CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Dairy ingredients-based emulsions and beta-carotene delivery
Authors	Lu, Wei
Publication date	2018
Original Citation	Lu, W. 2018. Dairy ingredients-based emulsions and beta-carotene delivery. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2018, Wei Lu. - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2026-10-30 04:55:32
Item downloaded from	https://hdl.handle.net/10468/6761



UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh



Teagasc Food Research Center, Moorepark

and

Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork



ucc

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Dairy Ingredients-based Emulsions and beta-Carotene Delivery

Thesis presented by

Wei Lu, BS, MS

For the degree of

Doctor of Philosophy

University College Cork

School of Food and Nutritional Sciences

Supervisors: Dr. Song Miao (Teagasc), Prof. Alan L. Kelly (UCC)

June 2018

TABLE OF CONTENTS

DECLARATION I

DEDICATION II

AKNOWLEDGEMENT III

ABSTRACT V

CHAPTER ONE 1

Introduction & Literature Review 1

Abstract 2

1. Introduction 3

2. Fundamental of Food Emulsions 4

2.1 Definition 4

2.2 Preparation and Stability 4

2.3 Properties of Emulsions 9

3. Emulsions as Encapsulation and Delivery Carriers 12

3.1 Overall Introduction 12

3.2 Structured Emulsion Based Delivery Systems 15

4. Digestion of Emulsion-based Delivery Systems 18

4.1 Digestion of Oil Droplets in GIT 18

4.2 Influence of Oil Droplets Structures on Lipid Digestion and Bioavailability of Components Encapsulated in Emulsions 20

5. Safety 27

6. Objectives of This Study 31

References 33

CHAPTER TWO 41

Correlation of Emulsion Structure with Cellular Uptake Behaviour of Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure 41

Abstract 42

1. Introduction 43

2. Material and Methods 46

2.1 Materials 46

2.2 Preparation of Emulsions with Different Droplet Size 46

2.3 Preparation of Emulsions with Different Emulsifiers 47

2.4 Characterization of Particle Size and Zeta Potential of Emulsions 47

2.5 Cryo-Scanning Electron Microscopy 47
2.6 Determination of Encapsulation Efficiency and Loading Capacity 48
2.7 Creaming Stability 49
2.8 Effect of pH on Droplet Size and Surface Charge of Emulsions 49
2.9 Cytotoxicity Assay 49
2.10 Caco-2 Cellular Uptake Assay 50
2.11 Extraction and Quantification of β-carotene 51
2.12 HPLC Analysis of β-carotene 51
2.13 Statistical Analysis 52
3. Results and Discussion 53
3.1 Droplet Size and Surface Charge 53
3.2 Creaming Stability of Emulsions 55
3.3 Cryo-SEM Observation of Emulsion Droplets 57
3.4 Effect of pH on Droplet Size and Surface Charge 59
3.5 Cytotoxicity of Emulsions 60
3.6 Cellular Uptake of β-carotene in Oil Droplets by Caco-2 cells 61
4. Conclusions 66
References 68
CHAPTER THREE 72
Improved Bioavailability of Encapsulated Bioactive Nutrient Delivered through Monoglyceride-structured O/W Emulsions 72
Abstract 73
1. Introduction 74
2. Material and Methods 77
2.1 Materials 77
2.2 Emulsion Preparation 77
2.3 Characterization of Droplet Size and Surface Charge 78
2.4 Determination of Displacement of Proteins from Interface by MG 78
2.5 Creaming Stability 79
2.6 Rheological Analysis 80
2.7 *In vitro* Simulated Gastrointestinal Tract (GIT) Digestion 80
2.8 Bioaccessibility of β-carotene after GIT 81
2.9 Cellular Uptake of β-carotene by Caco-2 cells 82
2.10 Extraction and HPLC analysis of β-carotene 83

2.11 Statistical analysis 83
3. Results and Discussion 83
3.1 Droplet Size and Surface Charge 83
3.2 Displacement of Protein by MG 84
3.3 Viscosity 86
3.4 Creaming Stability 87
3.5 Properties of Emulsions in GIT 89
3.6 Bioaccessibility and Cellular Uptake of β-carotene 91
4. Conclusion 97
References 98
CHAPTER FOUR 101
Bioaccessibility and Cellular Uptake of β-Carotene Encapsulated in Model O/W Emulsions: Influence of Initial Droplet Size and Emulsifiers 101
Abstract 102
1. Introduction 103
2. Material and Methods 105
2.1 Materials 105
2.2 Emulsion Preparation 106
2.3 Rheological Analysis 106
2.4 Creaming Stability 107
2.5 *In vitro* Simulated GIT Digestion 107
2.6 Bioaccessibility of β-carotene 107
2.7 Cellular Uptake by Caco-2 Cells 108
2.8 Extraction of β-carotene 108
2.9 HPLC Analysis of β-carotene 109
2.10 Statistical Analysis 109
3. Results and Discussion 109
3.1 Characterization of Emulsions 109
3.2 Properties of Emulsions in GIT Digestion 112
3.3 Bioaccessibility of β-carotene 116
3.4 Cellular Uptake of β-carotene 117
4. Conclusions 121
References 122
CHAPTER FIVE 124

Improved Emulsion Stability and Modified Nutrient Release by Structuring O/W Emulsions Using Konjac Glucomannan 124
Abstract 125
1. Introduction 126
2. Material and Methods 128
2.1 Materials 128
2.2 Preparation of Emulsions 128
2.3 Droplet Size and Surface Charge 129
2.4 Creaming Stability 129
2.5 Rheological Analysis 129
2.6 Fourier Transforms Infrared Spectroscopy (FT-IR) 130
2.7 Stability of Emulsions at Different pH Values and Temperatures 130
2.8 Freeze-Thaw Test 130
2.9 Simulated Gastrointestinal Tract (GIT) Digestion 131
2.10 Laser Scanning Confocal Microscope Observation 132
2.11 Release of Encapsulated β-carotene 132
2.12 Quantification of β-carotene 133
2.13 Statistical Analysis 133
3. Results and Discussion 134
3.1 Droplet Size and Surface Charge 134
3.2 Rheological Analysis 135
3.3 Fourier-Transform Infrared spectroscopy (FT-IR) Analysis 137
3.4 Creaming Stability 139
3.5 Stability of Emulsions at Different pH 142
3.6 Thermal Stability 143
3.7 Freeze-Thaw Stability 144
3.8 GIT Digestion 146
3.9 Release of Encapsulated β-carotene after GIT 147
4. Conclusion 150
References 151
CHAPTER SIX 153
Preparation of Re-dispersible Dry Emulsions Embedded with Bioactive Nutrients: Influence of Emulsion Compositions and Drying Methods 153
Abstract 154

1. Introduction 155
2. Material and Methods 157
2.1 Materials 157
2.2 Preparation of Emulsions for Freeze Drying 158
2.3 Preparation of Emulsions for Spray Drying 159
2.4 Droplet Size and Surface Charge 160
2.5 Creaming Stability 160
2.6 Rheological Analysis 160
2.7 Differential Scanning Calorimetry 160
2.8 Laser Scanning Confocal Microscope Observation 161
2.9 Scanning Electronic Microscope Observation 161
2.10 Quantification of β-carotene 162
2.11 Re-dispersibility of Dry Emulsions 162
2.12 Statistical analysis 163
3. Results and Discussion 163
3.1 Microscope Observation 163
3.2 MG Crystals in Dry Emulsions 169
3.3 Droplet size and Surface Charge of Re-constituted Emulsions 175
3.4 Viscosity 178
3.5 Creaming Stability 180
3.6 Re-dispersion of Dry Emulsions 182
4. Conclusions 186
References 188
CHAPTER SEVEN 190
General Discussion, Conclusions and Prospects 190
1. General Discussion 191
1.1 Initial Droplet Size 192
1.2 Oil Phase 193
1.3 Interface 193
1.4 Water Phase 194
1.5 Dry Emulsions 196
2. Overall Conclusions 196
3. Prospects 198

References 202
APPENDIX 204

DECLARATION

I hereby declare that the work submitted in entirely my own and has not been submitted to any other University or higher education institute, or for any other academic award in this University.

_____ Date: _____

Wei Lu

DEDICATION

To my parents, aunts, sister, and cousins

AKNOWLEDGEMENT

First of all, I would like to express my sincere gratitude to my chief supervisor, Dr. Song Miao in Teagasc Food Research Centre, Moorepark, for giving me the opportunity to pursue my Ph.D. through his supervision. His support helped me to go through with the Ph.D. program successfully and to be involved in a broader academic community. I also would like to thank my academic supervisor, Professor Alan L. Kelly from University College Cork for his valuable advices and discussion on my project and manuscripts.

I am grateful to the external examiner Professor Paola Pittia and the internal examiner Professor Yrjö Roos for their detailed discussion and constructive comments on the research carried out, which greatly contribute to an improved quality of the thesis.

I am thankful to Like Mao in our group, people in Moorepark, Professor Catherine Stanton, Professor Tim Guinee, Dr. Snehal Gite, Dr. Rita Hickey, Mr. Alberto Corrochano, Dr. Sean Hogan, Dr. Andre Brodkorb, Dr. Kamrul Haque, Mr. Bernard Corrigan, Ms. Martina OBrien, Ms. Helen Slattery, Ms. Catherine Mollins, Ms. Lisa Zychowski, Ms. Laura Saez Benita, Mr. Mattia Boiani, Ms. Ana Isabel Mulet Cabero, and Dr. Valentyn Maidannyk, and Pierce Maguire and Professor Hongzhou Zhang in Trinity College Dublin, for their support on my experiments, and the access to some instruments and the guidance in operations. Thanks to all other people for their help on my Ph.D. research.

I am also thankful to all my friends and workmates met in Moorepark, Jia Hou, Junfu Ji, Xu Lu, Hongliang Zeng, Sophie Gaspard, Ram Raj Panthi, Pooja Chalise Panthi, Arunima Gulati, Prabin Lamichhane, Archana Bista, Bhavya Panikuttira, Ashwini Shevade, Dinesh Thapa, Vincenzo Pennone, Longtao Zhang, Hairong Bao,

Kaibo Deng, Qingsong Zou, and etc, for their contributions to my colourful life in Ireland.

Finally, I'd like to give my deep thankfulness to all my family, my parents, aunts, uncles-in-law, sister and her husband, and cousins, for their constant love and support. It was your accompany that encouraged me to work more effectively, and made my life full of enjoyment. Also big thanks to all my relatives and friends of my family in China, for keeping it running well while I was absent.

Wei Lu

June 2018

ABSTRACT

Instability and water-insolubility of many bioactive nutrients greatly limit their oral bioavailability and thus their health benefits. Therefore, the delivery of these compounds requires protective mechanisms. Emulsion-based delivery systems are becoming some of the most ideal microencapsulation carriers for these lipophilic bioactive components, and tailored structures of emulsions potentially contribute to a better control of the stability and bioavailability of instable and poorly water-soluble bioactive components.

The current study mainly investigated four model O/W emulsions with different initial droplet size, oil phase compositions, emulsifiers, and water phase compositions. A representative lipophilic bioactive nutrient, β-carotene, was encapsulated into these model emulsions. Emulsion properties, and the *in vitro* digestion, release, bioaccessibility and absorption by enterocytes of encapsulated β-carotene were investigated. Re-dispersible dry forms of these model emulsions containing β-carotene were also prepared, and their microstructures, re-dispersibility, and the properties of their reconstitutions were characterized.

A whey protein isolate (WPI) stabilized emulsion with small initial droplet size showed better creaming and pH stability and higher cellular uptake of β-carotene than that with large initial droplet size. After passing through the simulated gastrointestinal tract (GIT) digestion, initial droplet size significantly influenced the emulsion properties (e.g., droplet size and distribution and surface charge), but did not significantly affect the bioaccessibility and cellular uptake of β-carotene.

Monoglycerides (MG) in the oil phase showed competitive absorption on the droplets surface with WPI, leading to reduced droplet surface charge. MG significantly increased the viscosity and creaming stability of WPI-stabilized

emulsions. MG also significantly promoted the bioaccessibility and cellular uptake of β-carotene by Caco-2 cells (p<0.05).

Emulsions stabilized with different emulsifiers of WPI, sodium caseinate, or tween 80, showed different droplet sizes, surface charges, creaming and pH stability, and cellular uptake of β-carotene without passing through the GIT. Selection of emulsifiers also significantly modified the emulsion properties when exposure to the GIT digestion, and the bioaccessibility and cellular uptake of β-carotene after the GIT digestion (p<0.05).

Incorporation of KGM into the water phase of emulsions greatly improved the creaming and pH stability of WPI-stabilized emulsions, and significantly decreased the oiling-off of the emulsions during a freeze-thaw test. Emulsions containing KGM in the water phase showed a lower final release rate of encapsulated β-carotene then the emulsion without KGM (p<0.05), and the release rate decreased with increasing KGM content.

Dried emulsions showed different morphologies and microstructures, depending on the drying method (spray-drying or freeze-drying), and the compositions of emulsions before drying. Dry emulsions showed fast re-hydration and good re-dispersibility in water. Compared with emulsions before drying, re-constituted spray-dried and freeze-dried emulsions showed shifted droplet size distribution to large and small size, respectively. Re-constituted emulsions containing KGM showed significantly decreased viscosity but increased creaming stability compared to emulsions before drying (p<0.05).

Overally, the present study provided useful information about different model O/W emulsions as delivery carriers for lipophilic components, and on how emulsion structures can be designed to modify the release of health-beneficial lipophilic

components and improve their oral bioavailability, which could be important in developing functional foods with sustained release, or improved oral stability and bioavailability of functional ingredients entrapped in food matrixes.

CHAPTER ONE

Introduction & Literature Review

Part of this section was published in:
Trends in Food Science and Technology, 2016, 47, 1-9.

Authors: Wei Lu, Alan L. Kelly, and Song Miao*

The work contained in this chapter was undertaken and written solely by myself with specific contributions from each co-author.

Abstract

Emulsion-based technologies have been widely used in the food, nutrition and pharmacy industries, and one major application of emulsions is in the microencapsulation and delivery carriers for lipophilic functional ingredients. Many emulsion-based delivery systems have been successfully developed in attempts to maintain the stability, modify the digestion behaviours in simulated gastrointestinal tract (GIT), control the release and improve the oral bioavailability of unstable lipophilic components. The compositions and microstructure of emulsion droplets can significantly influence the rate and extent of lipid digestion and thus the bioavailability of lipophilic components in emulsions. Therefore, the design of emulsion structures is particularly important when products with different properties in applications are desired. This review summarizes the application of emulsions as delivery systems and the relationship between emulsion structure and lipid digestion and bioavailability of encapsulated lipophilic components in emulsions. The definition, preparation, properties and stability of emulsions, and potential adverse health effects of food-grade nanoparticles (emulsions included), are also discussed.

Keywords: emulsion, delivery, structure, digestion, bioavailability

1. Introduction

A considerable number of natural and processed foods consist either partly or wholly as emulsions, such as milk, cream, beverages, soups, sauces, spreads, and butter (McClements, 2004). Emulsions show a wide diversity of physiochemical and sensory characteristics, depending on the ingredients and processing conditions used to create them. In spite of this diversity, there are a number of common properties of these products, which make it possible to study these products using a scientific discipline known as *emulsion science*. Emulsion science mainly focuses on the research of basic principles of emulsions, covering aspects from their preparation, and characterization to their structure-design and applications. Emulsion science combines physics, chemistry, biology and engineering, and fundamental principles of emulsion science were mostly derived from the disciplines of polymer science, colloid science, interfacial chemistry, and fluid mechanics (Evans, 1994; Hiemenz, 1997; Hunter, 2001). Along with the widely application of emulsions in different fields, especially the high-performance encapsulation and delivery carriers for lipophilic functional ingredients (e.g., flavors, nutrients or drugs), emulsion science has incorporated a variety of other disciplines, such as sensory science, nutrition science, pharmacology, and physiology. In the last ten years, more and more scientists have focused their research on emulsions, and a number of relevant achievements have been made, which accordingly contribute to a fast development of emulsion science, and inspire and encourage new scientists devoting to the research on emulsion science.

2. Fundamental of Food Emulsions

2.1 Definition

An emulsion consists of two immiscible liquids (e.g., oil and water), with one of the liquids dispersed as small spherical droplets in the other (McClements, 2004). A system with dispersed oil droplets in an aqueous phase is generally called an oil-in-water (or O/W) emulsion, while a system with dispersed water droplets in an oil phase is defined as water-in-oil (or W/O) emulsion. The component that make up the droplets in an emulsion is called dispersed, discontinuous or internal phase, whereas the component that make up the surrounding liquid is called the continuous or external phase. In terms of O/W emulsions, we can also simply refer to the droplets as the oil phase and the surrounding liquid as the water phase.

2.2 Preparation and Stability

Many emulsification methods can be used to prepare either O/W emulsions or W/O emulsions. An example is homogenization, which can converts two immiscible liquids into an emulsion using a homogenizer (Walstra, 1993). In the homogenization processing, mechanical forces (e.g., shear force, cavitation, and impact) are applied to rupture bulk phase into droplets and overcome surface tension or viscous stress to further rupture large droplets into small ones. Meanwhile, emulsifiers absorb onto the surface of newly-formed droplets to reduce the surface tension and form interfacial layers which can prevent the aggregate of droplets and produce stable emulsions (Schultz, 2004). High speed blenders, and high pressure valve homogenizers, are also widely used homogenization equipments.

Many other methods of making emulsions also exist, including phase inversion temperature, phase inversion composition, membrane emulsification, and flow-focus (Fryd & Mason, 2012).

Generally, emulsions are thermodynamically unstable systems that will eventually break down if they are left long enough. However, the rate of this process (kinetic stability) may be different between different emulsions. Thus, the term of "stability" of emulsions generally refers to the kinetic stability of emulsions. Despite emulsions are always thermodynamically unstable, many emulsions can still keep kinetically stable for months or even years. An emulsion that is kinetically stable has to have an activation energy (ΔG^*) that is significantly larger than the thermal energy of the systems (kT) (Friberg, 1997).

The kinetic stability of emulsions is mainly related to the dynamics and interactions of the droplets, since whether droplets move up, down or apart, collide with each other, remain separately or fuse together after collision, depends on the physiochemical nature and interaction between them (McClements, 2015). Therefore, an emulsion can reach a stable state after a balance of the different interactions between droplets (repulsive interaction and attractive interaction) is obtained. In addition, external factors, e.g., pH, ionic strength, temperature, and some physical (thermal processing, or centrifugation) and chemical processing (enzymatic hydrolysis) can affect the interaction balance, and thus the stability of emulsions.

Emulsion destabilization mainly refers to flocculation, creaming or sedimentation, coalescence, Ostwald ripening or emulsion break-down (**Figure 1-1**) (Lu et al., 2016).

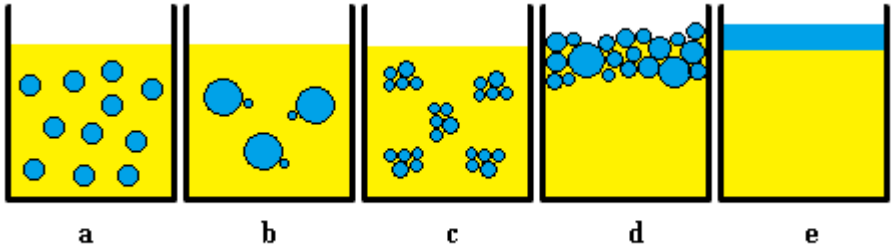


Figure 1-1. Destabilization of emulsions (a) Stable emulsion; (b) Coalescence; (c) Flocculation; (d) Creaming; (e) Break-down

Generally, droplets in an emulsion have a different density to the continuous phase, and thus a gravitational force acts on them (Dickinson & Stainsby, 1982). If the droplets have a lower density than continuous phase (e.g., O/W emulsions), they tend to move upward, which is referred to as *creaming* (**Figure 1-1d**). If their density is higher than the continuous phase, they tend to move downward, which is referred to as *sedimentation*. Generally, the creaming or sedimentation rate of isolated spherical particles in a liquid can be estimated by the following equation of Stokes' law:

$$V = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\eta} g r^2$$

where ρ_p is the mass density of the particles; ρ_f is the mass density of the fluid; g is gravitational acceleration; η is dynamic viscosity; and r is the radius of the particles. Based on this equation, emulsions with smaller average droplet size, more viscous continuous phase, or higher droplet density can accordingly show better creaming stability. An emulsion which has a calculated creaming rate that is less than 1 mm per day, can be considered as a stable emulsion towards creaming (Dickinson, 1992). However, Stokes' law can only be used to calculate the creaming velocity of an isolated rigid spherical particle suspended in an ideal liquid of infinite extent. In practice, many assumptions in Stokes' equation are invalid and thus lead to large

deviations between the creaming rate predicted by Stokes' law and the experimental results in emulsions. Therefore, some factors that can alter the creaming rate in emulsions should be also considered, including but not limited to droplet fluidity, polydispersity, droplet flocculation, non-Newtonian rheology of continuous phase, electrical charge, and fat crystallization (McClements, 2015).

Flocculation happens when two droplets are associated with each other, but without merging into one large droplet (**Figure 1-1c**). Flocculated droplets can be re-dispersed by blending or diluting the emulsions. Flocculation can accelerate the rate of gravitational separation (creaming or sedimentation) in dilute emulsions, which accordingly reduces their shelf life (Tan, 2004). Generally, in a colloidal dispersion containing monodisperse spherical particles, the rate of flocculation depends on two factors: collision frequency and the collision efficiency between the droplets (Evans, 1994). Any factors that increase the collision frequency increase the flocculation rate, and collisions between two droplets occur as a result of their own movement, which can be induced by Brownian motion, gravitational separation, or applied mechanical forces. The collision efficiency, a value between 0 (no flocculation) and 1 (every collision leads to flocculation) mainly depends on the hydrodynamic and colloidal interactions between droplets. Any factors that can influence the collision frequency and collision efficiency of droplets can be considered as potential strategies to improve the flocculation of emulsions, and the most effective way of decreasing the flocculation is to control the droplet interactions, in which the repulsive interactions are significantly greater than the attractive interactions. A wide range of methods have been developed to decrease the flocculation of droplets, such as increasing the viscosity of the water phase, using electrically-charged or sterically

emulsifiers, increasing the concentration of ionic emulsifiers, or introducing a second emulsifier (e.g., multilayer-interfacial droplets) (McClements, 2015).

Coalescence is the process whereby two or more liquid droplets merge together to form a single large droplet (**Figure 1-1b**). Coalescence can lead to rapid creaming or sedimentation of emulsions due to increased droplet size. Coalescence will occur when droplets are close enough and the surface thin films (consisting of continuous phase and interfacial layer) that separate them are destroyed. Thus, the coalescence rate is highly dependent on the properties of emulsifiers used to stabilize the emulsions. Factor affecting coalescence can be divided into two main groups, process parameters and composition and formulation of the emulsions. The main process parameters that influence the occurrence and the rate of coalescence are the mechanical shear forces and the time-temperature program applied during processing. The mechanical shear forces can affect the encounter frequency and capture efficiency of droplets while the temperature mainly determine the physical state of the oil droplets, all of which can accordingly lead to modified coalescence rate (Darling, 1982; Thanasukarn et al., 2004; van Boekel, 1981; Walstra, 2003). Components that used to create emulsions can also significantly influence the encounter frequency and the capture efficiency of emulsion droplets and thus the coalescence of emulsions, including fat volume fraction (Hinrichs & Kessler, 1997), particle size (Boekel., 1980), composition of the oil-water interfacial layer (Goff, 1997; van Boekel & Walstra, 1981), continuous phase composition (Boode, 1992; Thanasukarn et al., 2004), and oil phase composition (Darling, 1982; van Boekel & Walstra, 1981). All these factors can be controlled by proper experimental design to decrease the rate of coalescence of emulsions.

Ostwald ripening is the process whereby dispersed phase in small droplets migrate into the large droplets through continuous phase, resulting in growing of these large droplets over time (Kabal'nov & Shchukin, 1992). The concentration of solute (dispersed phase) around a small droplet is higher than that around a large one, and thus the concentration gradient of solute drives the them to transport from small droplets to large ones. Then, Ostwald ripening occurs. Overall effect of Ostwald ripening is an increase in the average radius of the emulsion droplets with time as the smaller droplets dissolve and redeposit their material onto the larger droplets. Many factors can influence the rate of Ostwald ripening (Taylor, 1998), such as volume fraction of dispersed phase (Enomoto et al., 1986), solubility of the dispersed phase (Kabal'nov et al., 1987), and interfacial tension and droplet size (Taylor & Ottewill, 1994). Generally, the rate of Ostwald ripening increases as the water-solubility of the oil phase increases and the radius of the oil droplets decreases.

2.3 Properties of Emulsions

Properties of emulsions prepared by homogenization are often largely influenced by the homogenization conditions used, including temperature, pressure and number of cycles (Yuan et al., 2008). These properties mainly include droplet size, surface charge, stability and rheology. For example, the droplet size of emulsions decreases with increasing homogenization pressure and cycles.

The concentration of droplets in an emulsion also plays a very important role in determining its properties, such as appearance, texture, stability, and viscosity. The droplet concentration is usually described in terms of the disperse phase volume fraction (ϕ), which is equal to the volume of emulsion droplets (V_D) divided by the total volume of the emulsion (V_E): $\phi = V_D / V_E$. Droplet concentration can also be

expressed as the disperse phase mass fraction (ϕ_m), which is equal to the mass of emulsion droplets (m_D) divided by the total mass of the emulsion (m_E): $\phi_m = m_D / m_E$.

Many important properties of emulsion-based systems are determined by the size of the droplets, e.g., appearance, stability, viscosity, texture, and digestion. Hence, it is very important to control, measure, and report the droplet size and the size distribution of emulsions. Several factors can influence the droplet size of emulsions, such as homogenization pressure and number of cycle as described above, and emulsifier type and concentration (Tcholakova & Ivanov, 2003). Generally, the droplet size of an emulsion decreases with an increasing homogenization pressure, number of cycles, and concentration of emulsifiers. Emulsions stabilized by low molecular emulsifiers (e.g., Tween) always show smaller droplet size than those stabilized by macromolecules, such as proteins, and polysaccharides (McClements, 2004).

The droplet interface consist of a narrow region of a few nanometers thick that coat each emulsion droplet, and contains a mixture of oil, water, and emulsifiers (Hunter, 2001). The interface plays a major role in determining many of the most important physicochemical properties of emulsions. Thus, research on emulsions is highly focussed on factors that can tailor the structure, thickness, rheology, and charge of the interfacial region, and on how these interfacial properties consequently influence the physicochemical properties of emulsions. The structure of the interfacial region is determined by the type and concentration of emulsifiers used prior to the emulsion formation, and by the treatments that occur after emulsion formation, for example protein-polysaccharide double-layer interface formed by introducing polysaccharide into protein-stabilized emulsions (Li et al., 2010). The selection of emulsifiers can also determine the surface charge of emulsion droplets

(McClements, 2015). Positively charged emulsifiers will produce positively-charged emulsion droplets, while negatively-charged emulsifiers will produce negatively-charged emulsion droplets. An emulsion with non-charged droplets can be obtained by using neutrally-charged emulsifier/surfactant. Furthermore, the surface charge of emulsion droplets can be influenced by the environment. For example, the surface charge of a protein-stabilized emulsion will change with environment pH, since protein molecules will be differently charged under different pH depending on its isoelectric point (pI). Protein molecule at pH value lower, higher, or equal to its pI will be positively-, negatively-, or neutrally-charged, respectively, which accordingly lead to similarly-charged emulsion droplets.

The rheology of emulsions is also very important in fully understanding their properties and thus obtaining an emulsion-based formulation or products with desired rheological properties before or after processing. Viscosity and viscoelastic properties are the main parameters that are used to characterize the rheology of emulsions. They can be influenced by the composition, concentration, or the status of the water and oil phase of emulsions. For examples, emulsions with a crystallized oil phase (e.g., monoglyceride-structured oil phase) always show higher viscosity than those with liquid oil phase (Mao et al., 2014). The control of rheology of emulsion can be easily obtained by selecting proper composition of oil phase, water phase, and emulsifiers.

3. Emulsions as Encapsulation and Delivery Carriers

3.1 Overall Introduction

Many bioactive nutrients, e.g., polyphenols, carotenoids, vitamins, fatty acids, flavonoids, and phytochemicals, can effectively contribute to the prevention and therapy for various human diseases. However, only a small proportion of these compounds taken orally are absorbed, due to insufficient gastric residence time, low permeability and water-solubility (Wildman, 2001). Instability of these compounds during processing, storage or in the digestive tract, also greatly limits their potential health benefits (Munin & Edwards-Levy, 2011). The delivery of these compounds therefore requires protection mechanisms that can maintain their chemical integrity and deliver them to the physiological target. The utilization of protected encapsulation and delivery system can achieve this goal.

A wide range of technologies have been developed to protectively encapsulate bioactive nutrients, including spray-drying, coacervation, emulsions, liposome, micelle, nanoparticles, freeze-drying, cocrystallization and yeast encapsulation (Fang & Bhandari, 2010; Munin & Edwards-Levy, 2011). Each of these carriers has its own specific strengths and weaknesses in encapsulation, protection delivery, cost, regulatory status, ease of use, biodegradability and biocompatibility. Among these, emulsions are becoming one of the most popular encapsulation and delivery system for bioactive nutrients, due to their advantages of wide range of encapsulation of lipophilic, hydrophilic and amphiphilic molecules, high-efficiency encapsulation, maintenance of chemical stability, and potential for controlled release of encapsulated molecules (McClements et al., 2007). Therefore, one of the major applications of emulsions is their use as the micro-, and nano-encapsulation and

delivery carriers, and many emulsion-based delivery systems has been developed as novel carriers for a wide range of functional ingredients, as summarized in **Table 1-1**.

Table 1-1. Examples of compounds that have been encapsulated and delivered through emulsions

Category	Representative Compounds	Reference
Polyphenols	Epigallocatechin gallate, EGCG Quercetin Resveratrol Curcumin Ellagic acid Catechin	(Lu et al., 2016) (Bhushani et al., 2016) (Galho et al., 2016) (Aditya et al., 2015)
Carotenoids	β-carotene Lycopene Lutein Astaxanthin Fucoxanthin	(McClements et al., 2007) (Mao et al., 2017); (Ha et al., 2015); (Salvia-Trujillo et al., 2015)
Vitamins	Vitamin A Vitamin D Vitamin E Folic acid	(Santos, 2011); (Guttoff et al., 2015); (Ozturk et al., 2014) (Jafari, 2017) (Parthasarathi, 2016)
Fish oils	ω-3 unsaturated fatty acids Docosahexaenoic acid, DHA Eicosapentaenoic acid, EPA	(McClements et al., 2007) (Gulotta et al., 2014) (Kumar et al., 2016) (Karthik & Anandharamakrishnan, 2016)
Flavonoids	Rutin	(Sharma et al., 2015)
Phytochemicals	Paclitaxel Pterostilbene	(Y. Sun et al., 2015) (Dias et al., 2007)
Drugs	Doxorubicin Carbamazepine Saquinavir	(Dluska et al., 2017) (Gutiérrez et al., 2008)

Encapsulation in emulsions can not only dramatically increase the water-solubility of lipophilic health-beneficial nutrients, but also can significantly improve their environmental stability towards temperature or light (Joye et al., 2014). Meanwhile, emulsion formulation can also significantly enhance the stability of nutrients in gastrointestinal tract (GIT) through protecting them from chemical/enzymatic oxidation or degradation in the stomach (Hatanaka et al., 2010; Lin et al., 2011), and then these encapsulated nutrients can safely reach the small intestine, where they are released and absorbed, leading to a significantly enhanced oral bioavailability of these nutrients.

However, emulsion-based delivery carriers also have some disadvantages. Liquid emulsions are dynamic unstable systems, and can cream after being placed for a certain period of time, which may influence the qualities of some emulsion-based liquid products, e.g., functional beverages. In addition, many emulsion formulations contain proteins or polysaccharides, which can be utilized by microorganisms, resulting in the bacterial contamination and spoilage of these emulsion-based products. Furthermore, previous studies have mainly focused on liquid emulsion systems, but liquid emulsions are not convenient for transportation and long-term storage. Therefore, some studies have explored the possibility of obtaining powdered emulsions by freeze-drying or spray-drying (Anwar & Kunz, 2011; Gharsallaoui et al., 2010; Jafari, 2017; Christensen, 2001). These studies provided some useful information, but much further work still needs to be done, especially on how to successfully obtain fine emulsion powders with relative high fat content but lower excipient (e.g., biopolymers in the water phase of liquid emulsions as wall materials) content.

3.2 Structured Emulsion-Based Delivery Systems

Since emulsions are widely used as microencapsulation and delivery systems in the food and pharmacy industries, many emulsion-based carriers with different structures have been developed, including conventional emulsions, multilayer emulsions, nanoemulsions, double-emulsions, solid lipid nanoparticles, filled hydrogel particles, micelles, and liposomes (**Figure 1-2**) (McClements & Li, 2010).

Several of widely used emulsion-based delivery systems with an aqueous continuous phase are discussed in this section. Their structures and properties are summarized in **Table 1-2.** By designing the formulation and preparation technologies, emulsion-based delivery systems with differently-structured droplets can be obtained, and these have been widely used in a variety of different applications, such as cosmetics, ice-cream-making, gelled foods, controlled release and/or targeted delivery of bioactive ingredients or drugs, or production of functional foods embedded with lipophilic nutrients with improved digestive stability and oral bioavailability.

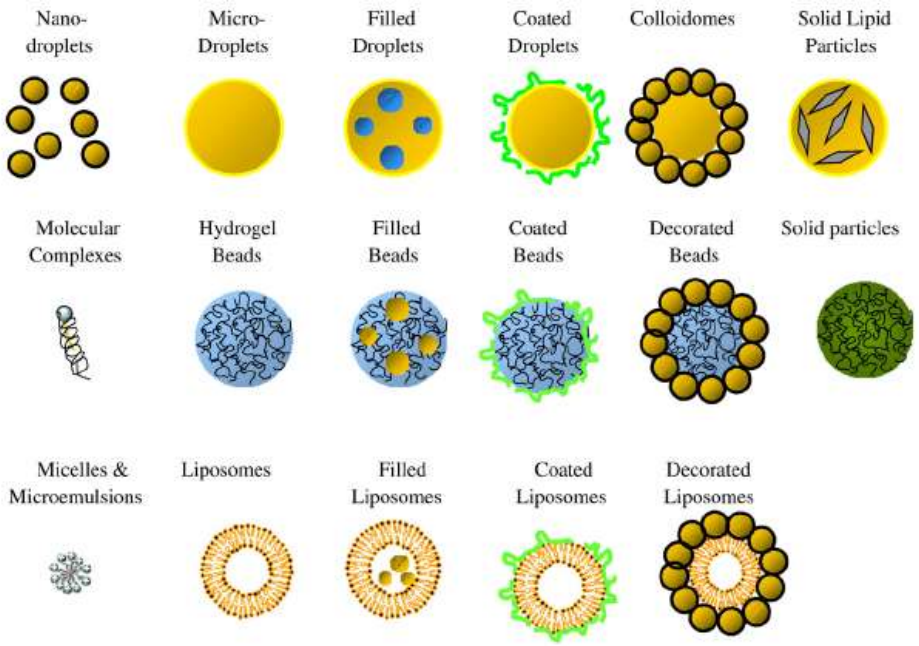
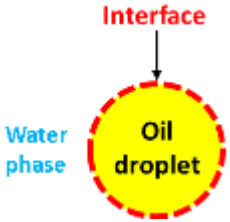
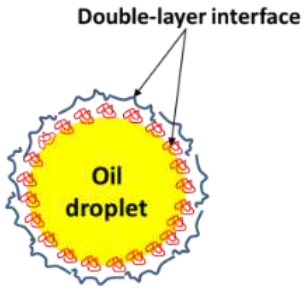
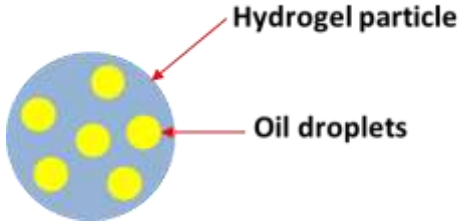


Figure 1-2. Examples of different kinds of structured-emulsion-based delivery systems (McClements & Li, 2010)

Table 1-2. Summary of structures and properties of some O/W emulsion-based delivery systems

Category	Structure	Properties	References
Conventional emulsions	Micro-oil-droplets dispersed in an aqueous phase, with an emulsifier-coated surface 	• Cloudy or opaque appearance, with an average diameter in the range 200 nm-100 μm • Thermodynamically unstable system, prone to gravitational separation depending on the formulation and properties (viscosity, and droplet size, surface charge and density) of emulsions • Positively-, neutrally-, or negatively-charged depending on emulsifiers selected and/or environmental pH • Better stability to Ostwald ripening than nanoemulsions	(McClements, 2004) (McClements et al., 2007) (McClements, 2010b)
Nanoemulsions	Nano-oil-droplets dispersed in an aqueous phase, with an emulsifier-coated surface 	• Transparent or slight turbid appearance with an average diameter in the range 10 nm-200 nm • Better gravitational separation and droplet aggregation stability than conventional emulsions, due to smaller droplet size • High surface area-to-volume ratio resulting in an accelerated chemical reaction at the oil-water interface, e.g., lipid digestion	(Mason et al., 2006) (Gupta et al., 2016) (McClements, 2012) (McClements & Rao, 2011)
Multilayer emulsions	Small oil droplets dispersed in an aqueous phase, with a multiple layer interface	• Similar physiochemical properties (e.g., rheology, optical property, and stability) as conventional emulsions with similar characteristics (e.g., compositions, concentration, size, and surface charge) • Multiple-layer coated droplets lead to improved stability	(McClements & Li, 2010) (Dickinson, 2011) (Burgos-Díaz et al., 2016)

		towards creaming, flocculation, coalescence, Ostwald ripening, extreme pH and freeze-thaw, and functional performance (protection of the chemical stability, and controlled release of encapsulated components)	
Solid lipid particles, SLP	Partly or fully solidified oil droplets dispersed in an aqueous phase, with an emulsifier-coated surface 	• Crystallised oil phase within droplets • Different internal structures in lipid droplet by controlling lipid composition and ratio, emulsifier type, and cooling conditions • Controlled location of different phases in lipid droplets, e.g., the core could be solid and the shell liquid; structure and spatial organization of lipid crystals can greatly influence the encapsulation, protection, and delivery of lipophilic components	(Wissing et al., 2004) (Weiss et al., 2008) (Mehnert & Mäder, 2012)
Filled hydrogel particles	Oil droplets trapped within a hydrogel particle that is dispersed within an aqueous medium 	• Cloudy or opaque in appearance with closely packed oil droplets in hydrogels • Prone to gravitational separation, flocculation and coalescence processes as conventional emulsions • Greater viscosity than conventional emulsion with similar oil concentration due to the fact that the water trapped inside the hydrogel can increase the effective volume fraction of the dispersed phase	(Weiss et al., 2005) (Lee et al., 2012) (Dickinson, 2012)

4. Digestion of Emulsion-based Delivery Systems

Emulsion-based delivery systems go through the gastrointestinal track (GIT) when taken orally, which can cause a complex series of physical and chemical changes to emulsions (**Figure 1-3**). Therefore, knowledge of basic physiochemical and physiological processes that occur when emulsions passes through GIT is of high importance to design effective emulsion-based delivery systems to control the digestion and release of lipophilic components.

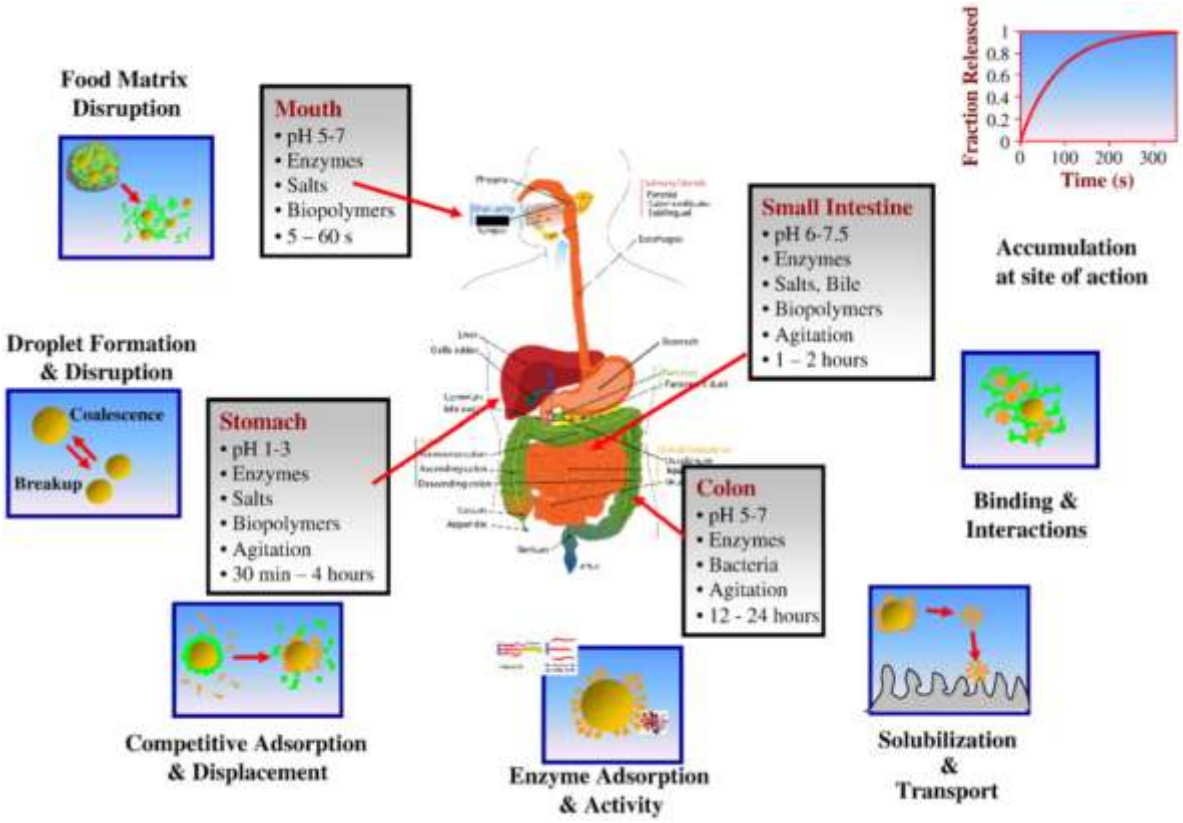


Figure 1-3. Schematic diagram of the complex physiochemical and physiological processes that may occur during digestion of emulsion and absorption of encapsulated components (Mcclements & Li, 2010)

4.1 Digestion of Oil Droplets in GIT

The digestion of emulsion droplets in GIT can be divided into three main phases, mouth, gastric, and intestinal phase (McClements, 2015). In mouth phase, droplets will be brought to body temperature rapidly, and mixed with saliva, containing saliva

amylase which will digest the starch in emulsions and mucus, which can interact with the emulsion droplets and lead to significant increases in droplet size (Mao & Miao, 2015). After undergoing the partial digestion, emulsion droplets will enter into stomach through the oesophagus through the action of peristalsis. The hydrochloric acid and pepsin in gastric juice then start to hydrolyse proteins or carbohydrates at the interfacial layer of emulsion droplets (Ensign et al., 2012) along with the further mechanical mixing of droplets and digestive enzymes by peristalsis, resulting in great changes in droplet size, and surface charge of emulsion droplets. After 1-2 hours in the stomach, emulsion droplets travel down into the duodenum, where droplets will mix with digestive enzymes from pancreas, e.g., trypsin and lipase and bile juice from the liver, and then pass through the small intestine. In this stage, digestive enzymes will further digest proteins and the oil in droplets, resulting in the release of encapsulated molecules, or fatty acids. Bile salts will then emulsify these lipophilic compositions and form bile-salts-stabilized micelles, which will be absorbed by enterocytes or lymphocytes. Emulsion droplets remain in the small intestine for more than 3 hours before entering into the large intestine or colon, and 95% absorption of nutrients occurs in the small intestine.

From the micro-perspective of a single emulsion droplets, taking protein-stabilized O/W emulsion as an example (Lu et al., 2018) , the digestion of emulsions mainly goes through four steps: (i) binding of proteinase (e.g., pepsin and trypsin) to the droplet surface and hydrolysis of interfacial protein layers by proteinase; (ii) binding of lipase to oil phase and hydrolysis of lipids by lipase; (iii) release of entrapped bioactive nutrients from hydrolysed oil phase; and (iv) formation of bile-salt emulsified micelles containing released lipophilic bioactive nutrients. Any factors that can influence these four digestion processes will influence the final formation of

the nutrient-loaded micelles, and thus the bioaccessibility of these encapsulated nutrients. Therefore, as previously reported, emulsion composition, e.g., oil phase, water phase, and emulsifiers, and emulsion properties, e.g., droplet size and surface charge can accordingly influence the digestion and release of entrapped nutrients in emulsions, which will be discussed in the following sections.

4.2 Influence of Oil Droplet Structures on Lipid Digestion and Bioavailability of Components Encapsulated in Emulsions

4.2.1 Oil-Water Interface

The composition of the interfacial layers (emulsifiers) can not only influence the physical and chemical stability of emulsions (Qian et al., 2012; Yin et al., 2009), but also can affect the rate of lipid digestion, the release, and thus the bioavailability of encapsulated components.

It has been reported that selection of different types of emulsifiers (soy lecithin, tween 80, and sodium caseinate) can influence the *in vitro* lipolysis and *in vivo* absorption of fatty acids of emulsified flaxseed oil (Couedelo et al., 2015). Mun et al., (2007) found that the amount of free fatty acids released per unit volume of emulsion decreased in the following order: proteins (caseinate and whey protein isolates)>phospholipids (lecithin)>non-ionic surfactants (Tween 20), which is probably attributed to the different rate of displacement of these emulsifiers by bile salts at the oil droplet surface. A sustained release of DHA from emulsions in the initial stage of digestion can be obtained by selecting proper emulsifiers when preparing DHA-loaded emulsions (Karthik & Anandharamakrishnan, 2016). The type of emulsifier had a significant influence on the release properties of β-carotene in emulsions, and WPI-stabilized emulsions showed the highest release rate of β-

carotene, followed by decaglycerolmonolaurate and soybean soluble polysaccharides-stabilized emulsions (Hou et al., 2014).

The digestion and release of lipophilic components encapsulated within emulsion-based delivery systems can be controlled by coating the lipid droplets with a multilayer interface. For example, emulsions with multilayer interface were successfully prepared using an interfacial electrostatic deposition method (Li et al., 2010). The multilayer interface is composed of an inner layer of globular protein (β-lactoglobulin), an intermediate layer of cationic polysaccharide (chitosan), and an outer layer of anionic polysaccharide (pectin or alginate). Compared with protein-stabilized single emulsions, polysaccharide-coated multilayer emulsions showed much slower velocity of lipid digestion and thus reduced lipid digestibility. Similar results were also observed in some other studies (Gudipati et al., 2010; McClements & Li, 2010). This may be mainly attributed to: (i) the single layer interface can easily be digested and displaced by bile salts and lipase, thereby facilitating the digestion of encapsulated lipids by lipase; (ii) multiple-layer-droplets have outer coatings that consist of compact and indigestible biopolymers (e.g., polysaccharides or proteins), which accordingly inhibit the access of lipase to the oil phase within droplets core (Mun, 2006); (iii) biopolymers in outer layers can bind bile salts and thus reduce the amount of bile salts available to adsorb to oil droplet surface, which potentially can slow down the digestion of lipids, since bile salts are needed to solubilize lipid digestion products out of the emulsion droplets to decrease the concentrations of end products below reaction equilibrium levels, thus allowing for the continued activity of lipase (Wickham, 1998).

Zeeb et al. (2015) also compared the digestion of lipids within multilayer emulsions and cross-linked multilayer emulsions. The rate and extent of lipid

digestion did not greatly differ between crosslinked and non-crosslinked multilayered emulsions. Free fatty acid profiles showed that the stability of emulsified oil droplets plays a major role in the rate and extent of lipid digestion, rather than the initial layer properties.

In addition, a previous study investigated the influence of the type of emulsifier used to form excipient emulsions on their stability and the bioaccessibility of powdered curcumin (Zou et al., 2015). The results showed that the amount of curcumin transferred into the excipient emulsions depended on both incubation temperature and emulsifier type. After passing through the GIT, The amount of curcumin in the raw digesta phase depended on emulsifier type (caseinate>WPI>tween 80), which was attributed to the ability of the proteins to protect the curcumin from chemical degradation in GIT. The bioaccessibility of curcumin also depended on emulsifier type (WPI≈tween 80>caseinate), which was probably attributed to the inhibition of lipid digestion by caseinate.

Furthermore, the emulsifier type can influence the diffusion process of lipophilic components (e.g., natural antimicrobials) into oil droplets, which accordingly lead to different effective concentration of these components in the dispersed phase and thus their bioactivities (Donsi et al., 2012).

Results described above are some examples; many other studies (Mun et al., 2007; Park et al., 2007; Porter et al., 2007; Mun, 2006) also have confirmed a significant influence of emulsifiers on the lipid digestion, release and bioaccessibility of lipophilic components in emulsions, which will not be discussed one by one.

However, some widely used emulsifiers, e.g., carboxymethylcellulose (CMC) or polysorbate-80 (tween 80), can negatively impact the gut microbiota, and promote colitis and metabolism syndrome (Chassaing et al., 2015), suggesting that more

natural and health-beneficial emulsifiers are probably desired for safe and novel applications in food, nutrition and pharmacy industries.

4.2.2 Oil Phase

Likewise, compositions and microstructures of the oil phase of emulsions can significantly influence and digestion and bioaccessibility of encapsulated lipophilic components in emulsions. For example, tween 20-stabilized emulsions with different type of carrier oils (long chain triglyceride, LCT; medium chain triglyceride, MCT; and orange oil) showed significant different rate and extent of lipid digestion, and the bioaccessibility of β-carotene encapsulated in these emulsions decreased in the order LCT>MCT>orange oil (Qian et al., 2012). A similar result, of increased bioaccessibility of encapsulated carotenoids with increasing chain length of fatty acids of triglycerides in oil phase of emulsions was also observed by Huo et al. (2007). These results are also in line with studies in the pharmaceutical industry showing that many lipophilic drugs have a higher bioaccessibility when LCT is used as a carrier oil rather than MCT in lipid-based delivery systems (Kossena 2003; Nielsen, 2001). Flavour oils, such as orange oil, do not contain triacylglycerol components and therefore cannot be digested into free fatty acids. As a result there are relatively few mixed micelles formed after digestion capable of solubilising and transporting lipophilic components (Fatouros & Mullertz, 2008). In addition, the free fatty acids (FFA) released by MCT digestion may be not able to assembly into mixed micelles with a large enough hydrophobic cavity to accommodate lipophilic components, e.g., β-carotene, a highly hydrophobic molecule with an approximately linear rod-like structure. However, LCT contains relative long chain fatty acids, which potentially can form sufficient-large micelles to accommodate these lipophilic components, and increase their dispersibility in micelles. All these factors may

explain why emulsions with a carrier oil of LCT showed much higher bioaccessibility of encapsulated lipophilic components than that with MCT and orange oil.

Many other studies also confirmed the significant influence of the type of carrier oils, such as flaxseed oil, Oliver oil, mineral oil, or the ratio of LCT and MCT, on the digestion and bioaccessibility of encapsulated lipid or lipophilic components in emulsions (Li et al., 2012; Salvia-Trujillo et al., 2013b; Salvia-Trujillo et al., 2015; Sun et al., 2015).

The physical state of the oil phase might also influence the digestibility and bioaccessibility of lipophilic components, since this can affect the accessibility of lipase to the ester bond in the triacylglycerols molecules. Many studies have investigated the influence of fat crystallization on the *in vitro* lipid digestion, and their findings suggested that solid lipid particles showed a slower rate of lipid digestion than liquid lipid particles (Bonnaire, 2008). In addition, some designed partly crystalline droplets underwent partial coalescence in the small intestine, due to the penetration of fat crystals from one droplet into liquid regions of another droplet (McClements & Li, 2010).

