

# Effect of Layer-by-Layer (LbL) Encapsulation of Nano-Emulsified Fish Oil on Their Digestibility *Ex Vivo* and Skin Permeability *In Vitro*

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**ABSTRACT:** Omega-3 rich fish oils are extremely labile, thus requiring control of oxidation and off flavor development. A recently proposed emulsification method, layer-by-layer (LbL) deposition, was found to be a plausible method to enhance the characteristics of bioactive ingredients, especially lipids. The present work was designed to test the possibility of enhancing the uptake and utilization of omega-3 fatty acids present in fish oil. The bioavailability of nano-emulsified fish oil was monitored in terms of intestinal absorption as well as skin permeability by using the everted intestinal sac model and Franz cell model. The skin permeability and intestinal absorption characteristics was significantly improved by LbL emulsification with lecithin/chitosan/low methoxypectin. Multilayer encapsulation along with nano-emulsification can be a useful method to deliver biologically active lipids and related components, such as fish oil. The protective effect of this tool from lipid oxidation still needs to be verified.

**Keywords:** encapsulation, everted intestine sac, fish oils, Franz cell, nano-emulcification

## INTRODUCTION

The beneficial effects of fish oils are related to their protective effect against numerous diseases such as cardiovascular disease, autoimmune disorders, ischemic myocardium, and arrhythmia (1), and the activities mainly are from omega-3 fatty acids. Omega-3 rich fish oils are extremely labile, thus requiring control of oxidation and off flavor development. In this context, encapsulation of marine omega-3 oil by a complex coacervation technique has been introduced as the most effective approach to delay its oxidation and extend the shelf life of omega-3-enriched food products (2,3). The bioavailability can also be notably enhanced when provided in a gelled emulsion as compared to those given in the free form (4).

Encapsulation has been studied and developed in the pharmaceutical and cosmeceutical industries as carrier systems for the protection and delivery of bioactive agents. Nano-encapsulations including nano-liposome, nano-emulsion, nanoparticles, and nano-dendrimers are

widely used and provides controlled release of core bioactive agents at the right place (5). Therefore, they enhance cellular uptake and effectiveness of the encapsulated material. Skin, hair, and mucosal surfaces are useful targets for the delivery of bioactive agents in the cosmeceutical industries. Encapsulation provides a great tool to the cosmeceutical formulator, providing great flexibility in the choice of delivery mechanisms (6).

A recently proposed emulsification method, layer-by-layer (LbL) deposition, was found to be a plausible method to enhance the characteristics of bioactive ingredients, especially lipids (7,8). In an *in vitro* study, the digestibility of emulsified lipids varies upon the cases, even within the same research group. The present work was designed to test the possibility of enhancing the uptake and utilization of omega-3 fatty acids present in fish oil. The bioavailability of nano-emulsified fish oil was monitored in terms of intestinal absorption as well as skin permeability using the everted intestinal sac model and Franz cell model.

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## MATERIALS AND METHODS

### Materials

The crude fish oil was kindly supplied by Doosan Co. Glonet BU (Youngin, Korea). The excipients of the emulsion were as follow: powdered chitosan (from crab shells, minimum 85% deacetylated) (Sigma-Aldrich Co., St. Louis, MO, USA), soybean lecithin (Junsei Chemical Co. Ltd., Tokyo, Japan), and low methoxyl pectin (LMP: from citrus peel, Sigma-Aldrich Co.).

### Preparation of nano-emulsified fish oil

Conventional fish oil (FO) was nano-emulsified (NO) by the method as discussed elsewhere. Nano-emulsion was developed to stabilize the fish oil. Using the biopolymer multiple layer-by-layer method, FO and NO were coated. A stock buffer solution was prepared by dispersing 100 mM sodium acetate and acetic acid in water and then adjusting the pH to 3.0. An emulsifier solution was prepared by dissolving 0.1 wt% lecithin into the stock buffer solution. The emulsifier solution was homogenized for 3 min at 11,000 rpm (primary homogenization). The oil/water emulsion obtained through the process of a primary homogenization was homogenized twice again using a M-110 Microfluidizer (Microfluidics, Newton, MA, USA) at 20,000 psi (secondary homogenization).

