The physicochemical and organoleptic evaluation of the nano/micro encapsulation of Omega-3 fatty acids in lipid vesicular systems

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ABSTRACT
Objective(s): Omega-3 fatty acids play a key role in maintaining human health. The present study aimed to reduce the fishy smell and taste of omega-3 fatty acids through the encapsulation of lipid vesicles. Materials and Methods: Different non-ionic surfactants from the sorbitan ester family and egg lecithin with cholesterol were utilized to form micro-niosomal and liposomal formulations in order to encapsulate omega-3. The size of the selected microparticulate suspension was reduced using the liposome extruder. In addition, the vesicular physical stability, encapsulation efficiency (EE), release profile, and organoleptic properties were evaluated. Results: All the amphiphiles formed omega-3 vesicles with masked omega-3 taste and smell. Span/Tween (ST) 60 niosomes had the highest EE (98.60%), while the physical stability of the liquid state forming the mixture (ST 20/cholesterol) was significantly lower compared to the other formulations. Moreover, the two-step release profile of omega-3 was achieved following entrapment in lipid bilayers. Conclusion: According to the results, lipid vesicular systems on the micro or nano-scale could be used to encapsulate and protect omega-3 for the production of functional foods with appropriate organoleptic properties.

Keywords: Nano-vesicles, Omega-3 Fatty Acids, Physical Stability, Release Profile, Size Analysis

INTRODUCTION
Numerous functional foods and nutraceuticals contain probiotics and various natural compounds, such as omega-3 fatty acids, curcumin, and crocin, which increase the nutritional value of food, promote health, and reduce the risk of diseases and healthcare costs [1]. Omega-3 (ω3) contains three major polyunsaturated fatty acids, including docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and α-linoleic acid (ALA), which is obtained from natural animal and botanical sources. ALA is an essential fatty acid that could only be obtained through dietary intake in mammals, and several studies have confirmed the positive impact of this element on health through reducing the risk of cardiovascular diseases, inflammation, arthritis, and dementia and improving the immune and cognitive function in humans [2]. The stabilization of these active and valuable nutraceuticals in food products during processing, packaging, storage, and consumption could be achieved by encapsulation a wide variety of colloidal drug delivery systems, such as lipid vesicles [3], micelles, micro/nanoemulsions, multiple emulsions, solid lipid nanoparticles, protein nanoparticles, and biopolymer microgels [4]. On the other hand, improving the taste of fish oil containing dairy foods has been reported to significantly decrease acidity and syneresis and peroxide values, while increasing the stability of the fatty acid content following encapsulation in lipid nano carriers [5]. Lipophilic compounds, natural and synthetic polymers, polysaccharides, and complex coacervates have been used successfully for the encapsulation of fish oil, omega-3, and...
omega-3 fatty acids. In a research in this regard, Ilyasoglu et al. [6] utilized the sodium caseinate–gum arabic (protein-polysaccharide) complex for the nano-encapsulation of EPA/DHA used for the enrichment of fruit juices. In the mentioned study, casein micelles and nanoparticles exhibited remarkable protective effects against DHA oxidation, demonstrating proper colloidal stability and bioactive conservation throughout the shelf life of various products at the temperature of 4°C [7]. In another study by Mozafari, nanoliposomal omega-3 was prepared and used as an additive in bread and milk, and the sensory evaluation of the products was conducted to determine the perceptible sensory difference/similarity between the control, non-encapsulated, microencapsulated, and nanoliposomal omega-3 samples [8]. The obtained results indicated no significant, detectable difference between the control and nanoliposomal omega-3 fishy flavor.

Recently, numerous amphiphilic compounds have been utilized as alternative ingredients in bilayer formation instead of phospholipids, which are commonly used in liposome preparation. Non-ionic surfactants are considered to be the most applicable materials for the formation of niosomes, which are a class of lipid vesicle systems, with higher temperature and oxidation stability compared to phospholipids [9]. Moreover, niosomes have been used for the encapsulation of various hydrophilic materials, such as vitamin C [10], amphiphilic molecules (e.g., insulin) [11], and lipophilic compounds (e.g., minoxidil) [12].