4.2.3 Water Phase

The composition and the microstructures of the water phase also can significantly influence the release and bioavailability of encapsulated lipophilic components in emulsions.

The physical state of the water phase of emulsions, e.g., gelation, can modify the release of entrapped lipophilic components and thus their oral bioavailability. Filled hydrogel beads are a type of emulsion with gel-structured water phase, which can be used to control the digestion and release of lipophilic components entrapped in oil

droplets. For example, filled hydrogel beads were successfully prepared by injecting emulsions containing alginate in the water phase into a calcium chloride solution, which can caused the alginate molecules to cross-link with each other through ion bridging, leading to the formation of alginate gels containing oil droplets (McClements & Li, 2010). The oil droplets encapsulated in the alginate gels showed a slow rate of lipid digestion, with <8% FFA being released during the first 25 min of digestion, demonstrating that this kind of emulsion-filled alginate gels can effectively retard lipid digestion, and thus potentially lead to a sustained release of encapsulated lipophilic components in oil droplets.

Several factors might result in this decreased rate of lipid digestion: (i) without alginate gel, the lipase can rapidly adsorb to the oil droplet surfaces and thereby quickly initiate the lipolysis process; (ii) when oil droplets are encapsulated in alginate gels, lipase must diffuse through the gel network to reach them. The diffusion rate of lipase will be affected by the pore size of the gel network and specific or non-specific interactions (e.g., electrostatic or hydrophobic interactions) of the lipase with the molecules that comprise the gel network (Gombotz & Wee, 2012); (iii) the movement of FFA away from the oil droplet surface may be slowed down by the gel network surrounding the oil droplets, which accordingly increases the local concentration of hydrolysates (free fatty acids) and thus decreases the rate of enzymatic hydrolysis rate of lipase (Fave, 2004; Gilchrist & Martin, 1983).

However, gelled emulsion-based carriers can also show advantages in delivering fish oil compared with conventional soft gel capsules. A study reported that fish oils rich in DHA and EPA delivered by gelled emulsions showed a significantly increased mean plasma levels in health young adults after oral administration, compared with that delivered through soft gel capsules (Haug et al., 2011). The

plasma C_{max} (μg/mL) level of EPA and DHA in gelled emulsions was significantly (p<0.05) higher than those from soft gel capsules while the t_{max} (h) of these in gelled emulsions was shorter than that in soft gel capsules, suggesting that improved oral bioavailability and fast absorption of EPA and DHA may be achieved by incorporating emulsified TAG fish oil in a gelled emulsion-based delivery system prior to oral ingestion.

4.2.4 Droplet Size

The size of the oil droplets reaching the small intestine varies widely depending on the properties of the droplets and the physicochemical processes occurring within the mouth, stomach, and small intestine phases of digestion, e.g., droplet digestion, flocculation, coalescence, and disruption (Li & McClements, 2010). The size of the oil droplets within the small intestine can affect their digestion rate, because the surface area of the droplet is closely related to their mean diameter. Recently, it was reported that, when administered directly into the small intestine, the effects of fat emulsions on gastrointestinal motility, hormone release, appetite, and energy intake, are related to their droplet size (Seimon et al., 2009). The findings have potential implications for the design of functional foods to maximize effects on the gut functions that are involved in the suppression of appetite, and could help to novel approaches to the prevention and management of obesity. It is therefore important to establish the influence of initial oil droplet size on the rate and extent of lipid digestion and on the bioaccessibility of encapsulated lipophilic components in emulsions.

The influence of droplet size (d_{32}=0.21, 0.70 or 2.2 μm) on the bioavailability of heptadecanoic acid and lipophilic nutraceutical (Coenzyme Q10) was investigated by Cho et al. (2014). Their results showed that small droplets (d_{32}=0.21 μm) were

digested more rapidly than large droplets (d_{32}=2.2 μm) using an *in vitro* simulated small intestinal digestion model (pH stat). A rat feeding study also showed that the bioavailability of the fatty acid and Coenzyme Q10 in small intestinal tissues significantly increased with reduced droplet size. Similarly, many other studies also confirmed the increased rate of lipid digestion and bioaccessibility of encapsulated lipophilic components with decreasing droplet size (Golding & Wooster, 2010; Helbig et al., 2012; Li & McClements, 2010; Nielsen et al., 2008; Parthasarathi, 2016; Salvia-Trujillo et al., 2017; Salvia-Trujillo et al., 2013a). This is mainly attributed to the fact that the surface area of lipid exposed to the surrounding aqueous phase increases as the mean oil droplet diameter decreases, which provides more sites for the lipase molecules to bind, leading to a faster and extended digestion of lipids, and thus an increased bioaccessibility of lipophilic components in emulsions. However, in most cases, only if the difference in droplet size is beyond 10-fold, can a reliable significantly difference in the bioaccessibility of encapsulated lipophilic components in emulsion be observed.

Furthermore, a comparison of lipase activity in emulsions with different emulsifiers and initial total surface area showed that lipase activity was equal (i.e. 4.5 U/mg for WPI-stabilized emulsion with a total surface area of 75.0 m^2 and gum arabic stabilized emulsions with total surface area 3.5 m^2), demonstrating that the choice of emulsifier has a larger effect on the hydrolysis of lipid in emulsions than the surface area (Helbig et al., 2012).

5. Safety

Nanotechnology opens a venue for the food industry to a number of new strategies for improving the quality, shelf life, safety, and health-benefits of foods. There is increasing concern from consumers, regulatory agencies, and the food industry about

the potential adverse effects (toxicity) of these nanotechnologies since they have been widely used in food industry. In particular, there is concern about the direct incorporation of artificial nanoparticles into foods, such as those used as delivery systems for pigments, flavors, preservatives, bioactive nutrients, and nutraceuticals, or those used to modify the optical, rheological, or flow properties of food products (McClements & Xiao, 2017).

Generally, the toxicity of ingested nanoparticles depends on their ability to damage cells or organs within humans, thereby adversely affecting human health or wellbeing. Cellular or organ damage can occur in various places within the gastrointestinal tract (GIT), as well as after absorption of the nanoparticles into the body (Buzea et al., 2007). Moreover, nanoparticles may damage the microbial cells that normally populate the human GIT, which could indirectly alter human health (Frohlich & Frohlich, 2016).

Nanoparticles in foods can be divided into two main categories, according to their composition, organic nanoparticles and inorganic nanoparticles. Inorganic nanoparticles are mainly composed of inorganic materials, such as silver, iron oxide, titanium dioxide, silicon dioxide, or zinc oxide (Pietroiusti et al., 2016). Organic nanoparticles are primarily composed of organic compounds, such as lipids, proteins, and/or carbohydrates. Since this thesis mainly focused on the dairy protein stabilized O/W emulsion-based delivery systems, organic nanoparticles are mainly discussed in this section.

Generally, organic nanoparticles are less toxic than inorganic ones, because they are often fully digested within the human GIT and are not bio-persistent. However, there might be certain circumstances where they could cause toxicity.

Altered location of bioactive release: The encapsulation of bioactive ingredients in nanoparticles may alter their location of release in the GIT. Consequently, the physiological response and biological impact of the bioactives may be altered, potentially leading to adverse health effects. For example, the encapsulation of digestible lipids in nano-laminated dietary-fibres may inhibit their digestibility in the upper GIT, leading to a high level of undigested lipids reaching the colon, which then be fermented by the colonic bacteria, and cause gastrointestinal problems (McClements, 2010a). Alternatively, antimicrobial agents are incorporated into nanoparticles that cannot be digested in the upper GIT. Then, the antimicrobial agent loaded nanoparticles reaches the colon, where they are digested and the released antimicrobial agent could accordingly alter the nature of the colonic microflora, and thus cause adverse health effects.

Enhancement of oral bioavailability: Many studies have shown that delivering bioactive agents within nanoparticles can greatly increase their bioavailability (Ting et al., 2014). However, excessively improving the bioavailability of some bioactive agents could cause health problems. For example, vitamin E is essential for maintaining human health. However, consumption of high doses of vitamin E may increase the risk of various chronic diseases (Miller, 2005). Many studies have not taken into account the issue of a significantly improved bioavailability of the bioactive agents delivered by nanoparticles when establishing their upper limits for the adverse health effects. Thus, the level where toxic effects are observed could be appreciably lower than published upper limits, because nanoparticle delivery systems can greatly increase the bioavailability of these bioactive agents (McClements & Xiao, 2017).

Interference with gut microbiota: Some inorganic nanoparticles that reach the colon can interact with colonic bacteria and alter their viability, thus changing the proportions of different bacterial species in the microbiota society (Frohlich & Frohlich, 2016; Pietroiusti et al., 2016). The constitution of the microbiota plays a very important role in maintaining human health and well-being (Consortium, 2012; Pflughoeft & Versalovic, 2012). Therefore, any changes in the gut microbiota induced by the presence of the food nanoparticles can potentially have adverse health effect. However, little is known about the influence of organic food-grade nanoparticles on the gut microbiota and further research need to be done.

6. Objectives of This Study

A number of studies described above investigate the influence of the emulsion structure on the digestion, release, bioaccessibility, and bioaccessibility of encapsulated lipophilic components in emulsions mainly through a simulated *in-vitro* GIT digestion model, besides several *in vivo* tests. However, these studies mostly focused on the amount of components that have been released and incorporated into micelles after intestinal phase digestion, but does not provide information on the consequent absorption of these micelles by enterocytes, especially those micelles with different surface structures derived from emulsion droplets with different emulsifiers before GIT digestion. Even though these micelle fractions contained same amount of lipophilic components, their different surface structures can still lead to significant different absorption by enterocytes. This may also be the main cause of the inconsistency observed between the bioaccessibility and the *in vivo* bioavailability of lipophilic components in emulsions (Salvia-Trujillo et al., 2017). Therefore, a cellular uptake assay is potentially needed to evaluate the cellular uptake of these nutrient-loaded micelles by enterocytes following the GIT digestion. This may contribute to a better prediction of the potential bioavailability of lipophilic components delivered through emulsion-based carriers, which is more closed to the real situation *in vivo*.

In the present study, model O/W emulsion based delivery systems with different oil phase, oil-water interface, and water phase, containing lipophilic bioactive nutrients (using β-carotene as an example) were prepared. A simulated *in vitro* GIT digestion model consisting of mouth, gastric, and intestinal phases combined with a cellular uptake test by enterocytes (Caco-2 cells) was employed to get a better understanding of the relationship between the emulsion structure and the oral

absorption of encapsulated lipophilic components in an attempt to achieve a potential controlled release and improved bioavailability of these compounds by designing the structure of emulsion-based carriers.

The study was divided into the following 5 main objectives:

(i) To investigate the effect of initial droplet size and selection of emulsifiers on the emulsion properties, emulsion stabilities, and the cellular uptake of encapsulated lipophilic components without passing through GIT;

(ii) To investigate the effect of the composition and microstructure of the oil phase on the digestion, release and cellular uptake of lipophilic components delivered through model O/W emulsions

(iii) To investigate the effect of different emulsifiers on the digestion, release and cellular uptake of lipophilic components delivered through model O/W emulsions;

(iv) To investigate the effect of the composition and the property of the water phase on the digestion, release and cellular uptake of lipophilic components delivered through model O/W emulsions;

(v) To investigate the effect of drying process (spray-, or freeze-drying) on the properties of liquid emulsions, and the influence of liquid emulsion formulas on the properties (e.g., microstructure and re-dispersibility) of powdered emulsions.

References

Aditya, N. P., Aditya, S., Yang, H., Kim, H. W., Park, S. O., & Ko, S. (2015). Co-delivery of hydrophobic curcumin and hydrophilic catechin by a water-in-oil-in-water double emulsion. *Food Chemistry, 173*, 7-13.

Anwar, S. H., & Kunz, B. (2011). The influence of drying methods on the stabilization of fish oil microcapsules: Comparison of spray granulation, spray drying, and freeze drying. *Journal of Food Engineering, 105*, 367-378.

Bhushani, J. A., Karthik, P., & Anandharamakrishnan, C. (2016). Nanoemulsion based delivery system for improved bioaccessibility and Caco-2 cell monolayer permeability of green tea catechins. *Food Hydrocolloids, 56*, 372-382.

Boekel., V. (1980). *Influence of fat crystals in the oil phase on stability of oil-inwater emulsions.* (Ph.D.), Agricultural University, Wageningen.

Boode, K. P. (1992). *Coalescence in oil-in-water emulsions.* (Ph.D.), Agricultural University, Wageningen.

Burgos-Díaz, C., Wandersleben, T., Marqués, A. M., & Rubilar, M. (2016). Multilayer emulsions stabilized by vegetable proteins and polysaccharides. *Current Opinion in Colloid and Interface Science, 25*, 51-57.

Buzea, C., Pacheco, I. I., & Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases, 2*, 17-71.

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature, 519*, 92-96.

Cho, H. T., Salvia-Trujillo, L., Kim, J., Park, Y., Xiao, H., & McClements, D. J. (2014). Droplet size and composition of nutraceutical nanoemulsions influences bioavailability of long chain fatty acids and Coenzyme Q10. *Food Chemistry, 156*, 117-122.

Christensen, K.L., Pedersen, G. P., & Kristensen, H.G. (2001). Preparation of redispersible dry emulsions by spray drying. *International Journal of Pharmaceutics, 212*, 8.

Consortium, H. M. P. (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*, 207-214.

Couedelo, L., Amara, S., Lecomte, M., Meugnier, E., Monteil, J., Fonseca, L., Pineau, G., Cansell, M., Carriere, F., Michalski, M.C., Vaysse, C. (2015). Impact of various emulsifiers on ALA bioavailability and chylomicron synthesis through changes in gastrointestinal lipolysis. *Food &Function, 6*, 1726-1735.

Darling, D. F. (1982). Recent advances in the destabilization of dairy emulsions. *Journal of Dairy Research, 49*, 695-712.

Dias, M. L., Carvalho, J. P., Rodrigues, D. G., Graziani, S. R., & Maranhao, R. C. (2007). Pharmacokinetics and tumor uptake of a derivatized form of paclitaxel associated to a cholesterol-rich nanoemulsion (LDE) in patients with gynecologic cancers. *Cancer Chemotherapy and Pharmacology, 59*, 105-111.

Dickinson, E. (1992). *Introduction to Food Colloids*. Oxford, UK: Oxford University Press.

Dickinson, E. (2011). Mixed biopolymers at interfaces: Competitive adsorption and multilayer structures. *Food Hydrocolloids, 25*, 1966-1983.

Dickinson, E. (2012). Emulsion gels: The structuring of soft solids with protein-stabilized oil droplets. *Food Hydrocolloids, 28*(1), 224-241.

Dickinson, E., Stainsby, G. (1982). *Colloids in Foods*. London, UK: Elsevier.

Dluska, E., Markowska-Radomska, A., Metera, A., Tudek, B., & Kosicki, K. (2017). Multiple emulsions as effective platforms for controlled anti-cancer drug delivery. *Nanomedicine, 12*, 2183-2197.

Donsi, F., Annunziata, M., Vincensi, M., & Ferrari, G. (2012). Design of nanoemulsion-based delivery systems of natural antimicrobials: effect of the emulsifier. *Journal of Biotechnology, 159*, 342-350.

Enomoto, Y., Tokuyama, M., & Kawasaki, K. . (1986). Finite volume fraction effects on Ostwald ripening. *Acta Metallurgica, 34*, 2119-2128.

Ensign, L. M., Cone, R., & Hanes, J. (2012). Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Advanced Drug Delivery Reviews, 64*, 557-570.

Evans, D. F., Wennerstrom, H. (1994). *The Colloidal Domain: Where Physics, Chemistry, Biology and Technology Meet*. New York: VCH Publishers.

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols-a review. *Trends in Food Science and Technology, 21*, 510-523.

Fatouros, D. G., & Mullertz, A. (2008). In vitro lipid digestion models in design of drug delivery systems for enhancing oral bioavailability. *Expert Opinion on Drug Metabolism & Toxicology, 4*, 65-76.

Fave, G., Coste, T.C., & Armand, M. (2004). Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability. *Cellular and Molecular Biology, 50*, 815-831.

Friberg, S. E. (1997). *Emulsion stability, in Food Emulsions* (3rd ed.). New York: Marcel Dekker.

Frohlich, E. E., & Frohlich, E. (2016). Cytotoxicity of Nanoparticles Contained in Food on Intestinal Cells and the Gut Microbiota. *International Journal of Molecular Sciences, 17*, 509.

Fryd, M. M., & Mason, T. G. (2012). Advanced nanoemulsions. *Annual Review of Physical Chemistry, 63*, 493-518.

Galho, A. R., Cordeiro, M. F., Ribeiro, S. A., Marques, M. S., Antunes, M. F., Luz, D. C., Hadrich, G., Muccillo-Baisch, A.L., Barros, D.M., Lima, J.V. (2016). Protective role of free and quercetin-loaded nanoemulsion against damage induced by intracerebral haemorrhage in rats. *Nanotechnology, 27*, 175101.

Gharsallaoui, A., Saurel, R., Chambin, O., Cases, E., Voilley, A., & Cayot, P. (2010). Utilisation of pectin coating to enhance spray-dry stability of pea protein-stabilised oil-in-water emulsions. *Food Chemistry, 122*, 447-454.

Gilchrist, T., & Martin, A. (1983). Wound treatment with Sorbsan-an alginate fibre dressing. *Biomaterials, 4*, 317-320.

Goff, H. D. (1997). Instability and partial coalescence in whippable dairy emulsions. *Journal of Dairy Science, 80*, 2620-2630.

Golding, M., & Wooster, T. J. (2010). The influence of emulsion structure and stability on lipid digestion. *Current Opinion in Colloid and Interface Science, 15*, 90-101.

Gombotz, W. R., & Wee, S. F. (2012). Protein release from alginate matrices. *Advanced Drug Delivery Reviews, 64*, 194-205.

Greg A. Kossena , B. J. B., Christopher J.H. Porter, and William N. Charman. (2003). Separation and characterization of the colloidal phases produced on digestion of common formulation lipids and assessment of their impact on the apparent

solubility of selected poorly water-soluble drugs. *Journal of Pharmaceutical Sciences, 92*, 634-648.

Gudipati, V., Sandra, S., McClements, D. J., & Decker, E. A. (2010). Oxidative stability and in vitro digestibility of fish oil-in-water emulsions containing multilayered membranes. *Journal of Agricultural and Food Chemisty, 58*, 8093-8099.

Gulotta, A., Saberi, A. H., Nicoli, M. C., & McClements, D. J. (2014). Nanoemulsion-based delivery systems for polyunsaturated (omega-3) oils: formation using a spontaneous emulsification method. *Journal of Agricultural and Food Chemisty, 62*, 1720-1725.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: formation, properties and applications. *Soft Matter, 12*, 2826-2841.

Gutiérrez, J. M., González, C., Maestro, A., Solè, I., Pey, C. M., & Nolla, J. (2008). Nano-emulsions: New applications and optimization of their preparation. *Current Opinion in Colloid and Interface Science, 13*, 245-251.

Guttoff, M., Saberi, A. H., & McClements, D. J. (2015). Formation of vitamin D nanoemulsion-based delivery systems by spontaneous emulsification: factors affecting particle size and stability. *Food Chemistry, 171*, 117-122.

Ha, T. V., Kim, S., Choi, Y., Kwak, H. S., Lee, S. J., Wen, J., Oey, I., Ko, S. (2015). Antioxidant activity and bioaccessibility of size-different nanoemulsions for lycopene-enriched tomato extract. *Food Chemistry, 178*, 115-121.

Hatanaka, J., Chikamori, H., Sato, H., Uchida, S., Debari, K., Onoue, S., & Yamada, S. (2010). Physicochemical and pharmacological characterization of alpha-tocopherol-loaded nano-emulsion system. *International Journal of Pharmaceutics, 396*, 188-193.

Haug, I. J., Sagmo, L. B., Zeiss, D., Olsen, I. C., Draget, K. I., & Seternes, T. (2011). Bioavailability of EPA and DHA delivered by gelled emulsions and soft gel capsules. *European Journal of Lipid Science and Technology, 113*, 137-145.

Helbig, A., Silletti, E., Timmerman, E., Hamer, R. J., & Gruppen, H. (2012). In vitro study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocolloids, 28*, 10-19.

Hiemenz, P. C., Rajagopalan, R. (1997). *Principles of Colloid and Surface Chemistry* (3rd ed.). New York: Marcel Dekker.

Hinrichs, J., & Kessler, H.G. (1997). Fat content of milk and cream and effects on fat globule stability. *J Food Sci, 62*, 992-995.

Hou, Z., Liu, Y., Lei, F., & Gao, Y. (2014). Investigation into the in vitro release properties of β-carotene in emulsions stabilized by different emulsifiers. *LWT - Food Science and Technology, 59*, 867-873.

Hunter, R. J. (2001). *Foundations of Colloid Science* (2nd ed.). Oxford: New York: Oxford University Press.

Jafari, E. A. S.-M. (2017). Spray drying of folic acid within nano-emulsions: Optimization by Taguchi approach. *Drying Technology, 35*, 1152-1160.

Joye, I. J., Davidov-Pardo, G., & McClements, D. J. (2014). Nanotechnology for increased micronutrient bioavailability. *Trends in Food Science and Technology, 40*, 168-182.

Kabal'nov, A. S., Pertzov, A.V., & Shchukin, E.D. (1987). Ostwald ripening in two-component disperse phase systems: Application to emulsion stability. *Colloids and Surfaces, 24*, 19-32.

Kabal'nov, A. S., & Shchukin, E.D. (1992). Ostwald ripening theory: applications to fluorocarbon emulsion stability. *Advances in Colloid and Interface Science , 38*.

Karthik, P., & Anandharamakrishnan, C. (2016). Enhancing omega-3 fatty acids nanoemulsion stability and in-vitro digestibility through emulsifiers. *Journal of Food Engineering, 187*, 92-105.

Kumar, D., Ali, J., & Baboota, S. (2016). Omega 3 fatty acid-enriched nanoemulsion of thiocolchicoside for transdermal delivery: formulation, characterization and absorption studies. *Drug Delivery, 23*, 591-600.

Lee, M. N., Chan, H. K., & Mohraz, A. (2012). Characteristics of pickering emulsion gels formed by droplet bridging. *Langmuir, 28*, 3085-3091.

Li, Y., Hu, M., Xiao, H., Du, Y., Decker, E. A., & McClements, D. J. (2010). Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings. *European Journal of Pharmaceutics and Biopharmaceutics, 76*, 38-47.

Li, Y., & McClements, D. J. (2010). New mathematical model for interpreting pH-stat digestion profiles: impact of lipid droplet characteristics on in vitro digestibility. *Journal of Agricultural and Food Chemistry, 58*(13), 8085-8092.

Li, Y., Xiao, H., & McClements, D. J. (2012). Encapsulation and Delivery of Crystalline Hydrophobic Nutraceuticals using Nanoemulsions: Factors Affecting Polymethoxyflavone Solubility. *Food Biophysics, 7*, 341-353.

Like Mao, D. W., Fuguo Liu & Yanxiang Gao. (2017). Emulsion design for the delivery of β-carotene in complex food systems. *Critical Reviews in Food Science and Nutrition, 58*, 770-784.

Lin, W., Hong, J. L., Shen, G., Wu, R. T., Wang, Y., Huang, M. T., Newmark, H.L., Huang, Q.R., Khor, T.O., Heimbach, T., Kong, A. N. (2011). Pharmacokinetics of dietary cancer chemopreventive compound dibenzoylmethane in rats and the impact of nanoemulsion and genetic knockout of Nrf2 on its disposition. *Biopharmaceutics & Drug Disposition , 32*, 65-75.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Lu, W., Zheng, B., & Miao, S. (2018). Improved emulsion stability and modified nutrient release by structuring O/W emulsions using konjac glucomannan. *Food Hydrocolloids, 81*, 120-128.

Lucile Bonnaire, S. S., Thrandur Helgason, Eric Andrew Decker, Jochen Weiss and David Julian McClements. (2008). Influence of Lipid Physical State on the in Vitro Digestibility of Emulsified Lipids. *Journal of Agricultural and Food Chemistry, 56*, 3791-3797.

Mao, L., & Miao, S. (2015). Structuring Food Emulsions to Improve Nutrient Delivery During Digestion. *Food Engineering Reviews, 7*, 439-451.

Mao, L., Calligaris, S., Barba, L., & Miao, S. (2014). Monoglyceride self-assembled structure in O/W emulsion: formation, characterization and its effect on emulsion properties. *Food Research International, 58*, 81-88.

Martin Wickham, M. G., John Leney, Peter D. G. Wilson, and Annette Fillery-Travis. (1998). Modification of a phospholipid stabilized emulsion interface by bile salt effect on pancreatic lipase activity. *Journal of Lipid Research, 39*, 623-632.

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., & Graves, S. M. (2006). Nanoemulsions: formation, structure, and physical properties. *Journal of Physics: Condensed Matter, 18*, R635-R666.

McClements, D. J. (2004). *Food emulsions: principles, practices, and techniques*. New York: CRC press.

McClements, D. J. (2010a). Design of nano-laminated coatings to control bioavailability of lipophilic food components. *Journal of Food Science, 75*, R30-42.

McClements, D. J. (2010b). Emulsion design to improve the delivery of functional lipophilic components. *Annual Review of Food Science and Technology, 1*, 241-269.

McClements, D. J. (2012). Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter, 8*, 1719-1729.

McClements, D. J. (2015). *Food Emulsions: principles, practices, and techniques* (3rd ed.). New York: CRC press.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science, 72*, R109-124.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*, 213-228.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food science and Nutrition, 51*, 285-330.

McClements, D. J., & Xiao, H. (2017). Is nano safe in foods? Establishing the factors impacting the gastrointestinal fate and toxicity of organic and inorganic food-grade nanoparticles. *npj Science of Food, 1*.

Mehnert, W., & Mäder, K. (2012). Solid lipid nanoparticles: Production, characterization and applications. *Advanced Drug Delivery Reviews, 64*, 83-101.

Miller ER, P.-B. R., Dalal D, Riemersma RA, Appel LJ, Guallar E. (2005). Meta-Analysis High-Dosage Vitamin E Supplementation May Increase All-Cause Mortality. *Annals of Internal Medicine, 142*, 37-46.

Mun, S., Decker, E. A., & McClements, D. J. (2007). Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. *Food Research International, 40*, 770-781.

Munin, A., & Edwards-Levy, F. (2011). Encapsulation of natural polyphenolic compounds; a review. *Pharmaceutics, 3*, 793-829.

Nielsen, F. S., Petersen, K. B., & Mullertz, A. (2008). Bioavailability of probucol from lipid and surfactant based formulations in minipigs: influence of droplet size and dietary state. *European Journal of Pharmaceutics and Biopharmaceutics, 69*, 553-562.

Nielsen, P.B., Mullertz, A., Norling, T., & Kristensen, H.G. (2001). The effect of α-tocopherol on the in vitro solubilisation of lipophilic drugs. *International Journal of Pharmaceutics, 222*, 217-224.

Ozturk, B., Argin, S., Ozilgen, M., & McClements, D. J. (2014). Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural surfactants: Quillaja saponin and lecithin. *Journal of Food Engineering, 142*, 57-63.

Park, G. Y., Mun, S., Park, Y., Rhee, S., Decker, E. A., Weiss, J., McClements, D.J., Park, Y. (2007). Influence of encapsulation of emulsified lipids with chitosan on their in vivo digestibility. *Food Chemistry, 104*, 761-767.

Parthasarathi, S., Muthukumar, S. P., & Anandharamakrishnan, C. (2016). The influence of droplet size on the stability, in vivo digestion, and oral bioavailability of vitamin E emulsions *Food &Function, 7*, 2294-2302.

Pflughoeft, K. J., & Versalovic, J. (2012). Human microbiome in health and disease. *Annual Review of Pathology, 7*, 99-122.

Pietroiusti, A., Magrini, A., & Campagnolo, L. (2016). New frontiers in nanotoxicology: Gut microbiota/microbiome-mediated effects of engineered nanomaterials. *Toxicology and Applied Pharmacology, 299*, 90-95.

Porter, C. J., Trevaskis, N. L., & Charman, W. N. (2007). Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nature Reviews Drug Discovery, 6*, 231-248.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. *Food Chemistry, 135*, 1440-1447.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry, 132*, 1221-1229.

Saehun Mun, E. A. D., Yeonhwa Park, Jochen Weiss, and D. Julian McClements. (2006). Influence of Interfacial Composition on in Vitro Digestibility of Emulsified Lipids Potential Mechanism for Chitosan's Ability to Inhibit Fat Digestion. *Food Biophysics, 1*, 21-29.

Salvia-Trujillo, L., Fumiaki, B., Park, Y., & McClements, D. J. (2017). The influence of lipid droplet size on the oral bioavailability of vitamin D2 encapsulated in emulsions: an in vitro and in vivo study. *Food &Function, 8*, 767-777.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013a). Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry, 141*, 1472-1480.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013b). Modulating beta-carotene bioaccessibility by controlling oil composition and concentration in edible nanoemulsions. *Food Chemistry, 139*, 878-884.

Salvia-Trujillo, L., Sun, Q., Um, B. H., Park, Y., & McClements, D. J. (2015). In vitro and in vivo study of fucoxanthin bioavailability from nanoemulsion-based delivery systems: Impact of lipid carrier type. *Journal of Functional Foods, 17*, 293-304.

Santos, O. D. H., Mezadri, Hygor, Aguiar, Tatiana A., Oliveira, Juliana S. . (2011). Hydrogel-Thickened Nanoemulsion with Green Coffee Seed oil for topical delivery of vitamin A. *Latin American Journal of Pharmacy, 30*, 1999-2003.

Schultz, S., Wagner, G., Urban, K., & Ulrich, J. (2004). High-Pressure Homogenization as a Process for Emulsion Formation. *Chemical Engineering and Technology, 27*, 361-368.

Seimon, R. V., Wooster, T., Otto, B., Golding, M., Day, L., Little, T. J., Horowitz, M., Clifton, P.M., Feinle-Bisset, C. (2009). The droplet size of intraduodenal fat emulsions influences antropyloroduodenal motility, hormone release, and appetite in healthy males. *The American Journal of Clinical Nutrition, 89*, 1729-1736.

Sharma, S., Sahni, J. K., Ali, J., & Baboota, S. (2015). Effect of high-pressure homogenization on formulation of TPGS loaded nanoemulsion of rutin - pharmacodynamic and antioxidant studies. *Drug Delivery, 22*, 541-551.

Slavka Tcholakova, N. D. D., Doroteya Sidzhakova,, & Ivan B. Ivanov, a. B. C. (2003). Interrelation between Drop Size and Protein Adsorption at Various Emulsification Conditions. *Langmuir, 19*.

Sun, Y., Xia, Z., Zheng, J., Qiu, P., Zhang, L., McClements, D. J., & Xiao, H. (2015). Nanoemulsion-based delivery systems for nutraceuticals: Influence of carrier oil type on bioavailability of pterostilbene. *Journal of Functional Foods, 13*, 61-70.

Tan, C. T. (2004). Beverage emulsions. In S. Friberg, Larsson, K., Sjoblom, J. (Ed.), *Food Emulsions: Principles, Practices, and Techniques* (4th ed.). New York: Marcel Dekker.

Taylor, P. (1998). Ostwald ripening in emulsions. *Adv Colloid Interface Sci, 75*, 107-163.

Taylor, P., & Ottewill, R.H. (1994). The formation and ageing rates of oil-in-water miniemulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 88*, 303-316.

Thanasukarn, P., Pongsawatmanit, R., & McClements, D.J. . (2004). Impact of fat and water crystallization on the stability of hydrogenated palm oil-in-water emulsions stabilized by whey protein isolate. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 246*, 49-59.

Tianyao Huo, M. G. F., Steven J. Schwartz, and Mark L. Failla. (2007). Impact of Fatty Acyl Composition and Quantity of Triglycerides on Bioaccessibility of Dietary Carotenoids. *Journal of Agricultural and Food Chemisty, 55*, 8950-8957.

Ting, Y., Jiang, Y., Ho, C., & Huang, Q. (2014). Common delivery systems for enhancing in vivo bioavailability efficacy of nutraceuticals. *Journal of Functional Foods, 7*, 112-128.

van Boekel, M. A. J. S., & Walstra, P. (1981). Stability of oil-in-water emulsions with crystals in the disperse phase. *Colloids and Surfaces, 3*, 109-118.

Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science, 48*, 333-349.

Walstra, P. (2003). *Physical Chemistry of Foods*. New York: Marcel Decker.

Weiss, J., Decker, E. A., McClements, D. J., Kristbergsson, K., Helgason, T., & Awad, T. (2008). Solid Lipid Nanoparticles as Delivery Systems for Bioactive Food Components. *Food Biophysics, 3*, 146-154.

Weiss, J., Scherze, I., & Muschiolik, G. (2005). Polysaccharide gel with multiple emulsion. *Food Hydrocolloids, 19*, 605-615.

Wildman, R. E. C. (2001). *Hand Book of Nutraceuticals and Functional Foods* (3rd ed.). Boca Raton: CRC Press.

Wissing, S. A., Kayser, O., & Müller, R. H. (2004). Solid lipid nanoparticles for parenteral drug delivery. *Advanced Drug Delivery Reviews, 56*, 1257-1272.

Yin, L.-J., Chu, B.-S., Kobayashi, I., & Nakajima, M. (2009). Performance of selected emulsifiers and their combinations in the preparation of β-carotene nanodispersions. *Food Hydrocolloids, 23*, 1617-1622.

Yuan, Y., Gao, Y., Zhao, J., & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International, 41*, 61-68.

Zeeb, B., Lopez-Pena, C. L., Weiss, J., & McClements, D. J. (2015). Controlling lipid digestion using enzyme-induced crosslinking of biopolymer interfacial layers in multilayer emulsions. *Food Hydrocolloids, 46,* 125-133.

Zou, L., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2015). Designing excipient emulsions to increase nutraceutical bioavailability: emulsifier type influences curcumin stability and bioaccessibility by altering gastrointestinal fate. *Food &Function, 6,* 2475-2486.

CHAPTER TWO

Correlation of Emulsion Structure with Cellular Uptake Behaviour of Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure

Published in *Journal of Agricultural and Food Chemistry*, 2016, 64, 8659-8666.

Authors: Wei Lu, Alan L. Kelly, Pierce Maguire, Hongzhou Zhang, Catherine Stanton, and Song Miao*

The work contained in this chapter was undertaken and written solely by myself with specific contributions from each co-author.

Abstract

In this study, an *in vitro* Caco-2 cell culture assay was employed to evaluate the correlation between emulsion structure and cellular uptake of encapsulated β-carotene. After 4h incubation, an emulsion stabilized with whey protein isolate showed the highest intracellular accumulation of β-carotene (1.06 μg), followed by that stabilised with sodium caseinate (0.60 μg) and Tween® 80 (0.20 μg), which are 13-, 7.5-, and 2.5-fold higher than that of free β-carotene (0.08 μg), respectively. Emulsions with small droplet size (239±5 nm) showed a higher cellular uptake of β-carotene (1.56 μg) than emulsion with large droplet size (489±9 nm) (0.93 μg) (p<0.01). The results suggested that delivery in an emulsion significantly improved the cellular uptake of β-carotene and thus potentially its bioavailability; uptake was closely correlated with the interfacial composition and droplet size of emulsions. The findings support the potential for achieving optimal controlled and targeted delivery of bioactive nutrients by structuring emulsions.

Key Words: bioactive nutrients, bioavailability, emulsion, encapsulation, delivery

1. Introduction

Instability and extreme water insolubility of lipophilic bioactive nutrients, e.g., β-carotene, lycopene, quercetin, vitamins, and ω-3 fatty acids, greatly limit their bioavailability and thus their potential health benefits. Hence, the delivery of these compounds requires a protective mechanism. Utilization of modern nano-encapsulation and delivery technology can achieve this (Borel & Sabliov, 2014; Joye et al., 2014). Among these, emulsions are ideal delivery systems due to their high encapsulation capability, maintenance of chemical stability, and controlled release of encapsulated molecules. Many emulsion-based delivery systems have been successfully developed to protectively encapsulate and deliver a variety of lipophilic bioactive nutrients (Karthik & Anandharamakrishnan, 2016; Lu et al., 2016; McClements & Li, 2010) and emulsion-based encapsulation and delivery of these compounds significantly improves their stability, solubility and bioavailability (Heo et al., 2016; Mao & Miao, 2015; McClements, 2010b).

Improved bioavailability of encapsulated bioactive nutrients in nanodelivery systems may be attributed to several potential mechanisms. *In vitro* simulated gastrointestinal tract (GIT) digestion assays showed that encapsulated lipophilic compounds in oil-in-water (O/W) emulsions form micelles during digestion more readily than pure free molecules and a positive correlation of the amount of bioactive nutrients loaded micelles with the *in vivo* absorption of bioactive nutrients by intestinal epithelial cells was observed (Salvia-Trujillo et al., 2015), suggesting that simulated GIT can potentially predict the *in vivo* bioavailability of encapsulated compounds in emulsions. Emulsion structure, e.g., droplet size (Salvia-Trujillo et al., 2013a), and oil phase (Uluata et al., 2015), can significantly influence the total amount of micelles loaded with bioactive nutrients in a simulated GIT digestion

assay. In addition, simulated GIT assays can provide information on the influence of emulsion compositions on the microstructural changes of emulsion droplets occurring during digestion (Hur et al., 2009; Salvia-Trujillo et al., 2013a), e.g., droplet size, surface charge and the competitive adsorption of bile salts. All these changes potentially lead to different absorption efficiency and thus bioavailability of encapsulated compounds in emulsions.

Encapsulation in an emulsion can improve bioavailability of lipophilic nutrients by increasing their plasma concentration after oral administration to animals (Siddiqui et al., 2009; Ting et al., 2015; Zhang et al., 2013); emulsion properties, including droplet size, stability, interface compositions, lipid types and compositions, significantly influence the *in vivo* digestion of lipids in the oil phase and thus the fate of the encapsulated compounds in oil phase (Golding & Wooster, 2010).

Enhanced cellular uptake is also considered as one of the potential mechanisms of improved bioavailability of bioactive nutrients following nanoencapsulation, since uptake of nanoparticles by digestive tract mucosa *via* mucosa-associated lymphatic tissues (MALT) is possible (Lu et al., 2012). In addition, encapsulation in an emulsion can significantly improve the cellular uptake of encapsulated molecules (Gaumet et al., 2010; Sun et al., 2010). Accordingly, it is hypothesized that emulsion droplets loaded with bioactive nutrients, as one type of nanoparticles, can be directly absorbed *via* monolayer cells and thus lead to higher bioavailability of these compounds. However, little is known about the correlation between emulsion structure, e.g., droplet size, interfacial compositions, or oil compositions, and cellular uptake of encapsulated molecules by enterocytes. Our previous studies confirmed the possibility of controlled release of flavor compounds from emulsions (Mao, Boiteux, et al., 2014; Mao, Calligaris, et al., 2014), but less is known about the encapsulation

of bioactive nutrients and their absorption efficiencies. In addition, simulated GIT digestion focuses on the amount of bioactive nutrients that have been incorporated into micelles during digestion, but does not provide information on the consequent absorption behaviors of these micelles through enterocytes cells. Therefore, an *in vitro* cellular uptake assay is potentially needed to evaluate the cellular uptake of these encapsulated bioactive nutrients before and after passing through simulated GIT digestion, which may contribute to the better understanding of the mechanisms of improved bioavailability of bioactive nutrients by emulsion delivery. This study may also contribute to the development of parenteral emulsions containing nutrients with improved delivery efficiencies. Parenteral emulsions have been safely in medical use for over five decades (Hormann & Zimmer, 2016) and they can be applied by intravenous or hypodermic injection without passing through the GIT digestion.

Above all, this study aimed to investigate the correlation of emulsion structure (droplet size and emulsifiers) with the cellular uptake of encapsulated lipophilic components by enterocytes without passing through the GIT with the objective of improving cellular uptake and thus potential *in vivo* bioavailability of these lipophilic components by altering the composition and properties of emulsion-based delivery systems. A model O/W emulsion was employed as a model delivery system and β-carotene was used as a model lipophilic bioactive nutrient. Caco-2 cells were used as the enterocytes.

2. Material and Methods

2.1 Materials

All-trans-β-carotene (>93%, UV), sodium caseinate, Tween® 80 (Polysorbate) Dulbecco's modified eagle's medium (DMEM) for tissue culture (containing 4.5 g/L D-glucose), penicillin and streptomycin (100×), fetal bovine serum, phosphate buffer solution and cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunflower oil (Solesta, >98% fat) was purchased from a local supermarket (ALDI, Fermoy, Co.Cork, Ireland), and whey protein isolate (70% β-lactoglobulin and 18% α-lactalbumin) was obtained from Davisco Food International (Le Sueur, MN, USA). The human colon carcinoma cell lines (Caco-2 cells, ATCC, Manassas, VA, USA) were grown in DMEM, and cells after 40-55 passages were used for this study. All other chemicals and reagents used were of HPLC grade and obtained from Sigma-Aldrich. Ultrapure water (18 mΩ) was prepared by a Milli-Q apparatus (Millipore Corp.).

2.2 Preparation of Emulsions with Different Droplet Size

Whey protein isolate (WPI) was dispersed (1.0%, w/w) in phosphate buffer (1/15 M, pH 7.0, PBS) containing 0.01% (w/w) sodium azide as water phase. The dispersions were stirred for 2 h and kept at room temperature overnight. β-carotene (0.2%, w/w in oil phase) was first dissolved in the sunflower oil (10% w/w in the final emulsions) at 140 °C for 30 sec and then mixed with water phase dispersions at a speed of 10,000 rpm for 1 min using an Ultra-Turrax (IKA, Staufen, Germany) followed by further high-pressure homogenization (APV 1000, SPX Flow Technology, Charlotte, North Carolina, USA) at 20 or 70 MPa to obtain WPI stabilized emulsions containing β-carotene with large (WPI-L) or small (WPI-S) oil droplets, respectively.

2.3 Preparation of Emulsions with Different Emulsifiers

WPI, sodium caseinate (SC), or tween 80 (TW) was dispersed (1.0%, w/w) in phosphate buffer (1/15 M, pH 7.0) containing 0.01% (w/w) sodium azide as water phase. The subsequent emulsion preparation was performed using the same process mentioned above except for a further high-pressure homogenization (M110-EH Microfluidizer, Microfluidics International Corp., Newton, MA, USA) step at 70 MPa for three passes.

2.4 Characterization of Particle Size and Zeta Potential of Emulsions

The mean particle size, and zeta potential of emulsion droplets were determined by dynamic light scattering (DLS) and particle electrophoresis technology using a laser particle analyzer (Nano-ZS, Malvern Instruments, Worcestershire, UK). Refractive indices of 1.47 for sunflower oil and 1.33 for ultrapure water were used and emulsions were diluted 1000-fold with PBS (pH 7.0) before testing.

2.5 Cryo-Scanning Electron Microscopy

Cryo-Scanning Electron Microscopy (Cryo-SEM) was employed to observe the real dispersion status of oil droplets in liquid emulsions. In addition, results from Cryo-SEM were used to confirm the droplet size obtained by DLS. The sample was deposited onto a sample holding cell, fixed in liquid nitrogen and the sample cell was then cleaved in situ in a Quorum Aquilo PP3010T Preparation Chamber. Furthermore, to remove the outer layers and potential contaminants, the sample underwent a sublimation step by holding at -120 °C for 5 min. Following this, the sample was sputter-coated with a thin layer of Pt, and loaded into the SEM chamber.

A Zeiss Ultra Plus with a Gemini column was used for high quality imaging. Samples were imaged at range of magnifications using a secondary electron (SE2)

detector with primary beam energy of 5 keV. Samples were fixed on contact with the liquid nitrogen, to obtain an observation environment which is approximate to the pre-frozen state.

2.6 Determination of Encapsulation Efficiency and Loading Capacity

The β-carotene contents in emulsions were determined by reversed-phase high-performance liquid chromatography (RP-HPLC), as outlined below. For the extract of BC outside of the droplets, the emulsion was first centrifuged at 15,000 g for 15 min and the separated 'cream layer' was removed. This process was repeated twice and then the remaining fraction was then centrifuged again at 15,000 g for 40 min, whereupon the bottom fraction was collected and extracted with n-hexane as the β-carotene fraction outside the oil droplets. The total β-carotene content of emulsions was obtained by de-emulsifying emulsions with ethanol (1:2, v/v) and then extracting with n-hexane (1:5, v/v) (Yuan et al., 2008); the de-emulsifying and extracting process was repeated three times. The hexane extracts were combined and dried under a stream of nitrogen gas, and dissolved with ethanol for HPLC analysis. The encapsulation rate and the loading capacity were calculated using the following equations:

$$\text{Encapsulation efficiency (\%)} = \frac{\textit{BC in total emulsions-free BC outside emulsions}}{\textit{BC in total emulsions}} \times 100\%$$

$$\text{Loading capacity (\%)} = \frac{\textit{BC in total emulsions-free BC outside emulsion droplets}}{\textit{quantity of total droplets (total oil content)}} \times 100\%$$

Where BC indicates β-carotene.

2.7 Creaming Stability

Creaming stability of emulsions was evaluated using Lumisizer (LUM GmbH, Berlin, Germany) as described by Mao et al. (Mao et al., 2012). Briefly, emulsions were transferred to measurement cells and analyzed by a light beam which scanned the cells vertically over the total length. The sensor received light transmitted through the sample, which showed a pattern of light flux as a function of the radial position at a given time. On the basis of the changes in the transmission signal, emulsion instability could be detected; for example, when creaming occurred, the transmission signal at the top of the sample decreased. In this study, emulsions containing β-carotene were centrifuged at 2,300 g at 25 °C with a scanning rate of once every 10 s for 1,000 s.

2.8 Effect of pH on Droplet Size and Surface Charge of Emulsions

The influence of pH on the mean particle size of emulsion droplets was evaluated. Emulsions were diluted with disodium hydrogen phosphate-citric acid buffer solutions with pH values ranging from 2.0 to 8.0, and maintained for 4 h at room temperature before particle characterization analysis. The mean particle size, size distribution, and zeta-potential of emulsion droplets were determined by Zetasizer, as described above.

2.9 Cytotoxicity Assay

Cell viability after treatment with emulsions was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Fischer et al, 2003). MTT, a yellow tetrazole, can be reduced to purple formaza in living cells. A solubilization solution (usually dimethyl sulfoxide, DMSO) can be added to dissolve the insoluble purple formaza product into a colored solution. Then, the absorbance of

this colored solution can be quantified by measuring at a certain wavelength (usually between 500-600 nm), and the absorbance is proportioned to the total amount of living cells (Mosmann et al, 1983).

Briefly, Caco-2 cells incubated (Cell Incubator, Sanyo) in DMEM containing 10% FBS were seeded at a density of 1.38×10^5 cells /well in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. Then, the medium was removed and 200 μL of medium diluted emulsions containing β-carotene was added. Pure medium (200 μL) was added to the control cells without treatment with an emulsion. After 24 h incubation, samples were removed and cells were washed by PBS buffer solution, 40 μL MTT in PBS (2.5 mg/mL) was then added to the wells and incubated for 2 h. The MTT was then removed and 100 μL DMSO was added to each well. The absorbance of each well was read with a Synergy HT microplate reader (BioTek, Winooski, VT, USA) at 570 nm. Cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{absorbance\ of\ emulsion\ treated\ cells}{absorbance\ of\ control} \times 100\%$$

2.10 Caco-2 Cellular Uptake Assay

Caco-2 cells were seeded in 6-well plate at a density of 3×10^5 cells /well. Cellular uptake experiments were performed 7-9 days after seeding. Emulsions containing β-carotene were diluted to a final β-carotene concentration of 10 μg/mL with medium (DMEM). β-carotene dissolved in DMSO was diluted to the same concentration (10 μg/mL) with medium as control. One mL emulsion containing β-carotene or control β-carotene solution was added to each well in a 6-well plate, which was then incubated at 37 °C and 5% CO_2. Before collection, cells were washed three times with phosphate buffer solution for tissue culture. Then, cells were collected at

several times intervals (0, 1, 2, 4 h), lysed, extracted and analyzed for β-carotene content by RP-HPLC.

Cell lysis followed the method described by Rahman et al. (Rahman et al., 2006) with minor modifications. Collected cells were dispersed in pure water and freeze-thawed twice at -80 °C, centrifuged at 3,000 g for 15 min at 4 °C, and the supernatant was immediately transferred to a pre-chilled tube at 4 °C. Cell lysis buffer (0.5 mL) was added to the pellets followed by lysis at room temperature for 15 min on a shaker. The lysate was centrifuged for 15 min at 15,000 g to pellet the cellular debris, and the supernatant was collected and combined with previous supernatant in pre-chilled tube. Combined supernatant was then extracted with ethanol/n-hexane and analyzed by HPLC.

2.11 Extraction and Quantification of β-carotene

β-carotene was extracted with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v). The extraction process was repeated three times for each sample (Yuan et al., 2008), and hexane fraction from each time of extraction was then combined and dried under a stream of nitrogen gas. Then, 0.4 mL ethanol was added to re-dissolve β-carotene for HPLC analysis.

For recovery test, one mL of β-carotene standard solution in ethanol was added to 1 mL sample, and the mixture was extracted as described above and analyzed by HPLC. The recovery of β-carotene was >96% in this study (data not shown).

2.12 HPLC Analysis of β-carotene

HPLC quantification of β-carotene was performed using an Agilent 1200 series system with a DAD UV-Vis detector (Agilent, Santa Clara, CA, USA). A reversed phase C_{18} column (4.6×250 mm, 5 μm, 300Å, Phenomenex) was employed. The

chromatography operation conditions mainly followed a previous study (Barba et al., 2006) with minor modification: column operation temperature at 30 °C; elution was performed with 90% ethanol and 10% acetonitrile from 0-30 min, flow rate was 1 mL/min, detection wavelength was 450 nm, and injection volume was 20 μL. The peak area of β-carotene on HPLC showed a good linear correlation with the β-carotene concentration in the range 0.01~10 μg/mL (data not shown).

2.13 Statistical Analysis

All the experiments were repeated at least three times. One-way analysis of variance (ANOVA) was employed to compare means of data. Significant differences were determined at the 0.05 level ($p<0.05$).

3. Results and Discussion

3.1 Droplet Size and Surface Charge

All emulsions had submicron droplet size ranging from 184-489 nm by DLS (**Table 2-1**); the emulsion stabilized by tween80 (TW) showed smaller average droplet size of 184 nm than that of emulsions stabilized by WPI, and sodium caseinate (SC). This is mainly attributed to the fact that non-ionic surfactants, e.g., tween, can adsorb more rapidly to the oil droplets and reduce the interfacial tension more effectively then biopolymers and/or proteins, e.g., WPI; therefore, they always form smaller droplets during homogenization (McClements, 2015).

Table 2-1. Mean Droplet Diameter (Z-average), Zeta Potential, Polydispersity Index (PdI), PdI width, Encapsulation Efficiency and Loading Capacity of Emulsions at pH 7.0 (mean±STD, n=3)

Emulsions	Z-average (nm)	Zeta potential (mV)	PdI	Encapsulation efficiency (%)	Loading capacity (%)
WPI	220 ± 6^{b}	-27.4 ± 2.2^{c}	0.226 ± 0.009^{c}	99.1 ± 0.5^{a}	1.96 ± 0.01^{a}
SC	204 ± 4^{b}	-29.7 ± 2.3^{c}	0.092 ± 0.01^{a}	99.3 ± 0.2^{a}	1.98 ± 0.02^{a}
TW	184 ± 4^{a}	-2.5 ± 0.2^{a}	0.168 ± 0.019^{b}	98.9 ± 1.1^{a}	2.01 ± 0.03^{a}
WPI-L	489 ± 9^{c}	-15.9 ± 0.7^{b}	0.289 ± 0.046^{d}	98.6 ± 0.3^{a}	2.03 ± 0.02^{a}
WPI-S	239 ± 5^{b}	-15.5 ± 1.0^{b}	0.200 ± 0.025^{c}	98.4 ± 0.6^{a}	1.97 ± 0.01^{a}

* WPI and SC indicate emulsions stabilized with whey protein isolate and sodium caseinate, respectively; WPI-S and WPI-L indicate WPI stabilized emulsions with small droplet size and large droplet size, respectively. Different letters indicate significant difference between values in a column ($p<0.05$)

Zeta potential (surface charge) is the potential of the shear plane of a colloid dispersion and it is closely related to the stability of the colloid dispersions. WPI- and SC-stabilized emulsions are negatively charged (**Table 2-1**), (-27.4±2.2 mV) and (-29.7±2.3 mV), respectively, due to the negatively charged protein molecules at pH 7.0, which is higher than their isoelectric point (pH 4.0-5.0). WPI-stabilized

emulsions with different droplet size showed no difference in surface charge, emulsion with large droplet size (WPI-L) of (-15.9±0.7) mV while emulsion with small droplet size (WPI-S) of (-15.5±1.0) mV. TW-stabilized emulsion (-2.5±0.2 mV) is almost neutrally charged; however, surface charge is less important for sterically stabilized emulsion, e.g., tween-stabilized emulsions, than for the electrically stabilized emulsions.