Nano-emulsions were prepared with different binding materials such as chitosan and LMP (Table 1). The biopolymers used in this experiment were aqueous chitosan solution (the secondary layered emulsion) and low methoxyl pectin solution (the tertiary layered emulsion). The fish oil nano-emulsion was diluted with aqueous chitosan solution to form a secondary emulsion using 0.1 M acetate buffer (pH 3.0). Secondary emulsions containing low methoxyl pectin solution were prepared by mixing the initial secondary emulsions with low methoxyl pectin solution. The emulsions were stored at 4°C overnight (12~15 h) in the dark.

**Table 1.** Fish oil concentration of native and nano-emulsified oils

No.	Sample	Concentration of the oils	Additional ingredients
FO1	Control	100.0%	—
FO2	FO	1.00%	Lecithin+DW
FO3	FO	0.33%	Lecithin+Chitosan solution
FO4	FO	0.17%	Lecithin+Chitosan solution+Low methoxyl pectin solution
NO1	Control	100.0%	—
NO2	NO	1.00%	Lecithin+DW
NO3	NO	0.33%	Lecithin+Chitosan solution
NO4	NO	0.17%	Lecithin+Chitosan solution+Low methoxyl pectin solution

FO, fish oil; NO, nano-emulsified oil.

### Animals

Male Sprague-Dawley rats weighing 180~220 g were maintained on commercial feed (Samyang oil & feed Co., Wonju, Korea) and given free access to water. The care and treatment protocols used for the experimental animals were conformed to the Korea University (KUIACUC-2013-184) guidelines for the ethical treatment of laboratory animals.

### Everted intestinal sac

Everted intestinal sac experiments were performed according to the method described by Tandon et al. (9) with some modifications. After overnight fasting, rats were killed by ether anesthesia. The abdomen was opened by a midline incision and the jejunum was taken quickly. After the underlying mesenterium was removed, the jejunum was flushed with ice-cold Krebs-Henseleit bicarbonate (KHB) buffer to remove intestinal contents. The composition of the buffer was NaHCO<sub>3</sub> 25 mM, NaCl 118 mM, KCl 4.7 mM, MgSO<sub>4</sub> 1.2 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.2 mM, CaCl<sub>2</sub> 1.2 mM, and Na<sub>2</sub>EDTA 9.7 mg/L. Glucose (10 mM) was added to the medium just before the start of the appropriate experiments. The jejunum was gently stretched and cut into segments (10 cm long each). Each of the sacs was carefully everted with a glass rod. One end was ligated with a conical rubber stopper that housed one port for removal and addition of the serosal fluid, and another port for continuous supply for 5% CO<sub>2</sub> and O<sub>2</sub> throughout the experiment. After being filled with 1 mL KHB buffer (inner compartment), the sacs were incubated in 29.5 mL KHB buffer (outer compartment) that was obtained from 0.5 mL fish oil sample at 37°C in a water bath. Transport of intestine of fish oil sample was expressed as P<sub>app</sub> (apparent permeability). P<sub>app</sub> was calculated from the following equation:  $P_{app} = (dQ/dt) \cdot V / (A \cdot C_0)$ , where dQ is the concentration in the inner compartment (mg/mL), dt is reaction time (h), V is volume of the solution in the inner compartment (cm<sup>3</sup>), A is membrane surface area (cm<sup>2</sup>), and C<sub>0</sub> is the concentration of inner and outer compartments (mg/mL). The concentration of the major fatty acids (C14:0, C16:0, and C18:0) was determined by gas chromatography.

