet diameter in contrast to the initial state.

Confocal images primarily suggested that in the gastric phase the droplet diameter increased both in conventional as well as nanoemulsion. The protein coating may be hydrolyzed by large amount of pepsin in the phase and the structure of the emulsion system got disrupted,[38] causing the oil droplets to aggregate in the soy-protein stabilized formulation. Furthermore, acidic pH and the gastric fluid's high ionic strength might weaken the electrostatic interactions, also promoting droplet aggregation.[39] Droplet aggregation was more profound in conventional emulsion compared to nanoemulsion due to its relatively larger droplet size and lower zeta potential favoring physical destabilization against acidic pH and high ionic strength. After entering into the intestinal stage, nanoemulsion manifested a notable reduction in droplet size and complete rupture of the oil droplets in comparison with the conventional one. Probably, the action of lipase was more pronounced in nanoemulsion owing to its higher surface area to volume ratio.[40]

Furthermore, reducing droplet diameter and keeping the protein concentration constant in the system, a thinner emulsifier layer was adsorbed on the droplet surface as less protein was available to cover up the larger interface.[41] The thinner layer was expected to provide smaller steric hindrance, to allow free access to lipase as well as to enhance hydrolysis. Hence, lipase completely ruptured the oil droplets of the nanoemulsion into relatively small entities compared to conventional emulsion, which showed higher resistance to lipase. The nanoemulsion droplets did not remain intact and showed distorted or non-uniform shape due to shattered droplets, while the conventional emulsion remained intact with minimal breakage. Moreover, in the intestinal phase the conventional emulsion showed little aggregation and comparative increase in diameter in respect to nanoemulsion. This may be because the tight interface membrane of protein is destroyed by the intestinal juice and at the same time it is hydrolyzed by the bile saline in the system leading to their

coalescence and upsurge in size. However, comprehensive work in future is needed to point out the detailed physicochemical phenomena accountable for the differences in lipid digestibility for protein-coated nano and conventional emulsion.

CONCLUSION

Our vigorous attempts have fulfilled the present study's prime objectives, *i.e.,* exploring soy protein as a natural food-grade emulsifier and assessing *in-vitro* lipid digestibility of sesame oil emulsion formulations in the simulated gastrointestinal environment. In the current investigation, the long-term shelf-life stability of fabricated nanoemulsion formulation was found to be remarkably improved compared to conventional emulsion, probably owing to the inherent physico-chemical characteristics. Morphological studies with transmission electron microscopy confirmed the considerable variance between conventional emulsion and nanoemulsion in terms of the size and distribution of droplets in the medium. Furthermore, the free fatty release kinetics as well as the confocal images captured during *in-vitro* digestion provided robust evidence in favor of nanoemulsion rather than conventional emulsion as an efficacious colloidal delivery system for lipophilic bioactive owing to its rapid digestion in a simulated intestinal environment. The present findings suggested prospective applications of other lipophilic bioactive and/or food grade compounds in a similar approach that would expect to bring emerging opportunities as well as positive outcomes in the frontier area of therapeutic nanodelivery.

ACKNOWLEDGEMENT

The authors would like to acknowledge the financial support from Indian Council of Medical Research for this research.