There were no significant differences in encapsulation efficiency (98.4%~99.3% by HPLC) and loading capacity (1.96%~2.03% by HPLC) between emulsions, indicating that almost all the β-carotene molecules are encapsulated in the emulsion droplets.

Emulsifiers refer to any surface-active molecules that are capable of absorbing to an oil-water interface and protecting emulsion droplets from aggregation (flocculation and/or coalescence). Emulsifiers vary widely in their ability to form and stabilize emulsions depending on their molecular and physicochemical characteristics, e.g., small molecules surfactants, or amphiphilic biopolymers (McClements, 2015). In this study, whey proteins, sodium caseinate and tween 80 were used as emulsifiers of O/W emulsions. For protein-stabilized emulsions, the major force for absorption to oil-water interface is the hydrophobic effect. When absorbing to the surface of newly-formed oil droplets during homogenization, proteins can adopt a conformation and expose their hydrophobic groups, which are located in the oil phase. The hydrophilic groups of proteins are located in the aqueous phase. Absorption reduces the contact area between the oil and water molecules at the oil-water interface, which lower the interfacial tension (McClements, 2015). In addition, protein-coated droplets are always identically

charged, which can produce a strong electrostatic repulsion force between droplets and thus prevent emulsions from flocculation or coalescence.

For tween 80-stabilized emulsions, tween 80 can also absorb to oil-water interface because they can adopt an orientation in which the hydrophilic part of molecule (polyoxyethylene groups) is located in the water, while the hydrophobic part (oleic acid) is located in the oil (Dickinson, 1992). Tween 80 is a nonionic surfactant, and it can stabilize emulsions by generating a number of short-range repulsive forces that prevent the droplets from coming to close together, such as steric, hydration, osmotic repulsion and thermal fluctuation interactions.

3.2 Creaming Stability of Emulsions

Optical integral transmission percentage of WPI-L increased rapidly and reached 32.0% after centrifugation at 2,300 g for 1,000 sec, while WPI-S showed a slow increase and reached only 13.7% (**Figure 2-1**), suggesting that emulsion with small droplet size is more stable towards creaming than emulsion with large droplet size under accelerated centrifugal force (McClements, 2015).

Emulsions with different emulsifiers demonstrated different integral transmission profiles. The TW-, and WPI emulsion significantly displayed a more rapid increase in integral transmission than SC-stabilized emulsions (**Figure 2-1**), indicating that SC-stabilized emulsion showed a better creaming stability than them. After centrifugation at 2,300 g for 1,000 s, integral transmission percentage of SC-, WPI-, and TW-stabilized emulsions reached 7.5%, 11.2%, and 12.7%, respectively.

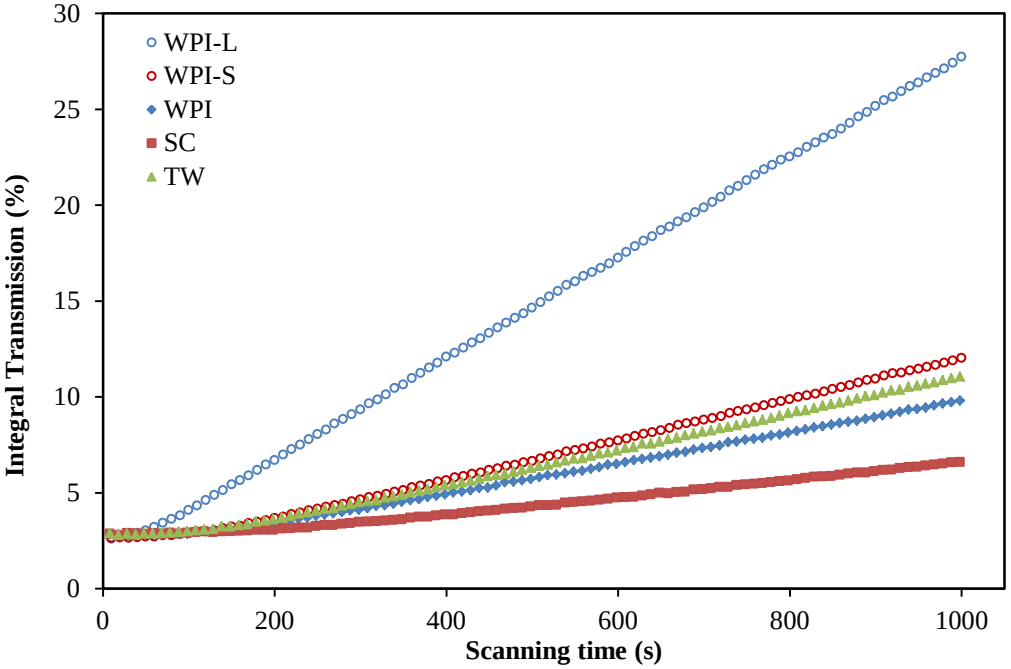


Figure 2-1. Time-dependent integral transmission profiles of different emulsions. Emulsions were centrifuged at 4,000rpm (2,300 g) and 25℃ at a scanning rate of once every 10 s for 1,000 s. WPI-L and WPI-S indicate emulsions stabilized by WPI with large and small droplet size, respectively. WPI, SC, and TW indicate emulsions stabilized with whey protein isolate, sodium caseinate, and tween80, respectively.

According to Stoke's law (McClements, 2015), the creaming velocity of an O/W emulsion is determined by several factors, e.g., droplet size, phase density, shear viscosity, or the concentration of emulsifier(Uluata et al., 2015). The TW-stabilized emulsion with smaller droplet size should have better creaming stability; however, that emulsion showed much wider size distribution (91-531 nm) (data now shown) and higher PdI (0.168±0.019) than the SC-stabilized emulsion, which had size distribution of (105-396 nm) (data now shown) and PdI of (0.092±0.01). Consequently, the large droplet fraction (of size 396-531 nm) of TW-stabilized emulsion potentially will show a higher creaming velocity, which may lead to an overall higher creaming velocity for the TW-stabilized emulsion than that of the SC-stabilized emulsion. In addition, the surface charge of TW-stabilized emulsion droplets is close to zero, which reduces inter-droplet repulsion when exposed to

centrifugal force during the integral transmission test used, and thus potentially leads to droplet aggregation. All these factors may help to explain why the TW-stabilized emulsion, with a smaller average droplet size, showed a lower creaming resistance than SC-stabilized emulsions.

Furthermore, even though zeta potential of TW-stabilized emulsion is close to zero, which may predict an instability of a colloid dispersion, it still can be considered as a stable emulsion ($\upsilon_{creaming}$<0.33 mm/day, data not shown), as emulsions with creaming velocity less than 1 mm/day can be considered as stable emulsion towards creaming instability (Dickinson, 1992). These results also confirm that zeta potential is not the key factor determining the stability of sterically stabilized emulsion (McClements, 2015).

3.3 Cryo-SEM Observation of Emulsion Droplets

Cryo-SEM technology was employed to obtain information on the existing state and morphology of WPI-, SC-, and TW-stabilized emulsion droplets prepared by microfluidizer at 70 MPa. Droplets dispersed well in all three emulsions, and no obvious droplet aggregation was observed. Droplets showed regular spherical particles with smooth surface (**Figure 2-2**). The cryo-SEM images suggested that three emulsions all demonstrated a polydisperse profile. The average droplet size and size distributions from estimation of cryo-SEM images were approximately consistent with that obtained by DLS and SC-stabilized emulsion showed a more uniform particle size distribution than that of TW-stabilized emulsion and WPI-stabilized emulsion, which matched the results of polydispersity index (PdI) values (SC<TW<WPI) obtained by DLS (**Table 2-1**). The results indicated a general alignment of cryo-SEM and DLS for the characterization of average droplet size, and

size distribution of emulsions, which was also observed by previous studies (Ghosh et al., 2013).

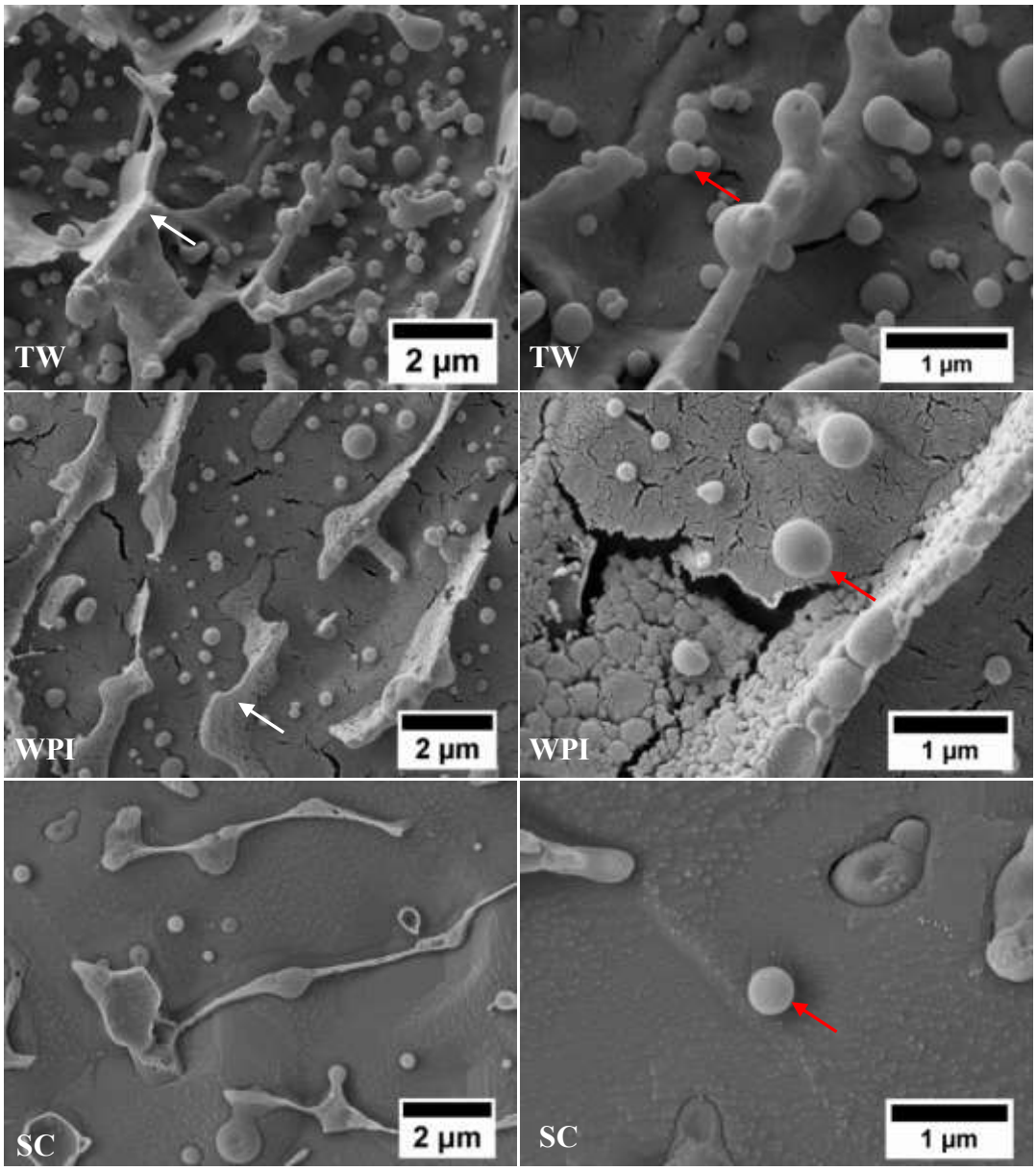


Figure 2-2. Cryo-SEM micrographs of emulsions droplets created by microfludizer. TW, WPI, and SC indicate emulsions stabilized by tween 80, whey protein isolate, and sodium caseinate, respectively. The red arrows in the micrographs indicate some of the examples of the emulsion droplets. The white arrows in the picture indicate larger features which are probably examples of ice formed during the cleaving step of sample processing.

3.4 Effect of pH on Droplet Size and Surface Charge

Droplet sizes of all emulsions remained unchanged at pH 2.0-3.0 and 5.5-8.0. However, droplet size of WPI-, and SC-stabilized emulsions (WPI-L, WPI-S, WPI and SC) dramatically increased at pH 4.0-5.0 (**Figure 2-3**), indicating a significant emulsion droplet aggregation, which attributed to the aggregation of proteins at pH close to their isoelectric point (pI) (pH 4.5-5.0 for whey protein and casein). TW-stabilized emulsion showed good stability over the range of pH values tested.

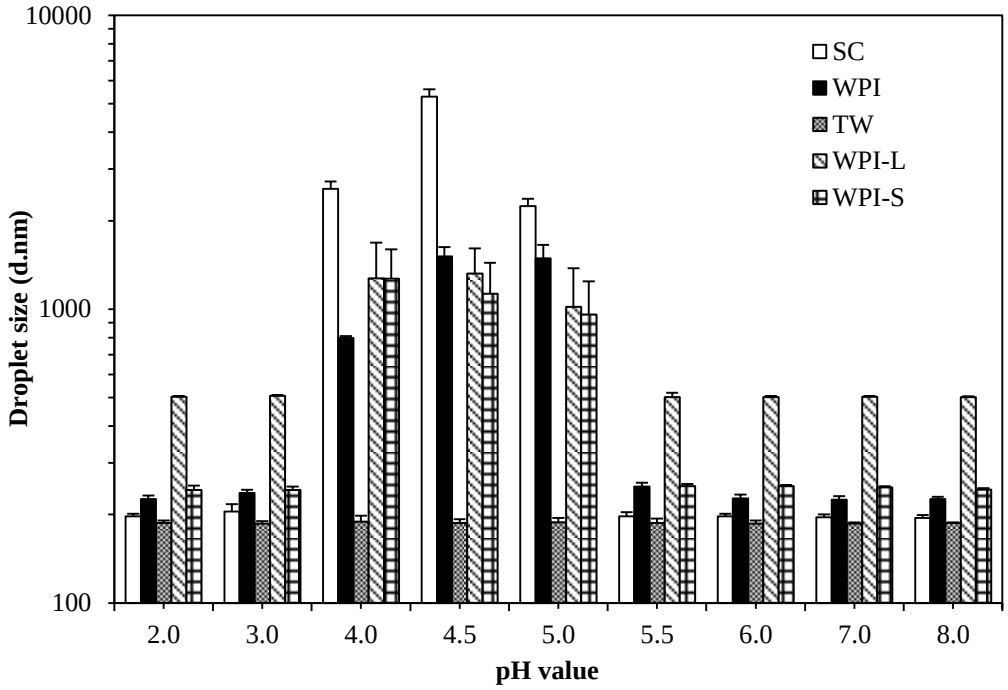


Figure 2-3. Droplet size of emulsions at different pH values. SC, WPI and TW indicate emulsions stabilized with sodium caseinate, whey protein isolate, and tween 80, respectively; WPI-L and WPI-S indicate WPI-stabilized emulsions with large droplet size and small droplet size, respectively.

Droplet surfaces of all emulsions were positively charged at pH 2.0-4.0, nearly non-charged at pH 4.5-5.0, and negatively charged at pH 5.5-8.0, except for the TW-stabilized emulsion, which was almost non-charged at all pH values (**Figure 2-4**). These variations are mainly attributed to the mechanism that protein molecules binding to the surface of the oil droplets as emulsifiers are positively charged at pH

values (2.0-4.0) below than their pI, nearly non-charged at pH values (4.5-5.5) close to their pI and negatively charged at pH values (5.5-8.0) above than their pI.

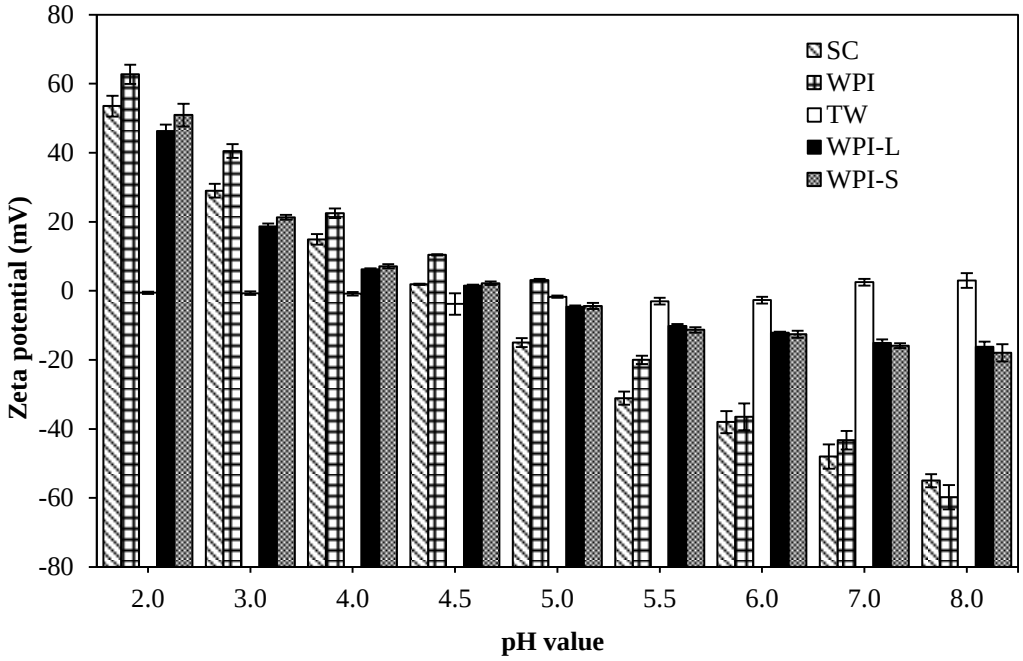


Figure 2-4. Zeta potential of emulsions at different pH values. SC, WPI and TW indicate emulsions stabilized by sodium caseinate, whey protein isolate, and tween 80, respectively; WPI-L and WPI-S indicate WPI-stabilized emulsions with large droplet size and small droplet size, respectively.

3.5 Cytotoxicity of Emulsions

Cell viability of Caco-2 cells after incubation with emulsions containing different concentrations of β-carotene showed no significant differences (p>0.05) with control group and was all higher than 90%, suggesting that these emulsions were nontoxic at these concentration (**Figure 2-5**).

Similar results were also observed by Weyenberg et al. (Weyenberg et al., 2007), using submicron emulsions stabilized with lecithin, sodium taurocholate, phosphatidylserine or polysorbate 80 (tween 80) on mouse fibroblast cells, HaCaT keratinocyte cells or J774 macrophage cells in a viability assay. In addition,

differences in droplet size and emulsifier did not significantly influence the *in vitro* cytotoxicity of emulsions containing β-carotene.

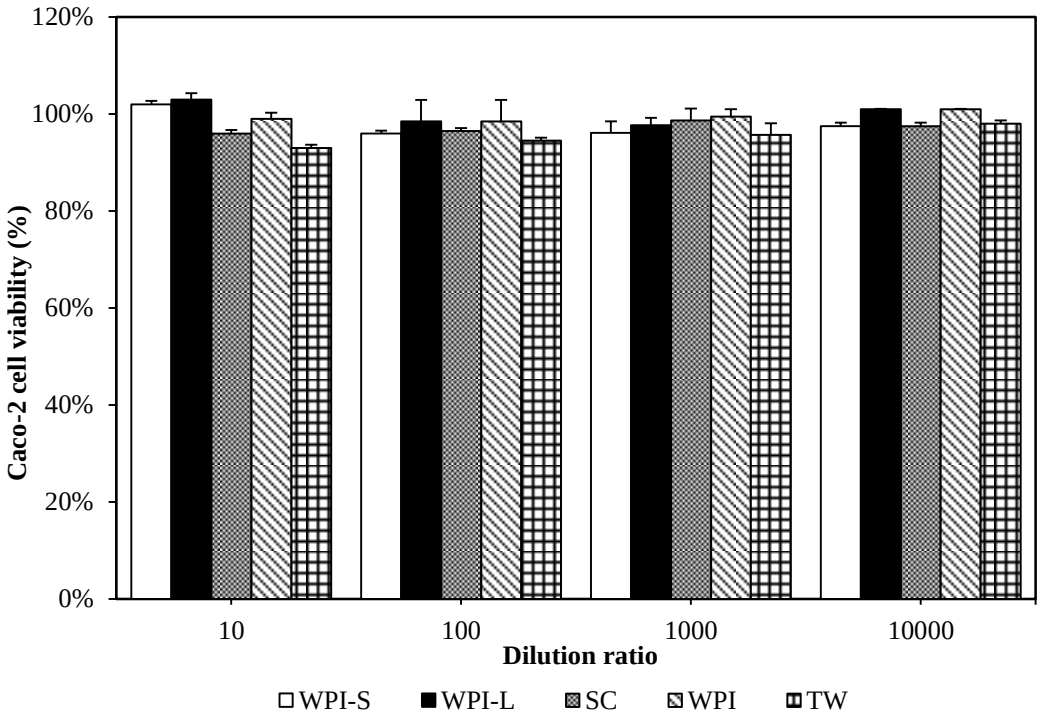


Figure 2-5. *In vitro* cytotoxicity of emulsions containing β-carotene at different concentrations on caco-2 cells by MTT assay. SC, WPI, and TW indicate emulsions stabilized by sodium caseinate, whey protein isolate, and tween 80, respectively; WPI-S and WPI-L indicate whey protein isolate stabilized emulsions with small and large droplet size, respectively.

3.6 Cellular Uptake of β-carotene in Oil Droplets by Caco-2 cells

Cellular uptake of β-carotene in all samples increased with the increasing incubation time, indicating a time-dependent cellular uptake behavior. Compared with control group (free β-carotene), cellular uptake of encapsulated β-carotene in emulsions was significantly higher (**Figure 2-6)**, suggesting an enhanced enterocyte cellular uptake of β-carotene following encapsulation in an emulsion.

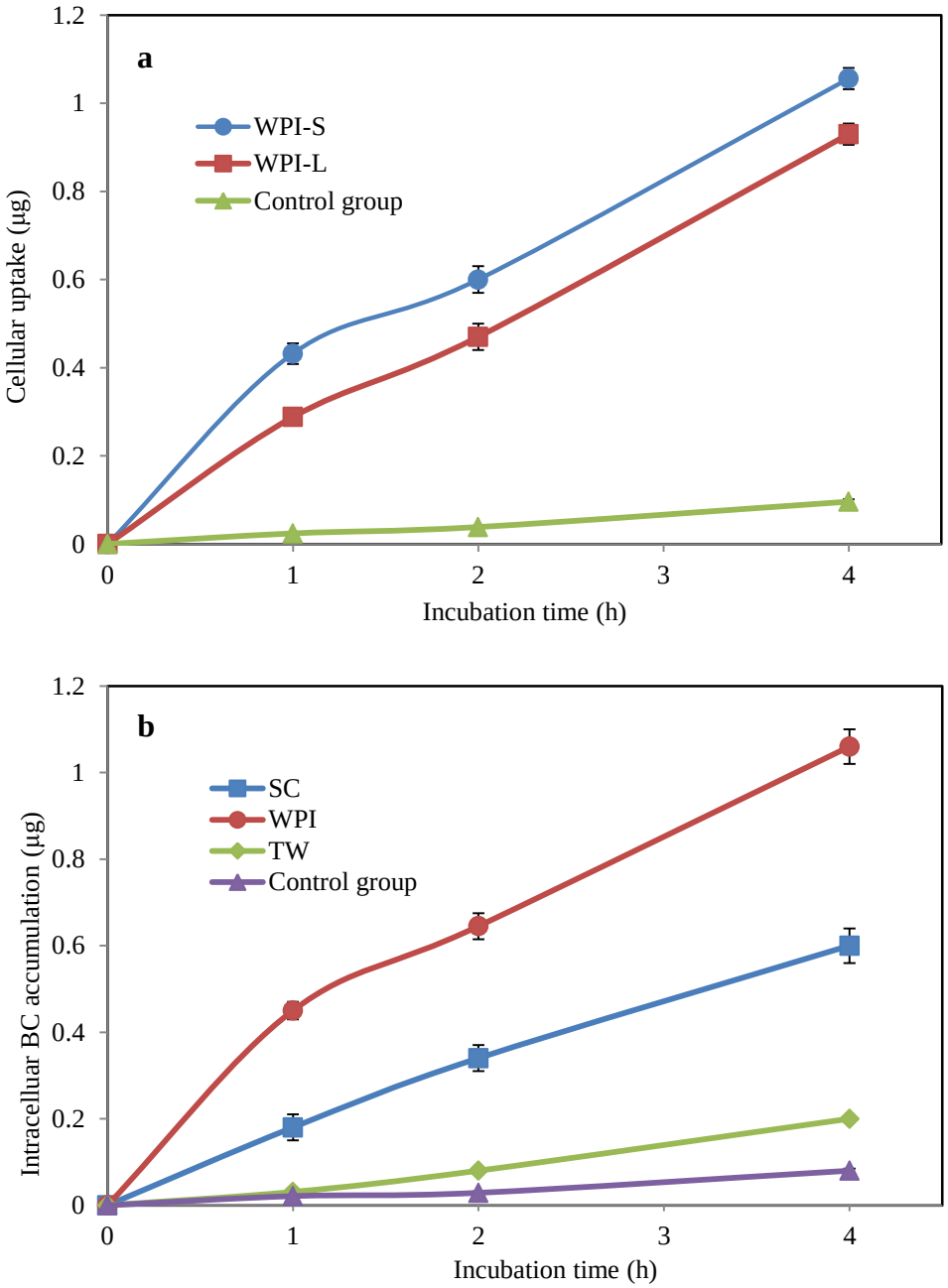


Figure 2-6. Cellular uptake of encapsulated β-carotene by caco-2 cells. (**a**) Emulsions stabilized by whey protein isolate with large (WPI-L) and small (WPI-S) droplet size, (**b**) Emulsions with different emulsifiers. SC, WPI, and TW indicate emulsions stabilized by sodium caseinate, whey protein isolate, and tween 80, respectively. Control group indicate β-carotene dissolved in DMSO.

After 4 h incubation, cellular uptake of β-carotene in WPI-stabilized emulsion with small (WPI-S) and large droplet size (WPI-L) reached 1.056 µg and 0.93 µg, respectively, which was 11-, and 9.6-fold greater than that for the control group (free

β-carotene without encapsulation), respectively (**Figure 2-6a)**. In addition, a significant increased cellular uptake of β-carotene with reduced droplet size was observed for all three time intervals (1, 2, and 4 h) (p<0.01), indicating a droplet-size-dependent cellular uptake of encapsulated β-carotene, which can be considered as one of the potential mechanisms of improved *in vivo* bioavailability of nanoencapsulated molecules with reduced particle size (Nielsen et al., 2008; Gershanik, 1998; Yap & Yuen, 2004). Furthermore, *in vitro* simulated GIT digestion assays have also confirmed improved bioavailability of BC-loaded emulsion with reduced droplet size (Salvia-Trujillo et al., 2013a), which may be attributed to an easier formation of micelles incorporated with β-carotene from emulsions with small droplet size compared to those with large droplet size, as has also been observed by previous studies (Nik et al., 2011; Nik et al., 2012).

The similar incubation-time-dependence and significantly improved cellular uptakes of encapsulated β-carotene were observed for emulsions with different emulsifiers. Intracellular β-carotene accumulation for the WPI-, SC-, and TW-stabilized emulsions reached 1.06, 0.60, and 0.20 μg after 4h, respectively, which were 13-, 7.5-, and 2.5-fold higher than that of control group (free β-carotene in DMSO) (**Figure 2-6b)**. For all three time intervals, the WPI-stabilized emulsion showed the highest β-carotene cellular uptake, followed by SC-, and TW-stabilized emulsions, suggesting that interfacial composition of emulsions can significantly influence the cellular uptake of encapsulated components. Higher cellular uptake of SCN emulsion than TW80 emulsion was also observed by Tan et al. (Tan et al., 2016). Similarly, Ribeiro et al. (Henelyta S. Ribeiro 2006) and Guri et al. (Gülseren et al., 2014), using different cell lines and bioactive nutrients, showed that cellular uptake of these encapsulated bioactive nutrients in emulsions was significantly

affected by interfacial compositions of the emulsions. In addition, β-carotene-loaded WPI protein nanoparticles displayed a higher cellular uptake and thus a higher intracellular antioxidant activity than sodium caseinate or soybean protein isolate (SPI) nanoparticles (Yi et al., 2015). Meanwhile, β-carotene-loaded WPI nanoparticles can resist gastric pepsin digestion but release β-carotene after intestinal trypsin digestion (Yi et al., 2014; Yi et al., 2015), suggesting that WPI-stabilized emulsion prepared in this study may be more suitable for lipophilic bioactive nutrients delivery than SC-, or TW-stabilized emulsion.

However, no significant differences in surface structure of three emulsion droplets were observed from the images of cryo-SEM (**Figure 2-2**); all three emulsion droplets showed uniform and smooth surface morphological structures. Different emulsifiers (e.g., whey protein, casein, or tween® 80) generally will lead to different surface structures of emulsion droplets; however, the resolution ratio of cryo-SEM may not be high enough to observe these differences in droplet surface structures at molecular level (<5 nm).

It has previously been shown that interactions of nanoparticles with intestinal cells increased with increasing surface hydrophilicity (Gaumet et al., 2010) and tween 80 showed a stronger binding to a hydrophobic surface but weaker binding to a hydrophilic surface than proteins, indicating a lower hydrophilicity than proteins (Joshi O, 2009). Consequently, it is inferred that emulsions stabilized with whey protein isolate or sodium caseinate may interact more easily with Caco-2 cells than emulsion stabilized with tween 80, which leads to an improved cellular uptake of encapsulated β-carotene. In addition, surface charge of nanoparticles has been shown to influence their interaction with intestinal mucosa and *in vivo* delivery efficiency (Gershanik, 1998). Charged nanoparticles were better than non-charged ones in

delivery efficiency, and positively charged were better than negatively charged (Poullain-Termeau et al., 2008). These factors may explain why the TW-stabilized emulsion showed a significantly lower cellular uptake of β-carotene than WPI and SC-stabilized emulsions. Moreover, in a simulated GIT digestion model system, transportation of lipophilic molecules from the oil phase of emulsions to the outer water phase, by forming mixed micelles stabilized by bile and phospholipids, was significantly affected by the interfacial properties of emulsions (Nik et al., 2012), indicating a close correlation of interfacial compositions of emulsions with the digestion, absorption and thus the bioavailability of encapsulated bioactive nutrients in emulsion-based delivery systems.

Furthermore, this chapter mainly focused on the influence of emulsifiers that were used to stabilize model O/W emulsions on the cellular uptake of encapsulated components. Hence, how to select different emulsifiers and their concentrations is very important to the study. Several reasons that WPI, SC, and TW were selected as the emulsifiers were discussed as follows: (i) emulsifiers that are commonly used to stabilize O/W emulsions can be divided into two main groups, natural or synthesised amphiphilic biopolymers (e.g., proteins or polysaccharides) and small-molecule surfactants (e.g., tween, spin, or monoglycerides) (McClements, 2015). Among them, dairy proteins (whey protein isolate and sodium caseinate) were selected as the biopolymer emulsifiers while tween 80 was selected as surfactant emulsifier in this study; (ii) Dairy proteins derive from cow milk, and they are natural edible safe ingredients and high-quality protein source for human beings. In addition, dairy proteins are widely used as emulsifiers for O/W emulsions (Livney, 2010), and Ireland is rich in milk and milk products (e.g., whey protein isolates, or sodium caseinate). Therefore, whey protein isolate and sodium caseinate were selected as

biopolymer emulsifier in this study, and their effects on the bioaccessibility and cellular uptake of encapsulated β-carotene was also investigated in **Chapter 4**; (iii) our previous studies successfully created tween 20-, and tween 80-stabilized O/W emulsions, which all showed good stability (Mao, 2014). Thus tween 80 was selected as a surfactant emulsifier in this study, and its effect on the bioaccessibility and cellular uptake of encapsulated β-carotene was also investigated in **Chapter 4**; (iv) Based on our previous results (Mao, 2014), 1% (w/w in final emulsions) of emulsifiers is an ideal concentration for emulsions with 10% of oil (w/w in final emulsions). Therefore, the concentration was used in this study, and our results also confirm this as described above.

4. Conclusions

Model O/W emulsions stabilized by whey protein isolate, sodium caseinate, or tween 80 possess good creaming stability. Emulsions droplets with different emulsifiers all showed regular spherical particles with smooth surface. Initial droplet size and emulsifiers can significantly influence the pH stability of emulsions. Encapsulation in an emulsion significantly improved the cellular uptake of β-carotene by enterocytes. Cellular uptake of encapsulated β-carotene is dependent on the initial droplet size and selection of emulsifiers. WPI-stabilized emulsion showed the highest cellular uptake of β-carotene, and WPI protein nanoparticles have been reported to resist gastric pepsin digestion but release β-carotene after intestinal trypsin digestion, suggesting that WPI-stabilized emulsions may be more suitable for delivery of those lipophilic bioactive nutrients which are sensitive to the gastric environment than sodium or tween 80 stabilized emulsions.

Overall, this goal of this study was to investigate the influence of the emulsion properties (droplet size) and compositions (emulsifiers) on the cellular uptake of encapsulated bioactive nutrients. The findings make it possible to improve the cellular uptake of bioactive nutrients and thus potentially their *in vivo* bioavailability by designing emulsion properties or compositions. The findings also contribute to the development of parenteral emulsions with desired delivery properties (e.g., regulating the absorption, distribution, and extended bioavailability of bioactive nutrients) through controlling their properties (e.g., droplet size) or compositions. Parenteral emulsions have been safely in medical use for over five decades (Hormann & Zimmer, 2016) and they can be used by intravenous or hypodermic injection without passing through the digestive tract. Certainly, for many oral cases, emulsion droplets will be exposed to digestion conditions in the mouth, stomach, small intestine and intestinal mucosa, before being absorbed by enterocyte cells, during which the droplet size and interfacial compositions of emulsion droplets will presumably change. Therefore, further work needs to be done regarding evaluation of cellular uptake of encapsulated bioactives in emulsion-based delivery systems, following exposure them to *in vitro* or *in vivo* digestion conditions prior to testing in the Caco-2 cell assay.

References

Barba, A. I. O., Hurtado, M. C., Mata, M. C. S., Ruiz, V. F., & Tejada, M. L. S. d. (2006). Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry, 95*(2), 328-336.

Borel, T., & Sabliov, C. M. (2014). Nanodelivery of bioactive components for food applications: types of delivery systems, properties, and their effect on ADME profiles and toxicity of nanoparticles. *Annual Review of Food Science and Technology, 5*, 197-213.

Dickinson, E. (1992). *Introduction to Food Colloids*. Oxford, UK: Oxford University Press.

Fischer, D., Y. Li., Ahlemeyer, B., Krieglstein, J., and Kissel, T. (2003). In vitro cytotoxicity testing of polycations influence of polymer structure on cell viability and hemolysis. *Biomaterials, 24*, 1121-1131.

Gaumet, M., Gurny, R., & Delie, F. (2010). Interaction of biodegradable nanoparticles with intestinal cells: the effect of surface hydrophilicity. *International Journal of Pharmaceutics, 390*, 45-52.

Ghosh, V., Mukherjee, A., & Chandrasekaran, N. (2013). Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity. *Ultrasonics Sonochemistry, 20*, 338-344.

Golding, M., & Wooster, T. J. (2010). The influence of emulsion structure and stability on lipid digestion. *Current Opinion in Colloid and Interface Science, 15*, 90-101.

Gülseren, İ., Guri, A., & Corredig, M. (2014). Effect of interfacial composition on uptake of curcumin–piperine mixtures in oil in water emulsions by Caco-2 cells. *Food &Function, 5*, 1218-1223.

Henelyta S. Ribeiro , J. M. M. G., Karlis Briviba ,Gerhard Rechkemmer ,Heike P. Schuchmann , and Helmar Schubert (2006). Cellular Uptake of Carotenoid-Loaded Oil-in-Water Emulsions in Colon Carcinoma Cells in Vitro. *Journal of Agricultural and Food Chemistry, 54*, 9366-9369.

Heo, W., Kim, J. H., Pan, J. H., & Kim, Y. J. (2016). Lecithin-Based Nano-emulsification Improves the Bioavailability of Conjugated Linoleic Acid. *Journal of Agricultural and Food Chemistry, 64*, 1355-1360.

Hormann, K., & Zimmer, A. (2016). Drug delivery and drug targeting with parenteral lipid nanoemulsions - A review. *Journal of Controlled Release, 223*, 85-98.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion. *Food Chemistry, 114*, 253-262.

Joshi O, M. J. (2009). Adsorption behavior of lysozyme and Tween 80 at hydrophilic and hydrophobic silica-water interfaces. *Applied Biochemistry and Biotechnology, 152*, 235-248.

Joye, I. J., Davidov-Pardo, G., & McClements, D. J. (2014). Nanotechnology for increased micronutrient bioavailability. *Trends in Food Science and Technology, 40*, 168-182.

Karthik, P., & Anandharamakrishnan, C. (2016). Enhancing omega-3 fatty acids nanoemulsion stability and in-vitro digestibility through emulsifiers. *Journal of Food Engineering, 187*, 92-105.

Lu, W., Guo, J., Zhou, J., Ke, L., Liu, S., Gao, G., Wang, H., Ding, W., & Rao, P. (2012). Hypothesis review: The direct interaction of food nanoparticles with the lymphatic system. *Food Science and Human Wellness, 1*, 61-64.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Malaki Nik, A., Wright, A. J., & Corredig, M. (2011). Micellization of Beta-Carotene from Soy-Protein Stabilized Oil-in-Water Emulsions under *In Vitro* Conditions of Lipolysis. *Journal of the American Oil Chemists' Society, 88*, 1397-1407.

Mao, L. (2014). *Novel Structured Emulsions For Delivering of Food Flavours.* (PhD), University College Cork, Cork, Ireland.

Mao, L., Boiteux, L., Roos, Y. H., & Miao, S. (2014). Evaluation of volatile characteristics in whey protein isolate-pectin mixed layer emulsions under different environmental conditions. *Food Hydrocolloids, 41*, 79-85.

Mao, L., & Miao, S. (2015). Structuring Food Emulsions to Improve Nutrient Delivery During Digestion. *Food Engineering Reviews, 7*, 439-451.

Mao, L., O'Kennedy, B. T., Roos, Y. H., Hannon, J. A., & Miao, S. (2012). Effect of monoglyceride self-assembled structure on emulsion properties and subsequent flavor release. *Food Research International, 48*, 233-240.

Mao, L., Roos, Y. H., & Miao, S. (2014). Flavour Release from Monoglyceride Structured Oil-in-Water Emulsions through Static Headspace Analysis. *Food Biophysics, 9*, 359-367.

McClements, & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*, 213-228.

McClements, D. J. (2010). Emulsion design to improve the delivery of functional lipophilic components. *Annual Review of Food Science and Technology,1*, 241-269.

McClements, D. J. (2015). *Food emulsions: principles, practices, and techniques* (3rd ed.). New York: CRC press.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(55-63).

Nielsen, F. S., Petersen, K. B., & Mullertz, A. (2008). Bioavailability of probucol from lipid and surfactant based formulations in minipigs: influence of droplet size and dietary state. *European Journal of Pharmaceutics and Biopharmaceutics, 69*, 553-562.

Nik, A. M., Langmaid, S., & Wright, A. J. (2012). Digestibility and beta-carotene release from lipid nanodispersions depend on dispersed phase crystallinity and interfacial properties. *Food &Function, 3*, 234-245.

Poullain-Termeau, S., Crauste-Manciet, S., Brossard, D., Muhamed, S., Nicolaos, G., Farinotti, R., Barthelemy, C., Robert, H., & Odou, P. (2008). Effect of oil-in-water submicron emulsion surface charge on oral absorption of a poorly water-soluble drug in rats. *Drug Delivery, 15*, 503-514.

Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols, 1*, 3159-3165.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013). Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry, 141*, 1472-1480.

Salvia-Trujillo, L., Sun, Q., Um, B. H., Park, Y., & McClements, D. J. (2015). In vitro and in vivo study of fucoxanthin bioavailability from nanoemulsion-based delivery systems: Impact of lipid carrier type. *Journal of Functional Foods, 17*, 293-304.

Siddiqui, I. A., Adhami, V. M., Bharali, D. J., Hafeez, B. B., Asim, M., Khwaja, S. I., Ahmad, N., Cui, H.D., Mousa, S.A., & Mukhtar, H. (2009). Introducing nanochemoprevention as a novel approach for cancer control: proof of principle with green tea polyphenol epigallocatechin-3-gallate. *Cancer Research, 69*, 1712-1716.

Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., Barnes, S., Grizzle, W., Miller, D., & Zhang, H. G. (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Molecular Therapy , 18*, 1606-1614.

Tan, T. B., Chu, W. C., Yussof, N. S., Abas, F., Mirhosseini, H., Cheah, Y. K., Nehdi, I.A., & Tan, C. P. (2016). Physicochemical, morphological and cellular uptake properties of lutein nanodispersions prepared by using surfactants with different stabilizing mechanisms. *Food &Function, 7*, 2043-2051.

Tatyana Gershanik, S. B., Simon Benita. (1998). Interaction of a self-emulsifying lipid drug delivery system with the everted rat intestinal mucosa as a function of droplet size and surface charge. *Pharmaceutical Research, 5*, 863-869.

Ting, Y., Jiang, Y., Lan, Y., Xia, C., Lin, Z., Rogers, M. A., & Huang, Q. (2015). Viscoelastic Emulsion Improved the Bioaccessibility and Oral Bioavailability of Crystalline Compound: A Mechanistic Study Using *in Vitro* and *in Vivo* Models. *Molecular Pharmaceutics, 12*, 2229-2236.

Uluata, S., Decker, E. A., & McClements, D. J. (2015). Optimization of Nanoemulsion Fabrication Using Microfluidization: Role of Surfactant Concentration on Formation and Stability. *Food Biophysics, 11*, 52-59.

Weyenberg, W., Filev, P., Van den Plas, D., Vandervoort, J., De Smet, K., Sollie, P., & Ludwig, A. (2007). Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application. *International Journal of Pharmaceutics, 337*, 291-298.

Yap, S. P., & Yuen, K. H. (2004). Influence of lipolysis and droplet size on tocotrienol absorption from self-emulsifying formulations. *International Journal of Pharmaceutics, 281*, 67-78.

Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., & Zhong, F. (2014). Controlled release of beta-carotene in beta-lactoglobulin-dextran-conjugated nanoparticles' in vitro digestion and transport with Caco-2 monolayers. *Journal of Agricultural and Food Chemistry, 62*, 8900-8907.

Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., & Zhong, F. (2015). Beta-carotene encapsulated in food protein nanoparticles reduces peroxyl radical oxidation in Caco-2 cells. *Food Hydrocolloids, 43*, 31-40.

Yuan, Y., Gao, Y., Zhao, J., & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International, 41*(1), 61-68.

Zhang, T., Zheng, Y., Peng, Q., Cao, X., Gong, T., & Zhang, Z. (2013). A novel submicron emulsion system loaded with vincristine-oleic acid ion-pair complex with improved anticancer effect: in vitro and in vivo studies. *International Journal of Nanomedicine, 8*, 1185-1196.

CHAPTER THREE

Improved Bioavailability of Encapsulated Bioactive Nutrient Delivered through Monoglyceride-Structured O/W Emulsions

Published in *Journal of Agricultural and Food Chemistry*, 2017, 65, 3048-3055.

Authors: Wei Lu, Alan L. Kelly, and Song Miao*

The work contained in this chapter was undertaken and written solely by myself with specific contributions from each co-author.

Abstract

Effects of monoglyceride (MG) on the properties of WPI-stabilized emulsions and the bioavailability of encapsulated β-carotene were investigated. Emulsions containing MG showed reduced surface charge, higher viscosity, and better creaming stability than an emulsion without MG. Exposure of emulsions to GIT digestion led to significant changes in droplet size and interfacial properties. Bioaccessibility of β-carotene in emulsions containing 1% MG (63.9%) and 2% MG (77.1%) were higher than that in emulsion without MG (53.4%) (p<0.05). All MG-emulsions demonstrated a better cellular uptake of β-carotene by Caco-2 cells than the emulsion without MG after the GIT (p<0.05). A significant increase in the cellular uptake of β-carotene with increasing MG content was observed, increasing from 0.109 μg/well for emulsion containing 0.5% MG up to 0.138 μg/well for emulsion containing 2% MG. The findings in this study confirm the potential of the model O/W emulsions with MG-modified oil phase as novel carriers for lipophilic nutrients with improved stability and oral bioavailability.

Keywords: monoglyceride, structured-emulsion, delivery, bioavailability

1. Introduction

Nanoemulsions have been widely used as novel delivery systems for lipophilic bioactive nutrients due to their high creaming stability, maintenance of physical and chemical stability, improved bioavailability and potential targeted delivery of encapsulated compounds (Hormann & Zimmer, 2016; Lu et al., 2016). Many nanoemulsion-based delivery systems for lipophilic bioactive nutrients have been developed, and encapsulated compounds, compared with free ones, show higher stability to extreme environments (pH or ionic strength) and gravitational separation, as well as improved *in vitro* and *in vivo* bioavailability (McClements & Rao, 2011).

There is a close correlation between the properties and compositions of emulsions and the bioaccessibility of encapsulated lipophilic bioactive nutrients in emulsions, which has been widely verified by many previous studies. Encapsulated lipophilic bioactive nutrients in emulsion-based carriers with different carrier oils (Qian et al., 2012; Salvia-Trujillo et al., 2015; Sun et al., 2015), droplet size (Cho et al., 2014; Salvia-Trujillo et al., 2013a), and emulsifiers (Hur et al., 2009), showed significant different bioaccessibility. This correlation also makes it possible to control the release or improve the oral bioavailability of these compounds by designing the emulsion-based delivery systems. Low molecular weight surfactants (LMWS) can significantly influence the properties of emulsions in several ways (Dickinson et al., 1989). Generally, LMWS can: (i) reduce the surface tension, and thus lead to the formation of smaller droplets during homogenisation; (ii) displace protein at the oil-water interface or interact with interfacial proteins, leading to a thicker and more strongly adsorbed layer; and (iii) increase the viscosity of the emulsions through the formation of crystal structures. Accordingly, LMWS may have effects on the digestion behavior and thus the bioavailability of encapsulated bioactive nutrients in

emulsion-based delivery systems by structuring emulsion droplets, which deserves further study.

Monoglycerides (MG) are one LMWS commonly used as an emulsifying ingredient in the food industry. MG can migrate rapidly to the surface of lipid droplets of an oil-in-water (O/W) emulsion due to their amphiphilic properties, or interact with proteins absorbed to droplets surface as stabilizers, to form a stronger membrane surrounding the droplets, preventing coalescence and flocculation (McSweeney et al., 2008). Thus, MG can effectively reduce the surface tension at the O/W interface (Doxastakis & Sherman, 1986) or the droplet size of protein-stabilized emulsions (Stephen et al., 2001). MG can also self-assemble into different crystalline structures in emulsion droplets, and modify the properties of emulsions modified (Yaghmur, 2005; Krog & Sparsø, 2004).

Our previous studies successfully prepared MG-structured emulsions with modified properties and demonstrated the great potential of the MG-structured emulsion in controlled release of encapsulated flavors from emulsions (Mao et al., 2014; Mao et al., 2012). Our previous study also confirmed the relation between emulsion structure, e.g., droplet size or interfacial layer, and the cellular uptake of bioactive nutrients within emulsion droplets without passing through simulated gastrointestinal tract (GIT) (Lu et al., 2016). Accordingly, it is hypothesized that lipophilic bioactive nutrients that are incorporated into MG-structured emulsion droplets will show significant differences in their bioaccessibility and cellular uptake behavior, compared with emulsions without MG. However, little is known about the influence of MG on the bioavailability of encapsulated bioactive nutrients after their exposure to GIT. Therefore, this study was designed to investigate the effect of MG on the *in vitro* bioavailability and cellular uptake of encapsulated bioactive nutrients

in MG-structured emulsions after passing through a simulated GIT consisting of mouth, gastric, and intestinal phases. Such investigations of the digestive fate of MG emulsion droplets and the encapsulated compounds will contribute to a better understanding of the influence of MG on the digestion, release, and cellular uptake of encapsulated lipophilic bioactive components in model O/W emulsions. Accordingly, this can help to improve delivery efficiency of emulsion-based carriers by designing proper emulsion structures. Furthermore, successful development of MG-structured emulsions makes it possible to design food nanoemulsion-based carriers with simple structures but high safety and digestibility, and wide scope for protective encapsulation and high-performance delivery of lipophilic functional nutrients or relevant medical delivery applications.

O/W emulsions stabilized with whey proteins isolate (WPI) were used as a model emulsion delivery system. There are two reasons why WPI was used as emulsifier in this study: (i) WPI is an easily obtained protein with low cost and is widely used as the emulsifier for fine emulsions (McClements, 2015); and (ii) WPI can potentially resist the digestion by pepsin in the stomach (Yi et al., 2014), and prevent the pepsin-induced break down of droplets and thus the release of encapsulated nutrients, which accordingly can protect the encapsulated nutrients from being exposure to extreme pH and enzymatic environment. β-carotene was used as a lipophilic bioactive nutrient, and the total amount of BC in micelle fractions and the cellular uptake of BC in micelles after intestinal phase were measured. The effect of MG on the properties of WPI-stabilized emulsions, e.g., droplet size, surface charge, displacement of proteins, viscosity, and creaming stability, was also investigated.

2. Material and Methods

2.1 Materials

All-trans-β-carotene (>93%, UV), pepsin (≥250 unit/mg), pancreatin (4×USP), bile salts, Dulbecco's modified eagle's medium (DMEM) (containing 4.5 g/L D-glucose), penicillin and streptomycin (100×), fetal bovine serum (FBS), phosphate buffer solution (PBS) and cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunflower oil (Solesta, >98% fat) was purchased from a local supermarket (ALDI, Fermoy, Co.Cork, Ireland), and whey protein isolate (70% β-lactoglobulin and 18% α-lactalbumin) was obtained from Davisco Food International (Le Sueur, MN, USA). Monoglyceride (glycerol monostearate, Danisco, Denmark) was purchased from Cloverhill Food Ingredients Ltd (Cork, Ireland). The human colon carcinoma cell lines (Caco-2 cells, ATCC, Manassas, VA, USA) were grown in DMEM, and cells after 86-96 passages were used for this study. All other chemicals and reagents used were of HPLC-grade and obtained from Sigma-Aldrich. Ultrapure water (18 mΩ) was prepared by a Milli-Q apparatus (Millipore Corp.).

2.2 Emulsion Preparation

WPI was dispersed (1.0%, w/w) in water containing 0.01% (w/w) sodium azide as aqueous phase. The dispersions were stirred for 2 h and kept at room temperature overnight. Glycerol monostearate (0.5, 1, or 2%, w/w in the final emulsion) was first dissolved in sunflower oil at 140 ℃, and β-carotene (0.2%, w/w in oil phase) was then added. This oil phase (10%, w/w) was then mixed with water phase dispersions at a speed of 10000 rpm for 1 min at room temperature using an Ultra-Turrax (IKA, Staufen, Germany) followed by further high-pressure homogenization (APV 1000, SPX Flow Technology, Charlotte, North Carolina, USA) at 80 MPa for 3 passes at room temperature to obtain final emulsions.

