# Development and characterization of PEGylated and carboxymethyl chitosan-coated fish oil liposomes

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## ABSTRACT

Fish oil is a rich source of omega-3 long chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The highly lipophilic nature of fish oil results in poor absorption and palatability, in addition to deterioration by oxidation, and these factors limit their use in functional foods. Therefore, protection of fish oil during processing and storage is important. In this study, different concentrations of carboxymethyl chitosan (CMCS)-coated and PEGylated liposomes containing fish oil were prepared and characterized. The CMCS coating increased the mean diameter whereas entrapment efficiency of liposomes did not showed any significant change when coated with CMCS and PEG. Similarly, there was no effect on zeta potential with polymer coating. The fish oil liposomes were freeze-dried to ensure long-term stability. The surface structure and characteristics of liposomes were determined. The Fourier transform infrared spectroscopy (FT-IR) showed that the hydrogen bonding formed between the CMCS and the carbonyl region of the liposomes' bilayer. Transmission electron microscopy images revealed the few spherical, most rectangular and nano-size particle distribution. This study can provide theories and practical information for further applications of fish oil into different pharmaceutical and nutraceutical products. Further *in vitro* release study can be carried out to understand the release behavior of polymer-coated fish oil liposomes.

**Keywords:** Fish oil, PEGylated liposome, Carboxymethyl chitosan

## 1. Introduction

Fish oil has received increasing attention in research because of their reported health benefits. Fish oil is a rich source of omega-3 long chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Davidson, 2013). In study conducted by Zhang et al. (1999), fish consumption was associated with a reduced risk from ischemic heart disease and stroke mortality. In addition, a dose-response

relationship exists between the frequencies of weekly fish intake and reduced cardiovascular disease risk factors (Kris-Etherton, Harris, and Appel, 2002). Several other health benefits, such as ameliorative effects on brain and nervous systems, and prevention of certain diseases including diabetes, inflammatory and autoimmune disorders have been reported with regular consumption of omega-3 fatty acids (Orchard et al., 2012).

The main sources of EPA and DHA are cold-water fish, and some algae; but their consumption is limited worldwide. Likewise, endogenous source show lower bioconversion rate from α-linolenic acid (0.3% EPA and <0.01% DHA) (Olloqui et al., 2018). The Food and Agriculture Organization (FAO) has recommended a daily preventive consumption from 250 mg of EPA and DHA per day, but occidental consumer has not reached this (Ghorbanzade et al., 2017). Nutritional daily intake can be obtained by diet supplements or enriched foodstuffs. Nevertheless, the highly lipophilic nature of these compounds results in poor absorption and palatability, in addition to deterioration by oxidation, limiting their use in functional foods (Chen et al., 2013). Therefore, protection of fish oil during processing and storage is important. Different strategies have been used to protect fish oil from oxidation. One effective approach is the use of a natural or synthetic antioxidant. Using of orally administered liposomes is a practical way as well (Ezhilarasi et al., 2013).

Liposomes are phospholipid bilayer structures enclosing an internal aqueous phase. It mimics feature of biological cells and can be used to deliver both hydrophilic and lipophilic drugs. The basic ingredients of liposomes are phospholipids and cholesterol. Depending on the needs and availability, different types of natural and manmade phospholipids can be used for the liposome preparation. Phosphate head group in phospholipids acts as hydrophilic part where as acyl hydrocarbon chain acts as hydrophobic part. Water loving drugs are encapsulated in the center and lipid loving drugs are entrapped in non-polar part of the liposomes. Cholesterol is normally used to stabilize the phospholipids. Liposomes are characterized into different types depending on their size and shape such as multilamellar vesicles, small unilamellar vesicles and large unilamellar vesicles (Samad, Sultana, & Aqil, 2007). However, liposomes that merely consist of amphiphilic phosphatidylcholine are poor in maintaining their shape mainly via hydrophobic interaction and are, therefore, not useful as a drug delivery system. Several surface modifications of liposomes with different polymers have been studied to enhance the stability of them. Many researchers started using different polymers for the preparation of nanoparticles and started witnessing the pros and cons of the same (Liechty et al., 2010). Due to the immense advantage and safety of biodegradable polymer, biodegradable polymer nanoparticles became the topic of interest. Biodegradable polymer nanoparticles have an advantage over non-biodegradable polymer nanoparticles that it is non-immunogenic, non-allergenic, less toxicity, no need to remove it from the body as the polymer degrades inside the body (Dhaliwal & Dosanjh, 2018).

