IMPROVING THE BIOACCESSIBILITY OF NARINGENIN USING
BIOPOLYMER DELIVERY SYSTEMS

By

LANG LIU

A dissertation submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey

In partial fulfillment of the requirements
For the degree of
Doctor of Philosophy
Graduate Program in Food Science

Written under the direction of
Dr. Qingrong Huang

And approved by

Chi-Tang Ho

Qingli Wu

Shiming Li

New Brunswick, New Jersey

October, 2021
ABSTRACT OF THE DISSERTATION

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By LANG LIU

Dissertation Director:

Dr. Qingrong Huang

In this dissertation, naringenin (5,7,4'-trihydroxyflavonone) (NAR) belongs to the category of citrus flavonoids. It possesses many excellent pharmacological effect, the preferred bioefficacies of naringenin are anti-oxidant, anti-inflammation, neuroprotective et al. Although NAR possesses many excellent pharmacological activities, it belongs to Biopharmaceutics Classification System (BCS) class II which a type of nutraceuticals that have poor water solubility and degrade easily, resulting a relatively low bioavailability. In order to improve the bioaccessibility of NAR, I designed a novel protein polysaccharide based nanocomplexes which were fabricated by beta-lactoglobulin (BLG) and oligochitosan (OCS). They are food derived materials that non-toxic, biodegradable and biocompatible to human body. BLG is a major component of whey protein in bovine milk. As an ideal encapsulation system, it shows a good affinity to various compounds and possesses peptide resistance property which has a better controlled release for bioactive compounds. OCS is derived from chitosan. Compared with chitosan, it has higher solubility and lower viscosity due to
its shorter chain, and its mucoadhesion property in order to improve its intestinal permeability enable OCS become a more favorable candidate.

In the first part of this research, because the most significant step of the encapsulation is the BLG and NAR binding process. So we investigated the interaction between BLG and NAR using both chemical experiments and computer simulations. For the chemical experiments, the fluorescence quenching study and circular dichroism were engaged and we can know the binding number between BLG and NAR and the secondary structure change from the macroscale dimension. Many detailed molecular level information can be obtained through the computer simulations which we cannot get them from the chemical experiments. By using Molecular Docking (MD), this is one molecule interaction, we can know the binding sites between BLG and NAR. By using Replica Exchange Monte Carlo (REMC), this can simulate multiple molecules interaction which is similar to the real drug delivery system, we can know the binding behaviors such as visualization trajectories of binding, probability and selectivity of binding, the weight and size changes of complexes, the top ranked prominent regions. Additionally, we can also know the binding number which can be mutually verified with the results of chemical experiments. Therefore, the research in this part connects the macroscale phase to the perspective of molecular level to obtain a more comprehensive understanding of the interaction between BLG and NAR.

In the second part of the research, BLG and OCS can self-assemble into nanoparticles through electrostatic interaction at pH 7.4 which close to the pH value
of the physiological environment of human body. The interaction between BLG and OCS have been investigated through pH-turbidity titration, dynamic light scattering (DLS), atomic force microscope (AFM), circular dichroism (CD). NAR was successfully encapsulated in the BLG/OCS nanoparticles, aiming to protect NAR from degradation under the environment of small intestinal (neural pH). The results showed that NAR first associated with BLG mainly through hydrophobic interaction. The BLG/NAR complexes then interacted with OCS by electrostatic interaction to form the nanoparticles with the particle size was in the range of 200-350 nm under pH 7.4. NAR was effectively encapsulated in the BLG/OCS nanocomplexes with more than 90% encapsulation efficiency. The cell viability of NAR loaded NPs was concentration-dependent, the more NAR loaded NPs added, the lower cell viability they would be. The cellular uptake was time dependent, at the time intervals of 6 to 9 hours, there was the largest amount of NPs endocytosis.

In the third part of this research, in order to increase the stability and loading capacity, the PE was applied and stabilized by BLG/OCS NPs. Because the driven force between protein and polysaccharide is electrostatic interaction, so at low pH, the particles will dissociate, resulting was not stable at low pH. To solve this problem, I used a natural crosslinker – genipin, to covalent crosslink BLG and OCS. When the genipin concentration reached 0.5 mg/ml, the particle size can be as low as 250 nm with the crosslinking time was 10 hours. The contact angle was between 30-40 degree when the crosslinking time was less than 20 hours which indicated that the pe can be regarded as stable. I also investigated the effect of protein concentrations, oil fraction
and crosslinking time on the microstructure change of GNPs based PE. The size of PE after crosslink did not change too much when the pH went from 2 to 7.4 by compared with the uncrosslinked samples. Which means the pH stability of PE has been greatly enhanced after crosslink with genipin. Then I used two in vitro methods – pH-lipolysis and Tim-1 to test whether the bioaccessibility of NAR has been improved or not after formulated. The tested bioaccessibility for pH-lipolysis was higher than Tim-1 measured. Because TIM-1 model has taken more factors into consideration. Such as peristalsis movement, GI residence time, passive absorption and elimination. Therefore, its result was a little lower than that of pH-lipolysis. However, no matter in which method, the bioaccessibility of NAR had been enhanced 3.5 times more than that by comparison with oil suspension samples and jejunum is the major organ for absorption.
ACKNOWLEDGEMENTS

I would like to express my great thanks to my advisor Dr. Huang, he opened the door to the science for me and let me feel the charm of it. In the last six years of doctoral, Dr. Huang gave me his patient guidance, support and encourage to help me overcome many confusions in the research. His solid academic foundation, rigorous attitude towards scientific research and optimistic personality have always influenced me. He is not only my respected mentor, but also my friend. I am honored to meet him, attend the lab of him and learn from him.

I sincerely acknowledge my committee members Dr. Chi-Tang Ho, Dr. Qing-Li Wu and Dr. Shiming Li. Thank them for their willingness to take time to guide my research and gave me many valuable suggestions. Dr. Li also has given me a lot of inspirations for my career from an industrial perspective.

There are many other people who have helped me in my research, all my labmates and all my friends. Special thanks to Dr. Weiping Jin for helping me with my study on protein and polysaccharide interaction when I was a freshman. Thanks to Dr. Xingran Kou for helping me with my study on naringenin encapsulation, and Dr. Zhenzhen Ge for helping me on cell study. Thanks to Dr. Benguo Liu on the study of molecular docking. Thanks to Dr. Yunqi Li, he opened his source code to us and has given me lots of valuable guidance, suggestions on the study of REMC. Thanks to my labmate – Yujia Cheng for assisting me on my experiments. Thanks to labmate, also my friend – Shiwei Su for supporting me and encouraging me. I can’t forget every day and night working in the lab we spent together, they are very precious memories for me.
Additionally, I would like to thank my beloved husband – Kangkang Li, he helped me a lot on the study of Monte Carlo simulation, and also thanks to his constant love and support. Thanks to my parents, they gave me the best education and unconditional support in life. Thanks to all the faculties, staff who taught me, helped me of the Department of Food Science, Rutgers.

Last but not least, I would like to thank China Scholarship Council’s financial support in the past four years so that I can focus myself on the research.
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Chapter I. INTRODUCTION

1.1 Introduction of Naringenin

Flavonoids belong to the group of polyphenolic compounds and they have a common characterized structure that is benzo-r-gpyrone structure. The concept of flavonoids is big, and it can also be categorized in detail such as flavonols, flavones, flavanones, isoflavones, catechins and anthocyanidins\(^1\). They are mainly concentrated in the fruits and vegetables (glycosides form), thus they are really important components in human’s daily diet. The flavonoids have been reported that they exhibit many kinds of biological activities and several researches have published that they play a significant role in lowering the risk of chronic diseases such as cardiovascular diseases and cancer\(^2\)\(^3\). Epidemiological studies revealed that a significant relationship between fruit and vegetable consumption with reducing the probabilities of some degenerative diseases such as inflammation, type II diabetes and cardiovascular diseases. Therefore, the bioavailability of flavonoids have been attracted many great interests all the time long.

Naringenin (5,7,4’-trihydroxyflavonone) belongs to the category of citrus flavonoids, which is a hydrophobic compound with a relatively low molecular weight that equals to 272.3. It is the predominant flavanone in immature orange fruit or the peels of grapefruits, which mainly occurs as the formation of glycosides – naringenin-7-rhamnoglucoside or naringenin-7-glucoside, and the bitterness of grapefruit juices can be attributed to the existence of naringenin\(^1\). Through the ingestion of human body, after the function of the intestinal
enzymes the naringenin can be disintegrated into an aglycone. Naringenin is difficult to be accessed to the intestinal and the bioavailability of it is only 4-5% through oral route. After oral administration, naringenin is mainly present as the free naringenin and monoglucuronide derivatives in plasma. It also distributes mainly in liver and brain. This is mainly due to the poor solubility and short residence time in GI tract. The major metabolite via oral administration is naringenin glucuronide.

**Figure 1-1.** The chemical structure of naringenin, the skeleton structure which has three hydroxyl groups at carbon 4', 5, 7.

Naringenin possesses a significant antioxidant property which has been reported by many publications. The imbalance of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, and the ability to neutralize them will lead to oxidative and nitrosative stresses which relate with various diseases. •OH, HO₂⁻, eaq, O₂•⁻, and H₂O⁺, these ROS which are generated excess will cause the damage of DNA, proteins and lipids resulting the dysfunction or malfunction of biological processes. There are some radioprotectors which can scavenge the free radicals to avoid the oxidative damage such as sulfhydral, but it is chemical synthetic antioxidant and it may cause some potential risks. The natural bioactive compounds from plants acting as antioxidants have been attracted more and more attention in the last two decades due to their low toxicity and high efficacy. Some
studies showed that naringenin can protect HaCaT cells from the damage of UV radiation induced through enhancing the DNA repair and augmenting the melanin production in murine melanoma (B16-F10) cell lines\textsuperscript{7-8}. The other study indicated that the reason is that naringenin has an ability to modulate the enzyme’s expression in the process of damaged DNA repair as well as it can scavenge free radicals\textsuperscript{9}.