### Skin permeation across Franz-type diffusion cell

Skin permeation was determined by the method of Sonavane et al. (10) with some modifications. The abdominal hair was removed from rats with an electric clipper and an electric razor 1 day before the study. Rats were anesthetized with ether anesthesia and decapitated. The abdominal skin was excised immediately. The excised skin was mounted in a Franz-type diffusion cell. In this study, 4.9 mL of 0.1 M sodium phosphate buffer (pH 7.4) was used as the receptor medium, and 100 µL of fish oil sample was placed on the donor side. The receptor medium

was kept at 37°C and stirred with a magnetic stirrer at 400 g. Aliquots (0.5 mL) of the receptor medium were withdrawn at 24 h. And after collection of the medium, 0.5 mL of fresh buffer was immediately added in the receptor cell. Transport of skin of fish oil sample was expressed as skin permeability (%).

$$\text{Skin permeability (\%)} = \frac{\text{fatty acid content (mg) in receptor cell}}{\text{fatty acid content (mg) in donor cell}} \times 100$$

The concentration of the major fatty acids (C14:0, C16:0, and C18:0) was determined by gas chromatography.

### Fatty acid analysis

The fatty acid methyl esters (FAMES) were prepared by saponification and methylation (11) and analyzed in a gas chromatograph (Varian 3900 GC, Varian, Inc., Walnut Creek, CA, USA) equipped with a supelco-2560 column (length 100 m, ID 0.25 mm, film thickness 0.2 µm) with helium carrier gas from 100°C increased at 3°C/min to 240°C. Injection temperature and the flame-ionization detector temperatures were set at 225°C and 270°C, respectively. Peak areas of the fatty acids were calculated as percentage of the total area, and the peaks were identified by comparing the retention times with those of a standard mixture of methyl esters.

### Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, USA). The differences among groups were evaluated statistically by Duncan's multiple-range test. The significance level of the statistical analyses was set at  $P < 0.05$ . All data were presented as means ± standard deviation (SD).

**Table 2.** Fatty acids composition of native fish oils (%)

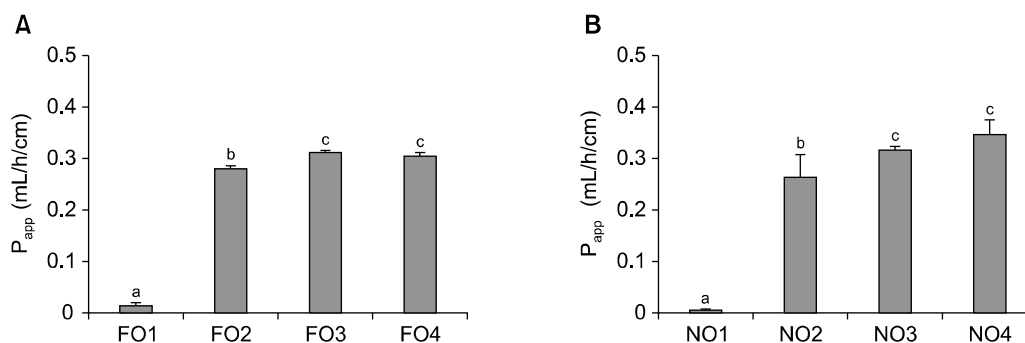
Fatty acid	FO	NO
12:0	0.06	0.03
14:0	3.77	3.56
16:0	23.89	23.45
16:1	5.31	5.15
18:0	5.85	5.96
18:1 (n-9)	15.11	19.87
18:1 (n-7)	0.03	0.09
18:2 (n-6)	0.07	0.11
20:0	0.41	0.34
18:3 (n-6)	0.06	0.05
20:1	1.16	1.96
18:3 (n-3)	0.03	0.50
20:2	0.28	0.30
20:3 (n-6)	0.13	0.17
20:4 (n-6)	2.29	2.34
20:5 (n-3)	6.40	7.24
22:5 (n-3)	1.43	1.77
22:6 (n-3)	33.72	27.12

FO, fish oil; NO, nano-emulsified oil.

## RESULTS AND DISCUSSION

The fatty acid composition of the fish oil used in this experiment is shown in Table 2. The absorption characteristics of NO as well as FO in the everted intestinal sac model is shown in Fig. 1 and Table 3. In both, FO as well as NO, the absorption in the everted intestinal sac was increased by LbL encapsulation. Specifically, the absorption rate of LMP and chitosan layered encapsulation was significantly higher than LbL encapsulated with different compositions. The absorption characteristics were also determined by measuring the representative fatty acid composition of absorbed lipids in the intestinal sac. There was no significant compositional difference in absorbed lipids in the intestinal sac.

