2015

FABRICATION, CHARACTERIZATION AND BIOLOGICAL FATE OF PHYTOCHEMICAL DELIVERY SYSTEM

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FABRICATION, CHARACTERIZATION AND BIOLOGICAL FATE OF PHYTOCHEMICAL DELIVERY SYSTEM

A Dissertation Presented

by

JINGJING CHEN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2015

The Department of Food Science
FABRICATION, CHARACTERIZATION AND BIOLOGICAL FATE OF PHYTOCHEMICAL DELIVERY SYSTEM

A Dissertation Presented

By

JINGJING CHEN

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Department of Food Science
DEDICATION

To my beloved families and respected teachers who always give me encouragements.
ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Dr. Xiao for his professional guidance and support to my research. Also for his wisdom, patience, and understanding. This study would not have been possible without his persistent help. To my committee members Dr. McClements, Dr. Decker and Dr. Wood, I am grateful for their suggestions. I would like to express my special appreciation to Dr. McClements for his academic suggestion and kindly help with my manuscript. Also for his generosity with chemicals and equipments in his lab. To Dr. Dale Callaham at Umass Central Microscopy facility for his assistance in using fluorescent and confocal microscopy. My study would not have been finished without their kindly support. To Dr. Jinkai, Mingfei, Fang, Jason, Hua, Cici, Zhengze, Minqi, Yue and Mingyue for their kindly help with my research. I thank other lab members Dr. Qi, Dr. Sun, Xian, Christina, Nok, Zili, Kun, Jiazhi, Tim, Will, Min and Xiaqiong for their support. Many thanks to the Food Science Department staffs: Jean, Fran, Deby and Dave. Warmest thanks to my friends Carlos, Rosa, Javier, Amira and Ran at Umass Campus Hotel for their friendliness. I would like to thank those kind-hearted strangers who helped me out when I was in trouble in the past four years. Last but not least, thank my beloved families for their unconditional support.
Polymethoxylflavones (PMFs) are a group of compounds with promising cancer preventing activities and many other health benefits. There’s a growing interest in fabricating delivery systems for PMFs as well as other phytochemicals due to their low water solubility. Firstly, we use nanoemulsion delivery system to encapsulate β-carotene. Sonication assisted method was developed to dissolve β-carotene to ensure minimum degradation. Powdered nanoemulsion was obtained after spray dry and freeze dry. Sample obtained after freeze dry showed better physiochemical characteristics. Then we use protein nanoparticle delivery system to encapsulate PMFs. The nanoparticle delivery system was fabricated by mixing the aqueous phase containing β-lactoglobulin with organic phase containing ethanol and tangeretin. Powder was obtained after vacuum evaporation of ethanol followed by freeze-drying. This powder can be easily dispersed into water and have similar property as freshly prepared, suggesting excellent applicability of the system as a convenient powder ingredient. Different delivery systems were made by mixing this powder with stock emulsion to represent various types of diets. These systems were then go
through in vitro digestion process. The delivery system contained 4% oil exhibited the highest bioaccessibility of tangeretin due to increased amount of mixed micelle formed after digestion in simulated small intestine. The following permeability determination experiments on Caco-2 cell monolayer model suggested digested sample with higher oil content has higher permeability than PMF that did not go through the digestion process. In order to further study the uptake and internalization of PMFs, a new approach to visualize polymethoxyflavones (PMFs) inside the cell and in mice colon using a fluorescence microscope was developed. 5, 3’, 4’-tridemethylnobiletin (THN) was used for further study due to strong fluorescent intensity upon conjugation with DPBA. Fluorescence spectroscopy indicated this conjugate has the maximum excitation wavelength of 490 nm and maximum emission wavelength of 570 nm. Both mass spectroscopy and Raman spectroscopy confirmed the reaction between one or two hydroxyl group on THN and diphenyl boron group on DPBA. This method could easily detect the PMFs in the single suspend cell or in the attached cell. It can also be used to visualize PMFs absorbed by mouse tissue such as colon.
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CHAPTER 1
INTRODUCTION

Polymethoxyflavones are a group of phytochemicals with many promising cancer prevention and other health benefiting activities. But due to the specific chemical structure, the water solubility of PMFs are very low. So it’s necessary to develop delivery systems to ensure sufficient bioactive compound could reach the small intestine so that enough compound could be absorbed. Besides, in recent years there’s an growing interest in nanoemulsion and nanoparticle based delivery system to increase the bioaccessibility and bioavailability of bioactive phytochemicals with low water solubility[1]. Related research would provide some insights for future application of functional food ingredients to improve the wellness of human being.

Among many different forms of delivery systems, polymer based delivery system has the advantage of stable, high encapsulate capacity, and may be able to realize controlled release. While nanoemulsion delivery system has the advantage of easy to fabricate, well-established techniques and versatile applications. After successful fabrication of nanoparticle or nanoemulsion delivery systems, it’s important to determine the biological fate of compound of interest inside human body. So in vitro digestion model was used in this study. Simulated gastrointestinal fluid was prepared with chemical reagents. The mouth, stomach environment was mimicked using 37 °C incubator. Small intestine environment was simulated using pH stat machine. The pH of the small intestine fluid was maintained at pH 7 using sodium
hydroxide titration. Mixed micelle would be tested on Caco-2 monolayer to see the permeability so as to indicate the final bioavailability of the protein delivery system. Despite the biological fate inside simulated gastrointestinal tract, the uptake of bioactive compounds inside the cell should also been determined. Since sometime delivery system may be applied through other ways instead of oral administration. Fluorescent detection is a powerful technique for quick and direct detection for compound of interest. It can be applied to fluorescent activate cell sorting to separate or enrich cells with particular feature based on the fluorescent after treated with the compound. It can also be used to track the cellular uptake of this compound inside the cell. In addition, it may be used for detecting the distribution of specific compound in animal tissue. In order to achieve fluorescent detection, a suitable fluorophore must be identified. We were able to find one fluorescent tag for our compound 5,3’, 4’-tridemethylnobiletin (THN). We used fluorescent spectrometer to detect the excitation and emission wavelength of this conjugate. We also tested the mass spectra of this fluorescent conjugate in order to elucidate the possible structure. This fluorescent staining method could be used to detect the uptake of THN with flow cytometer. Microscopy and Raman microscopy are two mostly used techniques to determine internalization of a compound or the change of carrier. Fluorescence microscopy uses the laser to excite a fluorophores. Different cell structure can be stained with corresponding fluorophores. Upon excitation with laser at different wavelength, different colored fluorescence can be recorded. Compound itself can also be tagged to a fluorophore, so that the location of this compound inside the cell can be tracked.
In conclusion, our long-term goal is to fabricate a delivery system that can effectively transport hydrophobic bioactive compounds to human body and understand the intracellular fate of these compounds. We will realize our goal by completing the following aims.

Specific aims are as follows:

1. **Fabrication of β-carotene nanoemulsion powders.** β-carotene nanoemulsion will be fabricated with high-energy microfluidization method. β-carotene will be dissolved in oil and optimal dissolving method will be developed to prevent β-carotene degradation. Both spray drying and freeze-drying method will be used to obtain dried nanoemulsion powder. These two methods will be compared in terms of powder property and encapsulated β-carotene content.

2. **Fabrication and characterization of polymethoxyflavone nanoemulsion delivery system.** 5-demethylnobiletin will be used as model compound to fabricate nanoemulsion delivery system. Different emulsifiers and oils will be used to make different nanoemulsion. These emulsions will be compared in terms of several physiochemical parameters. Nanoemulsion with best performance will be chosen for following permeability experiment to screen the oil content in the delivery system that may have best permeability.

3. **Fabrication of tangeretin loaded nanoparticle delivery system and biological fate determination.** The tangeretin loaded nanoparticle delivery system will be fabricated with antisolvent method. Different amount of tangeretin will be used to prepare this colloidal system. The concentration at which no crystal appears after preparation will be used in the following experiment. The delivery system will be
characterized for particle size, charge and morphology using zeta-sizer and transmission electron microscopy. The stability of this delivery system will be tested against environment stress such as salt, temperature and pH. The biological fate of the above tangeretin loaded zein nanoparticle colloidal system will be determined using in vitro digestion and Caco-2 cell monolayer model. Tangeretin nanoparticle system will be mixed with different amount of oil to represent different diet types. The bioaccessibility of these delivery systems will be determined through \textit{in vitro} digestion with simulated gastrointestinal fluid, including artificial saliva, simulated gastric fluid and pH-stat equipment mimicked small intestine environment. The bioavailability will be determined using digested micelle form in \textit{in vitro} digestion on simulated small intestine epithelium cell using Caco-2 monolayer.

\textbf{4. Fluorescent detection of PMF/PMF loaded zein nanoparticle inside the cell and in animal tissue.} Fluorescent tag will be chosen based on literature review of similar method used in flavonoid visualization in plant root. The conjugation between dye and PMF will be confirmed and characterized with Mass spectroscopy, Raman spectroscopy, and fluorescent spectroscopy. This method will be used to detect PMF in floating cells, attached cells and in PMF fed mouse tissue. The metabolites of THN and THN nanoparticles inside Caco-2 cell will also be identified. THN loaded nanoparticle delivery system will be fabricated with method described in aim 3. The intracellular localization of THN loaded zein nanoparticles will be determined using fluorescent detection method.
CHAPTER 2
LITERATURE REVIEW

2.1 Recent development on polymethoxyflavone study

2.1.1 Introduction

Polymethoxyflavones (PMFs) are a group of compound found in the tissues and peels of citrus fruits, such as tangerine, sweet orange and sour orange [2]. Their structures are similar to flavonoid, but with two or more methoxy groups on benzo-γ-pyrone backbone and also have a carbonyl group on C4 [3], as shown in Figure 1. Tangeretin and nobiletin are two major PMFs in the peels of citrus fruits. Study showed these two PMFs are highly permeable but poorly soluble [4]. The water solubility of polymethoxyflavones are generally very low due to multiple the existence of methoxy group. The oil solubility of some PMFs are also very low but still better than in water [5]. Hence there’s a growing interest in using delivery systems to increase the solubility of PMFs and with the ultimate goal of achieving increased bioavailability.

Figure 1. Structures of polymethoxyflavones.
2.1.2 Biological activities of PMFs

Accumulating studies has shown PMFs has many health promoting benefits, such as anticancer, anti-inflammation and anti-diabetes activities. Detailed information is listed below.

2.1.2.1 Antioxidant activities

Antioxidant activity is one of the most important activities of polymethoxyflavones. Many other health promoting activities of PMFs can be attributed to the antioxidant activities. Study showed raw fruit extracts which contains a mixture of PMFs can alleviate the organ injury (including heart, liver and kidney) of hypercholesterolemic rats due to their ability to regulate the antioxidant enzyme activities as well as lipid peroxidation level [6]. Most commonly found PMFs, tangeretin, was shown to have protective effect against Parkinson's disease due to neuroprotective function related with its antioxidant activity [7]. It can also decrease oxidative stress in rats with induced diabetes, mammary carcinoma and breast cancer [8-10].

2.1.2.2 Anti-inflammation

Inflammation activity is another important biological activity of PMFs. In vivo animal study showed pretreatment with 5-OH-HxMF could reduce the inflammation on mouse skin after topical application of tetradecanoylphorbol-13-acetate (a kind of skin tumor promoter)[11]. It was proposed that 3,5,6,7,8,3', 4'-heptamethoxyflavone may have anti-inflammatory effect on LPS treated mice hippocampus due inhibition of NF κ B and/or MARK[12]. Nobiletin can also alleviate the cognitive impairment related with age, oxidative stress and
hyperphosphorylation of tau in senescence-accelerated mice [13]. Recent study showed 5,3'-dihydroxy-3,7,4'-trimethoxyflavone and 5-hydroxy-3,7,4'-trimethoxyflavone from *Kamepferia parviflora* may have anti-allergic activity due to inhibition of RBL-2H3 cells by degranulation mechanism [14].

### 2.1.2.3 Anti cancer activities

PMFs were found to have anti-tumor effects, mainly due to their anti-proliferative and anti-carcinogenic activity [15]. For example, tangeretin and nobiletin could inhibit breast cancer and colon cancer cell growth by blocking cell growth at G1 phase [16, 17]. One PMF, 5-hydroxy-3, 6, 7, 8, 3', 4'-hexamethoxyflavone (5HHMF) was found to be able to inhibit the colony formation of human colon cancer cells potentially due to its ability to reduce β-catenin level in nuclear, as well as the inhibition of NF-κB nuclear translocation and potential angiogenesis activity [18]. 5-Demethyltangeretin was shown to inhibit the growth of human non-small cell lung cancer cell [19].

### 2.1.2.4 Obesity

Nobiletin, as another family member of PMFs, was shown to be able to improve the rat obesity and insulin resistance by modulating the gene expression related with lipid metabolism and insulin signaling activity [20]. Hydroxylated polymethoxyflavones could effectively inhibit the differentiation of fat tissue related cell line 3T3-L1. Animal study further confirmed hydroxylated polymethoxyflavones could reduce the body weight and lipid accumulation in liver for obesity mice. These results may indicate their potential activity against obesity [21]. Recent study
showed tangeretin could modulate lipid homeostasis in cardiac tissue of diabetic rats [9].

2.1.2.5 Other activities

Both tangeretin and nobiletin have antimicrobial activity against *Pseudomonas* due to their ability to decrease bacteria cell membrane permeability and prevent protein synthesis which will ultimately lead to cell death[22]. Recent study showed treatment of nobiletin could repair the short-term memory and recognition memory deficiency in mice, which may indicate it's potential in prevention and treatment of Alzheimer's disease [23]. Nolitetin may alleviate motor and cognitive impairment in Parkinson model mice[24]. Several PMFs had inhibition effect on the growth of human lens epithelial cells which may indicate their potential on preventing cataract formation[25].

**2.1.3 Bioavailability and metabolism of PMFs**

**2.1.3.1 Bioavailability**

The bioavailability was defined as the percentage of original administered compound that reaches the systemic circulation. The total bioavailability can expressed as:

\[ F = F_L \times F_A \times F_D \times F_M \times F_E \]

\( F_L \) is the amount of active compound liberated from the delivery system that is bioaccessible for epithelium cell[26]. A, D, M, E represents four stages of drug metabolism: absorption, distribution, metabolism and excretion. Hence, each part represents the fraction of unchanged active compound after absorption, distribution, metabolism and excretion [27]. In this equation, \( F_L \) is the fraction of
active compound released from the mixed micelle after digestion in gastrointestinal tract. Hence the amount of compound solubilized in the original delivery system has a major influence on the $F_L$ and finally to the bioavailability. For polymethoxyflavones, due to multiple methoxy groups on the ring, the water solubility are very low for PMFs (<100 µg/ml) [3]. A compound must be in the soluble form before it can be absorbed. Therefore it is very important to develop a potent system for the delivery of lipophilic nutraceutical PMFs. Animal study also confirmed that the oral bioavailability of a methoxyflavones mixture containing 5,7-dimethoxyflavone, 5,7,4’-trimethoxyflavone and 3,5,7,3’, 4’-pentamethoxyflavone range from 1 to 4%, which is very low[28]. However nobiletin dissolved in corn oil can be absorbed faster than crystal suspension administered to rats. The overall serum level of nobiletin was lower if administered in crystal suspension form [29]. The bioavailability can differ between different PMFs. For example, the total serum concentration of nobiletin was significantly higher (9.3 µg/mL) than tangeretin (0.49 µg/mL) when administered to rats by gavage [29].

Despite in vivo animal study, in vitro cell model can be used for quick screening of compounds with high bioavailability [30]. Caco-2 cell is cancer cell line originated from human colon. When maintained under certain condition it can differentiate into a monolayer, which could resemble human small intestine epithelial cell in terms of phenotype, morphology and characteristic enzymes [31]. Schematic representation can be seen in Figure 2. Caco-2 cells are usually grown on permeable filter supports and the apparent permeability coefficient ($P_{app}$) are determined to indicate compound permeability across human small intestinal
epithelium [32]. Compounds can be classified as well absorbed, moderately absorbed and poorly absorbed according to the apparent permeability coefficient. 

\[ P_{app} < 1 \times 10^{-6} \text{ cm/sec}, 1 - 10 \times 10^{-6} \text{ cm/sec and } > 10 \times 10^{-6} \text{ cm/sec} \]

can be classified as poorly, moderately and well absorbed compounds [33]. Interestingly, recent study showed the existence of structurally similar flavonoids such as quercetin and luteolin may prevent the absorption of nobiletin [34]. There's a growing interest in the study of using delivery system to increase the bioaccessibility and bioavailability of PMFs both in vitro and in vivo. Viscoelastic emulsion system was used to incorporate tangeretin. In vitro digestion model and in vivo model both suggested the higher bioaccessibility of tangeretin in viscoelastic emulsion than oil suspension [35]. Incorporation of PMFs into mixed micelle may result in increased uptake by Caco-2 cells. The bioavailability of PMFs was further increased by formation of chylomicron that encapsulate PMFs inside and transport into circulation through lymph system [36].
Figure 2. Schematic representation of Caco-2 monolayer. After culturing the Caco-2 cell on the transwell support for about 28 days, cell will differentiate and the morphology could represent small intestine epithelium cell so that it can be used as intestinal absorption model to predict bioavailability.

2.1.3.2 PMF metabolism

Nutraceutical metabolism can be divided into two phases. Phase I is the modification process where oxidation, reduction and hydrolysis occurs, mainly in the liver. The major enzyme participating in Phase I metabolism is cytochrome P450 (CYPs)[37]. Phase II is a conjugation process during which metabolites will conjugate with sulfate, glucuronic acid and some other charged substrates. Many studies had identified the metabolites of PMFs and the main enzymes that catalyze the production of various metabolites. Nobiletin is the one of the most studied PMFs in terms of metabolism. Six phase I metabolites have been reported [38, 39]. Besides, the transformation between nobiltetin metabolites and other PMFs was
also reported [40]. Information was summarized in Figure 3. Major enzymes that can catalyze the formation of different metabolites are listed in Table 1 [41]. Some PMFs metabolites may have higher anticancer activities than their parent compounds. 5-demethylnobiletin is one metabolites of nobiletin. Study showed the inhibition of non small cell lung cancer cell (NSCLC) growth was significantly higher for 5-demethylnobiletin than nobiletin [42]. Animal study further showed 5-demethylnobiletin can be metabolized into 5, 3'-didemethylnobiletin, 5, 4'-didemethylnobiletin and 5, 3', 4'-tridemethylnobiletin[43]. These three metabolites all showed higher inhibition against NSCLC growth. In particular, 5, 3', 4'-tridemethylnobiletin (THN) has the strongest activity. The IC$_{50}$ value of THN on H460 cell, h1299 NSCLC cell was 26 to 49 fold lower than its parent compound 5-demethylnobiletin. It was about 165 to 219 fold lower than the parent compound of 5-demethylnobiletin, i.e. nobiletin [44]. Study reported 5,7-DMF and 5,7,4'-TMF could be transformed into 5,7-dihydroxyflavone and 5,7,4'-trihydroxyflavone by human intestinal bacterium[45].
Figure 3. Transformation between some polymethoxyflavones.

Table 1 Major CYPs that catalyze the formation of different nobiletin metabolites.

<table>
<thead>
<tr>
<th>PMFs</th>
<th>CYPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>4’-OH-Nobiletin</td>
<td>CYP1A1, CYP1A2, CYP1B1</td>
</tr>
<tr>
<td>7-OH-Nobiletin</td>
<td>CYP3A4 (CYP3A5*)</td>
</tr>
<tr>
<td>6-OH-Nobiletin</td>
<td>CYP3A4 (CYP3A5*)</td>
</tr>
</tbody>
</table>

*CYP3A5 also catalyze the formation of 7-OH-Nobiletin and 6-OH-Nobiletin much more slowly.

2.2 Fabrication and characterization of delivery system

2.2.1 Introduction to delivery system

Delivery system is widely used to encapsulate, protect, or release hydrophobic nutrients, drugs and other flavors. The reason for using delivery system mainly include increased compound loading capacity, protecting the compound of interest from oxidation[46], achieving targeted release at certain gastrointestinal location.
and odor shielding [26]. There are many different kinds of delivery system such as liposomes, solid lipid nanoparticles, micelles, nanosuspensions, microemulsion, microgel, microcluster and nanoemulsion [47]. Lipid-based delivery system is the most commonly used[48]. Within many kinds of lipid based delivery system, nanoemulsion delivery system is one of the most effective way of encapsulating lipophilic compounds [49]. Because the small particle size contributes to the potential advantage of high stability, better appearance and better food matrix compatibility [50]. Study also showed the smaller the particle size, the better the uptake, therefore the better bioavailability [51]. Polymer based nanoparticle delivery system is another promising delivery system for transporting bioactive compound. The advantages of polymer based delivery system may include low toxicity, better binding capacity to bioactive compound and biodegradable property of the matrix [52]. For example biodegradable polymer poly (lactic-co-glycolic acid) or PLGA has been used as carrier to deliver dexamethasone for vascular injury and the system showed controlled release [53]. Studies also showed PLGA combined with cell penetrating peptide delivery system could provide increased cellular uptake for paclitaxel [54].