Cardiovascular diseases lead to a large amount of death reasons all around the world. A high concentration of low density cholesterol (LDL) in plasma acts an important role in the inducement of coronary heart diseases and atherosclerosis\textsuperscript{10}. One research showed that naringenin can influence the expression of the LDLr gene which has a great probability through the level of mRNA and protein\textsuperscript{10}. Many literatures have published that the ability to prevent LDL generation can also avoid the development of atherosclerosis. This is because LDL oxidation involved in almost all the stages of atherosclerotic\textsuperscript{11}.

The encapsulation technique can greatly enhance the solubility and protect the active compounds from degradation by outer environment. Because NAR is hydrophobic nutraceutical, the lipid based delivery has been at the center of attention which has been reported by lots of studies. Nanoemulsions\textsuperscript{12-16} have a high potential to modify the bioavailability of low soluble drugs through the oral route. These are prepared by combining oils, surfactants, hydrophilic solvents, and co-solvents that have the unique ability to form fine oil-in-water (o/w) colloidal dispersions. A NAR self-emulsifying nanoemulsion was prepared with triacetin (oily phase), tween 80 or span (surfactant) that improved NAR
pharmacokinetics. It possessed high dissolution rate and solubility improvement by optimization of this method. However, the synthetic emulsifier Tween or span or co-surfactant will cause toxic effect. According to another literature\textsuperscript{17}, NAR could be incorporated into SLNs to improve the stability and pulmonary bioavailability (2.53%). However, SLNs have some disadvantages due to their perfect crystalline structure. It has possibility of drug expulsion during the storage conditions. Initial burst release is an another drawback which usually occurs. Nanostructured lipid carrier (NLC)\textsuperscript{18-19} which is improved from SLNs was introduced to encapsulate NAR, but it involved in hot homogenization technique. Therefore, by using the lipid based delivery system for NAR cannot avoid complicated preparation techniques or synthetic surfactants. Protein and polysaccharide are food derived materials and the amphiphilic structure enable them as natural surfactants. They can be regarded as safety, biocompatibility and biodegradable which are consumers like. Additionally, the complexes are self-assembled and the preparation is very easy. Therefore, I designed a novel biopolymer based delivery system to overcome these limiting factors of bioavailability of NAR.

\textbf{1.2 Bioavailability of nutraceuticals}

Nutraceuticals are defined as the nutritional components which possess the therapeutic or physiological benefits. Nowadays they are considered as an important method for chronic diseases prevention\textsuperscript{20}. It can be classified as numerous lists, containing polyphenols, flavonoids, bioactive peptides, vitamins and so on. Lots of researches have been reported that
nutraceuticals have many bioactivities or bioefficacies such as anti-tumor, anti-inflammation, blood glucose and cholesterol regulating and cardiovascular diseases prevention, etc.

Oral administration is a favored route for chronic disease treatment and health promotion. It has lots of benefits due to it is the most convenient, also it requires low level of application skills, reducing medical costs and allows a more flexible intake schedule\textsuperscript{20}. However, most researches about the biofunctionalities of nutraceuticals were based on the intravenous injection or cell studies rather than oral administration. Nevertheless, many nutraceuticals digested through oral administration are incomplete which lead to large amount of bioactive compounds remaining unabsorbed or being excreted. This is because of that human body is such a complicated system, like the extreme acid environment in stomach, the trypsin exists in gastrointestinal tract and even though the nutraceuticals have been transported through intestinal lumen, they still face the challenge of first-pass metabolism. All the factors above may cause the transformation of their chemical structure, which can lead to the change of their bioactivities.

The definition of bioavailability defined by Food and Drug Administration (FDA) is that “The rate and the extent to which the therapeutic moiety is absorbed and becomes available to the site of drug action”. This definition can be simplified as the fraction of orally administered dose which eventually reaches the systemic circulation. The overall bioavailability can be described as the following equation which related to the fraction from the GI tract absorption and the remaining fraction that unaffected by the first pass metabolic activities.
\[ F = f_{\text{abs}} \times F_M^{21} \]

In which fabs represents the GI tract absorption, and the \( F_M \) means the remaining fraction unaffected by the first-pass metabolic.

\( F_{\text{abs}} \) can then be expressed using the following equation.

\[ f_{\text{abs}} = f_b \times f_T^{21} \]

In this equation, \( f_b \) means the amount of compounds that can be released into the intestinal lumen and be available for intestinal absorption. \( f_T \) represents the fraction of bioactive compounds that can be transported across the intestinal epithelial cells.

According to the introduction of bioavailability, we can easily know that the bioavailability by oral pathway is still low in large varieties of nutraceuticals. Because for hydrophobic compounds, the solubilities are poor and thus they are hard to be taken by the intestinal lining. For hydrophilic compounds, although they are soluble in aqueous environment, the ingredients still very hard to transport across the intestinal layer due to the major component of cell wall is phospholipid bilayer and the hydrophilic nutraceuticals can’t merge into cell very well. Besides, the transformation of their chemical structure can also lead to the loss of functionality even if the nutraceuticals can be absorbed by the intestinal, and further cause the bioactive compounds cannot reach the dosage concentration which cannot express their own bioactivities when arriving target sites\(^{20}\).
From all these information, we can conclude that several approaches to enhance the oral bioavailability of nutraceuticals.

**a. Protection of labile compounds**

It is easy to understand, from mouth to colon, the dynamic environment such as pH variation, ionic strength, enzyme degradation and mechanistic motilities may cause the instability.

**b. Extend the gastric retention time**

It will avoid the incomplete absorption of nutrients.

**c. Increase the aqueous solubility**

It can effectively augment the concentration getting into targeted action site.

**d. Improve the controlled release property**

It maintenances the constant dosage level to keep the meaningful physiological efficacy.

**e. Enhance the intestinal permeability**

It will allow more bioactive compounds getting into the systematic circulation.

Thus, based on the analysis of the limiting factors and approaches of nutraceutical bioavailability, the area of delivery systems design has attracted more and more attention and several significant improvements have been made. Delivery systems such as emulsions, coacervates, nanoparticles have been utilized as an efficient encapsulant to protect the active ingredients or flavor compounds from intense environment. The limited factors include instability, short retention time in GI tract, low aqueous solubility, release rapidly and low transport coefficient across the intestinal cells *etc.* Moreover, the materials for the
nutraceuticals encapsulation would better to hold a generally recognized as safe (GRAS) which means natural and non-toxic rather than using synthetic ingredients. Taking this point into consideration, the protein and polysaccharide nanoparticles seem like an appropriate choice for nutraceuticals delivery system. We will talk about it in detail in the following parts.

1.3 Protein/Polysaccharide complex and their applications

Proteins and polysaccharides exist together in many kinds of food systems. They are the two most important components in categories of biopolymers. The interaction between proteins and polysaccharides can lead to the phase separation, either soluble complexes (coacervation: liquid-liquid) or insoluble complexes (precipitation: liquid-solid). The very first research about complexes can be traced back to 1929 that composed by gelatin/ gum arabic coacervation which was investigated by Bungenberg de Jone and Kruyt. In history, the word “coacervate” origins from “acervus” which is Latin word that means the aggregation, and the prefix “co” means the union of the colloidal particles. Generally, the term “coacervate” has been widely used to describe a certain mixture solution where exists phase separation phenomenon, that one phase is rich in a certain macromolecules while the other phase is poor. According to the pioneering systematic research, the first practical application was the manufacturing of carbonless copying paper by Green and coworkers through utilizing gelatin/gum arabic system to encapsulate dyes as early as 1950s. During the last few decades, the complex coacervates have experienced diversified, multi-directional development. Owing to the advantages of food derived materials (protein/polysaccharide) and non-involvement of chemical reaction, the coacervates have been extensively applied in food,
nutraceutical, pharmaceutical and cosmetic industries nowadays. Here is Table 1 - 1 that lists some examples about the interaction between protein and oppositely charged polysaccharide in the studies of recent years.

**Table 1- 1.** Selected complexes between protein and polysaccharide

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Polysaccharides</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sodium caseinate</strong></td>
<td>Gum Arabic</td>
<td>Ye et al. (2006)(^{27})</td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>Low methoxyl pectin</td>
<td>Eghbal et al. (2017)(^{28})</td>
</tr>
<tr>
<td>caseinophosphopeptides</td>
<td>chitosan</td>
<td>Hu et al. (2012)(^{29})</td>
</tr>
<tr>
<td>(\beta)-lactoglobulin</td>
<td>pectin</td>
<td>Girard et al. (2003)(^{30})</td>
</tr>
<tr>
<td>gelatin</td>
<td>(\kappa)-carrageenan</td>
<td>Fang et al. (2006)(^{31})</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>pectin</td>
<td>Ru et al. (2012)(^{32})</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>(\kappa)-carrageenan</td>
<td>Chai et al. (2014)(^{33})</td>
</tr>
<tr>
<td>Whey protein</td>
<td>Gum arabic</td>
<td>Weinbreck et al. (2003a)(^{34})</td>
</tr>
<tr>
<td>Soybean protein isolate</td>
<td>chitosan</td>
<td>Huang et al. (2012)(^{35})</td>
</tr>
<tr>
<td>gelatin</td>
<td>Carboxymethyl cellulose</td>
<td>Lii et al. (2002)(^{36})</td>
</tr>
</tbody>
</table>
**1.3.1 Molecular interaction between protein and polysaccharide**

During the formation process of protein and polysaccharide complex coacervates, there will be involved in multiple interaction forces such as electrostatic interaction, hydrogen bond, hydrophobic interaction, covalent bond, *etc.* These interactions are responsible for holding biopolymers together that can also determine the way these biopolymers react with, the changes of microenvironment conditions and compositions in solution\textsuperscript{37}. However, the mechanism of thermodynamics in the protein and polysaccharide complexes formation is still in controversial. Some researchers stated that these interaction processes were driven by enthalpy through the method of electrostatic free energy decreasing\textsuperscript{38}, while some claimed that the complex formation was entropy-driven by condensed counterions releasement\textsuperscript{39}. A variety kind of interactions may exist between biopolymers, and next we will make a detailed discussion.

a. Electrostatic Interaction

The electrostatic interaction is considered as the most important force between oppositely charged protein and polysaccharide complex coacervates. Protein and polysaccharide belong to the category of polyelectrolytes, that they can change their electric characteristics such as polar, nonpolar, anionic, cationic and amphoteric\textsuperscript{37}. It will depend on the ionized degree of functional groups at a certain condition. Normally, the net charge on protein is positive when the pH is below isoelectric point (PI) which is the pH that the protein carries no charge. As pH goes up until above the PI value, the protein will be reversed to negative charge. As for
polysaccharide, either negative polysaccharides such as pectin, carrageenan, sodium alginate or positive polysaccharides – chitosan, oligochitosan, the charge properties of both kinds of polysaccharides depend on the pH of solution relative to the pKa of their ionized functional groups. At a particular range of pH, protein and polysaccharide will carry opposite charge and therefore attract with each other and we call it electrostatic attraction force, then the complex coacervates will be formed through this kind of force. Here is an example of electrostatic interaction between amino group of protein and carboxyl group of polysaccharide.