Carboxymethyl chitosan (CMCS) is a biodegradable chitosan derivative prepared by carboxymethylation of chitosan. It exhibits exceptional mucoadhesive properties, absorption enhancement and good aqueous solubility over a wide pH range. Therefore, surface modification of liposomes with CMCS may provide an alternative for materials already used in food and pharmaceutical industry (Mourya, Inamdar & Tiwari, 2010).

Polyethylene glycol (PEG) is nonionic hydrophilic polyester, which is prepared by polymerization of ethylene glycol monomer and its molecular weight ranges from 300-100,000 Da. Moreover, because of its hydrophilic nature, PEG is used to stabilize the nanoparticle in aqueous media, avoid aggregation due to steric hindrance in production, storage, as well as the application. In addition, PEG is found to increase solubility as well as it suppresses opsonisation and undergoes slower uptake by reticuloendothelial system (RES) in M cells on intestine (Kang et al., 2019).

Therefore, the aims of this study were to formulate fish oil nano-liposomes using thin film hydration method, coat them with different concentrations of CMC and further study the change in properties by doing PEGylation. Furthermore, the formulations were freeze-dried and studied their various characteristics.

## 2. Objectives of the study

The purposes of this study were to formulate and characterize fish oil liposomes.

## 3. Materials and methods

### 3.1 Chemicals

Tuna fish oil (containing EPA 45.9 mg/g and DHA 229.1 mg/g in triglyceride form) was bought from Nutritech Co., Ltd. (Thailand). Soybean phosphatidylcholine (SPC) and Tween 80 were purchased from Sigma-Aldrich (USA). Cholesterol (CH) and carboxymethyl chitosan (CMCS) (deacetylation degree 96.8%) were purchased from Anhui Minmetals Development Import & Export Co., Ltd. (China). Absolute ethanol (99.9%) was bought from Quality Reagent Chemical (New Zealand). Phosphate buffered saline (PBS, 0.05 M, pH 7.4) was bought from Corning (USA). Hexane and acetone were purchased from Fisher Scientific (UK). PEG (Mol. wt. 2000) was purchased from Merck & Co. (USA). All other chemicals used throughout this investigation were of analytical grade and no additional purification was carried out.

### 3.2 Preparation of liposomes

The coarse liposomes containing fish oil were prepared by the method of Wang et al. (2015) with some modification. Briefly, the mixture of SPC, CH, Tween 80, and fish oil (8:1:3.2:0.8, w/w/w/w) was dissolved in absolute alcohol in a 250 mL round bottom flask. The phase transition temperature of SPC is -20 to -30°C (Li et al., 2015). The organic phase was evaporated using a rotatory evaporator (Heidolph Instruments, Germany) at 45°C, resulting in a dry lipid film, which was dried further under nitrogen flow for 3 min. The lipid film was then hydrated with PBS (0.05 M, pH 7.4) at room temperature for 1 h in the dark in order to anneal any structural defects. After complete lipid hydration, the coarse liposomes (mean diameter >200 nm) were formed. The coarse liposomes were then added into an equal volume of CMCS solution (0.1%, 0.5%, 1.0% w/w, respectively) with and without PEG 2000, respectively, under constant stirring using a magnetic stirrer at room temperature for 2 h to

prepare the coarse CMCS and PEG-coated liposomes. In order to reduce the particle size and enhance encapsulation efficiency, the coarse liposomes were then subjected to probe sonication at 60% amplitude for 5 min without using pulse function. Before freeze-drying, the liposomes were kept at -80°C overnight. Then they were left for freeze-drying (Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 48 h.

### 3.3 Characterization of liposomes

#### 3.3.1 Particle size, polydispersity index (PDI) and zeta potential

The mean diameter and PDI of the prepared liposomes were determined using dynamic light scattering (DLS) method whereas zeta potential measurement was based on the principle of laser Doppler electrophoresis (Zetasizer Nano ZS, Malvern, UK) upon dilution with the same buffer used for their preparation to avoid multiple scattering phenomena. Note that 0.1 mL liposome suspension was diluted to 1 mL with buffer, put into a polystyrene latex cell, and measured at a detector angle of 90°C, wavelength of 633 nm, refractive index of 1.33, and viscosity value of 0.8880 mPas at 25°C. Similarly, zeta potential was determined using disposable folded capillary cell under similar conditions. All tests were done in triplicate.