2.2.2 Nanoemulsion delivery system

2.2.2.1 Introduction

Nanoemulsion is one of the most simple and widely used system to encapsulate, protect and delivery bioactive component such as flavors, vitamins, carotenoids and fatty acids. It is a system with one liquid dispersed evenly in the other liquid. These two liquids are usually immiscible to each other. Depending on the nature of the
continuous phase, nanoemulsion can be classified as water in oil nanoemulsion and oil in water nanoemulsion. Nanoemulsion usually has the particle diameter less than 100 nm. It is believed smaller particle could lead to better absorption, hence better bioavailability [55].

2.2.2.2 Nanoemulsion fabrication and characterization

**Composition:** Nanoemulsion was composed of two immiscible phases, i.e., water phase and oil phase. Emulsifier, water to oil ratio and homogenization condition all play important roles in the formation of small particles. Emulsifier can be dissolved in the oil phase or the water phase depending the polarity of the emulsifier. They can be small protein molecule, polysaccharide, phospholipid or non-ionic surfactant such as Tween 20. Different emulsifier type will not only influence particle size but also the digestibility of the delivery system[56]. Oil type also plays a very important role for the property of nanoemulsion. Since different have different viscosity. The stability of nanoemulsion is governed by the interfacial tension on the particle surface. Viscosity has a major impact on the easiness of oil droplet disruption. Closer viscosity between oil and aqueous phase may result in efficient disruption of oil droplet. This would effectively prevent interfacial tension decrease and result in better stability [57]. In addition, oil composition may have significant influence on the digestibility of the delivery system[58-61]. Long chain oil may result in a slower digestion rate than short chain oil due to slow production of free fatty acid[56]. For example, nanoemulsion made with non digestible oil such as orange oil, had significant low bioaccessibility than that prepared with digestible oil such as corn oil[58]. Oil in water nanoemulsion usually has a lipophilic core (Gharsallaoui, #10)
phase and a hydrophilic shell (emulsifier) as shown in Figure 4. The content of emulsifier would have a direct impact on the nanoemulsion droplet composition. In order to cover the surface of oil droplet with a defined particle size, certain amount of emulsifier must be reached.

**Figure 4.** Schematic representation of oil in water nanoemulsion structure.

**Fabrication:** Proper preparation method of nanoemulsion should be carefully selected to obtain desired nanoemulsion functionality. Generally speaking, there are two kinds of approaches, high-energy approach and low energy approach to fabricate nanoemulsion. High-pressure valve homogenizer, microfluidizer and ultrasonic homogenizer are three widely used equipment using high energy approach. The general principle is to use high-intensity energy (pressure or ultrasonic wave) to disrupt oil phase and water phase into small droplets. **High pressure valve homogenizer (Figure 5a)** is most widely used in the food industry. A coarse emulsion was firstly prepared before applying the homogenization
pressure. The coarse emulsion would be disrupted into small droplets by high pressure upon passed through a narrow valve. Homogenization condition can influence the characteristic of emulsion. Higher pressure and repeat processing may lead to smaller particle size due to increased force to break oil droplet into small particles and simultaneously covered by emulsifier to preventing from aggregating together[62]. In conclusion, it's important to optimize preparation condition to obtained nanoemulsion based delivery system with desired physiochemical characteristics. Microfluidizer (Figure 5b) is a equipment similar to high pressure valve homogenizer. After entering the receiving chamber, the feed will be separated into two small streams. Then the constant high pressure will drive these two streams into the reaction chamber and where they will collide with each other. Then the small droplet will be produced and covered with emulsifiers. Ultrasonic homogenizer (Figure 5c) use the strong mechanical vibration generated by ultrasonic wave to disrupt liquids into small droplets. Particle size of emulsion tends to decrease as treatment time and intensity increases. A major drawback of this method may be the uneven distribution of energy intensity may result in denaturation of protein-based emulsifier.
Figure 5. Principle of a: high pressure valve homogenizer; b: microfluidizer; c: ultrasonic homogenizer.
**Low energy approaches** include spontaneous emulsification and phase inversion methods. Phase inversion method can be further divided into phase inversion temperature method, phase inversion composition method and emulsion inversion point method. Spontaneous emulsification can occur when two phase of liquids are mixed together. This can happen when certain conditions are met. For example, the oil phase and the water phase are immiscible to each other. But if one liquid contains component that can also partially dissolved in the other liquid, when mixing together, this component may migrate from its original liquid phase to the other phase. This will result in increased interfacial area and appearance of emulsion droplets [50]. Phase inversion method often refers to the change of oil in water (O/W) emulsion to water in oil (W/O) emulsion or from W/O to O/W emulsion. The phase inversion temperature method depends on the change of emulsifier property at different temperature so that O/W can be transformed to W/O or vice versa. Phase inversion composition method depends on the change of system composition to alter the structure of the system. Sometimes salt can be added to the system to change the structure of the emulsifier[63]. Emulsion inversion point method does not rely on the change of temperature or composition of surfactant but the ratio of oil to water. Phase inversion happens when the content of one liquid reaches its critical content. These phase inversion methods has been used to make polymer particles and emulsions [64-67].

**Particle size:** Particle size is one of the most important parameter when characterize a nanoemulsion system. Particle size was determined by both preparation method and nanoemulsion composition. Particle has a direct influence
on the appearance, stability and potentially biological fate of the delivery system. Nanoemulsion with small particle size tend to be clear, because the particle diameter is smaller than the wavelength of visible light. On the other hand, nanoemulsion with larger particle size tend to be semi opaque or opaque. Particle size is usually determined with dynamic light scattering method and represented with particle size distribution.

**Charge:** Due to the electrical charge of ionic surfactant, ions from oil or aqueous phase, nanoemulsion droplet usually has specific electrical charge. The particle charge also has a great influence on the stability and functional performance of the delivery system. High absolute charge may indicate high stability of the droplet since the charge may prevent particles from getting together. Zeta potential is one of the most convenient way to represent particle charge.

**Appearance:** Nanoemulsion can be clear, opaque or semi opaque depending on the particle composition described before. It is important to determine the optical characteristic of nanoemulsion since it may influence what kind of food system it could be incorporated into. The optical property of nanoemulsion can be characterized using opacity.

**Stability:** Unlike microemulsion, nanoemulsion is thermodynamically unstable. Gravitational separation, Ostwald ripening, flocculation and coalescence may all lead to nanoemulsion break down [56]. It important to design nanoemulsion with certain characteristic so that possible particle aggregation can be avoided. For example, nanoemulsion prepared with ionic emulsifier tend to have high negative or high positive zeta potential, they are usually stronger than the van der Waals
interaction and hydrophobic interaction. The overall energy barrier tends to be high so that particle aggregation can be prevented.

2.2.3 Biopolymer based delivery system

2.2.3.1 Introduction

There’s an increasing interest in the study of using biopolymer nanoparticles to delivery functional ingredients or drugs[68]. The particle size and surface characteristic of biopolymer nanoparticles can be controlled so that certain desired functionality may be achieved. Biopolymer nanoparticles are usually made of food grade protein or polysaccharides such as gelatin, cellulose, whey protein starch and so on. In addition to these large molecules, water, lipids or sugar may also exist in the nanoparticles. For example, lipids can incorporated in the nanoparticles to dissolve lipophilic bioactives. Some ions may be added to facilitate the gelation of the matrix. Biopolymer delivery system can be fabricated with several physiochemical methods. For example, biopolymer particles can be formed by protein or polysaccharide gelation, or by molecular complex. Molecular complex can be further divided into single molecule or by mixed molecules. Some biopolymers can be fabricated using processing operation method such as extrusion, homogenization and injection methods[69].

2.2.3.2 Zein based nanoparticle delivery system

Zein is a protein from maize considered as prolamine due to its characteristic of insoluble in water but soluble in ethanol. This unique feature was due to the composition of more than 50% of nonpolar amino acid such as alanine, valine and phenylalanine[70]. There are four types of zein, α (19 and 22 kDa), β (14 kDa), γ (16
and 27 kDa) and δ (10 kDa) based on the solubility [71]. Of these four types of zein, α-zein has the highest solubility in alcohol and also major zein in maize so it is most widely used.

2.2.3.2.1 Application of zein as a bioactive compound carrier

Zein has many applications in food and biomedical industries as well as for drug delivery due to its unique characteristics[72]. Soluble in ethanol but insoluble in water can be utilized in antisolvent production of zein nanoparticle colloidal systems. Gelling property can be used to make zein hydrogels due it’s large molecular weight and unique structure. It’s adhesive nature can be utilized to for mucoadhesive compound delivery. Special helix structure can be used to realize controlled release of compound of interest. The inherent antioxidant property of zein can be used to protect compound of interest from microbial spoilage[73]. It is reported N-terminal of zein can translocate cell membrane, thus it can be a carrier for compound to cross cell membrane[74]. The mucoadhesion property of zein can increase absorption for zein based drug delivery system [75].

There are several different forms of zein based delivery system, mainly include film, fibers and nanoparticles [76]. Zein film is usually fabricated through solvent casting, extrusion method or spin casting. Raman Spectroscopy analysis showed zein film was formed through hydrophobic interaction supported by the vibrational modes at 1565 cm⁻¹ for amide I and at 1274 cm⁻¹ for amide II [77]. Due to grease and solvent resistance property, the film can be used in fruits, doughnuts, pies and cookie coating[78]. Zein fiber can be prepared through electrospin of zein solution into organic solution containing ethanol and isopropanol. A fiber with smooth surface
and diameter around 300 nm can be produced with this method. It has been used to encapsulate fish oil, ω-3 fatty acid, α-tocopherol, peppermint oil and gallic acid to achieve the aim of protecting, delivery as well as controlled release [76, 79-81]. Although zein itself could form nanoparticles due to its hydrophobic characteristic, studies indicate that when using certain emulsifier such as Vitamin E TPGS, the uptake of zein nanoparticle could be improved. Zein nanoparticles were usually prepared through antisolvent method of introducing organic zein solution to aqueous solution containing emulsifier or at controlled pH. The small particle size of bioactive compound loaded zein nanoparticles could favor targeted delivery and controlled release[82-87]. Study showed the uptake of 5-fluorouracil encapsulated in zein nanoparticle followed by intravenous administration could be 2.9 fold higher than the that dissolved in solution[85].

In addition to the delivery systems fabricated with zein along, there are matrix fabricated with zein in combination with other polymers such as chitosan, polyethylene glycol (PEG) and cellulose[88]. By using PEG-zein micelle, the loading capacity of curcumin could be 1000-2000 folds higher than dissolved in water. The use of this delivery system also results in better absorption than plain curcumin solution[89]. Recent study reported significantly higher curcumin solubility (17530 folds) with zein-mPEG-PVL (methoxy poly(ethylene glycol)-b-poly (8-valerolactone)) nanoparticle delivery system. Animal study also showed increased curcumin concentration in plasma for curcumin administered in nanoparticle delivery system other than plain curcumin solution control group [90].
Study showed cellular uptake of zein nanoparticle is an endocytosis process that depend on energy. The uptake of zein nanoparticle depend on emulsifier content [91]. So far, there’s only a few studies focus on the change of zein after zein based delivery system was applied to cell culture or to animal model.

2.2.3.2.2 Zein degredation:

Studies showed corn zein can be degraded at high temperature (40 °C) and high moisture under netural condition (50%-60%)[92]. Being a protein, zein microsphere can be degraded by protease such as pepsin, thermolysin and trypsin[93, 94]. If administered through the routes with no enzyme, the release of drug from zein microsphere was slow, indicating the resistance of zein from degradation without enzyme.

2.3 Biological fate of delivery system

2.3.1 Gastrointestinal tract

After oral intake, delivery system will encounter a series of different environmental conditions through human gastrointestinal tract. An understanding of these conditions would provide some implication of potential change occur to delivery system after digestion.

**Mouth:** Mouth has the pH of 5-7. After ingestion, the delivery system will be mixed with saliva. Mucin from saliva may coat on the surface of the particle in the delivery system. Due to short residence time in mouth, the environment will have less effect on the delivery system.

**Stomach:** Human stomach is an acidic environment, with the pH around 1 to 3. After passing through mouth, the delivery system will mixed with enzymes and
proteins in the stomach fluid. Delivery system made with protein emulsifier will aggregate together due to the low pH that may near the isoelectric point of protein. Lipid and protein from the delivery system may be digested by gastric lipase and gastric protease.

**Small intestine:** Small intestine has the pH around neutral. It is the main digestion location of human body. There are a lot of salts and enzymes in the small intestine fluid such as bile salts, phospholipids, colipase and pancreatic lipase. Lipid will be digested by lipase into monoglycerides and free fatty acids. The free fatty acids will be mixed with bile salts and phospholipids and form mixed micelles.

In vitro digestion (Figure 6) is a mostly used method to study the potential biological fate of different delivery systems.
Figure 6. Schematic representation of in vitro digestion process. Delivery system was firstly mixed with artificial saliva, incubate at certain temperature time, then mixed with gastric fluid, incubate for certain time and finally mixed with salt, lipase and bile salt.

2.3.2 Intracellular fate of bioactive compounds

After digestion in small intestine, the mixed micelle will be absorbed by epithelium cell. A detailed absorption process is illustrated in Figure 7. The monoglycerides, free fatty acid and bioactive component will be transported to epithelium cell surface in the form of mixed micelle. The existence of mucus layer on epithelium cell surface may favor the residence of mixed micelle on enterocyte cell surface. Mixed micelle was absorbed through two mechanisms: active process at low FFA concentration and pass process at FFA concentration[95]. Bioactive component inside the mixed micelle may first be released and then diffused across cell
membrane. Monoglyceride and free fatty acid will reassemble and form triglyceride inside enterocyte cells and may further be packed into chylomicrons. Bioactive compound inside chylomicrons will be transported through lymph circulation to different part of body.

Figure 7. Schematic representation of nanoparticle uptake and distribution by small intestine epithelium cells.

Advances in delivery system for bioactive nutraceuticals requires proper detection methods to locate the compound of interest inside the tissue or the cell and to detect possible interaction between carrier and cell structures. And finally understand the mechanism for uptake so that more effective delivery system can be developed.

Within various kinds of approaches, light microscopy is the mostly used method to
visualize cells. It could only provide some basic information such as cell morphology and cell condition other than specific structure information[96]. Other technology such as transmission electron microscopy and atomic force microscopy are also used to visualize cells. These two methods could only provide more detailed information about cell organelles. But they can only get information from sample surface, meaning cells must be properly treated and sectioned. Fluorescent microscopy and Raman microscopy are two mostly used methods for visualize cell and to locate delivery system inside cell.

2.3.3 Intracellular detection of bioactive compounds

2.3.3.1 Fluorescence microscopy

Fluorescence microscopy is a powerful tool for analyzing biological samples. By using sensitive and specific staining, distribution of bioactive compound and protein can be visualized under fluorescence microscope. The principle for fluorescence microscopy is when exposed to light of certain wavelength, the active functional group in the fluorophore will be excited and a light of longer wavelength will be emitted. The emitted signal than goes through a filter, so that specific signal at known wavelength can be captured [97]. Epifluorescence microscope and confocal microscopy are two most common microscope. Confocal microscope can offer better resolution due to the use of point illumination and a pinhole to eliminate signals that are out of focus. Both fluorescent microscopy and confocal microscope are well established method for detection of cellular components.

Different cellular components can be tagged with corresponding fluorophores. Then these fluorophores can be excited with laser with different wavelength. By taking
fluorescent image at different time intervals, the location for compound of interest may be determined. These information may provide some implication for the mechanism of compound or delivery system uptake. For cellular components, there are commercially available tags or immunohistochemistry markers. But for compound of interest or delivery system, certain fluorescent tag must be added before they could be observed under a fluorescent microscope.

There are several approaches for tagging target compound. The most ideal scenario to use the intrinsic fluorescent characteristic of compound or cell structure itself. Such as green fluorescent protein, when illuminated with light with proper wavelength, can emit green fluorescent. This characteristic has been widely used in nuclear localization, indication for live cell imaging, thrombin activity detection and disease virus protein localization [98-102]. Nutraceutical such as curcumin can exhibit fluorescence itself upon illumination of light at certain wavelength[103]. Many studies has used this unique feature to study the internalization and localization of liposomal curcumin [104], curcumin microemulsion [105], micelle [106] to normal and cancer cell [103]. For compounds that do not have intrinsic fluorescent property, several methods can be used to become "visible" inside the cell. One method is to prepare fluorescent conjugate before administering the compound to the cell. For example, fluorescein isothiocyanate (FITC) could be conjugated to insulin that was encapsulated PLGA nanoparticles. Then Caco-2 cell was treated with these nanoparticles. The location of the nanoparticle can be identified by illuminating fluorescent tag FITC [107]. Rhodamine123, another commonly used fluorophore was encapsulated in dextran sulfate-PLGA hybrid
nanoparticles and then was applied to breast cancer cell (MCF-7) to study the cellular uptake of vincristine sulfate [108]. Paclitaxel nanoparticle was conjugated with transferrin, so that the its retention inside the cell can be tracked by detecting fluorophore transferrin [109]. One major disadvantage of this approach is that incorporation of fluorophores inside the cell may cause some toxicity [110]. Another method is to tag the compound of interest with fluorophore or similar dye after treating the cell. Studies reported treating human keratinocytes with 2-aminoethyl diphenyl borate (DPBA, or Naturstoff reagent A) after incubation with cyanidin. Since cyanidin can form fluorescent conjugate with DPBA, so its location inside the cell can be identified [111]. This method is simple also cause no toxicity to the cell as long as no the study does not focus on the intracellular localization of living cells. In order to prevent quenching during laser illumination, anti fade medium can be used when preparing sample slides [112]. Although fluorescent microscopy can provide some knowledge in terms of intracellular location, more information are still needed to fully understand the biological fate of a delivery system.

2.3.3.2 Raman microscopy

Raman spectroscopy is an emerging tool for the analysis of cellular uptake of bioactive compounds. By analyzing Raman scattering fingerprint upon exposure to laser of different wavelength, the vibrational or rotational modes information of a system can be obtained[96]. These vibrational or rotational modes could provide many useful information in terms of cellular structure or compound that has been applied to the cell. Cell is mainly composed of protein, nucleic acids, carbohydrates and lipids. The characteristic Raman band position for nucleic acids is between 785-
788 cm$^{-1}$, protein 1002-1005 cm$^{-1}$, lipids and protein 1425-1475 cm$^{-1}$ and 2800-3020 cm$^{-1}$. There's a silent region between 1800-2800 cm$^{-1}$[96]. It would be ideal if the compound of interest has characteristic resonance in this region.

Raman technology has been used to study the cellular uptake of liposomes. The uptake can be visualized by using deuterated phospholipids. Since this deuterated phospholipids has CD stretch vibration in the silent region between 2000-2300 cm$^{-1}$[113].

Raman spectroscopy can also be used to monitor the digestion of the carrier system. For example, the degradation of PLGA nanoparticle inside the cell can be monitored by monitoring the shift of peak at 1768 cm$^{-1}$. Since the degradation of PLGA polymer starts from the hydrolysis of ester bond between lactic and glycolic acid[114]. Study showed PLGA nanoparticles inside the cell began to degrade 3 to 6 h after incubation. Combined with the increase of characteristic lipid peak at 1740 cm$^{-1}$, the author conclude the PLGA nanoparticle was included inside a vesicles [115]. Similar study reported the colocalization of PLGA nanoparticle and lipid body inside the cell [116].

