EFFECT OF MOLECULAR WEIGHT, CHARGE AND HYDROPHOBICITY OF WHEY PEPTIDES ON ENCAPSULATION EFFICIENCY AND SURFACE PROPERTIES OF SOY LECITHIN-DERIVED NANOLIPOSOMES

By

Aishwarya Mohan

Submitted in fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
April 2016

Table of Contents

List of Tables

List of Figures

Abstract

Low bioavailability and biostability of food protein-derived bioactive peptides can impede their commercialization as functional foods. Liposome encapsulation of bioactive peptides has been explored in order to circumvent this challenge, but there is a paucity of information on the effects of peptide structural properties on encapsulation. The impact of the molecular properties of whey peptides, including their molecular weight, net charge and hydrophobicity, on encapsulation efficiency (EE) and properties of the resulting nanoliposomes was studied. Soy lecithin-derived nanoliposomes were found to encapsulate the whey peptide fractions with high EE of >80%. The net negatively charged peptide fraction had a significantly lower EE than the other fractions, which can be attributed to electrostatic repulsion with the anionic phospholipid heads. Liposome properties (ζ-potential, particle diameter, polydispersity index) were not significantly altered by the different peptide molecular weight ranges. However, the hydrophilic peptide fraction resulted in unstable liposome suspension, with the lowest ζ-potential (-2.5±0.5 mV). Similarly, mean particle diameter was significantly higher for liposomes loaded with the cationic peptide fraction. Surface hydrophobicity and number of peptides per unit mass of the whey peptide fractions were also found to influence EE and liposome properties. Based on FTIR analysis, the peptide net charge and hydrophobicity did not affect their distribution in the core, surface and bilayer regions of the nanoliposomes, although the least hydrophobic low-molecular-weight peptide fractions interacted more strongly with choline on the liposome surface. These findings will support efforts towards the design and commercial production of encapsulated bioactive peptides with improved functional attributes.

List of Abbreviations Used

ANS	8-anilino-1-naphthalenesulfonic acid
ATR	Attenuated
AWP	Anionic whey peptide fraction
CAA	Cationic amino acids
CH	Casein hydrolysate
CWP	Cationic whey peptide fraction
EDUF	Electrodialysis with ultrafiltration membranes
EE	Encapsulation efficiency
FTIR	Fourier transform infrared spectroscopy
GIT	Gastrointestinal
HAA	Hydrophobic amino acids
HBW	Hydrophobic whey peptide fraction
HHR	Hydrophobic-hydrophilic amino acid ratio
HLW	Hydrophilic whey peptide fraction
PC	Phosphatidylcholine
PDI	Polydispersity index
P_E	Encapsulated peptides
P_I	Total peptides
PL	Phospholipid
P_{NE}	Non-encapsulated peptides
RAa	Relative abundance of total anionic residues
RA_c	Relative abundance of total cationic residues
TEM	Transmission electron microscopy
WPH	Whey protein hydrolysate
WPI	Whey protein isolate

Acknowledgments

I express my sincere thanks to my supervisor Dr. Udenigwe for his immense support, guidance and mentorship throughout the program. Sincere thanks to my committee members Dr. Bazinet and Dr. He for their valuable comments, suggestions and contribution for the completion of my thesis. I specially thank Dr. Bazinet and Jacinthe Thibodeau for hosting me at Univetsité Laval and helping me with the experiments. I am grateful to all the lab instructors in environmental sciences department; Anne LeLacheur, Margie Tate, Daryl Mullin and Paul McNeil for their patience and kindness towards my queries and needs. I convey my thanks to Sherryl Patton and Robin Spence of Intertape Polymer Group for providing access to instrument. I am also thankful to all my lab members, Subin Rajendran, Wei Xu, Maryam Thorabizadeh, Chinonye Udechukwu, Hannah Mawhinney, Stephani Collins, Holly Fisher and Amy Unicomb for their support and cooperation. I am grateful to Subin for his help during data collection and for proof reading my thesis document. I would like to express deepest gratitude to my parents Mohan and Narayani for their unconditional love and care.

Finally, I thank the funding agencies, Natural Sciences and Engineering Research Council of Canada (NSERC), Canada Foundation for Innovation (CFI) for the grants; Nova Scotia Research and Innovation Trust Fund (NSRIT) and Dalhousie University for the scholarships.

Chapter 1

Introduction

Non-communicable diseases, primarily cardiovascular disease, diabetes and cancer are reported to be responsible for 63% of global deaths (WHO report 2014). Managing non-communicable diseases through dietary or nutritional intervention has gained notable interest as a result of burgeoning knowledge on the association of food and health.[1] Additionally, consumer awareness of scientific evidence has shifted public preference towards a healthier diet in the form of functional foods. Ingredient such as polysaccharides, polyphenols, vitamins, peptides, fatty acids are currently being pursued for their bioactivity. Food-protein derived bioactive peptides are unique biomolecules with heterogeneous monomers within a single peptide. Mellander reported the first bioactive peptides (phosphopeptides) in 1950, since then our cognition of bioactive peptides has elevated drastically.[2]

Bioactive peptides are specific fragments of proteins, that are not limited to act as source of nutrients in the form of nitrogen and amino acids, but are capable of exhibiting single or multitude of positive physiological function(s) that ultimately influence overall health status.[1] Generally, functional peptides are considered to have anywhere from 2-20 amino acid residues and encrypted within the parent protein sequence.[1] Food-derived bioactive peptides are promising functional food ingredients, demonstrated to exhibit several bioactivities including antihypertensive, antioxidant, antidiabetic, antinflammatory, hypocholesterolemia, antimicrobial, anticancer, immune-modulatory and antithrombic.[3] To date, bioactive peptides have been derived from various food sources including, milk,[4] plant,[5] meat,[3] marine sources [1,6] and microalgae.[7] Recently, the scientific community is diverging from the use of dietary proteins to sustainable

sources including wastes and by-products. Nevertheless, milk remains the most predominant source of bioactive peptides,[2] particularly whey is a by-product of the cheese industry with several known bioactive peptides.[8]

Myriad of food-derived peptides with defined sequences have been identified to show specific bioactivities.[5] However, food-proteins hydrolysate and peptides are liable for proteolytic degradation on ingestion and can undergo potential interaction with other food components.[9] Low bioavailability and biostability, mainly contribute to the inability of most peptides to iterate the *in vitro* bioactivity *in vivo*.[10] Moreover, commercialization of bioactive peptides are also circumscribed by the stability issue, hence only a few bioactive peptide products are available for consumer use.[11] Peptides are chemically active biomolecules compared to proteins, with more free amino and carboxylic groups.[12] The reactive groups can covalently interact with other components in the food matrix, for instance free amino group can react with sugar giving rise to Maillard reaction products.[13] Furthermore, peptides have bitter taste due to the exposure of hydrophobic amino acids residues during hydrolysis.[9]

Therefore, delivery of bioactive ingredients has become indispensable to protect the bioactives from unfavourable environments.[12] It is obligatory for carriers of food bioactives to be biodegradable, edible and non-toxic.[13] The three types of carrier systems currently used are, polysaccharides, proteins and lipids.[9] The merits, demerits of each system is discussed in detail in Chapter 2. Lipid carriers were selected for this project owing to the mild preparation conditions, ease of preparation and low energy input processes. Several lipid based colloidal delivery systems including, microemulsions, nanoemulsions, solid lipid nanoparticles, liposomes have been studied. Liposome is of particular interest because of its compatibility with hydrophilic, lipophilic and amphipathic biomolecules.[16]

Liposomes are vesicular structures with one or more phospholipid bilayer formed spontaneously due to the exposure to aqueous surrounding.[17] Liposomes were first observed as swollen phospholipid system by Alec Bangham in 1965.[16] Realization of their structural resemblance to biological membranes and their ability to encapsulate molecules, has equipped us to adequately exploit liposomes in diverse areas of research and application. Particularly, cosmetic and pharmaceutical industries have meticulously used liposomes for protection and delivery.[9] Application of liposomes in food industry is steadily advancing as well, with the use of polysaccharide coating to improve the stability of liposomes.[18] It is known that the lipid composition alters the physicochemical properties of liposomes such as size, surface charge, rigidity and fluidity.[17] However, there is dearth of information regarding the interaction of the liposome with the active ingredients of different properties. Although the amino acid composition, sequence, structural and surface properties of food-protein hydrolysates/peptides are receiving attention, their role in designing carrier systems are relatively less studied.

Thus the aim of this study was to assess the impact of whey peptide properties such as molecular weight, net charge and hydrophobicity on the encapsulation efficiency and physicochemical properties of soy lecithin-derived nanoliposomes.

REFERENCES

(1) Harnedy, A.; Fitzgerald, R. J. Bioactive peptides from marine processing waste and shellfish : A review. **2011**, *4*.

(2) Korhonen, H.; Pihlanto, A. Food-derived Bioactive Peptides - Opportunities for Designing Future Foods. *Curr. Pharm. Des.* **2003**, *9* (16), 1297–1308.

(3) Bhat, Z. F.; Kumar, S.; Bhat, H. F. Bioactive peptides of animal origin : a review. **2015**,

52 (September), 5377–5392.

(4) Nongonierma, A. B.; Fitzgerald, R. J. The scientific evidence for the role of milk proteinderived bioactive peptides in humans : A Review. *J. Funct. Foods* **2015**, *17*, 640–656.

(5) Yoshikawa, M. Peptides Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides* **2015**, *72*, 208–225.

(6) Jo, C.; Khan, F. F.; Khan, M. I.; Iqbal, J. cr t. **2016**, *9129* (March).

(7) Wijesekara,I.; Kim, S. K. Application of marine algae derived nutraceuticals in the food industry. *Marine Algae Extracts: Processes, Products and Applications*. **2015,** 627-638.

(8) Walzem, R. L.; Dillard, C. J.; German, J. B. Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. *Crit. Rev. Food Sci. Nutr.* **2002**, *42* (4), 353–375.

(9) Mohan, A.; Rajendran, S. R. C. K.; He, Q. S.; Bazinet, L.; Udenigwe, C. C. Encapsulation of food protein hydrolysates and peptides: a review. *RSC Adv.* **2015**, *5* (97), 79270–79278.

(10) Segura-Campos, M.; Chel-Guerrero, L.; Betancur-Ancona, D.; Hernandez-Escalante, V. M. Bioavailability of Bioactive Peptides. *Food Rev. Int.* **2011**, *27* (3), 213–226.

(11) Hartmann, R.; Meisel, H. Food-derived peptides with biological activity: from research to food applications. *Curr. Opin. Biotechnol.* **2007**, *18* (2), 163–169.

(12) Van Lancker, F.; Adams, A.; De Kimpe, N. Chemical modifications of peptides and their impact on food properties. *Chem. Rev.* **2011**, *111* (12), 7876–7903.

(13) Mohan, A.; Udechukwu, M. C.; Rajendran, S. R. C. K.; Udenigwe, C. C. Modification of peptide functionality during enzymatic hydrolysis of whey proteins. *RSC Adv.* **2015**, *5* (118), 97400–97407.

(14) da Silva Malheiros, P.; Daroit, D. J.; Brandelli, A. Food applications of liposomeencapsulated antimicrobial peptides. *Trends Food Sci. Technol.* **2010**, *21* (6), 284–292.

(15) Fathi, M.; Martín, Á.; McClements, D. J. Nanoencapsulation of food ingredients using carbohydrate based delivery systems. *Trends Food Sci. Technol.* **2014**, *39* (1), 18–39.

(16) Allen, T. M.; Cullis, P. R. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* **2013**, *65* (1), 36–48.

(17) Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: classification, preparation, and applications. *Nanoscale Res. Lett.* **2013**, *8* (1), 102.

(18) Li, Z.; Paulson, A. T.; Gill, T. A. Encapsulation of bioactive salmon protein hydrolysates with chitosan-coated liposomes. *J. Funct. Foods* **2015**, *19*, 733–743.

Chapter 2

Literature Review: Encapsulation of food protein hydrolysates and peptides: a review

Aishwarya Mohan[1], Subin R.C.K. Rajendran[1], Quan Sophia He[2], Laurent Bazinet[3], Chibuike C. Udenigwe[1,*]

[1]Food Functionality and Health Research Laboratory, Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada

[2]Department of Engineering, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada

[3]Department of Food Science and Nutrition, and Institute of Nutrition and Functional Foods, Université Laval, Québec, Québec G1V 0A6, Canada

*Corresponding author

E-mail: cudenigwe@dal.ca; Phone: +1 902 843-6625; Fax: +1 902 843-1404

Mohan, A., Rajendran, S. R., He, Q. S., Bazinet, L., & Udenigwe, C. C. (2015b). Encapsulation of food protein hydrolysates and peptides: a review. *RSC Advances*, *5*(97), 79270-79278 – Published by the Royal Society of Chemistry

Abstract

Food protein hydrolysates and peptides are considered a category of promising functional food ingredients. However, commercial application of protein hydrolysates and their constituent peptides can be impeded by their low bioavailability, bitter taste, hygroscopicity and likelihood of interacting with the food matrix. Encapsulation as a delivery mechanism can be used to overcome these challenges for improving bioavailability and organoleptic properties of the peptides. Proteins, polysaccharides and lipids are the three carrier systems that have been utilized in food peptide encapsulation. The protein and polysaccharide systems mainly aim at masking the bitter taste and reducing hygroscopicity of protein hydrolysates, whereas the lipid-based systems are intended for use in enhancing bioavailability and biostability of encapsulated peptides. Spray drying technique is largely used to achieve microencapsulation in both protein and polysaccharide systems while, generally, liposomes are prepared by film hydration technique. However, it is seen that encapsulation efficiency (EE) of peptides using the liposome model is relatively lower since the entropy-driven liposome formation is uncontrolled and spontaneous. Achieving adequate EE through cost effective techniques is indispensable for encapsulation to be applicable to bioactive peptide-based product commercialization. Furthermore, the design of high quality functional foods requires detailed understanding of the release mechanism and kinetics, gastrointestinal stability, bioavailability and physiological bioactivity of the encapsulated peptide products.

Keywords: Encapsulation, Protein hydrolysate, Bioactive peptides, Biostability, Encapsulation efficiency

1.0. Introduction

Encapsulation is the process of packaging solid, liquid or gaseous materials in capsules of different carriers, which release the active compounds (by diffusion, dissolution, pH trigger, degradation, etc.) at various intervals depending on the stability of the capsule.[1] The pharmaceutical industry has extensively used encapsulation technology in drug delivery to achieve precise, controlled, stable and targeted delivery of the drug. The food industry has also embraced the process of encapsulation to overcome certain challenges arising as a result of growing demand for functional ingredients in food.[1] This review is focused on bioactive food protein hydrolysates and peptides, whose incorporation into functional foods can be hindered by several challenges such as bitter taste, hygroscopicity, hydrophobicity, reaction with the food matrix, incompatibility, limited bioavailability, and biostability.[2] Biostability and bioavailability are pivotal for achieving physiological benefits as the peptides need to reach their targets intact in order to exert their bioactivity. Encapsulation has been used in the food industry and for delivery of several bioactive compounds that are sensitive to environmental factors, such as polyphenols, carotenoids and omega-fatty acids.[3] Nevertheless, encapsulation is yet to be applied in the commercial production of bioactive food protein hydrolysates and peptides.

Bioactive peptides are different from other food bioactive compounds such as vitamins or polyphenols in that the chemical species within the protein hydrolysates are highly heterogeneous.[4] Consequently, bioactive peptides may need to be isolated from more complex matrices or fractionated prior to encapsulation. Most studies on bioactive peptides are focused on the discovery of new bioactivity and protein precursors and elucidation of mechanisms with limited attention given to their biostability and bioavailability. Encapsulation can be explored for the delivery of bioactive food peptides; however, it is seen that optimum conditions for encapsulation of other

compounds do not necessarily apply to bioactive peptides. Currently, there is a dearth of literature expounding various aspects of encapsulation in relation to food protein-derived bioactive peptides. Bioactive peptides are primarily encapsulated for the purpose of masking the bitter taste that result from exposure of taste receptors to hydrophobic amino acid residues generated from protein hydrolysis.[5] Another major objective of encapsulation is the reduction of hygroscopicity to ensure textural and storage stability of protein hydrolysates and peptides. Bioavailability and stability of the peptides are rarely investigated as major concerns despite strong evidence indicating that *in vitro* bioactivity are not always replicated in animal models and human subjects. The roles of several factors related to the process of encapsulation of food protein hydrolysates and peptides including the type of carrier system, method used for encapsulation, purity of wall/carrier material, core-to-wall ratio, and encapsulation conditions are still not clearly understood. This review highlights current advances in the process of encapsulation for food protein hydrolysates and peptides including factors that determine encapsulation efficiency (EE), and knowledge gaps that exist in the use of encapsulation for achieving the highest possible potential for food-derived bioactive peptides.

2.0. Need for peptide encapsulation

A primary challenge faced in translating food protein-derived bioactive peptides into commercial products is the susceptibility of peptides to gastrointestinal (GIT) digestion with the risk of losing their structural integrity and function when hydrolysed by GIT proteases and peptidases.[2,6] Bioavailability is used to depict the portion of the bioactive compound that is unchanged, absorbed and that reaches the systemic circulation.[3] Bioactive peptides, when orally administered, are subjected to peptic digestion in the stomach under acidic conditions,[7] followed by several alkaline pancreatic protease digestion in the intestinal phase before being absorbed through the enterocyte

cells. It has been understood that oral ingestion of bioactive peptides will expose them to the action of at least 40 different enzymes before reaching systemic circulation.[7] Several studies have demonstrated that most food protein-derived bioactive peptides containing more than 2-3 amino acid residues do not withstand simulated gastrointestinal enzymatic digestion.[7] However, the bioactivity of some peptides have been retained or even increased following simulated GIT proteolytic activities. Particularly, dairy-derived antihypertensive tripeptides VPP and IPP, already commercially available for consumption through functional foods, are among the very few peptides that are reported to be stable following GIT digestion. Protecting bioactive peptides from physiological modifications is essential in translating *in vitro* activities in animal models and humans. Therefore, encapsulation has become a relevant and important technology for enhancing the utilization of food-derived bioactive peptides for human health promotion.

3.0. Type of carrier systems for peptide encapsulation

The food industry is restricted to the use of carrier matrices that are edible, biodegradable, nontoxic and inexpensive.[3] Although there are separate extensive reviews on lipids,[8] polysaccharides[3] and protein-based[9] carriers for encapsulation of food-derived bioactive compounds, there is a need to discuss the different carriers with particular focus on their use for encapsulating food protein hydrolysates and peptides (Fig. 2.1).

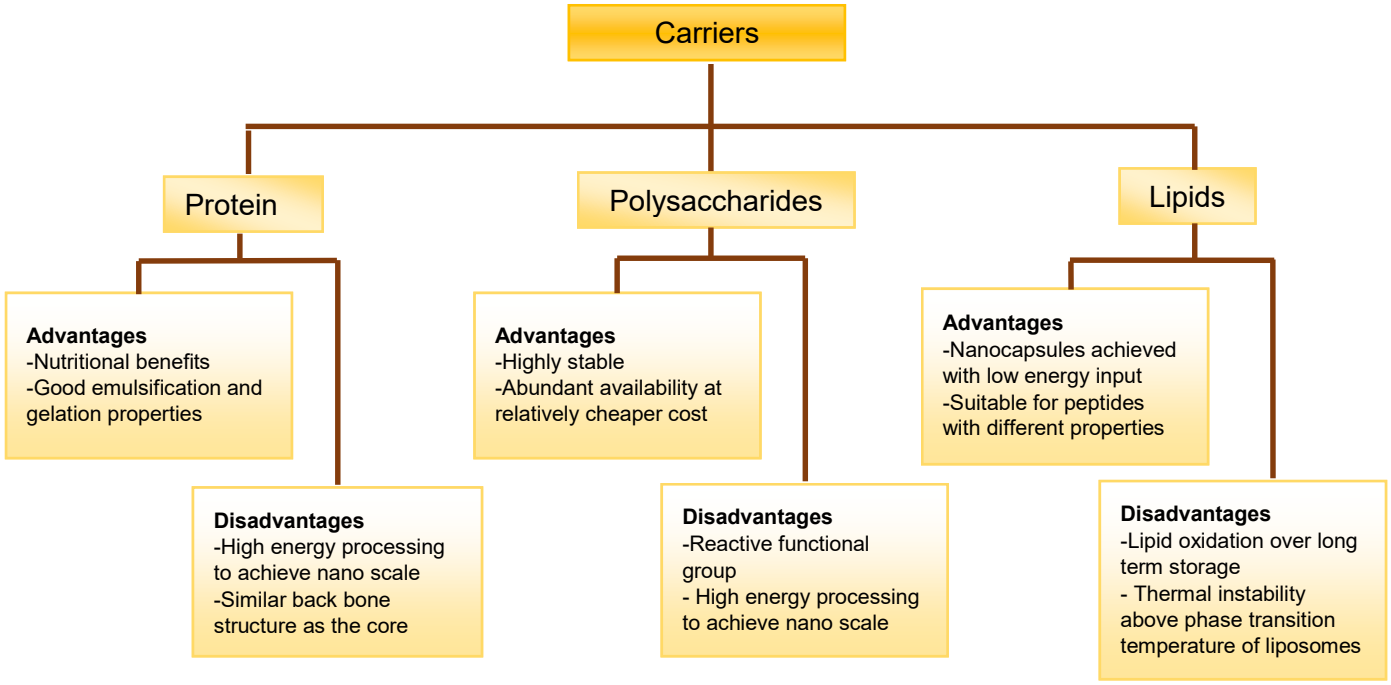


Fig. 2.1. Carriers used for encapsulation of protein hydrolysates and peptides

***3.1. Protein-based carriers*:** Encapsulation using the protein-based matrix is thought to be the most nutritionally beneficial system.[10] Despite the popularity of protein-based carriers for delivering other food bioactive compounds such as flavonoids, vitamins and β-carotene,[9] the use of protein carriers in bioactive peptide encapsulation is limited. Encapsulating bioactive core substance with a chemically similar material is challenging because of structural similarity; that is, the encapsulation shell is predicted to face instability issues similar to the encapsulated bioactive compound.[5] Recently, Wang *et al.* reported the use of native, acylated and high pressure-treated rapeseed protein isolate for the encapsulation of peptides derived from the same material.[11] The inclination towards the use of proteins for delivery of bioactive compounds is due their functional properties such as film and gel forming ability, emulsification and solubility, in addition to their nutritional benefit as sources of essential amino acids. Among the protein sources, soybean has been the predominant choice for bioactive peptide encapsulation (Table 2.1) whereas milk proteins are extensively used in the encapsulation of other non-peptide bioactives.[9] Milk caseins has been used for encapsulation of small hydrophobic compounds due their micellar structure in aqueous enviroment.[12] However, it appears that there is no clear rationale for selection of the protein carrier for food protein hydrolysate and peptide encapsulation. The encapsulation mechanism involving hydrophilic or hydrophobic interactions appear challenging to achieve with protein carriers considering the structural heterogeneity of the encapsulated peptide mixtures. Moreover, recent studies have reported successful encapsulation of dipeptide Phe-Trp and pentapeptide Leu-Trp-Met-Arg-Phe using $CaCl_2$ cross-linked whey protein microbeads of 1-2 mm diameter, resulting in equilibrium constants of 2.3 and 37, respectively for the peptides.[13,14] This demonstrates that the peptides are more distributed in the protein microbeads compared to the aqueous phase, with higher distribution and EE observed for the pentapeptide. Although not extensively used as carriers for

peptide encapsulation, milk proteins are well established as major sources of bioactive peptides. Furthermore, polysaccharides can be combined with the protein carriers to provide structural stability to the encapsulation (Table 2.1). Although a "top-down" approach, involving fragmentation of larger structures, has been proposed to accomplish nanoencapsulation,[9] only microencapsulation has been achieved to date when proteins are used for peptide encapsulation. Protein carriers have been shown to reduce the hygroscopicity of peptides,[10,15] although there are contrasting reports of increased hygroscopicity after encapsulation.[5] This variation could be attributed to physical and structural changes that can occur with the processing of proteins during encapsulation.