#### 3.3.2 Transmission electron microscopy (TEM)

TEM was employed to monitor the microstructure of liposomes using a negative staining method. In brief, the liposomes samples were diluted 10-fold with double distilled water to reduce the concentration of the vesicles. A drop of this solution was placed on a copper grid, allowed to air dry for 5 min at room temperature. The grid was then be negatively stained with 2% phosphotungstic acid aqueous solution for 1 min. Subsequently, the excess liquid was drawn off using filter paper. After air dried at room temperature, the liposomes were examined by transmission electron microscope (JEM-2100F, JEOL Ltd., Japan).

#### 3.3.3 Fourier transform infrared (FT-IR) spectroscopy

The infrared spectra of liposomes were obtained using FT-IR spectrometer (Nicolet 6700, Thermo Fisher Scientific, USA). Ten scans over the range of 400 to 4000 $cm^{-1}$ were performed at a resolution of 2 $cm^{-1}$ with the background scan subtracted.

#### 3.3.4 Entrapment efficiency (EE)

The EE of liposomes was determined according to the method of Wang et al. (2015) with some modification. The centrifugation technique was used to separate the unentrapped fish oil from liposomes. Briefly, the freeze-dried liposomes were dispersed into PBS (0.05 M, pH 7.4) (50 mg/mL) and centrifuged at 15,000 rpm for 20 min. The residue was extracted three times with hexane. The amount (mg) of entrapped fish oil in liposomes was determined at a wavelength of 241 nm using the UV/VIS spectrophotometer (UV-2600, Shimadzu, Japan) and

calculated according to a calibration curve of fish oil which was achieved from fish oil solutions in hexane with concentrations between 0.008 and 0.25 mg/mL. The % EE was determined by the following equation.

$$\% \text{ EE} = \frac{Q_t - Q_a}{Q_t} \times 100$$

Where, $Q_a$ is the weight (mg) of fish oil loaded in liposomes and $Q_t$ is total weight (mg) of fish oil in the prepared formulation.

### 3.4 Statistical analysis

All data are presented as the mean ± standard deviation (SD) from three independently carried out experiments. Statistical analysis was carried out using the one-way ANOVA (Microsoft Excel 2016, USA). P value < 0.05 was considered statistically significant.

## 4. Results and discussion

### 4.1 Effects of CMC concentration and PEGylating on characteristics of liposomes

The liposomes containing fish oil were coated with different concentrations of CMCS, and its effects on the physicochemical properties of liposomes were evaluated. The mean diameter, PDI, and % EE of liposomes with different CMCS concentration are listed in Table 1. It was found that the mean diameter increased with an increase in CMCS content. In case of PEGylating, particle size seemed to increase. There was no significant effect on the entrapment efficiency with polymer coating as shown in Table 1. It indicated that polymers were coated on the surface of liposomes and did not interface with internal structure of phospholipid bilayer of liposomes. Polydispersity is usually expressed as an index of particles diameter distribution in colloidal systems. The smaller the value of PDI, the more likely the particle diameter distribution is narrower, and thus particles show better uniformity in diameter (Ruozi et al., 2005). The PDI of liposomes with different CMCS concentration ranged from 0.25 to 0.53. It indicated that the liposomes had narrow size distribution. The zeta potential of liposomes was found in the range of -11 mV to -13 mV.