The Raman spectra of compound in solution have also been studied. This is mainly realized by tagging a specific group to the compound molecule so that peaks can be seen in the Raman silent region of cell. Alkyne groups is one of the mostly used tag due to their rare existence inside the cell and characteristic Raman peak in silent region[96]. This method has been used in live cell imaging to study the change of DNA, RNA, protein and phospholipids after treatment of alkyne tagged drug [117-119].
Surface enhance Raman spectroscopy is a label free method to increase Raman signal of some compound by using man-sized metal particle coated surface. This method could increase the Raman signal of some Raman insensitive compound. This technique has been widely used in the detection of trace amount of pesticide and low concentration of adenosine [120-123]. More importantly, SERS can be used to detect certain cell structures by conjugation of organelle specific antibody with silver or gold nanoparticles. Study has reported the use of Raman spectroscopy to monitor the intracellular release of doxorubicin. By using a biohybrid nanoparticle composed of gold nanoparticle, cell penetrating peptide, polyethylene glycol and cancer-targeting antibody, the increased uptake and targeted release of anti cancer drug doxorubicin can be realized [124]. Recently, a SERS method to was developed to get high-resolution cell image of nucleus, mitochondria and cytosol. It was achieved by incorporation of reporter molecule into a special gold nanoparticle intra gap and then conjugation with cell organelle specific peptide. When illuminated by light, reporter molecules showed strong Raman signal [125].
CHAPTER 3

FABRICATION OF β-CAROTENE NANOEMULSION POWDER

3.1 Introduction

β-carotene is an orange colored compound belongs to carotenoid family that can be found in many fruits and vegetables such as carrot, tomato and spinach[126]. β-carotene is sensitive to light, heat and oxygen due to multiple conjugated double bonds on its unique chemical structure[127]. This also makes it an important compound for human health because it is the precursor of vitamin A[128]. Besides, β-carotene is an important antioxidant. Studies showed it could lower oxidative stress for workers who were exposed to lead[129]. In addition, many studies has reported other functional activities of β-carotene such as reduce the risk of type 2 diabetes [130], lower metabolic syndrome in middle-aged adults[131], enhance immune system performance, as well as reduce the risk of cardiovascular disease[132].

β-carotene is insoluble in water, soluble in some organic solvent such as tetrahydrofuran and chloroform[133] and slightly soluble in oil [59]. So it’s necessary to find suitable food matrix to encapsulate β-carotene before delivered into human body. Nanoemulsion is one ideal delivery system for lipophilic compounds due to the unique composition of oil that can dissolve these compounds and small particle size that may lead to higher bioavailability [134]. In addition to conventional nanoemulsions in the liquid form, we also want to see the functional performance of dried nanoemulsion. Since dried nanoemulsion powder may be more versatile in terms of applications in different food system and may lower the
risk of deteriorative of the nanoemulsion due to reduced water content [135]. In this study, we will focus mainly on the application of spray drying and freeze drying to make dried β-carotene nanoemulsion powder. Spray drying is a major drying method that has been used for decades. The short drying time and high drying temperature makes it an ideal and economical drying technique for many heat sensitive food materials and pharaceuticals such as milk powder, vitamins and flavorings[136]. Freeze drying is the drying method that provide the optimal final product quality compared to other drying methods due to the use of low drying temperature during the process[137]. It utilizes the sublimation of water in frozen sample under vacuum to remove water. Freeze drying was widely used for food dehydration. It can largely retain the original structure of the food matrix. High cost and long process time are the two major disadvantages of freeze-drying. Sometimes freeze drying protectants are used due to freezing and desiccation stress during the process[138]. Such protectants can be cyclodextrin, gelatin and sucrose[139-141]. The use of protectants such as maltodextrin could also increase the glass transition temperature of freeze dried powders which will ultimately increase the stability of the product [142]. Another advantage of adding maltodextrin is that it could contribute to the dispersibility of the final dried powder[136]. The aim of this study was to develop an effective method obtain β-carotene nanoemulsion powder. First, both heat assisted dissolution method and sonication assisted dissolution in ice bath were used to dissolve β-carotene. The two methods were compared in terms of efficiency and the amount of β-carotene that can be dissolved. Then two drying methods for obtaining β-carotene nanoemulsion powder were compared in terms of
retention of β-carotene and generation of degraded product. The water content in the final dried powders were also compared.

3.2 Material and Methods

3.2.1 Material

β-carotene powder (with purity ≥ 93%), casein, butylated hydroxytoluene (BHT), tert-Butylhydroquinone (TBHQ), sodium azide were purchased from Sigma (Sigma-Aldrich Corporation, Missouri). Vegetable oil (Wesson, ConAgraFoods Inc, Nebraska) was purchased from a local supermarket. Maltodextrin Maltrin M180 was obtained from Grain Processing Corporation (Muscatine, Iowa). HPLC grade ethanol, methanol, isopropyl alcohol, acetonitrile and chloride were purchased from Fischer Scientific.

3.2.2 Dissolve of β-carotene

Temperature assisted dissolution: A certain amount of β-carotene was placed in a 1.5 mL tube, and then 1 g vegetable oil was added. Samples were mixed, then placed on water bath (50 °C) for 5 min. Another set sample was prepared same way but has 0.01% and 0.05% (w/w) BHT and TBHQ dissolved in the oil with nitrogen gas. Then samples were vortexed for 3 min on a vortex mixer. Samples were centrifuged at 20800 g for 30 min and supernatant were properly diluted with mobile phase before analyzed on a HPLC for β-carotene content.

Sonication assisted dissolution: A certain amount of β-carotene was placed in a 1.5 mL tube, and then 1 g vegetable oil was added. The mixture was mixed on a vortex mixer. Then it was placed on ice bath and sonicated for 30 s, 1 min and 2 min with a sonicator (model FB 505, Fisher Scientific, PA). Samples were centrifuged at 20800
g for 30 min and supernatant were properly diluted with mobile phase for HPLC analysis (see β-carotene content determination section) and analyzed on a HPLC for β-carotene content.

### 3.2.3 Fabrication of emulsion

Vegetable oil with β-carotene dissolved inside obtained by sonication was used as oil phase for preparation of nanoemulsion. 10 g oil was added to 100 g aqueous phase with 1% (w/w) of casein as emulsifier. Then the sample was mixed with a BioSpec 10810 hand mixer (Biospec Products, OK) for 1 min. Then nanoemulsion was fabricated with a M110 microfluidizer (Microfluidics, MA) at 12,000 psi for 3 passes. During the whole process from dissolving to mixing and microfluidization, β-carotene was kept against light when possible. A certain amount of fresh nanoemulsion was kept in the -20 °C freezer before analysis for β-carotene.

### 3.2.4 Stability of β-carotene nanoemulsion under elevated temperature

Freshly made β-carotene was mixed with sodium azide (w/w, 0.02%) kept in 50 water for a month against light. An aliquot of 200 μL solution was withdrawn from the test tube every other day. The samples were analyzed on a HPLC for β-carotene content.

### 3.2.5 Spray drying

Freshly made nanoemulsion was mixed with 50% maltodextrin solution (w/w) at the weight ratio of 4:1. Then the mixture was stirred on a magnetic-stirrer for 10 min to ensure complete mix. Both β-carotene nanoemulsion and β-carotene nanoemulsion-maltodextrin mixture were spray dried using a Buchi B290 mini spray dryer (Buchi Corporation, Switzerland). Air inlet temperature was set at 100
°C, 110 °C, 120 °C, outlet temperature 60 °C and the feed rate was 1mL/min.

Samples were collected and stored in glass bottles in -20 °C freezer against light.

### 3.2.6 Freeze-drying

Samples were prepared the same as those for spray dry. Freshly made nanoemulsion was mixed with 50 % maltodextrin solution (w/w) at the weight ratio of 4:1. Samples were dried in a Virtis pilot freeze dryer (25EL, SP Scientific, PA) for two days. The freeze dried powder was stored in glass bottles in a -20 °C freezer.

### 3.2.7 Characterization of fresh nanoemulsion and nanoemulsion powders

#### 3.2.7.1 Particle size and ζ- potential

Freshly prepared nanoemulsion was diluted 50 times with double distilled water before analyzed on a Zetasizer Nano ZS (Malvern Instruments, UK). Spray dried and freeze dried nanoemulsion powder were reconstituted to its original concentration and then diluted 50 times with double distilled water before analysis. The particle size was reported as intensity-weighted mean particle diameter (d_{3,2}).

#### 3.2.7.2 β-carotene content determination

β-carotene content was determined by HPLC equipped with a UV lamp in reference to a CDC Laboratory Procedure Manual (Vitamin A, Vitamin E, Gamma Tocophorol, Retinyl Palmitate, and Retinyl Stearate in Serum NHANES 1999-2000) with some modification. Mobile phase (1L) was made of 972 mL acetonitrile, 148 mL tetrahydrofuran and 60 mL of isopropanol alcohol and methanol mixture (1:1, v/v). For both fresh nanoemulsion, 100 mg of nanoemulsion was put in a 1.5 mL centrifuge tube. 500 μL of mobile phase was added to the tube and vortexed for 1 min. Then 500 μL of chloroform was added to the tube and vortexed for 1 min. The
sample was centrifuged at for 20 min. After centrifugation, there are two layers with colorless aqueous phase on top and yellowish organic phase and a thin protein layer in the middle. An aliquot of 100μL solution from the button was carefully collected from the bottom layer and mixed with mobile phase for HPLC analysis. Similarly, 10 mg of spray dried or freeze-dried powder was reconstituted into nanoemulsion by double distilled water and then extracted with 500 μL of mobile phase and 500 μL of chloroform. After centrifugation, solution in the bottom was collected and properly diluted for HPLC analysis.

3.2.8 Moisture content determination

The moisture content of spray dried and freeze dried powders were determined by oven dry method [143, 144]. A certain amount of dried powder was accurately weighed and put in a drying pan with cover. Then the sample was put in a forced draft oven for 24 h at 102 °C. Then the sample was cooled down in a desiccator before weighed again. Each sample has three replicas.

3.2.9 Statistical analysis

All data in this study are expressed as mean ± SD. Student’s t-test was used to determine the significance of difference between two groups. 5% significance level was used for all tests.

3.3 Results and Discussion

3.3.1 Comparison of heat assisted dissolution and sonication assisted dissolution

β-carotene is sensitive to heat, light and oxygen[127]. Degradation and isomerization may happen during processing [145]. It's important to monitor the
change of β-carotene in each stage of processing before getting the final product. Dissolve in oil is the first step of making β-carotene nanoemulsion powder. It is a critical step because it determines the maximum β-carotene content could be presented in the dried powder. So it’s important to ensure maximum solubility and minimum degradation during this step. Besides, if large amount β-carotene was degraded or isomerized at this step, the degraded product will exist and keep increasing during each process and the quality of the final powder will be affected.

Figure 8. Influence of antioxidants on β-carotene degradation. The degraded product content was expressed as the percentage of its peak area to that of β-carotene peak from HPLC profile. Each sample was repeated for three times for 3 min. All data represent mean ± SD(*, P<0.05, n=3).
For our experiment, we first use temperature-assisted method to dissolve β-carotene due to its low solubility at ambient temperature. A degraded product was obtained, it account for about 24% of the main β-carotene peak as shown in Figure 9. At first, we assumed it was due to oxidation during our operation. So we choose two antioxidants butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) to test if these antioxidants could prevent the formation of degraded product. As shown in Figure 8, antioxidants did not have significant effect in terms of β-carotene degradation prevention. The degraded product content was only slightly lower than that with no antioxidant added. So we conclude the degradation during dissolution in oil was not caused by oxidation. The other factor was heat. So we developed a sonication-assisted method to dissolve β-carotene in oil. As can be seen from Figure 9, more degradation product was produced when β-carotene was dissolved in oil with heat-assisted dissolution (24% of main peak area). While sonication assisted dissolution (1 min) only cause minimum (4% of main peak area) degradation. However, sonication for 3 min led to even more production of degradation product (Figure 9d). It account for about 30 % of the main peak. This could be due to long time exposure to heat generated during sonication. This further proved the conclusion that β-carotene degradation was mainly caused by high temperature. So in the following studies we use 1 min sonication as our experiment condition. Many studies showed β-carotene might go through isomerization and degradation at elevated temperature. There are several kinds of degraded products. Most abundant was all-E-5, 8-exoxy- β-carotene [146]. The isomerized product could be 13-Z- β-carotene and 15-Z- β-carotene [145]. In the future study, we would
use other analytical methods to identify the degraded product. Another major advantage of using sonication-assisted dissolution is that much more ϒ-carotene could be solubilized in vegetable oil. For example, 1.73 ± 0.27 % of ϒ-carotene (w/w) could be obtained with sonication-assisted dissolution while 1.21 ± 0.16 % (w/w) for heat assisted dissolution. Overall, sonication on ice bath offers a better way to dissolve ϒ-carotene that can minimize ϒ-carotene degradation by shortening dissolving time and at the same time increase solubility.

**Figure 9.** HPLC profile of ϒ-carotene dissolved with different methods. (a) standard, (b) heat-assisted dissolve, (c) sonication assisted dissolve for 1 min, (d) sonication assisted dissolve for 3 min.
3.3.2 Characterization of fresh nanoemulsion and nanoemulsion powders
dried with two different methods

3.3.2.1 Particle size, zeta potential and appearance of fresh, spray dried and
freeze dried β-carotene nanoemulsion.

The particle size and zeta potential information are listed in Table 2.

Table 2 Particle size (mean droplet diameter \(d_{32}\), nm) of fresh nanoemulsion and
reconstituted nanoemulsion.

<table>
<thead>
<tr>
<th></th>
<th>Fresh</th>
<th>Spray dry</th>
<th>Freeze dry</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle size</strong></td>
<td>231 ± 2(^a)</td>
<td>277 ± 13(^a)</td>
<td>230 ± 8(^b)</td>
</tr>
<tr>
<td><strong>Zeta-potential (mV)</strong></td>
<td>-64.5 ± 5.8(^a)</td>
<td>-49.3 ± 1.27(^b)</td>
<td>-54.05 ± 0.07(^b)</td>
</tr>
</tbody>
</table>

*Means with different letters are significantly different, p<0.05, n=3.

Freshly prepared β-carotene nanoemulsion has the average particle size of 231 ± 2
nm and \(\zeta\)-potential of -64.5 ± 5.8, indicating excellent stability. While the
reconstituted spray dried nanoemulsion powder has the particle size around 277±
13 nm. This may due to nanoemulsion surface change of the nanoemulsion caused
by heat and hot air during spray drying, when redispersed into water they may form
larger droplets [136]. \(\zeta\)-potential of this reconstituted solution was lowered due to
the added maltodextrin may bind to surfactant that was originally bound to
vegetable oil, so that the negative charge of the oil was reduced [147]. For freeze
dried nanoemulsion powder the particle size was 230 ± 8 nm, similar to that of fresh
nanoemulsion.

The appearance of spray dried and freeze dried samples can be seen from Figure 10
Spray dried β-carotene nanoemulsion was loose and was powder like. The freeze-
dried sample mixed with maltodextrin stayed as a whole piece after freeze-drying. It was crispy when pressed with a lab spatula and can be crushed into powders. The sample with no maltodextrin also maintained its original structure, but it has a creamy texture that was similar to moisturizer for skin. This was due to lack of maltodextrin. Spray dried powder was difficult to be dispersed into water. The higher the drying temperature, the difficult it would be. Although the particle size of reconstituted spray dried β-carotene nanoemulsion has relative small particle size, it was not stable after dispersed into water. As can be seen from Figure 10, after dispersed into water, there's a thin layer of oil containing β-carotene on top for powder obtained by spray drying at 120 °C. This may be due to the shrinkage of nanoemulsion droplets caused by high temperature, the oil inside come from inside the particle [148]. But there's less precipitated oil for sample obtained by spray drying at 110 °C and no oil on top for sample spray dried at 100 °C. On the contrary, freeze dried β-carotene nanoemulsion powders was easy to be dispersed into water. Beside, as the maltodextrin content in the mixture decrease, it became easier to be dispersed into water. This may also due to the interaction between maltodextrin and emulsifier [147]. As maltodextrin content decreased, more emulsifier would be able to act as stabilizer when dispersed into water. In conclusion, freeze-drying and spray drying at low temperature could be ideal for getting β-carotene nanoemulsion powder that has better stability after reconstitution.
Figure 10. Appearance of spray dried and freeze-dried β-carotene nanoemulsion power and reconstituted solution. Spray dried powder was obtained under three temperatures: 100 °C (a), 110 °C (b) and 120 °C (c). Freeze dried nanoemulsion powder was obtained with same freeze drying condition but with different maltodextrin content: (d) 20% (w/w) maltodextrin solution (50%, w/w), (e) 50 % maltodextrin solution and (f) no maltodextrin.

3.3.2.2 Moisture content of freeze dried nanoemulsion powder

The moisture content of spray dried and freeze dried β-carotene nanoemulsion powders are listed in Table 3. Powder obtained by spray drying at 100 °C has the water content of 3.21 ± 0.94 %, 4.17 ± 0.39 % when spray dried at 120 °C. While powder obtained from freeze-drying has the water content of 1.6 ± 0.59 %. In general, spray dried nanoemulsion powders have higher moisture content than freeze dried nanoemulsion powder. This was caused by short drying time and low
drying temperature that was not enough to remove all the moisture from the nanoemulsion [149]. We also observed that the powder from β-carotene nanoemulsion alone (not mix with maltodextrin solution) did not contain any moisture after freeze-drying. This may be due to the water binding activity of maltodextrin that can retain the moisture in the nanoemulsion even during freeze drying [147]. In conclusion, both methods could produce powdered β-carotene nanoemulsion with low water content.

Table 3 Moisture content of spray dried and freeze dried β-carotene nanoemulsion powder.

<table>
<thead>
<tr>
<th></th>
<th>SD 100 °C</th>
<th>SD 120 °C</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moisture content (%)</strong></td>
<td>3.21 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.17 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Different letter between groups indicates statistical significance, p<0.05, n=3.

3.3.3 β-carotene degradation during processing

3.3.3.1 β-carotene degradation during storage (fresh nanoemulsion)

In this experiment, β-carotene content was expressed as all the signals that can be detected under UV detector, no degradation and isomerization was considered. As can be seen from Figure 11, β-carotene content decreased rapidly at elevated temperature. There’s almost no β-carotene left after one month at 50 °C. As storage time increased, the color of the nanoemulsion became lighter, but no creaming phenomenon was observed, suggesting excellent physical stability of liquid nanoemulsion.
Figure 11. Stability of fresh β-carotene nanoemulsion stored at 50 °C for one month.

3.3.3.2 β-carotene degradation during spray drying and freeze-drying

Despite dissolution with sonication assisted approach, homogenization and spray drying process all generate heat that may lead to β-carotene degradation. It’s important to know how much β-carotene has left after each operation. In this study we use theoretical content to represent theoretically calculated β-carotene content in the product at a certain stage, while using experimental content to represent
tested value in the product. The initial $\beta$-carotene content in the oil used for fabricating nanoemulsion was $1.73 \pm 0.27 \%$. The $\beta$-carotene content in the nanoemulsion was $0.19 \pm 0.042 \%$, almost equal to theoretical content. This indicates there’s minimum loss during microfluidization process. This was due to the microfluidizer coil was immersed in ice bath that helped to reduce the heat generated during homogenization. So less isomerization and degradation happened. On the contrary, a lot of $\beta$-carotene was degraded during spray drying operation. As can be seen in Table 4, $\beta$-carotene degradation increased as the spray drying temperature increased. When spray drying with $100 \, ^\circ C$ gas, almost $73\%$ of $\beta$-carotene was lost. $79\%$ $\beta$-carotene of was lost when drying with $110 \, ^\circ C$ gas and $85\%$ when dried with $120 \, ^\circ C$ gas. Although spray drying for individual droplet was very fast, the time the powder stay in the receiving chamber was relatively long where it was exposed to high temperature drying gas. This was the major reason that caused the degradation of $\beta$-carotene. On the contrary, freeze-drying offers a better alternative for obtaining $\beta$-carotene nanoemulsion powder. The $\beta$-carotene content in the freeze-dried nanoemulsion powder (sample not mix with maltodextrin) was only slightly lower than theoretical value. This was mainly attributed to the low drying temperature used so that $\beta$-carotene degradation and isomerization could be avoided.
Table 4 β-carotene content in nanoemulsion powder dried at different temperature.

<table>
<thead>
<tr>
<th></th>
<th>β-carotene content (%)</th>
<th>Theoretical content(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD 100 °C</td>
<td>0.24 ± 0.1</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>SD 110 °C</td>
<td>0.18 ± 0.013</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>SD 120 °C</td>
<td>0.13 ± 0.005</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>Freeze dried sample</td>
<td>1.50 ± 0.02</td>
<td>1.57 ± 0.24</td>
</tr>
</tbody>
</table>

Freeze dried data was calculated from sample not mixed with maltodextrin.