2.3 Characterization of Droplet Size and Surface Charge

The droplet size and zeta potential of prepared emulsions were measured by dynamic light scattering (DLS) and particle electrophoresis technology using a laser particle analyzer (Nano-ZS, Malvern Instruments, Worcestershire, UK). Emulsions were diluted to the final oil content (w/w) of 0.01% before testing. The RI for particle was set at 1.47 and the viscosity and RI for the solution (water) were set at 0.8872 cP and 1.33, respectively. The Smoluchowski mode was used to measure zeta potential. The operation temperature for particle size and zeta potential measurement was 25 °C.

2.4 Determination of Displacement of Proteins from Interface by MG

For the quantification of droplet-surface-absorbed WPI, the emulsions were first centrifuged at 15,000 g for 15 min and the separated 'cream layer' was removed. This process was repeated twice and then the remaining fraction was then centrifuged again at 15,000 g for 40 min, whereupon the bottom fraction was collected as the serum fraction, filtered (0.45 μm) and applied to the HPLC for the quantification of free WPI in emulsions. The protein absorption can be calculated with the following equation (Tcholakova, 2002):

$$\Gamma\ (mg/m^2) = \frac{V_c(C_{ini}-C_{ser})}{SV_{oil}} \qquad \text{equation 1}$$

where V_c and V_{oil} are the volumes of the aqueous and oil phases (mL); C_{ini} and C_{ser} are the protein concentration (mg/mL) in the initial solution (prior to homogenization) and the concentration in the serum after the homogenization process, respectively; S is the surface area per unit volume of dispersed oil, which can be calculated with the following equation:

$$S\ (\mathrm{m^2/mL}) = \frac{A_{oil}}{V_{oil}} = \frac{4\pi {R_{32}}^2}{(\frac{4}{3})\ \pi {R_{32}}^3} = \frac{3}{R_{32}} \qquad \text{equation 2}$$

where A_{oil} and V_{oil} are the total surface area and volume of dispersed oil, respectively. R_{32} is the volume-surface radius. Based on the unit of S should be square meters per mL of oil, the unit of R_{32} should be present as microns (μm). Substituting equation 2 into equation 1, we obtain equation 3 as follows, by which the protein absorption was calculated in this study:

$$\Gamma\ (\mathrm{mg/m^2}) = \frac{V_c(C_{ini}\text{-}C_{ser})}{SV_{oil}} = \frac{V_c(C_{ini}\text{-}C_{ser})R_{32}}{3V_{oil}} \qquad \text{equation 3}$$

HPLC quantification of WPI was performed using an Agilent 1200 series systems with a DAD UV-Vis detector (Agilent, Santa Clara, CA, USA). A C_{18} column (4.6×150 mm, 5 μm, Agilent) was used following the chromatography operation condition described in a previous study (Fernandez & Kelly, 2016) with minor modification. Briefly, the mobile phases consisted of solvent A, acetonitrile with 0.1% TFA, and solvent B, water with 0.1% TFA. The flow rate was 1 mL/min, detection wavelength was 280 nm, injection volume was 10 μL, and gradient elution was performed from 20% A to 90% A within 20 min.

2.5 Creaming Stability

Emulsion stability was evaluated using a Lumisizer (LUM GmbH, Berlin, Germany) as described in our previous study (Lu et al., 2016). The sample-filled section of the test cell for Lumisizer (the section between cell bottom and 20 mm distance from the bottom) was selected for the calculation position of the integral

transmission percent and this sample-filled section is around 7 mm shorter than the light beam.

2.6 Rheological Analysis

Rheological measurements were performed using an AR 2000ex rheometer (TA Instruments). A DIN and concentric cylinder geometry (stator inner radius=15 mm, rotor outer radius=14 mm) was selected, and 20 mL of each sample was placed into the inner cylinder and equilibrated for 2 min before measurement. Viscosity testing was performed over a shear rate range of 0-200 s^{-1} at 25 °C.

2.7 *In vitro* Simulated Gastrointestinal Tract (GIT) Digestion

An *in vitro* simulated GIT digestion model consisting of mouth, gastric and intestinal phases as used to digest emulsion samples. Droplet size and zeta potential of nanoemulsions after each phase were measured by DLS. The simulated saliva fluid (SSF), gastric fluid (SGF), and intestinal fluid (SIF) were prepared following the method of Minekus et al.(2014).

SSF: KCI (1.126 g), KH_2PO_4 (0.503 g), $NaHCO_3$ (1.142 g), $MgCI_2$ (0.15 mM) and $(NH_4)CO_3$ (0.0058 g) were dissolved in deionized water and made to a final volume of 1,000 mL, with pH adjustment to 7.0.

SGF: KCI (0.515 g), KH_2PO_4 (0.122 g), $NaHCO_3$ (2.1 g), $MgCI_2$ (0.1 mM), NaCI (2.758 g) and $(NH_4)CO_3$ (0.048 g) were dissolved in 800 mL deionized water and pH was adjusted to 2.5 with HCI. Before use, pepsin (final 2,000 U/mL) was added to the SGF and the volume was adjusted to 1,000 mL with deionized water.

SIF: KCI (0.507 g), KH_2PO_4 (0.109 g), $NaHCO_3$ (7.138 g), $MgCI_2$ (0.33 mM), and NaCI (2.244 g) were dissolved in 800 mL water and pH was adjusted to 7.0. Before use, bile salts (10 mM) and pancreatin (trypsin activity of 100 U/mL) were

added to SGF and the volume was adjusted to a final volume of 1,000 mL with deionized water.

For the mouth phase digestion, emulsions were mixed with SSF (1:1, v/v), the pH was adjusted to 6.8 and the mixtures were incubated at 37 °C for 10 min with continuous agitation at 100 rpm.

For the gastric phase, the bolus sample from the mouth phase was mixed with the SGF (1:1, v/v). The pH of the mixture was adjusted to 2.5 and it was incubated at 37 °C for 2 h with continuous agitation at 100 rpm.

For the small intestinal phase, the bolus sample from the gastric phase was mixed with the SIF (1:1, v/v). The pH of the mixture was adjusted to 7.0 and it was incubated at 37 °C for 2 h with continuous agitation at 100 rpm. The amount of 0.1M NaOH necessary to maintain the pH (7.0) was added during 2 h.

2.8 Bioaccessibility of β-carotene after GIT

The amount of β-carotene in micelle fractions after simulated intestinal digestion was measured as the bioaccessibility of encapsulated β-carotene (Qian et al., 2012; Salvia-Trujillo et al., 2013a; Sun et al., 2015). Briefly, an aliquot of raw digesta from the intestinal phase was centrifuged at 4,000 rpm (2647 g) for 40 min at 4 °C and the supernatant was collected and considered as the micelle fraction, in which the bioactive compound is solubilized. 1 mL of the micelle fraction was extracted twice with ethanol/n-hexane. The top n-hexane layer containing the solubilized β-carotene was collected and analyzed by RP-HPLC as described below.

The bioaccessibility of encapsulated β-carotene was calculated using the follow equation:

$$\text{Bioaccessibility (\%)} = \frac{C_{\text{micelle}}}{C_{\text{initial}}} \times 100\%$$

where C_{micelle} and C_{initial} are the concentration of β-carotene in the micelle fraction and initial emulsion before digestion, respectively.

2.9 Cellular Uptake of β-carotene by Caco-2 cells

Cellular uptake of β-carotene-loaded micelles after intestinal phase digestion of GIT was also tested for the further evaluation of the bioavailability of encapsulated β-carotene. Caco-2 cells were seeded in 6-well plate at a density of 3×10^5 cells /well. Cellular uptake experiments were performed 5-7 days after seeding. Micelle fractions after intestinal phase digestion were diluted 20-fold with complete medium and added to each well in a 6-well plate, which was then incubated at 37 °C and 5% CO_2 for 3 h. Before collection, cells were washed three times with PBS buffer solution. Then, cells were collected, lysed, extracted and analyzed for β-carotene content by RP-HPLC.

The cell lysis followed the method described by Rahman (Rahman et al., 2006) with minor modifications. Collected cells were dispersed in pure water and freeze-thawed twice at -80 °C, centrifuged at 3,000 g for 15 min at 4 °C, and the supernatant was immediately transferred to a pre-chilled tube at 4 °C. Cell lysis buffer (0.5 mL) was added to the pellets followed by lysis at room temperature for 15 min on a shaker. The lysate was centrifuged for 15 min at 15,000 g to pellet the cellular debris, and the supernatant was collected and combined with previous supernatant in pre-chilled tube. Combined supernatant was then extracted with ethanol/n-hexane and analyzed by HPLC.

2.10 Extraction and HPLC analysis of β-carotene

β-carotene was extracted with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v) as described in **Chapter 2** (section2.10). The hexane extracts were combined and dried under a stream of nitrogen gas, and dissolved in 0.4 mL ethanol for HPLC analysis of β-carotene as described in **Chapter 2** (section 2.12). One mL of β-carotene standard solution in ethanol was added to 1 mL sample, and the mixture was extracted with ethanol/n-hexane and analyzed by HPLC to determine the recovery of β-carotene (>98% in this study, data not shown).

2.11 Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was employed to compare means of data. A t-Test was used to determine the differences between means. Significant differences were determined at the 0.05 level ($p<0.05$).

3. Results and Discussion

3.1 Droplet Size and Surface Charge

All emulsions showed droplet sizes between 210-225 nm (**Table 3-1**); all emulsions were negatively charged due to the emulsifier (WPI) being negatively charged at pH 7.0, which is higher than the isoelectric points of whey proteins (pH 4.0-5.0). No significant difference in droplet size between emulsions with or without MG was observed ($p>0.05$), showing that incorporation of MG into the oil phase did not influence the droplet size of final emulsions. However, compared with the emulsion without MG (-68.7±6.0 mV), the surface charge of MG-structured emulsions significantly decreased (-52±3.0 mV of emulsion with 2% MG). This may be attributed to the displacement of WPI from the water-oil interface by MG.

Table 3-1. Droplet Size, zeta potential, and polydispersity index (PdI) of monoglyceride (MG) structured emulsions (mean±STD, n=3)

MG (%, W/W)	Droplet size (d.nm)	Zeta potential (mV)	PdI
0	223±4[a]	-68.7±6.0[a]	0.225±0.04[a]
0.5	211±8[a]	-54.3±2.7[b]	0.228±0.03[a]
1	211±6[a]	-52.5±4.4[b]	0.241±0.01[b]
2	222±6[a]	-52.0±3.0[b]	0.281±0.03[c]

[a]Different letters indicate significant difference between values in a column (p<0.05)

3.2 Competitive Occupation of MG with WPI at Interface

In order to confirm the potential competitive occupation of MG with WPI at the water-oil interface, concentration of three main components of WPI, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA)(data not shown), in water phase of different emulsions were determined. Total absorption of WPI was calculated by summing the absorption of β-LG, α-LA, and BSA. Emulsion without MG showed the highest absorption of β-LG and α-LA of 1.72 mg/m^2 and 0.66 mg/m^2, respectively, followed by emulsions with MG content of 0.5%, 1%, and 2%, respectively (**Figure 3-1**), suggesting that MG can reduce the protein absorption at the interface and the extent of the reduction increased with increasing MG content. This may explain why emulsions containing MG showed reduced surface charge. However, reduced protein absorption at the interface by MG had no significant influence on the droplet size after storage for 30 days at 4 °C (data not shown). In addition, only about 40% of total WPI in the water phase was absorbed onto the surface of oil droplets as emulsifier after homogenization (data not shown), suggesting that WPI was present in excess, in terms of its use as emulsifier, and the level could be reduced (~50%) for those applications requiring low free protein levels in the continuous phase.

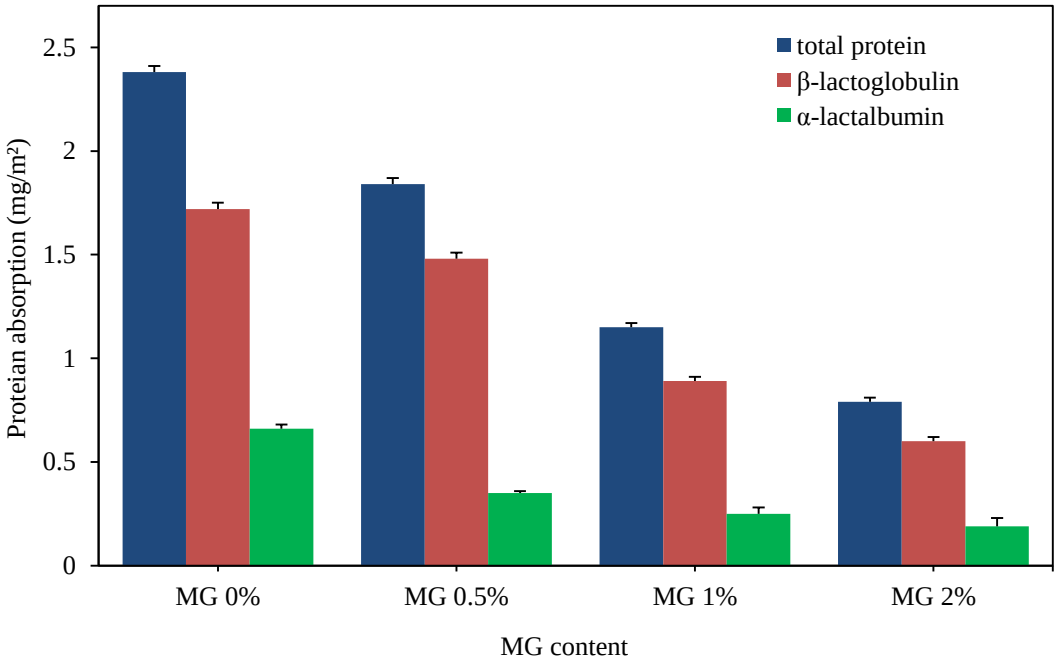


Figure 3-1. Protein absorption at the oil-water interface. A C_{18} column (4.6×150 mm, 5 μm, Agilent) was used. Mobile phases were solvent A, acetonitrile with 0.1% TFA and solvent B, water with 0.1% TFA. Flow rate was 1 mL/min, detection wavelength was 280 nm, and gradient elution was performed from 20% A to 90% A within 20 min. MG 0% was the emulsion without MG while MG 0.5%, MG 1%, and MG 2% were emulsions with an MG content (w/w) of 0.5%, 1%, and 2%, respectively.

Generally, proteins can more effectively reduce the interfacial tension at the same low concentration than non-polymeric surfactants. however, these surfactants can produce a lower equilibrium tension than proteins at the same high concentration, which is probably the main thermodynamic basis for the competitive displacement of proteins from the oil-water interface by various surfactants of different hydrophile-lipophile balance (Chen et al, 1993). The displacement of proteins by surfactants mainly depends on the type of proteins absorbed at the interface (Chen et al, 1993), and the concentration and type of surfactants (Chen et al., 1993; Pelan et al., 1997). For example, water soluble tween 60 was much more effective in displacing protein from the interface than oil-soluble surfactants, such as monoglyceride.

As an oil-soluble surfactant, MG can rapidly absorb onto the droplet surface once the water-oil interface is formed. In protein-stabilized emulsions, MG can also

displaced protein molecules from droplet surface and milk protein displacement by MG has been observed directly using confocal scanning laser microscopy (Heertje et al., 1990). The displacement of absorbed protein by MG was also observed in some other previous studies (Krog & Larsson, 1992; Pugnaloni et al., 2004). The displacement of proteins by MG accordingly lead to reduced protein absorption at the interface and potentially reduced surface charge of emulsion droplets as described above.

MG can also form protein-lipid complexes at the interface (Leenhouts1 et al., 1997). The mixture of MG and protein at the interface can form a complex film, which may strengthen the barrier properties of the interface. The interaction of proteins and MG can also modify the thermal properties of MG (Boots et al., 1999; Anker et al., 2002) and thus the properties of emulsions containing MG. Therefore, the influence of MG on the viscosity and creaming stability of WPI-stabilized emulsions was also investigated.

3.3 Viscosity

All emulsion samples showed a near Newtonian fluid behavior with a very low viscosity when no MG was included. After adding MG, a significant increase in viscosity was observed and the viscosity increased with increasing MG content (**Figure 3-2**). The increased viscosity was mainly attributed to the crystallization of MG inside oil droplets, which entrapped oil molecules, leading to a higher resistance to shear (Mao et al., 2014; Mao et al., 2012). The same increased viscosity of MG-structured emulsions stabilized with sodium caseinate (Davies et al., 2000) and Tween 20 (Mao et al., 2014) was also observed in previous studies. At very low stresses, the apparent viscosity of all emulsions decreased with increasing shear stress, which was similar for all emulsions. This decrease may suggest the presence

of weak attractive forces existing between droplets prior to application of shear forces. However, no flocculation was observed in these emulsion samples. Meanwhile, the viscosity curve from the shear rate 200 s^{-1} to 0 showed a good overlay with the curve from 0 to 200 s^{-1}, suggesting that the shear force applied in this test did not irreversibly influence the viscosity properties of these BC-loaded emulsions.

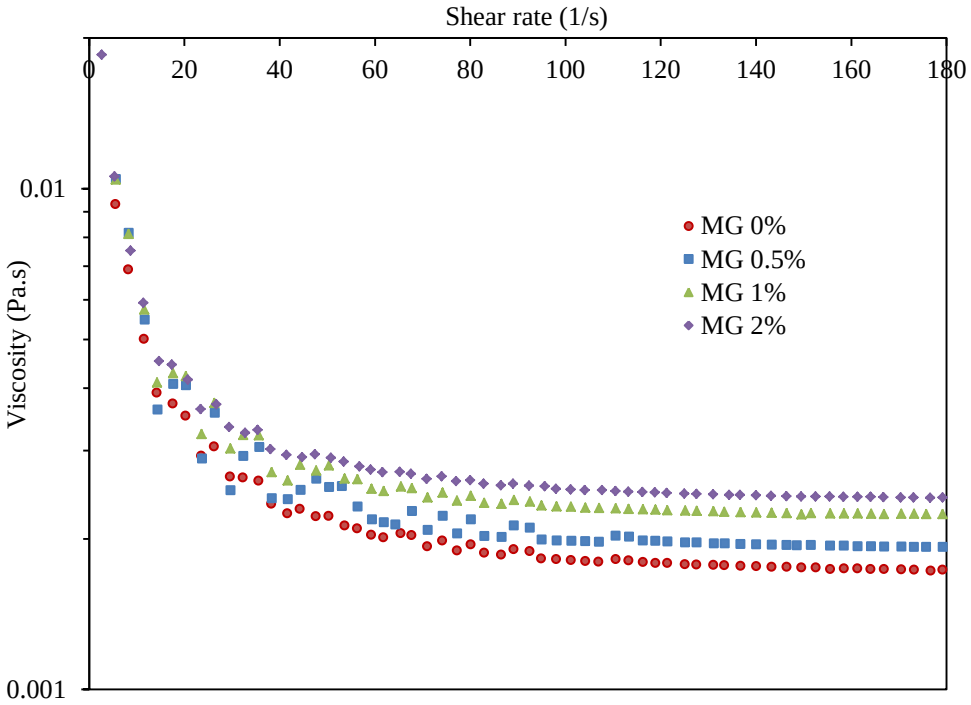


Figure 3-2. Viscosity of emulsions. MG 0% was the emulsion without MG while MG 0.5%, MG 1%, and MG 2% indicate emulsions with MG content (w/w) of 0.5%, 1%, and 2%, respectively.

3.4 Creaming Stability

Compared with MG-structured emulsions, integral transmission of the emulsion without MG increased more rapidly and reached 14.9% after centrifugation at 2,300 g for 1,200 s; integral transmission of MG-structured emulsions decreased with increasing MG content (**Figure 3-3**). After centrifugation at 2,300 g for 1,200 s, the emulsion with 2% MG showed the lowest integral transmission of 3.8%, followed by

emulsions with 1% MG (8.6%), and 0.5% MG (13.8%), respectively. These results suggested that addition of MG into the oil phase can significantly increase the creaming stability of emulsions and that this effect is MG-dose-dependent. MG-structured emulsions stabilized with Tween also showed the same increased creaming stability as emulsions without MG (Mao et al., 2012). According to Stoke's law, the increased creaming stability of MG-structured emulsions in this study may be mainly attributed to the following factors: (i) MG has a higher density (0.97 g/cm^3) than sunflower oil (0.919 g/cm^3), leading to a higher density of oil droplets of MG-structured emulsions; (ii) MG-structured emulsions had a higher viscosity (0.0024 Pa.s) than emulsion without MG (0.0017 Pa.s); and (iii) MG-structured emulsions showed narrower droplet size distribution than emulsion without MG.

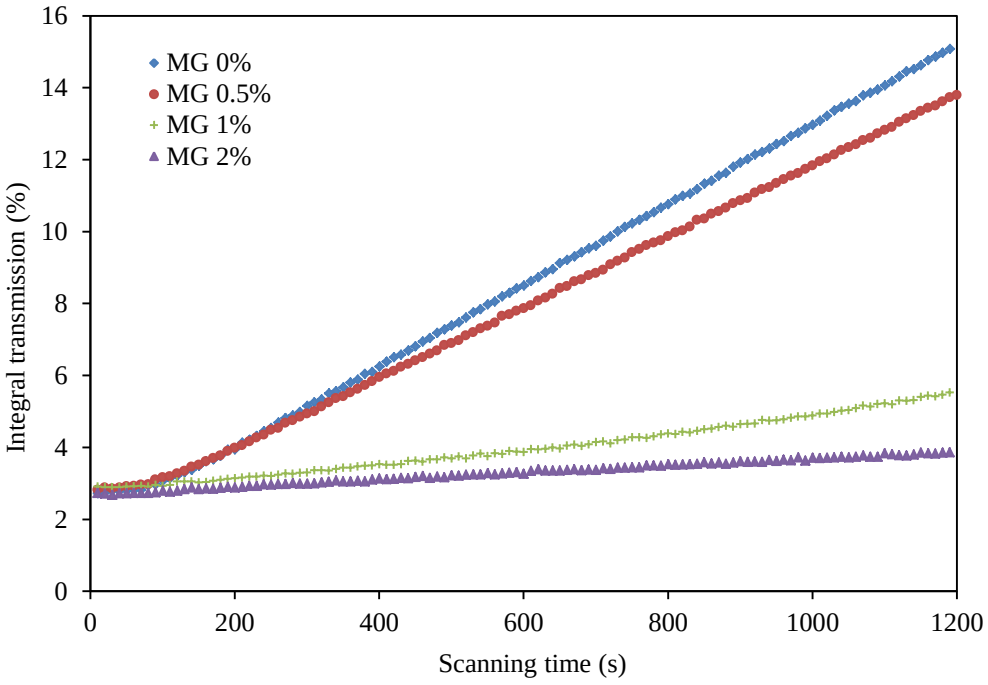


Figure 3-3. Time-dependent integral transmission profiles of emulsions. Emulsions were centrifuged at 2300 g and 25 ℃ at a scanning rate of once every 10 s for 1,200 s. MG 0% indicate the emulsion without MG while MG 0.5%, MG 1%, and MG 2% indicate emulsions with MG content (w/w) of 0.5%, 1%, and 2%, respectively.

3.5 Properties of Emulsions in GIT

When taken orally, emulsion droplets will be exposed to digestion conditions in the mouth, stomach, and intestine before being absorbed by enterocyte cells, during which the droplet size and interfacial compositions of emulsion droplets will presumably be changed. Thus, emulsion properties, e.g., droplet size and surface charge, after exposure to simulated GIT digestion were investigated.

Overall, the average particle size of all emulsions slightly increased after being exposed to a simulated mouth phase digestion, dramatically increased after gastric phase, and then significantly decreased after intestinal phase. Compared with the initial emulsions, after intestinal phase, digesta derived from emulsion without MG showed the smallest average particle size of 169 nm, followed by digesta derived from emulsions containing 0.5% (200 nm), 1% (275 nm), and 2% MG (368 nm) (**Table 3-2**).

In the protocol for simulated GIT digestion used in this study (Minekus et al., 2014), mucin was not included in the SSF fluid; mucin is widely considered as the main cause of increased droplet size of emulsions in the mouth phase (Salvia-Trujillo et al., 2013a). This may explain why a significant increase in droplet size after mouth phase was not observed in this study. The dramatic increase in droplet size after the gastric phase may be potentially attributed to the relatively high ionic strengthen of SGF (177 mM), which may have induced aggregation of emulsion droplets (McClements, 2015) and reduced surface charge at pH 2.5 due to the displacement of WPI from the water-oil interface by MG. Reduced surface charge accordingly led to decreased electrostatic repulsion force between droplets, which can potentially result in flocculation or coalescence of emulsions and thus increased droplet size. The subsequent decrease in droplet size of emulsions after the intestinal

Table 3-2. Droplet Size, zeta potential, and polydispersion Index (PdI) of monoglyceride (MG) structured emulsions in GIT digestion (mean±STD, n=3)

MG (%, w/w)	Droplet size (d.nm)			Zeta potential (mV)			PdI		
	Mouth phase	Gastric Phase	Intestinal phase	Mouth phase	Gastric Phase	Intestinal phase	Mouth phase	Gastric Phase	Intestinal phase
0	226±2^{a,b}	386±181^{a}	169±24^{a}	-53.3±2.7^{a}	48.0±9.8^{a}	-62.5±7.4^{a}	0.219±0.021^{a}	0.23±0.01^{a}	0.24±0.04^{a}
0.5	219±3^{a}	740±269^{b}	200±37^{b}	-50.6±1.6^{a,b}	31.0±8.8^{b}	-63.8±7.1^{a}	0.292±0.027^{b}	0.65±0.26^{b}	0.37±0.02^{b}
1	238±11^{b,c}	816±288^{c}	275±51^{c}	-48.5±2.4^{b}	20.0±4.2^{c}	-64.5±1.5^{a}	0.317±0.011^{b}	0.74±0.14^{c}	0.54±0.16^{c}
2	243±2^{c}	1045±69^{d}	368±14^{d}	-47.2±2.0^{b}	12.6±1.7^{d}	-66.1±1.0^{a}	0.376±0.01^{c}	0.84±0.05^{d}	0.54±0.02^{c}

^{a}Different letters indicate significant difference between values in a column (p<0.05)

phase was mainly attributed to the break-down of the large droplets from gastric phase by trypsin hydrolysis and then the formation of bile-salts-stabilized micelles with small particles.

Protein-stabilized emulsions are sensitive to the ionic strength and pH of the continuous phase. At low ionic strength, there may be a sufficiently high-energy barrier to prevent the droplets from coming close enough to aggregate. Along with the increase in ion concentration, screening of electrostatic interactions becomes more effective, and the energy barrier is no longer high enough to prevent the droplets from coming close enough, and hence the droplets tend to aggregate (McClements, 2015). The potential mechanism by which pH can influence the aggregation of emulsion droplets will be related to the surface charge of emulsions.

All emulsions were negatively charged after being exposed to simulated mouth phase and intestinal phase digestion, while they were all positively charged after the gastric phase (**Table 3-2**). The negative charge in the mouth phase is mainly attributed to the negatively charged proteins at the interface. However, after the intestinal phase, WPI at interface have been hydrolyzed by trypsin in SIF while some other anionic molecules, e.g., bile salts and phospholipids, as well as free fatty acids derived from oil digestion, will be absorbed onto the droplet/micelle surface, resulting in the negatively charged surface (Salvia-Trujillo et al., 2013a).

Compared with emulsion without MG, MG-structured emulsions showed a significant reduction in surface charge when being exposed to gastric digestion, and the surface charge of MG-structured emulsions decreased with increasing MG concentration (**Table 3-2**). This reduction may be attributed to the displacement of WPI from the water-oil interface by MG, as described above. This reduced surface charge can decrease the electrostatic repulsion between emulsion droplets and

potentially result in aggregation of droplets (McClements, 2015). This may explain why MG-structured emulsions showed a significant increase in droplet size after being exposed to gastric digestion.

3.6 Bioaccessibility and Cellular Uptake of β-carotene

After exposing emulsions containing β-carotene to simulated GIT digestion, the bioaccessibility of β-carotene was evaluated by quantifying the percent of β-carotene incorporated into the micelle fractions after the intestinal phase. The subsequent cellular uptake of β-carotene in micelles was also investigated.

3.6.1 Bioaccessibility

The percent of β-carotene in micelle fractions significantly increased with increasing MG content in emulsions, rising from 53.4% for the emulsion without MG to 77.1% for the emulsion with 2% MG (**Figure 3-4**). This increase may be attributed to two factors: (i) reduced protein absorption at the interface of emulsions containing MG leads to a faster break-down of the protein layer at the interface by trypsin hydrolysis and thus more rapid lipid hydrolysis by lipase; (ii) easily binding of lipase in pancreatin to the oil-water interface of emulsions containing MG due to reduced protein absorption at the interface. Both these factors potentially result in a higher rate of enzymatic hydrolysis of lipid by lipase and thus less undigested β-carotene-loaded oil droplets, which accordingly leads to the higher concentration of β-carotene in micelle fractions derived from MG-structured emulsions than that from the emulsion without MG.

The absorption of dietary lipophilic ingredients from food goes through several stages, starting with the release of compounds from the food matrix, followed by incorporation into lipid droplets under gastric emulsification and then solubilisation

within micelles formed in intestinal digestion process (Yonekura & Nagao, 2007). Accordingly, encapsulated BC in emulsions tends to be more bioavailable than BC in natural fruits and vegetables (Van het Hof, 2000) and emulsion structure (e.g., droplet size, oil compositions, surface charge, or interfacial layer) can significantly influence the digestion and absorption behaviors of encapsulated bioactive nutrients in emulsions (Cho et al., 2014; Salvia-Trujillo et al., 2015; Zou et al., 2015). Findings in this section also suggested that oil compositions and interface layer of oil droplets play an important role in determining the bioavailability of encapsulated bioactive nutrients, e.g., β-carotene in emulsions.

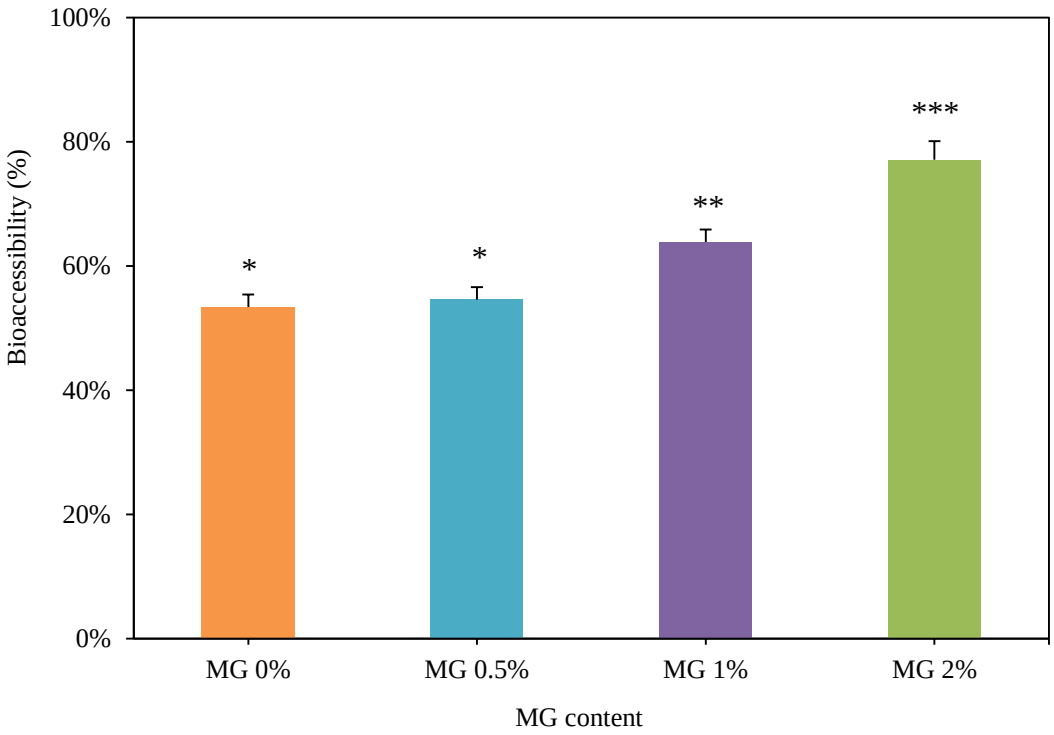


Figure 3-4. Bioaccessibility of β-carotene after GIT. MG 0% indicate the emulsion without MG while MG 0.5%, MG 1%, and MG 2% indicate emulsions with MG content (w/w) of 0.5%, 1%, and 2%, respectively. The different numbers of the symbol (*) indicate the significant difference between emulsion with different MG content (p<0.05).

In addition, the fatty acid chain length of lipid in the oil phase, e.g., MG, can influence the solubility of the encapsulated lipophilic nutrients, e.g., carotenoids. Generally, short-chain fatty acids show a better solubilizing ability of carotenoids than long-chain fatty acids (Borel, 1996; Roohinejad et al., 2015), suggesting that the selection of lipids, including oil phase and the lipophilic emulsifiers (e.g., MG), can potentially influence the loading capacity of the emulsions.

3.6.2 Cellular Uptake

The cellular uptake of β-carotene was evaluated by incubating the micelles containing β-carotene after intestinal phase of GIT digestion with Caco-2 cells. Compared with an emulsion without MG (0.085 μg/well), MG-structured emulsions showed higher cellular uptake of β-carotene. An emulsion with 2% MG showed the highest BC cellular uptake of 0.138 μg/well after 4 h incubation, followed by emulsions with 1% MG (0.119 μg/well), and 0.5% MG (0.109 μg/well) (**Figure 3-5**), respectively, suggesting that cellular uptake of β-carotene in MG-structured emulsions increased with increasing MG content. This increase may be attributed to the reduced particle size or different surface structure of micelles.

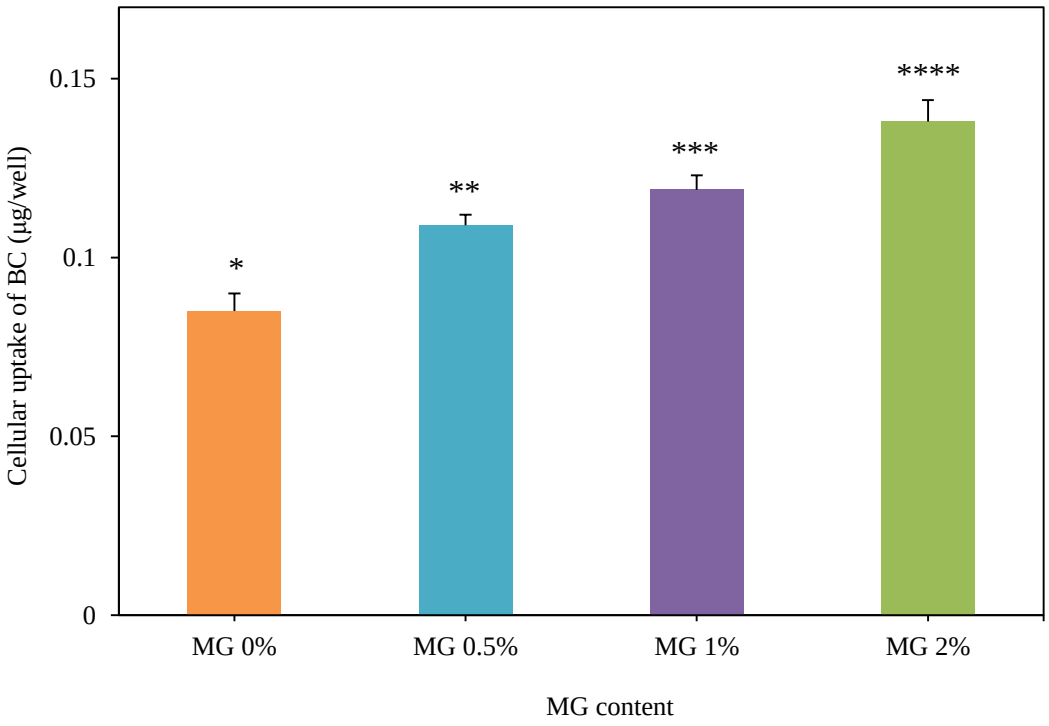


Figure 3-5. Cellular uptake of β-carotene in micelles. MG 0% indicate the emulsion without MG while MG 0.5%, MG 1%, and MG 2% indicate emulsions with an MG content (w/w) of 0.5%, 1%, and 2%, respectively. The different numbers of the symbol (*) indicate the significant difference between emulsion with different MG content (p<0.05).

The particle size and surface charge of micelles were thus tested by DLS. Generally, the cellular uptake of bioactive compounds in nanoparticles increases with reduced particle size. However, the average size of micelles with a higher cellular uptake of β-carotene was slightly higher than that of micelles with lower cellular uptake of β-carotene, and the surface charge of all micelles were not significantly different (**Table 3-3**), suggesting that this increased cellular uptake could not be attributed to the particle size and surface charge.

Table 3-3. Droplet size and zeta potential of micelle fractions after intestinal phase digestion (mean±STD, n=3)

Monoglycride (%, w/w)	Size (d.nm）	Zeta potential (mV)
0	194±8[a]	-64.6±0.9[a]
0.5	214±3[b]	-65.7±0.1[a]
1	236±12[c]	-65.0±0.2[a]
2	244±8[c]	-66.9±1.3[a]

[a]Different letters indicate significant difference between values in a column (p<0.05)

After intestinal phase digestion, MG potentially will be incorporated into the newly-formed micelles and the glycerol groups will migrate to the hydrophilic surface of the micelles. Each glycerol molecule contains two hydroxyl groups; thus, the outer layer of these micelles is highly hydrophilic. Furthermore, the brush-border membrane of enterocytes is separated from the bulk fluid phase in the intestinal lumen by an unstirred water layer (Phan, 2001), which mixes poorly with the bulk fluid phase in the intestinal lumen. Consequently, solute molecules in the bulk fluid phase gain access to the brush border membrane by diffusion across the unstirred water layer. For any molecule or nanoparticles, the rate of uptake by the small intestine is dependent on the number of molecules that are in close proximity to the brush border membrane and are therefore available for uptake. Molecules or nanoparticles with good water solubility can easily diffuse across the unstirred water layer, approach the brush border, and be uptaken. Hence, the increased cellular uptake of β-carotene may be mainly attributed to the hydroxyl-group-rich surface of MG micelles, leading to a higher hydrophilicity of these micelles and thus an easier approach to the brush border of Caco-2 cells, which accordingly results in a better cellular uptake of these β-carotene-loaded micelles by Caco-2 cells.

4. Conclusion

Monoglyceride (MG) in the oil phase showed competitively occupation at the oil-water interface with protein molecules, leading to reduced protein absorption and thus reduced surface charge of droplets. Emulsions containing MG showed similar droplet size but higher viscosity and better creaming stability than emulsion without MG. Exposure to simulated GIT digestion resulted in large changes in both droplet size and surface charge of emulsions. β-carotene encapsulated in emulsions containing MG showed significantly increased bioaccessibility and cellular uptake by enterocytes ($p<0.05$) than that in emulsion without MG, and the bioaccessibility and cellular uptake increased with increasing MG content. The findings of this study confirm the great potentials of MG-structured O/W emulsions as ideal carriers for lipophilic nutrients with improved stability and bioavailability, and contribute to a better understanding of the correlation between the microstructure of the oil phase of emulsion and the biological fate of encapsulated bioactive nutrients in emulsions. Furthermore, the findings also make it possible to potentially control the stability and release of lipophilic components by structuring the oil phase of emulsion-based delivery carriers.

References

Anan Yaghmur, L. d. C., Laurent Sagalowicz, Martin E. Leser, & Otto Glatter. (2005). Emulsified Microemulsions and Oil-Containing Liquid Crystalline Phases. *Langmuir, 21*, 569-577.

Boots, J.W.P., Chupin, V., Killian, J.A., Demel, R.A., & Kruijff, B. D. (1999). Interaction mode specific reorganization of gel phase monoglyceride bilayers by β-lactoglobulin. *Biochimica et Biophysica Acta (BBA), 1420*, 241-251.

Borel, P., Grolier, P., Armand, M., Partier, A., Lafont, H., Lairon, D., & Azais-Braesco, V. (1996). Carotenoids in biological emulsions solubility surface-to-core distribution and release from lipid droplets. *Journal of Lipid Research, 37*, 250-261.

Chen, J., Dickinson, Eric., & Iveson, Greaeme. (1993). Interfacial Interactions, Competitive Adsorption and Emulsion Stability. *Food Structure, 12*, 135-146.

Cho, H. T., Salvia-Trujillo, L., Kim, J., Park, Y., Xiao, H., & McClements, D. J. (2014). Droplet size and composition of nutraceutical nanoemulsions influences bioavailability of long chain fatty acids and Coenzyme Q10. *Food Chemistry, 156*, 117-122.

Davies, E., Dickinson, E., & Bee, R. (2000). Shear stability of sodium caseinate emulsions containing monoglyceride and triglyceride crystals. *Food Hydrocolloids, 14*, 145-153.

Dickinson, E., Mauffret, A., Rolfe, S. E., & Woskett, C. M. (1989). Adsorption at interfaces in dairy systems. *International Journal of Dairy Technology, 42*, 18-22.

Doxastakis, G., & Sherman, P. (1986). The interaction of sodium caseinate with monoglyceride and diglyceride at the oil-water interface and its effect on interfacial rheological properties. *Colloid and Polymer Science, 264*, 254-259.

Fernandez, A., & Kelly, P. (2016). pH-stat vs. free-fall pH techniques in the enzymatic hydrolysis of whey proteins. *Food Chemistry, 199*, 409-415.

Heertje, I., Nederlof, J., Hendricks, H. A. C. M., & Lucassen-Reynder, E. H. (1990). The observation of the displacement of emulsifiers by confocal scanning laser microscopy. *Food Structure, 9*, 305-316.

Hormann, K., & Zimmer, A. (2016). Drug delivery and drug targeting with parenteral lipid nanoemulsions - A review. *Journal of Controlled Release, 223*, 85-98.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion. *Food Chemistry, 114*, 253-262.

Krog, N., & Larsson, K. (1992). Crystallization at interfaces in food emulsions–a general phenomenon. *European Journal of Lipid Science and Technology, 94*, 55-57.

Krog, N. j., Sparsø, F.V. (2004). Food Emulsifiers: Their Structures and Properties. In K. L. S. E. Friberg, & J. Sjöblom (Eds.) (Ed.), *Food Emulsions* (4th ed., pp. 44-90). New York: Marcel Dekker Inc.

Leenhouts, J.M., Demel, R.A., Kruijff, B de., & Boots, J.W.P. (1997). Charge-dependent insertion of β-lactoglobulin into monoglyceride monolayers. *Biochimica et Biophysica Acta (BBA), 1330*, 61-70.

Lu, W., Kelly, A. L., Maguire, P., Zhang, H., Stanton, C., & Miao, S. (2016). Correlation of Emulsion Structure with Cellular Uptake Behavior of

Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure. *Journal of Agricultural and Food Chemistry, 64*, 8659-8666.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Mao, L., Calligaris, S., Barba, L., & Miao, S. (2014). Monoglyceride self-assembled structure in O/W emulsion: formation, characterization and its effect on emulsion properties. *Food Research International, 58*, 81-88.

Mao, L., O'Kennedy, B. T., Roos, Y. H., Hannon, J. A., & Miao, S. (2012). Effect of monoglyceride self-assembled structure on emulsion properties and subsequent flavor release. *Food Research International, 48*, 233-240.

Martin Anker, Jonas Berntsen, Anne-Marie Hermansson, & Stading, M. (2002). Improved water vapor barrier of whey protein films by addition of an acetylated monoglyceride. *Innovative Food Science & Emerging Technologies, 3*, 81-92.

McClements, D. J. (2015). *Food Emulsions: principles, practices, and techniques* (3rd ed.). New York: CRC press.

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrition, 51*, 285-330.

McSweeney, S. L., Healy, R., & Mulvihill, D. M. (2008). Effect of lecithin and monoglycerides on the heat stability of a model infant formula emulsion. *Food Hydrocolloids, 22*, 888-898.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Effer, L., Golding, M., Karakaya, S., Kirkhus, B., Feunteun, S.L., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Menard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function, 5*, 1113-1124.

Pelan, B.M.C., Watts, K. M., Campbell, I.J., & Lips, A. (1997). The stability of aerated milk protein emulsions in the presence of small molecule surfactants. *Journal of Dairy Science, 80*, 2631-2638.

Phan CT, T. P. (2001). Intestinal lipid absorption and transport *Frontiers in Bioscience, 6*, 299-319.

Pugnaloni, L. A., Dickinson, E., Ettelaie, R., Mackie, A. R., & Wilde, P. J. (2004). Competitive adsorption of proteins and low-molecular-weight surfactants: computer simulation and microscopic imaging. *Advances in Colloid and Interface Science, 107*, 27-49.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. *Food Chemistry, 135*, 1440-1447.

Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols, 1*, 3159-3165.

Roohinejad, S., Oey, I., Wen, J., Lee, S. J., Everett, D. W., & Burritt, D. J. (2015). Formulation of oil-in-water beta-carotene microemulsions: effect of oil type and fatty acid chain length. *Food Chemistry, 174*, 270-278.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013). Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry, 141*, 1472-1480.

Salvia-Trujillo, L., Sun, Q., Um, B. H., Park, Y., & McClements, D. J. (2015). In vitro and in vivo study of fucoxanthin bioavailability from nanoemulsion-based delivery systems: Impact of lipid carrier type. *Journal of Functional Foods, 17*, 293-304.

Stephen, E. R., Suzanne, F. R., & Robyn, H. L. (2001). Aggregation kinetics of heated whey protein-stabilised emulsions effect of low-molecular weight emulsifiers. *Food Hydrocolloids, 15*, 253-262.

Sun, Y., Xia, Z., Zheng, J., Qiu, P., Zhang, L., McClements, D. J., & Xiao, H. (2015). Nanoemulsion-based delivery systems for nutraceuticals: Influence of carrier oil type on bioavailability of pterostilbene. *Journal of Functional Foods, 13*, 61-70.

Tcholakova, S., Denkov, N. D., Ivanov, I. B., and Campbell, B. (2002). Coalescence in β-Lactoglobulin-Stabilized Emulsions: Effects of Protein Adsorption and Drop Size. *Langmuir, 18*, 8960-8971.

Van het Hof, C. W., JA Weststrate and Joseph G.A.J. Hautvast. (2000). Dietary Factors That Affect the Bioavailability of Carotenoids. *Journal of Nutrition, 130*, 503-506.

Yi, J., Lam, T. I., Yokoyama, W., Cheng, L. W., & Zhong, F. (2014). Controlled release of beta-carotene in beta-lactoglobulin-dextran-conjugated nanoparticles' in vitro digestion and transport with Caco-2 monolayers. *Journal of Agricultural and Food Chemistry, 62*, 8900-8907.

Yonekura, L., & Nagao, A. (2007). Intestinal absorption of dietary carotenoids. *Molecular Nutrition & Food Research, 51*, 107-115.

Zou, L., Zheng, B., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2015). Enhancing nutraceutical bioavailability using excipient emulsions: Influence of lipid droplet size on solubility and bioaccessibility of powdered curcumin. *Journal of Functional Foods, 15*, 72-83.

CHAPTER FOUR

Bioaccessibility and Cellular Uptake of β-Carotene Encapsulated in Model O/W Emulsions: Influence of Initial Droplet Size and Emulsifiers

Published in *Nanomaterials*, 2017, 7, 282-292.

Authors: Wei Lu, Alan L. Kelly, and Song Miao*

The work contained in this chapter was undertaken and written solely by myself with specific contributions from each co-author.

Abstract

The effects of the initial droplet size and emulsifier on the properties of emulsions and the digestion, release, and cellular uptake of encapsulated β-carotene in emulsions were investigated. Exposure to simulated gastrointestinal tract (GIT) significantly changed the droplet size, surface charge and composition of all emulsions, and these changes were dependent on their initial droplet size and the emulsifiers used. Whey protein isolate stabilized emulsion showed the highest β-carotene bioaccessibility, while sodium caseinate stabilized emulsion showed the highest cellular uptake of β-carotene after GIT. This inconsistency between the results of bioaccessibility and cellular uptake of β-carotene indicated that the cellular uptake assay is necessary for a reliable evaluation of the final bioavailability. The bioavailability of β-carotene calculated based on the results of bioaccessibility and cellular uptake showed the same order with the results of cellular uptake being sodium-caseinate>tween 80>whey-protein-isolate. The findings in this study contribute to a better understanding of the correlation between emulsion properties (e.g., droplet size) and compositions (e.g., emulsifiers) and the digestive fate of emulsion-encapsulated lipophilic components, which make it possible to achieve controlled delivery of these compounds by designing the structure of emulsion-based carriers.

Keywords: emulsion; β-carotene; digestion; cellular uptake; bioavailability

1. Introduction

Carotenoids are a class of natural pigments abundant in plants and fruits that can have many health benefits when consumed at proper levels. Previous studies have shown that carotenoids possess strong antioxidant activity and that intake of carotenoid-rich foods was correlated with the reduced risks of several chronic diseases, including cancers, cardiovascular diseases, age-related macular degeneration and cataracts (Gerster, 1993; Lintig, 2010). Several potential mechanisms have been proposed to explain these biological activities, e.g., scavenging free radicals and preventing oxidative damage, altering transcription activity or functioning as precursor of vitamin A (Qian et al., 2012). β-carotene is a representative member of the carotenoids family and has been widely studied due to its high pro-vitamin A activity. However, extreme water insolubility and instability greatly limit the health benefits of β-carotene. Therefore, the delivery of β-carotene requires an encapsulation and protection mechanism. Emulsions are ideal carriers for lipophilic nutrients, such as β-carotene, due to their ease of operation, maintenance of chemical stability, controlled release and potential for target delivery of encapsulated compounds (Lu et al., 2016).

Since emulsions are widely used as delivery systems for lipophilic nutrients (Lu et al., 2016; McClements et al., 2007), an in-depth understanding of the biological fate of emulsion droplets and encapsulated compounds in the digestive tract is necessary for optimizing the delivery efficiency of emulsions. The determination of the changes of droplet properties, e.g., size, surface charge and the subsequent release of encapsulated compounds during digestion, can also contribute to a better understanding of the mechanism of improved bioavailability by emulsion delivery. When being exposed to gastrointestinal tract (GIT) digestion, emulsions can show

great changes in their droplet size, surface charge or compositions (Lu et al., 2017b; Pool et al., 2013), due to the extremely acidic environment in the gastric phase or as a result of enzymatic hydrolysis in the mouth, gastric and intestinal phases; all of these changes can influence the digestion of emulsion droplets and thus the biological fate of nutrients within droplets.

Many previous studies have investigated the influence of emulsion structure, e.g., droplet size or emulsifiers, on the digestibility of lipid droplets in emulsions (Hur et al., 2009), the physical and chemical stability of emulsion-encapsulated nutrients (Qian et al., 2012) and the release of these encapsulated nutrients after passing through simulated GIT digestion (Hou et al., 2014). The bioaccessibility of these encapsulated nutrients in emulsions with different initial droplet size (Salvia-Trujillo et al., 2013a), emulsifiers (Mao et al., 2013) and oil compositions (Qian et al., 2012) was also well evaluated by measuring the content of nutrients in micelle fractions after GIT digestion. However, these studies did not investigate the absorption of these nutrient-loaded micelles by enterocytes, which is important for the evaluation of the bioavailability of encapsulated nutrients. This may also be the main cause of the inconsistency observed between the bioaccessibility and the in vivo bioavailability of emulsion-encapsulated nutrients (Salvia-Trujillo et al., 2017). In addition, Dairy proteins are widely used as food emulsion stabilizers due to their edibility, health benefits and good amphiphilic properties. Many studies have been done on dairy protein-stabilized emulsions. However, the information on the cellular uptake of encapsulated nutrients in dairy-protein-stabilized emulsions (e.g., whey protein isolate or casein) after passing through GIT was very limited. The comparison between different dairy proteins concerning their influence on the digestion behavior of emulsions containing nutrients in the GIT and the subsequent

enterocytes cell absorption of released nutrients after the GIT still needs further investigation. Furthermore, little is known about the influence of small initial droplet sizes (~1 μm) on the bioaccessibility of encapsulated nutrients.