![Electrostatic attraction](image)

**Figure 1-2.** The electrostatic interaction between amino group and carboxyl group

b. Hydrogen Bond

Hydrogen bond also plays an important role in the complex coacervates formation. It is an attraction force between hydrogen donors (*i.e.* H) and acceptors (*i.e.* N, O, F). Protein contains a large amount of hydrogen donors (H) and acceptors (N), and the polysaccharide also contains hydroxyl and carbonyl groups which may form hydrogen bonds with protein. They may exist within the same biomolecule (intermolecular) or between different biopolymers (intramolecular) to maintain the secondary structure of protein (helices, sheets). By compared with electrostatic interaction, the hydrogen bond has less impact by pH and ionic strength change, while temperature and some polar properties of solution have a relatively larger impact.
c. Hydrophobic Interaction

Hydrophobic interaction plays a significant role to determine the configuration and functionality of many kinds of biopolymers. The process of the hydrophobic interaction is that first folding protein into compact globular structure to avoid the contact between non-polar groups and water (polar) molecules. Second, the amphiphilic groups will be absorbed onto the interface and the hydrophilic groups will extend into water, the hydrophobic groups will reach out the inner protein itself. Third, the nonpolar groups of polysaccharide will bind to the hydrophobic patches of protein. Additionally, it has the same situation with hydrogen bond that ionic strength and salt concentration has little impact due to the hydrophobic interaction does not involve the ionic species. Furthermore, this kind of interaction is entropy-driven so that the temperature has a relatively larger impact. The change of polar microenvironment will also influence the hydrophobic interaction due to the alter of the biopolymers’ configuration. Here is an example of hydrophobic interaction.

![Hydrophobic Interaction](image)

**Figure 1-3.** The hydrophobic interaction between nonpolar molecules.

d. Covalent Bond

Covalent bond belongs to chemical interaction which is not as same as former three forces that belongs to the category of physical interaction. This kind of interaction can be initiated with heating, enzymes and chemical reagents. (e.g. maillard reaction is usually referred to the
protein and polysaccharide conjugates through heating under a certain condition\(^{44}\)). Moreover, the protein and polysaccharide can also form covalent bonds in the function of chemical reagents (\textit{i.e.} glutaraldehyde for protein) and enzymes (\textit{i.e.} transglutaminase for protein, laccase for polysaccharide). Here is an example for covalent bond formation.

![Covalent bond](image)

**Figure 1-4.** Thermal denaturation of globular proteins leads to disulfide bonds (covalent bonds) formation due to sulphydryl groups of proteins\(^{16,21}\).

### 1.3.2 Factors that Affect the Interaction Between Protein and Polysaccharide

Since we have investigated the major forces which may exist in the complex coacervates formation of protein and polysaccharide, several factors can be inferred such as pH, ionic strength, biopolymer ratios, temperature, \textit{etc.} may affect the interaction that have been reported by many researchers. Next I will select the most important three factors to discuss.

#### 1.3.2.1 pH

As we have discussed in previous section, protein and polysaccharide belong to the category of polyelectrolytes so that the change of pH can influence the ionization degree of functional groups of the biopolymers. Normally, the net charge on protein is positive when the pH is below PI value which is at that pH point, the protein carries no charge. As pH goes above PI value, the protein will be reversed to negative charge. As for the polysaccharide, the charge properties depend on the pH of solution relative to the pKa of their ionized functional groups.
Commonly, a majority of charged polysaccharides will carry positive charge when the pH is above their pKa, while as for the chitosan or oligochitosan (positive in nature), the pKa of amino acid is about 6.5 resulting negatively charged when pH is below pKa.

![Figure 1-5](image)

**Figure 1-5.** Schematic phase diagram of protein and polysaccharide complexes coacervate (pH vs salt concentration). Reproduce from reference²¹. Note that the black solid lines are hypothetical, that is the extension of the pHc dashed line.

Based on the Figure 1 - 5, I would like to make a statement about three typical pH values in order to let the following experiment part more clearly. First is pHc, the soluble complexes will be formed at this pH value. Although the protein and polysaccharide take same charge, they can attract with each other due to protein is amphiphilic, some patches of it are still in opposite charge by compared with polysaccharide. The pHo₁ is the pH value at where the starting point of insoluble complexes formation. The protein and polysaccharide also start to carry opposite charge here. The third important pH value is pHo₂ which means the dissociation of complexes coacervates due to the neutralization of polysaccharide.

Particularly, when protein and polysaccharide carry the same amount charge (that means the net charge is zero), the maximum coacervates will be yielded and corresponds to pHₘₐₓ.
1.3.2.2 Ionic Strength

Ionic strength plays a key role in the protein and polysaccharide complexes formation due to it can affect the charge density and further affect their interaction. It can be divided into two situations. First is that under the condition of high salt concentration, the net charge will be decreased because of interaction with micro-ions which present in solution, further reducing the electrostatic attraction between these macromolecules and hence affect the formation of complexes\(^{42}\). While at the low salt concentration, this screening effect is small. However, some researches\(^{34, 43}\) have reported that low concentration of NaCl can even promote the protein and polysaccharide coacervates formation.

1.3.2.3 Biopolymer Ratios

It is obviously that the protein to polysaccharide ratios can influence the charge density and further affect the complexes coacervates formation. In most cases, the maximum coacervates will be yielded under a specific ratio of protein to polysaccharide with a given pH and ionic strength. Soluble complexes will be formed due to non-neutralized charge exist in solution when one of the components is too much or too little\(^{42}\).

1.3.3 Applications of protein/polysaccharide complex coacervates

Three important applications of protein/polysaccharide system have been attracted more attention.

First is the purification of macromolecules. The separation process is that making a protein
medium, then adding the biopolymer to interact with the protein. Adjusting the pH, ionic strength and some other factors which can influence the interaction to obtain the maximum yield of coacervates. These insoluble coacervates will be centrifuged and filtrated and repeat the separation process above. Finally the macromolecules will be reclaimed.

Second, several low-fat products utilize the protein-polysaccharide complexes. The principle of this application is related to the nutritional and functional properties of these complexes. The nutritional property is easy to understand because the compositions of protein (amino acids) and the carbohydrate chain of the polysaccharide. The functionality can be ascribed to the hydration and gelation properties of the coacervate which are able to stimulate the meat fibrous texture. Additionally, the materials have a good compatibility to human body and they are biodegradable which can be regarded as environment friendly.

The most important application of protein and polysaccharide system is the micro or nano encapsulation of nutraceuticals. The principal of directly using micro-/nano- particles encapsulation is based on the complex formation of two oppositely charged biopolymers which is mainly driven by electrostatic attraction and hydrophobic interaction. The active compound can interact with the protein through the hydrophobic interaction first, then adding the polysaccharide to form the self-assembly particles. The process of nutraceuticals entrapment can be shown as the schematic graphic as below from a literature.
Figure 1-6. Nutraceuticals encapsulated with β-lactoglobulin-sodium alginate complexes, which is driven by electrostatic attraction\textsuperscript{23}.

Liang et al. reported that EGCG encapsulated with β-lactoglobulin/chitosan nanoparticles could prolong the release capabilities and residence time under stimulated gastrointestinal conditions\textsuperscript{44}. Teng et al. reported that the encapsulation efficiency of VD had a significant improvement (from 50.1\% to 96.8\%) after coating with carboxymethyl chitosan, moreover after coating with polysaccharide, there was a reduction of VD release in under stimulated gastric fluid and the release under stimulated intestinal condition had enhanced\textsuperscript{45}. Chen et al. reported that the fabrication of core-shell soy protein and soy polysaccharide nanoparticles could improve the properties of water dispersibility, stability and sustained release of curcumin\textsuperscript{46}. What’s more, besides the physical properties, the research of Izadi et al. had showed β-lactoglobulin/pectin nanoparticles could enhance the bioefficacy of antitumor with the advantage of low systemic toxicity especially for the treatment of colon cancer\textsuperscript{47}. Hu et al. also reported the encapsulation efficiency of EGCG in the caseinophosphopeptide/chitosan (CS-CPP) had been increased significantly by compared with CS-TPP nanoparticles and the rate of EGCG released had slowed down in a more controllable manner. Moreover, the results of CAA experiment suggested that the antioxidative of EGCG had increased after encapsulation with CS-CPP nanoparticles\textsuperscript{48}. 
1.4 Introduction of β-lactoglobulin

BLG is a major component of whey protein in bovine milk which takes up to 60%. Since BLG is a natural protein and is generally recognized as safe (GRAS), it has been widely investigated due to non-involvement of chemical reactions and good biodegradability by compared with other synthetic polymers. The sequence of BLG contains 162 amino acid residues with an average molecular weight of ~ 18.4kDa and pI value is about 5.1 - 5.2. The structure of it has been studied well that BLG is a relatively small globular protein that folded into a calyx with a structure of eight antiparallel β-strands and the α-helix located on the surface of β-barrel. BLG has a flexible structure that implies various forms of encapsulants, such as molecular complexes, nanoparticles, nanoemulsion and gel. Here is the structure diagram of BLG.