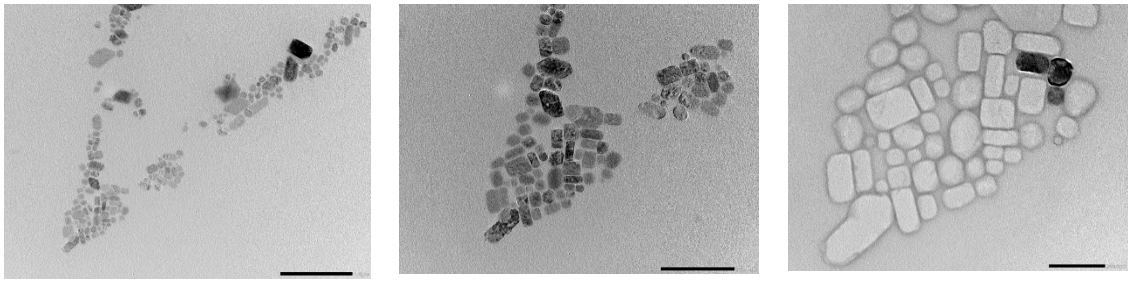
### 4.2 Morphology

TEM is an important method for the characterization of size and shape of nanoparticles as it can directly visualize single particles and even their inner architecture. The TEM micrographs of liposomes are shown in Figure 1. A negative stain does not penetrate the object, but coats the surface and surroundings, obscuring the object itself and all internal structural details, and giving a foot-print like appearance (Baxa, 2018). It was evident that the 1.0% w/w CMCS-coated liposomes were mostly elongated and few with spherical shape, which might be due to aggregation of particle (Baxa, 2018). The staining pattern of three images seems different which might be due to dehydration of the stain (Robson et al., 2018). The layer of CMCS was also visualized at focus of 500 nm. In

addition, the particles formed were well within 200 nm in agreement with particle size determined using DLS method. For better visualization of the liposomes, cryogenic (cryo)-TEM can be used for further study.

**Table 1.** Physicochemical characteristics of liposomes without and with different CMCS contents

| CMCS concentration (%w/w) | Mean diameter (nm) | Polydispersity index (PDI) | Zeta potential (mV) | EE (%) |
|---|---|---|---|---|
| 0.0 | 101.03 ± 3.78 | 0.25 ± 0.02 | -11.87 ± 0.64 | 95.98 ± 4.16 |
| 0.1 | 125.43 ± 0.89 | 0.25 ± 0.03 | -12.53 ± 1.45 | 96.71 ± 5.89 |
| 0.5 | 130.03 ± 5.23 | 0.48 ± 0.01 | -11.93 ± 0.75 | 96.93 ± 5.31 |
| 1.0 | 133.60 ± 2.98 | 0.53 ± 0.07 | -13.33 ± 0.77 | 97.78 ± 3.84 |
| PEG 2000 + 1.0 | 180.83 ± 2.70 | 0.41 ± 0.08 | -13.77 ± 0.74 | 97.92 ± 2.26 |



A B C

**Figure 1.** TEM micrographs of 1.0% w/w CMCS-coated liposomes at 1 µm (A), 500 nm (B) and 200 nm (C), respectively.

**4.3 FT-IR spectroscopy**

FT-IR is used for investigation of molecular structures. Therefore, it was used to explain the coating mechanism by exploring the possible interactions between CMCS and bilayer of liposomes. FT-IR spectra of SPC, fish oil liposome, CMCS, CMCS-coated liposomes, PEG, and PEGylated CMCS-coated liposomes are shown in Figure 2. The symmetrical stretching vibration absorption of C=O of the CMCS shifted from 1739.90 to 1739.18 $cm^{-1}$ with intensity reduced significantly after coating, indicating hydrogen bonding between the carbonyl region of the bilayer and CMCS. Moreover, the position of choline peak shifted from 990 to 985 $cm^{-1}$, suggesting an interaction of CMCS with the surface-exposed choline groups (Wang et al., 2015).

PEG exhibits absorptions those of a primary alcohol. Hence these absorptions, which comprise stretching and bending vibrations restricted to C-C stretching, C-O stretching, C-H stretching (methylene absorptions) and the C-H bending. The O-H stretching vibration was observed in the region 3000 $cm^{-1}$ exhibiting hydrogen bonded nature. Thus, PEG also formed H-bond with liposome indicated by the decrease in O-H stretching in the PEGylated liposomes.