To the best of our knowledge, this is the first report regarding the micro/nano niosomes and liposomes of omega-3 used for the taste masking of this valuable ingredient in functional foods.

**Preparation of Liposomes and Niosomes**

Various molar percentages (mol %) of the amphiphilic/cholesterol mixture (70/30, 60/40, and 50/50 mol% as 300 μmol of total lipids) were utilized for the preparation of liposome vesicles using the film hydration method [13]. In brief, the lipids and omega-3 (2% w/v in the final formulations) were dissolved in appropriate volumes of chloroform, and the organic solvent was evaporated using a rotary evaporator (Heidolf, Germany) at the temperature of 65°C. At the next stage, round-bottom flasks with inner thin lipid films were placed in a vacuum cabinet overnight for the removal of the chloroform residues. Afterwards, five milliliters of deionized water was added as the hydration medium at the temperature of 65°C for 30 minutes. The N₂ atmosphere was also utilized in the preparation of the liposomes in order to avoid phospholipid oxidation. Following that, the niosomal/liposomal suspension was placed in type I glass vials and stored at room temperature for 24 hours to achieve the complete hydration of the lipid vesicles. Further storage was carried out in a refrigerator (2-8°C) for up to six months.

**Assessment of Vesicle Morphology**

At this stage, an optical microscope (Olympus, Germany) was used to observe the formation and aggregation of multilamellar vesicles (MLVs), as well as the possible separation of the components. Moreover, some micrographs were captured using the digital port attached to the microscope in the DinoCapture 2.0 software.

**Size Measurement of the MLVs**

The static laser light scattering method (Malvern, Master Sizer 2000E, UK) was utilized for the evaluation of size distribution and determining the volume frequency of the lipid vesicles. The method presented the mean volume diameter of the vesicles (d_v), as well as the volume frequency size distribution curves for the microparticulate suspensions.

**Size Reduction for Nano-lipid Vesicle Preparation**

At this stage, the lipid vesicle formulations containing 30 mol% of cholesterol were gradually passed through 0.45-, 0.22-, and 0.1-micrometer polycarbonate membranes (MF-Millipore™ Membrane Filters, USA) using a manual liposome extruder (University of Monash, Australia). The
total passage number was within the range of 11-13 to achieve the desired size distribution.

**Zeta-potential and Size Analysis of Small Unilamellar Vesicles (SUVs)**

Due to the high scattering of the nano-sized lipid vesicles, photon correlation spectroscopy was applied (PCS; VASCO NP size analyzer; Coduan Technology, France) for the size analysis of the nano-niosomal and nano-liposomal formulations. Following that, the Z-average diameters of the nano-vesicles were measured and reported. In addition, the zeta potential of the selected formulations was measured using the WALLIS zeta potential analyzer (Coduan Technology, France).

**Physical Stability of the Vesicular Suspensions**

At this stage, the lipid MLV suspensions were maintained at the temperature of 2-8°C and defined time intervals for three months, and the vesicle morphology and size distribution were surveyed using light microscopy and Malvern particle size analyzer.

**Measurement of Omega-3 Encapsulation Efficiency (EE)**

For the separation of free omega-3 from the vesicular encapsulated drug, dialysis was performed using a cellulose acetate bag (molecular weight cutoff: 12KD) against 200 milliliters of 96% ethanol/deionized water (50/50 v/v). One milliliter of the vesicular suspension was preserved in the dialysis bag at the temperature of 37°C for four hours, and the concentration of omega-3 in the vesicles was measured after the disruption of the lipid bilayers by adding two milliliters of isopropyl alcohol via gas chromatography (GC; Shimadzu, Japan) at 193°C as the column temperature. Finally, The EE rate was calculated using the following equation:  

$$EE\% = \frac{(C_p \times 100)}{C_t}$$

Where $C_p$ shows the concentration of omega-3 in the dialysis bag, and $C_t$ represents the total drug concentration.