![BLG Structure Diagram](image)

**Figure 1-7.** The molecular structure diagram of BLG monomer.

BLG shows a good affinity for a variety of both hydrophobic and hydrophilic compounds, such as retinol, fatty acids, VD and EGCG. Various ligand bindings forces exist in the interactions, including hydrophobic interaction, hydrogen bonding, electrostatic interaction and even van der Waal force. They also lead to the transition of protein structure. According to the research of G.Kontopidis et al, the results showed that there were at least two pockets of a single BLG molecule that had an ability to bind with two different ligands at the same time.
By compared with prolamin protein (i.e. zein, wheat gluten), BLG has a good solubility due to it possesses a relatively low percentage of hydrophobic amino acids (molar ratio 53.4%)\(^{51}\). Such amphiphilic nature enables BLG to encapsulate both hydrophobic and hydrophilic compounds. Also the high water solubility can simplify the preparation process.

What’s more, BLG possesses peptide resistance property by compared with other common food derived proteins. First is the pepsin resistance which is the major protease of human’s stomach\(^{49}\). Pepsin’s cleavage sites locate at the hydrophobic patch of protein while BLG has a limited amount of hydrophobic amino acids\(^{55}\). Another factor can be attributed to the secondary structure of BLG. High content (\(>55\%\)) of rigid β-sheet structure and the existence of two disulfide bonds (Cys82 – Cys176, and Cys122 – Cys135/137) can protect protein from digestion\(^{55}\). Also, BLG can be digested slowly in small intestine with a similar situation of pepsin digestion\(^{51}\).

In summary, the properties of natural material, ligand binding, high water solubility, flexible structure and peptide resistance in gastrointestinal tract make BLG as an effective encapsulant for the controlled release of labile nutraceuticals.

### 1.5 Introduction of Oligochitosan

When we mention oligochitosan (OCS), we should refer to the original material – chitin, which is the second most abundant natural polymer (just after cellulose) in the world with white color. It is a polysaccharide that has a hard and inelastic structural and has been widely found in cell walls of fungi and exoskeletons of crustaceans\(^{56}\). The molecular structure of chitin is identified as N-acetyl-D-glucosamine units (GlcNAc) linked by β-1,4 bonds (Figure 3 - 7A). Chitosan (CS) is the N-deacetylated form of chitin, and it mainly contains D-glucosamine (GlcN) in its structure (Figure 3 - 7B) which can form NH\(3^+\) positively charged moiety at neutral pH after protonation. By compared with chitin, the molecular weight of CS is much smaller (larger than 100kDa) and can be dissolved in dilute organic acid solution at
low pH (also depends on the degree of deacetylation). The chemical or enzyme hydrolyzed product of CS is the OCS. The average molecular weight of it is less than 10kDa and the degree of polymerization is usually less than 50 - 55. Compared with CS, it has a higher solubility and lower viscosity properties due to its shorter chain length that enable OCS become a more favorable candidate. The structure is as similar as CS (Figure 1 - 8B) but with much smaller n number.

Figure 1- 8. The molecular structure of repeat units of A. chitin (GlcNAc) B. chitosan and oligochitosan (GlcN, with different n number).

OCS has been demonstrated to possess many excellent pharmacological activities, such as anti-inflammation, anti-tumor, anti-oxidize and so on. Chae et al. reported that OCS could transport through a monolayer Caco-2 cells, which is an in vitro model usually used in stimulating the intestinal epithelial. The rate of OCS absorption is mainly depend on the molecular weight which can lead to OCS has a better absorbability than that of CS. Additionally, Zeng et al. showed that OCS had a lower toxicity to Caco-2 cell lines than that of CS with ED₅₀ > 20 mg/mL which has been greatly enhanced by compared with 4.5 mg/mL for CS (22 kDa) and 3.2 mg/mL for CS (230 kDa). It has been demonstrated that the main distribution locations of OCS/CS are liver, spleen and kidneys through intraperitoneal or oral route. Also, the accumulation highly depends on molecular weight and deacetylation degree. Onishi & Machida reported that CS/OCS was degraded by lysozyme in plasma, liver, kidney and urine in both in vivo and in vitro model. What’s more, liver plays a major role in CS/OCS metabolism. Dong et al. found that more than 80% CS/OCS had been excreted in urine after administration. That result showed that CS/OCS was mainly eliminated in urine.
It has been generally accepted that one of the most important application of OCS is the drug delivery enhancer through epithelial barriers. As mentioned before, OCS can be absorbed by the intestinal epithelia and it can be protonated to form NH\textsubscript{3}\textsuperscript{+} positively charged moiety at physiological pH, which can lead to the mucoadhesion property promotion and stronger interaction between nanocarriers and epithelial cells (negative water layer), resulting the increased permeability across epithelial barriers\textsuperscript{65-67}. Moreover, Murata et al. reported that OCS coated nanocarriers could cause tight junction opening in epithelial monolayers that may conclude that OCS coated delivery system could enhance the permeability of nanocarriers across epithelial cells\textsuperscript{66}.

1.6 Introduction of Pickering Emulsion

The formation of emulsion can be referred to as the two immiscible liquids dispersion, like water and oil which are very common used liquids. It has a widely application in the area of food supplement, pharmaceutical and cosmetic industries\textsuperscript{68}. Emulsions are thermodynamically unstable system that they will be breakdown after a period of time due to a series of physicalchemical mechanisms, such as coalescence, gravitational separation, flocculation, phase separation and Ostwald ripening\textsuperscript{68-70}. Emulsified by some kinds of emulsifiers can greatly enhance the stability of emulsions due to the reduction of their surface tension. The conventional emulsion can be stabilized by either amphiphilic small molecule surfactants or the biopolymers such as protein, polysaccharide, or some other like phospholipids\textsuperscript{71}. Additionally, it can also be emulsified with silica or some inorganic emulsifiers. The selection of emulsifiers is very important in determining the formulation of the emulsion based products\textsuperscript{72}. 
In general, solid particles stabilized emulsions called pickering emulsion (PE). This concept was recognized since S. U. Pickering’s research\textsuperscript{73} and therefore obtained the name ‘Pickering emulsion’. Although it has been hundreds of years after proposition, this kind of researches about PE topic was understood in depth and also explored in applicable areas raised only in these recent two decades, especially it was found that it has an excellent stability against coalescence and Ostwald ripening\textsuperscript{74} which are two main reasons that lead to the instability of emulsions. It is different from the mechanism of the stabilization of conventional emulsion that requires the amphiphilic property for emulsifiers, as for PE, it requires the particles as stabilizer which can be partially wetted by both immiscible phases\textsuperscript{75} to gain sufficient efficiency for the interface absorption. The three phase contact angle $\theta$ was used to quantitative this property. The type of PE is determined by $\theta$. If it is located in the range of $0^\circ$ to $90^\circ$, that means the particles are hydrophilic that preferentially wetted by the water phase (O/W type PE). When it is $90^\circ$ to $180^\circ$, that means hydrophobic particles that preferentially wetted by the oil phase (W/O type PE). The contact angle means that the angle formed at the phase boundary like water and oil, and can be expressed by classical Young’s equation\textsuperscript{75}:

$$\cos \theta = (\gamma_{so} - \gamma_{sw})/\gamma_{ow}$$

where $\gamma_{ij}$ means the interfacial tension among solid particles, oil phase and water phase.

The energy required for detaching a spherical particle from the interface can be represented by the following equation\textsuperscript{74}: 
\[ E = \gamma \pi R^2 (1 - |\cos \theta|)^2 \]

For the O/W type PE, \( \gamma \) means the interfacial tension at the surface of water and oil phase; \( R \) is the particle radius; \( \theta \) is the three phase contact angle as we have discussed above.

**Figure 1-9.** Schematic picture of protein/polysaccharide particles locate at the interface of water-oil phases in the O/W type pickering emulsion with the contact angle of three phases (particle-water-oil).

From Figure 1 - 9 and the detachment energy equation, we can conclude that as long as the contact angle is not too close to 0° or 180°, especially for colloidal particles which locate in the range of 30° to 150°, the detachment energy will be several orders of magnitude larger than that of Brownian motion kinetic energy. If so, the particle adsorption can be regarded as irreversibly. This is in a significance different from the mechanism for conventional emulsions which are stabilized by dynamic equilibrium process.

Actually, as we have mentioned before, most of pickering emulsions are stabilized by the inorganic particles. However, the application of such pickering emulsions emulsifiers is limited in food and pharmaceutical industries due to their non-biodegradation and non-compatibility. Therefore, lots of efforts will be made to discover the food derived particles.
that can stabilize pickering emulsions. It is a challenge that most probably relate with the particles fabrication and proper wettability gaining. Because the biopolymers like protein and polysaccharide are the relatively complicated systems. They are easy to be affected by the surrounding environment that may result in the instability in the lifetime of pickering emulsions. Additionally, they have their own hydrophobic or hydrophilic properties that cannot lead to an appropriate partial wettability. Despite there are lots of challenges and inconveniences in introducing food grade biopolymers into the pickering emulsion system, the safety, organic, surfactant-free and ‘clean label’ which are the consumers’ perception pursuing have attracted more and more attention to study the food derived emulsifiers. Most of these works were related to either proteins\textsuperscript{78-80} or polysaccharides\textsuperscript{81-82}, while it was relatively less reported about using protein/polysaccharide particles as stabilizers in the pickering emulsions.