REFERENCES

1. Langyan S, Yadava P, Sharma S, Gupta NC, Bansal R, Yadav R, Kumar A. Food and nutraceutical functions of Sesame oil: an underutilized crop for nutritional and health benefits. Food Chemistry. 2022 Apr 16:132990.
2. Orsavova J, Misurcova L, Vavra Ambrozova J, Vicha R, MLcek J. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. Int J Mol Sci. 2015;16(6):12871–90.
3. Khanin P, Wasifur R, Sudhansu B, Bhabesh G. Sesame (Sesamum indicum L.), an underexploited oil seed crop: Current status, features and importance-A review. Agric Rev. 2017;38(3):223–7.
4. Aklakur M, Asharf Rather M, Kumar N. Nanodelivery: An Emerging Avenue for Nutraceuticals and Drug Delivery. Crit Rev Food Sci Nutr. 2016;56(14):2352–61.
5. Hsu DZ, Su S Bin, Chien SP, Chiang PJ, Li YH, Lo YJ, et al. effect of sesame oil on oxidative-stress-associated renal injury in endotoxemic rats: Involvement of nitric oxide and proinflammatory cytokines. Shock. 2005;24(3):276–80.
6. Vanti G. Recent strategies in nanodelivery systems for natural products: a review. Environ Chem Lett. 2021;19(6):4311–26.
7. Marzuki NHC, Wahab RA, Hamid MA. An overview of nanoemulsion: Concepts of development and cosmeceutical applications. Biotechnol Biotechnol Equip. 2019;33(1):779–97.
8. McClements DJ, Gumus CE. Natural emulsifiers — Biosurfactants, phospholipids, biopolymers, and colloidal particles: Molecular and physicochemical basis of functional performance. Adv Colloid Interface Sci [Internet]. 2016 Aug;234:3–26. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0001868616300380
9. Elzoghby AO, Samy WM, Elgindy NA. Protein-based nanocarriers as promising drug and gene delivery systems. J Control Release. 2012;161(1):38–49.
10. Sripriyalakshmi S, Jose P, Ravindran A, Anjali CH. Recent Trends in Drug Delivery System Using Protein Nanoparticles. Cell Biochem Biophys. 2014;70(1):17–26.
11. Tang CH. Emulsifying properties of soy proteins: A critical review with emphasis on the role of conformational flexibility. Crit Rev Food Sci Nutr. 2017;57(12):2636–79.
12. Vaishanavi S, Preetha R. Soy protein incorporated nanoemulsion for enhanced stability of probiotic (Lactobacillus delbrueckii subsp. bulgaricus) and its characterization. Mater Today Proc. 2020;40:S148–53.
13. Nishinari K, Fang Y, Guo S, Phillips GO. Soy proteins: A review on composition, aggregation and emulsification. Food Hydrocoll. 2014;39:301–18.
14. Zhang P, Hu T, Feng S, Xu Q, Zheng T, Zhou M, et al. Effect of high intensity ultrasound on transglutaminase-catalyzed soy protein isolate cold set gel. Ultrason Sonochem. 2016;29:380–7.
15. Keerati-u-rai M, Corredig M. Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. Food Hydrocoll. 2009;23(8):2141–8.
16. Lee SJ, Choi SJ, Li Y, Decker EA, McClements DJ. Protein-stabilized nanoemulsions and emulsions: Comparison of physicochemical stability, lipid oxidation, and lipase digestibility. J Agric Food Chem. 2011;59(1):415–27.
17. Ozturk B, Argin S, Ozilgen M, McClements DJ. Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural biopolymers: Whey protein isolate and gum arabic. Food Chem. 2015;188:256–63.
18. Zhang SB, Yan DQ, Jiang YS, Ding CH. Competitive displacement of interfacial soy proteins by Tween 20 and its effect on the physical stability of emulsions. Food Hydrocoll. 2021;113.
19. Jasmina H, Džana O, Alisa E, Edina V, Ognjenka R. Preparation of nanoemulsions by high-energy and lowenergy emulsification methods. In: IFMBE Proceedings. 2017. p. 317–22.
20. Kumar M, Bishnoi RS, Shukla AK, Jain CP. Techniques for formulation of nanoemulsion drug delivery system: A review. Prev Nutr Food Sci. 2019;24(3):225–34.
21. Teng F, He M, Xu J, Chen F, Wu C, Wang Z, et al. effect of ultrasonication on the stability and storage of a soy protein isolate-phosphatidylcholine nanoemulsions. Sci Rep. 2020;10(1).
22. Dey TK, Banerjee P, Chatterjee R, Dhar P. Designing of ω-3 PUFA enriched biocompatible nanoemulsion with sesame protein isolate as a natural surfactant: Focus on enhanced shelf-life stability and biocompatibility. Colloids Surfaces A Physicochem Eng Asp. 2018;538:36–44.
23. Tagne JB, Kakumanu S, Nicolosi RJ. Nanoemulsion preparations of the anticancer drug dacarbazine significantly increase its efficacy in a xenograft mouse melanoma model. Mol Pharm. 2008;5(6):1055–63.
24. Huber GM, Vasantha Rupasinghe HP, Shahidi F. Inhibition of oxidation of omega-3 poly-unsaturated fatty acids and fish oil by quercetin glycosides. Food Chem. 2009;117(2):290–5.