Theoretical value was calculated according to tested β-carotene content in the nanoemulsion and based on the assumption that there’s no water left in the powder. Despite β-carotene loss during these operations, β-carotene degraded product percentage increased as the whole process advances. **Figure 12** shows the percentage of degraded β-carotene after each processing. Initially, degraded β-carotene product only account for 3.7 ± 0.28 % of β-carotene in oil. After made into emulsion, it had a slight increase, accounting for 6.34 ± 0.01 % of β-carotene. This may be caused by little amount of heat generated during homogenization process. Since the coil was immersed in ice bath, most β-carotene stayed in its original form. After spray drying, the percentage of degraded β-carotene increased to 12.75 ± 0.5 % (Sample spray dried at 100 °C). This was caused by exposure to hot drying gas during spray drying. After freeze drying, there was a slight increase (7.89 ± 0.01 %) in the percentage of degraded β-carotene, indicating excellent β-carotene stability during freeze drying operation.
Figure 12. Change of degraded β-carotene percentage during preparation of spray dried and freeze dried β-carotene nanoemulsion powder. Different letter between groups indicates statistical significance, p<0.05, n=3.

3.4 Conclusions

In this study, we investigated the degradation of β-carotene during heat-assisted and sonication assisted dissolution, high-pressure homogenization (microfluidization), spray drying and freeze-drying. Our result showed β-carotene degradation was mainly induced by heat. Long time sonication and heat-assisted dissolution can cause the production of high amount of degraded product. Further
studies will be conducted to identify the structure of this degraded or isomerized product. Fresh nanoemulsion stored at 50 °C showed fast degradation. After 30 days storage, there’s no β-carotene left in the nanoemulsion. The recovery rate of β-carotene obtained from spray drying decreased as spray drying temperature increases. The recovery rate of β-carotene obtained by freeze-drying was higher than spray drying. In addition, freeze-drying has other advantages such as better powder dispersibility and better stability after reconstitution. In conclusion, we’ve developed a method to prepare dried β-carotene nanoemulsion powder with high compound loading efficiency and minimum degradation during processing.
CHAPTER 4

FABRICATION AND BIOLOGICAL FATE OF NANOEMULSION SYSTEM FOR THE DELIVERY OF 5-DEMTHOXYNOBILETIN

4.1 Introduction

Despite polymer based delivery system, lipid based delivery system is another mostly used method to increase the loading capacity water insoluble bioactive compounds. There are many different kinds of lipid based delivery system such as nanoemulsion, microemulsion, multilayer droplets, liposomes, solid lipid nanoparticles, filled liposomes, filled hydrogel beads, colloidomes and hydrogel beads[50].

Nanoemulsion is one of the most simple and widely used delivery system. It is believed smaller particle could lead to better absorption, hence better bioavailability[55]. It is formed by mixing oil phase and aqueous phase under high pressure homogenization. Emulsifier, water to oil ratio and homogenization condition all play important role in the formation of small particles. Emulsifier can be dissolved in the oil phase or the water phase depending the polarity of the emulsifier. They can be small protein molecule, polysaccharide, phospholipids or non-ionic surfactant such as Tween 20. Different emulsifier type will not only influence particle size but also the digestibility of the delivery system[56]. Oil type also plays a very important role for the property of nanoemulsion. The viscosity of carrier oil will contribute to the overall physiochemical characteristic of nanoemulsion. The stability of nanoemulsion is governed by the interfacial tension on the particle surface. Viscosity has a major impact on the easiness of oil droplet
disruption. Closer viscosity between oil and aqueous phase may result in efficient
disruption of oil droplet hence. This would effectively prevent interfacial tension
decrease and result in better stability [57]. While composition of oil may have great
influence on the digestibility of the delivery system[58-61]. Long chain oil may
result in a slower digestion rate than short chain oil due to slow production of free
fatty acid[56]. For example, nanoemulsion made with non digestible oil such as
orange oil, had significant low bioaccessibility than that prepared with digestible oil
such as corn oil[58]. Homogenization condition also affect delivery system property.
Higher pressure may lead to smaller particle size due to increased force to break oil
droplet into small particles and simultaneously covered by emulsifier to preventing
from aggregating together[62]. In conclusion, it’s important to optimize preparation
condition to get nanoemulsion based delivery system with desired physiochemical
characteristics.

Previous study showed, after dissolve certain amount of PMF in oil at elevated
temperature, upon cool down, PMF crystal appear in the oil. Small clusters of PMF
also appear after preparing these PMFs dissolved oil for emulsion[5]. Hence, there’s
a strong need to determine the saturation concentration of PMF in oil. So that the
concentration of PMF dissolved in oil phase can be controlled to ensure there’s no
crystallization formation after making into emulsion. The stability of PMF inside the
emulsion will also be determined.

In this study, we would also use the in vitro digestion model and Caco-2 cell
monolayer model described in the previous chapter to see the possible biological
fate of PMF transported through nanoemulsion delivery system.
4.2 Material and methods

4.2.1 Material

Corn oil and canola oil were purchased from a local supermarket (Mazola, ACH Food Companies, Inc., Memphis, TN). β-lactoglobulin was obtained from Daviso Foods International (lot JE 002-8-415, Le Sueur, MN), Tween 20, and Sodium caseinate. Hank’s balance salts (cat. no. H1387), uranylacetate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO). Other chemical such as sodium chloride (NaCl), sodium hydroxide (NaOH), calcium chloride (CaCl2), hydrochloric acid (HCl), HPLC grade methanol, tetrahydrofuran (THF), trifluoroacetic acid (TFA), acetonitrile (ACN) and HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was obtained from EMD Chemicals Inc. (Gibbstwon, NJ, USA). Double distilled water was made from Barnstead nanopure water system (Model D14031, Dubuque, Iowa, USA). DMEM (Dulbecco’s Modification of Eagle’s Medium) and non-essential amino acid was purchased from Mediatech (Mediatech, Inc., Manassas, VA).

4.2.2 Emulsion preparation

Oil phase: 5DN (0.075%, w/w) was dissolved in three different kind of oil: canola oil, medium chain triglyceride (MCT) and corn oil and stirred overnight to ensure 5DN was fully dissolved.

Aqueous phase: Each emulsifier (β-lactoglobulin, Sodium caseinate and Tween 20) was dissolved in double distilled water (1%, w/w) and stirred for at least 2 h to ensure full hydration.
**Emulsion preparation:** Oil phase and aqueous phase was mixed together at the weight ratio of 1:9 and mixed well with a hand blender (M133/1280, Biospec Products, Inc., ESGC, Switzerland) for 2 min. This coarse emulsion was then passed through a high-pressure homogenizer (Microfluidics M-110Y, Newton, MA) at 12,000 psi for 3 times.

4.2.3 **Determination of particle size and ζ-potential**

The particle size and ζ-potential of the particles of the nanoparticles were determined with a commercial dynamic light scattering and micro-electrophoresis device (Nano-ZS, Malvern Instruments, Worcestershire, UK). Freshly prepared samples were diluted 50 times with buffer solution (10mM phosphate buffer, pH 7) at room temperature before measurement. Freeze dried sample was redispersed into buffer solution (10mM phosphate buffer, pH 7), stirred for 5 min and then diluted 5 times with same buffer. The particle size data are reported as the intensity-weighted ("Z-average") mean particle diameter, while the particle charge data are reported as the ζ-potential.

4.2.4. **Saturation concentration of 5DN in MCT and in water**

A certain amount of 5DN (0.05% -2%, w/w) was dissolved in MCT and heated to 80°C to ensure full dissolution of the crystal. The samples were stored at room temperature for 72h. At last 10 µL of supernatant from each sample was collected for HPLC analysis. Saturation concentration of 5DN in water was conducted as previously described [49]. 5DN powder was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 1% (w/w) to form a stock solution. Then this solution was properly diluted with
double distilled water and forms a series of 5DN concentration in water (0.001% - 1%, w/w). The samples were thoroughly vortexed and stored at ambient temperature for 72 h. Then the samples were centrifuged, Then the samples were centrifuged (Centrifuge 5417R, Eppendorf co., Hamburg, Germany) at (20800g) for 15 min. The supernatants were collected and diluted accordingly before HPLC analysis.

4.2.5 Fabrication of emulsion with different particle sizes

A certain amount of 5DN was dissolved in MCT (0.25%, w/w) and heated to 80°C to ensure complete dissolve. β-lactoglobulin (1%, w/w) was dissolved in double distilled water and stirred for 2h. Then the two solutions was mixed together at the ratio of 1:9 (w/w). The mixture was homogenized with a hand blender for 2 min as described before. Then the coarse emulsion was passed through a microfluidizer at 6,000 psi for 1 pass. Sample was collected and the remaining emulsion was passed through the microfluidizer at 12,000 psi for 3 times.

4.2.6 Stability of 5DN emulsion

Emulsions was put in three test tubes and kept at 4°C, 20 °C and 37 °C respectively. These temperature conditions were chosen to simulate refrigeration condition, room temperature condition in mild and hot climates[57]. Particle sizes were measured every other day for a total period of 14 days. An aliquot of 100 μL of emulsion was also collected from the middle of the test tube and stored in a -20 °C refrigerator before determination of 5DN concentration. These samples were properly diluted and 5DN concentrations were measured with a HPLC machine.
4.2.7 Determination of 5DN using HPLC method

Sample preparation: The content of 5DN in different systems was determined using a HPLC method. A certain amount of sample was transferred into a 1.5 mL centrifuge tube. 400 µL of ethyl acetate was added to the tube and vortexed for 30 s. Then the mixture was centrifuged (1170 g) for 6 min. The ethyl acetate layer was collected and the remaining sample was prepared using the same procedure for a second time. The two ethyl acetate layers were combined and dried on a vacuum concentrator (Model: SVC 100H, Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, 100µL of mobile phase was added to the tube to dissolve the 5DN extracted by ethyl acetate. The tube was vortexed and centrifuged (1170 g) for at least 30 s to sediment non-dissolved substance. A 5DN standard solution was prepared by diluting 50 μM stock solution (in DMSO) with mobile phase to a series of desired concentration (5, 10, 20, and 50μM). An aliquots (90 µL) of solution were collected from the top and injected into a HPLC vial.

HPLC condition: Samples were analyzed on a HPLC system (CoulArray, Fisher Scientific, USA), which contains a binary solvent delivery system (Model 584), an auto-sampler (Model 542), a CoulArray 5600A Detector and a UV detector (Waters Model 526, MA, USA). A RP-Amide reversed-phase HPLC column (15cm×4.6mm id, 3μm) (AscentisExpress, Sigma-Aldrich, MO, USA) was used on this HPLC system. The mobile phase and detection conditions used were as described previously with some slight modifications [150]. The 50% mobile phase contained 50% water, 40% ACN, 10%THF, 0.05% TFA and 50 mM ammonium acetate. The 25% mobile phase was made up of 25% water, 40% ACN, 10%THF, 0.05% TFA and 50mM ammonium
acetate. The running time was 35 min, with a flow rate of 0.7 mL/min for 50% mobile phase and 0.3 mL/min for 25% mobile phase. The injection volume was 50μL.

4.2.8 In vitro digestion of 5DN nanoemulsion

*Mouth phase:* Oral condition was mimicked by mixing the delivery system with a simulated saliva fluid (SSF), which was prepared from various salts and mucin as described previously[151]. Delivery systems were mixed with SSF at the same volume, and then the mixture was adjusted to pH 6.8 and shaken continuously at 100 rev/min in an incubator at 37 °C for 10 min (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA).

*Stomach phase:* Simulated gastric fluid was prepared by mixing 2 g of NaCl and 7 ml of concentrated HCl and then making the volume up to 1 L using double distilled water [151]. Pepsin was then dissolved in this mixture (0.32%, w/v), the pH was adjusted to 1.2. It was then mixed with the sample from the mouth phase at the same volume. The pH of the mixture was then adjusted to 2.5 and incubated at 100 rev/min at 37 °C for 2 hr.

*Small intestine:* Small intestine conditions were simulated using a pH-stat automatic titration unit (Metrohm, USA Inc.). The pH of the samples was controlled by automatic pH monitoring and titration unit by using sodium hydroxide. The sample obtained from the stomach phase was put in a beaker in a 37°C water bath. The pH was adjusted to pH 7.0. Bile salt (187.5 mg in 4 ml pH 7 PBS buffer solution) was then added to the digesta, and the pH was adjusted to 7.0. Calcium chloride solution (110 mg dissolved in 1 ml double distilled water) was then added to this solution,
followed by adjusting of the pH precisely to 7.0. Finally, freshly prepared pancreatin lipase (60 mg lipase in 2.5 ml pH 7.0 PBS) was added to the solution. At the same time, the automatic titration of pH-stat was started. The free fatty acid released during digestion was calculated using the following equation[152]:

\[ \text{FFA\%} = 100 \times \left( \frac{V_{NaOH} \times m_{NaOH} \times M_{lipid}}{w_{lipid} \times 2} \right) \]

Here, \( V_{NaOH} \) is the volume of sodium hydroxide solution required to neutralize the free fatty acids (and other sources of \( H^+ \)) released during digestion; \( m_{NaOH} \) is the molar concentration of sodium hydroxide solution, which is 0.25 M in this study; \( M_{lipid} \) is the molecular weight of MCT, which is 500 g/mol; \( w_{lipid} \) is the amount of oil in the reaction system.

4.2.9 Caco-2 cell monolayer maintenance and permeability determination of digested micelle with different oil content.

4.2.9.1 Caco-2 cell monolayer

Caco-2 cell monolayer maintenance and permeability determination was conducted as described before [153]. Caco-2 cell were seeded on Costar transwell permeable support 0.4 µm Polycarbonate Membrane (Corning incorporated, Corining, NY) with the seeding density of \( 2.6 \times 10^5 \) cells / cm\(^2\) and incubated in a humidified incubator at 37°C with 5% CO\(_2\). The medium was changed 16 h after seeding, then medium on apical and basolateral compartment were changed every other day. This process was maintained for about 21 days until the transendothelial electrical resistance (TEER) of the filter was 260± 65Ω cm\(^2\). Medium was changed at least 24h before the permeability determination experiment.
4.2.9.2 5DN emulsion delivery system permeability determination

The digested samples with different particle sizes were filtered through a 400 nm membrane. Then were diluted with Hank's Balanced Salt (HBSS, pH 7.4, 37°C) to the certain 5DN concentrations for permeability determination experiment. 5DN dissolved in DMSO was used as control group. All the solutions used in this experiment were pre-warmed in the 37°C water bath. Caco-2 monolayer transwell was incubated with HBSS for 30 min before the experiment. Then both the apical and basolateral compartments were rinsed with HBSS (pH 7.4) for twice. An aliquot (2.5 ml) of each sample was added to the apical compartment and 1.5 to the basolateral compartment. Then this plate was incubated in the incubator. In each 30 min, 200 µL was withdrawn from the apical compartment without adding new sample and half volume of sample was taken from the basolateral compartment and replaced by same amount of HBSS. The concentration of 5DN and its metabolites in each sample were analyzed by the HPLC. Apparent permeability coefficient (cm s⁻¹) was calculated with the following equation:

\[ P_{app} = \frac{dQ/dt}{A} \frac{1}{C_0} \]

dQ/dt is the steady-state flux (µmol s⁻¹), A is the surface of the filter (4.67 cm²), C₀ is the concentration of 5DN added on each filter (µM).

4.3 Results and Discussion

4.3.1 Influence of oil and emulsifier on particle size

In this study we first made a series of nanoemulsions composed of different oil and emulsifier types. Three kind of oil were used, including two long chain triglycerides (LCT): canola oil, corn oil and medium chain triglyceride (MCT). In Figure 13,
emulsions with larger particle sizes were obtained, when the oil phase was canola oil. Emulsions made with MCT and corn oil had similar particle size. The difference in particle sizes produced with these three different oils are caused by disperse phase viscosity and / or interfacial tension [154]. Usually small particles are produced by the oil phase with low viscosity [50]. The viscosities of canola oil, MCT, corn oil at ambient temperature are 78.2, 52 and 26 mPa.s respectively. Emulsifier type can also influence the particle size. Small molecule surfactant tend to forming small droplet size, while large molecule such as proteins and polysaccharides tend to form large particle [50]. Among the three emulsifiers we used, Tween 20 is a small non-ionic molecule. Emulsion made with this surfactant has the smallest particle size when oil types were the same. Emulsions made with sodium caseinate have the largest particle size when compared with the one made with Tween 20 and β-lactoglobulin. Sodium caseinate and β-lactoglobulin are two large molecules originated from milk. Sodium caseinate can absorb to the interface and form the extended layer of about 10 nm thick, while β-lactoglobulin may partially unfold and form the layer of about 2 nm thick [155].
Figure 13. Influence of oil and emulsifier types on the particle size of nanoemulsion. Samples were made with same condition; only differ in the composition of oil and emulsifier types. All data represent mean ± SD (*, P<0.05, n=3). Study showed a similar PMFs, 5-demethyltangeretin has the highest saturation concentration in MCT than corn oil [49], therefore we choose MCT as our carrier oil for our further experiment. Previous in vitro digestion study showed that the digestibility of pancreatic lipase towards emulsion made with Tween 20 is lower than that made of proteins [156], so we choose the formulation of MCT and natural
ingredient β-lactoglobulin. In conclusion we have identified a potential 5DN delivery system made up of MCT and β-lactoglobulin, which may have relatively small particle size and good digestibility when consumed by human.

4.3.2 5DN saturation concentration

In order to determine the maximum amount of 5DN that can be dissolved in the MCT, we measured the saturation concentration of 5DN. The saturation concentration of 5DN in MCT was 0.26% (w/w). It is significantly higher than its saturation concentration in water, which is 0.01% (w/w). This may justify the use of nanoemulsion based delivery system to encapsulate PMFs.

4.3.3 Stability of nanoemulsion and 5DN

We use the nanoemulsion with the particle diameter of 176.9 nm for our stability experiment. Three temperatures conditions were used 4 °C, 20 °C and 37 °C. As shown in Figure 14, the particle size of the nanoemulsion increased with storage time when stored at 4 °C and 20 °C. But the droplet size didn't change that much for the emulsion that was stored in a 37 °C environment. There are several mechanisms that could cause the instability of nanoemulsion, including flocculation, coalescence of droplets and Ostwald ripening[50]. For our experiment, the particle size increase may be caused by the crystallization of 5DN. Although initially fully dissolved in the oil phase, 5DN may crystalize in the nanoemulsion due to supercooling effect. Because compound nucleation due to supercooling can occur more easily in a nanoemulsion than within the bulk oil [157]. For chemical stability, as can be seen in Figure 15, our compound 5DN is quiet stable when stored under different conditions in the emulsion systems. No degradation was observed.
Figure 14. Stability of emulsion under different temperature conditions. All data represent mean ± SD.
4.3.4 *In vitro* digestion and bioaccessibility of 5DN nanoemulsion containing different amount of oil.

As can be seen from Figure 16, free fatty acids were released very quickly after digestion process began. It was due to rapid absorption of lipase to the oil droplet surface. System with 4% oil reached the digestion plateau in about 10 min. 10% oil system reached the digestion plateau in about 25 min. The system containing 2% oil shows a slower digestion rate and longer digestion time. It may due to the absorption of bile salt to the droplet that preventing lipase from lipolysis. This
hypothesis may also explain the slower increase in free fatty acid production in the course of complete digestion process.

![Graph](image)

**Figure 16.** In vitro digestion profile of emulsion with different oil content.

As seen from **Figure 17**, the bioaccessibility of 5DN from 10% delivery system has the highest bioaccessibility. This was due to higher amount of free fatty acid release during digestion so that more PMF can be encapsulated inside the mixed micelle. The hydrophobic nature of 5DN may also contribute to its incorportaion into mixed micelle.
**Figure 17.** Bioaccessibility of digested micelle with different oil content. All data represent mean ± SD (Different letter between groups indicates statistical significance, p<0.05, n=3).

### 4.3.5 Permeability of 5DN emulsion across Caco-2 monolayer.

Absorption is the first step in drug metabolism may influence the overall bioavailability of this drug. Permeability determine the easiness for a compound In order to determine whether there is a correlation between particle size and permeability across human small intestine epithelium cell, we fabricated emulsion with different particle sizes. We obtain this emulsion by changing the pressure of the microfluidizer and the pass time emulsion goes through the chamber. For emulsion made with 6000 psi and 1 pass, the mean particle diameter was 274.7 nm.
When the pressure went up to 9000 psi and pass time increased to 3 times, the particle size decreased to 204.7 nm. While less than 12000 psi and 3 times condition, the droplet diameter decrease to 176.9 nm. We choose the emulsion with the smallest and the largest particle sizes for our permeability experiment. As can be seen from Table 5, in the delivery system with smaller particle size, 5DN was absorbed or transported to the basolateral compartment more quickly. It was also a little quicker than the system without a delivery system, i.e., the control group with 5DN dissolved in DMSO. Smaller sized particle may penetrate into the cell more easily than large particles[56]. For digested micelles from different oil containing system, the permeability coefficient did not differ to much with each other as well as compared to plain 5DN solution. It may because 5DN existed in the mixed micelle in soluble form. The mixed micelle may taken up by the cell through same manner when 5DN was in the dissolved form. All these delivery system are considered to be well absorbed (>10⁻⁶ cm/s)[33]. This study indicated 5DN from both raw nanoemulsion and digested micelle had similar high permeability as its plain solution in culture medium. This may further justify the application of nanoemulsion delivery system to increase the bioavailability of poorly water soluble bioactive component.
Table 5 The apparent permeability coefficient of different 5DN nanoemulsion delivery systems.