Generally, the process to build protein/polysaccharide particles stabilized pickering emulsions is firstly, fabricate protein/polysaccharide complexes through electrostatic interaction between the positive or negative groups. Then using these complexes with appropriate range of particle size have formed already to stabilize emulsions. Also, we should rationale design the selected contact angle which can yield emulsions belong to pickering emulsions. Besides the electrostatic interaction complexes formed, protein and polysaccharide complexes can also be formed through chemical reaction such as Maillard reaction. They are referred to as conjugates, we will not discuss about this part in our research.
1.7 Conclusions

The naringenin is a typical hydrophobic flavonoid that has many kinds of bioefficacies. We discussed the limiting factors of the low bioavailability and the potential approaches to improve. Based on all the analysis, we conclude that the BLG/OCS complexes can be a potential delivery system candidate for naringenin. We have introduced the mechanism of interaction between protein and polysaccharide. We know that the phase behavior of protein and polysaccharide complex coacervates is mainly depend on the ratios, pH and ionic strength. There are four kinds of forces existing in the coacervates that are electrostatic interaction, hydrogen bonds, hydrophobic interaction and covalent bonds for chemical reaction. Then we introduced the protein of beta-lactoglobulin (BLG) and the polysaccharide of oligochitosan (OCS). We know that the amphiphilic property enables BLG as a good candidate encapsulant for both hydrophilic and hydrophobic nutraceuticals. The enzyme resistance provides the controlled release property as a nutraceuticals delivery system. OCS possesese a higher solubility and lower cytotoxicity than chitosan which enable it as a more appropriate encapsulant. OCS also carries the positive charge that can open tight junction and interact with the epithelial cells, leading to more bioactive compounds transport across the cells.
1. Chapter II. Investigation of the Interactions between BLG and Naringenin (NAR)

2.1 Introduction

In our study, Naringenin (NAR) as a model hydrophobic nutraceutical, which is going to be encapsulated in BLG/OCS NPs. The process of using BLG/OCS complexes to encapsulate NAR was that mixing BLG and NAR together through hydrophobic or hydrogen bonds first and then adding OCS to form the core-shell structure to protect the nutraceutical. In order to better understand the structure and forces existing in the system, the fluorescence quenching (FQ) was used to monitor the microenvironment change of the fluorescent amino acids (AAs) of BLG and calculate the thermodynamic parameters after binding with NAR to determine the involved forces in the interactions. Molecular docking (MD) was used to stimulate the interaction between small molecule NAR and macromolecule BLG and the specific binding sites of hydrogen and hydrophobic interactions are also observed. CD was used to measure the secondary structure change of BLG after binding with NAR.

2.2 Materials and methods

2.2.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B.
was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments. Naringenin was purchased from Shanxi Huike Botanical Development Co., LTD (Xi’an, P.R. China). It was analytical grade and used it directly. Poly(ethylene glycol) with average molecular weight 200 (PEG - 200) was purchased from Sigma-Aldrich (CAS Number 25322-68-3).

For FQ and CD measurements, BLG stock solutions were prepared by dissolving in 10 mM phosphate buffer at pH equal to 7.4 to obtain a constant concentration. NAR stock solutions were made by first adding NAR into PEG - 200 until fully dissolved and then mixing by DI water at a fixed PEG - 200 concentration of 40 % (w/w). Samples were prepared by mixing BLG and NAR stock solutions and the highest PEG-200 concentration was 1%, which had no appreciable effect on the structure of BLG.

2.2.2 Methods

2.2.2.1 Fluorescence Quenching (FQ)

The fluorescence emission spectra and lifetime were measured by F-4600 FL Hitachi Spectrophotometer. The excitation wavelength (λ<sub>ex</sub>) was 280 nm with the emission spectra (λ<sub>em</sub>) were recorded from 300 – 550 nm. The split widths of both were fixed at 5 nm. BLG intrinsic fluorescence was measured at the constant concentration of 0.025 mg/mL and the NAR final concentrations varied from 9.2 uM to 73.5 uM by successive dilution. The measurements were conducted at 25, 30 and 35°C respectively.
2.2.2.2 Molecular Docking (MD)

To investigate the binding between NAR and BLG, the Surflex-dock method in Sybyl 8.1 software was applied. The protein crystal structure was downloaded from the Brookhaven Protein Bank (PDB) with the ID of 3NPO. The 3D structure of NAR was optimized at the B3LYP/6-31+G(d,p) level of theory by Gaussian 09. Before docking, the water crystallization of BLG was removed and hydrogen atoms were added. Additional, the amino acids were selected of 6 Å around the docking site. Both N - and C - terminal residues were charged with –NH2 and –COOH respectively to reflect the real status of BLG. The protocol bloat and threshold were set at 0.5 and 0.0 as well. Using scoring functions to evaluate the outputs and the results with highest C - score and T - score were selected.

2.2.2.3 Circular Dichroism (CD)

The changes of secondary structure of BLG after binding with NAR was studied by using Jasco J-1500 circular dichroism spectrum (Jasco Corporation, Rochester, New York USA) in a quartz cell with an optical path length of 1 mm. BLG and NAR were dissolved in 10 mM NaCl solutions at pH 7.4 before mixing. The concentration of BLG stock solution was 0.1 mg/mL and that of NAR was 1 mg/mL (containing 40% w/w PEG-200). Mixing different amount of NAR solutions (10, 20, 30, 40, 50 uL) to BLG solution respectively and measured with a scanning rate of 100 nm/min at 25 ºC. The CD spectra were calculated as the mean values of three times’ scanning in the far-UV regions (190 – 250 nm). The spectra obtained were converted by the Jasco Standard Analysis software. The percentages of different components
of BLG’s secondary structure were also calculated by the software.

2.3 Results and discussions

2.3.1 Fluorescence Quenching (FQ) Measurements

The method of fluorescence quenching has been widely used in the investigation of the interactions and binding between flavonoids or polyphenols and proteins or peptides due to the photophysical character of protein (fluorophore) which is sensitive to the polarity change of surroundings. Tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are the three main fluorescent amino acids that make contribution to the fluorescence property of proteins. BLG monomer contains two Trp residues and four Tyr residues. In which Trp is the most sensitive amino acid to polarity of local environment with a typical emission peak at 350 nm in water and an excitation wavelength at 295 nm. Tyr has a character emission peak at 303 nm, and however, it could be shifted up to 350 nm at certain conditions that depends on the ionization degree of phenolic hydroxyl groups. Therefore, if the protein has abundant Tyr, it could be excited at 280 nm (also co-excitation of both Trp and Tyr) due to the intensity of Trp yield is smaller than that of both Trp and Tyr.
Figure 2-1. Fluorescence quenching spectra of BLG in the presence of increasing concentrations of NAR from 9.2 uM to 73.5 uM with λex = 280 nm at (A)298 K (B)303 K (C)308 K. The insets are a plot of log[(F0-F)/F] versus log[Q] according to Stern-Volmer equation.
Table 2-1. Binding constant $K_A$, binding number $n$ and thermodynamic parameters (obtained from the insets of Figure 2 - 1) of NAR binding to BLG at different temperatures (calculated by using van’t Hoff equation).

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$K_A$ (mol$^{-1}$)</th>
<th>$n$</th>
<th>$R^2$</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>9.52x10^6</td>
<td>1.4461</td>
<td>0.9917</td>
<td></td>
<td></td>
<td>-39.878</td>
</tr>
<tr>
<td>303</td>
<td>1.32x10^7</td>
<td>1.4941</td>
<td>0.9925</td>
<td>36.758</td>
<td>257.169</td>
<td>-41.164</td>
</tr>
<tr>
<td>308</td>
<td>1.54x10^7</td>
<td>1.5242</td>
<td>0.9942</td>
<td></td>
<td></td>
<td>-42.450</td>
</tr>
</tbody>
</table>
In the FQ study, the pH of all the experiments was fixed at 7.4 that was close to the physiological environment of human body. The fluorescence quenching was studied under three different temperatures (25, 30 and 35 ℃) respectively. Figure 2 - 1 showed the fluorescence emission spectra quenched by a series of concentrations of NAR through successive adding NAR into BLG solution. We can see that with the concentrations of NAR increasing, the fluorescence intensity decreased accordingly due to the polarity environment of fluorescence amino acid of BLG had changed after binding with NAR. There was a noticeable red shift of the emission spectra that the apex wavelength shifted to larger with the increasing addition of NAR. This observation was also published in other research. The red shift means that the polarity microenvironment of Trp has increased. In BLG molecule, Trp19 faces into the hydrophobic core that is known to take 80% responsibility of total intrinsic fluorescence. The red shift would occur when Trp became to be exposed to water environment due to forming hydrogen bonds between the amino groups of BLG’s Trp residues and hydroxyls groups of NAR. Moreover, in Figure 2 – 1 (A), (B) & (C), it was almost all the λmax had shifted a lot (332 nm – 360 nm) indicated that the environment of BLG was very hydrophobic, which probably in the internal cavity. Therefore, we suggested that the binding sites of NAR were probably in the core of BLG.

When small molecules bind to the large macromolecules, the Stern-Volmer equation can be applied to describe the relationship between the logarithm of quencher concentrations and fluorescence intensity.
\[
\log \left( \frac{(F_0 - F)}{F} \right) = \log K_A + n \log [Q]
\]

$K_A$ means the binding constant and $n$ means the number of binding sites. $F_0$ and $F$ denote the steady state fluorescence intensity when in the absence and in the presence of quencher.

Based on this equation, the values of $K_A$ and $n$ can be obtained through the double-logarithm curve just shown as the insets of Figure 2 - 1 and tabulated as Table 2 - 1, which yielded $\log K_A$ as the intercept and the number of binding sites $n$ as slope. The results suggested that the binding constants increased with the temperature rising. All of the correlation coefficients were larger than 0.99 indicating that the results were reasonable. Moreover, the values of $n$ also increased with temperature rising up that the number of binding sites per BLG molecule went from 1.45, 1.49 to 1.52 with the temperature increased from 25, 30 to 35°C respectively.

Generally, there are mainly four types of interactions – electrostatic forces, hydrophobic bonds, hydrogen forces and van der Waals forces existing in the interactions between small molecules and macromolecules. Enthalpy change ($\Delta H$), entropy change ($\Delta S$) and free energy change ($\Delta G$) are three important thermodynamic parameters to determine the types of binding modes. If the temperature does not change significantly, $\Delta H$ and $\Delta S$ can be seen as a constant. They can be calculated through the Van’t Hoff equation:

\[
\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

Where $K$ is the binding constant at corresponding $T$ and $R$ is the gas constant. The Gibbs free
energy change (ΔG) can be expressed as the following equation:

\[ \Delta G = \Delta H - T\Delta S \]

All these parameters have been calculated and summarized in Table 3 - 1. Both ΔH and ΔS were positive, which indicated that the hydrophobic force may play an important role in the binding process. The negative ΔG suggested that the binding process was spontaneous.

2.3.2 Docking Studies
Figure 2-2. The diagrams stimulated by MD of NAR (ligand) binding with BLG (macromolecule); [A]. Binding poses by molecular binding; [B]. The specific amino acids binding sites of hydrogen bind; [C]. Hydrophobic interaction binding sites. ([B][C] were stimulated by a hybrid ONIOM method which can give a good accuracy and a low computational cost in the system of protein and its ligands treatment\textsuperscript{83}).