Table 2.1. Encapsulation of food protein hydrolysates and peptides using protein, polysaccharide and lipid carriers

	Hydrolysate/peptide	Core to wall ratio (w/w)	EE (%)	Size (μm)	Methodology	Ref
Protein matrix						
SPI	CH	2:8 and 3:7	-	9-11	Pre-dissolved SPI and CH mixed and homogenized followed by spray drying.	[5]
SPI + Pectin	CH	1:1:1; 1:1:2; 1:1:3	78-91	16-24	Coacervation: Aqueous CH was emulsified in soy oil to form w/o emulsion followed by emulsification in SPI at pH 8 to form o/w emulsion. Pectin slowly added to w/o/w emulsion and pH reduced to 4.4 at 40°C. Coarcervated material stored at 7°C and later freeze dried.	[32]
SPI + gelatin	CH	3:7 and 2:7	-	10-17	SPI dispersed in water at pH 8 was mixed with gelatin and then was homogenized with CH followed by spray drying.	[15]
WPC and WPC + sodium alginate	WPC hydrolysate	3:7	-	-	WPC and sodium alginate separately dissolved; WPC hydrolysate added under agitation until dissolved and spray dried; freeze dried or mechanically blended.	[10]
RPI	Rapeseed peptides	1:1, 1:2 and 2:1	63-99	5-16	Pre-dissolved native, acylated or high pressure-treated RPI was adjusted to pH 11.0, followed by the addition of the peptides and spray drying of the mixture.	[11]
WPI	Phe-Trp	0.2[a]	32	-	Peptides (0.2 g/L) were mixed with WPI microbeads (0.2 g) at volume ratios of 0.013-0.2 (bead-to-peptide solution). Mixtures were then stirred for 24 h. Encapsulation efficiency was dependent on volume ratio.	[13]
		0.4[a]	56	-		[14]
	Leu-Trp-Met-Arg-Phe	0.2[a]	89	-		[13]
		0.4[a]	95	-		[14]
Polysaccharide matrix						

	Hydrolysate/peptide	Core to wall ratio (w/w)	EE (%)	Size (μm)	Methodology	Ref
MD + gum arabic	Chicken hydrolysate	10: (1-3)	-	5-20	MD and gum arabic directly added to the hydrolysate and then spray dried.	[18]
MD	CH	1:9	-	13-15	Pre-dissolved MD and CH mixed and homogenized followed by spray drying.	[30]
Carboxymethylated gum + sodium alginate (1:1)	Hydrolysate of *Phaseoulus lunatus*	4:3	53-78	-	Gum and hydrolysate dispersed in water which was dropped into $CaCl_2$ solution to form beads. Beads were allowed to harden for 30 min.	[16]
Gum arabic	CH	1:9; 2:8; 3:7	-	16-20	Aqueous solution of gum and CH prepared followed by spray drying.	[28]
MD + cyclodextrin (1:1)	Whey protein hydrolysate	3:7	-	2.47	MD and CD were separately dispersed in water (pH 7) and mixed together with the hydrolysate, rotary evaporated and spray dried.	[23]
Chitosan	Polypeptide (*Spirulina platensis*)	1:2	49	0.15	Ionotropic gelation: Chitosan dissolved in acetic acid, centrifuged and polypeptide solution added. TPP added and stirred for 60 min and oven dried.	[21]
Liposome matrix						
PC	*Micropogonias furnieri* (fish) hydrolysate	1:5	80	0.263-0.266	Phospholipid (PL) dissolved in organic solvent and evaporated followed by hydration using hydrolysate in buffer. Heating, stirring, vortexing and sonicating in cycles.	[25]
PC	Sea bream scales collagen peptide fraction	1:31	74.6	0.066-0.21 nm	PL dissolved in organic solvent and evaporated followed by hydration with hydrolysate sample dissolved in buffer. Encapsulation by sonication.	[24]
PC + PG + cholesterol	CH	-	56-62	0.5-1.0	PL dissolved in organic solvent and evaporated followed by hydration using sample dissolved in buffer and EDTA. Encapsulation by sonication.	[26]
Lecithin	CH	1:7.5	30-46	0.5-5.0	Similar to Morais et al.[22] Also used sucrose as a cryoprotectant. Encapsulation by agitation and sonication.	[17]
Liposphere matrix						

	Hydrolysate/peptide	Core to wall ratio (w/w)	EE (%)	Size (μm)	Methodology	Ref
Stearic acid + PC	CH	-	66	3.8		[27]
Stearic acid + PC	CH	-	50-83	5.0	CH was added to melted stearic acid followed by the addition of PC pre-dissolved in buffer. Mixture was homogenized to form an emulsion and rapidly cooled to 20°C.	[19]
Stearic acid + PC	CH	-	50-83	5.0		[26]
Stearic acid + cupuacu butter	CH	-	73.9	2-10	Melted lipid phase (80% stearic acid + 20% cupuacu butter) was mixed with 4% polysorbate 80 at 80°C with agitation followed by cooling of the emulsion system to 20°C.	[20]

[a]Represent volume ratios (i.e. V_{bead}/V_{aq}, where V_{bead} is the volume of the protein microbeads and V_{aq} is the volume of the peptide solution
Soy protein isolate, SPI; casein hydrolysate, CH; whey protein concentrate, WPC; rapeseed protein isolate, RPI; whey protein isolate, WPI; maltodextrin, MD; phosphatidyl choline, PC; phosphatidyl glycine, PG

***3.2. Polysaccharide-based carriers*:** Polysaccharides are generally ideal for use as delivery agents because they are structurally stable, abundant in nature and inexpensive. The reactive functional groups of polysaccharides make them one of the best choices as carrier matrix.[3] On the other hand, under extreme conditions, such as high temperature, the polysaccharide wall is susceptible to reacting with the peptide core to form complex products (e.g. Maillard reaction products), which can be potentially toxic and also deplete the bioactive peptides. In order to circumvent this challenge, the reactive functional groups of polysaccharides have been modified by processes such as carboxymethylation to produce relatively inert carriers.[16] The colossal molecular structure of polysaccharides contributes to their stability as carriers during production and processing of encapsulated products. Polysaccharides derived from plants, animals and microbial sources, such as gum arabic, chitosan, cyclodextrin and maltodextrin, have been utilised for food protein and peptide encapsulation (Table 2.1). Although polysaccharides are mostly used in combination with protein carriers, Yokota *et al.* used disaccharides as cryoprotectants in the liposome encapsulation model.[17] In the study, addition of disaccharides was found to reduce the EE and increase the particle size of the products. Furthermore, the amount of polysaccharide carriers used was found to positively correlate with particle size of the encapsulated products.[18]

***3.3. Lipid-based carriers*:** Liposphere and liposome are two lipid-based systems that are currently used for encapsulating food protein hydrolysates and peptides. The former has a fatty acid inner layer and outer layer composed of the hydrophilic part of the fatty acid or phospholipid (PL), whereas the latter is a single or multiple concentric bilayer made of phospholipids constituting a vesicle.[4] Accordingly, lipospheres appear appropriate for encapsulating hydrophobic peptides that can interact with the hydrophobic inner layer of the carrier. A few studies have used lipospheres for the encapsulation of protein hydrolysates with moderate to high EE. For instance,

a combination of stearic acid and phosphatidyl choline (PC) was used to encapsulate casein peptide fractions by the melt process, and this resulted in different (50-83%) EE, even when the samples possess similar surface hydrophobicity.[19] This suggest that the EE of peptides in lipospheres can be affected by other factors. Similar EE (74%) was also reported for CH encapsulation in multi-component lipid carrier (stearic acid/cupuacu butter/polysorbate 80).[20] Peptide encapsulation was found to not affect the thermal behaviour of the capsules[20] and no considerable oxidation was observed during a 60-day storage of the encapsulated products.[19] The latter can be attributed to the predominant composition of saturated stearic acid and absence or small amounts of oxidatively-labile unsaturated fatty acids in the spheres.

Liposome is a more popular encapsulation carrier compared to the liposphere, which would be less preferred for food applications because of its high saturated fatty acid content, and the limited choice of substances that can be incorporated in its highly hydrophobic core. However, liposome is compatible with a wide variety of bioactive peptides. The aqueous core appears suitable for hydrophilic peptides and other compounds, while the interior of the bilayer is compatible with hydrophobic peptides. Moreover, amphiphilic peptides can exist at the interface between the shell and core of the liposome structure, which would interact with the hydrophobic and hydrophilic amino acid residues, respectively.[17] Liposome is similar to cell membranes and is therefore favourable for the delivery of bioactive compounds, which can otherwise be degraded by the digestive physiological environment. PC is the commonly used phospholipid for liposome preparation. The large, commercial-scale production that is possible in the case of lipid carriers is a distinct advantage of liposomes over other carrier systems.[8] Liposomes adapted from the pharmaceutical industry have certain shortcomings in functional food application. Particularly, the thermal instability of liposome encapsulated food peptide products beyond the phase transition

temperature of the phospholipid can limit their incorporation in thermally processed food. Besides, liposome preparation involves the use of cholesterol to increase the stability of the lipid bilayer, which is a health concern for application in functional foods. Yet another drawback of using liposome system in peptide encapsulation is the risk of lipid oxidation during production, processing and storage of the products. Consequently, the presence of lipids (especially unsaturated fatty acids) in the peptide-based functional foods can impact product shelf life and limit the choice of processing and storage conditions. Mild oxidation was reported for liposomes at high temperature and low pH,[21] although this needs to be reassessed when food protein hydrolysates and peptides are loaded in the capsules. Taken together, optimum conditions need to be developed to take advantage of the lipid-based system in food protein hydrolysate and peptide encapsulation considering the health and product quality challenges posed by the use of saturated and unsaturated lipids in lipospheres and liposomes, respectively.

4.0. Criteria for determining the quality of peptide encapsulation

***4.1. Particle size*:** The dispersibility and solubility of the encapsulated peptide product greatly depend on the particle size. Particle size of above 50 μm can significantly affect the solubility, dispersion and hence, the texture and feel of the food.[15] Encapsulation products can be either of micro or nano scale. Nanoencapsulation is advantageous because of its high surface area that can increase the solubility and bioavailability of the product. It is thought that the smaller size of the capsules enhances delivery or release of the active molecules.[8] Among the various carriers, the lipid-based systems are more efficient for preparing nanoencapsulated protein hydrolysate and peptide products compared to the protein or polysaccharide systems. Due to their large molecular structure, most encapsulation involving protein and polysaccharide carriers, or a combination of both, results in the production of microcapsules. The combination of proteins and polysaccharides

in encapsulation generates relatively large capsules, although all peptide encapsulation studies to date have yielded products with particle sizes under the threshold value of 50 μm (Table 2.1). However, Zhang *et al.* produced 150-nm nanoencapsulated spirulina protein hydrolysates using chitosan as carrier.[22] Apart from the type of carrier, the particle size of the encapsulated peptide products also depends on the method used for encapsulation.[9] Yang *et al*., in spite of using maltodextrin and cyclodextrin, were able to produce encapsulated products loaded with whey protein hydrolysates with particle sizes as small as 2.4 μm using the spray drying method.[23] Since spray drying is a destructive method of preparation, it is possible that the smaller particle size resulted from fragmentation of the capsules. Furthermore, the particle size of encapsulated peptides was found to depend on the core-to-wall ratio[18] (see section 5.3), but some studies have reported the absence of a particular

trend.[15,24]

***4.2. Zeta potential*:** Surface charge is one of the properties that convey the stability of encapsulated products. Stability enables the prediction of the behaviour of the encapsulated product in a food matrix. However, encapsulation performed for the purpose of masking the bitter taste of protein hydrolysates and peptides has not been focused on this surface property. Liposome-based encapsulation studies report high net negative zeta potential (surface charge) due to the presence of phospholipids, which have negatively charged hydrophilic heads. A decrease in the magnitude of the zeta potential would decrease the stability of the encapsulated product. Encapsulated protein hydrolysate and peptide products of low magnitude zeta potential have the tendency to aggregate in aqueous environment; a surface charge of ±30 mV is essential to form stable dispersion due to electrostatic repulsion of the particles.[25] Encapsulation of peptides using chitosan yielded a product with a high positive surface charge of +41.5 mV.[22] Although there is limited knowledge on surface

charge dynamics of encapsulated food protein hydrolysates and peptides, Mosquera *et al.* reported that simultaneously increasing the concentration of components of both the core (sea bream scale collagen peptide fraction) and the wall (PC) reduces zeta potential.[24] Most studies with polysaccharide and protein carriers did not report the zeta potential of the encapsulated protein hydrolysates and peptides. This information is particularly useful in evaluating the effects of the processing techniques utilized for these carriers, such as spray drying, on the encapsulated product stability. As discussed in section 5.4, mild processing techniques such as film hydration and ionotropic gelation have so far resulted in stable encapsulated products.[22,24]

***4.3. Encapsulation efficiency*:** EE can be defined as the amount of bioactive compound (peptide) trapped in the core or surface of the carrier compared to the initial amount of the bioactive material. Zavareze *et al.* measured EE of peptides indirectly by removing unencapsulated portion of the protein hydrolysate by centrifuging followed by estimation of peptide concentration using Lowry assay.[25] Membrane ultrafiltration has also been used to separate unencapsulated hydrolysate from the capsules prior to protein quantification.[15] Moreover, Morais *et al.* assessed the encapsulation rate of peptides in liposomes and lipospheres indirectly using second derivative spectrophotometry.[26] EE is an important factor to consider especially in producing commercial bioactive protein hydrolysate and peptide products. Although it was suggested that EE of over 50% increases the risk of leakage,[8] lower EE would lead to inefficient use of the bioactive materials and also imply that higher amount of encapsulated products would be required to attain the peptide quantities needed to exert physiological bioactivities. EE depends on the core-to-wall ratio, the conditions in which encapsulation is carried out, and encapsulation technique or production method utilized.[9] EE of microcapsules of protein hydrolysates and peptides prepared with polysaccharide carriers are occasionally reported (Table 2.1). Moreover, encapsulation using protein and

polysaccharide carriers have resulted in higher EE compared to lipid-based (particularly liposome) peptide encapsulation (Table 2.1), possibly since the former is controlled and involves high energy processes in entrapping or encapsulating the peptides. Liposome formation involves entropy driven, spontaneous and less controlled process. In general, techniques using high shear forces, pressure and high temperature result in higher EE, while mild preparation techniques such as film hydration and ionotropic gelation result in lower EE.

5.0. Factors that can affect encapsulation of peptides

The chemistry of the encapsulated bioactive material fundamentally affects the EE. Although, there is limited comprehensive knowledge about the impact of peptide structure on encapsulation, EE is also thought to partly depend on some other factors as discussed below.

5.1. Peptide charge: Encapsulation of casein-derived peptides using liposomes mostly resulted in low EE (14%), which is attributable to the phosphoserine residues in caseinophosphopeptides (Mohan & Udenigwe, unpublished data). PL and the phosphopeptides are highly negatively charged leading to molecular repulsion and reduced encapsulation. Similarly, liposomal encapsulation of negatively charged intact bovine serum albumin has also been reported to result in low EE of 34%.[27] Higher EE values have been reported for the encapsulation of protein hydrolysates from other sources that lack the phosphoserine residues[24,25], although the surface charge of the core materials was not reported.

5.2. Type and purity of carrier/wall material: Type and purity of carrier material are important factors that determine EE. High EE of 74-80% have been achieved using purified PC to form the liposomal carrier.[24,25] The small difference in EE can be due to the nature and different sources of the protein hydrolysates and peptides used for encapsulation. However, encapsulation of a similar protein hydrolysate with crude soy lecithin resulted in low EE of 46%.[15] Similarly, in

liposphere-based encapsulation studies, EE was no less than 50% and a maximum of 83% EE was obtained using purified PC and stearic acid.[19,26,28] Conversely, comparable EE have been obtained when crude cupuacu butter was used with stearic acid in encapsulating casein peptide fractions.[20] The high EE of liposphere encapsulation can generally be attributed to the affinity of hydrophobic peptides in the core to the hydrophobic stearic acid inner layer (in contact with the core), although this mechanism entails the exclusion of hydrophilic (possibly bioactive) peptides from the capsule. Apart from casein peptides, there is a dearth of information on encapsulation of other protein hydrolysates and peptides using lipospheres making it challenging to draw conclusions on the prospects of the carrier system. Hydrophobic interaction has also been reported to increase EE for peptide encapsulation using microbeads prepared from whey protein isolate as the carrier; the study found a linear relationship between hydrophobic column capacity factor (k, depicting molecular hydrophobicity) and equilibrium constant (K).[13] However, the study did not consider the role of peptide charge, which can also affect EE. Furthermore, acylation (by 47%) and high pressure treatment (200-400 MPa) of rapeseed protein isolate carrier resulted in high amount of secondary structure (α-helix and β-sheet) and increased Young's modulus, which led to higher EE compared to the native and 5% hydrolyzed protein carrier.[11]

The advantage of using purified carrier materials is the reduction in the amount of materials needed to achieve high EE. Most polysaccharide-based encapsulation uses purified or synthetic carrier materials. Despite the consistently high EE, the use of high-purity wall materials in protein hydrolysate and peptide encapsulation does not appear to be economically feasible for the functional food industry,[9] except perhaps for the protein carriers. Obtaining or purifying the wall material would add significant step to the production process and can increase the input and product

unit costs. There is a need for further research to identify and adapt processes that will lead to adequate EE for bioactive peptides without requiring high-cost input.

5.3. Core-to-wall ratio: Typically, encapsulation involves the use of large amounts of wall materials than the active core compounds. EE is largely influenced by the core-to-wall ratio and is found to always decrease with increase in the core concentration,[22,24] which can be due to overloading of the encapsulation system. Increase in the concentration of the wall material initially leads to increase in the EE until a certain point. For instance, Mosquera *et al.* reported a maximum EE of 74.6% at 1:31.5 (w/w) core-to-wall ratio (PC and sea bream scale protein hydrolysate);[24] the EE was found to decrease to 67% when the ratio was slightly changed to 1:38.5 (w/w). Interestingly, Zavareze *et al.* achieved 80% EE of fish protein hydrolysate using a much lower core-to-wall (PC) ratio of 1:5 (w/w),[25] which suggests possible dependence of EE on the nature and molecular composition of the encapsulated material. However, Subtil *et al.* found that increase or decrease of the amounts of the wall (gum arabic) or core materials (casein hydrolysate) did not affect other characteristics such as the capsule morphology.[29] In contrast, a few studies involving protein and polysaccharide carriers have reported that varying the core-to-wall ratio increases the mean particle size and alters the morphology of the encapsulated protein hydrolysate and peptide products.[11,18] However, there has been no observable link between core-to-wall ratio and particle size for liposome-based encapsulated protein hydrolysates and peptides.[24] The lack of relationship between liposomal size and concentration of the core or wall can be partly attributed to difference in the process used in encapsulation. Furthermore, core-to-wall ratio increase from 1:2 to 2:1 was found increase the mean particle size and decrease the spray dry yield of peptide products encapsulated with rapeseed proteins.[11] Volume ratio of the core and wall material is also important in determining EE. A recent study reported that high EE (up to 95%) can be achieved by increasing

the volume ratio of whey protein microbeads and peptides in aqueous solution (V_{bead}/V_{aq}) from 0.013 to 0.2.[13] To date, commonly used core-to-wall ratios are 3:7 and 2:8 for protein and polysaccharide carriers and 1:(5-10) for liposome carriers (Table 2.1).

***5.4. Techniques used for encapsulation*:** Currently, several techniques have been proposed for use in encapsulation of bioactive compounds such as coacervation, spray cooling, extrusion, supercritical fluid extraction, cocrystallization and inclusion.[9] Techniques involved in the nanoencapsulation of food ingredients utilizing lipid, protein and polysaccharide-based carrier systems have been extensively reviewed.[3,8,9] This review focuses only on the techniques currently applied and are relevant for encapsulation of food protein hydrolysates and peptides.

5.4.1. Film hydration: Liposome-based encapsulation of food protein hydrolysates and peptides mostly employs the film hydration technique. This option is popular due to the simple, yet effective mechanism where phospholipids self-assemble in response to energy input in the form of heat, agitation and sonication thereby trapping the aqueous core containing the peptides. The bilayer formation in liposome does not require the use of any sophisticated equipment except for the application of energy to drive the self-assembly. The disadvantage of liposome formation is that the uncontrolled assembly mechanism can lead to poor reproducibility and varying EE (Table 2.1). Moreover, organic solvents used in the liposomal encapsulation process need to be removed prior to use of the encapsulated products in functional foods, which introduces additional steps that can affect EE and the quality of the encapsulated products.

5.4.2. Spray drying: Both the protein and polysaccharide-based encapsulation frequently employs spray drying to achieve encapsulation due to the relatively low processing cost and ease of the technique.[15] This technique involves forming droplets and spraying at high temperature resulting in dried particles.[18] Unlike the liposome system, the spray drying process achieves

entrapment of bioactives in the protein and polysaccharide carriers rather than having a distinct wall and core.[15] Spray drying has been found to result in microspheres with the active material uniformly distributed in the carrier, which typically occurs when the carrier and core materials are similarly hydrophilic.[15] This phenomenon is expected to lead to high EE although no study has yet reported the EE of spray dried encapsulated protein hydrolysate and peptide microcapsules (Table 2.1). Moreover, concavities on the microspheres produced through spray drying are commonly observed due to the rapid evaporation of the solvent.[5,15,30] However, the high temperature used during spray drying can lead to denaturation of protein carriers[5] and possibly alter peptide structure due to their reactivity. For instance, non-enzymatic browning can occur if considerable amount of reducing sugar is present in the system. Spray drying technique can be considered more appropriate for micro- rather than nanoencapsulation. Rocha *et al.* adapted spray drying for encapsulation of peptides in protein hydrolysates for functional food application, and also reported to have successfully incorporated the encapsulated product in protein bars.[30]

5.4.3. Coacervation: This technique is considered effective for encapsulation since it is based on electrostatic attraction between the core and wall materials. The technique involves phase separation and deposition of coacervate phase on the core.[31] Unlike other encapsulation methods, coacervation has been used to achieve EE of up to 91.6% using similar amounts of core (soy protein and pectin) and wall materials (casein hydrolysate).[32] The affinity between the core and wall due to surface properties contributed to the resulting high EE. One caveat with achieving such high affinity between the core and wall is that the peptides can be tightly bound to the extent that it becomes difficult to release them when needed. The wall material should have compatible (opposite) charge with the core to be able to coacervate. For instance, anionic polysaccharides such as gum arabic or alginate can be used to coacervate cationic peptides, and vice versa. Another

technique used for peptide encapsulation include ionotropic gelation,[22] which is also a mild preparation method based on electrostatic interactions of the encapsulation materials.

5.5. Production condition: The peptide net charge is dependent on the pH of the medium during encapsulation, and this can influence the EE due to electrostatic effects. Encapsulation with both protein and polysaccharide-based carriers have been found to occur favourably at alkaline pH 8.[5,15,32] Moreover, Ruiz *et al.* reported that maximum EE was observed at pH 10 with dilute salt ($CaCl_2$) solution while the least EE was observed at neutral pH and high salt concentration.[16] Conversely, liposome formation has been found to result in higher EE when conducted at neutral pH.[17,24-26] Taken together, the size of the encapsulated product is determined by production parameters and inherent properties of the wall and core materials such as energy input per unit mass, surface tension and density.

