UNIVERSITI PUTRA MALAYSIA

PREPARATION, CHARACTERIZATION, *IN-VIVO* EVALUATION AND APPLICATION OF NANOLIPOSOMAL POLYUNSATURATED FOR FOOD ENRICHMENT

BABAK RASTI

FSTM 2013 12

PREPARATION, CHARACTERIZATION, *IN–VIVO* EVALUATION AND APPLICATION OF NANOLIPOSOMAL POLYUNSATURATED OIL FOR FOOD ENRICHMENT

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2013

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairman: **Jinap Selamat, PhD**
Faculty: **Food Science and Technology**

The ω–3 fatty acids, Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have low water-solubility and are very sensitive to oxidation; therefore there is a need for new methods to solubilize and protect such sensitive compounds. One approach for increasing the level of ω–3 in our diet is to increase the ω–3 content of food formulations with micro- and nano-encapsulated ω–3. The aim of the present research was to develop, optimize and characterize the formation of stable liposomal nanocomplexes for encapsulation and delivery of ω–3 FAs with high *in-vivo* bioavailability and suitability for food enrichment. Liposomes were prepared by Bangham/conventional thin-layer evaporation as reference method and Mozafari method (direct hydration without using organic solvents).

In the first study, the application of the response surface methodology (RSM) to develop an optimal preparation condition namely shear rate (600–1000 rpm), mixing time (30–60 min) and sonication time (10–20 min) for DHA and EPA nanoliposomes was studied. Fifteen lipid mixtures were generated by the Box–Behnken design and nanoliposomes were prepared by the Mozafari method. Nanoliposomes were characterized with respect to encapsulation/entrapment efficiency (EE) and particle size (PS). The results were then applied to estimate the coefficients of response surface model and to determine the optimal preparation conditions with maximum EE and minimum PS. The response surface analysis showed no significant ($p > 0.05$) lack of fit for the reduced models. The response optimization of experiments was the shear rate: 795 rpm; mixing time: 60 min; and sonication time: 10 min with the average diameter of 81.4 nm and EE of 100%. In the second study, using the optimum preparation conditions from the first study, the influence of liposome composition namely phospholipid (PL; 2–8 g), DHA and EPA (300–600 mg) and glycerol (1–3% w/w) on EE and PS was evaluated and optimized by RSM. The response optimizations of experiments were the PL: 6.87 g and ω–3: 300 mg. The optimal nanoliposome had an average diameter of 72.9 nm and EE of 100 %.

In the third study, the physical and relative oxidative stability of freshly prepared and stored liposomal and nanoliposomal systems of DHA and EPA were investigated. The effects of organic solvents on the oxidative stability of liposomal ω–3 produced by both

methods were compared. The highest physicochemical stability was observed in PUFA liposomes prepared by the Mozafari method, followed by conventional liposomes and bulk PUFAs. There was no significant ($p > 0.05$) change in physicochemical stability of nanoliposomal ω–3 during 10 months of cold storage (4 °C). Moreover, the comparison between liposomes (>200 nm) and nanoliposomes (50–200 nm) revealed that the surface charge, physical stability and oxidative stability of liposomal PUFAs increased as the size of the liposomes decreased.

In–vivo experiment was carried out, the fourth study, to determine whether EPA and DHA, esterified in triglycerides as oil or in PL as liposome and nanoliposome, exhibit comparable fates in plasma and liver lipids. PL and TG mixtures with close contents of EPA and DHA were administered to 80 male Sprague–Dawley rats for 8 weeks. Most relevant events occurred after 8 weeks for all treatments. However, significant ($p < 0.05$) increase in ω–3 content of plasma and liver was observed from the second week of the experiment. At that time, nanoliposomes and liposomes caused higher increase in the DHA and EPA contents of plasma and liver compared with oil. Liposome and fish oil feedings caused a marked increase in the amounts of ω–3 PUFA. However, nanoliposomes increased the ω–3 level in significantly ($p < 0.05$) higher amount compared with liposomes and oil.