It is known that BLG has the pockets with the internal cavity as the main binding sites for the
hydrophobic ligands. In the present study, the investigation of the interaction between NAR and BLG was studied by using molecular docking and the results were shown as Figure 2 - 2. Figure 2 - 2[A] was a schematic diagram to show the total binding and we can see that the active binding sites of BLG were just in the internal cavity. In Figure 2 - 2 [B], the results showed all the amino acids sequences of BLG that associated with NAR and hydrogen-bond interactions. There were two intermolecular H-bonds in the complexes between –OH groups of NAR and amino acids residues of BLG that were showed by the red dotted line. They were formed by the specific residues of Thr(4) and Lys(135) respectively. However, the interaction between NAR and BLG greatly depends on the hydrophobic interactions due to the hydrophobic internal cavity. From Figure 2 - 2 [C] we can see that there were considerably hydrophobic interactions existing in the complexes. The residues sequences in BLG were Leu(117), Glu(108), Asn(90), Leu(39), Val(41), Ile(71) and Ile(84). In conclusion, hydrophobic interaction played the major role in the interaction between NAR and BLG with minor hydrogen binding, which was also in consistence of the results of fluorescence quenching above.

2.3.3 Circular Dichroism (CD) Measurements

The bio-functionalities of protein are associated with their specific conformation. After binding with flavonoids, it is probably that the conformations of protein have changed. Generally, in proteins or peptides, the main photoactive groups are the peptide bonds, aromatic amino acid residues or disulfide bonds in the peptide chains. When the plane-polarized light passes through
these reactive groups, the photoactive center absorbs the left and right circularly polarized light in the plane circularly polarized light differently, resulting in a polarization vector difference, and the circularly polarized light becomes elliptically polarized light. Therefore, we used the far-UV CD spectra to test the secondary structure variations of BLG after interacting with different concentrations of NAR.
Figure 2-3. Far-UV CD spectra of 0.1 mg/mL BLG associated with different concentrations of NAR (from 9.2 uM to 73.5 uM) at pH 7.4.

Table 2-2. The components percentages of secondary structure changes of BLG treated with gradient concentrations of NAR.

<table>
<thead>
<tr>
<th>Samples</th>
<th>α-helix %</th>
<th>β-sheet %</th>
<th>β-turn %</th>
<th>random coil %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLG</td>
<td>18.7</td>
<td>38</td>
<td>19.8</td>
<td>23.5</td>
</tr>
<tr>
<td>BLG +10ul</td>
<td>23.2</td>
<td>36.6</td>
<td>14.1</td>
<td>26.1</td>
</tr>
<tr>
<td>NAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLG +20ul</td>
<td>19.8</td>
<td>33.8</td>
<td>21.2</td>
<td>25.2</td>
</tr>
<tr>
<td>NAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLG +30ul</td>
<td>24.5</td>
<td>26.5</td>
<td>20.6</td>
<td>28.4</td>
</tr>
<tr>
<td>NAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLG +40ul</td>
<td>22.3</td>
<td>27.6</td>
<td>27.4</td>
<td>22.7</td>
</tr>
<tr>
<td>NAR</td>
<td>BLG +50ul</td>
<td>26.5</td>
<td>23.2</td>
<td>28.5</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
</tbody>
</table>

Figure 2 - 3 showed the far-UV CD spectra of BLG with the different amounts of NAR. The negative band at 192 nm and positive band at 208 to 222 nm were the characterized peaks of the structure of α-helix and the negative band at 195 nm and positive and at 216 nm were the characterized peaks the structure of β-sheet. In our graph, we can see there were two relatively strong positive bands at 192 nm and 195 nm respectively. There was a broad negative band from 208 to 222 nm, which indicated that the α-helix and β-sheet structures constituted the major components of BLG secondary structure. There was an obviously reduction in negative bands without significant peaks shift with the concentration of NAR increased that indicated a decreased percentage of β-sheet structure. Then we used the associated software to calculate the exact values of the components percentages of secondary structure changes of BLG treated with gradient concentrations of NAR and tabulated as Table 2 - 2. Compared with free BLG, adding NAR induced a decrease of β-sheet. In conclusion, it was evident that the interaction between BLG and NAR caused a slight change of conformation of protein, especially for a loss of β - sheet structure.

2.4 Conclusions

In summary, we have investigated the interactions between BLG and NAR by using the
techniques fluorescence quenching, molecular docking and circular dichroism. The results of FQ spectra showed a red shift due to the microenvironments of Trp and Tyr residues of BLG had changed after binding with NAR and with the concentrations of NAR increasing, the binding constant $K_A$ and binding number $n$ per BLG molecule were also increased. Thermodynamics parameters indicated that the mainly forces involved in the complexes were hydrophobic and hydrogen binding and it was spontaneously reaction. The results of MD revealed the NAR was located in the internal cavity of BLG and the major force existing in the complexes was hydrophobic interaction. It also showed the specific amino acids residues of BLG associated with NAR through hydrogen and hydrophobic binding respectively. The CD far-UV regions spectra showed after binding with NAR, the secondary structure of BLG had changed and with the concentrations of NAR increasing, there was a reduction in the percentage of $\beta$ - sheet structure.
2. Chapter III. Replica Exchange Monte Carlo Simulation of the Interaction Between β-lactoglobulin and Narigenin

3.1 Abstract

The effect of molar ratio on the complex formation of β-lactoglobulin (BLG) and Narigenin (NAR) was studied by a kind of computer simulation model – Replica exchange Monte Carlo (REMC) simulation equipped with an orientation-enhanced hydrophobic interaction. The visualization of simulation could be obtained to see the BLG and NAR dynamic binding process. The addition of NAR could lead to two specific bindings and orientated BLG arrangement, while increasing the ratios of NAR to BLG could inhibit such selectivity and suppressed the self-aggregation of proteins. With the ratios increased, the BLG/NAR complexes changed from “big (54kDa) and less (6)” to “small (33kDa) and much (10)”, and most size of complexes formation could be ascribed to the hydrophobic interaction. The number of NAR per central protein also went up with ratios increased, but at low ratios, the number of NAR per BLG molecule was larger than the exact ratio we released in the simulation box due to only part of BLG molecules interacted with NAR, while at high ratios, the interactions were in saturated. These results provide many detailed information and dynamic visualization about the binding behaviors of bio-macromolecules and small bioactive molecules. As a computer based simulation method, the research connects the macroscale phase to the perspective of atomic level.
3.2 Introduction

From the previous study, we know that the most important step of protein polysaccharide encapsulation is the interaction between protein and the bioactive compound. The complex formation can greatly enhance both stability and the bioactivities\textsuperscript{92-93}. In most cases, the bioactivities are proportional to the loading amount of the nutraceuticals\textsuperscript{94}. Moreover, with more stable system, the bioactive compounds can exhibit their effects better. Meanwhile, the phenomenon of phase separation behaviors between protein and small active molecules has been widely investigated in the food and beverage industry. The typical one is that there will be haze or precipitates\textsuperscript{95} which are consumers not to be delighted to see and even will influence the quality control.

However, we have done lots of work to study the interaction between BLG and NAR, but in the real research, there are three ways to explore the unknown area, including doing experiment, theoretical analysis and computer simulation. Because the protein is the biopolymer, it has a relatively complicated structure and is very sensitive to the change of environment. The theoretical approach cannot handle such sophisticated situations so that it cannot cover all the possibilities to provide a more accurate prediction or description, just as Figure 3-1 showed\textsuperscript{96}. Thus, in the macromolecules involved system, the computer simulation is also an important approach in the scientific research. Based on this point, in this chapter we will use the Replica Exchange Monte Carlos simulation to simulate the interaction between BLG and NAR under
the effect of different molar ratios.

**Figure 3-1.** The relationship among experiments, theoretical approach and computer simulation to understand the real system\(^{124}\).

Generally, it is believed that the main driving forces of the protein/flavonoid interaction are the hydrophobic interaction and hydrogen bonds (hydrophobic interaction is more dominated)\(^{97}\), and our previous results of study can also provide an evidence to prove this statement. The calculation for hydrophobic interaction uses the Lennard-Jones potential\(^{98}\) which equals to several \(k_B T\) (\(k_B\) is the Boltzmann constant, \(1k_B T = 0.593\) kcal/mol when at room temperature). While the energy for a single hydrophobic interaction pair is less than that of a hydrogen bond (tens of \(k_B T\) level). But it seems that the hydrogen bonds will be restricted by the number of donors and acceptors. According to the previous fluorescence quenching study, it is an indirect method to judge which force in the complexes in qualitative way. So the quantification of these two interactions involved in the complexes to the contributions to the whole system is still under discussion. Additionally, the flavonoid is regarded as bridge to crosslink the protein molecules
when there is only little amount of flavonoid in the protein-flavonoid complexes\textsuperscript{99-101}. So when we added NAR to the BLG solution, the particle size would increase greatly first because of this mechanism. However, the fractions of flavonoid have not been quantified. Meanwhile, in the previous study we used molecular docking to identify the binding sites in the BLG molecule when interacting with NAR, we knew that NAR was located in the hydrophobic pocket of BLG and the detailed binding residues sequence. Actually, we used the standard protein and flavonoid structures in the database in that study. While BLG is a highly structured protein, it will display different shapes in the different environment. Such as it will exist as monomer when pH < 3.5 and pH > 7.5, as dimer when pH is in the range of 5.5 - 7.5, as tetramer in the pH range of 3.5 - 5.5\textsuperscript{102}. In the following computer simulation study, we will fixate the pH to 7.4 which is similar to the microenvironment of human body, also use the thermodynamic equilibrium process step by step to stimulate the real environment of system. The MC simulation will provide a more comprehensive perspective to study the interaction between protein and flavonoid.

In our study, we used Replica Exchange Monte Carlo (REMC) method to stimulate the process of BLG/NAR interaction. In order to know this method better, first we should introduce some background about Monte Carlo method.

Monte Carlo is a computer simulation sampling method which is used to estimate the properties of a certain distribution through examining the random samples from this distribution\textsuperscript{103}. A very typical example for the use of Monte Carlo is the $\pi$ calculation and is shown as Figure 3 - 2.
Figure 3-2. π plotting by python language, from left top to right down, the input size changed from $N = 100$, $N = 1000$, $N = 10000$, $N = 100000$.