<table>
<thead>
<tr>
<th>Delivery system*</th>
<th>$P_{app} \times 10^{-5}$ cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$2.51 \pm 0.23^c$</td>
</tr>
<tr>
<td>6K</td>
<td>$1.12 \pm 1.38^a$</td>
</tr>
<tr>
<td>12K</td>
<td>$2.88 \pm 0.1a$</td>
</tr>
<tr>
<td>2% oil</td>
<td>$2.28 \pm 0.07^c$</td>
</tr>
<tr>
<td>4% oil</td>
<td>$1.85 \pm 0.17^bc$</td>
</tr>
<tr>
<td>10% oil</td>
<td>$2.53 \pm 0.15^c$</td>
</tr>
</tbody>
</table>

*: 6K: Nanoemulsion made under 6000 psi and then applied directly to the cell. 12K: Nanomeulsion made under 12000 psi and then applied directly to the cell. 2% oil, 4% oil and 10% oil represented different oil containing delivery system went through in vitro digestion, the digested micelles were applied to Caco-2 cells. Different letter between groups indicates statistical significance, $p<0.05$, n=3.

4.4 Conclusions

The purpose of this study was to design a system for the delivery of lipophilic bioactive compound polymethoxylated flavones. Our results showed lipid-based nanoemulsion system was one of the effective means for deliver PMFs. Among the oils and emulsifiers we used, the combination of β-lactoglobulin and MCT can generate the smallest particle size. This delivery system was stable for at least two weeks at ambient temperature as well as under both hot and cold climate conditions. Our compound 5DN was stable in this delivery system when stored under these conditions and no degradation was observed.
Particle size has a major impact on the permeability of the delivery system across Caco-2 cell simulated small intestinal epithelial cell. Emulsion with the smaller particle size can be absorbed faster than emulsion with large particle size. The digested micelle from delivery system containing different amount of oil didn’t show significant difference in permeability. In conclusion, we have successfully fabricated a stable delivery system to encapsulate lipophilic compound.
CHAPTER 5
TANGERETIN-LOADED PROTEIN NANOPARTICLES FABRICATED FROM ZEIN/β-
LACTOGLOBULIN: PREPARATION, CHARACTERIZATION AND FUNCTIONAL
PERFORMANCE

5.1 Introduction

Tangeretin (5,6,7,8,4’-pentamethoxyflavone, shown in Figure 1) is a flavonoid (polymethoxyflavone or PMF) found in citrus fruits. There has been considerable interest in the use of PMFs as functional ingredients in foods and pharmaceuticals because the potential of their beneficial activities, such as anti-carcinogenic activities and anti-inflammatory activities [3, 158, 159]. However, the extensive application of PMFs is currently limited because they have low water-solubility, which makes them difficult to be incorporated into liquid food products [3]. In addition, the low water-solubility of PMFs means they may be present in foods as crystals (rather than soluble form), which may limit their bioavailability [160]. Studies in the pharmaceutical industry suggest that most lipophilic compounds must be in the form of a molecular dispersion (soluble form) before they can be absorbed through the biological membranes of human intestinal tract [161]. It is therefore important to develop effective delivery systems to encapsulate, protect and release PMFs to the targeted site.

In this section, we focused on the fabrication of food-grade colloidal delivery systems containing protein nanoparticles as a means of encapsulating and delivering tangeretin, a representative PMF. We chose protein nanoparticles because they are particularly suitable for commercial applications because they can
be prepared from food-grade ingredients, the manufacturing process can often easily be scaled up, and they are biodegradable [52]. In addition, powders could be formed from protein nanoparticle suspensions by spray drying methods, which are already widely used in the food industry [162]. Many different types of proteins can potentially be used to fabricate colloidal delivery systems for bioactive food components, including soy proteins, caseinate, whey proteins, and zein [69]. The unique molecular structure and physicochemical properties of each of these protein types make them suitable for different types of applications [163]. A variety of different preparation methods can be used to fabricate protein nanoparticles, including controlled thermal denaturation, extrusion methods, gel disruption methods, molding methods, and antisolvent precipitation methods [69].

We used a hydrophobic protein (zein) as a core for forming protein nanoparticles based on antisolvent precipitation method. Zein is a GRAS (generally recognized as safe) food-grade protein that is mainly found in corn kernels [85]. Zein is soluble in high concentration alcohol solutions but insoluble in water, which provides the potential for creating protein particles that encapsulate lipophilic bioactive agents [52]. The lipophilic bioactive agent and zein are initially dissolved in an alcohol solution, which was then introduced into an aqueous solution. Zein precipitates when the water-soluble alcohol moves into the surrounding aqueous phase, leading to the formation of a protein nanoparticle or micro-particle that encapsulates the bioactive agent. This method has been successfully used to encapsulate oils, such as fish oil, flax oil, and essential oils [164-166] and lipophilic bioactives, such as curcumin [73]. We chose β-lactoglobulin as an emulsifier because it is a model
globular protein with well-defined properties that is already widely used in the food industry. In this study, we examined the possibility of using zein nanoparticles to encapsulate tangeretin (a representative PMF), and to determine its stability to various environmental stresses that it may experience in commercial food and beverage products. To the author’s knowledge, this is the first time that zein nanoparticles have been used as a delivery system for tangeretin, which is an important nutraceutical suitable for incorporation into functional foods and beverages. The knowledge gained from this study should therefore be useful for the design of food-grade delivery systems for lipophilic bioactive.

5.2 Material and Methods

5.2.1 Material

Tangeretin (purity 98.4%) was purchased from Bepharm Ltd. (Shanghai, China). Corn oil (Mazola) was purchased from a local supermarket (ACH Food Companies, Inc., USA). β-lactoglobulin [54 JE 002-8-415] was obtained from Daviso Foods International (Le Sueur, MN). The manufacturer reported the composition of this powder to be 97.4% total protein, 92.5% β-lactoglobulin (β-lg), and 2.4% ash. Zein (purity 92%, w/w) and uranyl acetate was purchased from Sigma-Aldrich (St Louis, MO). Other chemicals, such as sodium chloride (NaCl), sodium hydroxide (NaOH), calcium chloride (CaCl₂), hydrochloric acid (HCl), HPLC grade methanol, tetrahydrofuran (THF), trifluoroacetic acid (TFA), and acetonitrile (ACN) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Double distilled water was
prepared using a Nanopure water purification system (Model D14031, Thermo Scientific, USA).

5.2.2 Preparation of tangeretin loaded zein nanoparticle

The tangeretin-loaded zein colloidal system was prepared using a liquid-liquid dispersion method described previously [167]. The schematic representation of this procedure is shown in Figure 18.

**Figure 18.** Schematic representation for preparing tangeretin loaded zein colloidal system.

Organic phase: Zein and tangeretin (mass ratio 25:1, 0.5g/0.02g) were dissolved in 20 ml of ethanol solution (90%, v/v) and stirred (Corning Stirrer PC-420, Corning Inc., USA) for at least 2 hours to ensure full dissolution. The criteria for choosing this
ratio was based on our preliminary experiments to ensure there is enough zein to encapsulate tangeretin and no tangeretin crystals were formed during preparation. Aqueous phase: β-β-lactoglobulin was dissolved in phosphate buffer saline (10 mM, pH 7) solution (3%, w/v) and stirred for at least 2 hours to ensure complete hydration.

Colloidal system preparation: The organic phase was introduced into the aqueous phase drop by drop under constant stirring at 1,000 rpm (organic phase: aqueous phase= 1:3, v/v). The organic solvent was then removed using a rotary evaporator (Rotavapor R110, Buchi Crop., Switzerland). A sample containing no β-lactoglobulin was made using the same method and was regarded as a control. The resulting tangeretin-loaded zein colloidal suspensions were dried using a freeze dryer (VirTis Genesis Lyophilizer, Virtis genesis company inc., USA) and kept in a refrigerator before analysis. In order to determine the maximum amount of tangeretin that could be encapsulated into the zein nanoparticles, a series of different colloidal systems (organic phase containing 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.0% tangeretin) were made using the same method. The encapsulation efficiency of tangeretin was calculated using the following formulation:

\[
\text{Encapsulation efficiency (\%)} = \frac{C_{\text{Colloidal}}}{C_{\text{Total}}} \times 100\%
\]

Where, \(C_{\text{Colloidal}}\) is the concentration of tangeretin in the colloidal suspension, and \(C_{\text{Total}}\) is the concentration of tangeretin in the total system. The value of \(C_{\text{Colloidal}}\) was measured by extracting the tangeretin from the dried powders and measuring its
concentration using HPLC, where as the value of \( C_{\text{Total}} \) was known from the initial amount of tangeretin added to the system.

**5.2.3 Tangeretin concentration determination**

The content of tangeretin in different systems was determined using a HPLC method. For dried powders, samples were first reconstituted to their initial concentrations using PBS buffer (10mM, pH 7). After dilution, 100 µL of sample was transferred into a 1.5 mL centrifuge tube. 400 µL of ethyl acetate was added to the tube and vortexed for 30 s. Then the mixture was centrifuged (1,170 g) for 6 min. The ethyl acetate layer was collected and the remaining sample was prepared using the same procedure for a second time. The two ethyl acetate layers were combined and dried on a vacuum concentrator (Model: SVC 100H, Thermo Fisher Scientific Inc., USA). Then 100µL of mobile phase was added to the tube to dissolve the tangeretin extracted by ethyl acetate. The tube was vortexed and centrifuged (1,170 g) for at least 30 s to remove non-dissolved material. A tangeretin standard solution was prepared by diluting 50 µM of stock solution (dissolved in DMSO) with mobile phase to a series of desired concentration (5, 10, 20, and 50 µM). An aliquot (90 µL) of solution were collected from the top of the tube and analyzed using a CoulArray HPLC system (Thermo Scientific Inc., USA) that contains a Model 584 binary solvent delivery system, an Model 542 auto-sampler, a CoulArray 5600A detector and a Model 526 UV detector (Waters, USA). A RP-Amide reversed-phase HPLC column (15 cm×4.6 mm id, 3 µm, Ascentis Express, Sigma-Aldrich Corp., USA) was used on this HPLC system. The mobile phase and detection conditions used were as described previously with some modifications [150, 168]. The 50% (B) mobile
phase contained 50mM ammonium acetate, 0.05% TFA, 10% THF, 40% ACN and 50% water. The 25% (A) mobile phase was made up of 25 mM ammonium acetate, 0.05% TFA, 5%THF, 20% ACN and 75% water. The injection volume was 50 μL. The running time was 35 min and a linear gradient elution mode was used, i.e., 10%, 100% and 100% mobile phase B at time 0 min, 20 min and 30 min. UV detector was set at 330 nm to detect the signal of tangeretin. The equation was \( y = 0.3228x + 0.14 \), \( R^2 = 0.9971 \).

5.2.4 Characterization of tangeretin-loaded zein nanoparticles

Particle size and ζ-potential: The particle size and ζ-potential of the particles in the colloidal dispersions were determined using a commercial dynamic light scattering and micro-electrophoresis device (Nano-ZS, Malvern Instruments, UK). Freshly prepared samples were diluted 5 times with buffer solution (10 mM phosphate buffer, pH 7) at room temperature before measurement. Freeze-dried samples were re-dispersed in buffer solutions (10mM phosphate buffer, pH 7), stirred for 5 min, and then diluted 5 times with the same buffer. The particle size data are reported as the intensity-weighted ("Z-average") mean particle diameter, while the particle charge data are reported as the ζ-potential.

Visual and microstructure observation: The appearance of tangeretin-loaded zein colloidal dispersions, dried powders, and other samples were recorded with a digital camera (Canon PowershotG12, NY). Microstructure of nanoparticles in solutions and powders were observed using an optical microscope (Nikon Eclipse E400, Nikon Crop., Japan). For aqueous samples, 7 μL of solution was placed on a
microscope slide and covered by a cover slip. For dried samples, powders were spread evenly on the microscope slide and covered by a cover slip.

Transmission electron microscopy: Samples were prepared with negative staining for TEM observation. Tangeretin-loaded zein colloidal suspensions were diluted with PBS buffer (10 mM, pH 7) until the solution was semi-opaque. A drop of this solution was applied to the grid surface and kept for about 2 min. Excess sample was removed with a wedge of filter paper. Then the grid was immersed in a drop of negative stain solution uranyl acetate (2%, w/w), quickly taken out and left to stand for 45 s. Excess uranyl acetate was removed with a wedge of filter paper. The grid was then observed under a transmission electron microscope (Tecnai 12, FEI Company, USA).

5.2.5 Stability of tangeretin loaded zein colloidal system

**pH:** The pH of freshly prepared colloidal suspensions were adjusted to the desired values (from pH 3 to 7) using NaOH or HCl solution. The mixtures were then transferred to glass tubes and stored at ambient temperature overnight.

**Salt:** Colloidal suspensions were diluted with the same volume of concentrated salt solutions to form a series of samples with different salt concentrations (0 to 500 mM NaCl). The resulting mixtures were then stirred for 30 min and stored at ambient temperature overnight.

**Temperature:** Samples with no salt and 150 mM salt were incubated in water bath for 30 min (30 °C to 90 °C) and then stored at ambient temperature overnight.

The particle size, ζ-potential and appearance of the samples were then measured.
5.2.6 Data Analysis

All experiments were carried out at least three times and the results were reported as averages and standard deviations of these measurements. Student's t-test was used to determine the difference between two groups. One way ANOVA was used to determine the statistic difference for more than two groups. A 5% significant level was used for all the study.

5.3 Results and Discussion

5.3.1 Characterization

In this study, we first determined the role of emulsifier (β-lactoglobulin) in the formation of stable colloidal suspensions of zein. In the absence of emulsifier, we observed that some material coagulated on the wall of the test tubes during the preparation procedure, which was not observed when β-lactoglobulin was present (Figure 19). These visual observations were supported by particle size distribution measurements (Figure 19). Colloidal suspensions prepared without emulsifier had a bimodal particle size distribution with a population of particles > 1000 nm. This suggests that some of the zein particles formed clumps that adhered to the sides of the container, presumably through hydrophobic interactions. It should be noted that dynamic light scattering cannot detect particles greater than about 5000 nm, and therefore the large zein particles that were visible in this system would not show up in the particle size distributions. All the particles in the colloidal suspensions prepared in the presence of β-lactoglobulin were relatively small, and the particle size distribution was narrow. Presumably, the β-lactoglobulin molecules adsorbed to any hydrophobic patches on the surfaces of the zein particles, and therefore
prevented precipitation. Previous studies have also shown that a protein emulsifier (caseinate) could prevent the aggregation of zein particles [169]. Hence, the emulsifier plays an important role in the formation and stabilization of zein colloid suspensions.

![Figure 19](image)

**Figure 19** Particle size distribution and appearance of suspensions of tangeretin loaded protein nanoparticles made without and with β-lactoglobulin (β-lg) in the initial aqueous solution.
Figure 20. Structure of tangeretin-loaded protein nanoparticles: (a) visual appearance after freeze drying; (b) optical microscopy image of freeze dried powder (dimensions 0.3 mm × 0.22 mm); (c) visual appearance after rehydration in PBS buffer; (d) transmission electron microscopy image of rehydrated protein nanoparticles (scale bar = 1000 nm); (e) optical microscopy image of structure of rehydrated powder (dimensions 0.3 mm × 0.22 mm).

The freshly prepared zein particles had a mean particle diameter of 462 ± 24 nm in the aqueous colloidal suspensions. After evaporation, the mean particle diameter decreased to 249 ± 4 nm, which can be attributed to removal of ethanol that was originally present inside the particles. The morphology of tangeretin-loaded nanoparticles depended on the nature of the system (Figure 20). The freeze-dried samples were white powders (Figure 20a) that contained irregularly shaped particles when observed using optical microscopy (Figure 20c). After the samples were rehydrated in PBS buffer, the colloidal suspension had a similar appearance as
the freshly prepared samples (Figure 20b). The TEM image shows that these particles had well-defined spherical shapes, with diameters around 200 nm (Figure 20d), which was in agreement with the dynamic light scattering measurements (d = 263 ± 13 nm). No crystals were observed by optical microscopy after they were rehydrated (Figure 20e). The encapsulation efficiency of tangeretin in the protein nanoparticles was found to be around 73%. In summary, we were able to prepare a homogeneous colloidal suspension containing protein nanoparticles with tangeretin encapsulated inside. This system had good water-dispersibility after lyophilisation, thus it has potential to be incorporated into a variety of food and beverage products.

To determine the maximum amount of tangeretin that can be encapsulated in the zein nanoparticles, we made a series of protein nanoparticle suspensions that contained different amounts of tangeretin, while keeping other parameters constant. There was a slight increase in mean particle diameters of these systems with increasing tangeretin concentration as determined by dynamic light scattering (Figure 21). Nevertheless, the mean particle diameter was relatively insensitive to tangeretin concentration in the initial organic phase (d = 350 ± 20 nm), which suggests this bioactive component did not have a major influence on protein nanoparticle formation. However, optical microscopy clearly showed that there were large crystals in samples containing relatively high tangeretin concentrations, i.e., > 0.4% w/v in the initial organic phase (Figure 21). We assumed that these crystals were formed due to the supersaturation of tangeretin in the mixture of organic phase and aqueous phase [157]. The fact that the dynamic light scattering did not indicate the presence of these large crystals was probably because they
settled to the bottom of the measurement cells prior to analysis. In addition, the crystals were too large to be detected by dynamic light scattering since this method relies on movement of the particles due to Brownian motion. Based on these findings, we used 0.2% w/v tangeretin in the organic phase in the subsequent experiments to ensure no crystals were formed during preparation of the protein nanoparticles.

![Figure 21](image.jpg)

**Figure 21.** Influence of tangeretin concentration on the mean particle diameter of tangeretin-loaded protein nanoparticles (scale bar = 50 µm). The insert shows optical microscopy images of systems made with different tangeretin concentrations: 0.1%; 0.2%; 0.4%; and 0.8%. The image represents an area of 0.3 mm × 0.22 mm. Notice crystals were formed at higher tangeretin concentrations.
5.3.2 Stability of tangeretin-loaded zein colloidal suspensions

It is important to test the stability of delivery systems under different environmental conditions because this determines the type of commercial products it can be incorporated into, as well as their behavior in the human gastrointestinal tract [69]. For this reason we examined the influence of a number of different environmental stresses on the stability of the tangeretin-loaded protein nanoparticles.

5.3.2.1 Ionic strength

Delivery systems may be dispersed within aqueous solutions containing different types and amounts of electrolytes within commercial products and within the human gastrointestinal tract, and so it is important to establish the influence of ionic strength on their properties. Tangeretin-loaded protein nanoparticles were stable to aggregation at relatively low salt concentrations (< 50 mM), but exhibited extensive aggregation at higher levels, as indicated by an increase in mean particle diameter and the formation of a white sediment at the bottom of the test tubes (Figure 22). As the salt content increased, the sediment layer became thicker and the solution above it became clearer indicating that more extensive particle aggregation had occurred. The mean particle diameter increased from around 310 nm in the absence of salt to over 4500 nm in the presence of 500 mM NaCl. These effects can be attributed to the influence of ionic strength on the colloidal interactions between the protein nanoparticles. In the absence of salt, there will be a relatively strong electrostatic repulsion between the nanoparticles, which prevents them from aggregating [170, 171]. In the presence of salt, the electrostatic interactions
between the nanoparticles are screened and so the attractive interactions (e.g., van der Waals and hydrophobic) dominate the repulsive interactions (e.g., electrostatic and steric), leading to extensive nanoparticle aggregation [170]. These results suggest that the protein nanoparticles will remain stable in low-viscosity commercial products with relatively low salt concentrations (such as fortified waters or soft drinks), but may aggregate and sediment in products with high levels of salt. It also suggests that they may be susceptible to aggregation after ingestion since human gastrointestinal fluids have relatively high ionic strengths (approximately 100 mM in stomach and 140 mM in small intestine) [172, 173]. On the other hand, they may be suitable for utilization in high viscosity, gelled or solids foods, since then particle sedimentation is less of a problem.
Figure 22. Influence of ionic strength on the stability of tangeretin loaded protein nanoparticles. At higher salt concentrations, the mean particle diameter increases and sediments are formed in the bottom of the test tubes. All data represent mean ± SD.