25. Garrett DA, Failla ML, Sarama RJ, Craft N. Accumulation and retention of micellar β-carotene and lutein by Caco-2 human intestinal cells. J Nutr Biochem. 1999;10(10):573–81.
26. Pinsirodom P, Parkin KL. Lipase Assays. Curr Protoc Food Anal Chem. 2001;00(1).
27. Pinheiro AC, Lad M, Silva HD, Coimbra MA, Boland M, Vicente AA. Unravelling the behaviour of curcumin nanoemulsions during *in-vitro* digestion: Effect of the surface charge. Soft Matter. 2013;9(11):3147–54.
28. Belhaj N, Dupuis F, Arab-Tehrany E, Denis FM, Paris C, Lartaud I, et al. Formulation, characterization and pharmacokinetic studies of coenzyme Q10 PUFA's nanoemulsions. Eur J Pharm Sci. 2012;47(2):305–12.
29. Gaikwad SG, Pandit AB. Ultrasound emulsification: Effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size. Ultrason Sonochem. 2008;15(4):554–63.
30. Modarres-Gheisari SMM, Gavagsaz-Ghoachani R, Malaki M, Safarpour P, Zandi M. Ultrasonic nano-emulsification – A review. Ultrason Sonochem [Internet]. 2019 Apr;52:88–105. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1350417718312690
31. Wu MH, Yan HH, Chen ZQ, He M. Effects of emulsifier type and environmental stress on the stability of curcumin emulsion. J Dispers Sci Technol. 2017;38(10):1375–80.
32. D. Wang, X. S. Sun, G. Yang, Y. Wang. Improved Water Resistance of Soy Protein Adhesive at Isoelectric Point. Trans ASABE. 2009;52(1):173–7.
33. Skoglund S, Hedberg J, Yunda E, Godymchuk A, Blomberg E, Odnevall Wallinder I. Difficulties and flaws in performing accurate determinations of zeta potentials of metal nanoparticles in complex solutions - Four case studies. PLoS One. 2017;12(7).
34. McClements DJ, Jafari SM. General Aspects of Nanoemulsions and Their Formulation. Nanoemulsions Formul Appl Charact. 2018;3–20.
35. Choi SJ, McClements DJ. Nanoemulsions as delivery systems for lipophilic nutraceuticals: strategies for improving their formulation, stability, functionality and bioavailability. Food Sci Biotechnol. 2020;29(2):149–68.
36. McClements DJ. Nanoemulsions versus microemulsions: Terminology, differences, and similarities. Soft Matter. 2012;8(6):1719–29.
37. Li Y, Mcclements DJ. New mathematical model for interpreting ph-stat digestion profiles: Impact of lipid droplet characteristics on *in-vitro* digestibility. J Agric Food Chem. 2010;58(13):8085–92.
38. Liu Y, Liu C, Zhang S, Li J, Zheng H, Jin H, et al. comparison of different protein emulsifiers on physicochemical properties of β-carotene-loaded nanoemulsion: Effect on formation, stability, and *in-vitro* digestion. Nanomaterials. 2021;11(1):1–18.
39. Mao Y, McClements DJ. Influence of electrostatic heteroaggregation of lipid droplets on their stability and digestibility under simulated gastrointestinal conditions. Food Funct. 2012;3(10):1025–34.
40. Armand M, Borel P, Ythier P, Dutot G, Melin C, Senft M, et al. Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase: an *in-vitro* study. J Nutr Biochem. 1992;3(7):333–41.
41. Yi J, Li Y, Zhong F, Yokoyama W. The physicochemical stability and invitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. Food Hydrocoll. 2014;35:19–27.