Skin permeation across Franz-type diffusion cell is shown in Fig. 2 and Table 4. Skin permeability of fish oil



**Fig. 1.** Absorption characteristics of nano-emulsified fish oil in the intestinal everted sacs model. (A) fish oil (FO) and (B) nano-emulsified oil (NO). Values are the mean ± SD. Means with different letters (a-c) above bars are significantly different between samples of FO at  $P < 0.05$  by Duncan's multiple range test. Transport of intestine of FO sample was expressed as apparent permeability (P<sub>app</sub>). The P<sub>app</sub> was calculated from the following equation:  $P_{app} = (dQ/dt) \cdot V / (A \cdot C_0)$ .

**Table 3.** Absorption characteristics of nano-emulsified fish oil in the intestinal everted sacs model

	FO1	FO2	FO3	FO4	NO1	NO2	NO3	NO4
14:0	—	0.01±0.00	0.04±0.03	0.02±0.01	—	0.01±0.02	0.02±0.01	0.01±0.00
16:0	0.01±0.01	0.14±0.00	0.14±0.02	0.15±0.01	—	0.13±0.01	0.15±0.00	0.19±0.04
18:0	—	0.13±0.00	0.13±0.02	0.14±0.01	0.01±0.00	0.12±0.01	0.14±0.01	0.15±0.01
Total	0.01±0.01 <sup>a</sup>	0.28±0.01 <sup>b</sup>	0.31±0.00 <sup>c</sup>	0.31±0.01 <sup>c</sup>	0.01±0.00 <sup>a</sup>	0.26±0.04 <sup>b</sup>	0.32±0.01 <sup>c</sup>	0.35±0.03 <sup>c</sup>

Values are the mean±SD.

Means with different letters (a-c) are significantly different between samples of fish oils at  $P<0.05$  by Duncan's multiple range test.

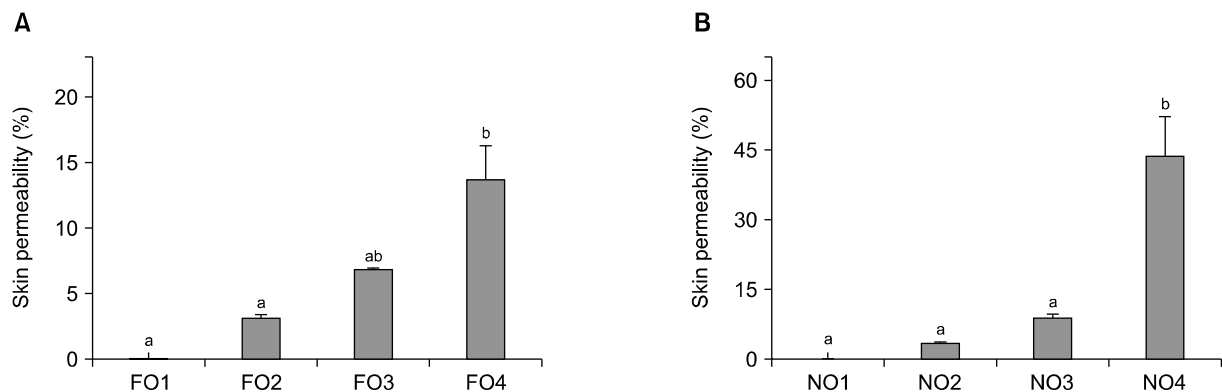
FO, fish oil; NO, nano-emulsified oil.

is calculated and the values are the sum of the 3 kinds of representative fatty acids (C14:0, C16:0, and C18:0). No significant difference was observed in terms of skin permeability by lecithin and/or chitosan encapsulation. Notably, lecithin/chitosan encapsulation with LMP significantly increased skin permeability. When the fish oil was given as nano-emulsions with the chitosan solution (the secondary layered emulsion), there was an increase in the uptake of FO and NO by 22% and 48%, respectively as compared to the oil given in native form. Similarly, when fish oil was given as nano-emulsions with the chitosan solution and LMP solution (the tertiary layered emulsion) there was also an increase in the uptake of FO and NO by 21% and 53%, respectively as compared to the oil given in native form. Thus, intestinal everted sacs absorbed significantly higher levels of oil in nano-emulsions form with chitosan and/or LMP as compared to the

oils given in native form.