Finally, application of nanoliposomal ω–3 in bread and milk was compared with un–encapsulated and microencapsulated ω–3. Objective discrimination sensory test was conducted to determine the perceptible sensory difference or similarity between un–encapsulated (fish oil), microencapsulated and nanoliposomal ω–3 enriched food. Results of the sensory evaluation showed no significant detectable difference ($p > 0.05$) between the control and nanoliposomal ω–3 enriched samples. In contrast, samples enriched with fish oil or microencapsulated ω–3 showed significant ($p < 0.05$) detectable fishy flavor. Moreover, significantly ($p < 0.05$) higher ω–3 % recovery and lower peroxide and anisidine values were observed in nanoliposomal ω–3 enriched samples in comparison with other samples. In conclusion, we have successfully developed a safe, effective and reproducible method for protection, delivery and application of ω–3 FAs in food system. However, the safety of the nanoliposomes after ingestion in humans should be evaluated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENYEDIAAN, PENCIRIAN, *IN-VIVO* PENILAIAN DAN PENGGUNAAN NANOLIPOSOM LEMAK POLITAKTEPU UNTUK MEMPERKAYAKAN MAKANAN

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Omega-3 asid lemak, asid eicosapentaenoic (EPA) dan asid docosahexaenoic (DHA), ini mempunyai keterlarutan dalam air yang rendah dan sangat sensitif kepada pengoksidaan; oleh itu, kaedah baru adalah diperlukan untuk melarutkan dan melindungi sebatian tersebut. Salah satu pendekatan untuk meningkatkan tahap ω–3 dalam diet kita adalah dengan meningkatkan kandungan ω–3 dalam rumusan makanan dengan ω–3 mikro dan nanopengkapsulan. Tujuan kajian ini adalah untuk membangun, mengoptima dan mencirikan pembentukan nanokompleks liposom yang stabil untuk nanopengkapsulan dan penyampaian ω–3 FAs dengan biokedapatan *in-vivo* dan kesesuaian yang tinggi untuk penkayaan makanan.Liposom telah disediakan dengan merujuk kepada kaedah Bangham/penyejatan lapisan nipis konvensional dan kaedah Mozafari (penghidratan langsung tanpa menggunakan pelarut organik).

Dalam kajian pertama, penggunaan kaedah permukaan sambutan (RSM) untuk mendapatkan satu cara persediaan optimum iaitu kadar ricih (600–1000 rpm), masa pencampuran (30–60 min) dan masa sonikasi (10–20 min) untuk DHA dan EPA nanoliposomes telah dikaji. Lima belas campuran lipid telah dijana oleh reka bentuk Box–Behnken dan nanoliposom telah disediakan oleh kaedah Mozafari.Pencirian nanoliposom merujuk kepada pengkapsulan/perangkap kecekapan (EE) dan saiz zarah (PS). Keputusan yang diperolehi digunakan untuk menganggarkan pekali model permukaan sambutan dan untuk menentukan cara persediaan optimum dengan EE yang maksimum dan PS yang minimum. Analisis permukaan sambutan tidak menunjukkan kekurangansesuai yang ketara ($p > 0.05$) untuk model yang dikurangkan. Pengoptimuman balas eksperimen adalah kadar ricih: 795 rpm; masa pencampuran: 60 min dan masa sonication: 10 min dengan diameter purata 81.4 nm dan EE 100%. Dalam kajin kedua, dengan menggunakan cara persediaan optimum dari kajian pertama, pengaruh komposisi liposom iaitu phospholipid (PL; 2–8 g), DHA dan EPA (300–600 mg) dan gliserol (1–3% w/w) keatas EE dan PS telah dinilai dan dioptimumkan dengan menggunakan RSM. Pengoptimuman balas eksperimen adalah PL: 6.87 g dan ω–3: 300 mg. Nanoliposom yang dioptimumkan mempunyai diameter purata 72.87 nm dan EE 100%.

Dalam kajian ketiga, kestabilan fizikal dan kestabilan oksidatif relatif bagi liposom yang baru disediakan dan yang telah disimpan serta sistem nanoliposoma DHA dan EPA telah disiasat.Kesan pelarut organik terhadap kestabilan oksidatif liposomal ω–3 yang dihasilkan oleh kedua–dua kaedah telah dibandingkan. Kestabilan fizikokimia tertinggi diperhatikan dalam PUFA liposom yang disediakan oleh kaedah Mozafari, diikuti oleh liposom konvensional dan PUFA pukal. Tiada perubahan yang signifikan ($p > 0.05$) didapatibagi kestabilan fizikokimia pada nanoliposoma ω–3 sewaktu 10 bulan di dalam penyimpanan sejuk (4 °C). Selain itu, perbandingan antara liposoma (> 200 nm) dan nanoliposom (50–200 nm) mendedahkan bahawa caj permukaan, kestabilan fizikal dan kestabilan oksidatif PUFA liposom meningkat dengan penurunan saiz liposom.