**Omega-3 Release from the Lipid Vesicles**

All-glass Franz diffusion cell was used to evaluate the delivery of omega-3 from the niosome and liposome formulations and determine the release profile through the cellulose acetate membrane at the temperature of 37°C. The receptor phase was composed of 15 milliliters of 96% ethanol and deionized water (50/50 v/v). In addition, free omega-3 solution was used as control to compare the effects of the lipid vesicles on drug transfer through the synthetic membrane. Sampling in the receptor phase was performed at defined time intervals (up to 240 min), and omega-3 content was analyzed via GC.
Organoleptic (sensory) Assessment of Omega-3 Formulations

At this stage, 10 untrained male and female volunteers aged 22-50 years participated in the sensory acceptance test.

To this end, one milliliter of the free omega-3 solution or niosomal/liposomal suspension was provided to the testers, and they would grade the fishy odor of the formulation within a scale of 1-10. All the testers randomly received five test samples at 10-minute intervals.

RESULTS

The film hydration method was applied for the formation of the niosomal and liposomal MLVs (Fig 1a-d).

No omega-3 droplets or cholesterol crystals were observed before the filtration of the lipid vesicular suspensions.

Table 1 shows the mean volume diameter (d_v) and EE of the prepared niosomes and liposomes. Size reduction of the selected formulations was also achieved using the filtration method (Table 2).

Fig 2 depicts the physical stability of the niosomes and liposomes during three months of refrigerated storage. Fig 3 shows the Release profiles of the selected formulations as the two-phase delivery of omega-3. The sensory (organoleptic) evaluation of the omega-3 lipid vesicles was carried out by human volunteers, and the results will be elucidated in the Discussion section.

DISCUSSION

Vesicle Formation Capability of the Applied Surfactants

According to the findings of the current research, the encapsulated active ingredient, hydration medium composition, and lipid vesicle preparation affected the capability of surfactant reconstruction into the bilayer structures [14]. Furthermore, omega-3 as the lipid-soluble compound could be successfully encapsulated in the liposomes and niosomes, which were composed of lecithin, sorbitan esters (Span™), polyxylated sorbitan esters (Tween™), and cholesterol (Table 1).

Table 1. Mean Volume Diameter (dv) and Encapsulation Efficiency Percentage (EE %) of Prepared Omega-3 Lipid Vesicular Formulations in Deionized Water (Sizes measured by laser light scattering technique; mean±SD; n=3)

<table>
<thead>
<tr>
<th>Formulations (mol %)</th>
<th>Mean d_v (µm)±SD</th>
<th>Mean EE%±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST20/Cholesterol (35:35:30)</td>
<td>9.27±0.47</td>
<td>79.81±5.48</td>
</tr>
<tr>
<td>ST20/Cholesterol (30:30:40)</td>
<td>6.51±0.24</td>
<td>75.26±7.13</td>
</tr>
<tr>
<td>ST20/Cholesterol (25:25:50)</td>
<td>7.87±0.78</td>
<td>83.67±4.92</td>
</tr>
<tr>
<td>ST40/Cholesterol (35:35:30)</td>
<td>7.54±0.34</td>
<td>87.51±6.23</td>
</tr>
<tr>
<td>ST40/Cholesterol (30:30:40)</td>
<td>7.67±0.29</td>
<td>89.33±7.46</td>
</tr>
<tr>
<td>ST40/Cholesterol (25:25:50)</td>
<td>8.45±0.11</td>
<td>91.20±5.69</td>
</tr>
<tr>
<td>ST60/Cholesterol (35:35:30)</td>
<td>4.87±0.10</td>
<td>91.27±4.34</td>
</tr>
<tr>
<td>ST60/Cholesterol (30:30:40)</td>
<td>4.45±0.13</td>
<td>95.75±6.09</td>
</tr>
<tr>
<td>ST60/Cholesterol (25:25:50)</td>
<td>7.97±0.21</td>
<td>98.60±5.65</td>
</tr>
<tr>
<td>Lecithin/Cholesterol (70:30)</td>
<td>4.25±0.53</td>
<td>76.31±7.25</td>
</tr>
<tr>
<td>Lecithin/Cholesterol (60:40)</td>
<td>5.65±0.41</td>
<td>83.46±5.08</td>
</tr>
<tr>
<td>Lecithin/Cholesterol (50:50)</td>
<td>6.53±0.21</td>
<td>81.78±6.21</td>
</tr>
</tbody>
</table>