6.0. Release and gastric stability of encapsulated peptides

High affinity of the core and wall materials is paramount to the formation of stable encapsulated peptide products that can withstand food processing and storage conditions with limited diffusion losses of the core materials. Contrary to EE, a recent study demonstrated that the release kinetics of peptides encapsulated in protein microbeads in aqueous environment was inversely proportional to the peptide hydrophobicity with average release rate constants of 0.1 and 0.014 min^{-1} for Phe-Trp and Leu-Trp-Met-Arg-Phe, respectively, after 1 h.[14] Conversely, the modification of rapeseed protein by acylation and high pressure treatment that resulted in higher EE were found to increase the % release of the encapsulated peptide compared to the native protein after 24 h using the dynamic dialysis method.[11] This indicates weaker interaction of the peptides with the modified protein carrier. Although theoretically promising, the dearth of experimental information on the biostability of encapsulated protein hydrolysate and peptides makes it difficult to evaluate the

prospects of encapsulation in oral delivery of bioactive peptides. A myriad of bioactive peptides derived from various food proteins have been reported and it is becoming increasingly apparent that the focus needs to be shifted to the translation of the peptides into commercial functional food products. Studies focused on characterizing the digestion and release of encapsulated peptides during gastrointestinal processing are crucial in understanding the effect of encapsulation on biostability. One study evaluated the biostability of bioactive peptides encapsulated with a carboxymethylated gum and sodium alginate, and found minimal (up to 10%) and maximal (up to 60%) release of protein materials after simulated gastric and intestinal digestion phases, respectively.[16] The released peptides at the intestinal phase can then be presented for absorption into the enterocytes and subsequently into circulation where they are still susceptible to further peptidolytic modification. Therefore, it is imperative to assess the digestion kinetics and biostability of encapsulated peptides, and their bioavailability in different physiological sites to ensure the release of the intact bioactives at appropriate time and target location.

7.0. Challenges and future prospects of peptide encapsulation

The heterogeneity of protein hydrolysates containing diverse range of peptides with different net charge, hydrophobicity, molecular weight and surface properties makes it challenging to achieve high and uniform EE. Enhancing the EE is particularly important to avoid the use of large quantities of the encapsulated protein hydrolysates and peptides in attaining the desired amount of the actual active material.[30] Purifying the peptides from protein hydrolysates can improve the condition; however, it requires high-end processing techniques that can be uneconomical for small and medium-sized food industry.[9] However, some techniques are showing promise for use in purifying peptides or concentrating bioactive fractions at a large scale and low cost.[33,34] Some

promising techniques currently used for the delivery of drug and other bioactive compounds have the potential to be extended to food protein hydrolysates and peptides. For instance, proliposomes, which are used for drug delivery, can be used to overcome the quality issues associated with liposomes such as oxidation, aggregation and phospholipid hydrolysis.[35]

Future studies should focus on detailed and balanced evaluation of encapsulated peptides derived from all the carrier types for biostability, organoleptic properties and bioavailability. The applicable techniques would have to achieve practical EE without requiring expensive processing steps and carrier materials. Forthcoming research should also be focused on understanding the effect of encapsulation on the functionality and stability of encapsulated peptide products, digestion kinetics, release rate, and compatibility with the food and physiological matrices. It is noteworthy that spray dried microspheres have been reported to be highly resistant to mechanical fractures.[5] Although, peptide release from electrostatically-driven encapsulation (film hydration, coacervation) appear relatively easier to achieve, it is imperative to characterize the underlying mechanism and release profile of encapsulated peptide products to facilitate their use in product development. Furthermore, *in vivo* studies using animal models and human subjects are needed to confirm the effectiveness of encapsulation in enhancing bioavailability and in retention of bioactivity after oral consumption of the products as food.

8.0. Conclusion

Encapsulation of bioactive food compounds is well-positioned to facilitate the design of better and efficient functional foods. This is essential in advancing the research on bioactive food protein hydrolysates and peptides and to develop the market of the peptides as natural health products and nutraceuticals. To achieve high EE, the choice of the carrier material used is dependent on the encapsulation and processing techniques, environment and chemistry of the peptides, although

more work is needed to delineate the impact of the latter on EE. Apart from high EE, knowledge of digestion and release kinetics, and the morphology of encapsulated peptide products is paramount to obtaining applicable functional materials for food formulation.

Acknowledgement. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC). A.M. and S.R.C.K.R. are recipients of the Nova Scotia Research and Innovation Graduate Scholarships.

References

1. C. P. Champagne and P. Fustier. *Curr. Opin. Biotechnol.,* 2007, 18, 184.
2. C. C. Udenigwe. *Trends Food Sci. Tech*. 2014, 36, 137.
3. M. Fathi, Á. Martin and D. J. McClements. *Trends Food Sci. Tech.,* 2014, 39, 18.
4. D. J. McClements. Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds. CRC Press, 2014, 572 pages
5. S. E. M. Ortiz, A. Mauri, E. S. Monterrey-Quintero, M. A. Trindade, A. S. Santana and C. S. Favaro-Trindade, *LWT Food Sci. Technol*. 2009,42, 919.
6. G. Picariello, G. Iacomino, G. Mamone, P. Ferranti, O. Fierro, C. Gianfrani. A. Di Luccia, and F. Addeo. *Food Chem*., 2013, 139, 203.
7. M. Segura-Campos, L. Chel-Guerrero, D. A. Betancur-Ancona, and V. M. Hernandez-Escalante. *Food Rev. Int*., 2011, 27, 213.
8. M. Fathi, M. R. Mozafari and M. Mohebbi. *Trends Food Sci Tech.,* 2012, 23, 13.
9. G. M. Tavares, T. Croguennec, A. F. Carvalho and S. Bouhallab. *Trends Food Sci. Tech*., 2014, 37, 5.
10. J. J. Ma, X. Y. Mao, Q. Wang, S. Yang, D. Zhang, S. W. Chen and Y. H. Li. *LWT. Food*

Sci. Technol., 2014, 56, 296.

11. Z. Wang, X. Ju, R. He, J. Yuan and L.Wang. *Food BioprocessTechnol*., 2015, 8, 1305.
12. M. J. Sáiz-Abajo, C. Monzales-Ferrero, A. Moreno-Ruiz, A. Romo- Huald, and C. J. Gonzalez-Navarro. *Food Chem*., 2013, 138, 1581.
13. G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan. *Food Chem.,* 2014, 160, 46.
14. G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan. *Food Chem.,* 2015, 180, 150.
15. C. S. Favaro-Trindade, A. S. Santana, E. S. Monterrey-Quintero, M. A. Trindade and F. M. Netto. *Food Hydrocolloids*, 2010, 24, 336.
16. J. C. Ruiz Ruiz, M. R. Segura Campos, D. A. Betancur-Ancona, and L. A. Chel-Guerro *ISRN Biotech*., 2013.
17. D. Yokota, M. Moraes and S. C. Pinho. *Braz*. *J. Chem*. *Eng*., 2012, 29, 325.
18. L. E. Kurosawa, K. J. Park and M. D. Hubinger. *J. Food Eng.,* 2009, 94, 326.
19. C. Barbosa. H. A. Morais, F. M. Delvivo, H. S. Mansur, M. C. De Oliveira and M. P. Silvestre. *J. Sci. Food. Agri.,* 2004, 84, 1891.
20. J. C. Silva and S.C. Pinho. *Int. J. Food Stud.,* 2013, 2, 48.
21. J.A.A. Zhang and J. Pawelchak. *Eur*. *J. Pharm*. *Biopharm*., 2000, 50, 357.
22. B. Zhang and X. Zhang. *Biotechnol. Prog.,* 2014, 29, 1230.
23. S. Yang, X. Y. Mao, F. F. Li, D. Zhang, X. J. Leng, F. Z. Ren and G. X. Teng. *Eur*. *Food Res*. *Technol*. 2012, 235, 91.
24. M. Mosquera, B. Giménez, I. M. da Silva, J. F. Boelter, P. Montero, M. C. Gómez-Guillén and A. Brandelli. *Food Chem*., 2014, 156, 144.

25. E. Da Rosa Zavareze, A. C. Telles, S. L. Mello El Halal, M. Da Rocha, R. Colussi, L. M. De Assis, L. A. S. De Castro, A. R. G. Dias and C. Prentice-Hernandez. *LWT Food Sci. Technol.,* 2014, 59, 841.

26. H. A. Morais, C. M. Da Silva Barbosa, F. M. Delvivo, H. S. Mansur, M. C. De Oliviera and M. P. C. Silvestre. *J. Food Biochem*., 2004, 28, 21.

27. W. Liu, A. Ye, C. Liu, J. Han and H. Singh. *Food Chem*., 2015, 175, 16.

28. C. M. D. S. Barbosa, H. A. Morais, D. C. F. Lopes, H. S. Mansur, M. C. D. Oliveira and M. P. C Silvestre. *Braz. J. Pharm. Sci.,* 2002, 38, 361.

29. S. F. Subtil, G. A. Rocha-Selmi, M. Thomazini, M. A. Trindade, F. M. Netto and C. S. Favaro-Trindade. *J. Food Sci. Technol*., 2014, 51, 2014.

30. G. A. Rocha., M. A. Trindade, F. M. Netto and C. S. Favaro-Trindade. *Food Sci. Technol. Int*., 2009, 15, 407

31. S. Gouin. *Trends Food Sci. Tech.,* 2004, 15, 330.

32. D. V. Mendanha, S. E. M. Ortiz, C. S. Favaro-Trindade, A. Mauri, E. Monterrey-Quintero and M. Thomazini. *Food. Res. Int.,* 2009, 42, 1099.

33. L. Bazinet, and L. Firdaous. *Recent Patents on Biotechnology*, 2013, *7*, 9.

34. A. Doyen, C. C. Udenigwe, P. L. Mitchell, A. Marette, R.E. Aluko, and L. Bazinet. *Food Chem.*, 2014, 145, 66.

35. B. Zheng, L. Teng, G. Xing, Y. Bi, S. Yang, F. Hao, G. Yan, X. Wang, R. J. Lee, L.Teng and J. Xie. *Eur. J. Pharm. Sci.,* 2015, 77, 254.

Chapter 3

Encapsulation of bioactive whey peptides in soy lecithin-derived nanoliposomes: Influence of peptide molecular weight

Aishwarya Mohan[a], David Julian McClements[b], Chibuike C. Udenigwe[a,*]

[a]*Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada* [b]*Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA*

*Corresponding author cudenigwe@dal.ca, Phone +1 (902) 893-6625,

Fax +1 (902) 893-1404

Running title: Effect of peptide molecular weight on their encapsulation in liposomes

Submitted to *Food Chemistry* on February 16, 2016

Abstract

Encapsulation of peptides can be used to enhance their stability, delivery, and bioavailability. This study focused on the effect of the molecular weight range of whey peptides on their encapsulation within soy lecithin-derived nanoliposomes. Peptide molecular weight did not have a major impact on encapsulation efficiency or liposome size. However, it influenced peptide distribution amongst the surface, core, and bilayer regions of the liposomes as determined by electrical charge (ζ-potential) and FTIR analysis. The liposome ζ-potential depended on peptide molecular weight, suggesting that the peptide charged groups were in different locations relative to the liposome surfaces. FTIR analysis indicated that the least hydrophobic peptide fractions interacted more strongly with choline on the liposome surfaces. The results suggested that the peptides were unequally distributed within the liposomes, even at the same encapsulation efficiency. These findings are important for designing delivery systems for commercial production of encapsulated peptides with improved functional attributes.

Keywords: Bioactive peptides; Nanoliposomes; Molecular weight; Encapsulation; Stability; Bioavailability

1. Introduction

Bioactive peptides derived from food proteins are strong candidates for functional food production due to their health-promoting properties, such as antioxidative, antihypertensive, immunomodulating, hypolipidaemic, and anticancer activities (Udenigwe & Aluko, 2012). However, one of the major challenges in translating bioactive peptides into commercial products is their relatively high chemical reactivity, which can negatively impact their stability during processing and under physiological conditions (Mohan, Udechukwu, Rajendran, & Udenigwe, 2015a; Udenigwe, 2014). As a result, the biological activity of the peptides is reduced before they can reach their intended target within the human body (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Encapsulation of active ingredients within colloidal particles is a powerful means of protecting them from degradation and for enhancing their bioactivity (McClements, 2014). Numerous different types of colloidal delivery systems have been developed, including microemulsions, nanoemulsions, emulsions, solid lipid nanoparticles, liposomes, biopolymer nanoparticles, and hydrogel beads, each with its own advantages and limitations (McClements, 2015a, 2015b). Liposomes are particularly suitable for encapsulation of peptides because they contain polar, non-polar, and amphiphilic regions within the same colloidal particle (Allan & Cullis, 2013). The pharmaceutical industry routinely uses liposome-based delivery systems to encapsulate, protect, and deliver bioactive peptides for utilization as drugs to prevent or treat diseases (Malam, Loizidou, & Seifalian, 2009; Pisal, Kosloski, & Balu-Iyer, 2010). On the other hand, there are far fewer examples of liposomes being successfully utilized to encapsulate bioactive peptides in the food industry. A number of studies have shown that antimicrobial peptides can be encapsulated within liposomes, which can improve their chemical stability and antimicrobial activity (Carmona-Ribeiro & Carrasco, 2014;

Malheiros, Daroit, & Brandelli, 2010; Malheiros, Daroit, da Silveira, & Brandelli, 2010). In addition, it has been shown that encapsulation of ghrelin (a bioactive peptide) into liposomes improved its chemical stability, and increased the length of time that this appetite suppressing hormone remains in the blood (Moeller, Holst, Nielsen, Pedersen, & Ostergaard, 2010). Liposomes have also been used to encapsulate bioactive peptides derived from hydrolysis of fish proteins (Zavareze, et al., 2014). Liposome encapsulation has also been shown to be an appropriate method of reducing the bitterness associated with many bioactive peptides (Mohan, Rajendran, He, Bazinet, & Udenigwe, 2015b).

Peptide bioactivity is strongly influenced by their interactions with the surrounding solvent and with specific target tissues, which will depend on the molecular structure and physiochemical properties of the peptides used. Evidence suggests that smaller peptides possess higher bioavailability and bioactivity than larger ones (Udenigwe & Aluko, 2012). However, there is currently limited knowledge of how the molecular weight of peptides impacts their ability to be encapsulated by liposomes. One would expect that the molecular dimensions of peptides would influence their incorporation and location within the liposome structure, and therefore impact their retention, protection, and release characteristics. In addition, the size of the peptide molecules may impact the aggregation stability of liposomes, which would affect their ability to be utilized as delivery systems in many food products (McClements, 2014). The objective of this study was therefore to evaluate the impact of the molecular weight of whey peptide fractions on the encapsulation efficiency and physicochemical properties of liposomes. We hypothesize that variations in peptide-solvent interactions, surface hydrophobicity, and free amino content (number of peptides) for peptide fractions with different molecular weights will impact their inclusion and location in the liposomes.

2. Materials & Methods

2.1. Whey protein hydrolysis

Whey protein isolate (WPI) was obtained from Bulk Barn Foods Ltd. (Truro, NS, Canada). A 5% (w/v) WPI suspension was hydrolyzed using papain from papaya latex at an enzyme-to-substrate ratio of 1:100 (w/w). The mixture was maintained for 5 h at the enzyme's optimum conditions (65°C and pH 7.0) to allow hydrolysis to occur. Hydrolysis was then terminated by heating the mixture at 95°C for 15 min to inactivate the enzyme. The hydrolysate was cooled to room temperature, centrifuged at 5,000×g for 10 min, and then the supernatant was lyophilized to obtain the whey protein hydrolysate (WPH) powder.

2.2. Peptide fractionation by membrane ultrafiltration

WPH powder was reconstituted in 10 mM sodium phosphate buffer (pH 7.0) and passed through an ultrafiltration membrane with a molecular weight cut-off (MWCO) of 1 kDa using an Amicon Stirred Cell (EMD Millipore Corporation. Darmstadt, Germany) under constant nitrogen passage of 40 psi. After 4 h, the resulting permeate (<1 kDa peptide fraction) was collected and the retentate (>1 kDa peptides) was passed through a 3-kDa MWCO membrane, under similar conditions, to recover the 1-3 kDa peptide fraction in the permeate. The same procedure was followed with the 5 and 10 kDa MWCO membrane to obtain the 3-5, 5-10, and >10 kDa peptide fractions. The peptide fractions were freeze dried and then stored at -20 °C until further experiments.

2.3. Free amino group determination

The amount of free amino groups in the whey peptide fractions was determined using the O-phthaldehyde method as reported by Udenigwe, Wu, Drummond, and Gong (2014). Briefly,

33 µL of 1 mg/mL peptide sample was mixed with 250 µL of the O-phthaldehyde reagent in a 96-well plate and the absorbance of the mixture was then measured at 340 nm. Serine was used as standard and the amount of free amino groups was expressed as milliequivalent serine NH_2/g.

2.4. Surface hydrophobicity determination

Surface hydrophobicity of the whey peptide fractions was determined using a fluorescent hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid. The fluorescence of the aqueous solutions containing 0.0009–0.015% peptides was measured at an excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the fluorescence versus concentration plot was taken to be the surface hydrophobicity of the peptides.

2.5. Encapsulation of the whey peptide fractions

Liposome encapsulation of the different whey peptide fractions was conducted using the film hydration method (Mosquera et al., 2014). Soy lecithin (Bulk Barn Foods Ltd., Truro, NS, Canada) was ground to a fine powder and then dissolved (1% w/v) in chloroform. This was followed by rotary evaporation at 60°C, to remove the organic solvent, until a thin lipid layer was formed in the round bottomed flask. This flask was placed in a desiccator overnight for complete removal of chloroform. The thin lipid layer was hydrated with 10 mM phosphate buffer (pH 7.0) containing the whey peptide fractions (1 mg/mL), under agitation, followed by sonication for 30 min. Empty liposomes were prepared as control by hydrating the lipid film using phosphate buffer without the peptides.

2.6. Encapsulation efficiency determination

The encapsulation efficiency (EE) was defined as the ratio of encapsulated peptides (P_E) to total peptides (P_I) expressed as a percentage: $EE = 100 \square P_E/P_T$. The amount of encapsulated

peptides was indirectly determined by measuring the amount of non-encapsulated peptides (P_{NE}): $P_E = P_T - P_{NE}$. The value of P_{NE} was determined by passing the prepared samples through a 100kDa ultrafiltration membrane under 10 psi pressure to separate the peptides encapsulated within the liposomes from the non-encapsulated peptides in the surrounding aqueous phase. The protein content of the filtrate was measured by the Lowry method using a Bio-Rad Protein Assay kit (Richmond, CA, USA).

2.7. Particle characteristics

The mean particle diameter, polydispersity index, and ζ-potential of the empty and peptide-loaded liposomes were determined using a combined dynamic light scattering/particle electrophoresis instrument (Zetasizer Nano Series Nano-ZS, Malvern Instruments Ltd., Malvern, UK). The liposomes were suspended in deionized water and measurements were taken after placing the sample suspension in disposable capillary cells.

2.8. Fourier transform infrared spectroscopy (FTIR)

Attenuated Total Reflectance (ATR) FTIR spectra of the lyophilized samples were obtained as an average of 16 scans in the region of 4000-600 cm^{-1} using an FTIR spectrometer (Spectrum One, Perkin Elmer, CT, USA). Qualitative differences in the spectral peaks of the empty and peptide-loaded liposomes were determined.

2.9. Transmission electron microscopy (TEM)

The empty and peptide-loaded liposomes were loaded on a carbon film-coated 200 mesh copper grid (EMS, PA, USA) and allowed to stand for 10 min followed by the removal of excess sample with filter paper. Sample-loaded grids were stained using 2% uranyl acetate and allowed to stand for 2 min. Excess stain was then washed off by immersing the grid in deionized water.

Finally, the grids were allowed to dry for 1 h and then visualized with a Model Tecnai-12 microscope (Philips Electron Optics, Netherlands).

2.10. Statistical Analysis

Experiments, except protein hydrolysis and fractionation, were carried out in triplicate. Results are expressed as the mean ± standard deviation of these measurements. Tests for significant differences between samples were conducted by one-way analysis of variance followed by a Holm-Sidak multiple comparison test (Sigmaplot 12.1, Systat Software, San Jose, CA, USA). Correlation between the peptide properties, encapsulation efficiency, and liposome properties was analyzed by Spearman's rank order correlation (r_s).

3. Results and Discussion

3.1. Effect of whey peptide molecular weight on encapsulation efficiency

The encapsulation efficiency is a measure of the amount of peptides loaded into the nanoliposomes. Typically, a high EE is desirable because it can increase the amount of bioactive delivered and reduce costs, although overloading can lead to leakage of the bioactive materials, which is undesirable (Mohan et al., 2015b). Heterogeneity of peptides makes it challenging to predict their behavior during encapsulation (McClements, 2014; Mohan et al., 2015b). In this study, an EE of over 90% was achieved for whey peptide fractions with different molecular weight ranges (Fig. 3.1). Peptide encapsulation in liposomes has been demonstrated to result in lower EE when compared to encapsulation in protein and polysaccharide matrices (Mohan et al., 2015b). Notably, our findings represent the highest EE reported for food-derived peptides in liposome carriers. This is contrary to the lower EE of 40-60% reported for casein peptides in liposomes (Yokota, Moraes, & Pinho, 2012; Morais, Da Silva Barbosa, Delvivo, Mansur,

Cristina De Oliveira, & Silvestre, 2004), which was attributed to limited peptide-carrier interaction due to the repulsion of the negatively charged casein phosphopeptides and the anionic head groups of the phospholipids in the liposomes (Mohan et al., 2015b). Similarly, lower EE has been reported for negatively charged bovine serum albumin (Liu, Ye, Liu, Liu, Han, & Singh, 2015), but encapsulation of marine protein-derived peptides in liposomes has resulted in a relatively higher EE of up to 80% (da Rosa Zavereze et al., 2014, Mosquera et al., 2014). Based on these findings, it is apparent that the molecular properties of peptides impact their encapsulation in liposomes.

The molecular weight range of the whey peptide fractions did not have significant ($p>0.05$) impact on their EE (Fig. 3.1). This was unexpected since the whey peptide fractions contained different numbers, sizes, and hydrophobicities of peptides. For example, the number of peptides per unit mass increased as the average molecular weight of the fractions decreased (Fig. 3.2A), and the >10 kDa peptides had 10-times higher surface hydrophobicity (S_o) than the other fractions (Fig. 3.2B). The structural and compositional variations were expected to impact peptide inclusion in the liposomes and their resulting EE. The <1 kDa fraction appeared to have slightly higher EE followed by the 5-10 kDa fraction, while the 3-5 kDa had relatively lower EE. Although not significant, this trend correlated with S_o (r_s=-0.6) suggesting that the surface hydrophobicity of the peptides contributes to the effectiveness of their encapsulation. There was a weak relationship between free amino groups and EE (r_s =0.2), which suggests that inclusion in the liposomes was not affected by the number or size of the peptides in the different fractions. Mosquera et al. (2014) reported a decrease in EE with increasing concentration of a collagen peptide fraction in phosphatidyl choline-derived nanoliposomes. The structural heterogeneity of the whey peptides within each fraction limits our understanding of their specific interactions with

the phospholipid system and the resulting influence on peptide entrapment within the liposomes. However, subtle changes, beyond EE, can occur due to the different molecular weights of the whey peptides resulting in differences in liposome structure and properties.