Therefore, this study was designed to investigate the bioaccessibility and cellular uptake of an encapsulated lipophilic nutrient (β-carotene) in emulsions with different initial droplet sizes (~1 μm) and emulsifiers (whey protein isolate, sodium caseinate and Tween 80) by the simulated GIT digestion system and the Caco-2 cellular uptake assay. The changes of emulsion properties, such as droplet size and surface charge, during GIT digestion were also tested.

2. Material and Methods

2.1 Materials

All-trans-β-carotene (>93%, UV), sodium caseinate, tween 80 (Polysorbate 80), pepsin (≥250 unit/mg), pancreatin (4×USP), bile salts, Dulbecco's Modified Eagle's Medium (DMEM) (containing 4.5 g/L D-glucose), penicillin and streptomycin (100×), fetal bovine serum (FBS), phosphate-buffered solution (PBS) and cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunflower oil (Solesta, >98% fat) was purchased from a local supermarket (ALDI, Fermoy, Co.Cork, Ireland), and whey protein isolate (70% β-lactoglobulin and 18% α-lactalbumin) was obtained from Davisco Food International (Le Sueur, MN, USA). All other chemicals and reagents used were of AR-grade and obtained from Sigma-Aldrich.

2.2 Emulsion Preparation

2.2.1 Preparation of Emulsions with Different Droplet Sizes

A continuous phase was prepared by dissolving whey protein isolate (WPI) (1.0%, w/w) in water containing 0.01% (w/w) sodium azide (anti-bacterial agents). The oil phase was prepared by dissolving β-carotene (0.2%, w/w in oil phase) in the sunflower oil (10%, w/w) at 140 °C for 15 s and then mixed with the continuous phase at a speed of 10,000 rpm for 1 min using an Ultra-Turrax (IKA, Staufen, Germany) followed by further homogenization (APV 1000, SPX Flow Technology, Charlotte, North Carolina, USA) at 20 or 70 MPa.

2.2.2 Preparation of Emulsions with Different Interfaces

WPI, sodium caseinate (SCN) or tween 80 (TW80) was dispersed (1.0%, w/w) in water containing 0.01% (w/w) sodium azide as continuous phases. The subsequent emulsion preparation was performed using the same process mentioned above with high-pressure homogenization at 70 MPa.

2.2.3 Characterization of Droplet Size and Surface Charge

The mean droplet size, and zeta potential of emulsions were determined by dynamic light scattering (DLS) using a laser particle analyser (Nano-ZS, Malvern Instruments, Worcestershire, UK). Emulsions were 1000-fold diluted before testing.

2.3 Rheological Analysis

Rheological properties of emulsions were determined using an AR 2000 ex rheometer (TA Instruments, Crawley, UK). A concentric cylinder geometry (gap=5920 μm) were selected. A viscosity test was performed over a shear rate range of 0-200 s^{-1} at 25 °C.

2.4 Creaming Stability

The creaming stability of different emulsions was evaluated using a Lumisizer (LUM GmbH, Berlin, Germany) as described previously (Mao et al., 2012). In this study, emulsions were centrifuged at 2,300 g at 25 °C with a scanning rate of once every 10 s for 1,200 s.

2.5 *In vitro* Simulated GIT Digestion

An *in vitro* simulated GIT digestion method employed in a previous study (Lu et al., 2017b) was used to digest emulsions. The digesta after each phase (mouth, gastric and intestinal phase) were sampled for the determination of droplet size and zeta potential. The simulated saliva fluid (SSF), gastric fluid (SGF) and intestinal fluid (SIF) were prepared as described previously (Lu et al., 2017b).

Mouth phase: Emulsions were mixed with SSF (1:1, v/v), and the pH was adjusted to 6.8 and incubated at 37 °C for 10 min with continuous agitation at 100 rpm.

Gastric phase: The digesta from the mouth phase were mixed with the SGF (1:1, v/v), and the pH of the mixture was adjusted to 2.5. The mixture was then incubated at 37 °C for 2 h with continuous agitation at 100 rpm. The enzyme activity of pepsin in the final mixture was 2,000 U/mL.

Small intestinal phase: The digesta sample from the gastric phase was mixed with the SIF (1:1, v/v). The pH of the mixture was adjusted to 7.0, and it was incubated at 37 °C for 2 h with continuous agitation at 100 rpm. The enzyme activity of pancreatin (based on trypsin) in the final mixture was 100 U/mL.

2.6 Bioaccessibility of β-carotene

The bioaccessibility of β-carotene after the intestinal phase was evaluated as described previously with minor modification. An aliquot of raw digesta from the

intestinal phase was centrifuged at 2,700× g for 40 min at 4 °C, and the supernatant was collected and considered as the micelle fraction, in which the bioactive compound is solubilized. Aliquots of 2 mL of the raw digesta or the micelle fraction were extracted twice with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v). The top layer containing the solubilized β-carotene was collected and analysed by RP-HPLC as described below. The bioaccessibility of encapsulated β-carotene was calculated using the following equation:

$$\text{Bioaccessibility (\%)} = \frac{C_{\text{micelle}}}{C_{\text{initial}}} \times 100\%$$

where C_{micelle} and C_{initial} are the concentration of β-carotene in the micelle fraction after intestinal phase digestion and initial emulsion before GIT digestion, respectively.

2.7 Cellular Uptake by Caco-2 Cells

Caco-2 cells were seeded in a 6-well plate at a density of 3.5×10^5 cells $well^{-1}$, and cellular uptake experiments were performed 5-7 days after seeding. Micelle fractions of different BC-loaded emulsions after the intestinal phase were 20-fold diluted with complete medium. One millilitre of diluted samples was added to each well in a 6-well plate, which was then incubated at 37 °C and 5% CO_2 for 4 h. Before collection, cells were washed three times with PBS buffer solution. Then, cells were collected, lysed, extracted and analysed for β-carotene content by RP-HPLC.

2.8 Extraction of β-carotene

β-carotene was extracted from the micelle fraction or raw digesta emulsion systems with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v) as described

in Chapter 2 (section 2.10). The hexane extracts were combined and dried under a stream of nitrogen gas and dissolved in 0.6 mL ethanol for HPLC analysis.

2.9 HPLC Analysis of β-carotene

The concentration of β-carotene was determined as described in **Chapter 2** (section 2.12). A reversed phase C18 column (4.6 × 250 mm, 5 μm, 300Å, Phenomenex) was used; the operation temperature was 30 °C; elution was performed with 90% ethanol and 10% acetonitrile from 0 to 15 min; the flow rate was 1 mL/min; the detection wavelength was 450 nm; the injection volume was 20 μL. The peak area of β-carotene on HPLC showed a good linear correlation with the β-carotene concentration in the range of 0.05~5 μg/mL (data not shown).

2.10 Statistical Analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was employed to compare means of data. A *t*-test was used to determine the differences between means, and significant differences were determined at the 0.05 level ($p < 0.05$).

3. Results and Discussion

3.1 Characterization of Emulsions

Emulsions showed a reduced droplet size with increasing homogenization pressure (HP) used during their preparation (**Table 4-1**), which was as observed in many previous studies (Gupta et al., 2016), and no significant difference in droplet size of emulsions stabilized by whey protein isolate (WPI), sodium caseinate (SCN) and tween 80 (TW80), processed at similar homogenization pressures, was observed. Droplets of WPI- and SCN-stabilized emulsions were negatively charged, which is

mainly attributed to the protein molecules being negatively charged at pH (7.0), which is higher than their isoelectric point (pH 4.0-5.0). WPI-stabilized emulsions with different droplet sizes (produced at different homogenization pressures) showed similar surface charges. Droplets of TW-stabilized emulsion were also negatively charged, but showed a much lower zeta potential (-25 mV) than that of the emulsions stabilized with proteins (around -53 mV).

Table 4-1. Droplet size, zeta potential (ZP), polydispersity index (PdI), viscosity and creaming index (CI) of emulsions

Emulsions	Size (d.nm)	ZP(mV)	PdI	Viscosity (mPa.s)	CI
WPI-L	472 ± 20^{a}	-53.2 ± 1.7^{a}	0.24 ± 0.07^{a}	1.78 ± 0.02^{b}	0.327 ± 0.007^{a}
WPI-S	205 ± 4^{b}	-52.7 ± 0.6^{a}	0.24 ± 0.03^{a}	1.76 ± 0.02^{b}	0.169 ± 0.003^{c}
SCN	223 ± 12^{b}	-52.1 ± 0.7^{a}	0.18 ± 0.02^{b}	1.94 ± 0.02^{a}	0.111 ± 0.002^{d}
TW80	227 ± 12^{b}	-25.1 ± 0.5^{b}	0.22 ± 0.01^{a}	1.72 ± 0.02^{b}	0.193 ± 0.005^{b}

*WPI-L and WPI-S indicate emulsions stabilized by whey protein isolate with large and small initial droplet sizes; SCN and TW80 indicate emulsions stabilized by sodium caseinate and tween 80. Different superscript letters indicate significant differences between values in a column ($p < 0.05$)

All emulsions showed very low viscosity. The SCN emulsion showed the highest viscosity, followed by WPI- and TW80-stabilized emulsions, respectively (**Table 4-1**). WPI emulsions with large or small droplets did not significantly differ in viscosity. The viscosity of emulsions can be influenced by the proportion of the oil phase and emulsifiers (Choudhury et al., 2014; Sharma et al., 2015) and increases with increasing oil content, owing to the increased interfacial tension with water (Shafiq et al., 2007).

The SCN-stabilized emulsion showed the best creaming stability ($p<0.01$), followed by WPI- and TW-stabilized emulsion, respectively (**Figure 4-1**). The WPI-stabilized emulsion with a small droplet size showed better creaming resistance than that with a large droplet size ($p<0.01$). These results suggested that the creaming

stability of emulsions is dependent on their initial droplet size and interfacial composition.

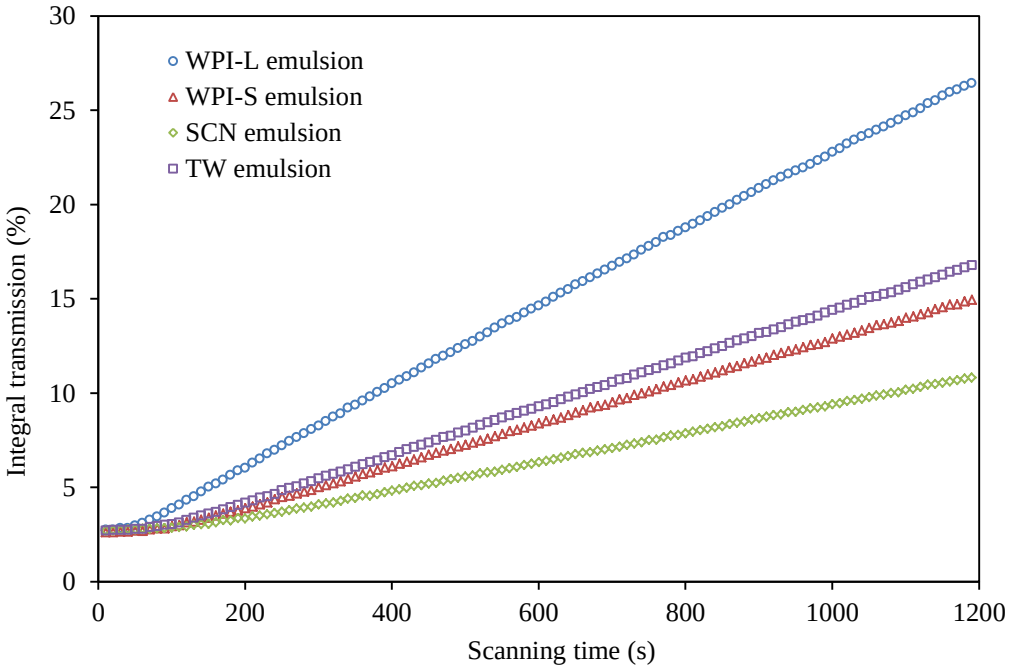


Figure 4-1. Integral light transmission of different emulsions. WPI-L and WPI-S indicate emulsions stabilized by whey protein isolate with large and small droplet sizes, respectively. SCN and TW emulsions indicate emulsions stabilized by sodium caseinate and tween 80, respectively.

According to Stokes' law, creaming velocity (V) is related to the radius of the particle (r), the viscosity (μ) and density of the particle (ρ_p) and the continuous phase (ρ_f). Emulsions with smaller droplet sizes, higher viscosity or higher particle density are thus expected to show better creaming stability. In this study, the SCN-stabilized emulsion showed higher viscosity and a narrower size distribution (**Figure 4-2**), as well as a lower PdI (**Table 4-1**) than WPI and TW80 emulsions, which may explain why the former emulsion showed the best creaming stability.

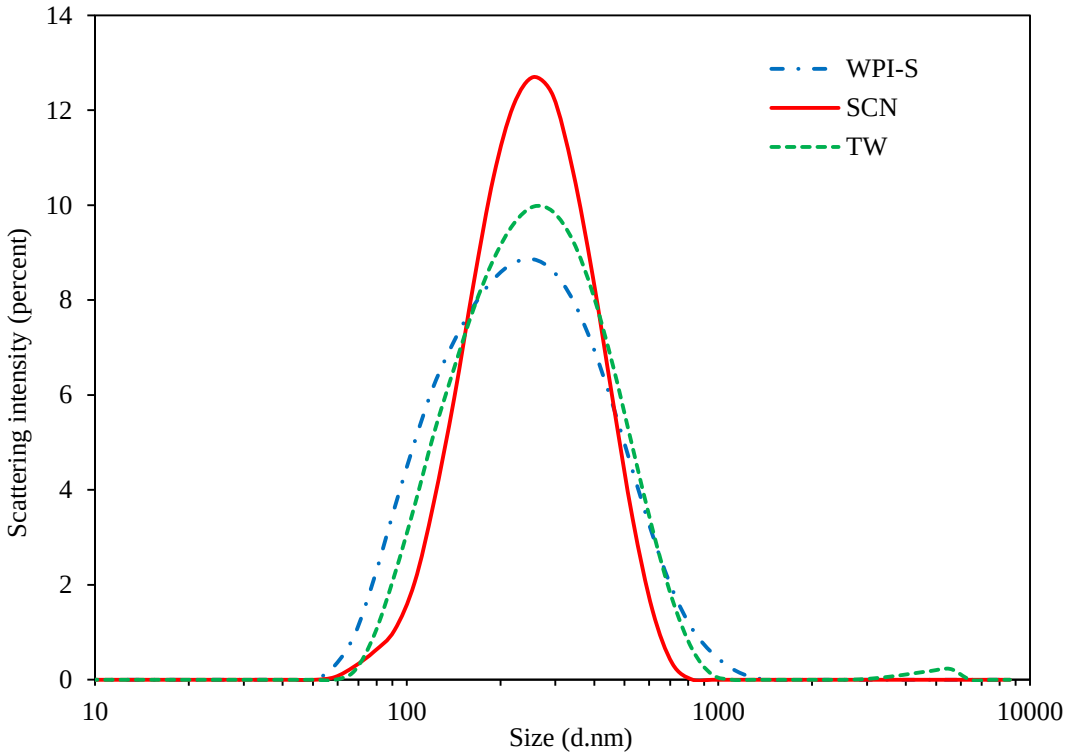


Figure 4-2. Size distribution of emulsions with different emulsifiers. WPI-S indicates whey protein isolate-stabilized emulsion with small droplet size; SCN indicates sodium caseinate-stabilized emulsion; TW indicates tween 80-stabilized emulsion.

3.2 Properties of Emulsions in GIT Digestion

Exposure to GIT digestion can result in great changes in the properties of emulsions, e.g., droplet size and surface charge, which accordingly will influence the digestion and absorption of nutrients incorporated into emulsions. Thus, the droplet size and surface charge of β-carotene-loaded emulsions after being exposed to GIT were investigated.

All emulsions showed only a slight increase in droplet size after exposure to simulated mouth digestion (**Table 4-2**). This is mainly attributed to the absence of mucin from the SSF used in this study because mucin is the main cause of the increase in droplet size during mouth-phase digestion (Salvia-Trujillo et al., 2013a).

Table 4-2. Particle size and surface charge of emulsions after being exposed to simulated GIT digestion

Emulsion	Droplet size (d.nm)			Zeta potential (mV)			Polydispersity index (PdI)		
	Mouse phase	Gastric Phase	Intestinal phase	Mouse phase	Gastric Phase	Intestinal phase	Mouse phase	Gastric Phase	Intestinal phase
WPI-S	224 ± 11^{b}	774 ± 16^{b}	148 ± 12^{a}	-51.7 ± 0.6^{a}	17.6 ± 0.9^{a}	-64.3 ± 7.0^{a}	0.20 ± 0.02^{b}	0.71 ± 0.03^{b}	0.38 ± 0.01^{a}
WPI-L	471 ± 11^{a}	1256 ± 242^{a}	153 ± 9^{a}	-53.3 ± 1.6^{a}	11.1 ± 0.5^{b}	64.0 ± 0.4^{a}	0.31 ± 0.09^{a}	1.0 ± 0.00^{a}	0.32 ± 0.04^{a}
SCN	224 ± 13^{b}	747 ± 20^{b}	166 ± 8^{a}	-55.1 ± 0.4^{a}	9.0 ± 0.5^{b}	-60.5 ± 3.3^{a}	0.19 ± 0.00^{b}	0.70 ± 0.07^{b}	0.23 ± 0.00^{c}
TW80	229 ± 6^{b}	233 ± 8^{c}	157 ± 9^{a}	-14.3 ± 0.7^{b}	0.51 ± 0.0^{c}	-62.1 ± 1.0^{a}	0.16 ± 0.04^{b}	0.19 ± 0.01^{c}	0.29 ± 0.04^{b}

*WPI-L and WPI-S indicate emulsions stabilized by whey protein isolate with large and small droplets, respectively; **S**CN and TW80 indicate emulsions stabilized with sodium caseinate and tween 80, respectively. [a]Different superscript letters **i**ndicate significant differences between values in a column ($p<0.05$)

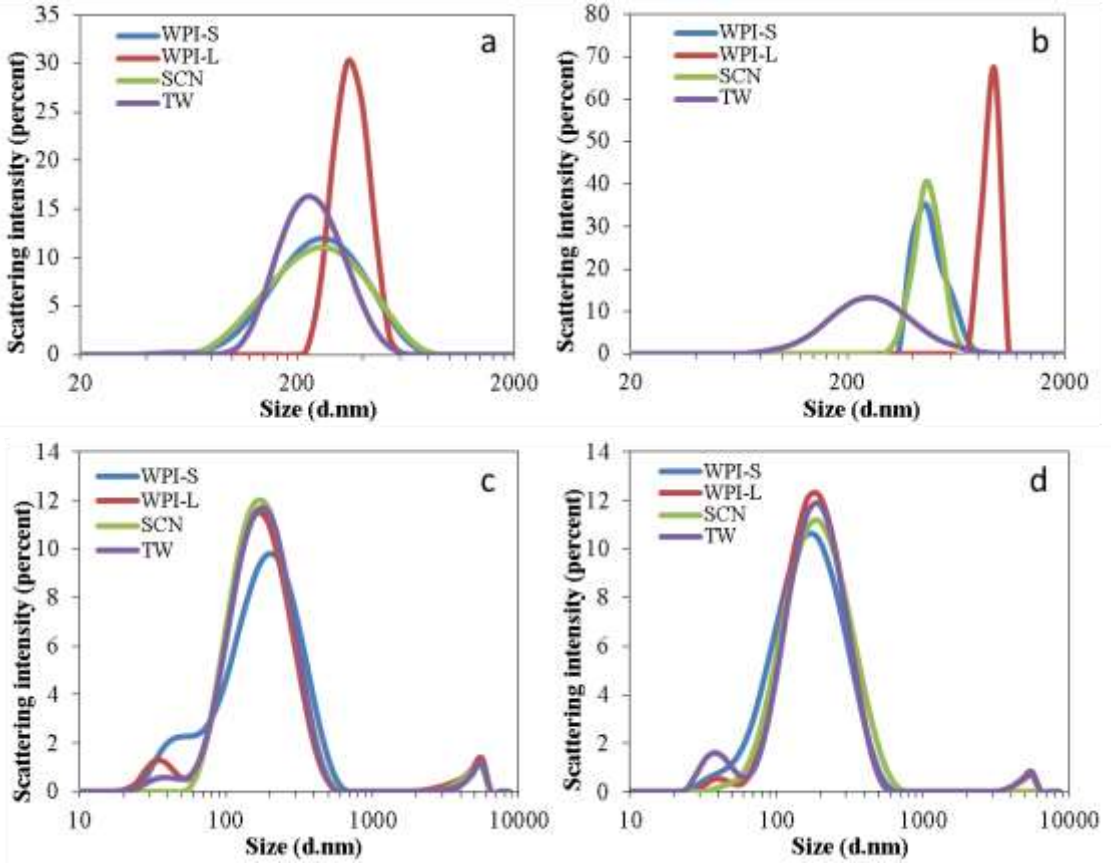


Figure 4-3. Size distribution of emulsions after passing through simulated GIT digestion. (**a**) Mouth phase; (**b**) Gastric phase; (**c**) Intestinal phase; (**d**) Micelle fractions. WPI-S and WPI-L indicate whey protein isolate-stabilized emulsions with small and large droplet sizes, respectively; SCN indicates sodium caseinate-stabilized emulsion; TW indicates tween80-stabilized emulsion.

After the gastric phase, a dramatic increase in average droplet size (**Table 4-2**) and size distribution (**Figure 4-3b**) of all emulsions was observed, except for the TW80-stabilized emulsion. The WPI-stabilized emulsion showed a larger average droplet size (774 nm) at this point than that of the SCN-stabilized emulsion (747 nm). The WPI-stabilized emulsion with large initial droplets showed a larger droplet size (1256 nm) than that with the small initial droplets (774 nm). The results suggested that the initial emulsion structure, e.g., emulsifiers and droplet sizes, can greatly influence the properties after being exposed to simulated gastric digestion. The dramatic increase in droplet size during this process is potentially attributed to several factors, including the low pH, incubation at 37 °C, ionic strength and the

hydrolysis of interfacial proteins by pepsin. However, incubation at 37 °C for 2 h did not increase the droplet size of WPI- and SCN-stabilized emulsions (data not shown), and the previous study also confirmed that dairy protein-stabilized emulsions were stable at pH<4.0 (Lu et al., 2016). Mao et al. (2013) found that WPI-stabilized multilayer emulsion droplets aggregated significantly in a NaCI solution of strength ≥150 mM because the relatively high ion strength can potentially reduce the electrostatic repulsion between droplets (McClements, 2015) and lead to their aggregation. Furthermore, pepsin in SGF can hydrolyse WPI and SCN at the oil-water interface and result in partially break-down of the interfacial layer and, thus, the aggregation of oil droplets. These findings suggest that the increased droplet size of emulsions during the gastric phase digestion may be mainly induced by the ionic strength (177 mM) in SGF and the hydrolysis of proteins at the interface by pepsin.

Compared with the gastric phase, the droplet size of all emulsions dramatically decreased after the intestinal phase (**Table 4-2**). The WPI-stabilized emulsion showed the smallest average droplet size of 148 nm after intestinal phase digestion, followed by TW- (157 nm) and SCN-stabilized (166 nm) emulsions, respectively. No significant difference between the WPI-stabilized emulsion with small and large initial droplet sizes was observed. The decrease in droplet size was mainly attributed to the rapid break-down of droplets due to the hydrolysis of proteins (WPI and SCN) and Tween 80 by trypsin and lipase, respectively, and the subsequent formation of small micelles stabilized by bile salts (**Figure 4-3c,d**).

All emulsions were negatively charged after mouth phase digestion, which is mainly attributed to the protein emulsifiers (WPI and SCN) being negatively charged at pH 6.8, which is above their isoelectric point (pI). The SCN-stabilized emulsion had the highest surface charge, of -55.1 mV, followed by the WPI- and TW-

stabilized emulsions, at -51.7 mV -24.3 mV, respectively. No significant difference in surface charge between WPI-stabilized emulsions with small (-51.7 mV) and large (-53.3 mV) droplet sizes was observed (Table 2).

WPI-, and SCN-stabilized emulsions were positively charged after the gastric phase (Table 2), as expected because pH 2.5 is below their pI. The WPI-stabilized emulsion had a higher surface charge than the SCN emulsion (9.0 mV) after the gastric phase, and the WPI-stabilized emulsion with small initial droplets showed a higher surface charge (17.6 mV) than the emulsion with a large initial droplet size (11.1 mV). The TW-stabilized emulsion was almost neutrally charged after the gastric phase.

After the intestinal phase, all emulsions were negatively charged, and there was no significant difference in charge between different emulsions. This is mainly attributed to the enzymatic hydrolysis of proteins (WPI and SCN) and Tween 80 at the droplet surface by trypsin and lipase and the subsequent absorption of other anionic molecules, e.g., bile salts, to the droplet/micelle surface, resulting in a uniformly negatively-charged surface (Salvia-Trujillo et al., 2013a).

3.3 Bioaccessibility of β-carotene

The WPI-stabilized emulsion (WPI-S) showed the highest (p<0.05) bioaccessibility of 58.5%, followed by the SCN- and TW-stabilized emulsions of 56.5% and of 41.3%, respectively (**Figure 4-4a**). No significant difference between WPI-stabilized emulsions with small (WPI-S) and large initial droplet sizes (WPI-L) was observed. This may be mainly attributed to the initial droplet size in this study (d<0.4 μm) being not as large as in previous studies (Cho et al., 2014; Salvia-Trujillo et al., 2013a), in which significant differences in the bioaccessibility of emulsion-

encapsulated nutrients in large and small droplets were observed. However, when the initial droplet size was below 1 μm, this difference becomes less significant.

Generally, initial droplet size and interfacial composition can significantly influence the bioaccessibility of nutrients incorporated into emulsions (Qian et al., 2012; Salvia-Trujillo et al., 2013a). The bioaccessibility increases with reduced initial droplet size due to fast lipid digestion by lipase. The interfacial compositions can also significantly influence the lipid digestion, and thus the release of encapsulated components. For example, Mun et al. (2007) found that the lipid digestion of emulsions with different emulsifiers decreased in the order of proteins (caseinate and whey protein isolate)>lecithin>tween 20. In addition, the digestion and release of lipophilic components within emulsions can also be controlled by coating the oil droplets with a double-layer interface, e.g., protein-polysaccharide double layer interface. Compared with protein-stabilized single emulsions, polysaccharide-coated multilayer emulsions showed much slower velocity of lipid digestion and thus reduced release of encapsulated lipophilic components (Li et al., 2010).

Tween 80 can be hydrolysed by lipase (Plou et al., 1998) in intestinal phase digestion and can act as a competitive substrate with lipid inside the oil droplets, which accordingly may decrease the hydrolysis rate of oil and thus potentially decrease the release of encapsulated β-carotene. This may explain why the TW-stabilized emulsion showed a lower bioaccessibility than those stabilized with WPI and SCN.

3.4 Cellular Uptake of β-carotene

In order to further evaluate the bioavailability of encapsulated β-carotene in emulsions, to understand why there is an inconsistency between the results of *in*

vitro bioaccessibility and the *in vivo* bioavailability of encapsulated components in emulsions in previous study (Salvia-Trujillo et al., 2017), a Caco-2 cell culture assay was employed to investigate the cellular uptake of β-carotene after GIT.

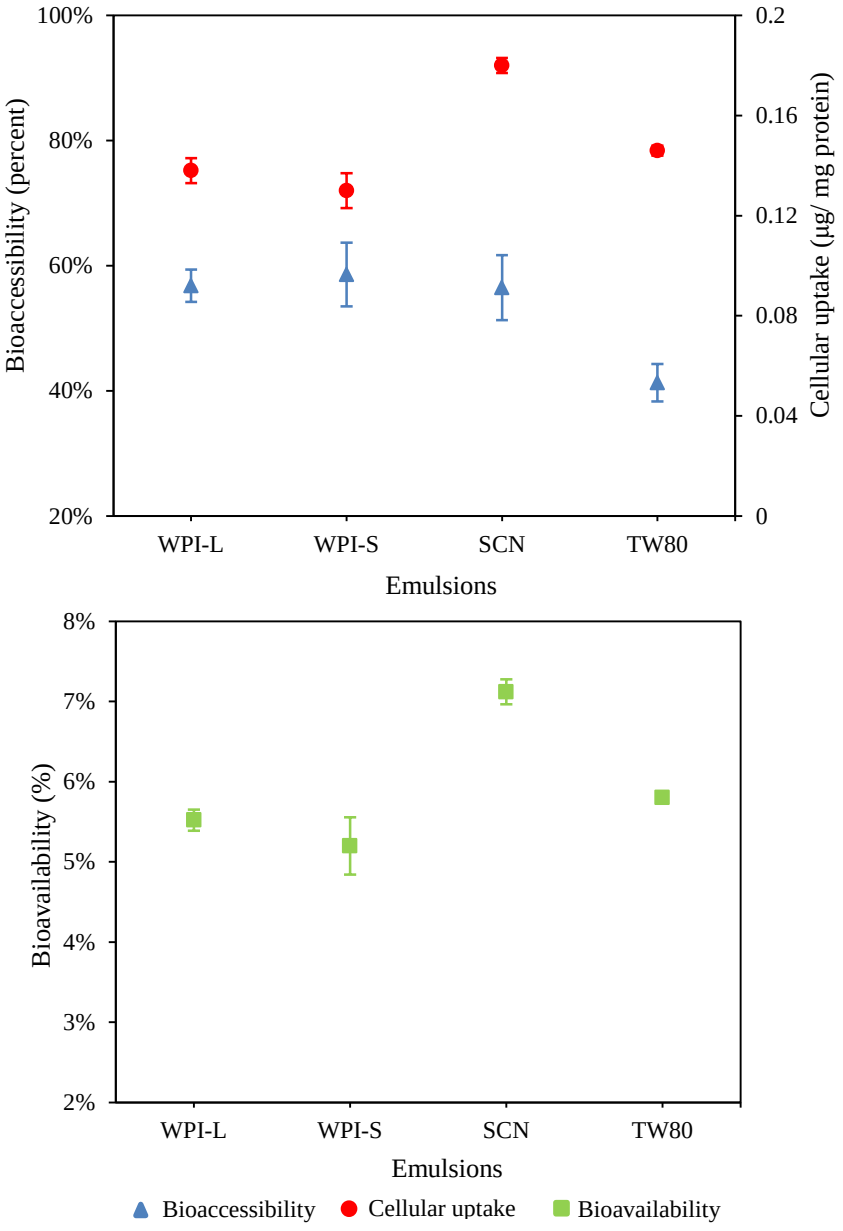


Figure 4-4. (**a**) Bioaccessibility and cellular uptake of encapsulated β-carotene; (**b**) Bioavailability of encapsulated β-carotene based on the results of bioaccessibility and cellular uptake. WPI-S and WPI-L indicate whey protein isolate-stabilized emulsions with small and large droplet sizes, respectively; SCN indicates sodium caseinate-stabilized emulsion; TW80 indicates tween 80-stabilized emulsion.

The SCN-stabilized emulsion showed a significantly higher (p<0.05) cellular uptake of β-carotene (0.180 μg/mg protein) than TW80- (0.146 μg/mg protein), and WPI-stabilized (0.130 μg/mg protein) emulsions (WPI-S) (**Figure 4-4a**), which is obviously different with the results of bioaccessibility described above. This may explain why an inconsistency between the results of *in vitro* bioaccessibility and in vivo bioavailability was observed. Generally, increased cellular uptakes of nanoparticles are mainly attributed to their reduced particle size and different surface structures. However, the micelle fraction of SCN-stabilized emulsion showed even a larger average droplet size than that of WPI- and TW-stabilized emulsions and the surface charge of all of the micelles was not significantly different (p>0.1) (**Table 4-3**), indicating that increased cellular uptake of encapsulated BC in the SCN-stabilized emulsion could not be attributed to the droplet size and surface charge.

Table 4-3. Particle size and zeta potential (ZP) of micelle fractions from different emulsions and the in vitro bioavailability and cellular uptake of encapsulated β-carotene after passing through GIT (mean ± STD, n = 2)

Micelles	Size (d.nm)	ZP(mV)
WPI-L	158 ± 3^{a}	-65.0 ± 0.5^{a}
WPI-S	142 ± 6^{b}	-64.2 ± 0.7^{a}
SCN	160 ± 10^{a}	-61.1 ± 3.3^{a}
TW80	156 ± 7^{a}	-63.0 ± 1.0^{a}

*WPI-L and WPI-S indicate micelles from emulsions stabilized by whey protein isolate with large and small droplet sizes after GIT, respectively; SCN and TW80 indicate micelles from sodium caseinate-, and tween80-stabilized emulsions after GIT, respectively. [a]Different superscript letters indicate significant difference between values in a column (p<0.05)

As is known, casein shows better surface activity than whey proteins (α-lactalbumin and β-lactoglobulin) (Livney, 2010), which is mainly attributed to their different amino acid sequences. After hydrolysis by pepsin and trypsin, SCN may produce more peptides that have amphiphilic structures than WPI, and these peptides can bind to the surface of newly-formed β-carotene-loaded micelles, facilitating the

interaction of micelles with Caco-2 cells and, thus, increasing the cellular uptake of β-carotene. This may explain why SCN-stabilized emulsion showed a higher cellular uptake of BC than WPI-stabilized emulsion in this study.

No significant difference between WPI- and TW80-stabilized emulsions was observed. Although WPI-stabilized emulsions with different initial droplet sizes showed significantly different micelle sizes after intestinal phase digestion, also no significant difference in cellular uptake of β-carotene was observed between them.

Based on the results of *in vitro* bioaccessibility and cellular uptake, the bioavailability of β-carotene in this study can be calculated according to the following equation (Joye et al., 2014):

$$\text{Bioavailability (\%)} = F_C \times F_B \times F_A \times F_M$$

where F_C is the fraction of β-carotene before passing through GIT; F_B is the bioaccessibility, which is the fraction of β-carotene in micelles after intestinal phase digestion in this study; F_A is the absorption, which is the cellular uptake fraction of β-carotene in this study; F_M is the metabolism, which is the fraction of β-carotene in a bioactive form after the metabolism within GIT, epithelium cells, blood circulation system or liver. This study did not refer to the test of metabolism within blood circulation and liver. Hence, F_M was not used in the calculation of the bioavailability.

As is shown in **Figure 4-4b**, the results of the bioavailability of different emulsions showed the same variation tendency with the results of cellular uptake (**Figure 4-4a**). No significant difference in bioavailability of β-carotene between WPI-stabilized emulsions with large and small droplet sizes was observed. SCN-stabilized emulsion showed the highest of 7.2%, followed by TW80- and WPI-stabilized emulsions of 5.8% and 5.2%, respectively, which showed the same order as the results of cellular uptake of β-carotene (**Figure 4-4b**), indicating that increased

bioavailability of these emulsion-encapsulated nutrients may be mainly attributed to their increased cellular uptake of nutrient-loaded micelles after passing through GIT. The cellular uptake assay is accordingly considered as a necessary assay for a better evaluation of the in vitro bioavailability of encapsulated nutrients.

4. Conclusions

The choice of emulsifier, between whey protein isolate (WPI), sodium caseinate (SCN) and Tween 80 (TW80) significantly influenced the creaming stability, surface charge and viscosity of β-carotene-loaded emulsions. The SCN-stabilized emulsion showed the highest creaming stability and viscosity in all emulsions. Passing emulsions through simulated GIT led to great changes in their droplet size, surface charge and compositions, and these changes were dependent on initial droplet sizes and interfacial compositions. However, bioaccessibility and cellular uptake of encapsulated β-carotene after GIT were mainly dependent on the interfacial compositions (emulsifiers). The SCN-stabilized emulsion showed the highest cellular uptake of β-carotene, followed by TW80- and WPI-stabilized emulsions, respectively, which showed the same order as the results of the bioavailability of β-carotene, potentially indicating that the increased bioavailability of emulsion-encapsulated β-carotene is mainly attributed to their increased cellular uptake. In addition, an inconsistency between the results of the in vitro bioaccessibility and bioavailability of β-carotene was observed, which may be the main cause of the reported inconsistency between the results of the in vitro bioaccessibility and in vivo bioavailability of emulsion-encapsulated nutrients, suggesting that the cellular uptake assay is necessary for a reliable evaluation of the in vitro bioavailability and may be useful for predicting the in vivo bioavailability of emulsion-encapsulated compounds.

References

Cho, H. T., Salvia-Trujillo, L., Kim, J., Park, Y., Xiao, H., & McClements, D. J. (2014). Droplet size and composition of nutraceutical nanoemulsions influences bioavailability of long chain fatty acids and Coenzyme Q10. *Food Chemistry, 156*, 117-122.

Choudhury, H., Gorain, B., Karmakar, S., Biswas, E., Dey, G., Barik, R., Mandal, M., & Pal, T. K. (2014). Improvement of cellular uptake, in vitro antitumor activity and sustained release profile with increased bioavailability from a nanoemulsion platform. *International Journal of Pharmaceutics, 460*, 131-143.

Gerster, H. (1993). Anticarcinogenic effect of common carotenoids. *International Journal for Vitamin and Nutrition Research, 63*, 93-121.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: formation, properties and applications. *Soft Matter, 12*, 2826-2841.

Hou, Z., Liu, Y., Lei, F., & Gao, Y. (2014). Investigation into the in vitro release properties of β-carotene in emulsions stabilized by different emulsifiers. *LWT - Food Science and Technology, 59*, 867-873.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion. *Food Chemistry, 114*, 253-262.

Joye, I. J., Davidov-Pardo, G., & McClements, D. J. (2014). Nanotechnology for increased micronutrient bioavailability. *Trends in Food Science and Technology, 40*, 168-182.

Li, Y., Hu, M., Xiao, H., Du, Y., Decker, E. A., & McClements, D. J. (2010). Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings. *European Journal of Pharmaceutics and Biopharmaceutics, 76*, 38-47.

Livney, Y. D. (2010). Milk proteins as vehicles for bioactives. *Current Opinion in Colloid and Interface Science, 15*, 73-83.

Lu, W., Kelly, A. L., Maguire, P., Zhang, H., Stanton, C., & Miao, S. (2016). Correlation of Emulsion Structure with Cellular Uptake Behavior of Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure. *Journal of Agricultural and Food Chemistry, 64*, 8659-8666.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Lu, W., Kelly, A. L., & Miao, S. (2017). Improved Bioavailability of Encapsulated Bioactive Nutrients Delivered through Monoglyceride-Structured O/W Emulsions. *Journal of Agricultural and Food Chemistry, 65*, 3048-3055.

Mao, L., O'Kennedy, B. T., Roos, Y. H., Hannon, J. A., & Miao, S. (2012). Effect of monoglyceride self-assembled structure on emulsion properties and subsequent flavor release. *Food Research International, 48*, 233-240.

Mao, L., Roos, Y. H., O'Callaghan, D. J., & Miao, S. (2013). Volatile release from whey protein isolate-pectin multilayer stabilized emulsions: effect of pH, salt, and artificial salivas. *Journal of Agricultural and Food Chemistry, 61*, 6231-6239.

Mao, Y., Dubot, M., Xiao, H., & McClements, D. J. (2013). Interfacial engineering using mixed protein systems: emulsion-based delivery systems for encapsulation and stabilization of beta-carotene. *Journal of Agricultural and Food Chemistry, 61*, 5163-5169.

McClements, D. J. (2015). *Food emulsions: principles, practices, and techniques* (3rd ed.). New York: CRC press.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science, 72*, R109-124.

Mun, S., Decker, E. A., & McClements, D. J. (2007). Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. *Food Research International, 40*, 770-781.

Plou, F. J., Ferrer, M., Nuero, O. M., Calvo, M. V., Alcalde, M., Reyes, F., & Ballesteros, A. (1998). Analysis of Tween 80 as an esterase/lipase substrate for lipolytic activity assay. *Biotechnology Techniques, 12*, 183-186.

Pool, H., Mendoza, S., Xiao, H., & McClements, D. J. (2013). Encapsulation and release of hydrophobic bioactive components in nanoemulsion-based delivery systems: impact of physical form on quercetin bioaccessibility. *Food & Function, 4*, 162-174.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. *Food Chemistry, 135*, 1440-1447.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry, 132*, 1221-1229.

Salvia-Trujillo, L., Fumiaki, B., Park, Y., & McClements, D. J. (2017). The influence of lipid droplet size on the oral bioavailability of vitamin D2 encapsulated in emulsions: an in vitro and in vivo study. *Food & Function, 8*, 767-777.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013). Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry, 141*, 1472-1480.

Shafiq, S., Shakeel, F., Talegaonkar, S., Ahmad, F. J., Khar, R. K., & Ali, M. (2007). Development and bioavailability assessment of ramipril nanoemulsion formulation. *European Journal of Pharmaceutics and Biopharmaceutics, 66*, 227-243.

Sharma, S., Sahni, J. K., Ali, J., & Baboota, S. (2015). Effect of high-pressure homogenization on formulation of TPGS loaded nanoemulsion of rutin - pharmacodynamic and antioxidant studies. *Drug Delivery, 22*(4), 541-551.

von Lintig, J. (2010). Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. *Annual Review of Nutrition, 30*, 35-56.

CHAPTER FIVE

Improved Emulsion Stability and Modified Nutrient Release by Structuring O/W Emulsions Using Konjac Glucomannan

Published in *Food Hydrocolloids*, 2018, 81, 120-128.

Authors: Wei Lu, Baodong Zheng, and Song Miao*

The work contained in this chapter was undertaken and written solely by myself with specific contributions from each co-author.

Abstract

Functional konjac glucomannan (KGM) was used to modify the water phase of O/W emulsions containing a lipophilic bioactive compound (β-carotene). KGM greatly increased the viscosity of the water phase and thus the viscosity of final emulsions. Results of Fourier-transform infrared spectroscopy (FT-IR) showed that there is no significant non-covalent interaction between KGM and whey proteins in the water phase. KGM significantly improved the creaming and pH stability of whey-protein-stabilized emulsions (p<0.05), and significantly decreased the oiling-off of emulsions during freeze-thaw test. Emulsions with or without KGM all had good thermal stability at 80 °C. Microscopy observations indicated obvious aggregation of free proteins and oil droplets in gastric phase and an enzymatic-induced break-down of droplets, mainly in the intestinal phase of the simulated gastrointestinal tract (GIT) digestion. Emulsions containing KGM showed a lower final release rate of encapsulated β-carotene than emulsion without KGM (p<0.05), and the release rate decreased with the increasing KGM content. The findings of this study contribute to a better understanding of the influence of the water phase on the release of encapsulated compounds from emulsions, and make it possible to achieve controlled release of encapsulated compounds, and/or to deliver multiple functional ingredients in one carrier by structuring the water phase of emulsion with functional polymers.

Keywords: Structuring emulsions; Konjac glucomannan; Emulsion stability; Digestion; Nutrient release

1. Introduction

There has been a growing interest in the utilization of emulsions as the carriers for the encapsulation, protection and delivery of lipophilic functional nutrients. Many emulsion-based carriers have been successfully developed to protectively deliver a variety of bioactive nutrients, such as carotenoids (Lu et al., 2016; Lu et al., 2017b), fatty acids (Karthik & Anandharamakrishnan, 2016), polyphenols (Lu et al., 2016), and peptides (Niu et al., 2016).

Since emulsions have been widely used as novel carriers for functional nutrients, a better understanding of the factors that can influence the digestion behavior of emulsion droplets and thus the release of encapsulated bioactive nutrients becomes very important. The digestion of emulsion droplets is closely related to their structure, including the oil phase, interfacial layer, and water phase. Taking protein stabilized oil-in-water (O/W) emulsion as an example, the digestion process of protein-stabilized emulsion droplets containing bioactive nutrients mainly goes through four steps: (i) binding of proteinase to the droplet surface in the water phase, e.g., pepsin and trypsin; (ii) hydrolysis of interfacial protein layers by proteinase; (iii) hydrolysis of lipid in the oil phase by lipase and release of bioactive nutrients from lipid droplets; and (iv) formation of bile-salt emulsified micelles containing released lipophilic bioactive nutrients. Accordingly, the formula of water phase and interface can influence the final bioaccessibility by modifying the first two steps of the digestion, while the microstructure and composition of oil phase can influence the final bioaccessibility through affecting the later two steps of digestion. The effects of composition or microstructure of the oil phase and interface on the release of encapsulated bioactive nutrients from emulsions have been well investigated in previous studies (Lu et al., 2017b; Qian et al., 2012; Salvia-Trujillo et al., 2017).

However, little is known about the influence of the water phase on the digestion of emulsion droplets and thus the release of encapsulated nutrients from emulsions.

Natural polysaccharides are a class of biopolymers that have been widely used in the production of emulsions, due to their wide availability, good physical and chemical stability, edibility, and low cost. Previous studies have shown that introducing polysaccharide as a second stabilizer into the water phase can significantly enhance the stability of emulsions (Dickinson, 2011). Polysaccharides can generally modify the interface, rheology, or gelation properties of emulsions. Polysaccharides can also significantly enhance the stability of protein-stabilized emulsions by forming a polysaccharide-protein double-layer interface (Aoki et al., 2005; Guzey et al., 2004; Surh, 2005; Mao et al., 2015). In addition, polysaccharides can form network structures in the water phase, which can limit the mobility of oil droplets by steric hindrance and thus improve the creaming stability of emulsions (Lin et al., 2017). Such network structures in the water phase can potentially also influence the binding and interaction process of enzymes, the digestion velocity of emulsion droplets, and thus the release of encapsulated bioactive nutrients, as described above.

Konjac glucomannan (KGM) is a natural polysaccharide obtained from tubers of *Amorphophallus konjac* cultivated in Asia. KGM is reported to possess many health benefits, such as lowering the blood cholesterol and sugar levels, positively modulating gut microflora, promoting weight loss, and improving immune function (Arvill, 1995; Chua et al., 2010). However, there are few studies on using KGM to modify the water phase and thus influencing the release of encapsulated bioactive nutrients from emulsions. Introducing KGM into emulsions can also endow the final emulsions with new health benefits associated with KGM besides those contributed

by encapsulated molecules. This can accordingly achieve a delivery of multiple nutrients in one carrier.

From the above, this study was therefore proposed to investigate the effect of structuring water phase of model O/W emulsions by KGM on their properties, including droplet size, surface charge, creaming stability, pH stability, thermal stability, and freeze-thaw stability, and also the effect on the release of encapsulated bioactive nutrient (β-carotene) from model O/W emulsions containing KGM after passing through a simulated gastrointestinal tract (GIT) digestion.

2. Material and Methods

2.1 Materials

Konjac glucomannan powder was obtained from Konjac Food (Cupertino, CA, USA). Whey protein isolate (70% β-lactoglobulin and 18% α-lactalbumin) was purchased from Davisco Food International (Le Sueur, MN, USA). All-trans-β-carotene (>93%, UV), pepsin, pancreatin (porcine, 4×USP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunflower oil (Solesta, >98% fat) was purchased from a local supermarket (ALDI, Fermoy, Co.Cork, Ireland). All other chemicals and reagents used were of AR-grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Preparation of Emulsions

Whey protein isolate (WPI) was dispersed (2%, w/w) in Millipore water containing sodium azide as antimicrobial agent (0.01% w/w). The dispersion was stirred for 4 h and kept at 4 °C overnight for complete dissolution of WPI. The dispersion was then brought to 25 °C before adding konjac glucomannan (KGM) powder to make a KGM content of 0.05%, 0.1%, or 0.2% in the final emulsions. The

mixtures were stirred for 4 h for a complete dissolution of KGM and then centrifuged at 4,000 rpm (2,700 g) for 20 min before being used as the water phase. The oil phase was prepared by dissolving β-carotene (0.2%, w/w in oil phase) in sunflower oil (10%, w/w in final emulsion) at 140 °C which was then mixed with the water phase at 10,000 rpm for 2 min at room temperature using an Ultra-Turrax (IKA, Staufen, Germany) followed by further homogenization (APV 1000, SPX Flow Technology, Charlotte, North Carolina, USA) at 50 MPa for 3 passes at room temperature to obtain final emulsions.

2.3 Droplet Size and Surface Charge

The droplet size and zeta potential of KGM emulsions were measured by a laser particle analyzer (Nano-ZS, Malvern Instruments, Worcestershire, UK) as described in our previous study (Lu et al., 2016). Emulsions were diluted to the final oil content (w/w) of 0.01% before testing. The refractive index (RI) of samples was set at 1.47 for sunflower oil.

2.4 Creaming Stability

The creaming stability of emulsions was evaluated using Lumisizer (LUM GmbH, Berlin, Germany) as describe previously. Emulsions were centrifuged at 2,300 g at 25 °C with a scanning rate of once every 10 s for 1,200 s. Following the test, curves of the integrated transmitted light against time were plotted, and the slope of each curve was taken as the Creaming Index (CI).

2.5 Rheological Analysis

Rheological measurements were performed using an AR 2000ex rheometer (TA Instruments, Crawley, UK). A concentric cylinder geometry was selected, and 19 g of each sample was placed into the inner cylinder and equilibrated for 2 min before

measurement. Viscosity testing was performed over a shear rate range of 0-300 s^{-1} at 25 °C.

2.6 Fourier Transforms Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) technology was used to evaluate the molecular structure of KGM and WPI in water phase before and after homogenization. The IR spectra of the samples were recorded by FT-IR spectrophotometer (Bruker Corp, Billerica, Massachusetts, USA) using an attenuated total reflection (ATR) technique. The spectrum was scanned from 4000 cm^{-1} to 900 cm^{-1} with a resolution of 4 cm^{-1}. An average of 300 scans was recorded for each sample.

2.7 Stability of Emulsions at Different pH Values and Temperatures

The effect of KGM on the resistance of emulsions to extreme pH environments was evaluated. The KGM emulsions were brought to different pH values from 2.0 to 7.0 with HCI or NaOH solution, and maintained for 4 h at room temperature before droplet size, surface charge and creaming stability analysis by DLS and Lumisizer as described above.

The effect of KGM on the resistance of emulsions to thermal processing was evaluated following incubation at 25°C, 37 °C, or 80 °C for 2 h. The droplet size was then analyzed by Malvern Nanosizer as described above.

2.8 Freeze-Thaw Test

Freeze-thaw testing was applied to emulsions with the objective of assessing the possibility of frozen storage of liquid emulsions containing bioactive nutrients. In addition, the changes in properties of emulsions after the freeze-thaw processing can potentially contribute to the freeze-drying of liquid emulsions into solid products,

which can significantly facilitate the storage, transportation, and application of emulsions.

Liquid emulsions were kept at -20°C for 24 h and then thawed at 25°C for 2 h in a water bath. This cycle was repeated three times. Droplet size and surface charge was measured after each cycle by Malvern nanosizer. Creaming stability of emulsions after three cycles was measured with Lumisizer as described above.

Oiling-off of emulsions after 3 cycles of freeze-thaw was also evaluated by measuring the content of β-carotene in the free oil fraction. The thawed emulsions were centrifuged at 10,000 g for 10 min (25 °C). Free oil layer containing β-carotene on top of emulsions were collected and subjected to a second centrifuge at 4000 g for 10 min (25 °C). The supernatant (free oil) containing β-carotene was collected and extracted with ethanol/hexane. The content of β-carotene in hexane fraction was quantified by RP-HPLC as described below. The oiling-off was calculated based on the equation below:

$$\text{Oiling-off (\%)} = \frac{C_{\text{free oil}}}{C_{\text{initial}}} \times 100\%$$

where $C_{\text{free oil}}$ and C_{initial} are the concentration of β-carotene in the free oil fraction after 3 cycles of freeze-thaw and in the initial emulsion before freeze-thaw test, respectively.

2.9 Simulated Gastrointestinal Tract (GIT) Digestion

An *in vitro* simulated GIT model consisting of mouth, gastric and intestinal phases was used to digest β-carotene loaded emulsions. The simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared as described previously (Lu et al., 2017b) with minor modification.

For the mouth phase digestion, emulsions were mixed with SSF (1:1, v/v), the pH was adjusted to 6.8 and the mixtures were incubated at 37 °C for 10 min with continuous agitation at 150 rpm.For the gastric phase, the bolus sample from the mouth phase was mixed with the SGF (1:1, v/v). The pH of the mixture was adjusted to 2.5 and it was incubated at 37 °C for 2 h with continuous agitation at 150 rpm. The pepsin activity in the final mixture was 1,000 U/mL. For the small intestinal phase, the bolus sample from the gastric phase was mixed with the SIF (1:1, v/v). The pH of the mixture was adjusted to 7.0 and it was incubated at 37 °C for 2 h with continuous agitation at 150 rpm. The concentration of pancreatin (porcine, 4×USP) in the final mixture was based on the trypsin activity (25 U/mL).