Kajian *in–vivo* telah dijalankan dalam kajian keempat untuk menentukan sama ada EPA dan DHA, yang diestifikasi dalam trigliserida sebagai minyak atau dalam PL sebagai liposom dan nanoliposom, mempamerkan kesan setanding dalam plasma dan lipid hati. PL dan campuran TG dengan kandungan dekat dalam EPA dan DHA telah diberikan kepada 80 tikus jantan Sprague-Dawley selama 8 minggu. Peristiwa–peristiwa yang paling berkaitan berlaku selepas 56 hari untuk semua rawatan.Walau bagaimanapun, peningkatan yang ketara anda kandungan ω–3 dalam plasma dan hati diperhatikan pada minggu percubaan kedua. Pada masa itu, nanoliposom dan liposom, jikadi bandingkan dengan minyak, iatelah mengurangkan plasma lipid dan kandungan hati yang mempunyai komposisi FA yang telah diperkaya dengan ω–3, setanding dengan DHA dan EPA. Liposom dan pemakanan minyak ikan menyebabkan peningkatan yang ketara dalam jumlah ω–3 PUFA. Walau bagaimanapun, nanoliposomes meningkatkan jumlah tahap ω–3 yang ketara ($p < 0.05$) lebih tinggi berbanding dengan liposom dan minyak.

Akhirnya, aplikasi nanoliposomal ω–3 dalam roti dan susu telah dibandingkan dengan ω–3 yang tidak dikapsulkan dan dengan mikro pengkapsulan. Objektif diskriminasi ujian deria telah dijalankan untuk menentukan perbezaan jelas deria atau persamaan antara yang tidak dikapsulkan (minyak ikan), dengan mikro pengkapsulan dan nanoliposomal dalam makanan yang telah diperkaya dengan ω–3. Ujian segitiga telah digunakan dengan 6 orang penilai yang berpengalaman dengan 3 replikasi dalam 4 sesi yang berbeza. Analisis data dilakukan dengan menggunakan Chi-Square % ujian defektif. Keputusan penilaian deria menunjukkan tiada perbezaan yang signifikan ($p > 0.05$) diantara kawalan dan sampel yang diperkaya dengan nanoliposom ω–3. Sebaliknya, sampel yang diperkaya dengan minyak ikan atau mikro pengkapsulan ω–3 mempunyai kesan rasa hanyir yang signifikan ($p < 0.05$). Selain itu, % pemulihan ω–3 yang lebih tinggi dan ketara ($p < 0.05$) serta nilai peroksida dan anisidin yang lebih rendah dan ketara ($p < 0.05$) telah diperhatikan dalam sampel yang diperkayakan dengan nanoliposom ω–3 berbanding dengan sampel lain. Kesimpulannya, kajian ini telah memperkenalkan kaedah yang baru, selamat, berkesan dan boleh diulang untuk perlindungan, penghantaran dan penggunaan ω–3 FAs dalam industri makanan.

ACKNOWLEDGEMENTS

I wish to express my most appreciation, my profound gratitude and my genuine heartfelt thanks to my supervisor Prof. Dr. Jinap Selamat (Chairman) for her extraordinary level of concern for the well–being of her students. Many long discussions in her office inevitably led to a few answers, several questions, and a new perspective on life in general. Her kindness, knowledge, energy, quick smile, and frequent encouragement were very much appreciated.

A sincere expression of gratitude goes to all the members of my supervisory committee including Associate Professor Dr. M.R. Mozafari and Professor Dr. Mohd Yazid Abd Manap for their comments and invaluable advice and constructive suggestions and guidance throughout of the duration of this research project.