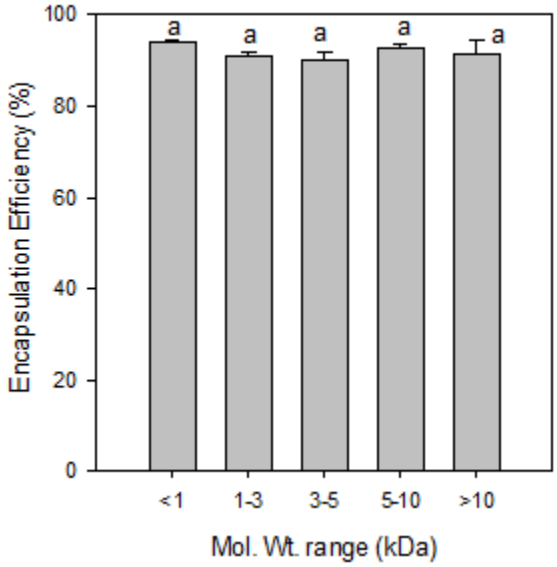


Fig. 3.1. Encapsulation efficiency of the different molecular weight whey peptide fractions in soy lecithin-derived nanoliposomes; same letter on the bars indicate that differences in the mean values were not statistically significant ($p>0.05$).

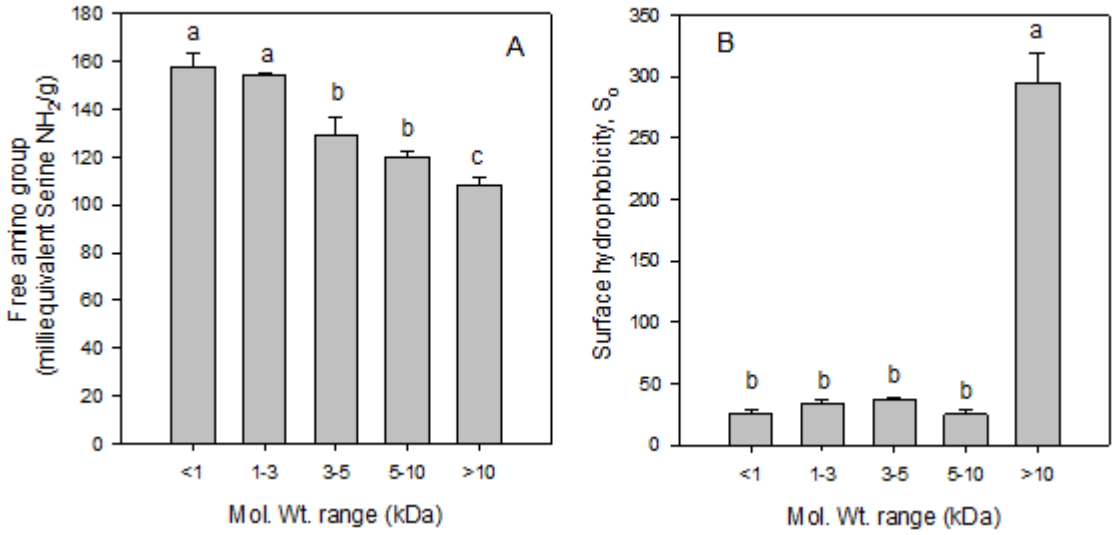


Fig. 3.2. (A) Free amino group, and (B) surface hydrophobicity of the different molecular weight whey peptide fractions; bars with different letters in each chart indicate that differences in the mean values were statistically significant ($p<0.05$).

3.2. Effect of whey peptide molecular weight range on the liposome properties

3.2.1. Mean particle size

Nanoliposomes with mean particle diameters less than 200 nm were produced from all whey peptide fractions, regardless of their molecular weight ranges, which indicates that the liposomes formed can be classified as medium to large unilamellar vesicles (Gómez-Hens & Fernández-Romero, 2005). The diameters of the liposomes produced in this study fall within the range previously reported for food peptide-loaded liposomes (Morais et al., 2004; Mosquera et al., 2014, 2016; Yokota et al., 2012). Interestingly, the mean diameters of the peptide-loaded liposomes were larger than those of the empty liposomes by about 30 to 90 nm (Table 3.1). Overall, the molecular weight of the peptide fractions did not have a statistically significant effect on the mean particle size of the liposomes. Nevertheless, it did appear that the size of the peptide-loaded liposomes tended to decrease with increasing peptide molecular weight (Table 3.1). This effect may have occurred because the incorporation of peptides into the phospholipid bilayer altered its optimum curvature, which favored different sized liposomes (Israelachvili, 2011). Previous studies have also shown that peptide composition can affect the size of peptideloaded liposomes (da Rosa Zaverze et al., 2014; Mohan et al., 2015b). The fact that there was not a significant difference between the liposome dimensions measured in our study may have been because of the fragile nature of the liposomes formed and their sensitivity to preparation conditions (Mohan et al., 2015b). Furthermore, the mean particle diameter of the liposomes positively correlated (r_s =0.7) with the free amino groups, supporting the fact that larger peptides led to smaller liposome sizes. The stained TEM images of the samples indicated the formation of liposomes in both the absence and presence of peptides (Fig. 3.3); however,

there was no obvious correlation between liposome dimensions and the molecular weight of the peptides used.

There was also no apparent relationship between the surface hydrophobicity of the peptide fractions and the mean particle size of the liposomes (r_s = -0.1), which suggested that differences in peptide hydrophobicity did not lead to large structural reorganization of the liposome structure.

3.2.2. Surface charge

The ζ-potential of nanoliposomes can be used as an indicator of their physical stability in aqueous suspensions (McClements, 2014; Mohan et al., 2015b). High positive or negative surface charges result in strong electrostatic repulsive interactions between liposomes, which prevents them from coming into close proximity and fusing together (da Silva Malheiros, Sant'Anna, Micheletto, da Silveira, & Brandelli, 2011). A surface charge of ±30 mV is typically taken to be sufficient to ensure the formation of a stable aqueous dispersion due to electrostatic stabilization. Previous studies have reported a wide range of ζ-potential values for food peptideloaded liposomes with magnitudes as low as -5.5 mV (da Rosa Zavareze et al., 2014) and -8.3 mV (Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007), and as high as -40.8 mV (Mosquera et al., 2014). This broad range of ζ-potentials can be attributed to differences in the type, composition and purity of the phospholipid material, as well as due to differences in solution conditions (such as pH and ionic strength). The surface charge magnitudes of the nanoliposomes prepared in this study from crude soy lecithin were higher than those of liposomes derived from purified phospholipids, albeit with different peptide compositions. Loading of the whey peptides was found to significantly ($p<0.05$) increase the magnitude of the liposome surface negative charge (Table 3.1). This change in liposome charge suggests that the

peptides were incorporated into the phospholipid bilayers, with some of their charged groups exposed to the surrounding aqueous phase. In addition, the presence of the whey peptides within the liposomes would be expected to increase the electrostatic repulsion between them and thereby increase their aggregation stability. This is contrary to previously reported decreases in surface charge magnitude when peptides were loaded in liposomes (Mosquera et al., 2016; da Silva Malheiros et al., 2011). As shown in Table 3.1, the ζ-potential of liposomes containing the high molecular weight peptides is significantly lower in magnitude compared to those of liposomes loaded with smaller peptides. An increase in the number of peptide molecules in the fractions (free amino groups) resulted in an increase in the magnitude of the anionic charge on the corresponding liposomes (r_s = -0.9). It is possible that there was a larger number of peptides with exposed charged groups incorporated into the liposomes for the lower molecular weight peptides. In addition, there was a strong correlation (r_s = -0.9) between the surface charge and mean particle diameter of the liposomes. This may have been because the smaller peptides became internalized within the phospholipid bilayers and altered their optimum curvature, as well as altering their charge characteristics. Although there was no apparent difference in encapsulation efficiency for different sized peptides, our findings indicate that loading smaller peptides into liposomes can improve their stability in aqueous suspensions by increasing the electrostatic repulsion between them. Cholesterol is often used to increase the stability of liposomes *via* its effect in modulating phospholipid bilayer fluidity (Liu et al., 2015; Mozafari, 2010). The empty and peptide nanoliposomes prepared in our study appear to be stable without the addition of cholesterol.

3.2.3. Polydispersity index (PDI)

As shown in Table 3.1, the PDI of the peptide-loaded liposomes were found to be higher than that of the control liposomes. This indicates that the empty liposomes were more uniformly dispersed than the peptide-loaded liposomes. Moreover, liposomes containing the higher molecular weight peptide fractions had lower PDI values indicating they formed more uniform colloidal dispersions. The liposomes loaded with the whey peptide fractions in this study are less uniformly dispersed than those reported previously in the literature (da Rosa Zaverze et al., 2014; Mosquera et al, 2014; da Silva Malheiros et al., 2011). We observed that the peptide heterogeneity, based on the number of peptides within the fractions, affects the uniformity of the liposome dispersion (r_s=0.7). In particular, an increase in peptide number (decrease in peptide size) resulted in less uniformly dispersed liposomes, and *vice versa*.

Table 3.1. Surface charge, particle size and polydispersity index of the empty liposomes and those loaded with the different molecular weight whey peptide fractions; numbers with different letters in rows indicate statistically significant mean values ($p<0.05$)

	Empty liposome	Mol. wt. range (kDa)				
		<1	**1-3**	**3-5**	**5-10**	**>10**
ζ-Potential (mV)	-55.0 ± 0.6[a]	-72.4 ± 1.7[b]	-69.1 ± 1.6[b,c]	-71.0 ± 0.4[b]	-67.6 ± 3.5[b,c]	-63.9 ± 3.9[c]
Mean particle diameter (nm)[1]	97.2 ± 5.9	178 ± 63	167 ± 50	189 ± 25	133 ± 39	127 ± 21
Polydispersity index	0.163 ± 0.004[a]	0.482 ± 0.074[a,b]	0.465 ± 0.250[a,b]	0.585 ± 0.031[b]	0.321 ± 0.173[a,b]	0.285 ± 0.138[a,b]

[1]The power of the performed statistical test, with $\alpha=0.05$, was below the desired power to observed statistically significant difference.

3.3. Peptide interactions with the liposome matrix

Information about the interaction of the peptides with the liposomes was obtained using spectroscopy. The FTIR absorption spectra of the empty and peptide-loaded liposomes were analyzed to elucidate the distribution of peptides within the liposomes, particularly their interaction with the outer/inner polar surface or hydrophobic bilayer regions of the liposomes. The wavenumbers of peaks representing particular functional groups found in the polar and nonpolar regions of the liposomes are presented in Table 3.2. The $(CH_3)_3N$ stretch of the surface choline group of the liposome polar region at 970 cm^{-1} was not affected when peptide fractions with molecular weights of >10, 1-3 and 3-5 kDa were loaded. This suggests little or no interaction of the peptides with the liposome surfaces. The higher surface hydrophobicities of the >10 kDa peptide fraction resulted in a higher distribution of the peptides in the hydrophobic bilayer region of the liposomes, with less interaction with the choline group. Similarly, the 3-5 and 1-3 kDa fractions were also relatively more hydrophobic than the >1 and 5-10 kDa, although not significantly different, and may have impacted their interaction with the polar head. Conversely, the <1 and 5-10 kDa peptide fractions were found to shift the choline band to lower wavenumbers (Table 3.2). This suggests possible distribution and interaction of the peptides with the inner or outer polar regions of the liposomes. New peaks were also observed in the liposomes loaded with the >1 and 1-3 kDa peptide fractions, which can be the vibration of new interactions. The PO_2 signals were also used to monitor peptide interaction with the polar region of the liposomes. The wavenumber of the PO_2 peak shifted in all the liposomes containing the peptide fractions, indicating some extent of interaction of the peptides with the polar region. Broad phosphate stretch of the quaternary amine group of choline, and the CH_2 and CH_3 stretch of the

hydrophobic fatty acids remained largely unaltered, even with our proposed interaction of the latter with the hydrophobic surface of the >10 kDa peptide fraction.

Table 3.2. Wavenumber of liposome functional groups derived from FTIR spectra of the empty liposomes and those loaded with the different molecular weight whey peptide fractions

	Empty Liposome	Mol. wt. range (kDa)				
		<1	**1-3**	**3-5**	**5-10**	**>10**
$(CH_3)_3N$	970	957 & 947	970 & 964	970	944	971
PO_2	1127	1149	1149	1152	1149	1146
Broad PO_2 stretch	1063	1064	1063	1063	1063	1062
CH_2	2854	2854	2854	2854	2854	2854
CH_3	2924	2924	2924	2925	2924	2926

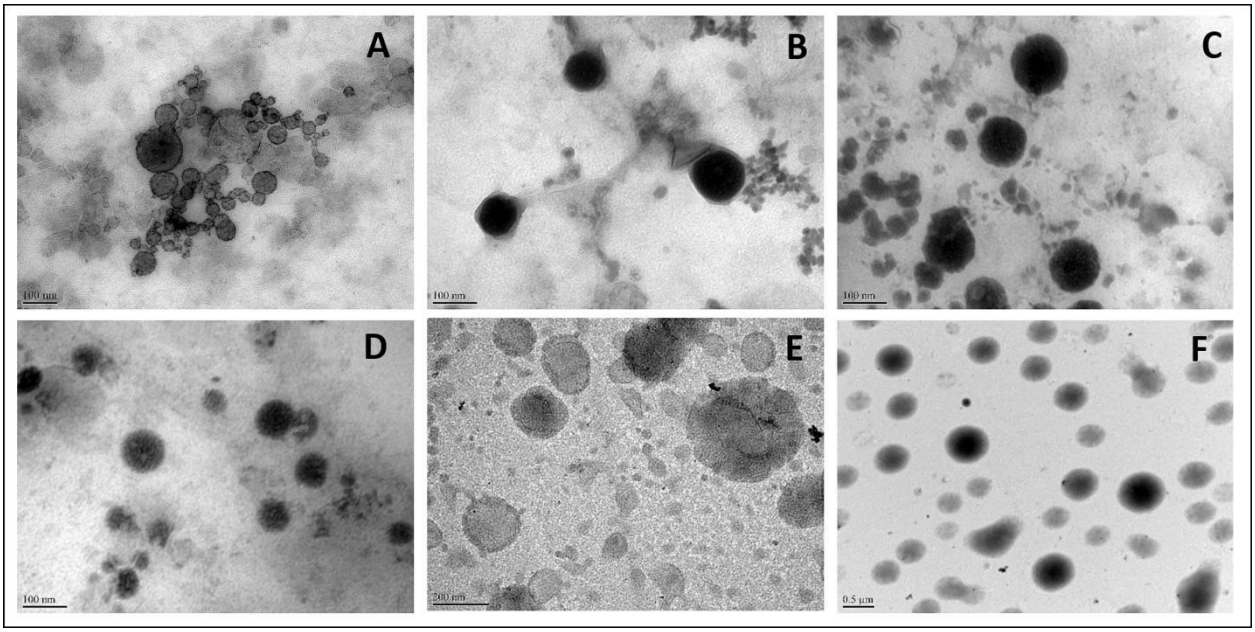


Fig. 3.3. Transmission electron microscopy of (A) the empty liposomes, and liposomes loaded with (B) <1 kDa, (C) 1-3 kDa, (D) 3-5 kDa, (E) 5-10 kDa, and (F) >10 kDa whey peptide fractions.

4. Conclusions

Encapsulation of food-derived peptides is often necessary to improve their physical stability, mask bitterness, or improve their bioavailability profiles. Bioactive peptides are available with a range of different molecular weights and therefore it was important to elucidate the influence of peptide size on their interactions within liposomes. Our study showed that different whey peptide fractions had similar encapsulation efficiencies in soy lecithin liposomes, despite differences in their molecular weights, molecular heterogeneities, and surface hydrophobicities. Nevertheless, incorporation of peptides with different molecular weights did influence the properties of the loaded liposomes formed, including their surface charge and size. The magnitude of the electrical charge and the mean particle diameter increased with decreasing peptide molecular weight, which may be due to differences in the number and location of the peptide molecules within the liposomes. Indeed, FTIR spectroscopy measurements indicated that the peptides were differentially distributed between the polar surface/core and hydrophobic bilayer of the liposomes. This is the first study to examine the impact of the structural properties of food peptides on their encapsulation in liposomes, and on the properties of the loaded liposomes formed. This information on the effect of molecular weight on liposomal encapsulation may facilitate the design of high quality delivery platforms for the production of encapsulated peptide products for utilization in functional food applications.

Acknowledgements. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant, and Canada Foundation for Innovation (CFI) and Nova Scotia Research and Innovation Trust Fund (NSRIT) through an Infrastructure

Grant. A.M. is a recipient of a Nova Scotia Research and Innovation Graduate Scholarship. We thank Sherryl Patton of Intertape Polymer Group (Truro, NS, Canada) for providing technical assistance and access to an FTIR spectrometer.

References

Allen, T. M., & Cullis, P. R. (2013). Liposomal drug delivery systems: from concept to clinical applications. *Advanced Drug Delivery Reviews*, *65*(1), 36-48.

Carmona-Ribeiro, A. M., & Carrasco, L. D. D. (2014). Novel formulations for antimicrobial peptides. *International Journal of Molecular Sciences, 15*(10), 18040-18083.

da Silva Malheiros, P., Sant'Anna, V., Micheletto, Y. M. S., da Silveira, N. P., & Brandelli, A. (2011). Nanovesicle encapsulation of antimicrobial peptide P34: physicochemical characterization and mode of action on *Listeria monocytogenes*. *Journal of Nanoparticle Research*, *13*(8), 3545-3552.

da Rosa Zavareze, E., Telles, A. C., El Halal, S. L. M., da Rocha, M., Colussi, R., de Assis, L. M., de Castro, L.A.S., Dias, A.R.G., & Prentice-Hernández, C. (2014). Production and characterization of encapsulated antioxidative protein hydrolysates from Whitemouth croaker (*Micropogonias furnieri*) muscle and byproduct. *LWT-Food Science and Technology*, *59*(2), 841-848.

Gómez-Hens, A., & Fernández-Romero, J. M. (2005). The role of liposomes in analytical processes. *TrAC Trends in Analytical Chemistry*, *24*(1), 9-19.

Israelachvili, J. (2011). *Intermolecular and Surface Forces, Third Edition* (Third Edition ed.). London, UK: Academic Press.

Liu, W., Ye, A., Liu, W., Liu, C., Han, J., & Singh, H. (2015). Behaviour of liposomes loaded with bovine serum albumin during in vitro digestion. *Food Chemistry*, *175*, 16-24.

Malam, Y., Loizidou, M., & Seifalian, A. M. (2009). Liposomes and nanoparticles: nano sized vehicles for drug delivery in cancer. *Trends in Pharmacological Sciences, 30*(11), 592-599.

Malheiros, P. D., Daroit, D. J., & Brandelli, A. (2010). Food applications of liposome encapsulated antimicrobial peptides. *Trends in Food Science & Technology, 21*(6), 284292.

Malheiros, P. D., Daroit, D. J., da Silveira, N. P., & Brandelli, A. (2010). Effect of nanovesicle encapsulated nisin on growth of Listeria monocytogenes in milk. *Food Microbiology, 27*(1), 175-178.

McClements, D. J. (2015a). Encapsulation, protection, and release of hydrophilic active components: Potential and limitations of colloidal delivery systems. *Advances in Colloid and Interface Science, 219*, 27-53.

McClements, D. J. (2015b). Nanoscale nutrient delivery systems for food applications: Improving bioactive dispersibility, stability, and bioavailability. *Journal of Food Science, 80*(7), N1602-N1611.

McClements, D. J. (2014). Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds, CRC Press, 572 pages.

Moeller, E. H., Holst, B., Nielsen, L. H., Pedersen, P. S., & Ostergaard, J. (2010). Stability, liposome interaction, and in vivo pharmacology of ghrelin in liposomal suspensions. *International Journal of Pharmaceutics, 390*(1), 13-18.

Mohan, A., Udechukwu, M. C., Rajendran, S. R., & Udenigwe, C. C. (2015a). Modification of peptide functionality during enzymatic hydrolysis of whey proteins. *RSC Advances*, *5*(118), 97400-97407.

Mohan, A., Rajendran, S. R., He, Q. S., Bazinet, L., & Udenigwe, C. C. (2015b). Encapsulation of food protein hydrolysates and peptides: a review. *RSC Advances*, *5*(97), 79270-79278.

Morais, H. A., Da Silva Barbosa, C. M., Delvivo, F. M., Mansur, H. S., Cristina De Oliveira, M., & Silvestre, M. P. C. (2004). Comparative study of microencapsulation of casein hydrolysates in lipospheres and liposomes. *Journal of Food Biochemistry*, *28*(1), 21-41. Mosquera, M., Giménez, B., da Silva, I. M., Boelter, J. F., Montero, P., Gómez-Guillén, M. C., & Brandelli, A. (2014). Nanoencapsulation of an active peptidic fraction from sea bream scales collagen. *Food Chemistry*, *156*, 144-150.

Mosquera, M., Giménez, B., Montero, P., & Gómez‐Guillén, M. C. (2016). Incorporation of liposomes containing squid tunic ACE‐inhibitory peptides into fish gelatin. *Journal of the Science of Food and Agriculture*, *96*(3), 769-76

Mozafari, M. R. (2010). Nanoliposomes: preparation and analysis. In *Liposomes* (pp. 29-50). Humana Press.

Pisal, D. S., Kosloski, M. P., & Balu-Iyer, S. V. (2010). Delivery of therapeutic proteins. *Journal of Pharmaceutical Sciences, 99*(6), 2557-2575.

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., & Hernandez-Escalante, V. M. (2011). Bioavailability of bioactive peptides. *Food Reviews International*, *27*(3), 213-226.

Taylor, T. M., Gaysinsky, S., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Characterization of antimicrobial-bearing liposomes by ζ-potential, vesicle size, and encapsulation efficiency. *Food Biophysics*, *2*(1), 1-9.

Udenigwe, C. C. (2014). Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science & Technology*, *36*(2), 137-143.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing, and potential health benefits. *Journal of Food Science*, *77*(1), R11-R24.

Udenigwe, C. C., Wu, S., Drummond, K., & Gong, M. (2014). Revisiting the prospects of plastein: Thermal and simulated gastric stability in relation to the antioxidative capacity of casein plastein. *Journal of Agricultural and Food Chemistry*, *62*(1), 130-135.

Yokota, D., Moraes, M., & Pinho, S. C. (2012). Characterization of lyophilized liposomes produced with non-purified soy lecithin: a case study of casein hydrolysate microencapsulation. *Brazilian Journal of Chemical Engineering*, *29*(2), 325-335.

Zavareze, E. D., Telles, A. C., El Halal, S. L. M., da Rocha, M., Colussi, R., de Assis, L. M., de Castro, L. A. S., Dias, A. R. G., & Prentice-Hernandez, C. (2014). Production and characterization of encapsulated antioxidative protein hydrolysates from Whitemouth croaker (Micropogonias furnieri) muscle and byproduct. *LWT-Food Science and Technology, 59*(2), 841-848.

Chapter 4

Liposome Encapsulation of Anionic and Cationic Whey Peptides: Influence of Peptide Net Charge on Nanoliposome Formation and Properties

Aishwarya Mohan[†], Subin R. C. K. Rajendran[†], Jacinthe Thibodeau[‡], Laurent Bazinet[‡], Chibuike C. Udenigwe[*,†]

[†]Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia B2N 5E3, Canada

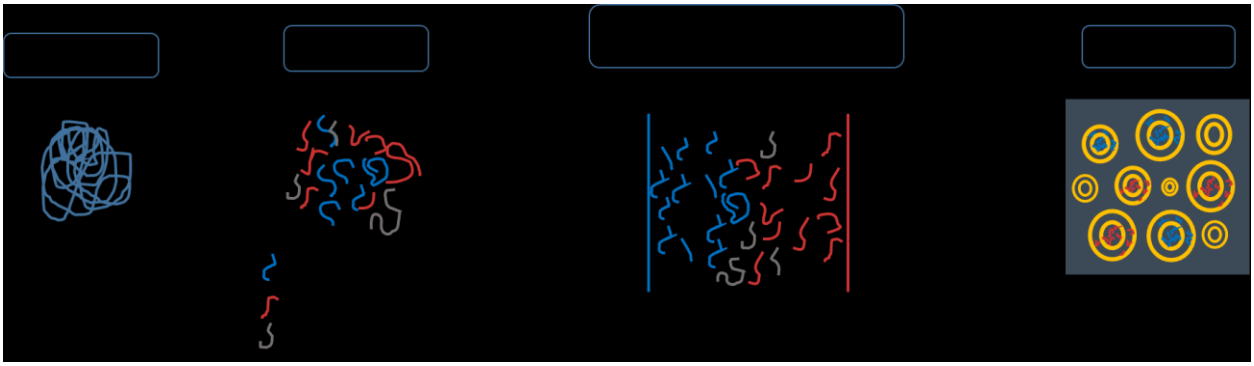
[‡]Department of Food Sciences, and Institute of Nutrition and Functional Foods, Université Laval, Québec, Québec G1V 0A6, Canada

*Corresponding Author

Email: cudenigwe@dal.ca. Phone: +1 (902) 893-6625. Fax +1 (902) 893-1404.