5.3.2.2 pH

Delivery systems may experience different pH environments when they are incorporated into commercial food and beverage products, or when they pass through the human gastrointestinal tract, e.g. mouth, stomach, small intestine, and
We therefore investigated the influence of pH on the stability and physicochemical properties of the tangeretin-loaded protein nanoparticles. It was postulated that the protein nanoparticles consisted of a hydrophobic zein core coated by an amphiphilic β-lactoglobulin layer, and so the net charge on the protein nanoparticles was dominated by β-lactoglobulin’s electrical characteristics. This hypothesis was supported by the pH dependence of the electrical charge on the protein nanoparticles. The ζ-potential changed from positive to negative as the solution was adjusted from pH 3 to 7, with a point of zero charge around pH 5 (Figure 23). This pH is close to the reported isoelectric point of β-lactoglobulin (pI ≈ pH 5) [69], and is considerably lower than the isoelectric point of zein (pI ≈ pH 6.2) [169]. The tangeretin-loaded zein nanoparticles were stable to aggregation at relatively low pH (< 4.5) and high pH (> 5.5), but were highly unstable at intermediate pH values (Figure 24). The good aggregation stability at low and high pH values can be attributed to a strong electrostatic repulsion between the particles at values far from the isoelectric point, whereas the poor stability at intermediate pH can be attributed to the weak electrostatic repulsion near the isoelectric point [171]. Previous studies have shown that charge reversal occurs when an amphiphilic protein (caseinate) was added to a suspension of zein particles, which also supports the idea of the amphiphilic proteins forming a thin layer around the water-insoluble zein core [169].
Figure 23. Influence of pH on the electrical charge of suspensions of tangeretin loaded protein nanoparticles. All data represent mean ± SD.
Figure 24. Influence of pH on the mean particle diameter and appearance of suspensions of tangeretin loaded protein nanoparticles. All data represent mean ± SD.

5.3.2.3 Temperature

The purpose of these experiments was to determine the influence of temperature on the stability of the tangeretin-loaded protein nanoparticles. This knowledge is important since delivery systems may experience variations in temperature during the manufacturing, storage, and utilization. In the absence of added salt (Figure 25), the size of the particles in the colloidal suspension remained relatively small from 30 to 60 °C (d < 500 nm), but at higher temperatures there was an appreciable increase in particle size. Previous differential scanning calorimetry studies have
shown that native β-lactoglobulin has a transition temperature around 70 °C, which was attributed to thermal denaturation of this protein [174, 175]. Protein denaturation exposes reactive non-polar and sulphydryl groups [176, 177], which increases hydrophobic attraction and disulfide bond formation within and between protein-coated particles [174].
Figure 25. Influence of temperature and salt addition on the mean particle diameter and appearance of suspensions of tangeretin-loaded protein nanoparticles. Light scattering measurements could not be made on the samples containing salt after high temperature processing since they formed gels (inset photograph). All data represent mean ± SD.

In the presence of salt (150 mM), the mean particle diameters were much greater than in its absence even at temperatures well below the thermal denaturation temperature, i.e., 30 °C (Figure 25). In addition, gelation was observed in samples that were held at temperatures around and above the thermal denaturation
temperature of β-lactoglobulin, i.e., > 70 °C. The addition of salt to the system will have decreased the electrostatic repulsion between the protein nanoparticles so that the net attractive forces (van der Waals and hydrophobic) dominated the net repulsive forces (electrostatic and steric). More extensive aggregation was observed above the thermal denaturation temperature because of the increase in the surface hydrophobicity of the β-lactoglobulin-coated particles after they unfold [178]. In general, gelation occurs when the concentration of particles exceeds a specific concentration so that a three-dimensional network can form throughout the system [179]. In summary, the zein nanoparticles were prone to extensive aggregation at elevated temperatures, particularly in the presence of salt.

5.4 Conclusions

The purpose of this study was to establish an effective method for the encapsulation and delivery of a lipophilic bioactive compound. Tangeretin has low water-solubility at ambient temperature and is therefore difficult to incorporate into many aqueous-based food and beverages. We therefore fabricated a colloidal system that could potentially deliver tangeretin in these types of products. We showed that tangeretin could be incorporated into relatively small protein-nanoparticles that consisted of a hydrophobic zein core and an amphiphilic β-lactoglobulin shell. These protein-nanoparticles behaved similarly to β-lactoglobulin-coated fat droplets under different environmental conditions: they were stable at low salt concentrations at pH values far from the isoelectric point, but aggregated at higher salt levels and pH values near the isoelectric point. In addition, they were stable to aggregation at temperatures below the thermal denaturation temperature of β-lactoglobulin, but
aggregated at higher temperatures, particularly in the presence of salt. These
delivery systems may be useful for incorporating bioactive flavonoids into aqueous-based food products. In future research, we intend to focus on the behavior of tangeretin-loaded protein nanoparticles under simulated gastrointestinal conditions so as to better understand the factors that affect the bioaccessibility and absorption of tangeretin.
CHAPTER 6

BIOLOGICAL FATE OF TANGERETIN LOADED ZEIN COLLOIDAL SYSTEM-A

STUDY OF IN VITRO DIGESTION AND PERMEABILITY

6.1 Introduction

Tangeretin represents a class of polymethoxy flavonoids (PMFs) found almost exclusively in citrus fruits, and particularly the peels of sweet and mandarin oranges [180]. PMFs have been shown to exhibit a variety of potential benefits for human health, such as enhancing biochemical events in mammalian cells [181], reducing serum triacylglycerol, very low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) levels [182]. PMFs can also inhibit the proliferation of certain types of cancer cells, such as lung, colon and breast cancer cells [18, 183, 184]. PMFs have also been demonstrated to modulate the liver and heart function of hypercholesterolemic of rats [6].

However the oral bioavailability of tangeretin is currently limited due to its poor water solubility, which is related to the presence of numerous methoxyl groups on the flavone backbone [3]. We have previously shown that zein nanoparticles and microparticles offer a suitable means of encapsulating and delivering tangeretin [185]. Colloidal particles can be formed from zein using antisolvent precipitation due to its high solubility in alcohol solutions but low solubility in water [186]. The zein and active component are dissolved in an alcohol solution, which is then injected into water, resulting in the spontaneous formation of zein particles containing the active component. This method has been successfully used to encapsulate lipids, such as fish oil, flax oil, and essential oils [164-166].
The purpose of the current study was to examine the impact of lipid nanoparticles on the potential gastrointestinal fate of tangeretin-loaded zein nanoparticles. It is well known that co-ingestion of digestible lipids can increase the oral bioavailability of lipophilic nutraceuticals and pharmaceuticals by altering their bioaccessibility, absorption, or transformation within the gastrointestinal tract [161, 187, 188]. Lipids may enhance the bioavailability of lipophilic molecules through a variety of mechanisms, including stimulating the secretion of digestive juices, increasing gastrointestinal transit times, enhancing their solubility within intestinal fluids through mixed micelle formation, increasing the permeability of the epithelium monolayer, controlling chemical or biochemical transformation, or altering the absorption route (portal vein versus lymphatic system). We therefore hypothesized that mixing tangeretin-loaded zein nanoparticles with digestible lipid nanoparticles would enhance the bioavailability of the lipophilic tangeretin molecules. The influence of lipids on the potential biological fate of the tangeretin was studied using a simulated gastrointestinal model that included mouth, stomach, and small intestine phases, combined with a Caco-2 cell model to study permeability [3].

An important aim of this study was to highlight the potential advantages of using combination delivery systems containing a mixture of different types of colloidal particles (in this case zein and lipid nanoparticles) rather than using single types of colloidal particle. This information may be useful in the rational design of oral delivery systems for food and pharmaceutical applications.
Figure 26. Graphic abstract of zein colloidal system digested with nanoemulsion.

6.2 Materials and Methods

6.2.1 Material

Tangeretin powder with a purity of 98.4% was obtained from Bepharm Ltd. (Shanghai, China). β-lactoglobulin (with a purity of 92.5%) was obtained from Daviso Foods International (lot JE 002-8-415, Le Sueur, MN). Corn oil was obtained from a local supermarket. Zein (purity 92%, w/w), bile extract (porcine, B8613), porcine pancreas (Type II, triacylglycerol hydrolase E.C. 3.1.1.1, PPL), Hank’s balance salts (cat. no. H1387) and uranyl acetate were purchased from Sigma-Aldrich (St Louis, MO). Pepsin (CAS: 9001-75-6), sodium chloride (NaCl), sodium hydroxide (NaOH), calcium chloride (CaCl₂), hydrochloric acid (HCl), HPLC grade
methanol, tetrahydrofuran (THF), trifluoroacetic acid (TFA), acetonitrile (ACN) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was obtained from EMD Chemicals Inc. (Gibbstwon, NJ, USA). Double distilled water was made from a water purification system (Model D14031, Barnstead Nanopure water system, Dubuque, Iowa, USA). DMEM (Dulbecco’s Modification of Eagle’s Medium) and non-essential amino acid was purchased from Mediatech Inc., (Manassas, VA). HEPES was purchased from Acros Organics (Geel, Belgium).

6.2.2 Preparation of tangeretin loaded zein nanoparticle.

The tangeretin-loaded zein nanoparticles were made using an antisolvent precipitation method described previously [167], with some slight modifications. The particles were prepared by injecting an organic phase into an aqueous phase. The organic phase consisted of zein and tangeretin (25:1, w/w) dissolved in 90% ethanol solution, while the aqueous phase consisted of β-lactoglobulin (3%, w/v) dissolved in PBS (10 mM, pH 7). The zein nanoparticles were formed spontaneously when the organic phase was injected into the aqueous phase (1:3, v/v) dropwise under constant stirring at 1000 rpm (Corning Stirrer PC-420, Corning Inc., USA). The ethanol in the mixture was then evaporated with using a vacuum rotary evaporator (Rotavapor R110, Buchi Crop., Switzerland). Then the sample was freeze dried (VirTis Genesis Lyophilizer, Virtis genesis company inc., USA) and kept in a refrigerator prior to further use.

6.2.3 Preparation of lipid nanoparticles

β-lactoglobulin was dissolved in 10 mM phosphate buffer (3%, w/w) and stirred for at least 2 hours to ensure full hydration. A coarse emulsion was prepared by mixing
20% corn oil with aqueous solution using a hand blender (M133/1280, Biospec Products, Inc., ESGC, Switzerland) for 2 min. This coarse emulsion was then passed through a high pressure homogenizer (Microfluidics M-110Y, Newton, MA) at 12,000 psi for 3 times.

6.2.4 Particle size and ζ-potential measurement

The particle size and ζ-potential of the nanoparticles were determined using a commercial dynamic light scattering and micro-electrophoresis device (Nano-ZS, Malvern Instruments, Worcestershire, UK). The samples were diluted 10 times in PBS buffer solutions (pH 7) at the same pH as the samples being analyzed at room temperature before measurement. The particle size is reported as the intensity-weighted ('Z-average') mean particle diameter, while the particle charge is reported as the ζ-potential.

6.2.5 Microstructure & visual observations

The microstructure of the colloidal delivery systems was observed using an optical microscope (Nikon Eclipse E400, Nikon Corp., Japan), and the resulting images were acquired using digital image processing software (Micro Video Instruments Inc., Avon, MA). Selected samples were also analyzed using transmission electron microscopy (JEOL JEM-2000FX, JEOL USA, Inc., MA, USA). The general appearance of the colloidal systems and digesta after different gastrointestinal stages were recorded by taking images using a digital camera (Powershot SD1300IS, Canon).

6.2.6 Potential gastrointestinal fate of tangeretin-loaded zein nanoparticles

An in vitro digestion model was used to study the potential behavior of the delivery systems under simulated gastrointestinal tract [189] conditions. Experiments were
carried out at different lipid contents by mixing the tangeretin-loaded zein nanoparticles with lipid nanoparticles to simulate different diet compositions (i.e. high versus low fat diets).

Different delivery systems were prepared by mixing the freshly prepared tangeretin-loaded zein nanoparticles and different amounts of stock nanoemulsion and phosphate buffer solution so that they differed only in oil content (0%, 2%, and 4%). The samples were then passed through a simulated GIT similar to that described earlier [190], but with some slight modifications.

**Mouth phase:** Oral conditions were mimicked by mixing the delivery systems with a simulated saliva fluid (SSF), which was prepared from various salts and mucin as described previously [151]. Delivery systems were mixed with SSF at a 1:1 volume ratio, and then the resulting mixture was adjusted to pH 6.8 and shaken continuously at 100 rev/min in an incubator at 37°C for 10 min (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA).

**Stomach phase:** Simulated gastric fluid was prepared by mixing 2 g of NaCl and 7 ml of concentrated HCl and then making the volume up to 1 L using distilled water [151]. Pepsin was then dissolved in this mixture (0.32 %, w/v), the pH was adjusted to 1.2, and the sample from the mouth phase was mixed with it at a 1:1 volume ration. The resulting mixture was then adjusted to pH 2.5 and incubated at 100 rev/min and 37 °C for 2 hr.

**Small intestine:** Small intestine conditions were simulated using a pH-stat automatic titration unit (Metrohm, USA Inc.). The sample collected from the stomach phase was placed in a container placed in a 37 °C water bath. The pH was adjusted to pH
7.0. Bile salt (187.5 mg in 4 ml pH 7 PBS buffer solution) was then added, and the pH was adjusted back to 7.0. Calcium chloride solution (110 mg dissolved in 1 ml double distilled water) was then added and again the pH was adjusted to 7.0. Finally, freshly prepared pancreatin lipase (60 mg lipase in 2.5 ml pH 7.0 PBS) was added to the solution. At the same time, the automatic titration of pH-stat was started. The free fatty acid released during digestion was calculated using the following equation [152]:

\[ \text{FFA\%} = 100 \times \left( \frac{V_{\text{NaOH}} \times m_{\text{NaOH}} \times M_{\text{lipid}}}{w_{\text{lipid}} \times 2} \right) \]  \hspace{1cm} (1)

Here, \( V_{\text{NaOH}} \) is the volume of sodium hydroxide solution required to neutralize the free fatty acids (and other sources of H\(^+\)) released during digestion; \( m_{\text{NaOH}} \) is the molar concentration of sodium hydroxide solution, which is 0.25 M in this study; \( M_{\text{lipid}} \) is the molecular weight of corn oil, which is 800 g/mol; \( w_{\text{lipid}} \) is the amount of oil in the reaction system.

### 6.2.7 Bioaccessibility determination

The samples obtained after in vitro digestion were collected and centrifuged (Centrifuge 5417R, Eppendorf co., Hamburg, Germany) (20200 g, 14000 rpm) at 4 °C for 40 min. The clear micelle phase on the top layer of the samples was filtered through a 220 nm syringe filter (EMD Millipore, Billerica, MA) and analyzed for the tangeretin content using the HPLC method described before [150]. The bioaccessibility was then calculated using the following equation:

\[ \text{Bioaccessibility\%} = \frac{C_{\text{Micelle}}}{C_{\text{Raw\ Digesta}}} \times 100\% \]  \hspace{1cm} (2)
Here, $C_{\text{Micelle}}$ is the concentration of tangeretin in the filtered micelle phase, and $C_{\text{Raw Digesta}}$ is the concentration of tangeretin in the raw digesta.

### 6.2.8 Cytotoxicity measurement of micelle phase

Caco-2 cells were seeded in 96-well plates at a density of 20,000 cells/well in 200 μL complete DMEM media (10% FBS, 1% antibiotic, 1% non-essential amino acid). After 24 h, cells were treated with different concentrations of micelle phase diluted with serum complete media. Tangeretin dissolved in DMSO was used as a control group and the final DMSO concentration in the medium was less than 1%. After incubation for 24 h, cells were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media in each well was replaced by 100 μL freshly prepared MTT solution (0.5 mg/mL dissolved in DMEM media). After 2 h incubation at 37°C, MTT solution was dumped and the reduced formazan dye was solubilized by adding 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570 nm using a plate reader (TECAN, Phenix Research Products, Candler, NC, USA) [191].

### 6.2.9 Tangeretin permeability determination using Caco-2 monolayers

Caco-2 cell monolayer maintenance and permeability determination was conducted as described before [153]. Caco-2 cells were seeded on transwell permeable supports containing 0.4 μm polycarbonate membranes (Corning Incorporated, Corning, NY) at a seeding density of $2.6 \times 10^5$ cells/cm². The medium was changed 16 h after seeding. The media in the apical and basolateral compartments were changed every other day. This process was maintained for about 21 days until the transendothelial electrical resistance of the filter was about 260 Ω cm².
The micelle phases were collected after *in vitro* digestion and filtered through a 220 nm membrane. They were then diluted with Hank’s Balanced Salt (HBSS, pH 7.4) to obtain tangeretin concentrations suitable for permeability determinations.

Tangeretin dissolved in DMSO was used as a control group. All the solutions used in this experiment were pre-warmed in a 37 °C water bath. The Caco-2 monolayer transwell was incubated with HBSS for 30 min before the experiment. Then both the apical and basolateral compartments were rinsed twice with HBSS. An aliquot (1.5 ml) of each sample was added to the apical compartment and 2.5 ml pre-warmed HBSS was added to the basolateral compartment. Then, this plate was placed in an incubator. Every 30 min, 200 μL of solution was withdrawn from the apical compartment without adding new sample and 100 μL of sample was taken from the basolateral compartment and replaced by same amount of pre-warmed HBSS. The concentration of tangeretin in each sample were analyzed using a HPLC method.

The apparent permeability coefficient (cm s⁻¹) was calculated with the following equation:

\[
P_{app} = \frac{(dQ/dt)(1/(AC₀))}{A}
\]

\[dQ/dt \text{ is the steady-state flux (μmol s}^{-1})\]
\[A \text{ is the surface area of the filter (4.67 cm}^2)\]
\[C₀ \text{ is the concentration of tangeretin added to apical compartment of each well (μM).}\]

**6.2.10 Data analysis**

All data in this study are expressed as mean ± SD. Student’s t-test was used to determine the significance of difference between two groups. One way ANOVA was used to analyze the significance of difference for more than three groups. 5% significance level was used for all tests.
6.3 Results and Discussion

6.3.1 Characterization of delivery systems

In this study, mixed delivery systems were prepared that contained a combination of tangeretin-loaded zein nanoparticles and digestible lipid nanoparticles. Knowledge of the initial characteristics of these nanoparticles is important to understand their subsequent behavior in the simulated GIT. We therefore measured the size, charge, and morphology of the particles using light scattering, electrophoresis, and microscopy. The mean particle diameters of the freshly prepared nanoemulsions, tangeretin-loaded zein nanoparticles, and their mixture were 197, 248, and 215 nm respectively (Figure 27). The ζ-potentials of the initial lipid nanoparticles (-29 mV), tangeretin-loaded zein nanoparticles (-25 mV) and mixed system (-52 mV) were all highly negative. The transmission electron microscopy (TEM) measurements suggested that the zein nanoparticles (smooth surfaces) and lipid nanoparticles (crinkly surfaces) existed separately in the mixture (Figure 27), which can be attributed to the strong electrostatic repulsion between them. We confirmed the nature of the different kinds of nanoparticles in the mixed systems by taking TEM images of samples containing only lipid nanoparticles, which had crinkly surfaces, and only zein nanoparticles which had smooth surfaces (data not shown). The crinkly appearance of the lipid nanoparticles is probably due to crystallization of the uranyl acetate dye on their surfaces during sample preparation for electron microscopy. Optical microscopy images of the mixed delivery systems confirmed that they had good stability to aggregation, with no evidence of large particles in the system (Figure 27). The colloidal delivery systems all had good
stability to gravitational separation (creaming or sedimentation), as demonstrated by the fact that no phase separation was observed after 24 hours incubation, which can be attributed to their small initial particle size and stability to aggregation.

Figure 27. Particle size distribution of fat droplets, protein nanoparticles, and the mixed system. The inset shows a transmission electron microscopy image of the mixed system.
6.3.2 Gastrointestinal fate of colloidal delivery systems

Colloidal delivery systems encounter a series of physicochemical and physiological environments as they pass through the various stages of the human GIT, such as changes in pH, ionic strength, agitation, enzyme activities, and surface active agents [192]. We therefore measured changes in the properties of mixed colloidal delivery systems (containing 2% fat) as they passed through the simulated mouth, stomach, and small intestine phases of the GIT model.