We generated a large number of points randomly and plotted them on a graph in any position within a square between the coordinate $(0,0)$ and $(1,1)$. They were labeled as blue color when they fell into the curve and as red color when they scattered outside of the curve. We kept track of the number of points both inside ($N_{inner}$) and total generated ($N_{total}$). Using the following equation, the $\pi$ value can be obtained.

$$\pi r^2 = \frac{\pi}{4}$$

$$\frac{\pi}{4} = \frac{N_{inner}}{N_{total}}$$
We can see that with the increase of input size from 100 to 100000, we can get a more accurate $\pi$ value. After running 100000 plots, the estimated value of $\pi$ only differs from the true value by 0.07%. From this example, it is clear to know that calculating $\pi$ value from a large number of samples is much easier than that directly from equation, especially for the situation that the random samples are easy to be generated, but the calculation equation is hard to get.

Biopolymers are difficult to be calculated by a certain equation due to a large size of random polymer coils and a relatively complicated structure which lead to a high level of freedom. Monte Carlo simulations can be used in this situation to generate lots of related configurations randomly to access thermodynamically quantities without solving the system analytically or performing exactly. The most important step for the process is how to obtain samples randomly. There are three sampling ways for Monte Carlo method:

a. **Accept/reject Sampling**: these samples are either accepted or rejected according to the target distribution.

b. **Importance Sampling**: the basic idea of it is replacing the original samples by adding weight in order to increase efficiency of Monte Carlo method.

c. **Markov Chain Sampling**: all the configurations generated should follow Markov chain which is a special sequential chain, that each configuration is the ground state for transferring to the new state of the next random configuration. A special property for Markov chain is ’No memory’, that means the new state depends only on the state just before it and irrelevant to any samples before the previous one.
Therefore, the former two are independently sampling, while Markov chain is the related sampling which can greatly increase the efficiency. The Metropolis-Hastings algorithm is a kind of Markov Chain Monte Carlo (MCMC) method to get a sequence of samples randomly from a probability distribution. The advantage of Metropolis algorithm is that it doesn’t follow an absolutely certain rule, the new state configuration is accepted with some probability even though the new energy is larger than that of the previous one. In one word, it can greatly reduce the amount of calculation. What’s the most important is that it can jump out of the locally optimal trap in order to obtain global minimal which is shown as Figure 3-3. We can see that if the configuration is not accepted with a certain probability ratio, it will stop at the local minimal rather than jumping out of the peak of the curve and trapped into the ‘locally optimal trap’ so that cannot reach the global minimal.

![Figure 3-3](image)

**Figure 3-3.** The energy of configuration change selected by the Metropolis-Hastings algorithm

Here are the steps for metropolis MCMC can be summarized as the following:\textsuperscript{107-109}:

1. Randomly generate a particle with a starting configuration and calculate its energy as $U(rN)$. 
2. Make a trial move of the particle by a small random displacement $r' = r + \Delta r$, then calculate the new system energy as $U'(r_N)$. By the way, this small move follows Markov Chain process, that the new state only depends on the previous one.

3. If $U'(r_N) \leq U(r_N)$, accept the new configuration; Elif $U'(r_N) > U(r_N)$, generate a new random number $\xi$ between 0 and 1, if $e^{-U'(r_N)/kT} < \xi \cdot e^{-U(r_N)/kT}$, that means accept it in a certain ratio and will transfer to a new configuration; otherwise reject it to keep the old configuration.

The Replica Exchange Monte Carlo (REMC) method is to maintain many independently replicas i.e., protein configurations. A different set of temperatures at different replicas and runs in Markov Chain process from the Boltzann distribution in energy space in order to simulating several different thermodynamic states in the same system. It allows replica exchanges to reach many local minima. Therefore, the random walk could be achieved by periodic exchanges of configurations through neighbouring replicas. In other word, all of these replicas with different temperature can be swapped with each other. The pronounced benefit of it is to speed up the whole simulation process. A certain replica with high temperature, that with high energy can go a large distance in one Monte Carlo step, while the low temperature replica can only go a small distance on one Monte Carlo step. So here is the question, if we only use high T replica, we cannot find the local minima easily, but if we swap it with low T replica, we can go a shorter distance step by step to find the local minima. Thus, this modification of Monte Carlo method has been found in greatly enhancing sampling of low-energy states and thereby providing the low-temperature systems with a source of trial
configurations which will cover a larger range of configuration spaces\textsuperscript{112}.

In our research, both protein molecules and flavonoid molecules are doing Brownian motion when they are in solution, also BLG is a kind of biopolymer which has a complicated structure and a highly freedom. We can simulate its stochastic process by using Metropolis-Hastings algorithm. Different replicas can also be swapped from each other like the following steps:

1. Constructing coarse grained (CG) model of the configuration for both protein and flavonoid molecules. CG model ignores the function of the large number of non-main groups or the side chain of polymers which can greatly extend the scope of molecular simulation.

2. Using a random number generator to generate the starting configuration and calculating its energy as $U(t)$.

3. Making random move trial in the coordinates of the particles which will give a new configuration (follows Metropolis MCMC) and calculating the new state energy as $U(t+\Delta t)$.

4. Comparing the energy change, if $U(t+\Delta t) \leq U(t)$, accept the new configuration; if not, go back to the original one to do the iteration.

5. Generating a new random number rand between 0 and 1, if rand $\leq \exp(-U(t+\Delta t)+U(t))$, accept the new configuration by a certain probability ratio; else go back to the original one to do the iteration.

6. Saving the new configuration, calculating the new state energy as $U(t)$. 
The steps from 3 to 5 are repeated until reaching the thermodynamic state equilibrium.

In the present study, the interaction between BLG and naringenin and the complex of them in different molar ratios were investigated under the condition of human physiological environment by using Replica Exchange Monte Carlo (REMC) simulation. This study can be divided into two parts: first is setting up the coarse grained model, second is the simulation part. Through this study, a lot of detail information such as the fraction of naringenin in the complexes, the distribution of binding sites can be viewed.

3.3 Methods

In this study, the coarse-grained (CG) models for protein and flavonoid were built up by using Replica Exchange Monte Carlo (REMC) method to ignore the shape of side chain or non-main groups. The size, pKa or partial charge and the hydrophobicity properties of each CG particle which corresponds to the ionized groups of each residues or rings were calculated and summarized. Normally, the interaction between protein and flavonoids will be involved in the force field including van der Waals force, electrostatic interaction and an orientation-enhanced hydrophobic interaction\textsuperscript{113-114} (methods newly developed in the cited literature). These forces will be engaged in REMC simulation.
3.3.1 Coarse-Grained Model

![Coarse-grained Model Diagram](image)

**Figure 3-4.** The energy landscape of all-atom model and coarse grained model\textsuperscript{143}.

From Figure 3 - 4, we can see that all-atom model exhibits very detail of the molecule. The molecular docking is based on this model that we downloaded the 3D structure of protein from the public database. The classical atom level modeling focuses with high accuracy on the local motions such as the folding process. As for the coarse grained model, the protein main chain will be represented by all heavy atoms and side chains are replaced by one or two united atoms per residue\textsuperscript{115}. It has a relatively low resolution by compared with that of all-atom model. But the computationally are more effective and it can be used in larger size systems. Besides, coarse grained model force field can smoothen out the energy landscape which can help to avoid the local minima traps in order to search for the global minima\textsuperscript{115}.
In the real drug system, it is impossible that only one protein molecule can interact with the functional small molecule, and we cannot adjust its pH or ionic strength. Additionally, it requires a high computing ability, while in the following REMC study, we will generate up to 300 BLG and NAR molecules, the representation of the simulation is the change of coordinate position. If we record each residue of each molecule, the computing will become a huge problem and obviously all-atom model is not suitable. Therefore, the coarse grained model will be set up first.

The structure of naringenin was drawn by using the software ChemOffice. The setting up of CG model of naringenin was based on the MM3 forced field which is an optimized conformation from MM2 force field with energy minimization\textsuperscript{116}. As for the protein modeling, the process is that the primary structure of beta-lactoglobulin was acquired through PDB (the public protein database) with code 2q2m. Then using the generator from CMBI to obtain the secondary structure of protein. Combining the solvent accessibility feature to generate CG model at a certain pH value (7.4 in this work). The CG models for BLG and NAR were drawn through PYMOL as Figure 3 - 5 & Figure 3 - 6.
Figure 3-5. Coarse-Grained (CG) model of beta-lactoglobulin (BLG). (a). The 3D structure of BLG was presented in cartoon mode. (b). CG model overlaid the 3D structure of BLG, that the residues level of protein was represented in sphere mode. (c). CG model of BLG.
Figure 3-6. Coarse-Grained (CG) model of naringenin (NAR). Each particle represented each ring and ignored the side chain groups. The vector (capsule like) of each sphere was in the geometric center of each ring which recorded the orientation of functional groups.