Submitted to *Journal of Agricultural and Food Chemistry* April 25, 2016.

GRAPHICAL ABSTRACT



ABSTRACT

Poor bioavailability of food-derived bioactive peptides can impede their application in functional foods. Liposome encapsulation has been used to protect the peptides from degradation during digestion and food processing. This study focuses on understanding the impact of net charge of whey peptides, separated by electrodialysis with ultrafiltration membrane (EDUF), on encapsulation efficiency (EE) and surface properties of the peptide-loaded liposomes. EDUF fractionation resulted in two fractions; one which contained anionic whey peptides (AWP) with a relative abundance of 30.4% anionic amino acid residues and the other was cationic whey peptides (CWP) with 8.5% cationic amino acid residues. AWP liposomes was observed to have lower encapsulation efficiency than encapsulated CWP, which had more peptides per unit mass and 5 times more surface hydrophobicity than AWP. Similarly, encapsulated CWP had larger mean particle diameter and higher polydispersity index compared to AWP-loaded liposomes. However, the effect of net charge of the fraction on surface charge (ζ-potential) of the liposomes was not apparent. FTIR spectra of the CWP- and AWP-loaded liposomes were largely similar and showed interaction of the peptides with the phosphate group of the liposomes. The peptide net charge did not appear to influence their distribution in different regions of the nanocapsules. Apart from peptide net charge, differences in EE and physicochemical properties of the liposomes can be due to a combined influence of the number of peptides per unit mass, their homogeneity, and surface hydrophobicity. These findings are relevant to developing efficient liposomal systems for bioactive peptide delivery.

Keywords: Bioactive peptides, Anionic peptides, Cationic peptides, Liposomes, Encapsulation, Electrodialysis with ultrafiltration membrane

INTRODUCTION

Bioactive peptides are one of the promising functional food ingredients that have been studied since the last two decades. Peptides are unique bioactives because the individual properties of each amino acid residue can act as a part of the single peptide unit to express physiological activity.[1] However, the susceptibility of peptides to gastrointestinal digestion[2] can limit their application in functional foods despite the potential health benefits. Consequently, encapsulation can be pursued for commercial application of the myriad of bioactive peptides currently identified from a wide range of food sources.

Delivery agents are considered to have the ability to modify the digestion kinetics of active ingredients thereby increasing the bioavailability and biostability of the otherwise digestion-labile bioactives.[3] Among existing delivery matrices, liposomes have been used to achieve high encapsulation efficiency (EE) due to their mild preparation conditions and ability to entrap hydrophilic, lipophilic and amphiphilic bioactive compounds.[1] Colloidal systems are among the most widely studied successful and efficient delivery systems. Liposomal systems consist of single or multiple layers of spontaneously self-assembled phospholipids that form vesicles as a result of exposure to aqueous environment.[4] These vesicular carriers are widely used for drug delivery in the pharmaceutical industry, and for delivering flavour compounds, enzymes, vitamins, proteins and antioxidants in the food industry.[5] The pharmaceutical sector has progressed remarkably with the development of second generation liposomes such as stealth liposomes (long-circulating PEGylated liposomes), triggered-release liposomes, and ligand-targeted liposomes to deliver drugs through specific receptor-mediated endocytosis.[6] Liposomal properties are now being explored in the food industry for the delivery of food bioactives. However, there is limited information on the interaction between encapsulation carriers and the bioactive ingredient being encapsulated,

especially for bioactive peptides.[1] A recent study reported the encapsulation of structurally diverse peptide mixture, and also the improved peptide-liposome stability by coating the liposomes with chitosan.[7] Moreover, liposomal delivery of antimicrobial and antioxidant peptides of microbial origin such as nisin, bacteriocin-like peptides and pediocin is another active field of research.[8–12] Despite the prospects of encapsulation, heterogeneity of peptides in food protein hydrolysates is considered to be a major challenge in ensuring encapsulation.[1,13] This underscores the need to characterize the effect of peptide physiochemical properties on encapsulation. The net charge of proteins and peptides has been suggested to impact their encapsulation in liposomes,[1,3] although their specific impact on encapsulation efficiency and liposome properties have yet to be elucidated. This study aimed to evaluate the impact of the net charge of whey peptides on their encapsulation efficiency and properties of the resulting liposomes including zeta potential, particle size, and interaction with different regions of the liposomes.

MATERIALS AND METHODS

Whey Protein Hydrolysis. An aqueous suspension of the whey protein isolate (5%, w/v) was hydrolysed using papain at an enzyme-to-substrate ratio of 1:100 (w/w), under optimum pH and temperature of 7.0 and 65°C, respectively. Hydrolysis was terminated after 5 h by incubating the mixture at 95°C for 15 min. The resulting hydrolysate was cooled to room temperature and centrifuged at 5,000 × g for 10 min. The supernatant was collected as whey protein hydrolysate (WPH), freeze dried and stored at -20°C for further analysis.

Peptide Fractionation by Electrodialysis with Ultrafiltration Membranes (EDUF). The whey hydrolysate was separated using EDUF based on the process reported by Doyen et al.[14] The EDUF

cell comprised of Neosepta CMX-SB cationic and AMX-SB anionic membrane (Tokoyuma Soda Ltd, Tokyo Japan) along with two cellulose ester ultrafiltration membranes of 20 kDa molecular weight cut-off (Spectrum Laboratories Inc, CA, USA). The whey protein hydrolysate was suspended in deionized water at a protein concentration of 4% and loaded into the feed compartment. Two recovery compartments, one for anionic and one for cationic peptides, were filled with KCl (2 g/L) solution. Electrode rinsing solution, $NaSO_4$ (20 g/L), was used to fill the last compartment. The feed and recovery compartments were adjusted to pH 7.0 and the initial conductivity was noted. The solutions were circulated using centrifugal pumps at a constant rate of 2 L/min. The electrode rinsing solution was maintained at a flow rate of 4 L/min, and separation was carried out for 6 h while maintaining the pH and conductivity at the initial values. The anionic and cationic recovery compartments were collected as anionic whey peptides (AWP) and cationic whey peptides (CWP), respectively. Both fractions were lyophilized, desalted by dialysis for 24 h using a 100-500 Da MWCO dialysis tube, and stored at -20°C until further analysis.

Relative Abundance of Cationic and Anionic Amino Acid Residues. LC-MS/MS analysis was performed on the recovered AWP and CWP fractions at the SPARC Biocentre (Toronto, ON, Canada) using EASY-nLC nano-LC System (Thermo Fisher, San Jose, CA, USA) as previously reported.[15] The relative abundance of total cationic and anionic residues in AWP and CWP fractions is expressed as percentage of ratio between summation of cationic or anionic residue content per peptide and the summation of each peptide content in the fraction by the following formula:

Relative abundance of total cationic residues (RA_c) % = $\sum(P_{c1}+P_{c2}+\ldots+P_{cn})/\sum(P_{t1}+P_{t2}+\ldots+P_{tn}) \times 100$; where P_{cn} is the cationic content of any given peptide in the fraction; P_{tn} is the total content of each peptide in the fraction, as represented by area under the retention-time curve.

Similarly,

Relative abundance of total anionic residues (RA_a) % = $\sum(P_{a1}+P_{a2}+..+P_{an})/ \sum(P_{t1}+P_{t2}+.. +P_{tn}) \times 100$; where P_{an} is the anionic content of any given peptide in the fraction; P_{tn} is the total content of each peptide in the fraction, as represented by area under the retention-time curve.

The cationic or anionic content of a given peptide is calculated by: $P_{cn(or)an} = N_{c(or)a}/N_t \times P_{tn}$; where $N_{c/a}$ is the number of cationic (or) anionic residues in a given peptide; N_t is the total number of amino acid residues in the given peptide and P_{tn} is the area under the retention-time curve of the given peptide. Glu and Asp were used for determining abundance of anionic residues and the cationic counterpart of the same was calculated using Arg and Lys. Histidine was excluded from cationic residues since the fractionation was carried out in neutral pH, at which His assumes no charge.

Free Amino Group. The free amino group of the peptide fractions were determined as previously reported.[16] Briefly, 250 µL of the O-phthalaldehyde reagent was mixed with 33 µL of the sample (1 mg/mL) and the absorbance was then measured at 340 nm. Serine was used as the standard and free amino group was expressed as milliequivalent serine NH_2/g protein.

Surface Hydrophobicity (S_o). S_o of AWP and CWP was determined as previously reported[17] using 8-anilino-1-naphthalenesulfonic acid (ANS) as the hydrophobic probe. Briefly, 100 µL of the peptide fraction (0.0009-0.015%) was mixed with 100 µL of ANS (0.04 mM), and fluorescence was then measured at excitation and emission wavelengths of 390 and 470 nm, respectively. S_o was calculated as the slope of the sample concentration vs. fluorescence plot.

Encapsulation of Charged Fractions. AWP and CWP were encapsulated by film hydration method. Soy lecithin powder was dissolved in chloroform (1%, w/v) and rotary evaporated at 60°C

to form a thin lipid film at the bottom of the flask. The lipid film was left overnight in a desiccator and then hydrated with the peptide solution (1 mg/mL, in 10 mM phosphate buffer, pH 7.0) at 60°C under agitation (150 rpm). Thereafter, the resulting liposome product was homogenized by sonicating for 30 min. The encapsulated product was used for further analysis.

Encapsulation Efficiency (EE). The ratio of amount of the loaded and initial peptides, in percentage, was determined to be the EE. The amount of the loaded peptide was indirectly determined by passing the encapsulation product through a 100-kDa ultrafiltration membrane at a constant pressure of 10 psi, to separate the non-encapsulated peptides from the encapsulated product. The protein content of the filtrate was determined using a Bio-Rad Protein Assay kit (Richmond, CA, USA).

Nanoliposome Characterization. The ζ-potential, mean particle diameter and polydispersity index of the liposome nanocapsules were determined using Nano Series Nano-ZS Zetasizer (Malvern Instruments Ltd., Worcestershire, UK) based on dynamic light scattering and Doppler shift velocimetry. The CWP- and AWP-loaded liposomes were diluted in MilliQ water in capillary tubes prior to obtaining the measurements.

Fourier Transform Infrared Spectroscopy (FTIR). The lyophilized peptide-loaded liposomes and control liposomes were used to perform the Attenuated Total Reflectance (ATR) FTIR spectroscopy. The infrared spectra was obtained as an average of 16 scans between 4000-600 cm^{-1} using FTIR (Spectrum One, Perkin Elmer, CT, USA). Spectral peaks of peptide-loaded and empty liposome was qualitatively compared.

Transmission Electron Microscopy (TEM). The encapsulated AWP and CWP samples were applied over a 200-mesh carbon film-coated copper grid, and excess sample was removed after 10

min. Uranyl acetate (2%, w/v) was then used to achieve negative staining of the sample-loaded grid followed by removal of excess stain by quick immersion of the grid in deionized water. The stained grids were dried for 1 h before imaging the liposomes with Tecnai-12 microscope (Philips Electron Optics, Netherlands).

Statistical Analysis. Peptide encapsulation was performed in triplicate and all the results were expressed as mean ± standard deviation. The significance of difference between the mean values were determined using one way analysis of variance followed by Holm Sidak multiple comparison test. All the statistical analysis were performed using SigmaPlot (Systat Software, San Jose, CA, USA).

RESULTS AND DISCUSSION

Relative Abundance of Total Cationic and Anionic Residues (RA_c and RA_a) in the peptide fractions. The relative strength of anionic and cationic charge of AWP and CWP, respectively, helps to ascertain the effective fractionation of whey hydrolysate by the peptide charge. Moreover, it can facilitate the interpretation of physicochemical properties of peptide-loaded liposomes, distribution and interaction of the peptides with the nanovesicles. The RA_a in AWP was 22 units of percentage (3.5-times) higher than RA_c (**Figure 1A**). However, RA_c of CWP was only 1.3 units greater than the RA_a (**Figure 1B**), implying that AWP is substantially more anionic than the cationic attribute of CWP. The major constituents of whey proteins, β-lactoglobulin (β-lg) and α-lactalbumin (α-la), contain more acidic amino acid residues (46 residues) than basic residues (32 residues), which could have accounted for the strong anionic attribute of the AWP. Although the Asp portion in RA_a of CWP and AWP fractions was similar, the contribution of Glu to the latter was considerably higher than that of the former. The increased Glu content can be attributed to the higher Glu content of β-lg (compared to Asp), which constitutes 50–80% of whey protein. It is also

noteworthy that Glu with deprotonated carboxylic group at neutral pH is fivefold more hydrophilic than deprotonated Asp.[18] Similarly, among the cationic residues, the contribution of Lys to RA_c of both the fractions was higher than that of Arg. However, Lys proportion was 2.5 times in the RA_c of CWP fraction than in the AWP fraction. Arg contribution was as low as 1.6% and 2.6% in the RA_c of AWP and CWP, respectively. β-Lg and α-la together contain just four Arg residues (Uniprot KB), which is considered to be responsible for the low abundance of Arg in the peptide fractions. Therefore, the highly abundant charged amino acid residues in the parent proteins are also reflected in the composition of the EDUF fractions.

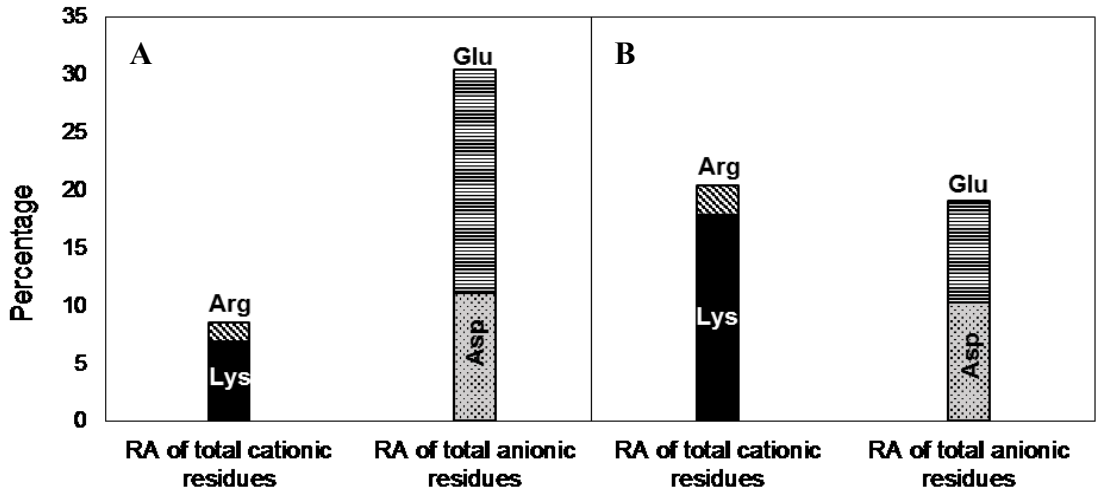


Figure 4.1. Relative Abundance (RA) of cationic and anionic residues in (A) Anionic Whey Peptide (AWP) (B) Cationic Whey Peptide (CWP)

Impact of Net Peptide Charge on Encapsulation Efficiency. The amount of bioactive material encapsulated in liposomes indicates the EE, which varies widely for different peptides due to their molecular diversity.[1] The heterogeneity of peptides in protein hydrolysates can make it challenging to understand the effect of peptide properties on EE and liposome properties. Electrostatic interactions are expected to play a major role in the interplay between peptides and the liposomal structure. Electrostatic repulsion between the hydrophilic phosphate group of phospholipids and the negatively charged AWP with 30% RA_a was expected to decrease liposome EE, similar to the case of encapsulated casein phosphopeptides.[19,20] Accordingly, as shown in **Figure 2**, the EE of encapsulated AWP (88.3%) in our study was found to be significantly lower than that of liposomal CWP (92.9%). However, the EE of AWP liposomes was considerably higher than the EE reported for encapsulation of other anionic bioactive peptides, [19,20] and even cationic peptides.[21] It is possible that the interactions of individual amino acid residues, including lower amounts of Lys and Arg (8.48%) with one another affected the extent of electrostatic repulsion in a net negatively charged protein or peptide. Moreover, the phospholipid composition can also affect the peptides interaction with the vesicle.[22] Nisin, a cationic amphiphilic bacteriocin peptide, was reported to have broad range of EE ranging between 34-94% depending on the type of phospholipid used in encapsulation.[22,23] Electrostatic interaction between the active ingredient and the lipid can be a key factor when anionic (phosphatidylinositol, phosphotidylglycerol) or cationic (stearylamine) lipids are used. Neutral or zwitterionic lipids such as phosphatidylcholine is reported to be least affected by electrostatic attraction or repulsion, resulting in higher EE than other anionic or cationic lipids.[10] Phosphatidylcholine, being the major phospholipid in soy lecithin, is predicted to be responsible for the high EE observed for AWP.

CWP was found to have higher number of peptides per unit mass than AWP (based on free amino group content, **Figure 3**), indicating that CWP contained smaller peptide sequences compared to AWP. To date, it is unclear how the number of peptides per unit mass of protein affects EE and peptide-loaded liposome properties. Plausible increase in cationic charge with higher number of peptides could increase the EE. Furthermore, smaller peptides can decrease the stearic hindrance and increase interaction due to solvent accessibility. However, the peptide-peptide interaction can also be promoted. Similarly, the S_o of CWP was 5-fold higher than that of AWP. As mentioned earlier, the higher AWP Glu content, which is more hydrophilic than Asp, and the higher CWP Lys content, whose long side chain is slightly more non-polar than Glu, are considered to be responsible for the lower S_o of AWP. It has been suggested that hydrophobic interactions underlies the entrapment of peptides into the nanovesicles when neutral phospholipids are used for liposome preparation.[11,24] This is suggestive of a major role of S_o along with the net charge in influencing CWP encapsulation within the liposomes.

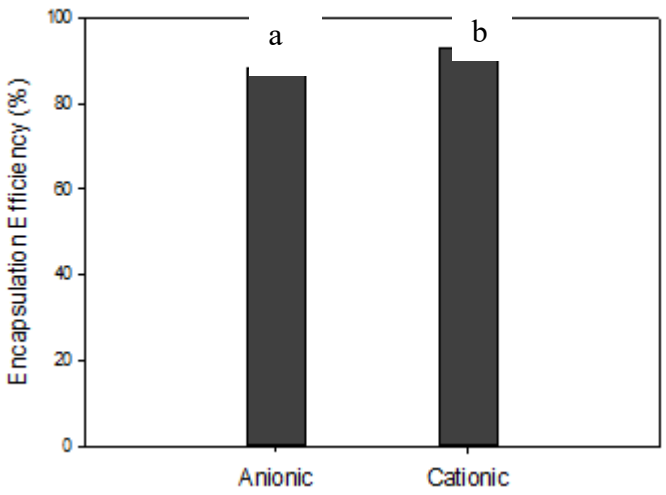


Figure 4.2. Encapsulation efficiency of the anionic and cationic whey peptide fractions in soy lecithin-derived nanoliposomes; different letter on the bar indicates that the difference in the mean value is significant ($p<0.05$).

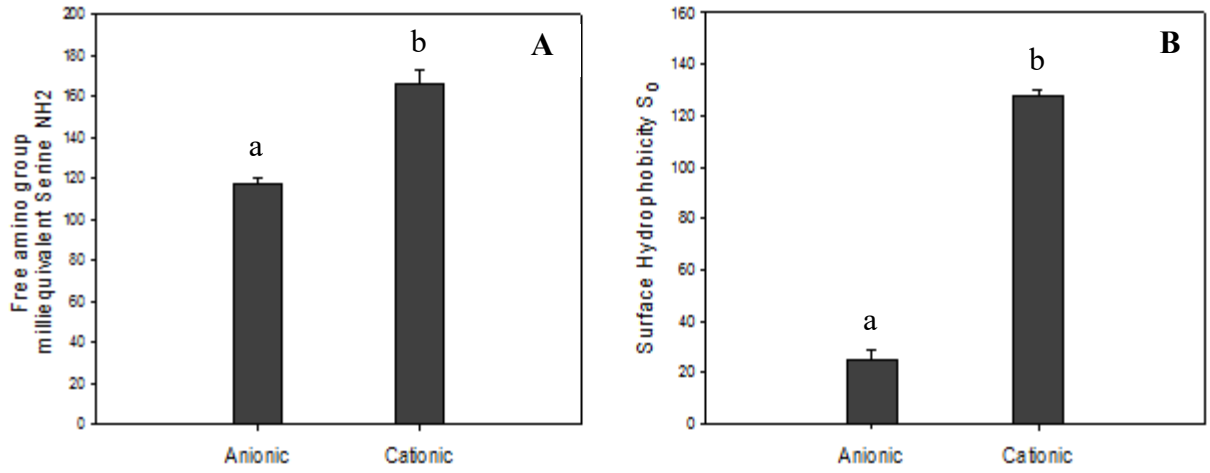


Figure 4.3. (A) Free amino group, and (B) surface hydrophobicity of anionic and cationic whey peptide fractions; bars with different letters in (A) and (B) indicate that differences in the mean values were statistically significant ($p<0.05$).

Impact of Net Peptide Charge on Liposome Properties.

Surface Charge (ζ-potential). The ζ-potential of the whey peptide-loaded nanoliposomes was lower than the minimum surface charge required to form a stable suspension (± 30 mV) as can be observed in **Table 1**. L-carnosine, an anionic dipeptide was reported to increase the negative surface charge of liposomes prepared using neutral phospholipids, thereby increasing their stability.[24] A similar report has attributed the increase in zeta potential of liposomes to the net anionic charge of encapsulated bovine serum albumin. In contrast, AWP encapsulated liposomes in our study had a lower magnitude of ζ-potential. The ζ-potential of AWP and CWP-loaded liposomes decreased in magnitude of surface charge compared to empty liposomes (**Table 1**). The surface charge of both encapsulated fractions were not significantly different from each other; nevertheless, AWP-loaded liposomes had a slightly higher ζ-potential than encapsulated CWP. Considerable amount of anionic residues (RA_a = 19%) in CWP fraction could have resulted in similar ζ-potential for the fractions. An observable trend was absent regarding the influence of peptides on liposomal surface charge. Some studies have also demonstrated the decrease in liposome surface charge in the presence of peptides,[7] whereas others have reported otherwise.[3,24] It can be hypothesized that the charge of exposed amino acid side chains influences the ζ-potential of encapsulated product; however, structural interactions of liposomes and peptides needs to be further elucidated.