2.10 Laser Scanning Confocal Microscope Observation

The morphology of the initial and digested emulsion droplets was observed using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Baden-Württemberg, Germany). All the images were taken using a 63 x oil-immersion objective and simultaneous dual-channel imaging, He-Ne laser (excitation wavelength at 633 nm) and an Argon laser (excitation wavelength at 488 nm). A mixture of two dyes, Fast green (0.1 %, w/w in water) and Nile red (0.1 %, w/w in propanediol) was used to color and detect protein and lipid, respectively. Initial/digested sample (500 µL) was gently mixed with 50 µL of mixed dye.

2.11 Release of Encapsulated β-carotene

The amount of β-carotene in micelle fractions after the intestinal phase of GIT was measured as the final release rate of encapsulated β-carotene. Briefly, an aliquot of raw digesta after the intestinal phase digestion was centrifuged at 4,500 rpm (2978 g) for 40 min at 4 °C and the middle layer was collected and considered as the micelle

fraction. One mL of the micelle fraction was extracted twice with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v). The upper n-hexane layer containing the solubilized β-carotene was collected and analyzed by RP-HPLC as described below.

The final release rate of encapsulated β-carotene was calculated using the follow equation:

$$\text{Release rate (\%)} = \frac{C_{\text{micelle}}}{C_{\text{initial}}} \times 100\%$$

where C_{micelle} and C_{initial} are the concentration of β-carotene in the micelle fraction and initial emulsion before digestion, respectively.

2.12 Quantification of β-carotene

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to quantify β-carotene. An Agilent 1200 series system with a DAD UV-Vis detector (Agilent, Santa Clara, CA, USA) and a reversed phase TSKgel ODS-100v C_{18} column (4.6×250 mm, 5 μm, TOSOH) was employed.

Chromatography conditions: column operation temperature at 30 °C; elution was performed with 90% ethanol and 10% acetonitrile from 0-30 min, flow rate was 1 mL/min, detection wavelength was 450 nm, and injection volume was 20 μL.

2.13 Statistical Analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was employed to compare means of data. A t-Test was used to determine the differences between means. Significant differences were determined at the 0.05 level ($p<0.05$).

3. Results and Discussion

3.1 Droplet Size and Surface Charge

As is shown in **Table 5-1**, an emulsion without konjac glucomannan (KGM) had an average droplet size of 252 nm, while emulsions containing KGM showed significantly increased droplet size with increasing KGM content from 0.05% to 0.2% (p<0.05). KGM, as a natural polysaccharide, is dispersible in water and can form a network structure with different particle size depending on its molecular weight and concentration (Lazaridou et al., 2003). This network structure can potentially increase the average droplet size of emulsions. However, the droplet size test was performed after 1000-fold dilution of emulsions and KGM at this extreme low concentration (<0.0002%) is unlikely to form a network structure.

There is another possibility to explain the increased droplet size of KGM-emulsions. The peak of the size distribution shifted to a slightly higher value (data not shown), indicating that there may be some flocculation or coalescence of the droplets, which were forced close enough at high KGM concentrations induced by depletion attraction between droplets (Ye et al., 2004). Slight aggregation of droplets was also observed in our previous research (Lu et al., 2016).

Table 5-1. Droplet size, zeta potential, polydispersity index (PdI) and creaming index (CI) of emulsions containing KGM

KGM (%, w/w)	size (d.nm)	zeta potential (mV)	PdI	CI (%/min)
0	252±9^{d}	-65.0±5.4^{a}	0.24±0.02^{a}	0.64±0.01^{b}
0.05	280±8^{c}	-60.4±2.2^{a}	0.21±0.02^{b}	0.52±0.02^{c}
0.1	306±17^{b}	-59.3±2.6^{a}	0.21±0.01^{b}	0.55±0.02^{c}
0.2	350±24^{a}	-59.3±0.7^{a}	0.20±0.02^{b}	1.70±0.04^{a}

*KGM indicates konjac glucomannan. ^{a}Different letters indicate significant difference between values in a column (p<0.05)

All emulsions were negatively charged and no significant difference in surface charge between different emulsions was observed (p>0.05). Generally, KGM is a non-charged polysaccharide and binding of KGM to the surface of WPI-stabilized emulsion droplets should lead to their significantly reduced surface charge. However, surface charge of emulsions containing KGM showed almost no difference from that of the emulsion without KGM (**Table 5-1**), potentially demonstrating that there is very limited binding of KGM to the droplet surface.

3.2 Rheological Analysis

The viscosity of emulsions significantly decreased with the increasing shear rate (~50 1/s), indicating a shear-thinning property. The KGM emulsion showed higher viscosity than emulsion without KGM, and the viscosity of KGM emulsions increased with increasing KGM content (**Figure 5-1**). Several factors can potentially influence the viscosity of emulsions, such as oil content, viscosity of water phase, droplet size, or surface charge (McClements, 2015). In this study, increased viscosity of KGM emulsions can be mainly attributed to two factors: (i) increased viscosity of water phase (WPI-KGM dispersion); and (ii) flocculation of droplets by depletion force induced by non-absorbed polysaccharide KGM. As shown in **Figure 5-2**, the viscosity of the water phase (WPI-KGM dispersions) also decreased with increasing shear rate, and a similar increase in viscosity with increasing KGM content was seen, suggesting that KGM can significantly increase the viscosity of the water phase and thus the viscosity of final emulsions. In addition, KGM, as a biopolymer can potentially generate a depletion force between droplets and induce flocculation of droplets at certain concentrations (Mao, 1995), which accordingly can increase the viscosity of emulsions. This point will be further discussed in the creaming stability section below.

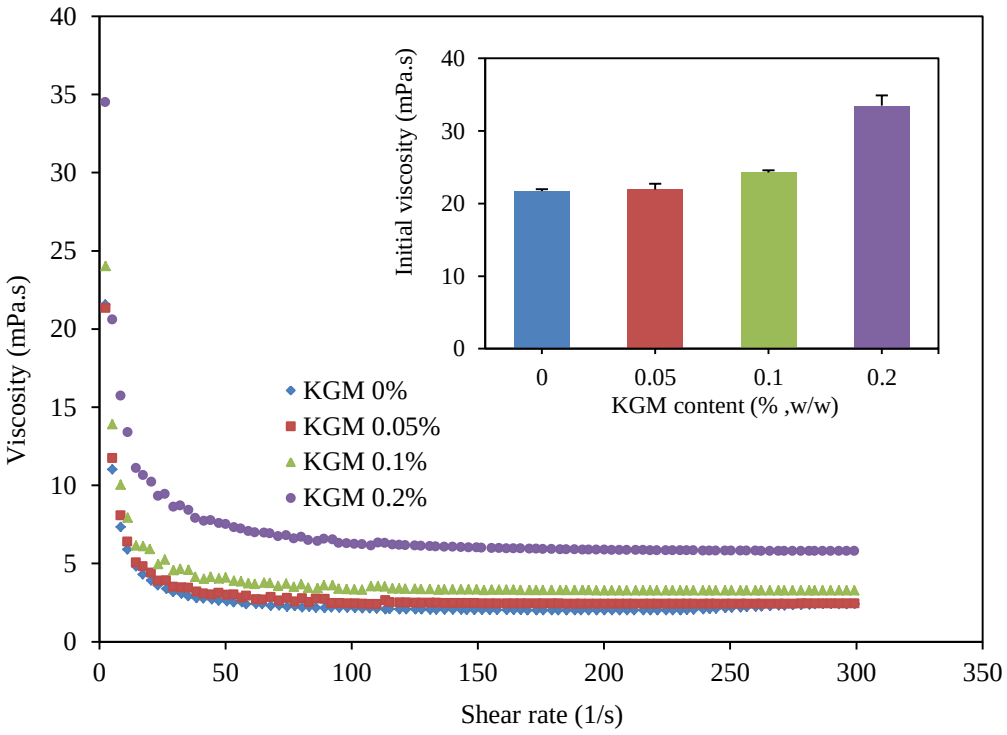


Figure 5-1. Viscosity of emulsions. KGM 0% indicates emulsion without konjac glucomannan (KGM), while KGM 0.05%, KGM 0.1%, and KGM 0.2% indicates emulsions with KGM content (w/w) of 0.05%, 0.1%, and 0.2%, respectively. Insert: initial viscosity at very low shear rate (~2.5 1/s)

Generally, KGM can disperse in water and form highly viscous suspensions at pH values of 4.0-7.0 due to its high molecular weight, ranging from 200-2,000 kDa (Chua et al., 2010; Villay et al., 2012). The viscosity of WPI-KGM suspensions after homogenization decreased significantly (**Figure 5-2**). This is mainly attributed to the mechanical de-polymerization and/or de-polymerization-coupled conformation of KGM by homogenization (Villay et al., 2012). The molar-mass distribution is the primary parameter that influences the viscosity of polysaccharide in solution. Homogenization can lead to mechanical degradation (de-polymerization) of polysaccharides, and produce fractions with low molecular weight or low polydispersity, which accordingly lead to decreased viscosity.

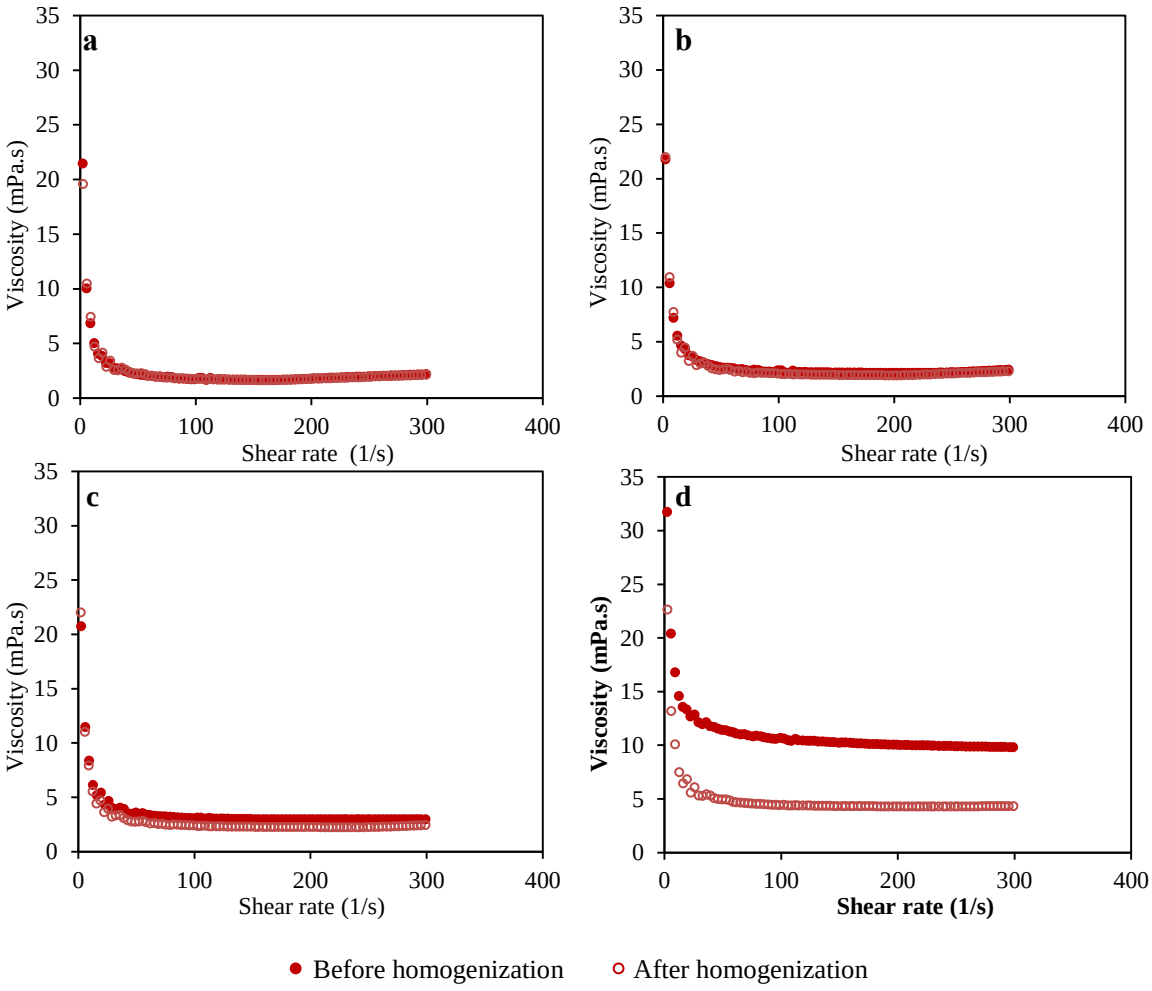


Figure 5-2. Viscosity of whey protein isolate (WPI) and konjac glucomannan (KGM) mixed solutions before and after homogenization at 50 MPa. (**a**) WPI solution without KGM; (**b**) WPI solution with 0.05% (w/w) KGM; (**c**) WPI solution with 0.1% KGM; (**d**) WPI solution with 0.2% KGM.

3.3 Fourier-Transform Infrared spectroscopy (FT-IR) Analysis

Generally, polysaccharides can form double layer structures at the interface of protein-stabilized emulsion through electrostatic attraction, hydrogen bond or hydrophobic interactions with protein. However, KGM is a non-charged polysaccharide and cannot form complexes with protein by electrostatic attraction. Our preliminary research also indicated that there is no significant hydrophobic or hydrogen bond interaction between WPI and KGM at different pH values (2-8) and temperatures (up to 90℃) (data not shown).

However, little was known about the interaction between WPI and KGM when passing through homogenization. Hence, FT-IR was employed to investigate the influence of homogenization process on their structures and potential interactions. The spectra of WPI-KGM dispersions before and after homogenization were collected within wavelengths of 900-4000 cm^{-1} and spectra between 1350-1700 cm^{-1} were analysed, since this zone covers the main characteristic absorption peaks of WPI (Chen et al., 2012). The spectra of WPI (**Figure 5-3**) indicated the presence of characteristic absorption peaks at 1645, 1548, 1456, and 1400 cm^{-1} corresponding to the C=O stretching and the bending of N-H, C-H, C-N bonds, respectively (Barth, 2007; Chen et al., 2012). Compared with WPI before homogenization, the spectra of WPI after homogenization did not show significant difference.

KGM exhibited a characteristic absorption peak of the β-1,4-linked glycosidic bond at 895 cm^{-1} and a characteristic peak of the enlargement of pyranoid rings at 808 cm^{-1} (Gao et al., 2014). Hence, KGM showed almost no significant absorption within wavelength of 900-4,000cm^{-1}. Spectra of WPI-KGM dispersions also showed no significant difference compared with pure WPI dispersion before and after homogenization (**Figure 5-3**). All these results suggested that: (i) homogenization did not induce significant changes in the molecular structure of WPI; and (ii) homogenization processing did not induce obvious non-covalent interactions, e.g., electrostatic attraction, hydrophobic interaction or hydrogen bond, between WPI and KGM. However, the droplet size (data not shown) of WPI-KGM dispersions significantly decreased after homogenization, which was mainly attributed to the mechanical de-polymerization of KGM by homogenization, as described above (Villay et al., 2012).

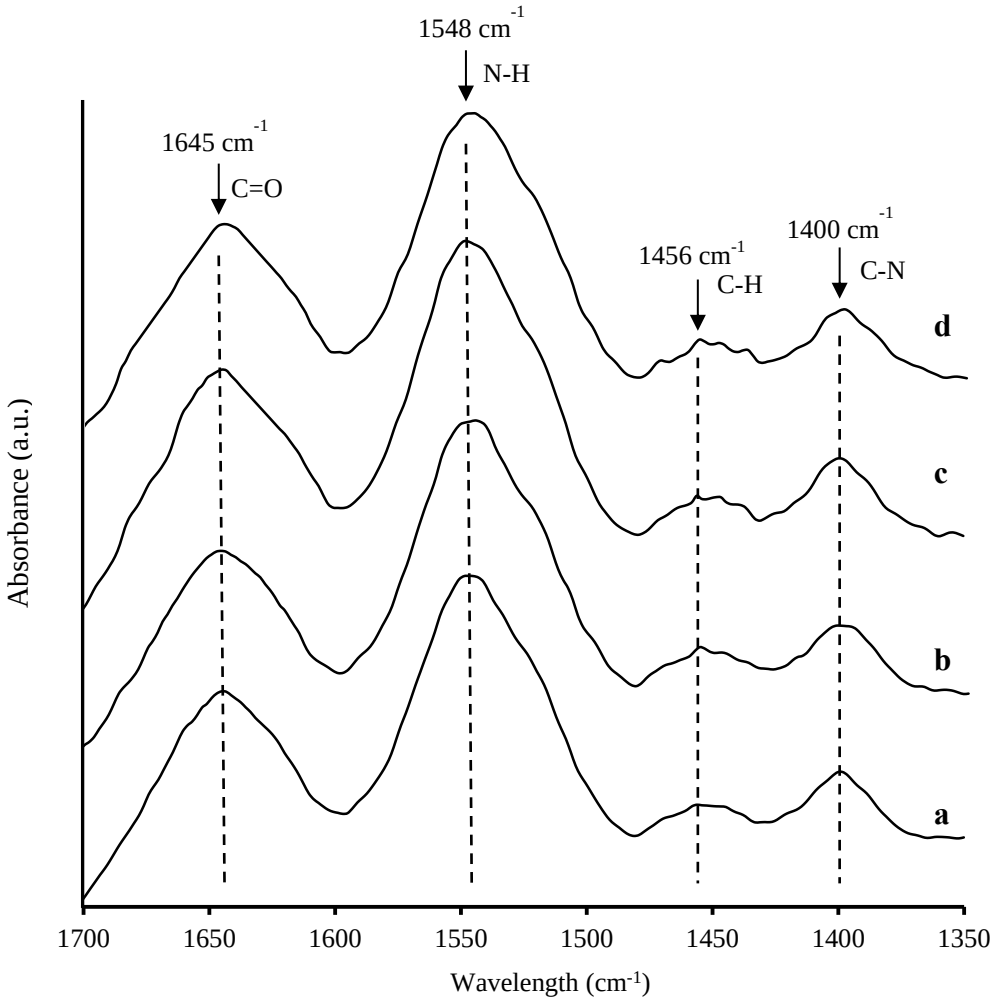


Figure 5-3. Fourier-transform infrared (FT-IR) spectra of (a) WPI (2%, w/w), (b) WPI (2%, w/w) after homogenization , (c) a mixture of WPI (2%, w/w) and KGM (0.2%, w/w) and (d) a mixture of WPI (2%, w/w) and KGM (0.2%, w/w) after homogenization. WPI indicates whey protein isolate. KGM indicates konjac glucomannan.

3.4 Creaming Stability

Creaming index (CI) was used to describe the rate of light transmission change. It is calculated based on the curve of the integrated transmitted light against time. A higher CI value indicates a lower creaming stability of emulsion. Hence, emulsion containing 0.05% KGM showed the highest creaming stability, followed by emulsions containing 0.1% KGM and the emulsion without KGM (**Table 5-1**). KGM can improve the creaming stability of emulsions by increasing the viscosity of

the water phase as described above (**Figure 5-1**). In addition, KGM at these concentrations (0.05%, or 0.1%) can potentially form a network structure in water phase. Even though, these network structures can be destroyed by mechanical homogenization as described above, the chain-structure of KGM can still limit the creaming of emulsions due to the increased steric hindrance and force of friction between droplets and the continuous phase.

However, emulsion containing 0.2% KGM showed very poor creaming stability, which was mainly attributed to the depletion flocculation of emulsion droplets by non-absorbed KGM (Klinkesorn et al., 2004; Mao, 1995). Non-adsorbed polymers can generate an attractive osmotic force between droplets. This osmotic force increases with increasing concentration of polymer until it is large enough to overcome the repulsive forces between droplets and cause flocculation of droplets. Droplet flocculation can also increase the viscosity of emulsions by decreasing the internal packing of droplets within flocs due to increased effective volume fraction of the particles based on Dougherty-Krieger equation (McClements, 2015). This may explain why emulsion with high content of KGM showed increased viscosity, as described above (**Figure 5-1**).

Table 5-2. Creaming index (CI) of emulsions at pH 5.0 or after freeze-thaw, oil-off of emulsions after freeze-thaw, and droplet size of emulsions at different temperatures

KGM content (%, w/w)	CI at pH 5.0 (%/min)	CI after freeze-thaw (%/min)	Oiling-off after freeze-thaw (%)	Size (d.nm)		
				25 °C	37 °C	80 °C
0	9.11±0.05[a]	0.76±0.00[b]	10.2±0.03[a]	252±8[d]	254±5[d]	257±2[d]
0.05	7.77±0.03[b]	0.70±0.00[c]	6.7±0.02[b]	271±10[c]	269±9[c]	266±6[c]
0.1	5.98±0.03[c]	0.62±0.00[d]	4.8±0.00[c]	313±6[b]	312±6[b]	314±6[b]
0.2	3.88±0.04[d]	1.36±0.01[a]	2.4±0.02[d]	334±6[a]	328±6[a]	331±6[a]

*KGM indicates konjac glucomannan. [a]Different letters indicate significant difference between values in a column (p<0.05)

3.5 Stability of Emulsions at Different pH

Emulsions were all stable at pH 2-4 and pH 6-7. The emulsion without KGM showed a significantly increase in droplet size at pH 5 (**Figure 5-4a**). This is mainly attributed to the aggregation of whey proteins at this pH value, which is close to its isoelectric point (IEP). This increase in droplet size accordingly resulted in poor creaming stability of these emulsions (**Table 5-2**). Emulsions containing KGM showed significantly less increase in droplet size at pH 5 (p<0.05) than the emulsion without KGM, depending on the content of KGM. Accordingly, these KGM-containing emulsions with reduced droplet size at pH 5 showed better creaming stability than the emulsion without KGM (**Table 5-2**). These results suggested that KGM can significantly improve the pH stability of WPI-stabilized emulsions.

KGM is a very stable polysaccharide across a wide range of pH values. KGM, with a chain-like structure, can fill in the space between free protein molecules and oil droplets in emulsions and potentially can isolate oil droplets from each other, which accordingly reduces the Brownian-motion-induced contact of proteins and oil droplets and thus their aggregation at pH value close to the IEP of proteins. The higher of the KGM content, the higher density of the filled space, and thus the more significant inhibition of aggregation.

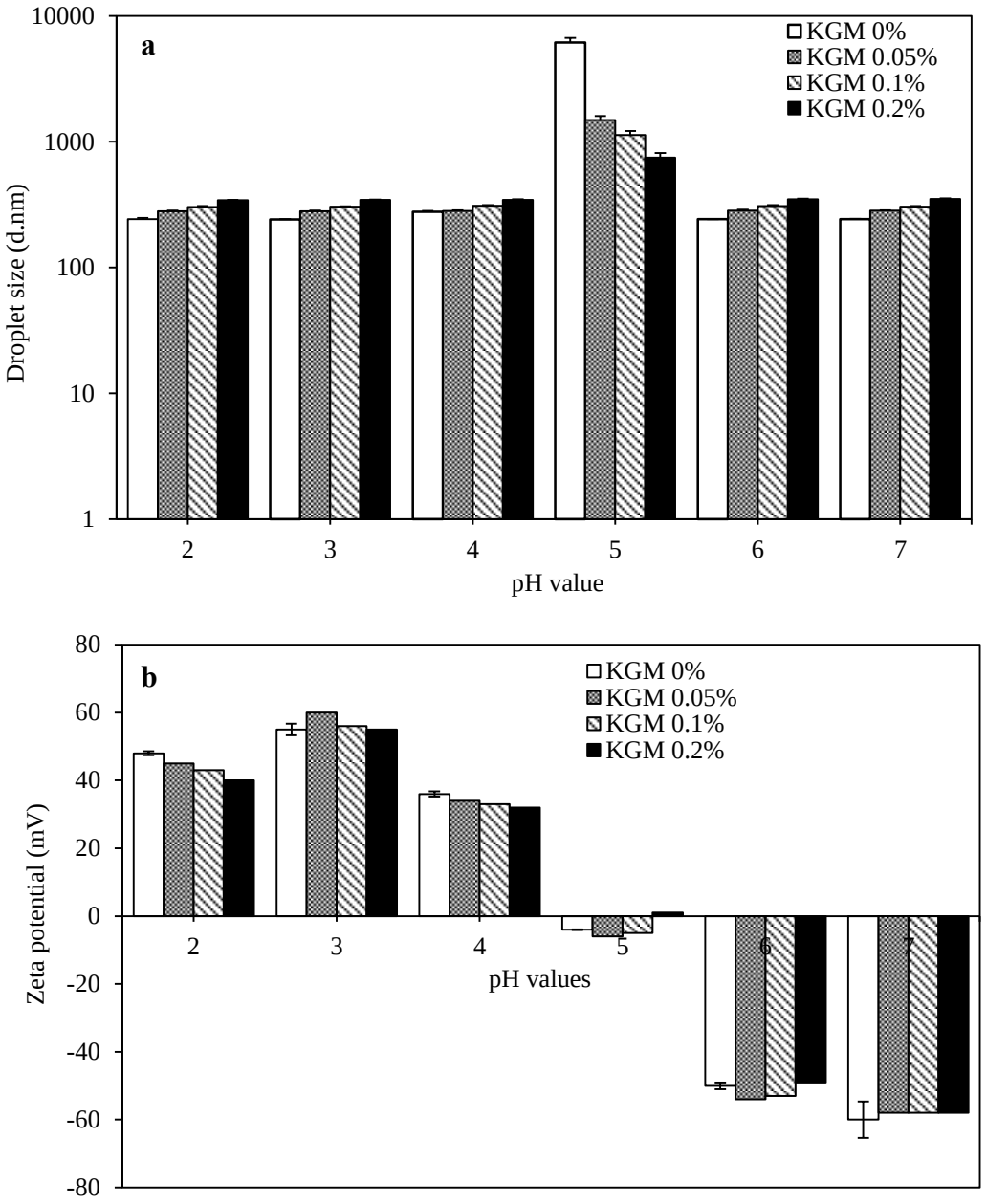


Figure 5-4. Droplet size (**a**) and zeta potential (**b**) of emulsions at different pH values. KGM 0% indicate emulsion without konjac glucomannan (KGM). KGM 0.05%, KGM 0.1%, and KGM 0.2% indicates emulsions with KGM content of 0.05%, 0.1%, and 0.2%, respectively.

3.6 Thermal Stability

The thermal stability of emulsions at different temperatures was also investigated. After 2 h incubation at elevated temperature (37℃ or 80℃), the droplet size of all emulsions showed almost no difference compared to the droplet size of emulsions at

25℃ (**Table 5-2**), suggesting that emulsions in this study had good thermal stability at temperatures up to 80℃.

3.7 Freeze-Thaw Stability

After the first cycle of freeze-thaw, the droplet size and surface charge of all emulsions significantly decreased (p<0.05) (**Table 5-3**). Then, the droplet size increased with the increasing freeze-thaw cycles and the final droplet size of all emulsions after three cycles were smaller than their initial droplet size. No significant difference in surface charge after second and third cycles of freeze-thaw was observed, except for the emulsion without KGM. Oiling-off of emulsions was also clearly observed after 3 cycles of freeze-thaw (**Table 5-2**) and KGM successfully reduced the oiling-off of the emulsion. Compared with the emulsion without KGM (10.2%), only 2.4% of the oil was released from the emulsion containing 0.2% KGM after 3 cycles of freeze-thaw (p<0.01).

Ice crystals are formed during freezing of emulsions, and the ice penetration can potentially lead to the break-down of the protein layer surrounding the oil droplets, and thus lead to oiling-off of emulsions. Combined with the results above, it is assumed that (i) freezing led to the break-down of large emulsion droplets, leading to the oiling-off of emulsions, a narrower size distribution and a smaller average droplet size, and (ii) freezing can also induce desorption of whey proteins from the droplet surface, leading to the reduced surface charge of emulsions after first cycle of freeze-thaw. However, the above findings suggested that KGM can help to maintain the stability of the interfacial protein layer and significantly protect the emulsion from oiling-off, probably by slowing down the formation of ice crystals (Mao et al., 2015).

Table 5-3. Droplet size and surface charge of emulsions after freeze-thaw processing

Freeze-thaw cycle	Size (d.nm)				Zeta potential (mV)			
	KGM 0%	KGM 0.05%	KGM 0.1%	KGM 0.2%	KGM 0%	KGM 0.05%	KGM 0.1%	KGM 0.2%
0	252±9[a]	280±8[a]	306±17[a]	350±24[a]	-65.0±5.4[a]	-60.4±2.2[a]	-59.3±2.6[a]	-59.3±0.7[a]
1	202±4[b]	226±2[b]	266±18[b]	302±18[b]	-57.3±0.1[b]	-52.6±1.4[b]	-53.1±2.1[b]	-52.8±0.7[b]
2	216±5[c]	249±8[c]	288±16[c]	308±13[b]	-52.5±1.8[c]	-54.5±1.8[b]	-53.9±1.8[b]	-52.4±1.3[b]
3	227±6[d]	273±9[d]	307±9[d]	314±10[b]	-52.5±1.7[c]	-54.2±1.4[b]	-51.4±0.1[b]	-54.5±0[b]

* Freeze-thaw test was done at -20°C, followed by thawing at 25°C. KGM 0% indicate emulsion without konjac glucomannan (KGM). KGM 0.05%, KGM 0.1%, and KGM 0.2% indicates emulsions with KGM content (w/w) of 0.05%, 0.1%, and 0.2%, respectively. [a]Different letters indicate significant difference between values in a column (p<0.05)

Table 5-4. Droplet size and zeta potential of emulsions containing KGM following different phases of gastrointestinal tract digestion

KGM content (w/w, %)	Size (d.nm)				zeta potential (mV)			
	initial	mouth phase	gastric phase	intestinal phase	initial	mouth phase	gastric phase	intestinal phase
0	252±9[d]	248±1[d]	1505±361[a]	192±56[a]	-65±5.4[a]	-64.5±4.9[a]	18.0±1.4[a]	-70.5±6.4[a]
0.05	280±8[c]	288±1[c]	1624±452[a]	185±31[a]	-60.4±2.2[a]	-58.5±0.7[a]	15.5±0.7[a,b]	-71.0±2.8[a]
0.1	306±17[b]	305±4[b]	1598±423[a]	178±19[a]	-59.3±2.6[a]	-59.5±2.1[a]	14.0±0[b]	-76.5±4.9[a]
0.2	350±24[a]	352±11[a]	1057±204[a]	200±38[a]	-59.3±0.7[a]	-59.0±1.4[a]	9.0±0[c]	-77.5±3.5[a]

*KGM indicates konjac glucomannan. [a]Different letters indicate significant difference between values in a column (p<0.05)

3.8 GIT Digestion

As described in our previous studies (Lu et al., 2017a, 2017b), passing emulsions through simulated gastrointestinal tract (GIT) digestion can greatly modify their properties. The average droplet size of all emulsions significantly increased after gastric phase and then decreased after intestinal phase (**Table 5-4**), which was also confirmed by the microscopy observation. As is shown in **Figure 5-5**, after the mouth phase, no major difference in droplet shape and size was observed. After the gastric phase, significant aggregation of proteins (green fluorescence) was observed, which was mainly attributed to the exposure of the hydrophobic domain of whey proteins induced by pepsin hydrolysis and relatively high ionic strength in gastric phase. The process of protein aggregation also clustered some oil droplets (red fluorescence) and formed larger complexes. In addition, hydrolysis of WPI at the interface by pepsin resulted in aggregation of some oil droplets. These two factors may explain the dramatic increase in droplet size after the gastric phase. After the intestinal phase, almost all proteins at the interface were hydrolysed by trypsin, leading to the collapse of oil droplets and the hydrolysis of oil by lipase. The hydrolysis of whey protein and sunflower oil also led to the quenching of most of the green and red fluorescence, respectively. Only very few intact oil droplets were captured.

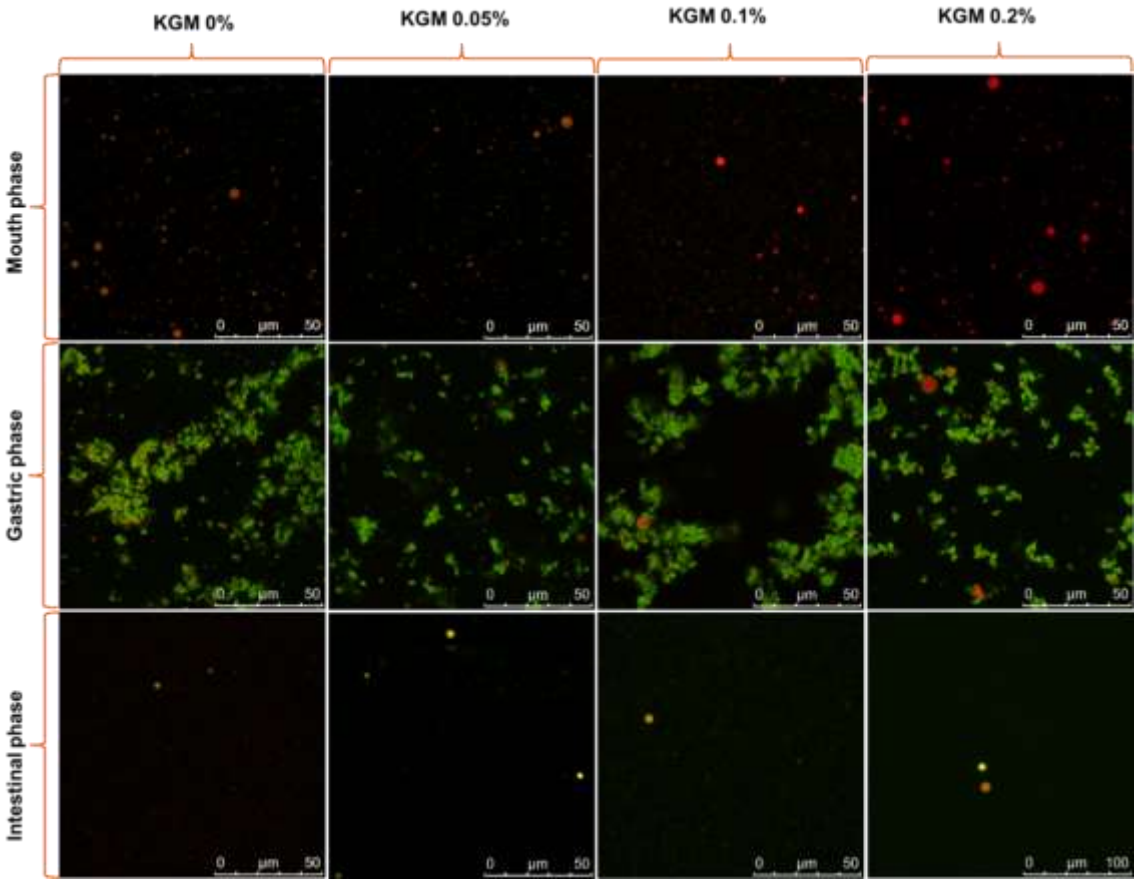


Figure 5-5. Confocal scanning microscope observation of emulsion droplets after being exposed to simulated gastrointestinal tract (GIT). KGM 0% indicate emulsion without konjac glucomannan (KGM). KGM 0.05%, KGM 0.1%, and KGM 0.2% indicates emulsions with KGM content (w/w) of 0.05%, 0.1%, and 0.2%, respectively.

3.9 Release of Encapsulated β-carotene after GIT

Adding KGM to the water phase of emulsions significantly modified the release of β-carotene from emulsion droplets after passing through GIT. Emulsion without KGM showed a final β-carotene-release-rate of 64.5%. β-carotene release rate of emulsions containing KGM were generally inferior to that of emulsion without KGM, and the release rate decreased with increasing KGM content (**Figure 5-6**). Emulsions containing 0.1% KGM and 0.2% KGM showed significant lower β-carotene release rate than emulsion without KGM (p<0.05). The results indicated

that KGM can potentially slow down the release of β-carotene from emulsion droplets, which is dependent on the content of KGM in final emulsions.

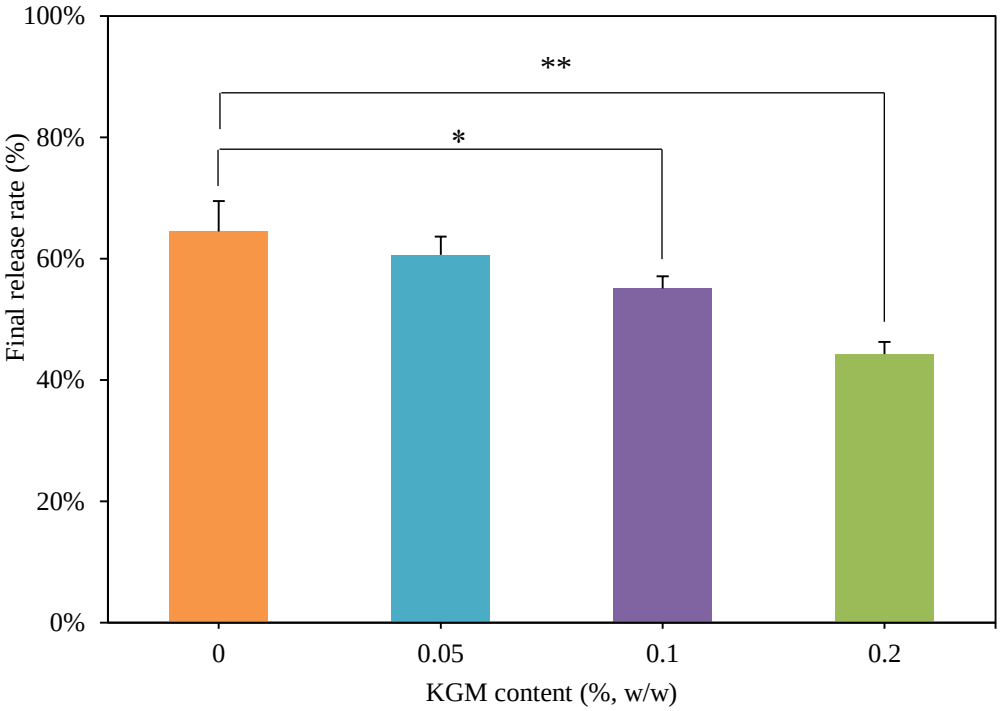


Figure 5-6. Release rate of encapsulated β-carotene after GIT (* p<0.05, ** p<0.01). KGM indicates konjac glucomannan.

As discussed in the introduction section, any factors that influence the four steps of the digestion process of emulsion droplets can potentially modify the final release of encapsulated ingredients from emulsions. Our previous studies also showed that emulsions with maltodextrin in the water phase showed significantly different release profiles of lipophilic food flavors due to modified mobility within the water phase (Mao et al., 2014). In this study, KGM was used to formulate the water phase of model O/W emulsion. Emulsions with KGM showed more a viscous water phase than emulsion without KGM. In addition, KGM shows a chain-like molecular structure in water, which facilitates intermolecular cross-linking into a gel-like structure. All these properties can potentially interfere with the digestion steps of the hydrolysis of interfacial protein layer and the hydrolysis of oil phase by steric

hindrance effect, leading to slower release of encapsulated ingredients from emulsion droplets. In addition, movement of produced free fatty acids (FFAs) and protons away from the oil droplet surface may be slowed down by the gel-like structure surrounding the oil droplets, which accordingly increased the local concentration of hydrolysates (free fatty acids) and thus decreased the rate of enzymatic hydrolysis rate of lipase (Fave, 2004; Gilchrist & Martin, 1983). All these factors may explain why emulsions with KGM in the water phase showed decreased release rates of encapsulated β-carotene with increasing KGM content in this study. The findings also suggest that it is feasible to achieve a controlled/sustainable release of encapsulated functional ingredients from emulsions by structuring the water phase of emulsions with natural biopolymers.

However, KGM can be hydrolysed and utilized by the microorganisms in the gut (Nakajima & Matsuura, 1997). Hence, the *in-vivo* digestion behaviour and release profile of encapsulated ingredients in emulsions with KGM in the water phase need to be further studied.

4. Conclusion

No significant non-covalent interaction between konjac glucomannan (KGM) and whey proteins before and after homogenization was observed by FT-IR. KGM increased the viscosity of WPI stabilized emulsions, and significantly improved the creaming and pH stability of emulsions and protected emulsions from oiling-off after freeze-thaw process. The digestion and break-down of emulsion droplets mainly happened in the intestinal phase of a simulated gastrointestinal tract (GIT), as evaluated by confocal laser scanning microscopy. KGM decreased the release rate of β-carotene after GIT, and the release rate decreased with increasing KGM content.

Model O/W emulsions with better stability and controlled release of β-carotene were obtained by simply structuring the water phase with a health-beneficial polysaccharide, KGM. Findings in this study make it possible to design emulsion-based functional food products or drug carriers with potential controlled or sustainable release of functional ingredients inside by structuring the water phase of emulsions with natural edible biopolymers.

References

Aoki, T., Decker, E. A., & McClements, D. J. (2005). Influence of environmental stresses on stability of O/W emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. *Food Hydrocolloids, 19*, 209-220.

Arvill, A. B., Lennart. (1995). Effect of short-term ingestion of konjac glucomannan on serum cholesterol in healthy men. *The American Journal of Clinical Nutrition, 61*, 585-589.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta (BBA), 1767*, 1073-1101.

Chen, B., Li, H., Ding, Y., & Suo, H. (2012). Formation and microstructural characterization of whey protein isolate/beet pectin coacervations by laccase catalyzed cross-linking. *LWT - Food Science and Technology, 47*, 31-38.

Chua, M., Baldwin, T. C., Hocking, T. J., & Chan, K. (2010). Traditional uses and potential health benefits of Amorphophallus konjac K. Koch ex N.E.Br. *Journal of Ethnopharmacology, 128*, 268-278.

Dickinson, E. (2011). Mixed biopolymers at interfaces: Competitive adsorption and multilayer structures. *Food Hydrocolloids, 25*, 1966-1983.

G Fave, T. C., and M Armand. (2004). Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability. *Cellular and Molecular Biology, 50*, 815-831.

Gilchrist, T., & Martin, A. (1983). Wound treatment with Sorbsan-an alginate fibre dressing. *Biomaterials, 4*, 317-320.

Guzey, D., Kim, H. J., & McClements, D. J. (2004). Factors influencing the production of o/w emulsions stabilized by β-lactoglobulin-pectin membranes. *Food Hydrocolloids, 18*, 967-975.

Jeonghee Surh , Y. S. G., Eric A. Decker , and D. Julian McClements. (2005). Influence of Environmental Stresses on Stability of Emulsions Containing Cationic Droplets Stabilized by SDS Fish Gelatin Membranes. *Journal of Agricultural and Food Chemistry, 53*, 4236-4244.

Karthik, P., & Anandharamakrishnan, C. (2016). Enhancing omega-3 fatty acids nanoemulsion stability and in-vitro digestibility through emulsifiers. *Journal of Food Engineering, 187*, 92-105.

Klinkesorn, U., Sophanodora, P., Chinachoti, P., & McClements, D. J. (2004). Stability and rheology of corn oil-in-water emulsions containing maltodextrin. *Food Research International, 37*, 851-859.

Lazaridou, A., Biliaderis, C. G., & Izydorczyk, M. S. (2003). Molecular size effects on rheological properties of oat β-glucans in solution and gels. *Food Hydrocolloids, 17*, 693-712.

Lin, D., Lu, W., Kelly, A. L., Zhang, L., Zheng, B., & Miao, S. (2017). Interactions of vegetable proteins with other polymers: Structure-function relationships and applications in the food industry. *Trends in Food Science and Technology, 68*, 130-144.

Lu, W., Kelly, A. L., Maguire, P., Zhang, H., Stanton, C., & Miao, S. (2016). Correlation of Emulsion Structure with Cellular Uptake Behavior of Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure. *Journal of Agricultural and Food Chemistry, 64*, 8659-8666.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Lu, W., Kelly, A. L., & Miao, S. (2017a). Bioaccessibility and Cellular Uptake of beta-Carotene Encapsulated in Model O/W Emulsions: Influence of Initial Droplet Size and Emulsifiers. *Nanomaterials, 7*, 282-292.

Lu, W., Kelly, A. L., & Miao, S. (2017b). Improved Bioavailability of Encapsulated Bioactive Nutrients Delivered through Monoglyceride-Structured O/W Emulsions. *Journal of Agricultural and Food Chemistry, 65*, 3048-3055.

Mao, L., Roos, Y. H., & Miao, S. (2014). Study on the rheological properties and volatile release of cold-set emulsion-filled protein gels. *Journal of Agricultural and Food Chemistry, 62*, 11420-11428.

Mao, L., Roos, Y. H., & Miao, S. (2015). Effect of maltodextrins on the stability and release of volatile compounds of oil-in-water emulsions subjected to freeze–thaw treatment. *Food Hydrocolloids, 50*, 219-227.

McClements, D. J. (2015). *Food emulsions: principles, practices, and techniques* (3rd ed.). New York: CRC press.

Nakajima, N., & Matsuura, Y. (1997). Purification and characterization of konjac glucomannan degrading enzyme from anaerobic human intestinal bacterium, Clostridium butyricum-Clostridium beijerinckii group. *Biosci Biotechnol Biochem, 61*(10), 1739-1742.

Niu, Z., Conejos-Sanchez, I., Griffin, B. T., O'Driscoll, C. M., & Alonso, M. J. (2016). Lipid-based nanocarriers for oral peptide delivery. *Advanced Drug Delivery Reviews, 106*(Pt B), 337-354.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. *Food Chemistry, 135*, 1440-1447.

Salvia-Trujillo, L., Fumiaki, B., Park, Y., & McClements, D. J. (2017). The influence of lipid droplet size on the oral bioavailability of vitamin D2 encapsulated in emulsions: an in vitro and in vivo study. *Food & Function, 8*, 767-777.

Villay, A., Lakkis de Filippis, F., Picton, L., Le Cerf, D., Vial, C., & Michaud, P. (2012). Comparison of polysaccharide degradations by dynamic high-pressure homogenization. *Food Hydrocolloids, 27*, 278-286.

Y. Mao, M. E. C., H.N.W. Lekkerkerker. (1995). Depletion force in colloidal systems. *Physica A, 222*, 10-24.

Ye, A., Hemar, Y., & Singh, H. (2004). Enhancement of coalescence by xanthan addition to oil-in-water emulsions formed with extensively hydrolysed whey proteins. *Food Hydrocolloids, 18*, 737-746.

Zhao Gao, Rongxin Su, Renliang Huang, and, W. Q., & He, Z. (2014). Glucomannan-mediated facile synthesis of gold nanoparticles for catalytic reduction of 4-nitrophenol. *Nanoscale Research Letters, 9*, 8.

CHAPTER SIX

Preparation of Re-dispersible Dry Emulsions Embedded with Bioactive Nutrients: Influence of Emulsion Composition and Drying Methods

Under submission

Authors: Wei Lu, Alan L. Kelly, and Song Miao*

Abstract

Dry forms of emulsions were obtained by spray-, and freeze-drying of liquid model O/W emulsions containing maltodextrin (MD) and/or konjac glucomannan (KGM). Dry emulsions (powders) showed different morphologies and surface microstructures, depending on the drying method (spray-drying or freeze-drying), and the formulas of emulsions. Monoglyceride (MG) existed in dry emulsion powders mainly as β-polymorph crystals. Dried emulsions showed good re-dispersibility in water (>85%), and freeze-dried emulsions showed faster re-hydration than spray-dried ones, immediately re-dispersing into droplets after contacting water. Compared with liquid emulsions before drying, re-constituted spray-dried emulsions showed shifted size distribution to large particle size while re-constituted freeze-dried emulsions showed shifted size distribution to small particle size. Re-constituted emulsions containing KGM showed significantly decreased viscosity (p<0.05) but increased creaming stability (p<0.05) than their liquid emulsions before drying. The finding of this study confirmed the feasibility of preparing dry forms of model O/W emulsions, which has good re-dispersibility in water and contain lipophilic components in oil phase and low level of wall materials (MD and/or KGM) in water phase. The findings also confirmed the potential of these dry emulsions as novel delivery carriers for lipophilic components.

Key words: dry emulsion, reconstitution, maltodextrin, konjac glucomannan

1. Introduction

Emulsions have been widely used for different objectives in the food, nutrition, and pharmacy industries (McClements, 2015). One of their major applications is in use as the encapsulation and delivery carriers for a variety of health-beneficial nutrients. Emulsions have become a preferred option when selecting an ideal carrier for lipophilic or hydrophilic functional ingredients due to their ease of preparation, edibility, maintenance of the physical and chemical stability of encapsulated compounds, potential controlled release and target delivery, and low cost. Emulsion-based carriers for many lipophilic nutrients, such as carotenoids (McClements et al., 2007), polyphenols (Lu et al., 2016), vitamins (Parthasarathi, 2016), ω-3 fatty acids (Karthik & Anandharamakrishnan, 2016), and probiotics (Gbassi & Vandamme, 2012), have been developed. Emulsion-encapsulation can not only increase the stability and extend the shelf-life of these nutrients products, but also significantly improve their oral bioavailability, which accordingly overally expands the application of these unstable health-beneficial nutrients.

However, emulsions are dynamically unstable systems, which limit their shelf-life. In addition, transportation, storage and packaging of liquid emulsions can incur high cost. Hence, strategies must be applied to increase the long-term stability (shelf-life) of emulsions, decrease their transportation, and storage cost, and thus improve their potential applications. Drying emulsions into powders (Gharsallaoui et al., 2010) is an ideal way to achieve these.

Dry emulsions can be prepared by drying liquid oil-in-water (O/W) emulsions. Drying process potentially can promote the instability of emulsions by altering their interfacial properties (Gharsallaoui et al., 2010). It is therefore important to select emulsions that are stable to drying, and accordingly are possible to be converted into

powders. Compositions and microstructure of liquid emulsions can influence the drying process of liquid emulsions, the properties of obtained dry emulsions (Jafari, 2017), and thus potentially the properties of reconstituted dry emulsions. These properties, e.g., droplet size, surface charge and stability, are very important to the application of dry emulsions since the structure of emulsion droplets is closely related to their digestion and absorption behaviour in the gastrointestinal tract (GIT) (Lu et al., 2017a, 2017b; Lu et al., 2018; McClements & Li, 2010). Hence, how to maintain the uniformity of emulsion properties before and after drying becomes a critical issue in the drying of emulsions.

Many strategies had been developed to obtain good quality spray-, or freeze-dried emulsions. For example, researchers have used polysaccharide-stabilized multilayer emulsions combined with high content (from ~8% to ~30%, w/w in final emulsions) of maltodextrin as protection wall materials in drying liquid emulsions (Gharsallaoui et al., 2010; Jang et al., 2014). Furthermore, many studies used lab-scale mini spray-driers, which show a different drying process and lower efficiency in drying compared with large-scale pilot spray-driers, making them a poor model for the pilot tests and subsequent industrial production of dry emulsions. However, little has been done on the pilot-level spray-drying of model O/W emulsions containing lipophilic bioactive nutrients.

Our previous studies showed that emulsion-based carriers with monoglyceride (MG) in the oil phase (Lu et al., 2017b) can significantly improve the bioavailability of encapsulated bioactive nutrients, while emulsions with konjac glucomannan (KGM) in the water phase (Lu et al., 2018) demonstrated a sustained-release of entrapped nutrients. In addition, MG can form a dense crystallized shell on the oil droplet surface, and stabilize the droplets; KGM with a long-chain molecular

structure can form a cross-link structure in water, which potentially can act as the protective skeleton and wall material of emulsion droplets during the drying process. However, the effects of MG and KGM during the drying process of emulsions and their effects on the properties of reconstituted emulsions haven't been reported.