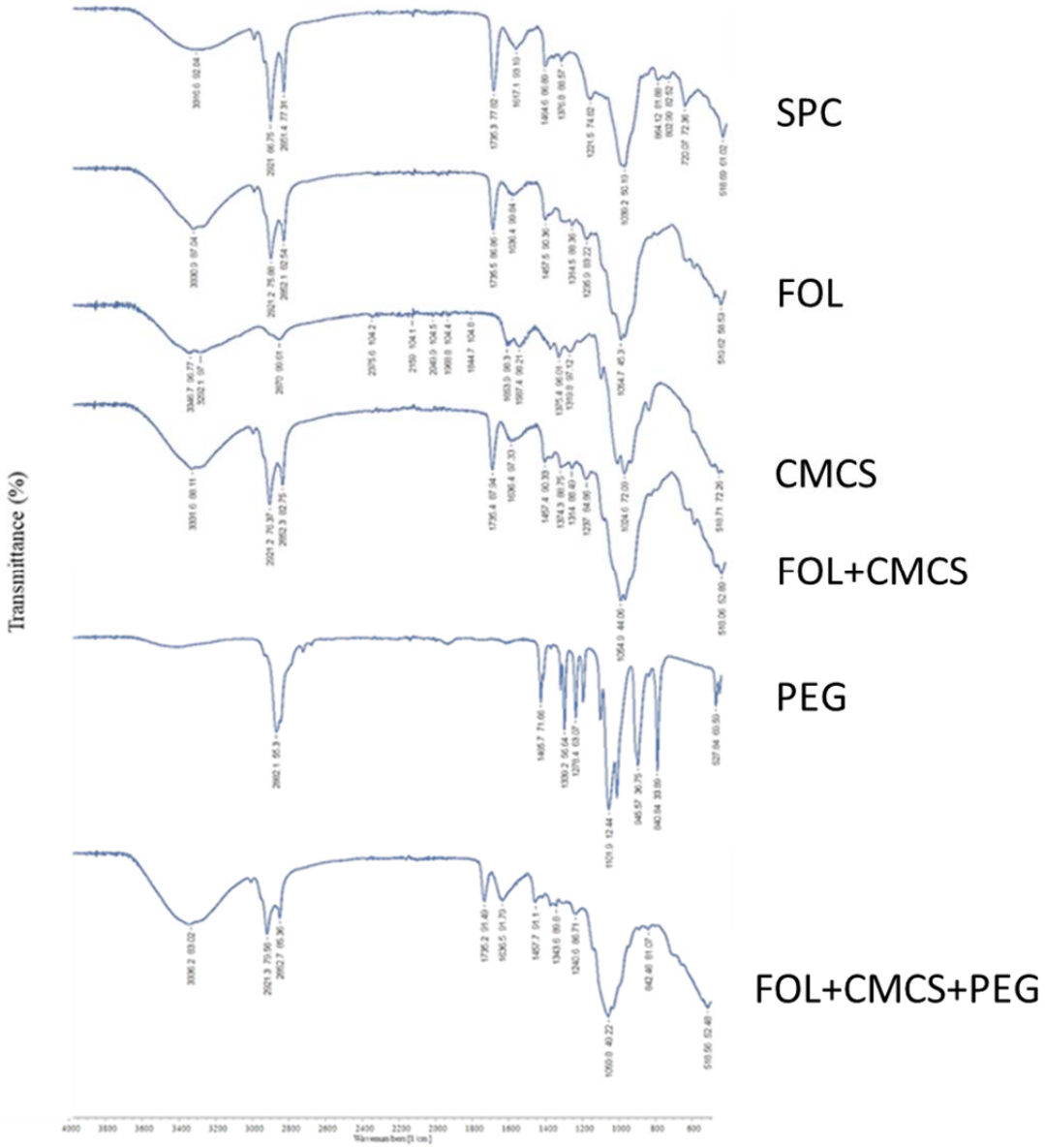



**Figure 2.** FT-IR spectra of SPC (soybean phosphatidylcholine)**,** FOL (fish oil liposome), CMCS (carboxymethyl chitosan)**,** FOL+CMCS (fish oil liposome-coated with CMCS)**,** PEG (polyethylene glycol)**,** FOL+CMCS+PEG (fish oil liposome-coated with CMCS and PEG).

**6. Conclusion**

The liposomes containing fish oil were prepared, and their characteristics were determined by TEM and FT-IR. CMCS coating increased the mean diameter whereas the % EE of liposomes did not show any significant difference, and the vesicle population was still homogeneous as judged by the low PDI. The novel point of this study is the use of two polymers, CMCS and PEG together, for coating the liposomes and study its impact on characteristics of liposomes with future view of studying its impact on the gastrointestinal stability, *in vitro* release and bioactivity of liposomes in simulated gastric and intestinal conditions.

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