Mean Particle Diameter. Encapsulation of AWP and CWP resulted in nanocapsules with mean particle diameter of less than 150 nm, which comes under the classification of medium-large unilamellar vesicles. Unfractionated whey peptide-loaded liposome had higher mean diameter in comparison to empty liposomes (**Table 1**) prepared under similar conditions. The CWP-loaded liposomes were significantly larger than those derived from AWP. Higher number of peptides per

unit mass of CWP is predicted to expand the vesicle, as larger number of peptides are entrapped, resulting in the larger mean particle diameter of CWP liposomes. It is unclear whether the higher S_o of CWP influenced the particle size of the liposomes *via* hydrophobic interactions. Factors other than peptide net charge can also influence the mean particle diameter of the nanoliposomes, such as sonication during liposome formation [25] and lipid composition of the bilayer.

Polydispersity Index (PDI). PDI value indicates the homogeneity of particle size distribution in the suspension.[25] Lower PDI indicates a narrow size distribution of particles in the suspension and vice versa. The larger CWP liposomes were also observed to have a higher PDI compared to AWP loaded liposomes, and it is likely that peptide charge and number of peptides per unit mass of the fractions affected the liposome dispersion. A homogenous size distribution was observed in empty liposomes with substantially lower PDI than the two fraction-loaded liposomes. Maherani *et al.*[24] have reported PDI as low as 0.09 for liposome encapsulated antioxidant dipeptide, L-carnosine. It is challenging to achieve a narrow size distribution for food protein hydrolysates due to heterogeneous peptide properties. Unlike the AWP-loaded liposomes, the CWP liposomes had a higher PDI than the values reported for other protein hydrolysates.[4,25] A high PDI value, especially value > 0.7 triggers the tendency of particles in the suspension to aggregate/coalesce and deposit as sediments, which can negatively impact texture and stability of food/ drug formulations.

Table 4.1. Surface charge, particle size and polydispersity index of the liposomes loaded with anionic and cationic whey peptides, and empty liposomes.

	Zeta Potential (mV)	Particle Size (nm)	PDI*
Empty Liposome	-55 ± 0.6[a]	97.2 ± 5.9[b]	0.163 ± 0.004
Anionic	-24.5 ± 6.4[b]	106.2 ± 8.6[b]	0.367 ± 0.062
Cationic	-18.6 ± 6.2[b]	147.4 ± 22.4[a]	0.462 ± 0.079

Numbers with different letters in rows indicate statistically significant mean values ($p<0.05$).

*The power of the performed statistical test, with $\alpha=0.05$, was below the desired power to observed statistically significant difference.

Interaction of Anionic and Cationic Whey Peptides with the Liposome structure. The FTIR spectral peaks of the empty liposomes and encapsulated CWP and AWP were compared to deduce the distribution and interaction of peptides in the liposomes. As shown in **Table 2**, the choline peak of the empty liposomes was slightly shifted to a higher wavenumber in liposomes containing both CWP and AWP. The downfield PO_2 stretch was shifted to a lower wavenumber in both AWP and CWP-loaded liposomes. However, the PO_2 stretch of encapsulated CWP product resulted in two peak maxima (**Table 2**). The asymmetrical broad phosphate stretch at 1063 cm^{-1} also shifted to a lower and higher wavenumber in the AWP and CWP liposomes, respectively. It can be observed from **Table 2** that CWP and AWP interact with the choline and phosphate groups of the liposome hydrophilic head. However, peptide interaction with the phosphate group was more pronounced than with choline. Notably, interaction of AWP fraction with the phosphate group suggests that the net anionic charge does not preclude the peptide interaction with the liposome surface.

Despite the net cationic nature of CWP, peptide interactions with the phosphate group of liposomes were not largely different from that of the encapsulated AWP. Arg residue, containing the guanidine side chain, is capable of directly interacting with the anionic phosphate head and polar region *via* strong cationic charge and multiple hydrogen bonding moieties.[26] Moreover, the multidentate hydrogen bonding ability of Arg allows these residues to simultaneously interact with more than one phosphate group.[26] Contrastingly, the amino group of Lys side chain can only form monodentate hydrogen bonds with single phosphate group. Therefore, as Arg content (< 3%) in both fractions is low, similar interaction and distribution profile is observed in the CWP/AWP prepared liposomes. In addition, higher S_o and number of peptides per unit mass of CWP is predicted to facilitate peptide-peptide interactions that leads to aggregation.[27] Aggregation of CWP could also have limited interaction of peptides with the polar region of the nanovesicle. The limited

interactions with the liposome structure can be advantageous during release as strong electrostatic interaction may render the peptides unavailable for release and absorption.[9] Moreover, profound electrostatic interaction between the peptides and the vesicle can affect the morphology of the liposomes sometimes leading to leakage of the encapsulated materials.[12] The high S_o of CWP did not affect the FTIR peak maximum of the hydrophobic region of the bilayer (**Table 2**). Additionally, the encapsulated AWP and CWP were observed under TEM to confirm the formation of the vesicles (**Figure 3**). Contrary to the argument that encapsulation of cationic peptides with positive charge at the C-terminal would modify the spherical liposomes to a rod-shaped morphology[10], spherical liposomes were observed under TEM (**Figure 4**).

Table 4.2. Wavenumber of liposome functional groups obtained from FTIR spectra of the liposomes loaded with whey peptide fractions of different net charge and empty liposomes.

	Empty liposome	**AWP liposomes**	**CWP liposomes**
$(CH_3)_3N$	970	972	972
PO_2	871	861	835 & 866
Broad PO_2 stretch	1063	1066	1068
CH_2	2854	2853	2853
CH_3	2924	2924	2924

AWP, anionic whey peptide fraction; CWP, cationic whey peptide fraction

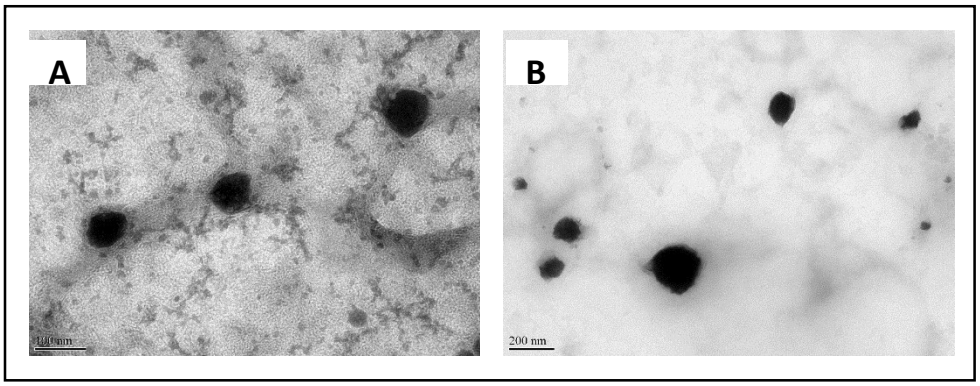


Figure 4.4. Transmission electron microscopy of liposomes loaded with (A) anionic whey peptide fraction (B) cationic whey peptide fraction.

CONCLUSION

Fractionation of whey hydrolysate on the basis of charge, yielded fractions with net anionic and cationic charge. The impact of net charge of whey peptide fractions on encapsulation efficiency and properties of their liposomes was elucidated. Anionic whey peptides had a significantly lower EE than the cationic peptides. However, it appears that electrostatic repulsion did not have substantial effect on liposome encapsulation as previously suggested for other negatively charged peptides. Factors other than the peptide net charge such as the properties of individual amino acid residues, number of peptides per unit mass, presence of zwitterionic phospholipids, surface properties of the peptides, and the encapsulation method is predicted to have also affected the encapsulation. Encapsulation of the whey peptides resulted in a decrease in the liposome surface charge compared to empty liposomes, irrespective of the peptide net charge. The mean particle diameter was higher for CWP liposomes, probably the polar and non-polar regions enlarged due to more number of peptides in the fraction. Based on FTIR spectroscopy, AWP and CWP were both observed to interact with the phosphate group of the liposomes. The findings indicate that the net charge of whey peptides in our study does not play a major role in determining their encapsulation in liposomes prepared from soy lecithin. This information will facilitate design of efficient peptide delivery systems for functional food applications.

ACKNOWLEDGEMENTS

This project was financially supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant, and Canada Foundation for Innovation (CFI) and Nova Scotia Research and Innovation Trust Fund (NSRIT) through an infrastructure grant. A.M.

and S.R.C.K.R. are recipients of Nova Scotia Research and Innovation Graduate Scholarships. The authors thank Sherryl Patton and Robin Spence of Intertape Polymer Group (Truro, NS, Canada) for providing access to FTIR spectrometer.

REFERENCES

(1) Mohan, A.; Rajendran, S. R. C. K.; He, Q. S.; Bazinet, L.; Udenigwe, C. C. Encapsulation of food protein hydrolysates and peptides: a review. *RSC Adv.* **2015**, *5* (97), 79270–79278.

(2) Segura-Campos, M.; Chel-Guerrero, L.; Betancur-Ancona, D.; Hernandez-Escalante, V. M. Bioavailability of Bioactive Peptides. *Food Rev. Int.* **2011**, *27* (3), 213–226.

(3) Liu, W.; Ye, A.; Liu, W.; Liu, C.; Han, J.; Singh, H. Behaviour of liposomes loaded with bovine serum albumin during in vitro digestion. *Food Chem.* **2015**, *175*, 16–24.

(4) Mosquera, M.; Giménez, B.; Montero, P.; Gómez-Guillén, M. C. Incorporation of liposomes containing squid tunic ACE-inhibitory peptides into fish gelatin. *J. Sci. Food Agric.* **2016**, *96* (3), 769–776.

(5) Mozafari, M. R.; Khosravi-Darani, K.; Borazan, G. G.; Cui, J.; Pardakhty, A.; Yurdugul, S. Encapsulation of Food Ingredients Using Nanoliposome Technology. *Int. J. Food Prop.* **2008**, *11* (4), 833–844.

(6) Allen, T. M.; Cullis, P. R. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* **2013**, *65* (1), 36–48.

(7) Li, Z.; Paulson, A. T.; Gill, T. A. Encapsulation of bioactive salmon protein hydrolysates with chitosan-coated liposomes. *J. Funct. Foods* **2015**, *19*, 733–743.

(8) de Mello, M. B.; da Silva Malheiros, P.; Brandelli, A.; Pesce da Silveira, N.; Jantzen, M. M.; de Souza da Motta, A. Characterization and Antilisterial Effect of Phosphatidylcholine Nanovesicles Containing the Antimicrobial Peptide Pediocin. *Probiotics Antimicrob.*

Proteins **2013**, *5* (1), 43–50.

(9) da Silva Malheiros, P.; Daroit, D. J.; da Silveira, N. P.; Brandelli, A. Effect of nanovesicle-encapsulated nisin on growth of Listeria monocytogenes in milk. *Food Microbiol.* **2010**, *27* (1), 175–178.

(10) da Silva Malheiros, P.; Micheletto, Y. M. S.; Silveira, N. P. da; Brandelli, A. Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Res. Int.* **2010**, *43* (4), 1198–1203.

(11) Teixeira, M. L.; dos Santos, J.; Silveira, N. P.; Brandelli, A. Phospholipid nanovesicles containing a bacteriocin-like substance for control of Listeria monocytogenes. *Innov. Food Sci. Emerg. Technol.* **2008**, *9* (1), 49–53.

(12) Laridi, R.; Kheadr, E. .; Benech, R.-O.; Vuillemard, J. .; Lacroix, C.; Fliss, I. Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *Int. Dairy J.* **2003**, *13* (4), 325–336.

(13) McClements, D. J. *Nanoparticle- and Microparticle-based Delivery Systems: Encapsulation, Protection and Release of Active Compounds*; CRC Press, 2014.

(14) Doyen, A.; Husson, E.; Bazinet, L. Use of an electrodialytic reactor for the simultaneous β-lactoglobulin enzymatic hydrolysis and fractionation of generated bioactive peptides. *Food Chem.* **2013**, *136* (3-4), 1193–1202.

(15) Udenigwe, C. C.; Udechukwu, M. C.; Yiridoe, C.; Gibson, A.; Gong, M. Antioxidant mechanism of potato protein hydrolysates against in vitro oxidation of reduced glutathione. *J. Funct. Foods* **2016**, *20*, 195–203.

(16) Mohan, A.; Udechukwu, M. C.; Rajendran, S. R. C. K.; Udenigwe, C. C. Modification of peptide functionality during enzymatic hydrolysis of whey proteins. *RSC Adv.* **2015**, *5*

(118), 97400–97407.

(17) Mohan, A.; Udenigwe, C. C. Towards the design of hypolipidaemic peptides: Deoxycholate binding affinity of hydrophobic peptide aggregates of casein plastein. *J. Funct. Foods* **2015**, *18*, 129–136.

(18) Young, V. R.; Ajami, A. M. Glutamate: An Amino Acid of Particular Distinction. *J. Nutr.* **2000**, *130* (4), 892 – .

(19) Yokota, D.; Moraes, M.; Pinho, S. C. Characterization of lyophilized liposomes produced with non-purified soy lecithin: A case study of casein hydrolysate microencapsulation. *Brazilian J. Chem. Eng.* **2012**, *29* (2), 325–335.

(20) Barbosa, C. M. S.; Morais, H. a.; Delvivo, F. M.; Mansur, H. S.; De Oliveira, M. C.; Silvestre, M. P. C. Papain hydrolysates of casein: Molecular weight profile and encapsulation in lipospheres. *J. Sci. Food Agric.* **2004**, *84* (14), 1891–1900.

(21) Imran, M.; Revol-Junelles, A.-M.; Paris, C.; Guedon, E.; Linder, M.; Desobry, S. Liposomal nanodelivery systems using soy and marine lecithin to encapsulate food biopreservative nisin. *LWT - Food Sci. Technol.* **2015**, *62* (1), 341–349.

(22) da Silva Malheiros, P.; Daroit, D. J.; Brandelli, A. Food applications of liposome-encapsulated antimicrobial peptides. *Trends Food Sci. Technol.* **2010**, *21* (6), 284–292.

(23) Malheiros, P. S.; Cuccovia, I. M.; Franco, B. D. G. M. Inhibition of Listeria monocytogenes in vitro and in goat milk by liposomal nanovesicles containing bacteriocins produced by Lactobacillus sakei subsp. sakei 2a. *Food Control* **2016**, *63*, 158–164.

(24) Maherani, B.; Arab-Tehrany, E.; Kheirolomoom, A.; Cleymand, F.; Linder, M. Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant l-carnosine. *Food Chem.* **2012**, *134* (2), 632–640.

(25) da Rosa Zavareze, E.; Telles, A. C.; Mello El Halal, S. L.; da Rocha, M.; Colussi, R.; Marques de Assis, L.; Suita de Castro, L. A.; Guerra Dias, A. R.; Prentice-Hernández, C. Production and characterization of encapsulated antioxidative protein hydrolysates from Whitemouth croaker (Micropogonias furnieri) muscle and byproduct. *LWT - Food Sci. Technol.* **2014**, *59* (2), 841–848.

(26) Schmidt, N.; Mishra, A.; Lai, G. H.; Wong, G. C. L. Arginine-rich cell-penetrating peptides. *FEBS Lett.* **2010**, *584* (9), 1806–1813.

(27) Gong, M.; Mohan, A.; Gibson, A.; Udenigwe, C. C. Mechanisms of plastein formation, and prospective food and nutraceutical applications of the peptide aggregates. *Biotechnol. Reports* **2015**, *5*, 63–69.

Chapter 5

Soy lecithin-derived nanoliposomes loaded with whey peptides – effect of peptide molecular hydrophobicity on encapsulation efficiency and physicochemical properties of liposomes

Aishwarya Mohan, Chibuike C. Udenigwe*

Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, NS, B2N 5E3, Canada

*Corresponding author

E-mail: cudenigwe@dal.ca; Phone: +1 902 843-6625; Fax: +1 902 843-1404

Submitted to *Food & Function* April 27, 2016

Abstract

Liposome encapsulation of food-derived bioactive peptides is a potential, yet relatively less explored area of research. Hence the focus of the present study is to understand the underlying influence of whey peptides' molecular hydrophobicity and surface properties on the encapsulation efficiency and physicochemical properties of soy lecithin-derived liposomes. Peptide fractionation based on hydrophobicity increased the hydrophobic amino acid content (HAA) of the hydrophobic whey peptide fraction (HBW) by 1.36-fold than the hydrophilic whey peptide (HLW) fraction. Molecular hydrophobicity did not alter the EE and the mean particle diameter of HLW and HBW. However, EE of both peptide fractions were significantly higher than that of the crude whey protein hydrolysate. Moreover, ζ-potential, a measure of surface charge, was different in the fractions compared to the hydrolysate with HLW having the least stable suspension followed by WPH and then HBW, with highest ζ-potential. Peptides from the two fractions and hydrolysate appeared to be distributed both in the polar region and lipophilic region of the bilayer with shifts in FTIR spectral peaks of choline, phosphate group and CH_2. The differences in the peptide surface and molecular hydrophobicity possibly influenced the peptide distribution in the liposomes. Understanding the influence of peptide hydrophobicity is essential in improving and adapting this delivery technique for bioactive peptides.

Keywords: hydrophilic peptides, hydrophobic peptides, liposomes, encapsulation, molecular hydrophobicity, surface hydrophobicity, whey protein

1. Introduction

Liposomes are small spherical vesicles formed by self-assembly of one or more phospholipid bilayers in an aqueous environment.[1] Owing to their similarity to biological membranes, liposomes find several applications including use as a biomembrane model, analytical tool, and therapeutic agent.[1] However, among the wide range of uses, its application in delivery or as a carrier has received considerable interest. Specifically, the use of liposomes for drug delivery has been extensively explored.[2] Currently, several therapeutic formulations enclosed in liposome are commercially available for clinical treatment of various diseases.[3] This application of liposomes has extended its relevance to food industry.[4] The nano/microcarrier has been investigated for delivery of unstable or sensitive components such as enzymes, proteins, flavour compounds, bioactive molecules, and vitamins.[5] The application of liposomes in the food industry has been facilitated by the biocompatibility, biodegradability and safety of these carriers.

Bioactive peptides derived from food proteins are promising functional food ingredients due to their broad spectrum of bioactivities including antioxidant, antihypertensive, anti-inflammatory, hypolipidemia, and immunomodulatory activities.[6] Hence, bioactive peptides have been considered for use in food-based management of non-communicable diseases such as cardiovascular disease, chronic inflammation, and cancer. However, some challenges faced in applying peptides as functional ingredients include issues with their bioavailability and biostability in the gastrointestinal tract.[4] Due to instability of peptides, encapsulation has become essential for their protection and efficient delivery. Bioactive peptides are highly chemically reactive and sensitive biomolecules;[7] therefore, it is preferable to encapsulate them with mild delivery agents that can be formed without the use of extreme preparation conditions. Protein and polysaccharide carriers, although highly stable, can possibly result in covalent interactions with

the peptides, thereby altering their structure and functionality, and also making it difficult to release them from the carriers. Liposomes are preferred for their mild preparation conditions and the interactions of the peptides with the liposomes are largely based on hydrophobic and electrostatic interactions. Although liposomes have been extensively studied for the delivery of peptides of microbial origin,[1,8–11] the technique is yet to be extensively utilized for delivery of food protein-derived bioactive peptides.[4] The heterogeneity of peptides within protein hydrolysates is thought to result in low encapsulation efficiency and to impact other properties of encapsulation, hampering its application in food peptide-liposome encapsulation compared to other delivery techniques. To date, there is a dearth of literature on the effect of peptide hydrophobicity on liposome encapsulation. Peptide hydrophobicity is particularly important as it has been linked to bioactivity, and the structure of liposomes can interact with the lipophilic peptides during encapsulation *via* hydrophobic interactions. Thus, the present study focuses on the effect of molecular hydrophobicity of whey peptide fractions on encapsulation efficiency and physicochemical properties of the liposomes.

2. Materials & Methods

2.1. Whey protein hydrolysis

Whey protein isolate was purchased from Bulk Barn Ltd. (Truro, NS, Canada). A 5% (w/v) suspension of the protein was made and hydrolyzed with papain, a plant derived protease obtained from papaya latex. Hydrolysis was carried out for 5 hours under optimum temperature and pH of 65°C and 7.0, respectively. The hydrolysis was terminated by heating to 95°C for 15 min. Thereafter, the hydrolysate was allowed to cool and then centrifuged at 5000 × g for 10 mins. The supernatant was collected and lyophilized to get the hydrolysate.

2.2. Separation of hydrophobic and hydrophilic fraction

The hydrophobic fraction of the whey protein hydrolysate was separated using activated carbon by following the method previously reported by Udenigwe & Aluko,[12] with modifications. A 10 mg mL^{-1} solution of WPH in phosphate buffer (7.0, 10mM) was mixed with activated carbon (2.5%, w/v). The mixture was vortexed intermittently for 15 min and then centrifuged at 5000 × g for 30 min. The supernatant was filtered through Whatman No.1 filter paper. The filtrate was further centrifuged at 20000 × g for 30 min to completely remove the activated carbon. The supernatant was collected as hydrophilic whey peptides (HLW) fraction. The residual activated carbon from the initial centrifugation was mixed with 40 mL of >90% methanol followed by vortex mixing and centrifugation as described above to obtain activated carbon free-supernatant containing hydrophobic whey peptides (HBW). Thereafter, methanol was removed from the extract by rotary evaporation at 50°C. The HBW fraction was then suspended in phosphate buffer (pH 7.0, 10 mM), lyophilized and both fractions were stored at -20°C until further analysis. The two fractions (HLW, HBW) and WPH were used for encapsulation using soy lecithin-derived liposomes.

2.3. Amino Acid analysis

Amino acid composition of HLW and HBW was analyzed by the SPARC BioCentre, The Hospital for Sick Children, Toronto, Canada. The samples were initially dried in a vacuum centrifugal concentrator and then hydrolyzed using 6 N HCl/1% phenol at 110°C for 24 h. Precolumn derivatization was performed with phenylisothyocyanate at room temperature on the acid hydrolyzed samples using the Pico-Tag method. Reverse phase high performance liquid chromatography with Waters ACQUITY UPLC (Milford, MA) on a 10-cm C-18 column (0.21 ×

10 cm) was used to analyse the amino acid derivatives suspended in phosphate buffer. The peaks were observed with a UV detector at 254 nm and amino acid composition was calculated as mole%.

2.3. Free amino content determination

The free amino content of HLW and HBW fractions was determined as previously reported by Mohan et al.[7] Briefly, 33 µL of sample (1 mg mL^{-1}) was mixed with 250 µL of O-phthaldehyde reagent in 96-well plate and the absorbance was read at 340 nm. L-Serine (0.1 mg mL^{-1}) was used as standard and the free amino content was obtained as milliequivalent serine NH_2/g protein, and used as an indicator of number of peptides per unit mass of the fraction.

2.4. Surface hydrophobicity (S_o) determination

Surface hydrophobicity of HLW, HBW fractions and WPH was determined as previously reported,[13] using the fluorescent hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS). 100 µL of sample (0.0009–0.015%) was mixed with 100 µL of 4 mM ANS and the excitation and emission was measured at 390 and 470 nm, respectively. The concentration vs. fluorescence graph was plotted, and the slope of the linear curve was derived and used as the surface hydrophobicity (S_o).

2.5. Liposome encapsulation of peptide fraction

WPH, HLW and HBW were encapsulated in soy lecithin-derived liposomes by film hydration method.[14] Crude soy lecithin (Bulk Barn Foods Ltd., Truro, NS, Canada) was powdered using a coffee grinder and dissolved at 1% (w/v) in chloroform. The lecithin solution was transferred to a round bottom flask and rotary evaporated at 60°C until a uniformly dispersed lipid film was formed in the bottom of the flask. The prepared lipid films were left in the desiccator overnight to ensure complete removal of chloroform. The lipid layer was hydrated under agitation (150

rpm) with phosphate buffer (10 mM, pH 7.0) containing each of the whey peptide fractions or the hydrolysate (1 mg mL^{-1}), and the suspension was then sonicated for 30 min to obtain a homogenous dispersion of liposomes. The encapsulated product was then used for further analysis.