Table 2. Mean Volume Diameter (dv) of Filtered Omega-3 Lipid Vesicular Formulations in Deionized Water (Sizes measured by photon correlation spectroscopy; mean±SD; n=3)

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Composition (mol %)</th>
<th>Mean Volume Diameter (nm)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Span/Tween 40/Cholesterol</td>
<td>35:35:30</td>
<td>273±42</td>
</tr>
<tr>
<td>Span/Tween 60/Cholesterol</td>
<td>35:35:30</td>
<td>494±69</td>
</tr>
<tr>
<td>Lecithin/Cholesterol</td>
<td>70:30</td>
<td>159±17</td>
</tr>
</tbody>
</table>

Considering the appropriate hydrophilic lipophilic balance (HLB) and critical packaging parameter (CPP) of Span/Tween or lecithin, these amphiphilic substances have also been used for the entrapment of myrtle essential oil [15] and fish oil [5]. Therefore, the selected lipid composition in the present study resulted in the supramolecular formation of all the applied amphiphile/cholesterol compositions (Table 1).

Fig 2. Size Changes in Some Lipid Vesicles Containing Omega-3 during Three Months of Refrigerated Storage as Physical Stability Indicator

Size Analysis of the Lipid Vesicles

In the present study, static laser light scattering was used for the size analysis of the prepared liposomes and niosomes on the micron scale, and PCS was employed for the nanoscale lipid vesicles. In the presence of 30% cholesterol, the reduction of the HLB values was associated with the decreased mean volume diameters of the vesicles in contrast to oral paclitaxel and α-tocopherol niosomes (Table 1) [16, 17].

This heterogenic finding may have resulted from the use of the bi-surfactant mixture in the current research and mono-surfactant (polyoxyethylene alkyl ether, Span or Tween) in the other studies in this regard.

According to the current research, the effect of cholesterol concentration on the mean volume diameter of the prepared gel-state niosomes (Span/Tween 40 or 60) and lecithin liposome was dissimilar to the liquid-state formulation (Span/ Tween 20) (Table 1).

Correspondingly, the increasing of cholesterol mole% led to size increment in gel-state vesicles and size reduction in the liquid-state niosomes. Furthermore, cholesterol intercalation into the amphiphilic gel- or liquid-state bilayers altered the entire structural behavior [18], physical stability, and solubilization capability of the lipid bilayers [19]. In the gel-state niosomes and lecithin liposome, increased cholesterol resulted in the higher number of the bilayers and the subsequent larger vesicles (Table 1).

Fig 3. Release Profile of Omega-3 from Filtered Lipid Vesicles

Size Reduction of the Prepared Lipid Vesicles

Some lipid vesicle preparation methods (e.g., heating proposed by Mozafari) could be used to directly prepare nano-sized vesicles [8].