**Mouth:** The mean diameter of the particles in the system increased from around 215 to 424 nm after incubation in the simulated saliva fluids (Figure 29), which suggests that some particle aggregation occurred. This observation was supported by the optical microscopy images, which clearly showed evidence of extensive particle aggregation (Figure 28a). The origin of this effect can be attributed to particle flocculation induced by the presence of mucin in the artificial saliva. Mucin is a large glycoprotein that can promote particle aggregation under oral conditions through both bridging and depletion mechanisms [193]. The magnitude of the negative charge on the particles decreased when they moved from the initial to the mouth phases (Figure 30), which can be attributed to electrostatic screening effects by salts in the simulated saliva, as well as to possible adsorption of mucin molecules to the lipid droplet surfaces.
Figure 28. Microscopic image of initial delivery system (a) and delivery system after digestion in (b) mouth, (c) stomach, (d) small intestine.

Stomach: There was a large increase in the mean particle diameter measured by light scattering (Figure 29) and evidence of extensive particle aggregation in the optical microscopy images (Figure 28) when the samples moved from the mouth to the stomach phases. A number of different physicochemical phenomena may contribute to the instability of the nanoparticles within the gastric environment. First, both the zein and lipid nanoparticles used in this study were coated with a globular protein (β-lactoglobulin) that has an isoelectric point around pH 5. Consequently, the nanoparticles will have passed through a point of zero charge when they moved from the simulated mouth (pH 7) to the simulated gastric (pH 2)
fluids, which may have promoted irreversible aggregation due to a reduction in electrostatic repulsion. Second, anionic mucin molecules from the saliva may have caused bridging flocculation of the cationic protein-coated nanoparticles in the stomach. Third, the relatively high ionic strength of the gastric fluids may have reduced any electrostatic repulsion between the nanoparticles leading to aggregation. Fourth, hydrolysis of the β-lactoglobulin coating around the nanoparticles by digestive enzymes (pepsin) may have reduced their aggregation stability. The electrical charge on the nanoparticles in the stomach was close to zero (Figure 30). One might expect nanoparticles coated by β-lactoglobulin to be strongly positively charged at pH 2 because this is well below their isoelectric point. The fact that the charge was near zero may have been because anionic mucin adsorbed to the droplet surfaces, thereby neutralizing some of the positive charge from the proteins. In addition, some of the surface proteins may have been digested by the pepsin, which would have altered the surface charge. The droplets in the emulsion were highly unstable to gravitational separation (Figure 29) under gastric conditions, which can be attributed to the relatively large particle size caused by droplet aggregation. Interestingly, we did not observe a sediment layer in these systems suggesting that the zein nanoparticles associated with the lipid nanoparticles and the overall density of the flocs formed was less than that of water.

Small Intestine: The mean particle diameter remained relatively large after incubation in the small intestinal fluids (Figure 29), but the large aggregates formed within the gastric fluids appeared to have largely dissociated (Figure 28). In the presence of pancreatin, the triacylglycerol molecules in the lipid droplets will be
converted to a monoacylglycerol [194] and two free fatty acids (FFAs) by pancreatic lipase. These MAG and FFAs will combine with phospholipids and bile salts to form mixed micelles (micelles and vesicles) that can solubilize lipophilic compounds, and then carry them through the mucous layer to the small intestine cell surfaces [161]. The pancreatic lipase used in this study was a crude extract that also has protease activity. Consequently, the β-lactoglobulin and zein in the protein nanoparticles would have been fully or partially hydrolyzed, thereby releasing the tangeretin in the intestinal fluids. As a result, the digesta is likely to contain a complex mixture of different types of colloidal particles, including undigested lipid particles, undigested protein particles, micelles, vesicles, and insoluble matter. The high negative charge on the particles in these samples can therefore be attributed to the anionic nature of the free fatty acids, bile salts, phospholipids, and proteins at neutral pH conditions (Figure 30).
Figure 29. Mean particle diameters and appearances of mixed systems initially containing protein nanoparticles and lipid nanoparticles (2% oil) after passage through various stages of a simulated gastrointestinal tract (Christensen, #70). The inset shows the appearance of the system at different digestion stages. (Means with different letters are significantly different, p<0.05).
Figure 30. Mean particle charges of mixed systems initially containing protein nanoparticles and lipid nanoparticles (2% oil) after passage through various stages of a simulated gastrointestinal tract (Christensen, #70). The inset shows the appearance of the system at different digestion stages. Different letter between groups indicates statistical significance, p<0.05, n=3.

6.3.3 Digestibility of colloidal delivery systems

In this section, we used the pH-stat method to measure the digestion of the various colloidal delivery systems. As mentioned earlier, the crude pancreatic lipase extract used in this study has both lipase and protease activity, and therefore we would
expect both the proteins and lipids to be digested. The amount of alkaline solution required to maintain the solution at pH 7.0 throughout the digestion period was measured (Figure 31), and then this information was used to calculate the percentage of free fatty acids (FFA) released from the samples containing lipid nanoparticles. Prior to calculating the FFAs for these samples, the volume of alkaline solution titrated into the lipid-free solutions was subtracted to take into account changes in pH induced by protein digestion.
Figure 31. pH-Stat titration curves carried out under simulated small intestinal conditions for mixed colloidal delivery systems containing tangeretin-loaded zein nanoparticles and different concentrations of lipid nanoparticles (0 to 4% oil).

The volume of alkaline solution titrated into the samples increased rapidly during the first few minutes of digestion, and then increased more gradually at longer digestion times (Figure 31). The fact that an appreciable increase in volume was observed for the system containing no lipids can be attributed to the hydrolysis of proteins (β-lactoglobulin and/or zein). For both systems containing lipid
nanoparticles, there was a rapid initial increase in FFAs released during the first 10 minutes, and then a relatively constant value was reached at longer times (Figure 32). These results suggest that the lipid phases were fully digested within the small intestine stage, thereby leading to the formation of free fatty acids and monoacylglycerols that could form mixed micelles to solubilize the tangeretin. The fact that the delivery system initially containing 4% oil had twice as much digestible lipid as the one containing 2% oil would be expected to lead to more mixed micelles.
Figure 32. Calculated free fatty acids released under simulated small intestinal conditions for mixed colloidal delivery systems containing tangeretin-loaded zein nanoparticles and different initial concentrations of lipid nanoparticles (2 or 4% oil).

6.3.4 Tangeretin bioaccessibility

The amount of a lipophilic bioactive component solubilized within the mixed micelle phase is normally taken as a measure of its bioaccessibility [195]. We therefore analyzed the amount of tangeretin in the mixed micelle phase obtained by
centrifuging the digested sample collected at the end of the GIT model. Normally after centrifugation, the digested sample has three layers: a creamy layer at the top containing any undigested lipid; a clear layer at the middle containing the mixed micelles; and a pellet at the bottom containing dense insoluble materials, such as calcium soaps, undigested proteins, and precipitated compounds [161]. In our study, all of the fat in the delivery systems was fully digested, and so only two layers were observed: the mixed micelle phase and the pellet.

The bioaccessibility of the tangeretin increased as the concentration of co-ingested lipid phase increased (Figure 33), being around 15, 26, and 37 % for delivery systems initially containing 0, 2 and 4% oil. As mentioned earlier, this effect can be attributed to the increased level of mixed micelles available to solubilize any lipophilic tangeretin molecules released from the digested zein nanoparticles [196]. At the highest fat content used in this study, the tangeretin concentration in the micelle phase was determined to be $12.4 \pm 1.8 \, \mu M$, while the saturation concentration of tangeretin in pure water is around $0.93 \pm 0.02 \, \mu M$, which clearly shows that the digested lipids were able to greatly increase tangeretin solubilization in the aqueous intestinal fluids.
Figure 33. Influence of initial oil content on tangeretin bioaccessibility in mixed colloidal delivery systems containing tangeretin-loaded zein nanoparticles and lipid nanoparticles (Means with different letters are significantly different, p<0.05).

Further information about the properties of the mixed micelle phase was obtained by analyzing the particle size using dynamic light scattering. The mean diameters of the particles in the micelle phase for delivery systems initially containing 0, 2 or 4% oil were 61.5, 69.1 and 112.5 nm, respectively. In the absence of fat, these particles may have been micelles formed by the bile salts and phospholipids in the simulated
small intestinal fluids. In addition, there may have been other forms of particles present in this phase, including protein nanoparticles that were not fully digested. In the presence of fat, the increase in particle size may have been due to the presence of mixed micelles consisting of bile salts, phospholipids, free fatty acids, and monoacylglycerols. The mixed micelle phase resulting from lipid digestion typically contains a combination of small micelles and large vesicle structures [197]. These mixed micelles are able to transfer the lipophilic bioactive components across the mucous layer so that they can be absorbed by epithelium cells [161, 198].

6.3.5 Cytotoxicity of tangeretin on Caco-2 cells

When performing cell permeability studies it is important to ensure that the material being tested does not appreciably alter cell viability. An MTT assay was therefore carried out to establish the potential toxicity of the mixed micelle phases collected from the simulated GIT on the Caco-2 cell monolayers. Tangeretin dissolved within DMSO did not significantly decrease cell viability over a relatively wide concentration range (Figure 34), e.g., at 0.1 μM almost all the cells were alive, and even at 20 μM the cell viability was still around 80%. Prior to analysis the mixed micelle phases were diluted with buffer solution to obtain a range of tangeretin concentrations. Consequently, both the tangeretin and mixed micelle concentrations in these samples varied. Our results suggest that the mixed micelles had an appreciable impact on cell viability, since when compared at the same tangeretin concentrations they caused more decrease in cell viability than the control (Figure 34). For example, cell viability was close to 100% at 0.1 μM tangeretin (0.1% v/v mixed micelle phase), but only 20% at 20 μM tangeretin (20%
v/v mixed micelle phase). This effect may be caused by increased levels of bile salts in the system, since it has previously been reported that these surface active lipids promote cell death by binding to the mitochondrial membrane, which causes loss of cytochrome activity and mitochondrial membrane potential [199]. For this reason, we used a tangeretin concentration of 5 μM in our permeability studies to ensure that the mixed micelles did not promote a significant decline in Caco-2 cell viability.
Figure 34. Influence of tangeretin dissolved in DMSO or mixed micelles on Caco-2 cell viability. Cells were seeded at 20,000 cells/well on a 96 well plate 24 hours before treatment. The samples were diluted using serum complete media to obtain a range of tangeretin concentrations (*, p<0.05).

6.3.6 Permeability of digested tangeretin micelle on Caco-2 cell monolayer

The permeability of the Caco-2 cells increased with increasing fat content in the mixed colloidal dispersions, as demonstrated by the faster rate of tangeretin loss from the apical side of the cells for the samples initially containing 4% oil (Figure
In addition, the calculated apparent permeability coefficients ($P_{app}$) of the tangeretin were ($18.1 \pm 4.5) \times 10^{-6}, (17.1 \pm 1.8) \times 10^{-6}, (19.3 \pm 2.0) \times 10^{-6}$, and $(26.8 \pm 1.8) \times 10^{-6}$ cm/s for the control group, micelles from 0% oil, micelles from 2% oil, and micelles from 4% oil, respectively (Table 6). It has been reported that a substance has a high permeability when its $P_{app}$ is greater than $10 \times 10^{-6}$ cm/s [3], and so all the samples analyzed in this study had high permeability. Nevertheless, the permeability of tangeretin did increase with increasing oil content in the initial delivery system, which can be partly attributed to the ability of the mixed micelles formed by lipid digestion products to increase the intestinal solubility of tangeretin [161, 188]. In addition, free fatty acids and emulsifiers are known to enhance intestinal permeability by increasing the dimensions of the tight junctions between epithelium cells [200].

**Table 6** Caco-2 monolayer permeability of digested phases from delivery systems initially containing tangeretin nanoparticles and different oil contents (delivered in the form of lipid nanoparticles). The control consisted of tangeretin dissolved in DMSO.

<table>
<thead>
<tr>
<th>Delivery System</th>
<th>$P_{app} \times 10^{-6}$ cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$18.1 \pm 4.5^a$</td>
</tr>
<tr>
<td>0% oil</td>
<td>$17.1 \pm 1.8^a$</td>
</tr>
<tr>
<td>2% oil</td>
<td>$19.3 \pm 2.0^a$</td>
</tr>
<tr>
<td>4% oil</td>
<td>$26.8 \pm 1.8^b$</td>
</tr>
</tbody>
</table>

*Different letter between groups indicates statistical significance, p<0.05, n=3.
Figure 35. Tangeretin concentration in the apical compartment of Caco-2 transwell cells at different times for different delivery systems. Control: tangeretin dissolved in HBSS; 0% oil: digested phase from tangeretin nanoparticles with no oil; 2%: digested phase from tangeretin nanoparticles mixed with 2% oil; 4%: digested phase from tangeretin nanoparticles mixed with 4% oil. The oil was delivered as lipid nanoparticles.
6.4 Conclusions

This study shows that the bioavailability of tangeretin encapsulated within zein nanoparticles can be increased by mixing them with lipid nanoparticles. This result supports the notion that mixed delivery systems containing combinations of different kinds of nanoparticles may have advantages over single-nanoparticle systems. One kind of nanoparticle may be designed to encapsulate and protect the bioactive agent in a product during storage, whereas the other kind of nanoparticle is designed to increase its bioavailability within the gastrointestinal tract. Light scattering and microscopy measurements showed that mixed colloidal delivery systems could be successfully prepared containing a combination of tangeretin-loaded zein nanoparticles and lipid nanoparticles. These systems appeared to be stable to aggregation and gravitational separation due to their high particle charge and small particle size. When these mixed colloidal delivery systems were passed through a simulated GIT the protein nanoparticles are digested by proteases thereby releasing the tangeretin molecules, whereas the lipid nanoparticles are digested by pancreatic lipases thereby forming mixed micelles. In the absence of lipid digestion products, some of the tangeretin molecules would have been solubilized in simple micelles formed by bile salts and phospholipids, whereas the rest may have formed crystals that remained in the insoluble matter. Conversely, in the presence of lipid digestion products (free fatty acids and monoacylglycerols) the solubilization capacity of the small intestinal fluids for the tangeretin molecules is increased. The absorption of the tangeretin by Caco-2
monolayers also increased in the presence of lipid nanoparticles, which was attributed to a higher level of tangeretin in the micelle phase, and a possible increase in cell permeability. The results of this study have important implications for the design and fabrication of colloidal delivery systems to increase the bioavailability of hydrophobic nutraceuticals and pharmaceuticals.
CHAPTER 7

FLUORESCENT DETECTION OF POLYMETHOXYFLAVONES IN CELL CULTURE
AND MOUSE TISSUE

7.1 Introduction

Polymethoxyflavones (PMFs) are group of compounds found mostly in citrus fruits. There’s an increasing interest in the study of PMFs in recent years, due to various health-benefiting effects reported. Such as anti cancer, anti inflammation activity antioxidant activity[180], antiangiogenic activity [201]. 5-Demethyltangeretin (5-hydroxy-6, 7,8, 4'-tetramethoxyflavone), a hydroxylated PMFs showed strong inhibition on lung cancer cell [42]; 5-hydroxy-6,7,8,4'-pentamethoxyxyflavones showed activity against colon cancer [18]. The water solubility of most PMFs are relatively low due to the presence of multiple methoxy groups on the flavonoid skeleton. Some studies reported the use of nanoemulsion or nanoparticles delivery to increase the bioavailabilities of hydrophobic PMFs [5, 185, 202]. So far, the fate of these PMFs in the delivery system inside the cell as well as in the tissue is not fully understood. In order to further explore the intracellular activities of PMFs inside the cells or in the animal tissue after successful delivery with these systems, it’s necessary to use some methods to locate the PMFs inside the cell or animal tissue. Fluorescent detection is one of the most commonly used methods for intracellular trafficking. It is ideal if the compound of interest itself can be fluorescent so no fluorophore would be needed. Curcumin is one of these compounds that have board band fluorescence in various solutions due to phenolic group and conjugated double bonds of its chemical structure [203, 204]. This distinct feature has been used to
study the transport of liposomal curcumin to living cell [105], the uptake of curcumin, curcumin microemulsion[104] and micelle [106] by normal cell and cancer cell [103]. For compounds that cannot give fluorescence by themselves, there are several ways to visualize them inside the cell or tissue. One approach is to make fluorescent conjugate before applying to the cell. For example, fluorescein isothiocyanate (FITC) labeled insulin encapsulated PLGA nanoparticles was prepared before adding to Caco-2 cells to study the uptake of insulin by the cell [107]. Rhodamine123 was encapsulated in dextran sulfate-PLGA hybrid nanoparticles to study the uptake by breast cancer cell (MCF-7) [108]. Transferrin-conjugated paclitaxel nanoparticle was used to track the retention of bioactive compound inside the cell [109]. The disadvantage of this method is that these conjugated compounds may cause toxicity to the cells due to the incorporation of fluorophores[110]. Another method is to stain the compound after applied to the cell or to the animal. For example, some study reported adding 2-aminoethyl diphenyl borate (DPBA, or Naturstoff reagent A) to human keratinocytes after incubating with cyanidin for 24 hours to locate the distribution of cyanidin inside the cell [111]. This approach is simple and doesn’t cause toxicity because fluorophore was added after cells were fixed. For both methods, anti fade medium could be used to prevent fluorescence quenching when observed under a fluorescence or confocal microscope [112].

The purpose of this study was to investigate the fluorescence of 5, 3', 4'-tridemethylnobiletin (THN) by using 2-aminoethyl diphenyl borate (DPBA). This compound was mostly used to visualize flavonoids in the plants [205-208]. Our
preliminary experiment showed this compound could also form fluorescent conjugates with THN when observed with a fluorescent microscope. We use fluorescence spectroscopy, mass spectroscopy and Raman spectroscopy to characterize the THN-DPBA conjugate. Our previous animal study also showed 5-demethylnobiletin (5DN) can be converted to THN by mouse. And THN showed much more higher anti cancer activities than its parent compound 5DN [209]. So we also use colon of cancer-induced mouse with a 5DN enriched diet to see the application of this method for detecting the distribution of PMFs in animal tissue. This is the first study focusing on fluorescence detection of PMFs in cell culture and animal model. It may provide some implication for the future study of cancer prevention of PMFs in the cell as well as in animal model. We would want to use zein nanoparticle delivery system describe in Chapter 6 to encapsulate THN. We will use Caco-2 cell model to determine permeability of THN loaded zein nanoparticle. We will also use fluorescent microscope to locate possible intracellular distribution of this delivery system.

7.2 Materials and Methods

7.2.1 Materials

PMFs such as 5-demethylnobiletin, 5,3’, 4’-tridemethylnobiletin, were synthesized and purified by our own lab. Each compound has the purity of higher than 98%. Formaldehyde, methanol, ethanol, glycerol, phosphate buffer saline (PBS) were purchased from Fischer Scientific (Fairlawn, NJ, USA), dulbecco’s modified eagle medium (DMEM) was obtained from Mediatech (Mediatech, Inc., Manassas, VA); azoxymethane (AOM), dextran sodium sulfate (DSS), 2-Aminoethyl diphenyl borate
(DPBA), hanks balanced salt (HBSS) were purchased from Sigma-Aldrich (St Louis, MO). Caco-2 cell was purchased from American Type Culture Collection (ATCC, Rockville, MD) Double distilled water was prepared with a Model D14031 Nanopure water purification system (Thermo Scientific, USA). β-lactoglobulin (with a purity of 92.5%) was obtained from Daviso Foods International (lot JE 002-8-415, Le Sueur, MN). Zein (purity 92%, w/w), bile extract (porcine, B8613), Hank’s balance salts (cat. no. H1387) and uranyl acetate were purchased from Sigma-Aldrich (St Louis, MO). Hydrochloric acid (HCl), HPLC grade methanol, tetrahydrofuran (THF), trifluoroacetic acid (TFA), acetonitrile (ACN) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was obtained from EMD Chemicals Inc. (Gibbstwon, NJ, USA). Double distilled water was made from Barnstead nanopure water system (Model D14031, Dubuque, Iowa, USA).

7.2.2 Preparation and characterization of sample compound

**Fluorescence spectroscopy:** THN was dissolved in methanol at the concentration of 20 μM. It was then mixed with DPBA solution (20 μM dissolved in double distilled water) at the same volume. Sample was then analyzed in a PTI fluorescence spectrometer (PTI Inc, NJ, USA). Both excitation and emission spectra were recorded.