Emulsified with lecithin, the chitosan solution and low methoxyl pectin solution showed higher permeation rates in SFA levels compared to the other samples ( $P<0.05$ ). When fish oil was given as nano-emulsions with the chitosan solution and LMP solution (the tertiary layered emulsion), there was also an increase in the permeation of FO and NO by 37% and 53%, respectively as compared to the oil given in native form. NO showed higher absorption rate rather than conventional fish oil. Also, NO with the chitosan solution or LMP solution showed higher absorption rate of SFA levels ( $P<0.05$ ).

Microencapsulation techniques have drawn considerable attention in the food industry. Chitosan, a food grade biopolymer, has been proposed as an effective encapsulating ingredient. The positive charge across a wide range of pH is the most valuable characteristics as an encapsu-



**Fig. 2.** Absorption characteristics of nano-emulsified fish oil in the Franz cell model. (A) fish oil (FO) and (B) nano-emulsified oil (NO). Values are the mean±SD. Means with different letters (a,b) above bars are significantly different between samples of FO at  $P<0.05$  by Duncan's multiple range test.

**Table 4.** Absorption characteristics of nano-emulsified fish oil in the Franz cell model

	FO1	FO2	FO3	FO4	NO1	NO2	NO3	NO4
14:0	0.00±0.00	0.22±0.06	0.21±0.04	0.42±0.17	0.00±0.00	0.13±0.04	0.22±0.08	2.66±0.62
16:0	0.01±0.00	1.79±1.48	3.76±0.48	7.57±1.31	0.01±0.00	1.12±0.06	3.16±0.87	32.90±6.56
18:0	0.01±0.00	0.81±0.22	2.32±0.43	5.43±1.89	0.01±0.01	0.89±0.06	2.57±0.65	9.67±4.99
Total	0.02±0.00 <sup>a</sup>	2.83±1.64 <sup>a</sup>	6.29±0.08 <sup>ab</sup>	13.42±3.37 <sup>b</sup>	0.02±0.01 <sup>a</sup>	2.15±0.03 <sup>a</sup>	5.95±1.43 <sup>a</sup>	45.23±12.17 <sup>b</sup>

Values are the mean±SD.

Means with different letters (a,b) are significantly different between samples of fish oils at  $P<0.05$  by Duncan's multiple range test.

FO, fish oil; NO, nano-emulsified oil.

lating ingredient. Chitosan encapsulated lipids showed significantly improved thermal stability (12-15), but the bioavailability of chitosan encapsulation is still arguable (7).

Encapsulation has evolved remarkably in the pharmaceutical and cosmeceutical fields for the protection and delivery of bioactive agents. Skin, hair, and mucosal surfaces are useful targets for the delivery of bioactive agents in the cosmeceutical industry providing great flexibility in the choice of delivery mechanism (6). A recently proposed emulsification method, LbL deposition, was found to be a plausible method to enhance the characteristics of bioactive ingredients, especially lipids (8). In this study, the possibility of enhancing the uptake and utilization of omega-3 fatty acids present in fish oil was tested by using LbL emulsification.

Another study showed that chitosan encapsulation does not impact the digestibility of encapsulated lipids (8). Chitosan encapsulation provides additional health benefits such as reduced serum cholesterol levels in both animals and humans. The bioavailability of nano-emulsified fish oil was monitored in terms of intestinal absorption as well as skin permeability using the everted intestinal sac model and Franz cell model. Skin permeability was significantly improved by LbL emulsification with chitosan/LMP. Our results suggest that multilayer encapsulation along with nano-emulsification can be a useful method to deliver biologically active lipids and related components, such as fish oil. The protective effect of this tool from lipid oxidation still needs to be verified.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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