For these reasons, this study was therefore conducted to investigate the effects of MG and KGM combined with maltodextrin on the drying process and subsequent reconstitution of microencapsulated lipophilic bioactive nutrients in model oil-in-water (O/W) emulsion-based carriers. A pilot-scale spray-drying system was employed to dry liquid emulsions, in an attempt to fill the gap between studies on the lab-scale spray-drying and pilot spray-drying. Meanwhile, a lab-scale freeze drier was also used to investigate the freeze-drying process of these emulsions. β-carotene was used as an example of lipophilic bioactive nutrients.

2. Material and Methods

2.1 Materials

All-trans-β-carotene (>93%, UV) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Whey protein isolate (70% β-lactoglobulin and 18% α-lactalbumin) was purchased from Davisco Food International (Le Sueur, MN, USA). Sunflower oil (Solesta, >98% fat) was purchased from a local supermarket (ALDI, Fermoy, Co.Cork, Ireland). Monoglyceride (glycerol monostearate, Danisco, Denmark) was purchased from Cloverhill Food Ingredients Ltd (Cork, Ireland). Konjac glucomannan (KGM) powder was obtained from Konjac Food (Cupertino, CA, USA). MALTRIN® M180 maltodextrin (DE 16.5-19.5) was obtained from Grain Processing Corporation (Muscatine, IA, USA). All other chemicals and reagents used were of AR-grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Preparation of Emulsions for Freeze Drying

Whey protein isolate (WPI) was dispersed (2%, w/w in final emulsion) in distilled water containing sodium azide as antimicrobial agent (0.01% w/w). The dispersion was stirred for 4 h and kept at 4 °C overnight for complete dissolution of WPI. The oil phase was prepared by dissolving β-carotene (0.05%, w/w in oil phase) or β-carotene (0.05%, w/w in oil phase) and monoglyceride (2%, w/w in final emulsion) in sunflower oil (10%, w/w in final emulsion) at 140 °C, followed by cooling and mixing at room temperature with the water phase (WPI dispersions) at 10,000 rpm for 2 min using an Ultra-Turrax (IKA, Staufen, Germany) followed by further homogenization (APV 1000, SPX Flow Technology, Charlotte, North Carolina, USA) at 50 MPa for 3 passes, also at room temperature, to obtain primary emulsions.

The primary emulsions were mixed (1:1, w/w) with 12% or 4% maltodextrin (MD), 0.3% konjac glucomannan (KGM), or maltodextrin-KGM (0.3% KGM with 12% or 4% MD) solution. The mixtures were then stirred for 1 h at room temperature to obtain final emulsions. Thirty mL of each final emulsion was added to a plastic tube, which was then frozen at -80°C for 48 hours in a freezer. These frozen plastic tubes were then transferred into glass flasks. After that, these glass flasks were connected to a vacuum freeze-dryer (FreeZone 6 liters Benchtop Freeze Drying System, Labconco Corporation, Kansas city, MO, USA), by which these frozen emulsions were dried. The drying process was performed at -40°C for 72h. The obtained freeze-dried emulsion powders were re-constituted with distilled water (25°C) to the same volume (30 mL) as before freeze drying. The properties, e.g., droplet size, surface charge, creaming stability, and viscosity of emulsions before freeze drying and the reconstituted emulsions from freeze-dried powders were investigated.

2.3 Preparation of Emulsions for Spray Drying

The compositions and preparation of emulsions for spray drying was similar to that of emulsion for freeze-drying described above with some modifications. The oil phase containing β-carotene (0.05%, w/w in oil phase) with or without MG was mixed with water phase with emulsifier (WPI, 2%, w/w in final emulsions) at 10,000 rpm for 4 min at room temperature using an Ultra-Turrax (IKA, Staufen, Germany) followed by further two-stage homogenization (TwinPanda 400, GEA Mechanical Equipment Italia, Parma, Italy) for 3 passes at room temperature to obtain primary emulsions. The pressure was 35 MPa for the first stage and 7 MPa for the second stage.

Primary emulsions with different compositions were mixed (1:1, w/w) with maltodextrin (MD) (12% or 4%), or maltodextrin-KGM (0.3% KGM with 12% or 4% MD) solution. The mixtures were then stirred for 1 h at room temperature to obtain final emulsions. About 5 kg of each final emulsion were dried by a pilot ANHYDRO spray dryer with a centrifugal atomizer (Copenhagen, Denmark) at Teagasc Food Research Centre (Moorepark, Fermoy, Co. Cork, Ireland). The inlet temperature was 185 °C; the outlet temperature was 85 °C.

The obtained spray-dried emulsion powders were reconstituted with distilled water (pre-heated to ~50 °C) to the same total solids content as it was before spray-drying, and stirred for 2 hours before being kept for overnight at room temperature. The properties, e.g., droplet size, surface charge, creaming stability, and viscosity, of these re-constituted emulsions were characterized.

2.4 Droplet Size and Surface Charge

The droplet size and zeta potential of KGM emulsions were measured by a laser particle analyzer (Nano-ZS, Malvern Instruments, Worcestershire, UK) as described in our previous study (Lu et al., 2016). Emulsions were diluted to the final oil content (w/w) of 0.01% before testing. The refractive index (RI) of samples was set at 1.47 for sunflower oil.

2.5 Creaming Stability

The creaming stability of emulsions was evaluated using a Lumisizer (LUM GmbH, Berlin, Germany) as described previously (**Chapter 2, section 2.7**). Emulsions were centrifuged at 2,300 g at 25 °C with a scanning rate of once every 10 s for 1,200 s. Following the test, curves of the integrated transmitted light against time were plotted, and the slope of each curve was taken as the light transmission rate or Creaming Index (CI).

2.6 Rheological Analysis

Rheological measurements were performed using an ARG2 rheometer (TA Instruments, Crawley, UK). A concentric cylinder geometry was used, and 20 mL of each sample was placed into the inner cylinder and equilibrated for 2 min before measurement. Viscosity testing was performed over a shear rate range of 0-300 s^{-1} at 25 °C.

2.7 Differential Scanning Calorimetry

The melting and crystallization behaviors of monoglycerides (MG) in spray-dried or freeze-dried emulsion powders were analyzed using a DSC Q2000 differential scanning calorimeter (TA Instruments, Crawley, UK). Approximately 4-6 mg of each powder was weighted in a Tzero pan, which was sealed with a Tzero hermetic

lid. An empty pan was used as a reference. Samples underwent two thermal cycles. In the dehydration cycle, samples were heated from 25 °C to 100 °C at a heating rate of 5 °C/min, followed by a cooling step from 100 °C to 25 °C at a rate of 10 °C/min. Afterwards, samples were heated up to 100 °C at a heating rate of 5 °C/min. Based on this second cycle of thermal process, melting temperature (T_m) and heat of fusion (ΔH) were measured.

2.8 Laser Scanning Confocal Microscope Observation

Leica TCS SP5 confocal laser scanning microscope (CLSM; Leica Microsystems CMS GmbH, Wetzlar, Germany) was used for powder particles visualization. Spray-dried and freeze-dried powder particles were placed onto a glass slide and labeled using a mixture of Fast Green and Nile Red. The dye mixture containing Fast Green (0.1 g/L) and Nile Red were dissolved in polyethylene glycol (0.1 g/L) mixed in a ratio 1:40 of Fast Green to Nile Red, which allowed diffusion of the dye molecules into the particles whilst not influencing the particle morphology and preventing solubilization. Dual excitation at 488 nm/633 nm was used. The confocal images of each systems were taken using 63x oil immersion objective with numerical aperture 1.4 z-Stacks were obtained in order to generate a three-dimensional structure of the particle and to identify surface lipid staining. Red and Green pseudo-colored pictures (8-bit), 512x512 pixels in size, were acquired using a zoom factor of 1-3.

2.9 Scanning Electronic Microscope Observation

Each spray-dried and freeze-dried emulsion powders were attached to double-sided adhesive carbon tabs mounted on scanning electron microscope stubs, and then coated with chromium (K550X, Emitech, Ashford, UK). Scanning electron microscopy images were collected using a Zeiss Supra 40P field emission SEM

(Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV. Representative micrographs were taken at 200×, 500×, 1000×, 5000× and 10000× magnification.

2.10 Quantification of β-carotene

β-carotene was extracted from re-constituted emulsions with ethanol/n-hexane (sample: ethanol: n-hexane=1:2:10, v/v) as described in Chapter 2 (section 2.10). The n-hexane extracts were combined and dried under a stream of nitrogen gas, and dissolved in ethanol for HPLC analysis.

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to quantify β-carotene as described in Chapter 2 (section 2.12). Briefly, an Agilent 1200 series system with a DAD UV-Vis detector (Agilent, Santa Clara, CA, USA) and a reversed-phase TSKgel ODS-100v C_{18} column (4.6×250 mm, 5 μm, TOSOH) was employed. Chromatography conditions: column operation temperature at 30 °C; elution was performed with 90% ethanol and 10% acetonitrile from 0-30 min, flow rate was 1 mL/min, detection wavelength was 450 nm, and injection volume was 20 μL.

2.11 Re-dispersibility of Dry Emulsions

The dried emulsion powders were re-constituted with distilled water to the same total solids content as it was before drying, and stirred for 2 hours at room temperature before testing. Re-dispersibility of dry emulsions was calculated based on the following equation:

$$\text{Re-dispersibility (\%)} = \frac{\textit{β-carotene content in reconstituted emulsions}}{\textit{β-carotene content in emulsions before drying}} \times 100$$

2.12 Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was employed to compare means of data. A t-Test was used to determine the differences between means. Significant differences were determined at the 0.05 level ($p<0.05$).

3. Results and Discussion

3.1 Microscope Observation

3.1.1 Spray-dried Emulsions

To study the morphological observations of spray-dried emulsions, scanning electronic microscope (SEM) was employed. Spray-dried emulsions containing maltodextrin (MD) and konjac glucomannan (KGM) (**Figure 6-1a,b,c**) showed approximately spherical particles with concavo-convex and striae structures on the surface, while spray-dried emulsions containing monoglyceride (MG) had a smoother surface with granular bulges. It was also observed that the oil droplets were well encapsulated within the wall materials (MD and KGM) (**Figure 6-2a,b**). In addition, an empty-inside structure of these dry emulsion particles was observed (**Figure 6-3**).

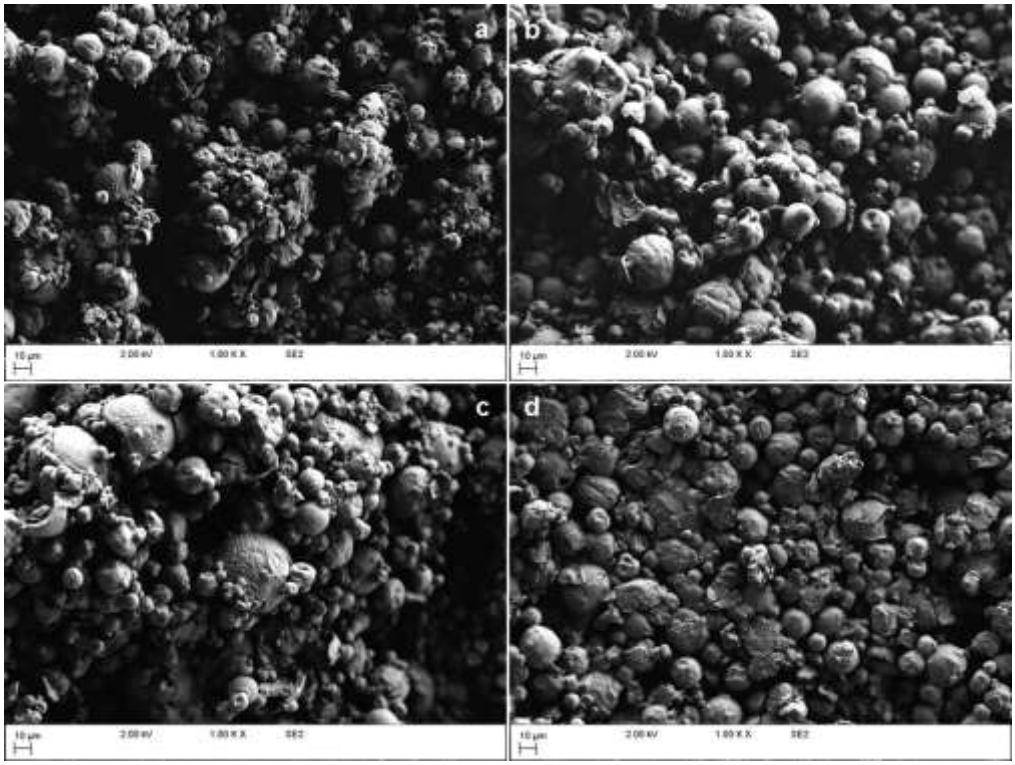


Figure 6-1. Scanning electron microscopy images of spray-dried emulsions: (a) 6% maltodextrin (MD); (b) 6% MD and 0.15% konjac glucomannan (KGM); (c) 2% MD and 0.15% KGM; (d) 6% MD and 1% MG.

Based on the observation of emulsion particles by SEM, the distribution of particle sizes mainly falls in the range of 3~35 μm, and dry emulsions containing MG had a more uniform size distribution but larger average particle size than those of emulsions without MG (**Figure 6-1**). Similar results were also obtained by Mastersizer (**Figure 6-4**). Spray-dried emulsions containing both MD and MG showed the largest average particles size, followed by emulsions containing MD and KGM, and emulsions containing MD, respectively. Emulsions with MG in the oil phase contained large particles (red arrow) and the addition of MD significantly reduced the particle size of emulsions with MG (data not shown).

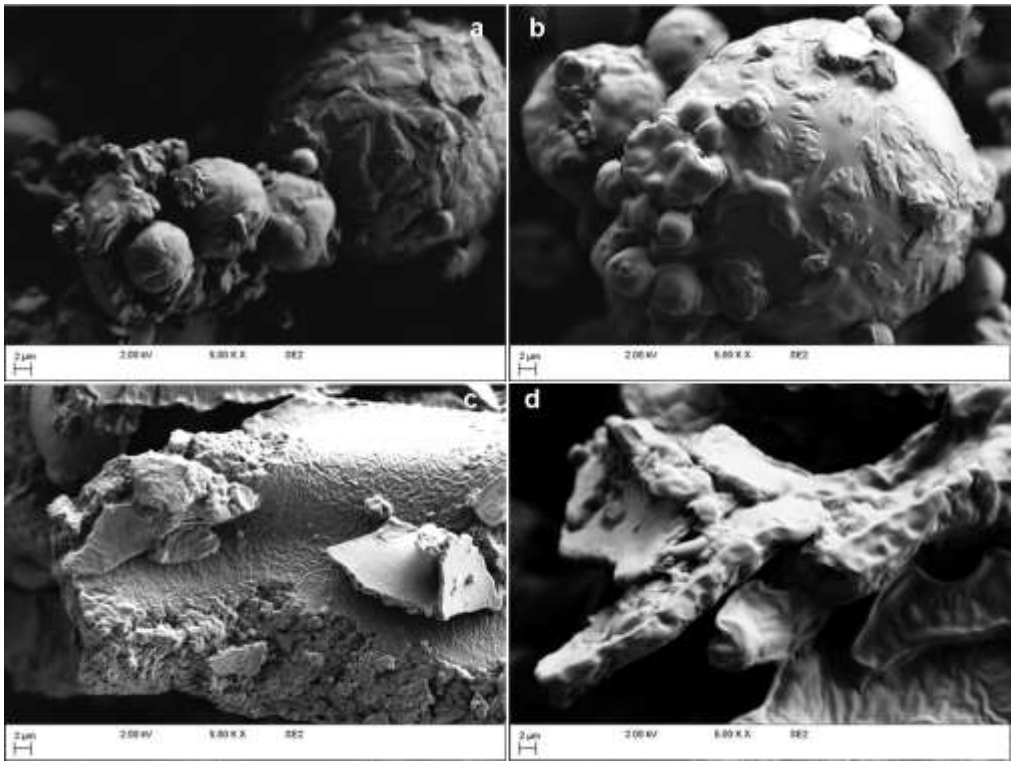


Figure 6-2. Scanning electron microscopy images of: (a) spray-dried emulsion with 6% maltodextrin (MD); (b) spray-dried emulsion with 2% MD and 0.15% konjac glucomannan (KGM); (c) freeze-dried emulsion with 6% MD; (d) freeze-dried emulsion with 0.15% KGM.

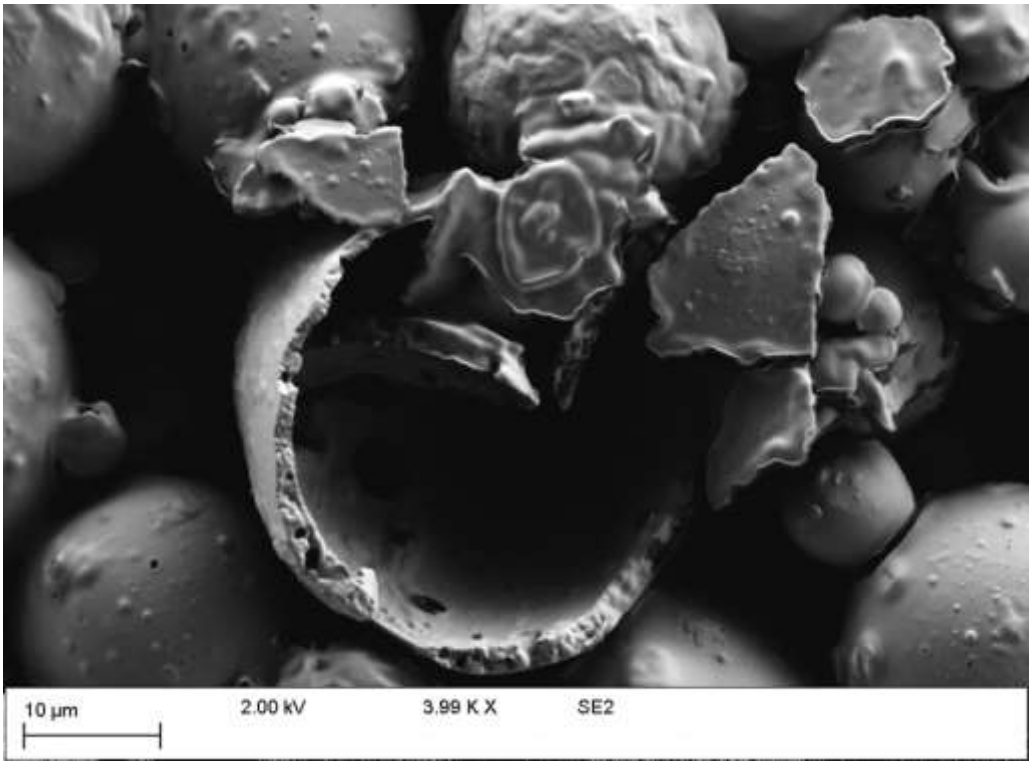


Figure 6-3. Scanning electron microscopy images of particles in a spray-dried emulsion containing maltodextrin (MD) and monoglyceride (MG).

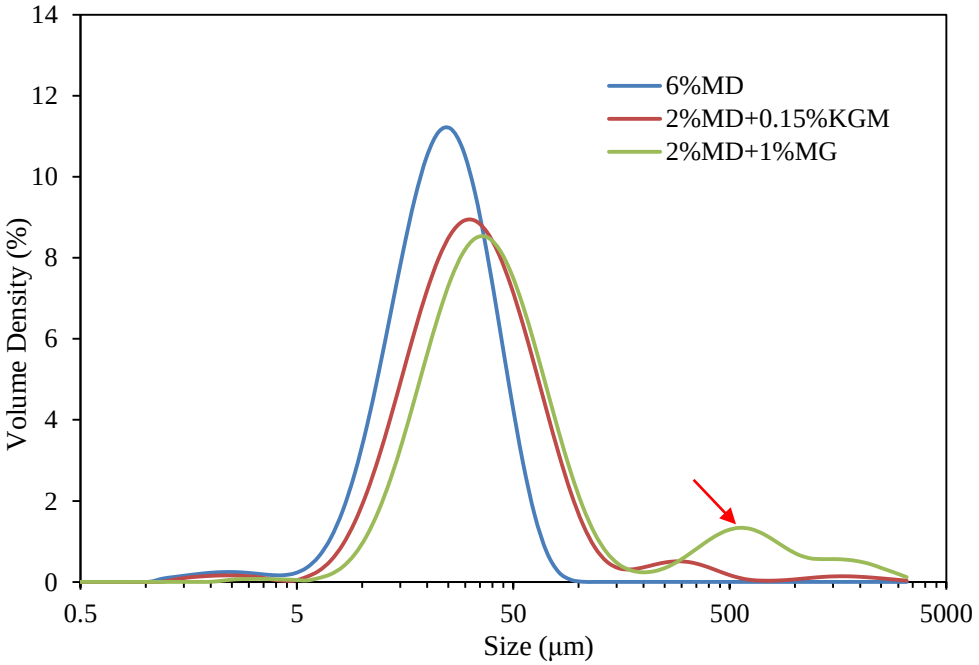


Figure 6-4. Particle size distribution of spray-dried emulsion powders. 6%MD, (2%MD+0.15%KGM), and (2%MD+1%MG) indicate spray-dried emulsion containing 6% MD, 2%MD and 0.15%KGM, and 2%MD and 1%MG, respectively;

Confocal laser scanning microscope (CLSM) was also used to further investigate the distribution of oil droplets in powders. As described above, an irregular spherical particle with porous surface and hollow structures was observed (**Figure 6-5**). The wall materials (MD and/or KGM) embedded with oil droplets formed the shell layer of these particles (**Figure 6-3 & Figure 6-5**).

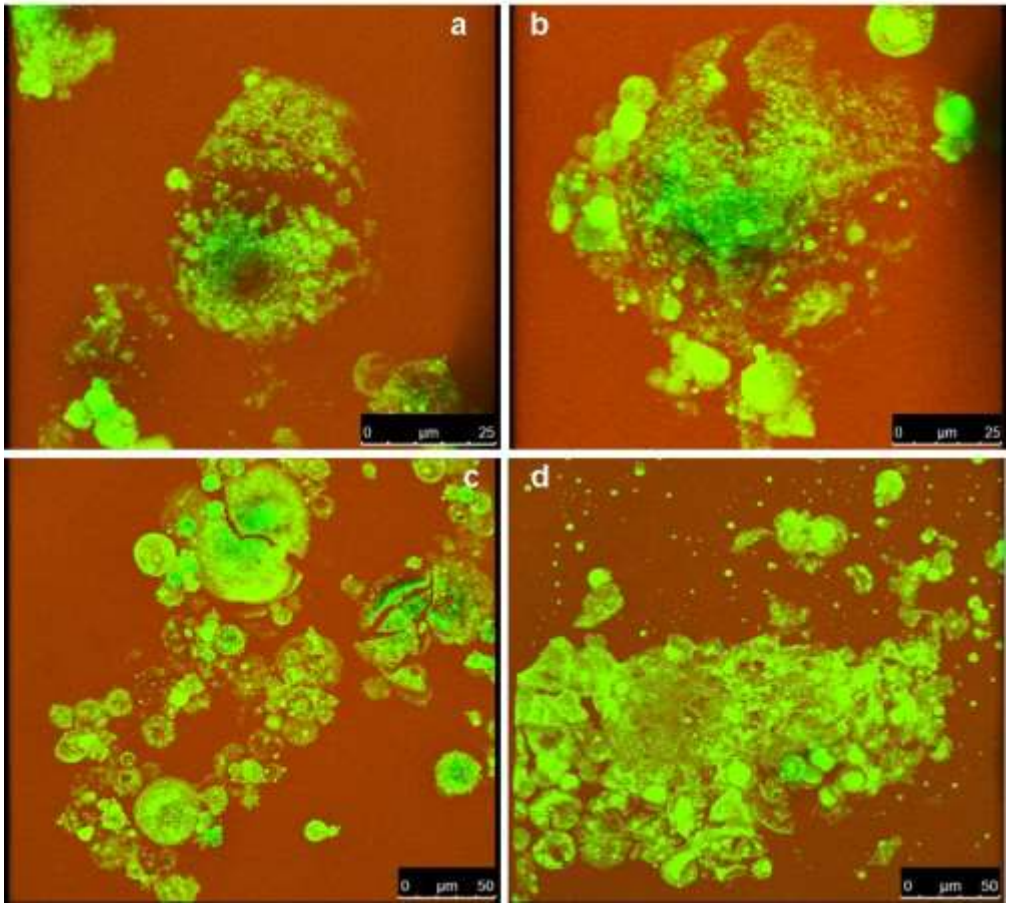


Figure 6-5. Confocal laser scanning microscope of spray-dried emulsions containing (a) 6% maltodextrin (MD), (b) 2% MD and 0.15% konjac glucomannan (KGM), (c) 6% MD and 0.15% KGM, (d) 6% MD and 1% monoglyceride (MG). Mixed dyes of Nile red and fast green were used to dye fat and protein, respectively; Green color indicates fat; Red color indicates proteins.

3.1.2 Freeze-dried Emulsions

Freeze-dried emulsions showed significantly different morphologies compared with spray-dried ones. Freeze-dried emulsions showed different flake shapes with smooth or folded surfaces depending on different emulsion formulas. Freeze-dried emulsion containing 6% MD had a very smooth surface (**Figure 6-6a**) while the emulsion containing 0.15% KGM showed a coarse particle surface scattered with flakes (**Figure 6-6b**); emulsion containing both MD and KGM showed a syncretic combination of these two structures (**Figure 6-6c**). All dry emulsions containing MD and KGM in the water phase as wall material showed an good encapsulation of oil

droplets into wall materials during freeze-drying process (**Figure 6-2c,d**); however, emulsion with MG did not show a well-encapsulation of oil droplets into wall materials, and many oil droplets scattered outside of wall materials and aggregated (**Figure 6-6d**).

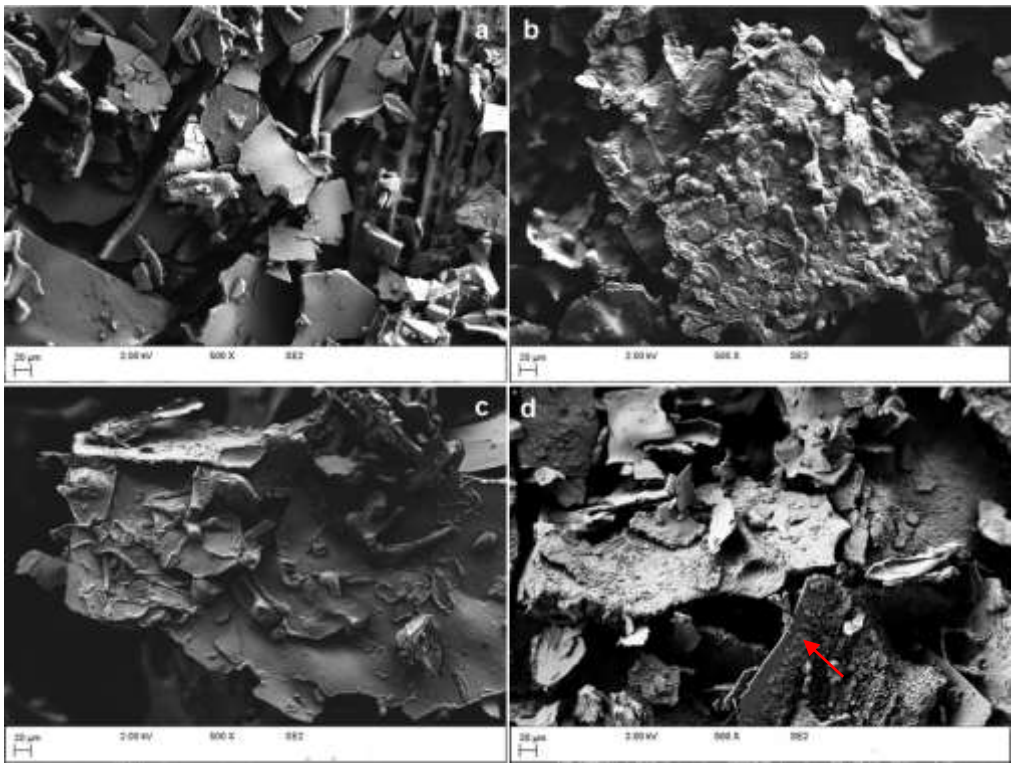


Figure 6-6. Scanning electron microscopy images of freeze-dried emulsions containing (a) 6% maltodextrin (MD), (b) 0.15% konjac glucomannan (KGM), (c) 2% MD and 0.15% KGM, (d) 6% MD and 1% monoglyceride (MG). Red arrow indicates aggregated oil droplets.

CLSM observation showed that freeze-dried emulsions immediately re-dispersed into fine oil-in-water emulsions with similar appearance as before freeze-drying, and regular spherical oil droplets could be clearly observed (**Figure 6-7**), indicating very good re-dispersibility of freeze-dried emulsions. Very few intact powder flakes could be captured.

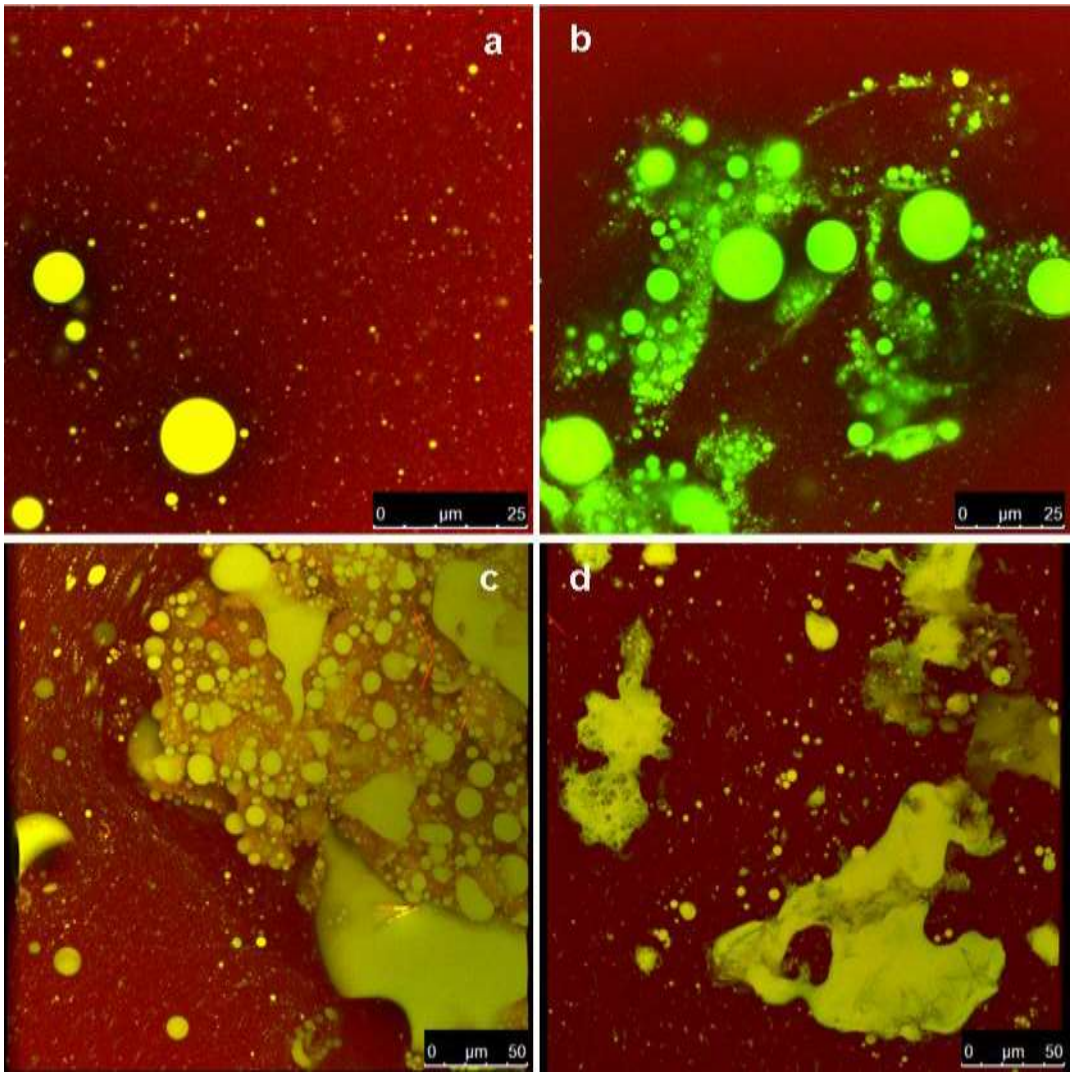


Figure 6-7. Confocal laser scanning microscope of freeze-dried emulsion powders containing (a) 6% maltodextrin (MD), (b) 0.15% konjac glucomannan (KGM), (c) 2% MD and 0.15% KGM, (d) 6% MD and 1% monoglyceride (MG). Mixed dyes of Nile red and fast green were used to dye fat and protein, respectively; Green color indicates fat; Red color indicates proteins.

3.2 MG Crystals in Dry Emulsions

Differential scanning calorimetry (DSC) was employed to investigate the crystallization and melting behaviour of dry emulsion, since some of them contained monoglyceride (MG), which can form crystals inside oil droplets at low temperatures and melt at high temperatures, which can potentially influence the properties of dry emulsions and their re-dispersibility.

3.2.1 Spray-dried Emulsions

Figure 6-8 shows the melting and crystallization of control sample, MG dispersed in sunflower oil. In order to observe all different types of crystal polymorphs, the range of temperature tested was 0.0 °C-120.0 °C. In the first heating step, a melting peak with a maximum temperature at 72.3 °C was observed, which corresponded to the melting of β polymorph. Two big melting peaks and a very small peak during the second heating steps with maximum temperatures at 63.7°C, 40.7°C, and 33.5°C were observed, which might correspond to the melting of α, sub-α_1 and sub-α_2 MG crystals, respectively (Hagemann, 1988; Vereecken et al., 2009). Similarly, the cooling process showed three crystallization peaks with maximum temperatures at 57.4 °C, 36.5 °C, and 18.1 °C, which accordingly corresponds to the formation of α, sub-α_1 and sub-α_2 MG crystals, respectively.

However, pure MG powders showed significantly different maximum melting and crystallization temperatures (data not shown) compared with MG powders dissolved in sunflower oil as describe above (**Figure 6-8**). MG powders showed a maximum melting temperature of β-polymorph at 77.8 °C, and maximum melting temperature of α and sub-α_1 polymorph at 72.4 °C and 40.0 °C, respectively. These results suggested that a phase transition behavior of MG is largely dependent on their physical state, e.g., as powder or dispersion in oils.

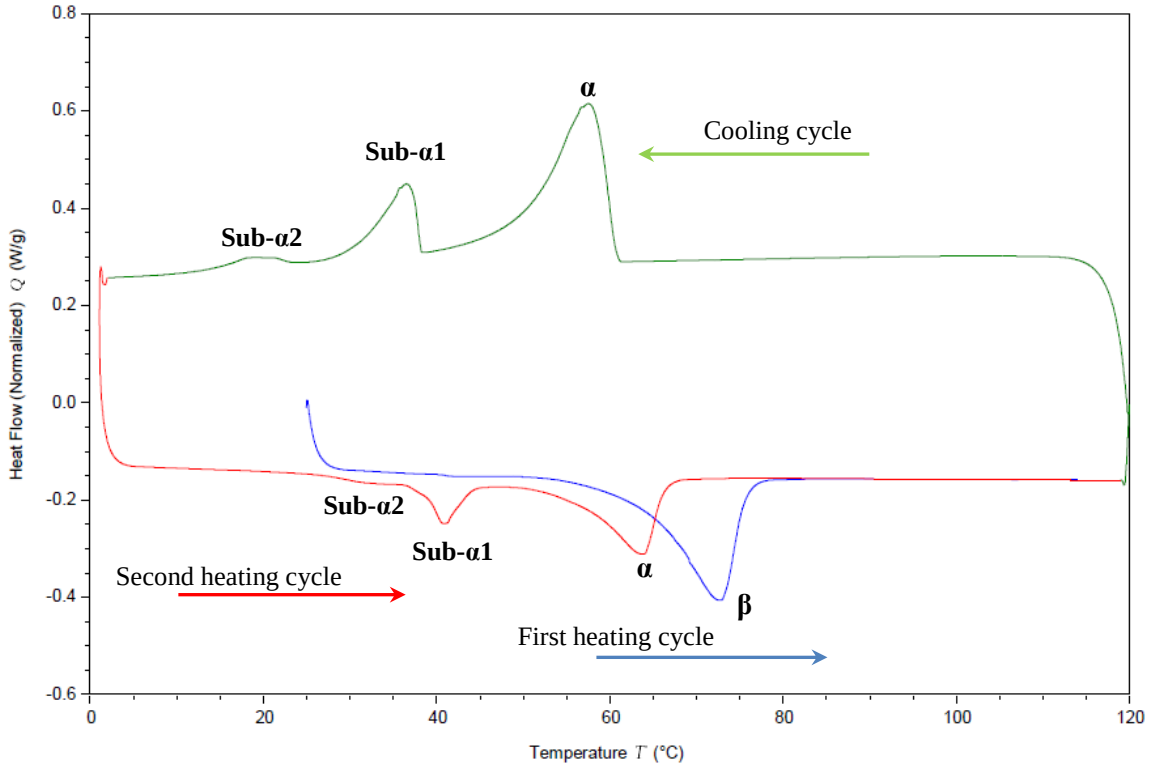


Figure 6-8. Crystallization analysis of MG dispersed in sunflower oil. Samples underwent following thermal cycles: heating from 25.0 °C to 120.0 °C (5 °C/min), equilibration at 120.0 °C for 5 min, cooling to 0.0 °C (10 °C/min), and heating up to 120.0 °C (5 °C/min).

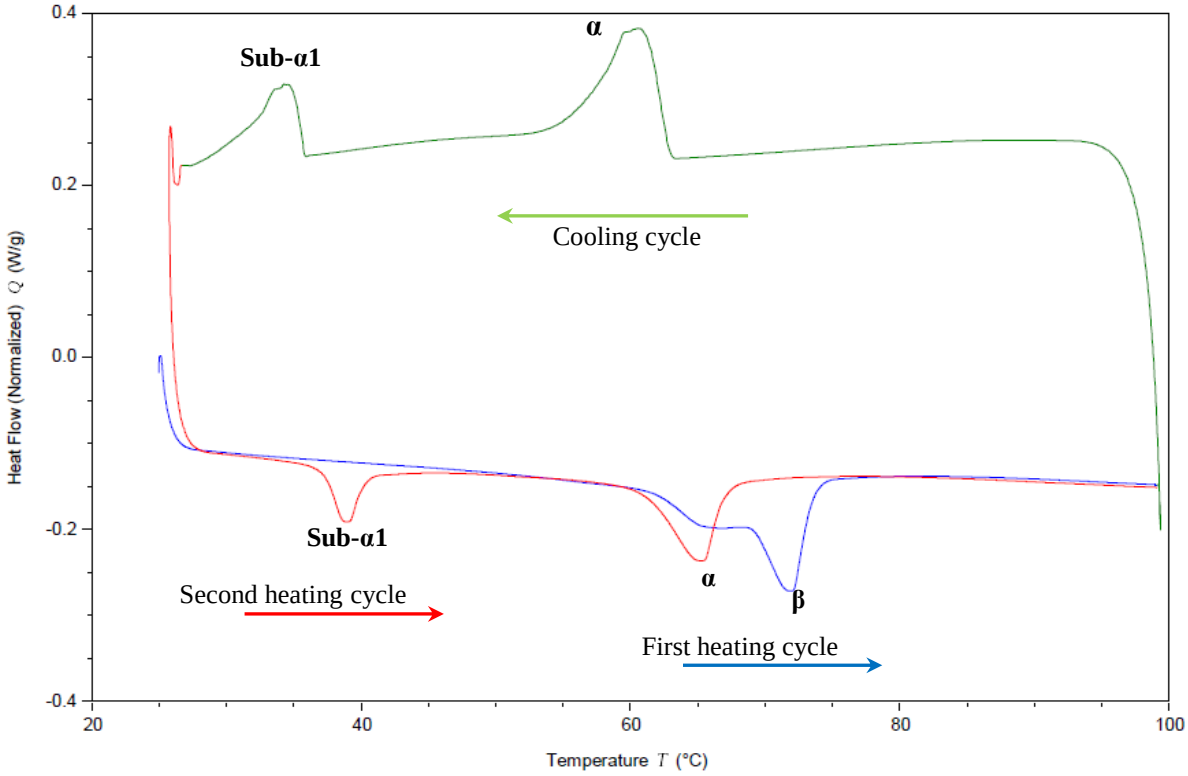


Figure 6-9. Melting and formation of MG crystals in spray-dried emulsion powders containing 6% maltodextrin (MD) and 1% monoglyceride (MG). Samples underwent the following thermal cycles: heating from 25.0 °C to 100.0 °C (5 °C/min), cooling to 25.0 °C (10 °C/min), and heating up to 100.0 °C (5 °C/min).

In the temperature range tested (25.0-100.0 °C) for powdered emulsions, no phase transition (fusion or crystallization) was observed in powders without MG (data not shown).

MG in spray-dried emulsion powders showed a similar fusion and crystallization behavior (**Figure 6-9**) as that of MG dispersed in sunflower oil. Based on results in **Figure 6-8** and previous studies (Hagemann, 1988; Vereecken et al., 2009), one melting peak of β-polymorph in the first heating step at 72.0 °C, and two melting peaks of sub-α_1 polymorph at 38.7 °C and α-polymorph at 65.3 °C in the second heating cycle were observed. In addition, these two peaks in the second heating cycle also correspondence to the melting of the same MG crystals (sub-α_1 and α polymorph) formed during cooling cycles at a peak temperature of 60.6 °C and 34.4 °C. The differences in the maximum melting and crystallization temperatures of MG crystals in sunflower oil (**Figure 6-8**) and spray-dried powders (**Figure 6-9**) also suggested that the interaction of MG with other compositions in spray-dried emulsions powders, e.g., whey protein isolate and β-carotene, can potentially alter their phase transition behavior.

Spray-dried emulsions containing MG and different concentration of MD showed similar fusion and crystallization peak temperatures (data not shown), indicating that MD did not influence the fusion and crystallization behaviour of MG.

3.2.2 Freeze-dried Emulsions

Freeze-dried emulsions without MG also did not show any significant phase transition, similar to spray-dried emulsions without MG described above (data not shown). Freeze-dried emulsion containing MG, however, showed different melting and crystallization temperatures and enthalpy compared with those of spray-dried emulsions containing MG (**Table 6-1**), suggesting that different processing

technologies, e.g., freeze-drying or spray-drying, can affect the formation and melting of MG crystals.

Formation and melting of MG crystals in emulsions can also be influenced by other factors, such as selection of emulsifiers, storage temperature and duration (Mao et al., 2014). In addition, MG can form different polymorphs (Hagemann, 1988; Vereecken et al., 2009), and a transition between different polymorphs can also be observed (Mao et al., 2014). This may explain why only β-polymorph MG crystals in dry emulsion powders can be observed in the first heating cycle but two other polymorphs, α-polymorph and sub-α_1 polymorph can be observed in the second heating cycle (**Figure 6-8 & Figure 6-9**). It is because the transition of α-polymorph and sub-α_1 polymorph (formed during the cooling of the emulsions) to the β-polymorph during storage.

Table 6-1 Maximum melting and crystallization temperature (T_m) and enthalpy (ΔH) of MG crystals in spray-dried and freeze-dried emulsions containing 6% maltodextrin (MD) and 1% monoglyceride (MG) (n=3).

	Spray-dried emulsions					Freeze-dried emulsions				
	First heating	Cooling		Second heating		First heating	Cooling		Second heating	
T_m (°C)	72.1±0.0	34.4±0.4	60.4±0.1	38.8±0.0	65.4±0.0	71.3±0.0	35.3±0.3	57.4±0.0	41.0±0.3	63.1±0.1
ΔH (J/g)	3.3±0.2	2.0±0.1	4.3±0.3	1.7±0.0	4.4±0.1	4.7±0.1	0.9±0.0	3.3±0.1	2.1±0.1	2.2±0.1

Table 6-2 Mean droplet size, zeta potential (ZP), and polydispersity index (PdI) of liquid emulsions and their re-constituted equivalents after spray-drying

	Before spray-drying			After re-constitution		
Emulsions	Size (d.nm)	ZP (mV)	PdI	Size (d.nm)	ZP (mV)	PdI
6%MD	250±7	-27.9±0.6	0.194±0.044	265±9	-33.2±0.3	0.283±0.016
6%MD +0.15%KGM	278±7	-27.6±0.6	0.191±0.044	311±20	-34.9±0.7	0.377±0.007
2%MD +0.15%KGM	239±9	-29.1±0.6	0.220±0.014	284±1	-35.5±1.2	0.290±0.050
6%MD +1%MG	233±5	-34.7±0.7	0.312±0.005	261±10	-36.4±1.2	0.306±0.031
2%MD +1%MG	184±14	-31.3±0.8	0.314±0.011	265±7	-35.1±1.4	0.314±0.011

* MD indicates maltodextrin; KGM indicates konjac glucomannan; MG indicates monoglyceride

3.3 Droplet size and Surface Charge of Re-constituted Emulsions

3.3.1 Spray Dried Emulsions

Spray-drying causes a significantly increase in the droplet size of re-constituted emulsions, which potentially influence their stability and functionalities. In this study, good reconstitution from spray-dried emulsions was achieved by formulating the water phase of emulsion before drying with very low level of maltodextrin (MD) and health-beneficial konjac glucomannan (KGM).

The average droplet size of re-constituted spray-dried emulsions varied from 260 nm to 310 nm (**Table 6-2**). Although droplet size of reconstitutions was slightly larger than that of emulsions before drying, such differences in droplet size did not significantly influence the digestion and bioavailability of bioactive nutrients encapsulated in emulsions (Lu et al., 2017a). Droplet size distributions of reconstituted emulsions containing KGM were shifted towards smaller particle sizes, while other reconstituted samples showed a shift towards larger particle sizes (**Figure 6-10a**)

Surface charge of re-constituted spray-dried emulsion showed different values of increase (**Table 6-2**). Emulsion containing 6% MD and 0.15% KGM had a highest increase in surface charge, while the emulsion containing 6% MD and 1% MG had a lowest increase in surface charge. Increased surface charge of re-constituted emulsions can potentially improve their stability, due to increased electrostatic repulsion between droplets.

Table 6-3. Mean droplet size, zeta potential (ZP), and polydispersity index (PdI) of liquid emulsions and re-constituted freeze-dried emulsions (n=3)

	Before freeze-drying			After re-constitution		
Emulsions	Size (d.nm)	ZP (mV)	PdI	Size (d.nm)	ZP (mV)	PdI
6%MD	220±5	-33.1±0.3	0.223±0.020	212±3	-32.2±0.5	0.260±0.008
0.15%KGM	219±6	-33.5±0.3	0.235±0.016	208±19	-33.5±0.2	0.261±0.023
6%MD+0.15%KGM	217±4	-32.0±0.6	0.238±0.019	210±5	-32.9±0.6	0.258±0.022
2%MD+0.15%KGM	219±3	-33.6±0.7	0.237±0.014	207±6	-35.3±0.7	0.274±0.023
6%MD+1%MG	228±3	-29.8±0.4	0.341±0.006	3229±911	-37.8±2.8	0.874±0.191

* MD indicates maltodextrin; KGM indicates konjac glucomannan; MG indicates monoglyceride

3.3.2 Freeze-dried Emulsions

Compared with emulsions before freeze drying, average droplet size of all re-constituted freeze-dried emulsions slightly decreased except for the emulsion containing MG, the size of which dramatically increased from 228 nm to 3229 nm (**Table 6-3**). Similarly, droplet size distributions of all re-constituted emulsions were shifted towards smaller particle sizes (**Figure 6-10b**). Re-constituted emulsion containing MG also showed a significantly increased surface charge (p<0.05) while no significant difference in surface charge was observed for other re-constituted emulsions, compared with that of emulsion before drying.

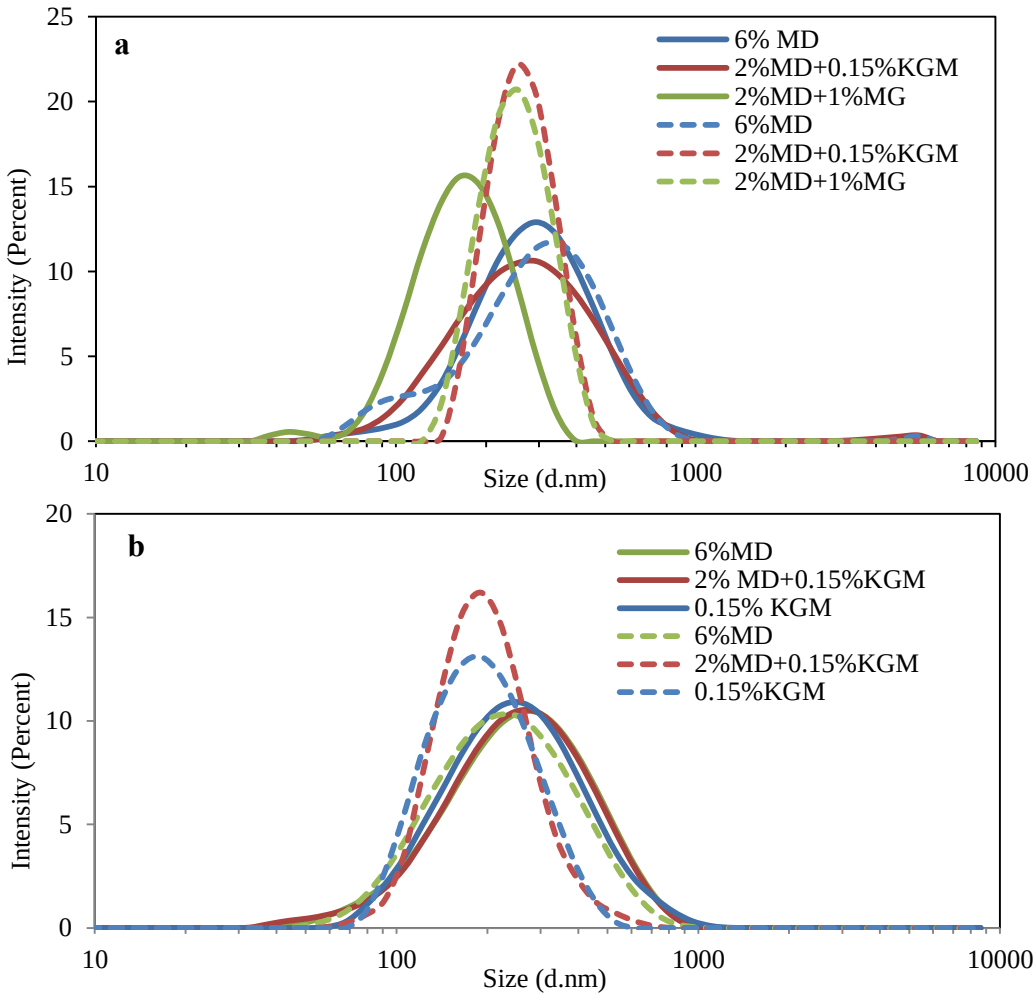


Figure 6-10. Size distribution of re-constituted (**a**) spray-dried emulsions and (**b**) freeze-dried emulsions. Dashed lines indicate reconstituted emulsions; Solid lines indicate emulsions before drying; KGM indicates konjac glucomannan; MD indicates maltodextrin; MG indicates monoglyceride.

3.4 Viscosity

Viscosity of all emulsions and re-constituted emulsions decreased with increasing shear rate, which was also observed in our previous studies (Lu et al., 2017a, 2017b; Lu et al., 2018) (**Chapter 3**, **Chapter 4**, and **Chapter 5**), indicating that weak attractive forces exist between the droplets. Emulsions containing KGM had significantly higher viscosity than those without KGM, and a higher content of MD lead to higher viscosity of emulsions containing both KGM and MD (**Figure 6-11a**, **Figure 6-12a** and **Figure 6-13**).

Re-constituted spray-dried emulsions containing KGM showed significantly decreased viscosity than emulsions before drying (p<0.05), while no significant differences were observed for other re-constituted emulsions (**Figure 6-11**). The same result was also observed on the re-constituted freeze-dried emulsions (**Figure 6-12**). This is probably attributed to two reasons: (i) de-polymerization of KGM during the drying process, producing fractions with low molecular weight, accordingly resulting in decreased viscosity (Villay et al., 2012); (ii) the absorption of KGM to the surface of droplets during the drying process, which reduced the concentration of KGM in the water phase of re-dispersed emulsions, and thus led to a decreased viscosity of emulsions.