**Raman Spectroscopy:** THN powder, DPBA powder were analyzed with Raman microscope. Conjugate was obtained by mixing THN solution (dissolved in methanol) and DPBA solution (dissolved in double distilled water) at the same molar ratio. Then 200 μL of mixture was transferred on to a glass slide and dried in dark. A DXR/SERS (surface-enhanced Raman scattering) Raman spectrometer was
used in this experiment (Thermo Scientific, MA, USA). The laser power was 24 mV and the exposure time was 2s. The excitation wavelength was 780nm and all the spectra were measured in the range between 0 and 2000 cm$^{-1}$.

**Mass spectroscopy:** THN, DPBA and THN-DPBA conjugate were dissolved in HPLC grade methanol and was analyzed on a JEOL-700 MStation (JEOL Ltd, Tokyo, Japan).

**7.2.3 Fluorescence detection of PMF in floating cells**

Caco 2 cells were seeded on 6-well plate at the seeding density of 50*10$^4$ at least 24 h before the experiment. The cells were incubated with culture media containing 10 μM THN for 2 h. Then the cells were trypsinized for 5 min, transferred to a plastic flow cytometry tube, cell pellet was obtained after centrifugation (1000 g, 5 min). The cells were then washed with ice cold PBS by gentle vortexing, centrifuged at 1000 g for 5 min. Then the cells were fixed with 2% of formaldehyde for 10 min. Then the supernatant was discarded after centrifugation (1000 g, 5 min). The cells were washed with PBS, then centrifuged, the supernatant were discarded. Then the cells were incubated with staining solution (DPBA dissolved in 0.25% Triton X-100) in dark for 15 min. After incubation, the cells were washed with PBS for three times in dark. The PBS solution of last wash was kept in the tube, protected from dark. The samples were analyzed with BD LSR II Flow Cytometer (BD Bioscience, CA, USA).

**7.2.4 Fluorescent detection of PMF in attached cells**

Caco-2 cells were seeded on pretreated and UV light sterilized 25 mm cover glasses placed inside a 6-well plate at the seeding density of 50*10$^4$. When more than 80% confluent, cell culture medium was removed, and then incubated with culture media or HBSS (Hanks Balance Salt, pH 7.4) containing 10μM of THN. After incubating 2h,
media was removed and the cells were washed three times with ice cold PBS, the supernatant was discarded. Cells incubated with PMF for different time (0.5h, 1h, 1.5h, 2h) were also obtained. Then the cells were fixed with formaldehyde (2%, w/w) for 10 min. The cells were washed with PBS again and then incubated with dye solution (DPBA dissolved in Triton X-100) in dark for 15 min. Cells treated for 2h but washed with Triton X-100 solution only was also prepared. The slides were then washed PBS for three times, 5 min each. At last, the slides were incubated with DAPI (1μg/ml in PBS) solution for 1 min in dark. After rinsing with PBS, the cover glasses were mounted with anti-fade mounting medium on a slide and sealed with nail polish, dried in dark. The slides were observed under a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Same parameter was used for analyzing cells treated with PMF for different time.

7.2.5 Fluorescence detection of PMF in mouse colon tissue

Colon tissue used in this study was obtained from an animal experiment in our lab. The animal experiment procedure was approved by Institutional Animal Care (permission number, 2011-0066). Male CD-1 mice (5 week old) were treated with AOM through intraperitoneal injection at the dose of 12 mg /kg body weight. Then the mice were injected with DSS every four days for four times. The mice were sacrificed at 27 weeks. Colon tissues were harvested, quickly washed with ice cold PBS and fixed in formaldehyde. These samples were then embedded with paraffin and cut into slice with a slicing machine, then coated on a slide. The paraffin on the slide was removed by immersing in xylene 3 times, each for 5 min. The tissues were then rehydrated by been immersed in 100%, 95%, 70% and 50% of ethanol, each
for 5 min. At last the slide was washed with double distilled water before it can be used. The slide was incubated with staining solution in a humidified chamber in dark for 20 min. Then the slide was rinsed with PBS buffer. A cover glass was mounted on top of the tissue section with freshly made anti-fade mounting medium. The cover glass was sealed with nail polish and then dried in dark. The slide was observed under with Zessis confocal laser scanning microscope (Zeiss510 META, Carl Zeiss AG, Jena, Germany).

7.2.6 Intracellular fluorescent detection of THN in zein nanoparticles

**Preparation of nanoparticle:** THN loaded zein nanoparticle was prepared same as tangeretin loaded zein nanoparticle. Basically, the colloidal system was prepared by mixing two different solutions: an organic phase, which was made up of zein and THN (25:1, w/w) dissolved in 90% ethanol solution; and an aqueous phase, which contained β-lactoglobulin (3%, w/v) dissolved in PBS (10 mM, pH 7). THN nanoparticles was formed simultaneously when the organic phase was introduced to the aqueous phase (1:3, v/v) drop wise under constant stirring of 1000 rpm (Corning Stirrer PC-420, Corning Inc., USA). The ethanol in the mixture was be then evaporated with a vacuum rotary evaporator (Rotavapor R110, Buchi Crop., Switzerland). Then the sample was dried with a freeze dryer (VirTis Genesis Lyophilizer, Virtis genesis company inc., USA) and kept in the refrigerator for further use.

**Intracellular detection:** The fluorescent staining of THN loaded zein nanoparticle will be used as described before. Caco-2 cells were seeded on pretreated and UV light sterilized 25 mm cover glasses placed inside a 6-well plate at the seeding
density of 50*10⁴. When more than 80% confluent, cell culture medium will be removed, and then incubated with culture media or HBSS (Hanks Balance Salt, pH 7.4) containing 10 μM of THN. After incubating 2h, media will be removed and the cells will be washed three times with ice cold PBS, the supernatant was discarded. Then the cells will be fixed with formaldehyde (2%, w/w) for 10 min. The cells were washed with PBS again and then incubated with dye solution (DPBA dissolved in Triton X-100) in dark for 15 min. The slides will be then washed with PBS for three times, 5 min each. After rinsing with PBS, the cover glasses will be mounted with anti-fade mounting medium on a slide and sealed with nail polish, dried in dark. The slides will be observed under a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

7.3 Results and Discussion

7.3.1 Characterization of PMF-DPBA conjugates

We’ve conducted some preliminary experiment to screen some PMFs that may conjugate to the DPBA. THN, 5, 3', 4'-tridemethylflavone (5, 3', 4'-trihydroxy-6, 7, 8-trimethoxylflavone) shows most strong fluorescence. So we use THN as our model compound to conduct the following experiments.

Fluorescence spectroscopy: The conjugate has the maximum excitation peak around wavelength of 490 nm and emission peak around wavelength of 570 nm. Spectra were not shown.

Raman Spectroscopy: As can be seen in Figure 36 THN, DPBA and THN-DPBA conjugates have different Raman patterns. THN: 1550 cm⁻¹ and 1600 cm⁻¹ was the ring stretch of benzene derivatives. Peak at 790 cm⁻¹ and 1210 cm⁻¹ was the para-
disubstituted benzenes ring vibration. The peaks at 520 cm\(^{-1}\) and 590 cm\(^{-1}\) represent the ring stretch (ring B-C, see Figure 36)[210]. DPBA Peak at 615 cm\(^{-1}\) was the monosubstituted benzene. Peak around 1590 cm\(^{-1}\) was the NH\(_2\) scissors of primary amines. Boronic acid (H\(_3\)BO\(_3\)) has the peak at about 1166 cm\(^{-1}\) when excited at 514.5 nm[211]. Peak around 1050 cm\(^{-1}\) was the stretching of C-B for diphenyl phenylboronate[212]. The broad and short peak near 1330 cm\(^{-1}\) was the stretch of asymmetric stretching of B-O[213]. After conjugating with THN, the peak between B-O became sharper and stronger. This may due to the reaction between hydroxyl group on the THN and B on the DPBA, so new B-O linkage was formed. Raman spectrum further confirmed that the linkage between THN and DPBA was formed through O on the hydroxyl group of the THN and B on the DPBA. As can be seen from Figure 36, the spectrum of the THN-DPBA was not simple overlay of individual compound, this indicate the formation of new conjugate. In addition, the characteristic peak of THN has a blue shift when mixed with DPBA (Figure 36 the shaded area), this indicate the introduction of new functional group with higher energy. In conclusion, Raman analysis proved the formation of conjugate between THN and DPBA.
**Figure 36.** Raman spectrum of DPBA, THN and THN-DPBA conjugate.

**Mass Spectroscopy:** Negative mode mass spectroscopy was used in this study. This mode of mass spectroscopy can provide the highest sensitivity in terms of molecular structure[194]. THN has the molecular weight of 360.31 while DPBA was 225.09. The peak at 358.9 was the molecular weight of THN after lost of hydrogen (THN⁻)(**Figure 37**). The fragment at m/z 328.8 was the loss of OCH₃ from A ring on THN. The fragment at m/z 523.2 was the sum of THN⁻ + 164.3, which was the molecular weight of two benzene ring and one boron. This means the conjugate was formed between -OH group THN and B-O bond on DPBA. The fragment at m/z 523.2 has the strongest signal; this confirmed the conjugates formed through the reaction between THN and DPBA. The fragment at m/z 687.3 was the sum of THN⁻ and 164.3*2, this indicate a small part of conjugate was formed between one THN and two DPBA molecules through the B-O bond. The mass spectra showed there’s
reaction between THN and DPBA, the fluorescence was attributed to this new conjugate.

![Mass spectrum of the [M-H]⁻ ions of THN-DPBA conjugate.](image)

**Figure 37.** Mass spectrum of the [M-H]⁻ ions of THN-DPBA conjugate.

7.3.2 Fluorescence detection of PMF in floating cells

Flow cytometer detects specific physical and chemical feature of each individual cell[214]. In this experiment, the fluorescent intensity of each cell was detected. Three groups of samples were analyzed. One treated with THN only, one stained
with dye only and another was treated with THN and then stained with dye. As can be seen from Figure 38, the cytograms of these three samples falls into three different regions. Each region represents different fluorescent intensity. Sample treated with DPBA only showed the lowest fluorescent intensity, while sample treated with THN and then stained with dye has the strongest intensity. As shown in Figure 39, the uptake of THN by Caco-2 cells was in a dose dependent manner. When treated with same time, more cells with higher fluorescent intensity could be detected. This further confirmed the fluorescent conjugate formed between THN and DPBA. Meanwhile, it also provides some implication for future study on chemoprevention with PMFs. For example, this method could be used in the fluorescence activated cell sorting to divide the cells according to the their fluorescence intensity. Cells that can absorb more PMF can be collected and analyzed for specific biological feature. Certain protein for transform PMFs may be enriched.
Figure 38. Cytometric analysis of Caco-2 cells treated with THN and DPBA. The setting used for dye Alexa fluor 488-A was used for activating the THN-DPBA fluorescent conjugate. (a) Cells stained with DPBA only; (b) Cells treated with THN only; (c) Cells treated with THN and stained with DPBA. Gate P2 and P3 defined the cytogram into three different regions so cells inside each region were thought as different from cells in other regions.
Figure 39. Cytometric analysis of Caco-2 cells treated with THN at different concentration. The setting used for dye Alexa fluor 488-A was used for activating the THN-DPBA fluorescent conjugate. (a) Cell treated with 1 µM THN; (b) Cells treated with 2 µM; (c) Cells treated with 10 µM; (d) Cell treated with 20 µM THN.

7.3.3 Fluorescence detection of PMF in attached cells

Our experiment showed only cells treated with THN and dye showed fluorescence. As can be seen from Figure 40, cells only treated with THN without DPBA don’t show fluorescence when observed under a fluorescent microscope. Cells treated with THN dissolved in HBSS but not stained dye also didn’t show fluorescence. This excludes the possibility of fluorescence may come from the culture medium during incubating. The uptake of THN for attached cells at different time was shown in Figure 41. The incubation time of Caco-2 cell with THN range from 0.5h, 1h, 1.5h
and 2 h. It can be seen from the graph, the intensity increases as treatment time extends. In the first 30 min, only a small portion of THN was internalized by the cells. The uptake of THN occurs mostly from first hour after treatment. The fluorescence can be used to represent the internalized THN by Caco-2 cell. This method could be used to further track the uptake and distribution of THN in different delivery systems for various kinds of cancer cells, which will eventually contribute to the understanding of cancer preventing mechanism of PMFs.

**Figure 40.** Bright field and fluorescent image of cell treated without and with DPBA. (a) Bright field image for cells treated with THN only; (b) Bright field image for cells treated with THN and DPBA; (c) Fluorescent image for cells treated THN only; (d) Fluorescent image for cells treated with THN and stained with DPBA.
**Figure 41.** Fluorescence of THN in Caco2 cell at different time intervals. (a) 30min, (b) 60min, (c) 90min, (d) 120 min. Scale bar: 100 μm.

### 7.3.4 Tissue distribution of PMF in mouse colon

In this experiment, 5DN was mixed into mice feed to see the possible anti cancer effect of 5DN. The same animal experiment showed THN is one of the metabolite for 5-demethylnobiletin (5DN) in mouse tissue [209]. Mouse colon tissue analysis result indicated THN account for 20% of 5DN metabolites. Other two metabolites are 5, 3′-didemethylnobiletin, 5, 4′-didemethylnobiletin. These metabolites were produced through Phase I metabolism. However, Phase II metabolites were also identified in mouse tissue, urine and feces. So the result of this experiment only indicate DPBA could react with THN, THN glucuronides or both. The formation of fluorescent conjugates between THN glucuronides and DPBA need to be further verified.
After sacrificing the mice, different organs such as stomach, and small intestine with food inside, colon with feces inside and blood were collected. Due to the long term feeding of 5DN (27 weeks), the metabolites accumulated in all the tissues of mouse. So we were able to use fluorescent staining to locate these metabolites. As shown in Figure 42, control group (colon obtained from mouse with no 5DN in diet but stained with DPBA, colon obtained from mouse with 5DN in the diet but not stain with DPBA) has only a little background fluorescence. But colon sample of 5DN fed mouse also stained with DPBA showed strong fluorescence. As shown in Figure 43 (a), detailed morphology of mouse colon can be visualized under fluorescent microscope. Figure 43 (a) showed that THNs (THN, THN glucuronides or both) were widely distributed in mouse colon tissue, including mucosa, colon epithelium cell and muscular layer. After long term feeding, the metabolites of 5DN was widely distributed in the tissue. Figure 43 (b) and (d) represented the colonic crypt. It can be seen the fluorescence was stronger in the colon epithelium cell membrane than other locations. This may indicate that cell membrane was preferably localized by the hydrophobicity of 5DN its metabolites. This may due to the existence of low density lipoprotein in the cell membrane that can associate with hydrophobic nutraceuticals [215]. Figure 43 (c) showed the morphology of one hyperplasia. The hyperplasia cells can be seen clearly with this method. This fluorescence method could also be used to quantify the THN in mouse colon as well as other organs. Other fluorescent dye such as alcian blue for goblet cells[216], cytokeratin A1/A3 for cytokeratin, Hoechst 33342 for nuclear[217].
Figure 42. Fluorescence microscopic image of mouse colon. (a) Control sample with no DPBA, (b) Sample treated with DPBA, (c) Colon from mouse with no THN in the diet but stained with DPBA, (d) Colon sample from mouse with THN in the diet and not stained with DPBA. These images were taken with same confocal microscopic parameters. Scale bar: 200 μm.
**Figure 43.** Fluorescence image of mouse colon sample. (a) Normal colon, (b) (d) colon crypt of mouse colon, (c) hyperplasia. (a) Taken with a 40× lens, scale bar: 200 μm; (b)(c)(d) represents same sample taken with a 63× lens, scale bar: 20 μm.

### 7.3.5 Intracellular fluorescent detection of THN in zein nanoparticles.

As shown in **Figure 44 and 45**, we were able to use this fluorescent method to locate the distribution of THN loaded zein nanoparticles in Caco-2 cells. As shown in the figure, the majority of nanoparticles were internalized into the cells. This may due to the ability of zein to translocate cell membrane due to the existence of proline rich peptides in its N-Terminal domain. Further studies need to be done to study the uptake mechanism of THN loaded zein nanoparticles.
Figure 44 Confocal fluorescent images of Caco-2 cells after treating with THN loaded zein nanoparticles by 2h.
Figure 45. Z-stacked confocal fluorescent microscopy image of Caco-2 cells after treating with THN loaded zein nanoparticles for 2h.

7.4 Conclusions

A new method for visualizing distribution of PMFs inside cell and mouse tissue was developed. Bioactive compound was stained after treating the cell. The compound doesn’t need to conjugate to the stain before adding to the cell, which eliminates the possibility of causing toxicity to the cell. This method could be potentially used in the study on effect of THN on individual or for attached cell as well as its uptake and distribution in animal tissue. It may also be used to detect THN glucuronide as one conjugated metabolite from THN, however further experiment need to be done to
test this hypothesis. In addition, by using this method, fluorescence activated cell sorting could be utilized to distinguish and enrich cells with different response to PMFs, hence protein or DNA of interest could be intensified. This would eventually contribute to the understanding of anticancer mechanism of PMFs.
CHAPTER 8

CONCLUSION

This study focused on the fabrication, characterization and biological fate determination of different delivery systems. As well as to develop a fluorescent method to detect bioactive compound polymethoxyflavone inside the cell and in animal tissue.

We successfully fabricated nanoemulsion delivery systems for hydrophobic phytochemicals such as β-carotene and polymethoxyflavones. β-carotene is sensitive to heat, light and oxidation. Antioxidant such as BHT and TBHQ did not showed significant effect in preventing β-carotene degradation. Sonication on ice bath offered a better solution for dissolving β-carotene with minimum degradation. After sonication for 1 min, more β-carotene could be dissolved in oil and no degradation appeared. Besides, sonication for 3 min will result in production of degraded β-carotene. Both spray dry and freeze dry were used to obtain β-carotene nanoemulsion powder. Freeze dry is a better method to produce dried β-carotene nanoemulsion powder. Since dried powder produced by freeze dry has better dispersibility and could retain majority of β-carotene after drying.

It is important to understand the biological fate of nanoemulsion and nanoparticle delivery systems. We used in vitro digestion model to simulate human gastrointestinal tract environment and access the behavior of these delivery system. Our results demonstrated that oil content in the delivery system played a important role in the bioaccessibility of bioactive compounds. This was due to the increased
amount of mixed micelle formed during in vitro digestion that could encapsulate more bioactive compounds inside. Bioaccessibility represents the percentage of compound available for absorption in small intestine. We further examined the amount of bioactive compound that permeabilize through small intestine epithelium cell. We use Caco-2 monolayer model simulated human small intestine epithelium cell to test the permeability of digested micelle. Our result showed micelle digested from delivery system with high oil content tend to have better permeability across cell monolayer.

In order to further track the distribution of polymethoxyflavone inside the cell inside the cell and inside animal tissue, we developed a easy fluorescent method to visualize polymethoxyflavones using fluorescent microscope. 5, 3', 4'-tridemethylnobiletin (THN) was conjugated to 2-aminooethyl diphenyl borate (DPBA) under simple reaction condition. Comparison between individual compound and conjugate indicate the introduction new functional group with higher energy level for the conjugate. Mass spectroscopy confirmed the formation of new conjugate between THN and DPBA. Fluorescent spectroscopy showed this conjugate had the maximum excitation and emission wavelength at 490 nm and 570 nm. We then examined the application of this method in detecting THN in floating cells. Blank cells, cells with only THN and cells with both THN and DPBA fell into three different areas. This may indicate the specificity of signal for cells that contain THN. We also tested the use of this method for detecting THN in attached cells. Our results showed the uptake of THN was in a time dependent manner. In the first two
hours, the fluorescent increased as treatment time went longer. This method could also be used to detect THN in the tissue of animals with a PMF rich diet.

In conclusion, we've fabricated nanoemulsion and nanoparticle delivery system to encapsulate hydrophobic phytochemicals. Dried nanoemulsion powder was produced by freeze drying and spray drying. Freeze drying is a ideal method for obtain dried β-carotene nanoemulsion powder with high recovery rate and better physiochemical property. Biopolymer colloidal system was also fabricated to encapsulate hydrophobic polymethoxyflavones. Dried powder can be obtained by freeze drying. This delivery system has good stability under different environment conditions and may had the potential to be incorporated into several food systems as functional ingredient. Our result also suggest when taken up with "high fat" diet, the PMF inside this delivery system showed better bioaccessibility and also subsequent permeability through simulated human small intestine epithelium cell. We further developed a fluorescent method to track the distribution of PMF inside the cell and in mouse tissue. In a word, this study would provide some insight on how to use nanoemulsion and nanoparticle to encapsulate and protect hydrophobic phytochemicals, as well as the knowledge of biological fate of these delivery system inside human body.


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