Last but not least, I would like to express my deepest thanks to my beloved parents and brothers for their patience and continuous support and encourage. There are many to mention individually, but I grateful to them all.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACUC	Animal Care and Use Committee
ALA	α–linolenic acid
ANOVA	Analysis of variance
AOCS	American Oil Chemists' Society
ARA	Arachidonic acid
ATP	Adenosine tri–phosphate
ca.	Approximately
COX	Cyclooxygenase
C.V.	Coefficients of variation
d	Day
DDS	Drug delivery system
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
e.g.	For example
EE	Entrapment efficiency
EFTEM	Energy filtered transmission electron microscopy
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
FID	Flame ionization detector
FM	Fish meal
GC	Gas chromatography
GI	Gastrointestinal
GIT	Gastrointestinal tract
GMi	Monosialoganglioside
HDL	High density lipoproteins
I.S.	Internal standard
LA	Linolenic acid
LC	Loading capacity
LCPUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoproteins
LNA	Alpha-linolenic
LOX	Lipooxygenase
LUV	Large unilamellar vesicle
MA	Malonaldehyde
MDA	Malondialdehyde
MHP	Monohydroperoxide
MLV	Multilamellar vesicle
MRI	Magnet resonance imaging
MUFA	Monounsaturated fatty acid
N	Nitrogen
N	Normal
n	Omega
O/W	Oil–in–water
O/W/O	Oil–in–water–in–oil
OFN	Oxygen free nitrogen
P	Pressure
P	Probability

PC	Phosphatidylcholine
PDI	Polydispersity index
PE	Phosphatidylethanolamine
PEG	Poly(ethylene)glycol
PG	Phosphatidylglycerol
PL	Phospholipid
ppm	Parts per million
PS	Particle size
PTA	Phosphotungstic acid
PUFA	Polyunsaturated fatty acid
RES	Reticulo Endothelial System
rpm	Revolution per minute
RSD	Relative standard deviation
RSM	Response surface methodology
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SUV	Small unilamellar vesicles
TBARS	Thiobarbituric acid reactive substances
TEM	Transmission electron microscopy
TEP	1,1,3,3–tetraethoxypropane
v/v	Volume per volume
VPG	Vesicular phospholipid gels
vs.	Versus
W/O	Water–in–oil
W/O/W	Water–in–oil–in–water
w/v	Weight per volume
WHO	World health organization
x	Time
ζ	Zeta
ω	Omega

CHAPTER 1

INTRODUCTION

Omega–3 polyunsaturated fatty acids (ω–3 PUFA) have recently become a subject of intense interest in both human and animal nutrition. Studies have shown potential benefits of supplementation with long chain (LC) ω–3 fatty acids (FAs) for osteoarthritis (Hansen *et al.*, 2008), cardiovascular disease (Bovet *et al.*, 2007), renal disease (Brown *et al.*, 1998) and even learning and brain development (Innis, 2008). Many of these benefits are presumed to be related to the ability of ω-3 FAs to decrease inflammation by altering eicosanoid production. As numerous chronic disease processes are linked to aberrant inflammation, compounds that reduce inflammation have the potential to improve treatment and possibly even prevent these diseases. This potential has led to a plethora of ω–3 supplemented food items for human consumption ranging from bread to infant formula.

The benefits of ω–3 FAs have inspired researchers' interest in solving challenges related to the oxidative stability, storage and delivery of ω–3 FAs, particularly DHA and EPA (Zimet & Livney, 2009). Lipid peroxidation is thought to damage biological systems. Therefore, lipid peroxidation has received considerable attention in investigations into the dietary effects of PUFAs. The oxidative stability of each of these PUFAs is inversely proportional to the number of bis-allylic hydrogen in the molecule; therefore, highly unsaturated FAs such as DHA and EPA are more easily oxidized in the presence of air, with a resulting decrease in food quality. Moreover, a high intake of compounds such ω-3 PUFAs, which contain high hydroperoxides, aldehydes, and toxic oxidation products, may cause oxidative stress or an increased susceptibility to lipid peroxidation in the biological system. However, most studies have been carried out on neat oils and not on emulsified or other colloidal systems (Araseki *et al.*, 2002).

Different strategies have been used to protect PUFAs, from oxidation. Phospholipids have demonstrated antioxidant activity (Khan & Shahidi, 2000), and their synergistic effects have been observed in the protection of fish oil. In addition, incorporating PUFAs into phospholipids has been found to improve PUFA resistance to oxidation (Lyberg *et al.*, 2005). The use of phospholipids as food additives to stabilize PUFAs is, therefore, highly interesting. However, because there is a large variety of phospholipid, it is necessary to evaluate which phospholipid structures containing PUFAs offer the best protection against oxidation. Another approach to protecting PUFAs is encapsulation. The encapsulation of PUFA-containing oils, such as fish oil, significantly increases their oxidative stability, reduces the undesirable odors of volatile oxidation products (Klaypradit & Huang, 2008; Klinkerson *et al.*, 2006) and improves the odors of products enriched with fish oil (Castro *et al.*, 2004). Recently, researchers have reviewed the application of liposome-encapsulated bioactive food ingredients (Henna-Lu *et al.*, 2011; Da Silva Malheiros *et al.* 2010; Mozafari *et al.*, 2006). The use of liposomes, the multiple or single phospholipid bilayers surrounding an aqueous medium, as a biocompatible delivery system may be a suitable and promising method to increase the bioavailability and stability of PUFAs and to overcome their drawbacks.