However, the high temperature applied in these techniques may destroy the heat-labile ingredients; therefore, we used the film hydration method to prepare the MLVs, followed by the application of the liposome extruder and 100-nanometer polycarbonate membranes, in order to diminish the vesicle sizes within a nanoscale range.
Physical Stability of the Liposomes and Niosomes
Several pharmaceutical properties are effective in the physical stability of liposomes and niosomes, such as unchanged particle size, non-separation of the vesicular components, lack of phase separation, encapsulation maintenance of the entrapped material during storage, and minimal morphology alteration of the lipid vesicles [20].

In the present study, the liquid-state surfactant composition (Span/Tween 20 with 50 mole% cholesterol) had less stability compared to the other formulations as indicated by the statistically significant size change (P<0.05) (Table 2). The instability of the Span/Tween 20 niosomes has also been reported in the lipid vesicles of carvedilol, which is a lipophilic drug, due to the short chain (C12) of the hydrophobic segment of the surfactant molecule [21].

In a similar study, Ghorbanzadeh et al [5]. have reported higher omega-3 contents (docosahexaenoic acid, DHA, and eicosapentaenoic acid, EPA) in the yogurt fortified with nano-liposome-entrapped fish oil compared to yogurt containing free fish oil. In addition, the high physical stability of the niosomes and liposomes with the Span/Tween 40 or 60, lecithin, and 30 mole% of cholesterol resulted in the selection of these formulations for size reduction to the nanoscale via filtration.

Omega-3 EE
The high EE of lipid-soluble compounds (e.g., α-tocopherol) [17] in liposomes and niosomes is predicted due to the intercalation of these compounds into bilayer structures [22]. In this regard, Ramezani et al. [23] reported the high EE of minoxidil in transfersomes containing Tween 20 or 80 as the edge activator. In the present study, omega-3 was encapsulated from the minimum of 75.2% in lauryl (C12) chain surfactant niosomes (Span/Tween 20) to the maximum of 98.4% in stearyl (C18) chain amphiphilic vesicles (Span/Tween 60) (Table 1). Moreover, the increased hydrocarbon chain length decreased the HLB value and increased the EE of omega-3 in our formulations, which is in contrast to vitamin E entrapment in niosomes as reported by Basiri et al. [17]. The discrepancy in this regard could be due to the differences in the vesicle preparation methods, as well as heating and lipid film hydration.

Omega-3 Release
In lipid vesicular systems, the incomplete release of the entrapped compound occurs commonly as reported in case of insulin [24], ciprofloxacin [25], and ascorbic acid [10]. This could be attributed to the restricted volume of various cell receptor compartments, gradual decrease of the concentration gradients, interactions between the encapsulated compound and bilayer-forming materials, and lipid-solubility of the entrapped substance as omega-3 in the present study. Compared to the omega-3 solution, less than 25% of omega-3 was released from the selected vesicular systems.

Sensory (organoleptic) Properties
In a research in this regard, Muchow et al. [26] used lipid nanoparticles for the encapsulation of omega-3 to mask the taste and increase the oral bioavailability of fatty acids. Additionally, Rathod and Kairam [27] prepared calcium alginate beads to improve the taste quality of fish oil for use in dairy and non-dairy products. In the current research, 70% of the human volunteers announced no fishy odor in the encapsulated omega-3, while 20% sensed a better taste and smell of the entrapped omega-3 compared to the free omega-3 emulsion. Therefore, it could be concluded that the intercalation of the majority of omega-3 fatty acids in the bilayers of lipid vesicles could reduce the free lipids binding to the taste buds.

CONCLUSION
The main objective of this research was to prepare stable omega-3 containing lipid vesicles with appropriate taste and odor for addition to dairy products. According to the results, the gel-state lipid bilayers had higher stability compared to the liquid-state lipid bilayers (Span/Tween 20) as demonstrated by the slight size change during three months of refrigerated storage. Therefore, it could be concluded that nano-lipid vesicles are proper candidate for the fortification of dairy products owing to their acceptable taste-masking property, high physical stability, and maximum omega-3 EE.

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REFERENCES