2.6. Encapsulation Efficiency

The ratio of encapsulated peptides (P_E) and initial amount of peptides (P_I) used in liposome preparation was expressed in percentage as encapsulation efficiency (EE), i.e. $EE = 100 \times P_E/P_I$. The non-encapsulated peptides (P_{NE}) were separated from encapsulated peptides by passing the liposome preparation through an ultrafiltration membrane with a molecular weight cut-off of 100 kDa under a constant pressure of 10 psi. The encapsulated peptide concentration was indirectly estimated by determining the protein content of the filtrate: $P_E = P_I - P_{NE}$

2.7. Peptide-loaded liposome surface characterization

A zetasizer (Nano SeriesNano-ZS, Malvern Instruments Ltd., Malvern, UK) was used to determine the surface charge, mean particle diameter and polydispersity index (PDI) of the liposomes. The samples were suspended in cuvettes using deionized water and measurements were taken at appropriate dilutions.

2.8. Fourier transform infrared spectroscopy (FTIR)

The interaction of the whey peptide fractions with the phospholipid bilayer and the distribution of the peptides within the liposome were studied through the infrared spectra of the lyophilized nanoliposomes. Attenuated Total Reflectance (ATR) FTIR spectra of the liposomes were obtained as an average of 16 scans in the region of 4000-600 cm^{-1} using an FTIR spectrometer (Spectrum One, Perkin Elmer, CT, USA).

2.9. Transmission electron microscopy (TEM)

The peptide-loaded liposomes were applied as drops on the 200-mesh carbon coated copper grid and allowed to stand for a few minutes. Excess sample was then drawn off with a filter paper. A negative stain, uranyl acetate (2% w/v), was applied on the sample-loaded grid and allowed to stand for 2 min. Surplus stain was then removed by immersing the grid several times in deionized water. The prepared grids were allowed to dry for 1 h before visualizing under TEM (Model Tecnai-12, Philips Electron Optics, Netherlands).

2.10. Statistical Analysis

Apart from protein hydrolysis, fractionation and amino acid analysis, all the experiments and assays were performed in triplicate. Results are expressed as mean ± standard deviation. Significance of the difference between the means were determined by one way analysis of variance followed by Holm Sidak multiple comparison test. Statistical analyses were performed using Sigmaplot 12.1 (Systat Software, San Jose, CA).

3. Results & Discussion

3.1. Amino acid composition of the fractions

Amino acid composition of the hydrophilic and hydrophobic whey fractions were analyzed to determine their molecular hydrophobicity for ensuring the effectuation of the separation using activated carbon. The contents of hydrophobic amino acids (HAA) viz. Leu, Ile, Phe, Val and Met were higher in the HBW fraction, with lower contents of hydrophilic amino acids Asx, Glx, Ser and Lys contents compared to HLW (Fig. 5.1). The total HAA of HLW was 14 mole% lower than that of HBW. Furthermore, the hydrophobic-hydrophilic amino acid ratio (HHR) of HBW was calculated to be 2-fold higher than that of HLW (Table 5.1). Therefore, fractionation of

WPH by direct mixing with activated carbon resulted in fractions with different molecular hydrophobicity. It is also noteworthy that among the HAA of HBW, the contents of aromatic HAA residues (Tyr and Phe) doubled compared to HLW, and the aliphatic HAA content increased by a smaller margin. This can be attributed to the stronger affinity of aromatic amino acids to activated carbon compared to aliphatic amino acids.[12] Although HLW had higher content of cationic amino acids (CAA), HBW had a higher Arg content; the hydrophobicity of Arg is relatively higher than those the other CAAs and possibly enhanced the binding of the Arg containing peptides to activated carbon.

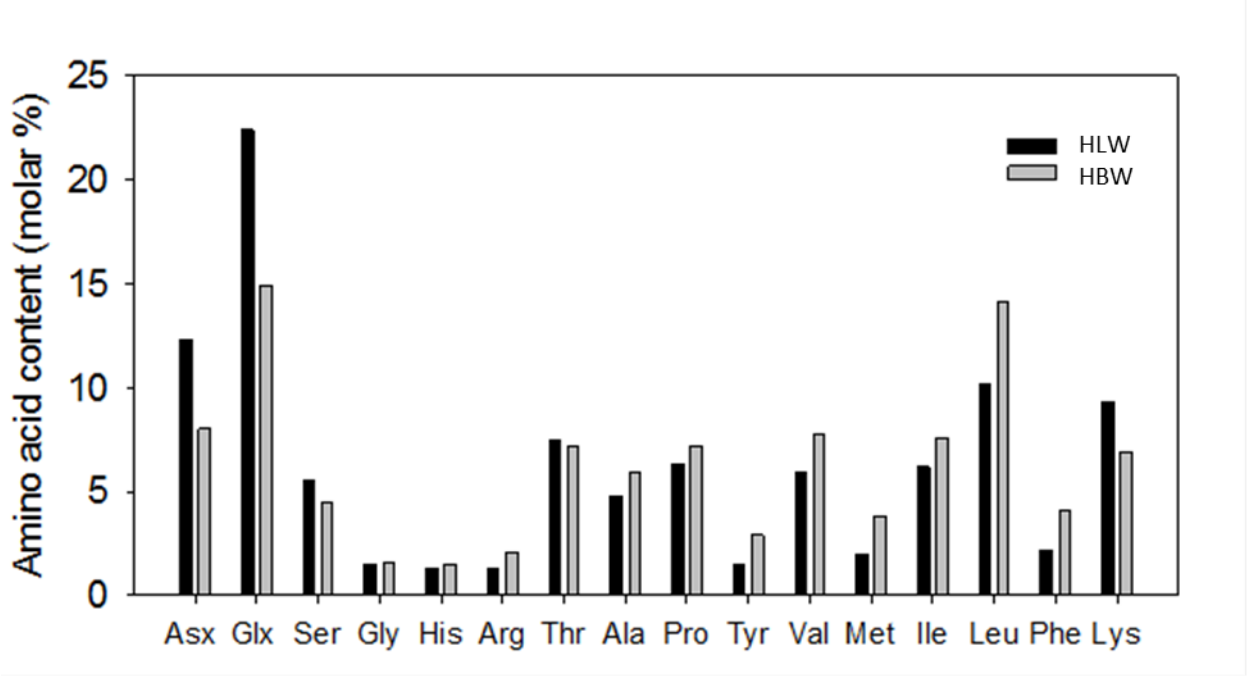


Fig. 5.1 Amino acid composition of hydrophilic (HLW) and hydrophobic (HBW) whey peptide fractions.

Table 5.1. Hydrophobic-hydrophilic amino acid ratio (HHR) and cationic amino acid (CAA) content of hydrophilic (HLW) and hydrophobic (HBW) whey peptide fractions.

	HLW	**HBW**
HHR	0.75	1.55
CAA	11.9	10.5

3.2. Effect of hydrophobicity on the encapsulation efficiency

The amount of compound trapped in liposomes is an important parameter indicating the efficiency of the encapsulation process.[4] EE of over 80% was achieved for the whey peptides and fractions, and these values were higher than the EE reported for liposome encapsulation of foodderived protein hydrolysates reported in the literature.[14–17] Nevertheless, the encapsulation of antimicrobial peptides of microbial origin such as nisin have resulted in high EE,[8] and similar results have been reported for therapeutic drugs with EE as high as 100%.[18] The EE of the whey peptide fractions separated based on their molecular hydrophobicity did not vary (Fig. 5.2). However, the EE of both fractions were significantly higher than that of WPH. A high content of HAA or high HHR value did not alter the entrapment of the peptide fraction in the nanocapsules based on EE. It is possible that the mechanism leading to encapsulation is different for the fractions with varying molecular hydrophobicity. Although the HAA content of HLW was lower, the surface hydrophobicity (S_o) was 1.5 times higher than that of HBW (Fig. 5.3B). The higher S_o of HLW is expected to enhance hydrophobic interaction of the peptides with hydrophobic region of the liposomes. On the other hand, the higher Arg content and lower amounts of Asp and Glu, which can be indirectly derived from the decrease in Asx and Glx contents of HBW may have facilitated the increased electrostatic interaction with the phosphate group of the bilayer. The contrast between the molecular hydrophobicity and surface hydrophobicity could have made the net effect on EE unnoticeable. Interestingly, the S_o of WPH was 1.5 and 2.2 folds higher than those of HLW and HBW, respectively, even though EE was significantly lower for WPH. Free amino group, which can be used as an indicator of the number of peptide per unit mass, was highest for WPH followed by HLW and then HBW (Fig. 5.3A). The reverse of this trend was observed in EE. It is possible that the higher number of peptides in the fractions,

indicating an increased heterogeneity, impaired EE since the different peptide properties compound their encapsulation.

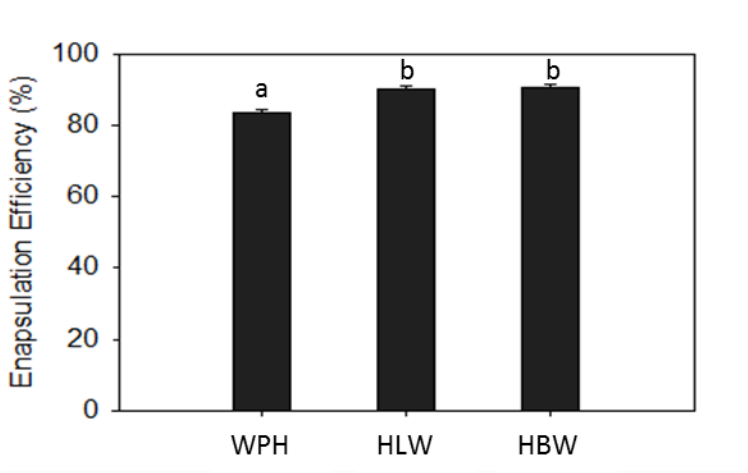


Fig. 5.2 Encapsulation efficiency (EE, %) of whey protein hydrolysate (WPH), hydrophilic (HLW) and hydrophobic (HBW) whey peptide fractions.

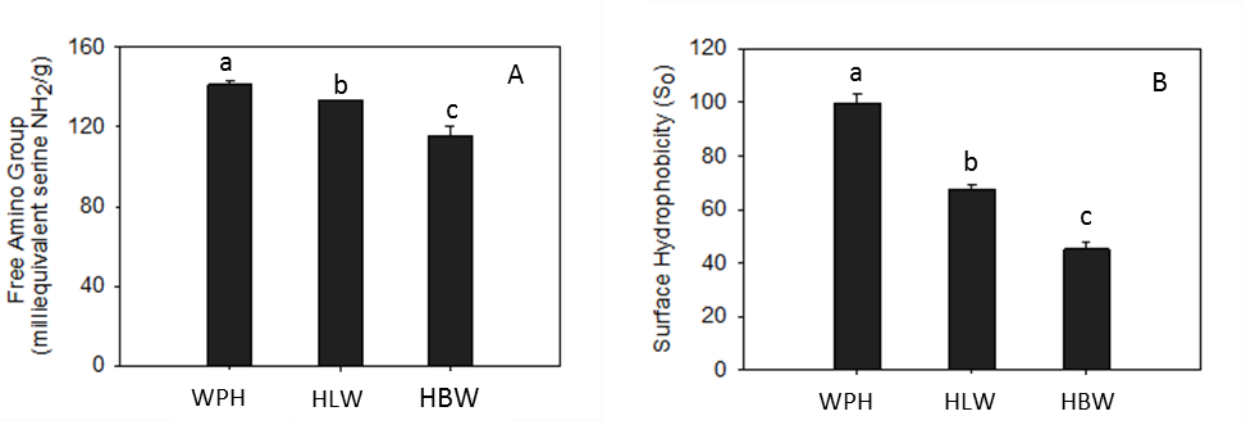


Fig. 5.3 (A) Free amino content expressed as milliequivalent serine NH_2/g protein, and (B) Surface hydrophobicity (S_o) of whey protein hydrolysate (WPH), hydrophilic (HLW) and hydrophobic (HBW) whey peptide fractions.

3.3. Effect of hydrophobicity on the physicochemical properties of the liposomes

3.3.1. Zeta potential (ζ). Surface charge or ζ-potential is an important parameter that indicates stability of the liposome suspension.[4] A higher magnitude of surface charge either in the positive or negative direction is essential for electrostatic repulsion of the vesicles in the suspension, preventing them from coalescing. Aqueous suspension with ζ-potential of ±30 mV is considered to be of good stability.[14] The surface charge of the peptide-loaded liposomes was found to be lower than that of the control liposomes, which was -55 mV (Table 5.2). Encapsulation of peptides and protein hydrolysates has been demonstrated to decrease the surface charge of liposomes when compared to empty liposomes.[14,15] Nevertheless, the low ζ-potential observed for HLW, with a value close to zero, indicates that the suspension is highly unstable. However, liposomes derived from HBW fraction had a higher ζ-potential. The surface charges of liposomes with WPH and HLW were 2 and 10 folds lower than those of liposomes with HBW, respectively. Although the low ζ-potential could partially be attributed to the net increase in CAA content of HLW, the increase in anionic amino acid residues (observed from Asx and Glx) is expected to have balanced the effect, which was not observed. The high S_o along with increased number of peptides could have triggered the peptide aggregation, thereby masking the effect of the acidic amino acid residues and making the suspension unstable. The ζ-potential of the WPH liposomes was found to fall between the values observed for liposomes containing HLW and HBW showing that it is a heterogeneous mixture of both the fractions. Apart from lipid composition of the liposomes, the variation in amino acid composition as a result of fractionation based on molecular hydrophobicity, along with surface properties of the peptides may have played important roles in determining the stability of the nanoliposomes.

3.3.2. Mean particle diameter. Medium-large sized unilamellar vesicles of less than 150 nm were obtained in this study (Table 5.2).[19] Small-large unilamellar vesicles are favourable for the delivery of bioactive compounds. The mean particle diameters of the encapsulated peptide fractions were not significantly different from each other, and also from that of the WPH liposomes. Molecular hydrophobicity of HBW, based on higher HAA content and HHR, did not contribute to any obvious changes in the liposome particle size. Notably, the standard deviation obtained for mean particle size of WPH liposomes was very high, which can be due to the heterogeneous nature of peptides within the hydrolysate along with the uncontrolled and spontaneous nature of liposome formation.[4]

3.3.3. Polydispersity index. PDI is a measure of homogeneity of the particle size distribution of a suspension. The value of PDI ranges from 0 to 1, and a low PDI indicates monodisperse population.[15] The PDI value of the liposomes containing WPH and the two peptide fractions were in the range of 0.30–0.35; similar values have been reported for peanut peptide-loaded liposomes.[20] Typically, PDI values for liposomes loaded with peptides and protein hydrolysates are approximately 0.2.[14,15,21] Although not significant, the HLW liposomes had higher PDI value than the HBW liposomes, plausibly due the different number of peptides per unit mass protein in the samples. The number of peptides was slightly higher ($p<0.05$) for WPH and its liposomes had a PDI value around the value observed for the HLW liposomes. Similar to the mean particle diameter, the standard deviation was relatively higher for PDI of WPH liposomes, possibly indicating the molecular diversity of the hydrolysate. However, the low surface charge of the liposomes and high S_o of the peptide fractions can have notable effects on PDI, possibly inducing aggregation.

Table 5.2. Zeta (ζ)-potential, mean particle diameter and polydispersity index of liposomes encapsulated whey protein hydrolysate (WPH), and the hydrophilic (HLW) and hydrophobic (HBW) peptide fractions.

	Zeta Potential (ζ) mV	**Mean Particle Diameter (nm)**	**Polydispersity Index (PDI)***
WPH	$-14.6 \pm 4.0^{a,b}$	127.5 ± 17.7^{a}	0.335 ± 0.099
HLW	-2.5 ± 0.5^{b}	115.2 ± 5.2^{a}	0.346 ± 0.033
HBW	-26.8 ± 2.8^{a}	121.8 ± 6.7^{a}	0.307 ± 0.032

Mean values that are significantly different have different alphabets in the column and numbers without statistical significance have same letters

* The power was below the desired level for the performed statistical test (with $\alpha = 0.05$) to observe significant difference among the mean.

3.4. Interaction of WPH, HLW and HBW with the nanovesicles

FTIR spectra was used to understand the molecular interaction between the peptides in HLW, HBW and WPH, and the liposome lipid bilayer. The spectra of empty liposomes was used as the control to observe any shift in peak frequency as a result of the peptides. The asymmetrical stretching vibrations of $(CH_3)_3N$ of choline was observed in the region between 950–980 cm^{-1} with the peak maximum at 970 cm^{-1}. Entrapment of protein hydrolysate and the two peptide fractions shifted the peak maximum of $(CH_3)_3N$ to a higher frequency (Table 5.3). Despite the high S_o, HLW was found to interact with choline on the liposome surface; this can be attributed to the net decrease in HAA and increase in hydrophilic amino acid residues in HLW. Similarly, increases in the contents of Asx and Glx, which partly represent anionic amino acids Asp and Glu, respectively, may have enhanced electrostatic interaction between the peptides and the liposome choline group. Moreover, the shift in $(CH_3)_3N$ peak are observed to be small, merely one or two wavenumbers.[15,22]

The PO_2 vibration can be observed at various peak maxima occurring in broad regions including 1000-1200 cm^{-1} and 740-900 cm^{-1}.[23] In our study, the region at the higher frequency was found to overlap with the broad PO_2 stretch of the quaternary amine group of choline with peak maximum at 1063 cm^{-1}. Therefore, the lower frequency region was used to observe the PO_2 stretch with peak apex at 871 cm^{-1}. The PO_2 stretch peak maximum was shifted to a lower frequency for the peptide-loaded liposomes for all the three samples (WPH, HLW and HBW). As mentioned earlier, the higher Arg content of HBW, which has stronger interaction than Lys, along with decreased content of Asx and Glx, could have electrostatically favoured the interaction of HLW, despite its higher molecular hydrophobicity. The phosphate stretch of the quaternary amine group was observed at 1063 cm^{-1} for the empty liposome, and this shifted to a

higher frequency for the liposomes containing the hydrolysate and two peptide fractions. The lipophilic region of the liposomes can be observed by the symmetrical stretching of CH_2 band at 2854 cm^{-1}. This band was slightly shifted to 2853 cm^{-1} in the peptide-loaded liposomes, suggesting the distribution of peptides in the bilayer region for all the samples. The asymmetrical stretching vibration of CH_3 at 2924 cm^{-1} did not show any change or interaction. The liposome images (Fig. 5.4) were used to confirm the formation of nanovesicles. Aggregates were observed in the HLW and HBW liposomes, plausibly due to the low surface charges of the liposomes.

Table 5.3. Peak maxima of liposome functional groups derived from FTIR spectra of the control liposomes, and liposomes loaded with whey protein hydrolysate and peptide fractions with different molecular hydrophobicity

	Empty Liposome	WPH	HLW	HBW
$(CH_3)_3N$	970	972	973	972
PO_2	871	869	866	859
Broad PO_2 stretch	1063	1065	1067	1067
CH_2	2854	2853	2853	2853
CH_3	2924	2924	2924	2924

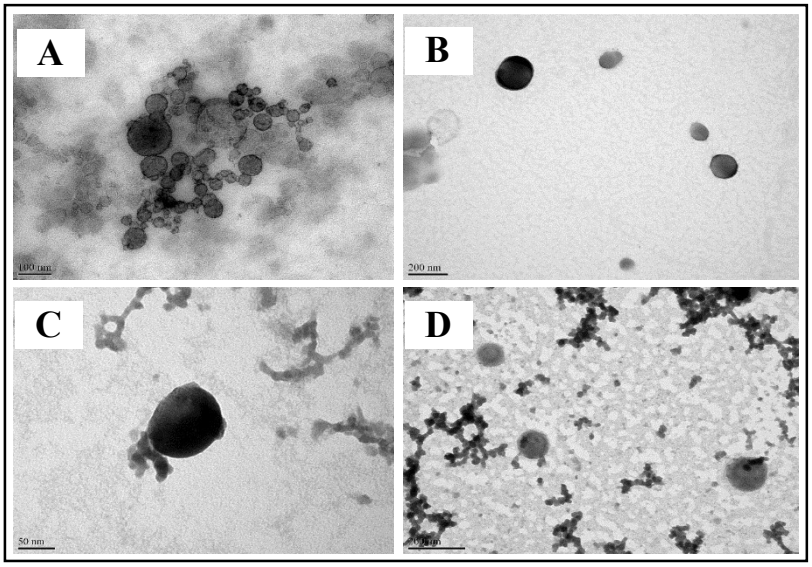


Fig. 5.4 Transmission electron microscopy of (A) empty liposomes, and liposomes loaded with (B) whey protein hydrolysate, (C) hydrophilic, and (D) hydrophobic whey peptide fractions.

4. Conclusions

In this study, increase in molecular hydrophobicity did not have an effect on the encapsulation efficiency of the whey peptide fractions in liposomes. However, fractionation of the whey peptides based on their hydrophobicity significantly increased the EE compared to liposomes loaded with WPH. Despite the decrease in the content of hydrophobic amino acid residues, the surface hydrophobicity of the hydrophilic whey peptides (HLW) was higher than that of the hydrophobic whey peptide fraction (HBW). Molecular hydrophobicity had no obvious effect on the particle size of the resulting liposomes. However, the liposome surface charge varied considerably with the HLW liposomes displaying the least surface charge. The polydispersity index of the encapsulated HLW was also higher, as was the free amino content (an indication of the number of peptides), indicating heterogeneity in size distribution of the liposomes. The interaction of peptides with the lipid bilayer is suggestive of the distribution of peptides in the polar and lipophilic regions of the liposomes irrespective of their molecular hydrophobicity. The net effect of increase in molecular hydrophobicity in the hydrophobic peptide fraction could plausibly be nullified due to the surface properties of the hydrophilic peptide fraction. The outcomes of this study indicate that the peptide surface properties play a significant role in determining the physicochemical properties of the peptide-loaded liposomes, and their interaction with the lipid bilayer. This insight will facilitate ongoing efforts in formulating better and efficient bioactive peptide delivery systems using liposomes.

Acknowledgment

This project was financially supported through Discovery Grant of C.U. from Natural Sciences and Engineering Research Council of Canada (NSERC) and Canada Foundation for Innovation

(CFI) and Nova Scotia Research and Innovation Trust Fund (NSRIT) through an Infrastructure Grant. Nova Scotia Research and Innovation Graduate Scholarship was awarded to A.M. We thank Sherryl Patton and Robin Spence of Intertape Polymer Group (IPG) for providing access to FTIR. We also thank Subin R. C. K. Rajendran for the assistance in data collection.

References

1 P. da Silva Malheiros, D. J. Daroit and A. Brandelli, *Trends Food Sci. Technol.*, 2010, **21**, 284–292.

2 A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S. W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi and K. Nejati-Koshki, *Nanoscale Res. Lett.*, 2013, **8**, 102.

3 T. M. Allen and P. R. Cullis, *Adv. Drug Deliv. Rev.*, 2013, **65**, 36–48.

4 A. Mohan, S. R. C. K. Rajendran, Q. S. He, L. Bazinet and C. C. Udenigwe, *RSC Adv.*, 2015, **5**, 79270–79278.

5 M. R. Mozafari, K. Khosravi-Darani, G. G. Borazan, J. Cui, A. Pardakhty and S. Yurdugul, *Int. J. Food Prop.*, 2008, **11**, 833–844.

6 C. C. Udenigwe and R. E. Aluko, *J. Food Sci.*, 2012, **77**, R11–R24.

7 A. Mohan, M. C. Udechukwu, S. R. C. K. Rajendran and C. C. Udenigwe, *RSC Adv.*, 2015, **5**, 97400–97407.

8 P. S. Malheiros, I. M. Cuccovia and B. D. G. M. Franco, *Food Control*, 2016, **63**, 158–164.

9 P. da Silva Malheiros, V. Sant'Anna, Y. M. S. Micheletto, N. P. da Silveira and A. Brandelli, *J. Nanoparticle Res.*, 2011, **13**, 3545–3552.