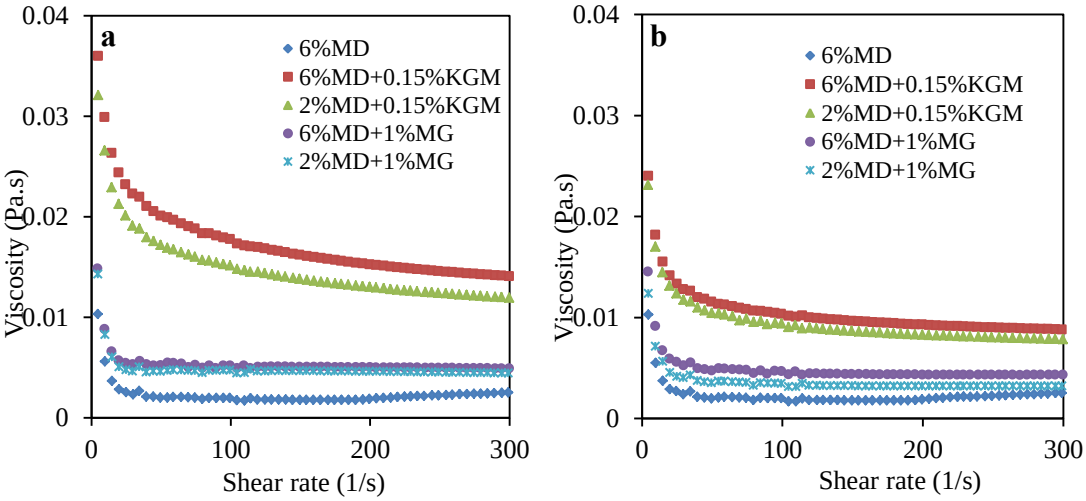


Figure 6-11. Viscosity of (**a**) emulsions before spray drying and (**b**) reconstituted spray-dried emulsions as a function of shear rate. KGM indicates konjac glucomannan; MD indicates maltodextrin; MG indicates monoglyceride.

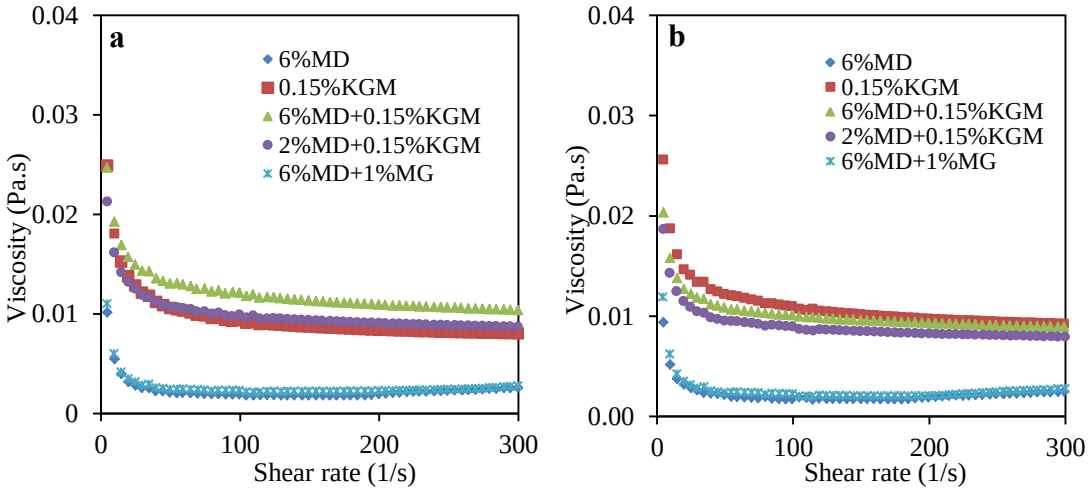


Figure 6-12. Viscosity of (a) emulsions before freeze-drying and (b) reconstituted freeze-dried emulsions, as a function of shear rate. KGM indicates konjac glucomannan; MD indicates maltodextrin; MG indicates monoglyceride.

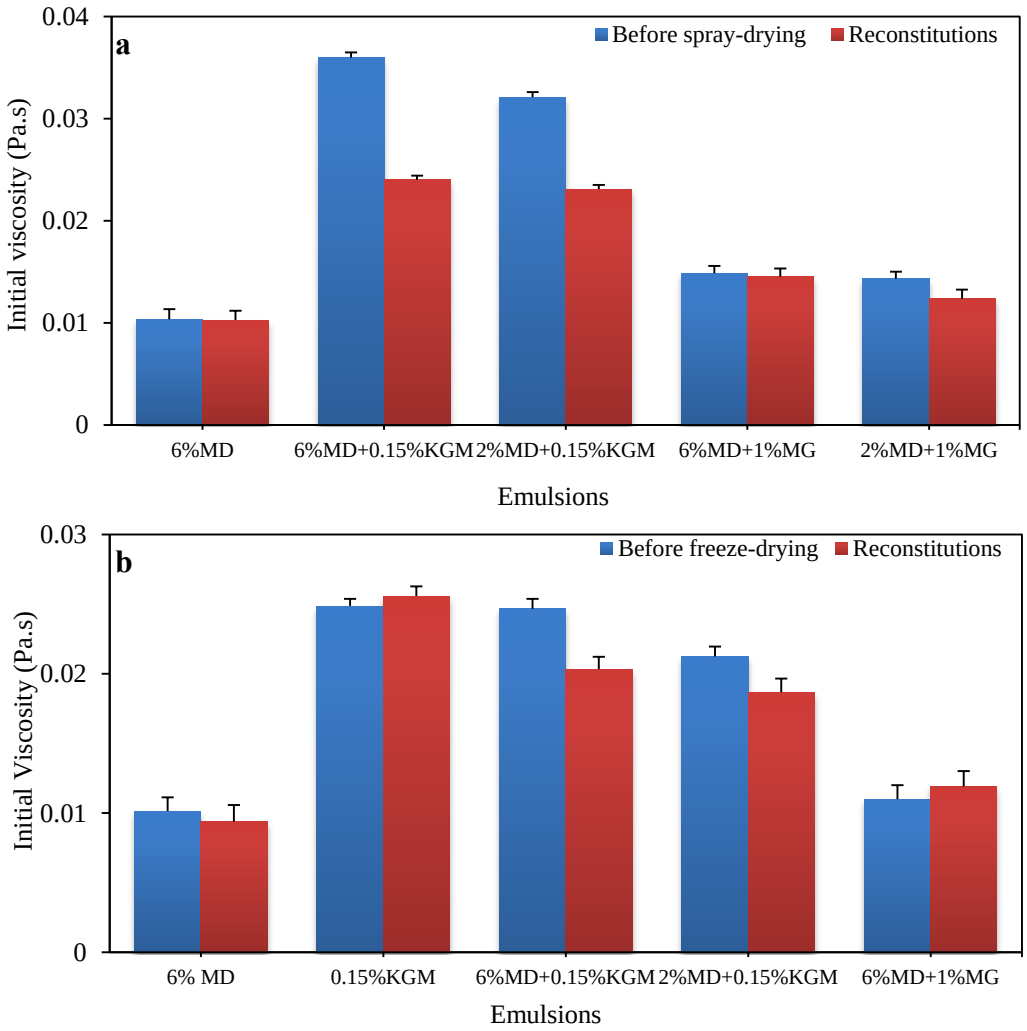


Figure 6-13. Initial viscosity of reconstituted (a) spray-dried emulsions and (b) freeze-dried emulsions. KGM indicates konjac glucomannan; MD indicates maltodextrin; MG indicates monoglyceride.

3.5 Creaming Stability

For the creaming stability test, curves of the integrated light transmission against time were plotted, and the slope of each curve was taken as the light transmission rate (%/second)/creaming index (CI). A higher value of this parameter indicates a lower creaming stability of emulsions.

Before drying, emulsions containing KGM (0.15%, w/w) showed better creaming stability than those without KGM (**Figure 6-14**), which was also observed in our

previous study (Lu et al., 2018). This is mainly attributed to the depletion flocculation of emulsion droplets by non-absorbed KGM, which can generate an attractive osmotic force between droplets. This osmotic force increases with increasing concentration of KGM until it is large enough to overcome the repulsive forces between droplets and cause their flocculation. Combined with our previous results (**Chapter 5**), the concentration of KGM that can cause significantly flocculation of emulsions is probably between 0.1%-0.15% (w/w).

Reconstituted spray-dried emulsions containing KGM showed even better creaming stability than their original emulsions before drying, while other reconstituted spray-dried emulsions without KGM had a lower creaming stability than their original emulsions (**Figure 6-14a**). The same results were also observed on reconstituted freeze-dried emulsions. In addition, creaming stability of emulsion containing MG dramatically increased nearly 20-fold after freeze-drying (**Figure 6-14b**) and a significant creaming layer in the top was observed. This is mainly caused by the aggregation of droplets after reconstituting, as described above (**Table 6-3**).

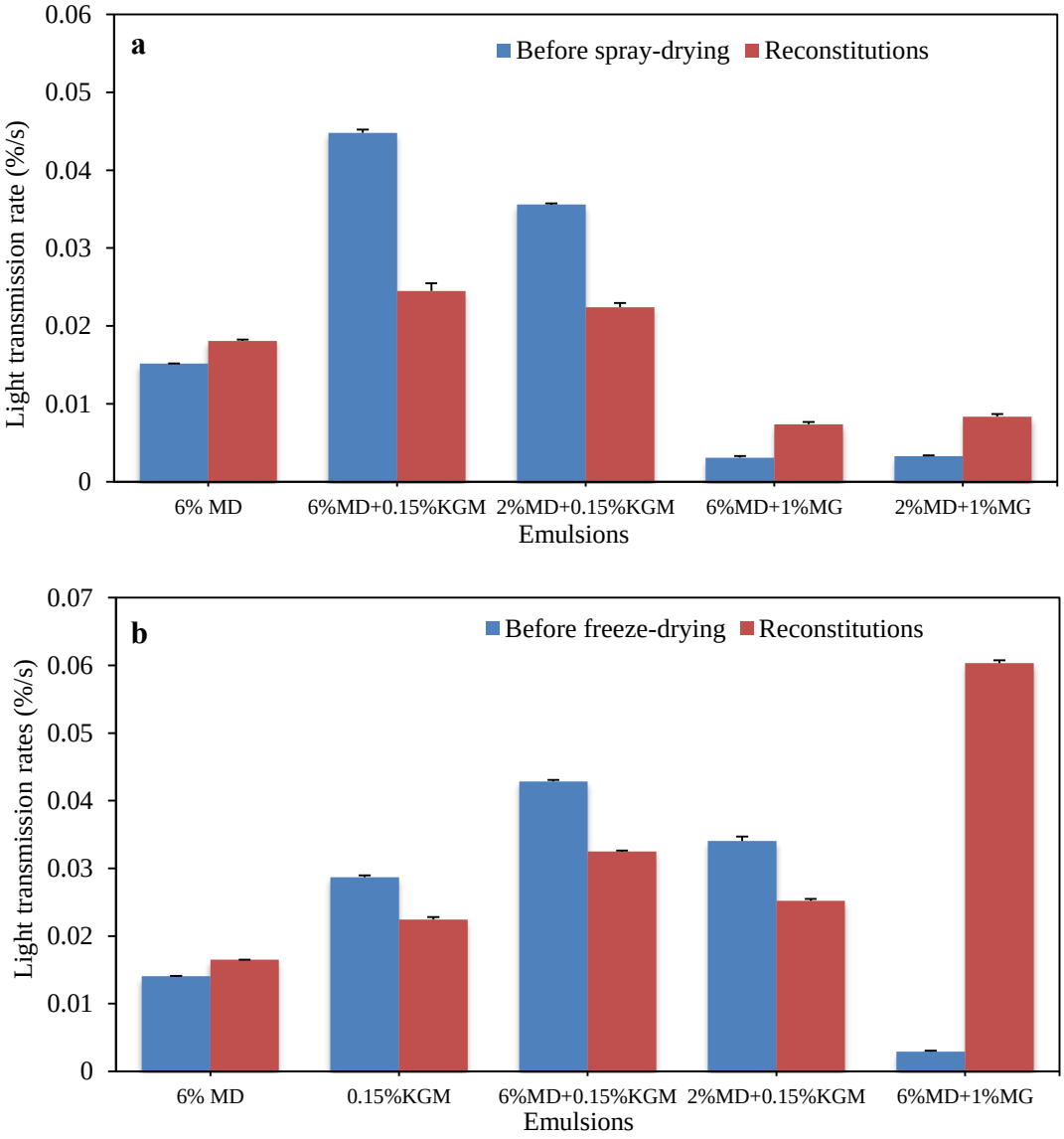


Figure 6-14. Creaming stability of reconstituted (a) spray-dried emulsions and (b) freeze-dried emulsions. KGM indicates konjac glucomannan; MD indicates maltodextrin; MG indicates monoglyceride.

3.6 Re-dispersion of Dry Emulsions

One of the most important properties of dried emulsions is their ability to re-disperse into fine emulsions in water with similar droplet size and distribution as they had before drying process. Thus, re-dispersibility of all dry emulsions with improved formulas using functional konjac glucomannan (KGM) combined with

maltodextrin (MD) was evaluated and the properties of re-constituted emulsions were also tested.

All spray-dried emulsions showed fast re-hydration in water at room temperature by visual observation. Freeze-dried emulsions showed even faster re-dispersing than spray-dried ones. All freeze-dried emulsions re-constituted into O/W emulsions with clearly visible spherical droplets by microscopy (**Figure 6-7**) immediately after contacting water, except for emulsion containing MG, which needed gentle shaking.

Many factors can influence the re-hydration of powders in water, e.g., composition, particle size, microstructure, and interactions between particles. For powders with similar formulas, re-dispersibility is mainly determined by particle size and microstructure. In this study, compared with a relatively complete spherical particle of spray-dried emulsion powders (**Figure 6-1**), the irregular flake-like shape of freeze-dried emulsion powders (**Figure 6-6**) was apparently better at facilitating the wetting and reconstitution of dried emulsions in water. In addition, the average size of particles of freeze-dried emulsions is significantly larger than that of spray-dried emulsions by SEM. Generally, powders with larger particle size can be more easily dissolved than those with smaller particles. All these results may be able to explain why freeze-dried emulsions showed much faster re-dispersion in water than spray-dried emulsions.

β-carotene dissolved in the oil phase of emulsions can be considered as natural dye for oil phase, which accordingly can be easily used as a calibration marker to quantify the oil fraction in re-constituted emulsions. As is shown in **Figure 6-15**, spray-dried emulsion all showed good re-dispersibility. More than 90% of oil was re-dispersed into oil droplets. The emulsion containing 6% MD showed the highest re-dispersibility of 96%, followed by emulsions containing MD and MG, and emulsions

containing MD and KGM, respectively, and no significant difference between these samples was observed. The results suggested that combined use of low levels of MD and KGM as wall materials during drying of emulsions still can effectively protect oil droplets as described above (**Figure 6-3**) and obtain good dry emulsion powders with high re-dispersibility in water.

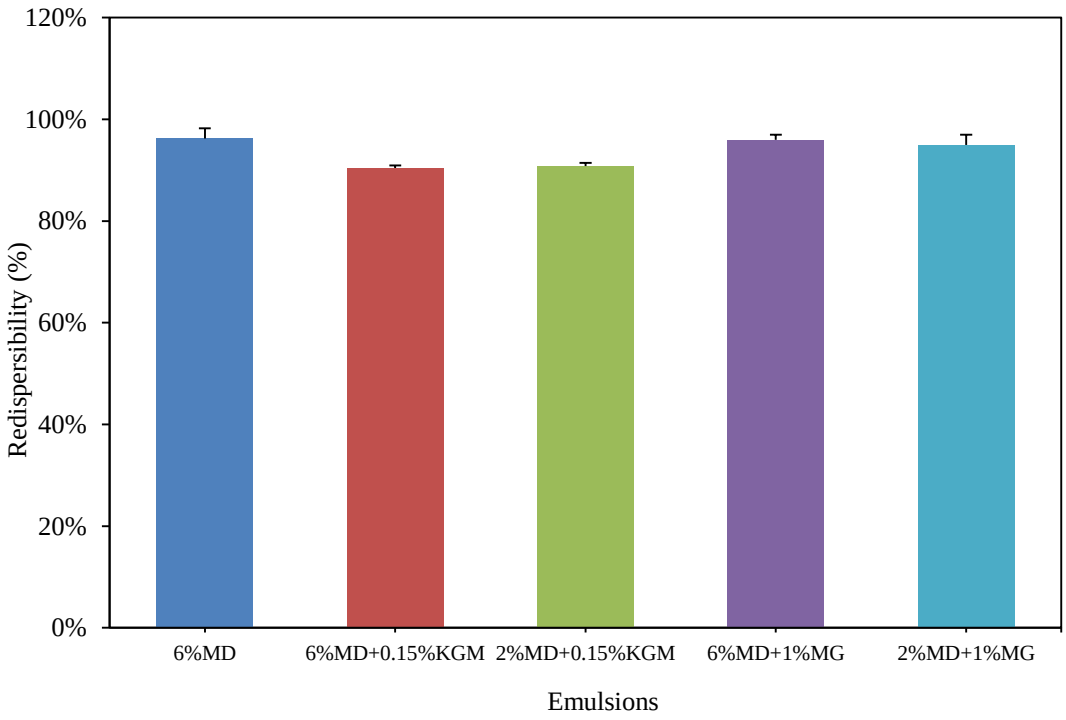


Figure 6-15. Re-dispersibility of spray-dried emulsions. MD indicates maltodextrin; KGM indicates konjac glucomannan; MG indicates monoglyceride.

Freeze-dried emulsions also showed good re-dispersibility (>85%) except for the emulsion containing MG, in which only 44% of oil phase was re-constituted into intact oil droplets (**Figure 6-16**). Emulsion containing only 0.15% of KGM showed the highest re-dispersibility of 96%. Emulsions containing both MD and KGM showed slightly lower re-dispersibility than emulsions containing only MD or KGM (p>0.05). The results confirmed the potential of KGM being used as protective wall material in the freeze-drying of liquid emulsions.

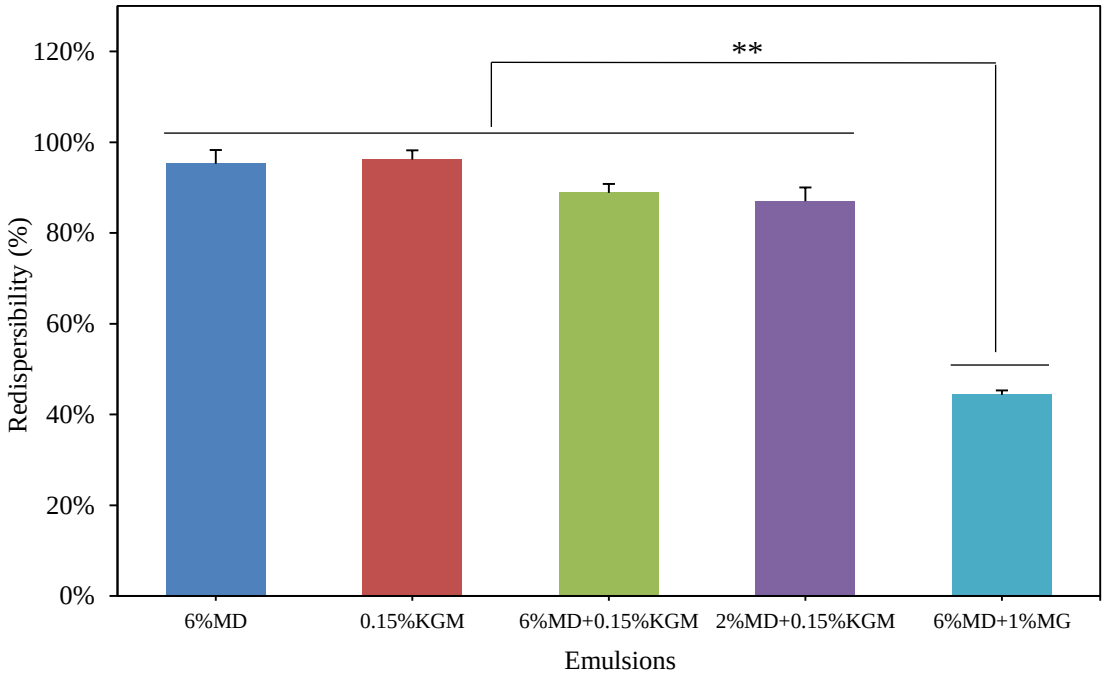


Figure 6-16. Re-dispersibility of freeze-dried emulsions. MD indicates maltodextrin; KGM indicates konjac glucomannan; MG indicates monoglyceride (** p<0.01).

Generally, wall materials, e.g., biopolymers, are added to the water phase of liquid emulsion before spray-drying or freeze-drying to obtain good dry emulsion powders. The main purpose of using wall materials is to coat oil droplets and protect them from being destroying during drying process. However, utilization of high level of wall materials will significantly increase the production cost and can also relatively decrease the concentration of functional components encapsulated in emulsions. Thus, it is valuable to develop optimized formulas of emulsions with lower levels of wall materials but great potential in creating good dry emulsion powders. The findings in this study provided helpful information on obtaining dry emulsions with high re-dispersibility by formulating the water phase of emulsions with very low level of MD and/or functional KGM.

4. Conclusions

Re-dispersible spray-dried and freeze-dried emulsions were obtained by formulating the water phase of liquid emulsions before drying with low dose of maltodextrin (MD) and/or konjac glucomannan (KGM). Dry emulsions (powders) showed different geometries and surface microstructures, depending on the dry methods (spray-drying or freeze-drying), and the formulas of emulsions. Spray-dried emulsions displayed approximately spherical hollow particles (mean diameter of 17-33 μm by laser diffraction) while freeze-dried emulsions had irregular flake-like shapes with larger size by SEM. Spray-dried emulsions containing monoglyceride (MG) showed a similar fusion and crystallization behavior with MG in sunflower oil. Freeze-dried emulsion containing MG, however, showed significant different melting and crystallization temperatures and enthalpy compared with those of spray-dried emulsions containing MG.

Dry emulsions showed fast re-hydration in water, and freeze-dried emulsions showed even faster re-dispersion than spray-dried emulsions, immediately re-dispersing into fine emulsions after contacting water, which is probably attributed to their flake-like shapes which can be more easily and rapidly wetted and dissolved in water compared with relatively complete spherical shapes of spray-dried emulsion particles. Re-dispersibility of dry emulsions was further evaluated by measuring the β-carotene content of liquid emulsion before drying and re-constituted emulsions. The results showed that all dry emulsions demonstrated a very high re-dispersibility of >85%, except for freeze-dried emulsion containing MG (44%), and re-constituted emulsions showed similar average droplet sizes and distributions with their liquid emulsions before drying. In addition, re-constituted emulsions containing KGM

showed significantly decreased initial viscosity (p<0.05) but increased creaming stability than their liquid emulsions before drying (p<0.05).

This study confirmed the potential of using low level of MD and/or KGM in the water phase of oil-in-water emulsion as wall materials to prepare spray-dried or freeze-dried emulsions containing bioactive nutrients. The results contribute to a better understanding of the influence of the water phase of emulsions on their drying, and make it possible to obtain stable dry emulsions and well-reconstituted emulsions by formulating the water phase with biopolymers.

References

Gbassi, G. K., & Vandamme, T. (2012). Probiotic encapsulation technology: from microencapsulation to release into the gut. *Pharmaceutics, 4*, 149-163.

Gharsallaoui, A., Saurel, R., Chambin, O., Cases, E., Voilley, A., & Cayot, P. (2010). Utilisation of pectin coating to enhance spray-dry stability of pea protein-stabilised oil-in-water emulsions. *Food Chemistry, 122*, 447-454.

Hagemann, J. (1988). Thermal behaviour and polymorphism of acylglyerides. In a. S. K. Garti N (Ed.), Crystallization and polymorphism of fats and fatty acids. (pp. 9-95). New York: Marcel Dekker.

Jafari, E. A. S.-M. (2017). Spray drying of folic acid within nano-emulsions: Optimization by Taguchi approach. *Drying Technology, 35*, 1152-1160.

Jang, D. J., Kim, S. T., Oh, E., & Lee, K. (2014). Enhanced oral bioavailability and antiasthmatic efficacy of curcumin using redispersible dry emulsion. *Bio-Medical Materials and Engineering, 24*, 917-930.

Karthik, P., & Anandharamakrishnan, C. (2016). Enhancing omega-3 fatty acids nanoemulsion stability and in-vitro digestibility through emulsifiers. *Journal of Food Engineering, 187*, 92-105.

Lu, W., Kelly, A. L., Maguire, P., Zhang, H., Stanton, C., & Miao, S. (2016). Correlation of Emulsion Structure with Cellular Uptake Behavior of Encapsulated Bioactive Nutrients: Influence of Droplet Size and Interfacial Structure. *Journal of Agricultural and Food Chemistry, 64*, 8659-8666.

Lu, W., Kelly, A. L., & Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science and Technology, 47*, 1-9.

Lu, W., Kelly, A. L., & Miao, S. (2017a). Bioaccessibility and Cellular Uptake of beta-Carotene Encapsulated in Model O/W Emulsions: Influence of Initial Droplet Size and Emulsifiers. *Nanomaterials, 7*, 282-292.

Lu, W., Kelly, A. L., & Miao, S. (2017b). Improved Bioavailability of Encapsulated Bioactive Nutrients Delivered through Monoglyceride-Structured O/W Emulsions. *Journal of Agricultural and Food Chemistry, 65*, 3048-3055.

Lu, W., Zheng, B., & Miao, S. (2018). Improved emulsion stability and modified nutrient release by structuring O/W emulsions using konjac glucomannan. *Food Hydrocolloids, 81*, 120-128.

Mao, L., Calligaris, S., Barba, L., & Miao, S. (2014). Monoglyceride self-assembled structure in O/W emulsion: formation, characterization and its effect on emulsion properties. *Food Research International, 58*, 81-88.

McClements, D. J. (2015). Food emulsions: principles, practices, and techniques (3rd ed.). New York: CRC press.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science, 72*, 109-124.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*, 213-228.

Parthasarathi, S., Muthukumar, S. P., & Anandharamakrishnan, C. (2016). The influence of droplet size on the stability, in vivo digestion, and oral bioavailability of vitamin E emulsions *Food &Function, 7*, 2294-2302.

Vereecken, J., Meeussen, W., Foubert, I., Lesaffer, A., Wouters, J., & Dewettinck, K. (2009). Comparing the crystallization and polymorphic behaviour of

saturated and unsaturated monoglycerides. *Food Research International, 42*, 1415-1425.

Villay, A., Lakkis de Filippis, F., Picton, L., Le Cerf, D., Vial, C., & Michaud, P. (2012). Comparison of polysaccharide degradations by dynamic high-pressure homogenization. *Food Hydrocolloids, 27*, 278-286.

CHAPTER SEVEN

General Discussion, Conclusions and Future Works

1. General Discussion

The present study investigated the effect of droplet size and formulas of the oil phase, the interface, and the water phase on properties and digestion of emulsions, as well as bioaccessibility and cellular uptake of lipophilic components in emulsions after passing through the GIT. The findings suggested that the stability and properties of emulsions in the GIT, as well as the bioaccessibility and cellular uptake of encapsulated lipophilic components after the GIT, were affected by the structure of emulsion-based carriers, which make it possible to control the release and improve the oral bioavailability of unstable lipophilic components by designing specific structures in emulsions.

The properties and structures of emulsions can be greatly influenced by their composition (e.g., oil, or type of emulsifiers), and preparation technologies that are used to create them. The structure of emulsion droplets can significantly affect their performance when being used as protective delivery carriers for lipophilic components, since a close correlation between the emulsion structure and the lipid digestion and release of encapsulated compounds in emulsions has been reported (Hou et al., 2014; Salvia-Trujillo et al., 2013a, 2013b). Thus, designing the structure of an emulsion-based carrier to achieve desired protection and delivery of target molecules appears to be particularly important in practical applications.

Many strategies can be used to control the digestion of lipid and the release of encapsulated lipophilic components in emulsions, e.g., modifying the structure of the oil phase, interface and water phase of emulsion-based delivery systems with different compositions (McClements & Li, 2010). These structured emulsions showed different microstructure in their oil phase (e.g., crystallized oil phase), interface (e.g., double-layer interface), or water phase (e.g., gelled water phase), and

thus different rates and extents of lipid digestion and release of encapsulated compounds.

1.1 Droplet Size

As was shown in this study, initial droplet size can significantly influence the cellular uptake of β-carotene loaded oil droplets by Caco-2 cells. Cellular uptake of β-carotene increased with reduced initial droplet size (p<0.05) (**Chapter 2**). However, after passing through the GIT, no significant difference in bioaccessibility and cellular uptake of β-carotene between emulsions with different initial droplet size was observed, suggesting that, after exposure to GIT digestion, differences in initial droplet size in a certain range (<5-fold) may not influence the rate and extent of digestion and subsequent cellular uptake of lipophilic components in emulsions (**Chapter 4**).

Generally, physicochemical properties of the delivery systems, including particle size, hydrophobicity, or charge will affect their absorption, distribution, metabolism, and excretion. Firstly, size is important for entry into cells, and cellular uptake mechanisms are partially dependent on size. Endocytosis is the process through which nanoparticles enter cells (Sahay et al., 2010). The common sizes of vehicles formed through endocytosis are between 60 nm and 1,000 nm, and particles larger than 500 nm are not endocytosed by enterocytes; thus, they are commonly excreted before reaching the bloodstream (Mitragotri1, 2010). Generally, the absorption of nanoparticles decreases with increasing size (Borel & Sabliov, 2014). This may explain why β-carotene encapsulated in emulsions with smaller droplet size showed higher cellular uptake than that in emulsions with larger droplet size.

1.2 Oil Phase

Likewise, compositions and microstructures of the oil phase of emulsions can significantly influence and digestion and bioavailability of encapsulated lipophilic components in emulsions. In this study, it was found that incorporation of monoglyceride (MG) into the oil phase of emulsion can significantly increase the bioaccessibility and cellular uptake of β-carotene, and the bioaccessibility and cellular uptake of β-carotene increased with increasing MG content (**Chapter 3**). After intestinal phase digestion, MG with free hydrophilic hydroxyl groups will potentially be incorporated into the newly formed micelles, and increase their hydrophilicity, and thus an easier approach to the brush border of Caco-2 cells (Phan, 2001), which accordingly results in a better interaction of these β-carotene loaded micelles containing MG with Caco-2 cells and thus improved cellular uptake.

Generally, bioaccessibility of encapsulated lipophilic components increases with increasing chain length of fatty acids of triglycerides in oil phase of emulsions (Tianyao Huo, 2007). The physical state of the oil phase can also influence the digestibility and bioaccessibility of lipophilic components, since this can affect the accessibility of lipase to the ester bond in the triacylglycerols molecules (McClements & Li, 2010).

1.3 Oil-Water Interface

The compositions of the interface of emulsion-based carriers for β-carotene can influence both the cellular uptake of β-carotene-loaded oil droplets before exposure to the GIT and the bioaccessibility and cellular uptake of β-carotene after passing through the GIT. In a test of direct absorption of β-carotene-loaded oil droplets by Caco-2 cells, after 4 h of incubation, an emulsion stabilized by WPI showed the

highest cellular uptake of β-carotene, followed by that stabilized with SCN and TW80 (**Chapter 2**). After exposure to GIT digestion, emulsions with different interfacial compositions showed different droplet aggregation and surface charge in the gastric phase digestion, and different bioaccessibility and cellular uptake of β-carotene-loaded oil droplets after the intestinal phase (**Chapter 4**).

As described in previous studies, the compositions and structure of the interfacial layers (emulsifiers) not only can influence the physical and chemical stability of emulsions (Qian et al., 2012; Yin et al., 2009), but can also affect the rate of lipid digestion, and the release and bioavailability of encapsulated components. A fast or sustained release of lipophilic components from emulsion-based delivery carriers can be controlled by designing specific structures of emulsions, e.g., selection of different emulsifiers, or formation of multilayer interfaces (Hou et al., 2014; Karthik & Anandharamakrishnan, 2016; Li et al., 2010).

1.4 Water Phase

In the study reported in **Chapter 5**, functional konjac glucomannan (KGM) was incorporated into the water phase of WPI-stabilized emulsions. KGM significantly increased the stability of WPI-stabilized emulsions towards creaming, pH-induced aggregation, and oiling-off during freeze-thaw test; KGM also modified the release of encapsulated β-carotene after GIT; emulsions containing KGM showed a lower final rate of release of encapsulated β-carotene than emulsion, without KGM, and the release rate decreased with increasing KGM content.

The physical state of the water phase of emulsions can modify the release of entrapped lipophilic components and thus their oral bioavailability. Oil droplets entrapped in the gelled water phase shows a decreased rate of lipid digestion

(McClements & Li, 2010). Several factors might result in this decreased rate of lipid digestion:

(i) Without gel in the water phase, the lipase can rapidly adsorb to the oil droplet surfaces and thereby quickly initiate the lipolysis process;

(ii) When oil droplets are encapsulated in alginate gels, lipase must diffuse through the gel network to reach them. The diffusion rate of lipase will be affected by the pore size of the gel network and specific or non-specific interactions (e.g., electrostatic or hydrophobic interactions) of the lipase with the molecules that comprise the gel network (Gombotz & Wee, 2012);

(iii) The movement of free fatty acids (FFAs) away from the oil droplet surface may be slowed down by the gel network surrounding the oil droplets, which accordingly increases the local concentration of hydrolysates (free fatty acids) and thus decreases the rate of enzymatic hydrolysis rate of lipase (Fave & Armand, 2004; Gilchrist & Martin, 1983).

However, gelled emulsion-based carriers can also show advantages in delivering fish oil compared with conventional soft gel capsules. A study reported that fish oils rich in DHA and EPA delivered by gelled emulsions showed a significantly increased mean plasma levels in health young adults after oral administration, compared with that delivered through soft gel capsules (Haug et al., 2011). The plasma C_{max} (μg/mL) level of EPA and DHA in gelled emulsions was significantly (p<0.05) higher than those in soft gel capsules, while the t_{max} (h) of these in a gelled emulsions was shorter than that in soft gel capsules, suggesting that improved oral bioavailability and fast absorption of EPA and DHA may be achieved by incorporating emulsified TAG fish oil in a emulsion-based carrier with gelled water phase prior to oral ingestion.

1.5 Dry Emulsions

Based on the findings of liquid emulsions, spray-dried and freeze-dried emulsions with good re-dispersibility were also prepared (**Chapter 6**), to achieve an extended shelf-life, and convenient and expanded application of lipophilic components delivered through emulsion-based delivery carriers.

Spray-dried and freeze-dried emulsions were obtained by formulating the water phase of emulsions with low dose of maltodextrin (MD) and/or konjac glucomannan (KGM). Dried emulsions showed different morphologies and surface microstructures, depending on the dry methods (spray-drying or freeze-drying), and the formulas of emulsions. All dry emulsions showed good re-dispersibility in water, and freeze-dried emulsions showed better re-dispersibility than spray-dried ones, immediately re-dispersing into O/W emulsions after contacting water. The finding of this study confirmed the potential of using low level of MD and/or KGM in the water phase as the wall materials when preparing dry emulsions, potentially reducing the cost of production, and making it possible to obtain stable dry emulsions that reconstituted well by structuring their water phase with functional biopolymers.

2. Overall Conclusions

The current study investigated the digestion, release and cellular uptake of encapsulated lipophilic components in model O/W emulsion based delivery carriers, and main conclusions in respect to the main objectives as described in **Chapter 1** are as follows:

- The initial droplet size and selection of emulsifiers (whey protein isolate, sodium caseinate, or tween 80) can significantly influence the emulsion stability (creaming and pH) and cellular uptake of encapsulated lipophilic components without passing through the GIT digestion;

- Incorporation of monoglyceride (MG) into the oil phase of emulsions can significantly improve emulsion creaming stability, modify the emulsion properties (droplet size and distribution, and surface charge) in GIT digestion, and improve the bioaccessibility and cellular uptake of encapsulated lipophilic components after passing through GIT;
- Interfacial compositions (emulsifiers) and initial droplet size can significantly influence the emulsion properties (droplet size and distribution, and surface charge) in the GIT digestion while the bioaccessibility and cellular uptake of encapsulated lipophilic components is mainly dependent on the selection of different emulsifiers (whey protein isolate, sodium caseinate, or tween 80), but not the initial droplet size;
- Incorporation of konjac glucomannan (KGM) into the water phase of WPI-stabilized emulsions can significantly increase their viscosity, and improve their creaming, pH, and freeze-thaw stabilities;
- KGM can slow down the release of encapsulated lipophilic components after GIT digestion and a lower final release rate was observed, confirming the potential of KGM in developing functional foods with sustained release properties;
- Dried emulsion powders showed good re-dispersibility (>85%) in water, and their re-dispersibility related to their compositions and microstructures;
- Re-constituted liquid emulsions from spray-dried powders showed shifted size distributions to large particle size while that of re-constituted liquid emulsions from freeze-dried powders showed shifted to the small particle size; KGM can significantly decreased the initial viscosity but increase the

creaming stability of re-constituted emulsions compared with those liquid emulsions before drying.

To summarize, the findings in the current study take a small step forward ton the study of emulsion-based delivery systems and contribute to a better understanding of the correlation between the emulsion properties and formulas with the digestion, release and absorption of encapsulated instable lipophilic bioactive components in emulsion-based carriers. The findings also confirm the potential of dry form of model emulsions as ideal delivery carriers for lipophilic components and make it possible to achieve desired delivery properties, e.g., sustained or potential target release, or improved bioaccessibility and cellular uptake, by designing proper emulsion-based delivery carries.

3. Further Works

Based on findings in this study and overall literature review, many emulsion-related subjects are worth exploring further.

First, the developments of edible emulsifiers without adverse health effects: A study on *Nature* (Chassaing et al., 2015) reported that two commonly used emulsifiers, namely carboxymethylcellulose and polysorbate-80, can induce low grade inflammation and obesity/metabolic syndrome in wild-type mice and promote robust colitis in mice predisposed to this disorder. This emulsifier-induced metabolic syndrome was associated with microbiota encroachment, altered species composition and increased pro-inflammatory potential. The results suggest that the broad use of emulsifying agents might be contributing to an increased societal incidence of obesity/metabolic syndrome and other chronic inflammatory diseases. Therefore,

naturally-derived safe emulsifiers with potential health benefits, e.g., food proteins and functional polysaccharides, are needed.

Second, parenteral nutrients: Intravenous emulsions have been in medical use for over 5 decades (Intra-lipid, approved in Europe in 1962). Parenteral nutrition emulsions supply critically ill patients with triglycerides, which cannot be fed orally. These triglycerides are metabolized to essential fatty acids in the human body to maintain its health. Emulsions are always composed of food materials and show safe metabolism. Emulsions also can significantly improve the water-solubility of hydrophobic active pharmaceutical ingredients. Many novel therapeutic agents have poor or no water solubility; formulating these substances as oil-in-water (O/W) emulsions can be an ideal way to overcome this hurdle and ensure the functionality of these substances (Chow et al., 2016; Hormann & Zimmer, 2016). In addition, parenteral emulsions can be used as delivery systems to target tissues in the body by designing their structures. Structured parenteral emulsions can be created to target to the liver (Dierling & Cui, 2005), lung and spleen (Nikonenko et al., 2014), brains (Madhusudhan et al., 2007; Wen et al., 2011), and tumors (Hormann & Zimmer, 2016). All these advantages make parenteral emulsions very promising delivery systems for lipophilic nutrients and drugs for clinical uses. Therefore, the development of safe parenteral nutrition emulsions with improved stability, reduced toxicity, and potential target delivery is worthy of further study.

Thirdly, dry emulsions: Dried emulsions have many advantages, including extended shelf life, reduced transportation cost, and ease of preparation and storage. However, little is known about the details in the rehydration of dry emulsions, and the digestion and release of encapsulated functional components from dry emulsions in the GIT; thus, more work need to be done on the relationship between the

microstructure of dry emulsions and their performance as microencapsulation carriers, since dry emulsions are receiving increasing attention for their potential as delivery systems for a variety of lipophilic components in specific applications.

Fourthly, the interaction of emulsion with food matrixes: In many cases, emulsions will be incorporated into other food matrix to create final functional foods. Hence, the influence of the environments in these food matrixes on the properties of emulsions and the release of encapsulated ingredients in emulsions should be further studied. In addition, the addition of emulsions can also influence the properties of food matrixes, such as texture, stability, and taste. The interaction of emulsions with environmental food materials and their influence on both emulsion and food matrixes is worth further study in the context of producing functional foods with stable physiochemical properties.

Lastly, applications in *Medicine* and *Immunology*: A recent publication on *Nature Materials* reported using Pickering emulsion to enhance vaccinations (Xia et al., 2018). As it is known, a major challenge in vaccine formulations is the stimulation of both the humoral and cellular immune response for well-defined antigens with high efficacy and safety. Adjuvant research has focused on developing particulate carriers to model the sizes, shapes and compositions of microbes or diseased cells, but not antigen fluidity and pliability. Particle-stabilized Pickering emulsions were then designed, which can retain the force-dependent deformability and lateral mobility of presented antigens while displaying high biosafety and antigen-loading capabilities. Compared with solid particles and conventional surfactant-stabilized emulsions, the optimized Pickering emulsions enhance the recruitment, antigen uptake and activation of antigen-presenting cells, potently stimulating both humoral and cellular adaptive responses, and thus increasing the

survival of mice upon lethal challenge. The pliability and lateral mobility of antigen-loaded Pickering emulsions may provide an easy, effective, safe and broadly applicable strategy to enhance adaptive immunity against infections and diseases. The findings also open a new venue to the extended application of emulsion-based technologies in disease and immune response areas.

References

Borel, T., & Sabliov, C. M. (2014). Nanodelivery of bioactive components for food applications: types of delivery systems, properties, and their effect on ADME profiles and toxicity of nanoparticles. *Annual Review of Food Science and Technology, 5*, 197-213.

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature, 519*, 92-96.

Chow, R., Bruera, E., Chiu, L., Chow, S., Chiu, N., Lam, H., McDonald, R., DeAngelis, C., Vuong, S., Ganesh, V., Chow, E. (2016). Enteral and parenteral nutrition in cancer patients: a systematic review and meta-analysis. *Annal of Palliative Medicine, 5*, 30-41.

Dierling, A. M., & Cui, Z. (2005). Targeting primaquine into liver using chylomicron emulsions for potential vivax malaria therapy. *International Journal of Pharmaceutics , 303*, 143-152.

Fave, G., Coste, T. C., & Armand, M. (2004). Physicochemical properties of lipids: new strategies to manage fatty acid bioavailability. *Cellular and Molecular Biology, 50*, 815-831.

Gilchrist, T., & Martin, A. (1983). Wound treatment with Sorbsan-an alginate fibre dressing. *Biomaterials, 4*(4), 317-320.

Gombotz, W. R., & Wee, S. F. (2012). Protein release from alginate matrices. *Advanced Drug Delivery Reviews, 64*, 194-205.

Haug, I. J., Sagmo, L. B., Zeiss, D., Olsen, I. C., Draget, K. I., & Seternes, T. (2011). Bioavailability of EPA and DHA delivered by gelled emulsions and soft gel capsules. *European Journal of Lipid Science and Technology, 113*, 137-145.

Hormann, K., & Zimmer, A. (2016). Drug delivery and drug targeting with parenteral lipid nanoemulsions - A review. *Journal of Controlled Release, 223*, 85-98.

Hou, Z., Liu, Y., Lei, F., & Gao, Y. (2014). Investigation into the in vitro release properties of β-carotene in emulsions stabilized by different emulsifiers. *LWT - Food Science and Technology, 59*, 867-873.

Karthik, P., & Anandharamakrishnan, C. (2016). Enhancing omega-3 fatty acids nanoemulsion stability and in-vitro digestibility through emulsifiers. *Journal of Food Engineering, 187*, 92-105.

Li, Y., Hu, M., Xiao, H., Du, Y., Decker, E. A., & McClements, D. J. (2010). Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings. *European Journal of Pharmaceutics and Biopharmaceutics , 76*, 38-47.

Madhusudhan, B., Rambhau, D., Apte, S. S., & Gopinath, D. (2007). 1-O-alkylglycerol stabilized carbamazepine intravenous o/w nanoemulsions for drug targeting in mice. *Journal of Drug Targeting , 15*, 154-161.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science , 159*, 213-228.

Mitragotri1, J.-W. Y. a. S. (2010). Polymer particles that switch shape in response to a stimulus. *Proceedings of the National Academy of Science of the United States of America, 107*, 11205-11210.

Nikonenko, B., Reddy, V. M., Bogatcheva, E., Protopopova, M., Einck, L., & Nacy, C. A. (2014). Therapeutic efficacy of SQ641-NE against Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy, 58*(1), 587-589.

Phan CT, T. P. (2001). Intestinal lipid absorption and transport *Frontiers in Bioscience, 6*, 299-319.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry, 132*(3), 1221-1229.

Sahay, G., Alakhova, D. Y., & Kabanov, A. V. (2010). Endocytosis of nanomedicines. *Journal of Control Release, 145*(3), 182-195.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013a). Influence of particle size on lipid digestion and beta-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry, 141*(2), 1472-1480.

Salvia-Trujillo, L., Qian, C., Martin-Belloso, O., & McClements, D. J. (2013b). Modulating beta-carotene bioaccessibility by controlling oil composition and concentration in edible nanoemulsions. *Food Chemistry, 139*(1-4), 878-884.

Tianyao Huo, M. G. F., Steven J. Schwartz, and Mark L. Failla. (2007). Impact of Fatty Acyl Composition and Quantity of Triglycerides on Bioaccessibility of Dietary Carotenoids. *Journal of Agricultural and Food Chemisty, 55*, 8950-8957.

Wen, C.-J., Yen, T.-C., Al-Suwayeh, S. A., Chang, H.-W., & Fang, J.-Y. (2011). In vivo real-time fluorescence visualization and brain-targeting mechanisms of lipid nanocarriers with different fatty ester: oil ratios. *Nanomedicine, 6*(9), 1545-1559.

Xia, Y., Wu, J., Wei, W., Du, Y., Wan, T., Ma, X., An, W., Guo, A., Miao, C., Yue, H., Li, S., Cao, X., Su, Z., & Ma, G. (2018). Exploiting the pliability and lateral mobility of Pickering emulsion for enhanced vaccination. *Nature Materials, 17*(2), 187-194.

Yin, L.-J., Chu, B.-S., Kobayashi, I., & Nakajima, M. (2009). Performance of selected emulsifiers and their combinations in the preparation of β-carotene nanodispersions. *Food Hydrocolloids, 23*(6), 1617-1622.

APPENDIX

Chromatograms of β-carotene and whey protein isolates &

Publications

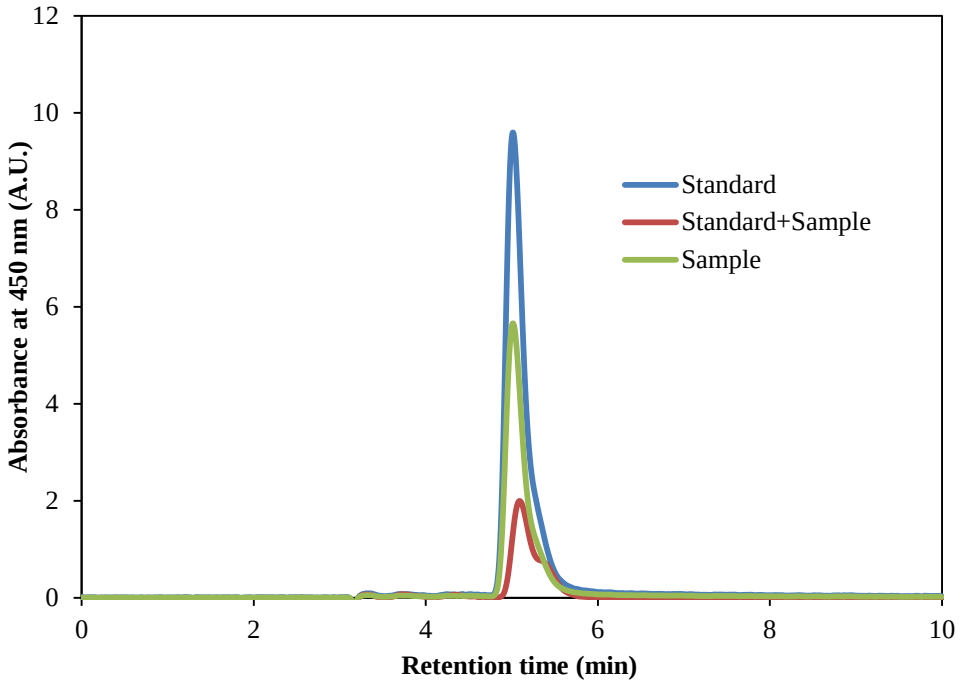


Figure s-1. Chromatogram of β-carotene. Standard: β-carotene dissolved in ethanol; Sample: β-carotene that was absorbed by enterocytes; Standard+Sample: mixture of Standard and Sample (1:1, v/v); mobile phase: ethanol: acetonitrile= 90:10 (v/v); flow rate: 1ml/min; column: C18 (Phenomenex, Jupiter, 5μm, 4.6*250 mm); detection wavelength: 450nm; injection volume: 20 μL.

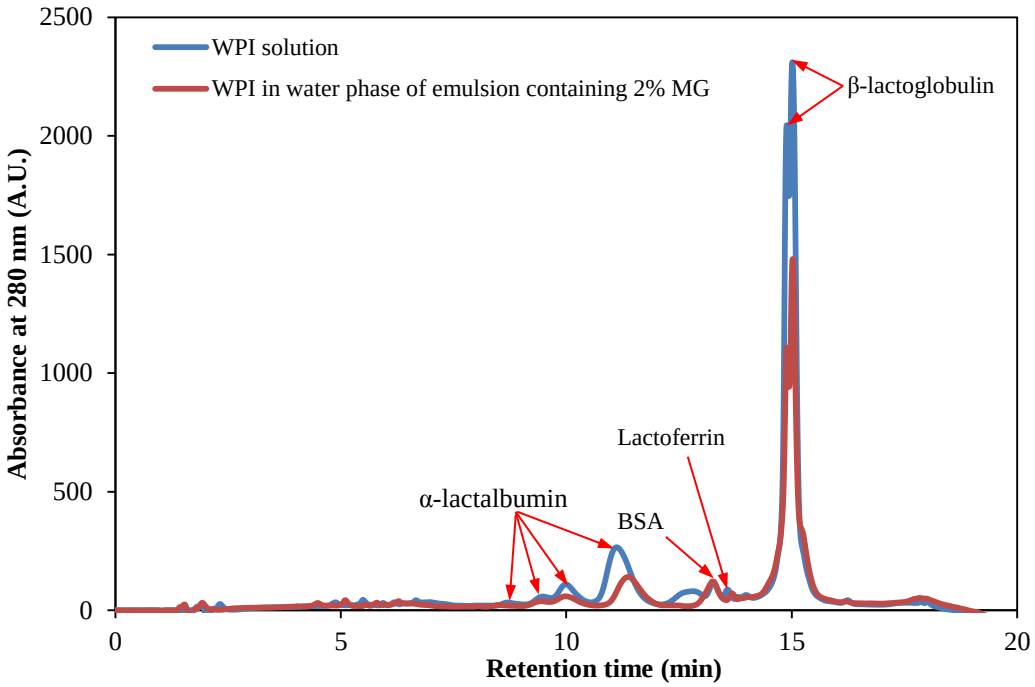


Figure s-2. Chromatogram of whey protein isolate (WPI). BSA indicates bovine serum albumin. MG indicates monoglyceride. Column: C18 (4.6×150 mm, 5 μm, Agilent); mobile phases, solvent A (acetonitrile with 0.1% TFA), and solvent B (water with 0.1% TFA); flow rate was 1 mL/min; detection wavelength was 280 nm; injection volume was 10 μL; gradient elution was performed from 20% A to 90% A within 20 min.