All biological membranes contain lipids as primary constituents. The building blocks of liposomes are surface-active amphiphiles with a head group that is strongly hydrophilic, coupled to a hydrophobic tail. Lipid molecules are insoluble in water and form colloidal dispersions. The vesicle size ranges from 20 nm to several dozen micrometers while the thickness of the membrane is about 4–5 nm. Because of their solubility properties the

structure of liposomes involves the ordering of lipid molecules in such a way, that the polar head is in contact with water while the non–polar hydrocarbon chain is hidden from water in the interior of the bilayer structures. Depending on the form factor of the lipid molecule they can form bilayers, micelles, or vesicles when in contact with water.

The ability to encapsulate and thereby segregate aqueous components led to a variety of applications of liposomes. These include their use in biological systems as quantized reagent packets for the delivery of deoxyribonucleic acid (DNA) vectors and genes (Kikuchi *et al.*, 1999), contrast agents for enhanced magnet resonance imaging (MRI) (Ayyagari *et al.*, 2006; Mulder *et al.*, 2006; Saito *et al.*, 2005), therapeutic agents (Abraham *et al.*, 2005; Gulsen *et al.*, 2005; Ramachandran *et al.*, 2006) , encapsulation of proteins and cells (Tan *et al.*, 2006), transport studies, model systems for the study of biological membranes and their fusion, investigation of membrane proteins that can be reconstituted in liposomes (Lasic, 1988),templates for the formation of solid hydrogel nanoparticles (Kazakov & Levon, 2006), or as protective coatings for enzymes entrapped in silica sol-gel biocomposites (Li & Yip, 2005).

Liposomes are especially interesting as transport vehicles for *in–vivo* applications such as drug delivery systems (DDSs) where they can achieve selective and high localization of active drug at the disease site (Mozafari *et al.*, 2007a). Due to their biphasic character, liposomes can act as carriers for both lipophilic compounds that are compartmentalized in the bilayer and hydrophilic compounds that are encapsulated in their aqueous interior.

We hypothesized that liposomal encapsulation will improve the oxidative stability, absorption and sensory properties (desirability) of ω–3 FAs. Therefore, the following objectives have been considered for the present study:

1. To optimize the preparation condition resulting in nanoliposomal ω–3 lipids with desirable physicochemical properties;

2. To optimize the chemical composition resulting in the nanoliposomal ω–3 lipids with desirable physicochemical properties;

3. To evaluate the storage stability of optimized liposomal and nanoliposomal ω–3 lipids;

4. To determine the absorption and distribution of nanoliposomal ω–3 lipids compared to liposomal and un-encapsulated FAs *in–vivo*; and

5. To determine the stability and perceptible sensory difference or similarity between un-encapsulated and nanoliposomal ω–3 lipids enriched food.

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The student, Babak Rasti was born in 1980. He obtained his primary education at Andisheh Primary School and continued his secondary education in Iran–zamin Guidance School and furthered in Sherafatian High School at Shiraz, Iran. He was accepted in Shiraz University in 1997 as an undergraduate student at the Faculty of Food Science and Technology and completed his degree in Bachelor of Food Science and Technology in 2001. Following 5 years of working in the industry, he completed his Master degreein Food Biotechnology in 2009 and continuously, pursues his PhD degree at the Faculty of Food Science and Technology, University Putra Malaysia (UPM).

LIST OF PUBLICATIONS

1. Rasti, B., Selamat, J., Mozafari, M.R. & Yazid, M. (2012). Comparative study of the oxidative and physical stability of liposomal and nanoliposomal polyunsaturated fatty acids prepared with conventional and Mozafari methods. *Food Chemistry*, 135: 2761–2770.

2. Rasti, B., Selamat, J., Mozafari, M.R. & Yazid, M. (2014). Optimization on preparation condition of polyunsaturated fatty acids nanoliposome prepared by Mozafari method. *Journal of Liposome Research*, DOI: 10.3109/08982104.2013.839702.