10 M. B. de Mello, P. da Silva Malheiros, A. Brandelli, N. Pesce da Silveira, M. M. Jantzen and A. de Souza da Motta, *Probiotics Antimicrob. Proteins*, 2013, **5**, 43–50.

11 Y. Zou, H.-Y. Lee, Y.-C. Seo and J. Ahn, *J. Food Sci.*, 2012, **77**, M165–70.

12 C. C. Udenigwe and R. E. Aluko, *J. Agric. Food Chem.*, 2010, **58**, 4762–4768.

13 A. Mohan and C. C. Udenigwe, *J. Funct. Foods*, 2015, **18**, 129–136.

14 M. Mosquera, B. Giménez, I. M. da Silva, J. F. Boelter, P. Montero, M. C. Gómez-Guillén and A. Brandelli, *Food Chem.*, 2014, **156**, 144–50.

15 E. da Rosa Zavareze, A. C. Telles, S. L. Mello El Halal, M. da Rocha, R. Colussi, L. Marques de Assis, L. A. Suita de Castro, A. R. Guerra Dias and C. Prentice-Hernández, *LWT - Food Sci. Technol.*, 2014, **59**, 841–848.

16 D. Yokota, M. Moraes and S. C. Pinho, *Brazilian J. Chem. Eng.*, 2012, **29**, 325–335.

17 C. M. S. Barbosa, H. a. Morais, F. M. Delvivo, H. S. Mansur, M. C. De Oliveira and M. P. C. Silvestre, *J. Sci. Food Agric.*, 2004, **84**, 1891–1900.

18 N. Dos Santos, K. A. Cox, C. A. McKenzie, F. van Baarda, R. C. Gallagher, G. Karlsson, K. Edwards, L. D. Mayer, C. Allen and M. B. Bally, *Biochim. Biophys. Acta*, 2004, **1661**, 47–60.

19 A. Gómez-Hens and J. Manuel Fernández-Romero, *TrAC Trends Anal. Chem.*, 2005, **24**, 9–19.

20 K.-J. Gong, A. Shi, H. Liu, L. Liu, H. Hu, Y. Yang, B. Adhikari and Q. Wang, *Food Funct.*, 2016.

21 Z. Li, A. T. Paulson and T. A. Gill, *J. Funct. Foods*, 2015, **19**, 733–743.

22 D. Pentak, *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.*, 2014, **122**, 451–60.

23 J. M. Nzai and A. Proctor, *J. Am. Oil Chem. Soc.*, 1998, **75**, 1281–1289.

Chapter 6

Discussion and Conclusions

Encapsulation of food protein-derived bioactive peptides is an essential delivery technique to enhance their structural stability and bioavailability. Peptide heterogeneity within protein hydrolysates is seen as a challenge when designing efficient peptide delivery systems. Peptide molecular structure and surface properties can influence their interaction with the carrier system. The present research project was focused on uncovering the influence of some of these peptide properties on the carrier system. Soy lecithin-derived liposomes were used in this study to encapsulate whey peptides. Molecular weight and hydrophobicity had no observable effect on the encapsulation efficiency. In contrast, net anionic charge was found to decrease the encapsulation efficiency. Encapsulation of peptide fractions with different molecular weights increased the stability of peptide-loaded liposome suspension compared to control/empty liposomes. Liposomes encapsulating fractions of whey peptides separated based on charge and hydrophobicity had lower stability than control liposome. The mean particle diameter and dispersity were relatively higher for liposomes loaded with different molecular weight fractions in comparison to charge and hydrophobicity-based peptide fractions. It was seen that other properties such as surface hydrophobicity and number of peptides per unit mass may have also played important roles in determining encapsulation efficiency and liposome properties. Moreover, it appears that peptides in all the fractions showed higher interaction with the phosphate group than the choline group of the liposomes. Findings from this study has demonstrated that multiple peptide properties can simultaneously influence the physicochemical properties of liposomes more than their encapsulation efficiency. Future direction in this area of

research will be to elucidate the effects of the peptide structure on the digestion and release kinetics of the encapsulated peptides.

Bibliography

A. Harnedy and R. J. Fitzgerald, 2011, **4**.

H. Korhonen and A. Pihlanto, *Curr. Pharm. Des.*, 2003, **9**, 1297–1308.

Z. F. Bhat, S. Kumar and H. F. Bhat, 2015, **52**, 5377–5392.

A. B. Nongonierma and R. J. Fitzgerald, *J. Funct. Foods*, 2015, **17**, 640–656.

M. Yoshikawa, *Peptides*, 2015, **72**, 208–225.

C. Jo, F. F. Khan, M. I. Khan and J. Iqbal, 2016, **9129**.

F. Van Lancker, A. Adams and N. De Kimpe, *Chem. Rev.*, 2011, **111**, 7876–903.

A. Mohan, M. C. Udechukwu, S. R. C. K. Rajendran and C. C. Udenigwe, *RSC Adv.*, 2015, **5**, 97400–97407.

P. da Silva Malheiros, D. J. Daroit and A. Brandelli, *Trends Food Sci. Technol.*, 2010, **21**, 284–292.

M. Fathi, Á. Martín and D. J. McClements, *Trends Food Sci. Technol.*, 2014, **39**, 18–39.

T. M. Allen and P. R. Cullis, *Adv. Drug Deliv. Rev.*, 2013, **65**, 36–48.

Z. Li, A. T. Paulson and T. A. Gill, *J. Funct. Foods*, 2015, **19**, 733–743.

A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S. W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi and K. Nejati-Koshki, *Nanoscale Res. Lett.*, 2013, **8**, 102.

C. P. Champagne and P. Fustier. *Curr. Opin. Biotechnol.,* 2007, **18**, 184.

C. C. Udenigwe. *Trends Food Sci*. *Tech*. 2014, **36**, 137.

D. J. McClements. Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds. CRC Press, 2014, 572 pages.

S. E. M. Ortiz, A. Mauri, E. S. Monterrey-Quintero, M. A. Trindade, A. S. Santana and C. S. Favaro-Trindade, *LWT Food Sci*. *Technol*. 2009,**42**, 919.

G. Picariello, G. Iacomino, G. Mamone, P. Ferranti, O. Fierro, C. Gianfrani. A. Di Luccia, and F. Addeo. *Food Chem*., 2013, **139**, 203-212.

M. Segura-Campos, L. Chel-Guerrero, D. A. Betancur-Ancona, and V. M. Hernandez-Escalante. *Food Rev. Int*., 2011, **27**, 213-226.

M. Fathi, M. R. Mozafari and M. Mohebbi. *Trends Food Sci Tech.,* 2012, **23**, 13-27.

G. M. Tavares, T. Croguennec, A. F. Carvalho and S. Bouhallab. *Trends Food Sci. Tech*., 2014, **37**, 5-20.

J. J. Ma, X. Y. Mao, Q. Wang, S. Yang, D. Zhang, S. W. Chen and Y. H. Li. *LWT. Food Sci. Technol.,* 2014, **56**, 296-302.

Z. Wang, X. Ju, R. He, J. Yuan and L.Wang. *Food Bioprocess. Technol*., 2015, **8**, 1305-1318.

M. J. Sáiz-Abajo, C. Monzales-Ferrero, A. Moreno-Ruiz, A. Romo- Huald, and C. J. Gonzalez-Navarro. *Food Chem*., 2013, **138**, 1581-1587.

G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan. *Food Chem.,* 2014, **160**, 46-52.

G. J. O'Neill, T. Egan, J. C. Jacquier, M. O'Sullivan and E. D. O'Riordan. *Food Chem.,* 2015, **180**, 150-155.

C. S. Favaro-Trindade, A. S. Santana, E. S. Monterrey-Quintero, M. A. Trindade and F. M. Netto. *Food Hydrocolloids*, 2010, **24**, 336-340.

J. C. Ruiz Ruiz, M. R. Segura Campos, D. A. Betancur-Ancona, and L. A. Chel-Guerro *ISRN Biotech*., 2013.

D. Yokota, M. Moraes and S. C. Pinho. *Braz. J. Chem. Eng*., 2012, **29**, 325-335.

L. E. Kurosawa, K. J. Park and M. D. Hubinger. *J. Food Eng.,* 2009, **94**, 326-333.

J. C. Silva and S.C. Pinho. *Int. J. Food Stud.,* 2013, **2**, 48.

J.A.A. Zhang and J. Pawelchak. *Eur. J. Pharm. Biopharm*., 2000, **50**, 357.

B. Zhang and X. Zhang. *Biotechnol. Prog.,* 2014, **29**, 1230.

S. Yang, X. Y. Mao, F. F. Li, D. Zhang, X. J. Leng, F. Z. Ren and G. X. Teng. *Eur. Food Res. Technol*. 2012, **235**, 91.

M. Mosquera, B. Giménez, I. M. da Silva, J. F. Boelter, P. Montero, M. C. Gómez-Guillén and A. Brandelli. *Food Chem*., 2014, **156**, 144-50.

H. A. Morais, C. M. Da Silva Barbosa, F. M. Delvivo, H. S. Mansur, M. C. De Oliviera and M. P. C. Silvestre. *J. Food Biochem*., 2004, **28**, 21-41.

W. Liu, A. Ye, C. Liu, J. Han and H. Singh. *Food Chem*., 2015, **175**, 16-24.

C. M. D. S. Barbosa, H. A. Morais, D. C. F. Lopes, H. S. Mansur, M. C. D. Oliveira and M. P. C Silvestre. *Braz. J. Pharm. Sci.,* 2002, **38**, 361.

S. F. Subtil, G. A. Rocha-Selmi, M. Thomazini, M. A. Trindade, F. M. Netto and C. S. Favaro-Trindade. *J. Food Sci. Technol*., 2014, **51**.

G. A. Rocha., M. A. Trindade, F. M. Netto and C. S. Favaro-Trindade. *Food Sci. Technol. Int*., 2009, **15**, 407.

S. Gouin. *Trends Food Sci. Tech.,* 2004, **15,** 330-347.

D. V. Mendanha, S. E. M. Ortiz, C. S. Favaro-Trindade, A. Mauri, E. Monterrey-Quintero and M. Thomazini. *Food. Res. Int.,* 2009, **42**, 1099-1104.

L. Bazinet, and L. Firdaous. *Recent Pat. Biotechnol.*, 2013, *7*, 9.

A. Doyen, C. C. Udenigwe, P. L. Mitchell, A. Marette, R.E. Aluko, and L. Bazinet. *Food Chem.*, 2014, **145**, 66.

B. Zheng, L. Teng, G. Xing, Y. Bi, S. Yang, F. Hao, G. Yan, X. Wang, R. J. Lee, L.Teng and J. Xie. *Eur. J. Pharm. Sci.,* 2015, **77**, 254.

A. M. Carmona-Ribeiro, and L. D. D. Carrasco. *Int. J. Mol. Sci.,* 2014, **15**, 18040-18083.

Israelachvili, J. Intermolecular and Surface Forces, Third Edition (Third Edition ed.). 2011, London, UK: Academic Press.

Y. Malam, M. Loizidou, and A. M. Seifalian. *Trends in Pharmacol. Sci.,* 2009, **30**, 592-599.

P. D. Malheiros, D. J. Daroit, N. P. da Silveira, and A. Brandelli. *Food Microbiol.,* 2010, **27**, 175-178.

D. J. McClements, *Adv. Colloid Interface Sci.,* 2015a*,* **219**, 27-53.

D. J. McClements. *J. Food Sci.,* 2015b, **80**, N1602-N1611.

D. J. McClements. (2014). CRC Press, 572 pages.

E. H. Moeller, B. Holst, L. H. Nielsen, P. S. Pedersen, and J. Ostergaard. *Int. J. Pharm.,* 2010, **390**, 13-18.

M. R. Mozafari. (2010). Nanoliposomes: preparation and analysis. In *Liposomes* (pp. 29-50). Humana Press.

D. S. Pisal, M. P. Kosloski, and S. V. Balu-Iyer. *J. Pharm. Sci.,* 2010, **99**, 2557-2575.

T. M. Taylor, S. Gaysinsky, P. M. Davidson, B. D. Bruce, and J. Weiss. *Food Biophys.*, 2007, **2**, 1-9.

C. C. Udenigwe. *Trends Food Sci. Tech.*, 2014, **36**, 137-143.

C. C. Udenigwe, S. Wu, K. Drummond, and M. Gong. *J. Agri. Food Chem.*, 2014, **62**, 130-135.

Z. Li, A. T. Paulson, T. A. Gill. *J. Funct. Foods.,* 2015, **19**, 733–743.

P. da Silva Malheiros, Micheletto, Y. M. S.; Silveira, N. P. da; Brandelli, A. *Food Res. Int.* 2010, **43**, 1198–1203.

M. L. Teixeira, J. dos Santos, N. P. Silveira, and A. Brandelli. *Innov. Food Sci. Emerg. Technol.* 2008, **9**, 49–53.

R. Laridi, E. Kheadr, R. O. Benech, J. Vuillemard. C. Lacroix, and I. Fliss. *Int. Dairy J.* 2003, **13**, 325–336.

D. J. McClements. *Nanoparticle- and Microparticle-based Delivery Systems: Encapsulation, Protection and Release of Active Compounds*; CRC Press, 2014.

A. Doyen, E. Husson, and L. Bazinet. *Food Chem.* 2013, **136**, 1193–1202.

C. C. Udenigwe, M. C. Udechukwu, C. Yiridoe, A. Gibson, and M. Gong. *J. Funct. Foods* 2016, **20**, 195–203.

A. Mohan, and C. C. Udenigwe. *J. Funct. Foods.,* 2015, **18**, 129–136.

V. R. Young, and A. M. Ajami. *J. Nutr.* **2000**, *130* (4), 892 – .

M. Imran, A. M. Revol-Junelles, C. Paris, E. Guedon, M. Linder, and S. Desobry. *LWT - Food Sci. Technol.* 2015, **62**, 341–349.

B. Maherani, E. Arab-Tehrany, A. Kheirolomoom, F. Cleymand, and M. Linder. *Food Chem.* 2012, **134**, 632–640.

N. Schmidt, A. Mishra, G. H. Lai, and G. C. L. Wong. *FEBS Lett.* 2010, **584**, 1806–1813.

M. Gong, A. Mohan, A. Gibson, and C. C. Udenigwe. *Biotechnol. Reports* **2015**, *5*, 63–69.

A. Mohan, S. R. C. K. Rajendran, Q. S. He, L. Bazinet and C. C. Udenigwe, *RSC Adv.*, 2015, **5**, 79270–79278.

M. R. Mozafari, K. Khosravi-Darani, G. G. Borazan, J. Cui, A. Pardakhty and S. Yurdugul, *Int. J. Food Prop.*, 2008, **11**, 833–844.

C. C. Udenigwe and R. E. Aluko, *J. Food Sci.*, 2012, **77**, R11–R24.

P. S. Malheiros, I. M. Cuccovia and B. D. G. M. Franco, *Food Control*, 2016, **63**, 158– 164.

P. da Silva Malheiros, V. Sant'Anna, Y. M. S. Micheletto, N. P. da Silveira and A. Brandelli, *J. Nanoparticle Res.*, 2011, **13**, 3545–3552.

M. B. de Mello, P. da Silva Malheiros, A. Brandelli, N. Pesce da Silveira, M. M. Jantzen and A. de Souza da Motta, *Probiotics Antimicrob. Proteins*, 2013, **5**, 43–50.

Y. Zou, H.-Y. Lee, Y.-C. Seo and J. Ahn, *J. Food Sci.*, 2012, **77**, M165–70.

C. C. Udenigwe and R. E. Aluko, *J. Agric. Food Chem.*, 2010, **58**, 4762–4768.

E. da Rosa Zavareze, A. C. Telles, S. L. Mello El Halal, M. da Rocha, R. Colussi, L. Marques de Assis, L. A. Suita de Castro, A. R. Guerra Dias and C. Prentice-Hernández, *LWT - Food Sci. Technol.*, 2014, **59**, 841–848.

C. M. S. Barbosa, H. a. Morais, F. M. Delvivo, H. S. Mansur, M. C. De Oliveira and M. P. C. Silvestre, *J. Sci. Food Agric.*, 2004, **84**, 1891–1900.

N. Dos Santos, K. A. Cox, C. A. McKenzie, F. van Baarda, R. C. Gallagher, G. Karlsson, K. Edwards, L. D. Mayer, C. Allen and M. B. Bally, *Biochim. Biophys. Acta*, 2004, **1661**, 47–60.

A. Gómez-Hens and J. Manuel Fernández-Romero, *TrAC Trends Anal. Chem.*, 2005, **24**, 9–19.

K.-J. Gong, A. Shi, H. Liu, L. Liu, H. Hu, Y. Yang, B. Adhikari and Q. Wang, *Food Funct.*, 2016, **7**, 2034-2042.

Z. Li, A. T. Paulson and T. A. Gill, *J. Funct. Foods*, 2015, **19**, 733–743.

D. Pentak, *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.*, 2014, **122**, 451–60.

J. M. Nzai and A. Proctor, *J. Am. Oil Chem. Soc.*, 1998, **75**, 1281–1289.

APPENDIX

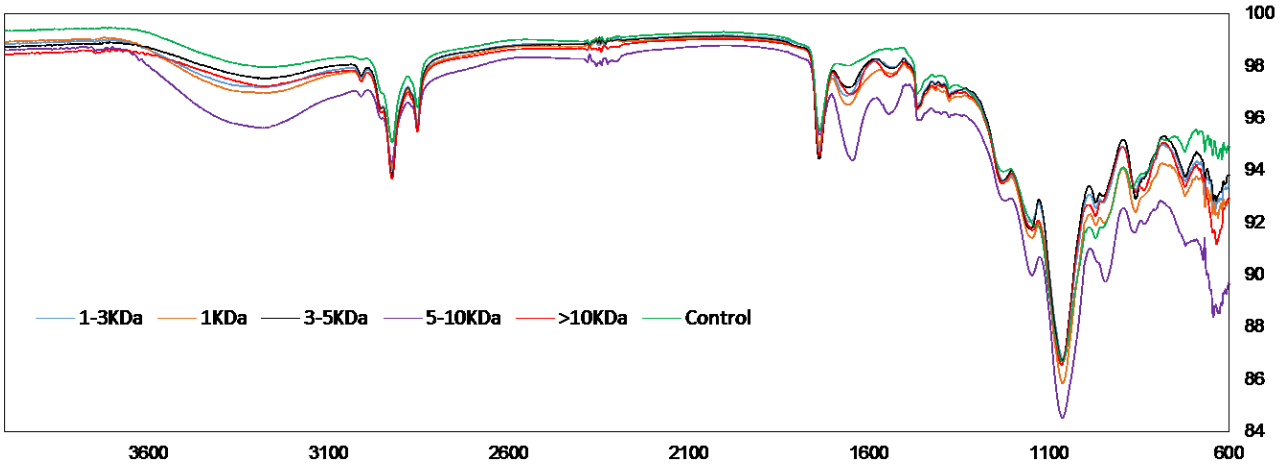


Fig A.1. FTIR spectra of control liposomes and liposomes loaded with <1kDa, 1-3kDa, 3-5kDa, 5-10kDa and >10kDa molecular weight whey peptide fractions

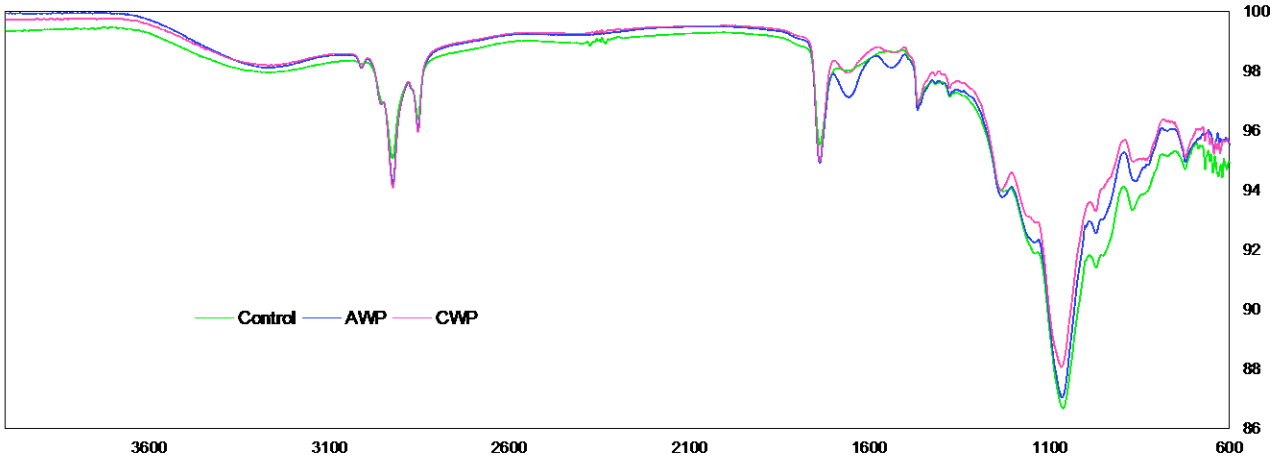


Fig A.2 FTIR spectra of control liposomes and liposomes loaded with anionic whey peptide (AWP) and cationic whey peptide (CWP) fractions

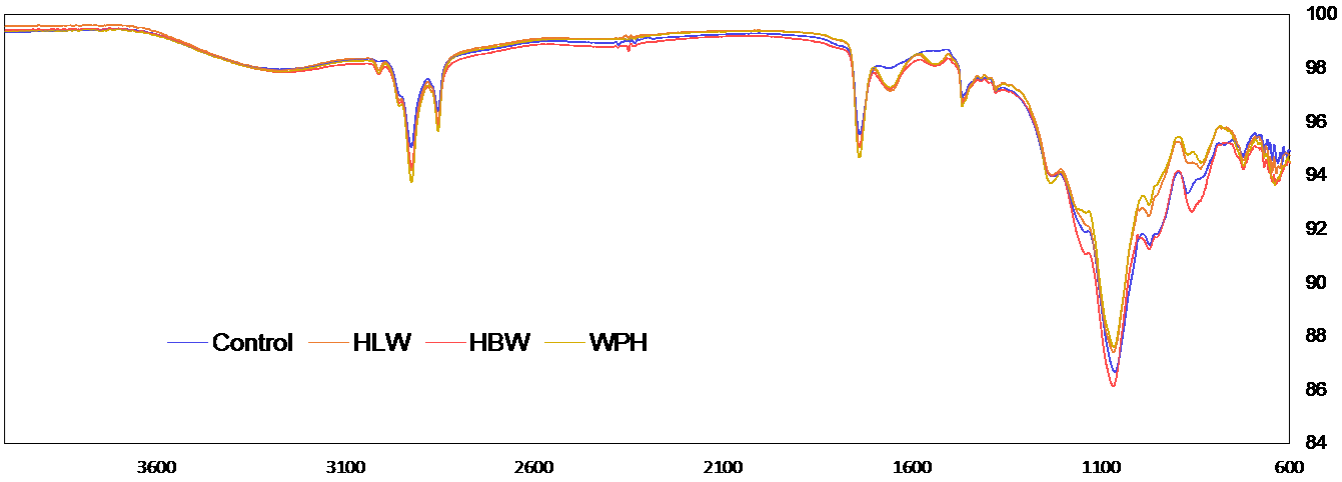


Fig. A.3 FTIR spectra of control liposomes and liposomes loaded with hydrophilic whey peptide (HLW), hydrophobic whey peptide (HBW) fractions and whey peptide hydrolysate (WPH)