As it was shown in Figure 3-6, for NAR model, the geometric center of each ring was the particles’ location. The size of each particle was equal to the summation of gyration radius of all atoms in the ring. Especially, there are two functional groups (hydroxyl groups) in the ring A and B. They will have strong interaction with protein so that we used a vector (capsule-liked in Figure 3-6) represented the orientation of π stacking and other hydrophobic interactions^{100,101,117-118}. It was from geometric center of CG particles to the geometric center of functional groups of the rings. It was the same to the CG model of protein. The particles of BLG was in residue-level of CG model which was based on the all-atom model with the addition of hydrogen using HAAD according to the all-atom structure PDB entry 2Q2M and was shown as Figure 3-5. Each residue was represented by a particle located at the geometric center of all atoms in the residue, similar to the NAR CG model, the size was also equal to the summation of gyration radius of all atoms. The partial charges brought by the residues or rings were calculated through Henderson-Hasselbalch acid-alkali balance equation^{119}. That for negatively charged group (e.g. –COO−) brings a partial charge of \( \frac{10^{pH-pK_a}}{1+10^{pH-pK_a}} \); For positively
charged group (e.g. –NH3+) brings a partial charge of \( \frac{(10^{pK_w-pH-pK_a})}{(1+10^{pK_w-pH-pK_a})} \) with pKw = 14 \( \text{[120]} \). The pKa for NAR ring A & B was cited from a literature \( \text{[121]} \) that pKa1 = 7.05 and pKa2 = 8.84. As for the hydrophobicity coefficient, here is a tool for calculating logP coefficient - Virtual Computational Chemistry Laboratory (VCCLAB) which includes ALOGPS2.1 program for logP calculation \( \text{[122]} \). Through a database of AAindex, we can find hydrophobicity of 20 amino acids through the website “https://www.genome.jp/dbget-bin/ www_bget?aaindex:FAUJ830101”. The size, partial charge and hydrophobicity coefficient for the CG particles of both BLG residues and NAR rings at pH 7.4 were summarized and listed in Table 3 - 1.
Table 3-1. Parameters of CG particles for BLG residues and NAR rings at pH 7.4

<table>
<thead>
<tr>
<th>CG particle</th>
<th>Size (Å)</th>
<th>partial charge</th>
<th>khyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLG Residues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1.26 – 1.39</td>
<td>-</td>
<td>0.00</td>
</tr>
<tr>
<td>A</td>
<td>1.4 – 1.49</td>
<td>-</td>
<td>0.31</td>
</tr>
<tr>
<td>S</td>
<td>1.59 – 1.66</td>
<td>-</td>
<td>-0.04</td>
</tr>
<tr>
<td>C</td>
<td>1.65 – 1.76</td>
<td>-</td>
<td>1.00</td>
</tr>
<tr>
<td>D</td>
<td>1.47 – 2.05</td>
<td>-1</td>
<td>-0.77</td>
</tr>
<tr>
<td>P</td>
<td>1.57 – 1.78</td>
<td>-</td>
<td>0.72</td>
</tr>
<tr>
<td>N</td>
<td>1.91 – 2.06</td>
<td>-1</td>
<td>-0.60</td>
</tr>
<tr>
<td>T</td>
<td>1.67 – 1.72</td>
<td>-</td>
<td>0.26</td>
</tr>
<tr>
<td>E</td>
<td>2.14 – 2.44</td>
<td>-0.999</td>
<td>-0.64</td>
</tr>
<tr>
<td>V</td>
<td>1.71 – 1.77</td>
<td>-</td>
<td>1.22</td>
</tr>
<tr>
<td>Q</td>
<td>2.19 – 2.46</td>
<td>-</td>
<td>-0.22</td>
</tr>
<tr>
<td>H</td>
<td>2.37</td>
<td>0.137</td>
<td>0.13</td>
</tr>
<tr>
<td>M</td>
<td>2.17 – 2.48</td>
<td>-</td>
<td>1.23</td>
</tr>
<tr>
<td>I</td>
<td>1.88 – 2.01</td>
<td>-</td>
<td>1.80</td>
</tr>
<tr>
<td>L</td>
<td>1.99 – 2.16</td>
<td>0.666</td>
<td>1.70</td>
</tr>
<tr>
<td>K</td>
<td>2.36 – 2.79</td>
<td>0.999</td>
<td>-0.99</td>
</tr>
<tr>
<td>R</td>
<td>2.82 – 3.06</td>
<td>1</td>
<td>-1.01</td>
</tr>
<tr>
<td>F</td>
<td>2.2 – 2.54</td>
<td>-</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Y</td>
<td>2.4</td>
<td>2.76</td>
<td>-0.001</td>
</tr>
<tr>
<td>W</td>
<td>2.7</td>
<td>2.86</td>
<td>-</td>
</tr>
</tbody>
</table>

Naringenin Rings

| A  | 2.11| -0.691 | 0.70 |
| B  | 1.92| -0.035 | 1.39 |
| C  | 1.93| -     | 0.00 |

### 3.3.2 Replica Exchange Monte Carlo (REMC) Simulation

In the REMC simulation process, we set up eight parallel replicas at a series of exponentially increased temperatures ranging from 0.4 to 0.8. In each replica, the configuration of BLG/BLG, NAR/NAR and BLG/NAR complexes will make a trial movement in every Monte Carlo Step (MCS). Random translation and random spin as two elementary motions were applied to each molecule, therefore there were six configurations that maybe occurred in this system. The configuration update followed the Metropolis rule, the formula is that:

\[
P(\text{accept}) = \min(1, \exp \left[ \frac{-E_1 - E_0}{k_BT_0Ti} \right])
\]

That \(E_0\) and \(E_1\) means the energy of current configuration and the next attempt configuration. \(k_B\) is the Boltzmann constant. \(T_0\) is the room temperature and \(T_i\) is the REMC factor which we set in advance.

In each 4000 MCS, the configuration of a certain replica will attempt to swap with one of the
neighboring replica’s configuration. Of course not all the attempted configuration will be accepted, the accepted ratio for the replica swap followed the Metropolis-Hastings rule, the formula is that:

\[
P(sw_{\text{accept}}) = (1, \exp \left[ -\frac{1}{kBT_0(E_j - E_i) \left(\frac{1}{T_j} - \frac{1}{T_i}\right)} \right])
\]

Here \(E_i\) and \(E_j\) means the energy of configuration of replica i and j.

Until now, the configuration of each molecule will be updated in each MCS and the replica will be swapped in every 4000 MCS. In this simulation, we then run iteratively for 600 times until the energy of replica at low temperature tends to be leveled off. So we know that a reasonable simulation process is strongly related to the temperature range, in other word, the energies of different replicas. Therefore, the temperature range and the temperature increase should be carefully optimized in order to achieve the energy convergence of the neighboring replicas and sampling accepted ratios. This is the very important step of the whole simulation process.

A 360 Å cubic box was set up and all the simulation would be started in this box which was similar to the container in the real experiment. It is much larger than the gyration radius of BLG which is 21.6 Å according to the SAXS\textsuperscript{123} to avoid the impact of finite size. The number of BLG molecules dispersed in the cubic box was fixed at 20 that corresponds to the concentration of 0.7 mM or 13 g/L. And the pH fixed at 7.4 which is close to the physiological environment of human body. In terms of the previous study, the molar ratio of BLG to NAR was changed from 1:1, 1:2, 1:5, 1:7, 1:10 to 1:15. If the concentration of NAR kept increasing, the complex would become insoluble precipitate. We will study the effect of different ratios on the
3.3.3 Energy Calculation

We have known the detailed information from CG modeling about size, partial charge and hydrophobicity coefficient. The REMC simulation was engaged in a force field to start the process which was in a combination with van der Waals, electrostatic interaction and hydrophobic interaction. The Metropolis rule for configuration movement and Metropolis-Hastings rule for replica swap required calculating the energy. There are three energies involved in this simulation. Additionally, the units of all the energies were represented by the thermal energy scale – \( k_B T \).

**Figure 3-7.** Parameters and scheme plot for energy functions \(^{141}\).
3.3.3.1 Van Der Waals

The van der Waals involved in the force field includes the attractive and repulsive terms which is defined as the following formula:

$$E_{\text{VDW}}(i,j) = E_{\text{VDW,rep}} + E_{\text{VDW,att}} = \varepsilon \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - 2 \varepsilon \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6}$$

$\varepsilon$ represents the depth of the van der Waals well which is equal to 0.12$k_B T$ referred to the average strength used in CHARMM22 force field\textsuperscript{124}. $\sigma_{ij}$ means the summation of the gyration radius of CG particles $i$ and $j$ which we can find the size value in Table 3 - 1. $r_{ij}$ is equal to the summation of the size of two CG particles plus 1.51 Å, that the average size of carbon atom.

3.3.3.2 Electrostatic Interaction

The energy function was accounted for the electrostatic interaction part of DLVO theory which can be calculated through the following function:

$$\beta_{\text{UDLVO}}(i,j) = \rho_i \rho_j e^{\frac{e^{\sigma_i}}{1 + e^{\sigma_i}}} e^{\frac{e^{\sigma_j}}{1 + e^{\sigma_j}}} \lambda \frac{e^{-r_{ij}}}{r_{ij}}$$

$\rho_i, \rho_j$ are the charge density of CG particles $i$ and $j$ at pH 7.4 in this simulation, respectively.

$\beta^{-1}$ is a parameter to united the unit to $k_B T$ which is the thermal energy scale at absolute temperature $T$. $\sigma_{ij}, r_{ij}$ we have described the meaning in the above van der Waals energy equation. $\lambda_D$ means the Debye screening length and $\lambda_B$ means the Bjerrum length.
3.3.3.3 Hyrophobic Interaction

This is a newly developed orientation-enhanced hydrophobic interaction\textsuperscript{125}, where it is proportional to the overlapped and solvent-accessible surface area\textsuperscript{113}. The energy function is as followed:

\[
E_{\text{hyd}}(i,j) = k_{\text{hyd}}(i,j) \gamma \frac{\pi}{r_{ij}} \left[ h_i r_{ij} - h_i (r_{ij} - h_i)^2 - h_j (r_{ij} - h_j)^2 \right] (1 - \cos \theta)
\]

\(k_{\text{hyd}}\) is the hydrophobicity coefficient of CG particles that we have listed in Table 8 - 1. \(\gamma\) is the surface tension coefficient that equal to \(-0.012k_B T/\text{Å}\textsuperscript{126}\). \(h\) means the hydration radius of each CG particle that equal to the size of CG particle plus the radius of a water molecule (1.41 Å). \(\theta\) means the angle between the two vectors of CG particles.

The total energy of each CG particle in the force field is the overlay of these three energy functions above. According to these formulas we can calculate the configuration energy, followed the rules of Metropolis and Metropolis-Hastings, the accepted ratio of configuration update and replica swap can be obtained to ensure the reasonable progress of the simulation.

3.3.4 Complexes Identification

We have defined a cutoff distance \(R_{\text{min}}\) which means that the distance between the CG particles of residues in proteins or rings in NAR. If the distance of two CG particles is less than \(R_{\text{min}}\), we can regard these two particles as complexes. So the complexes can be composed by BLG/BLG, BLG/NAR, NAR/NAR. We set \(R_{\text{min}}\) as 6, 12 Å to distinguish hydrogen or hydrophobic
interaction (short-range) and electrostatic interaction (long-range) which maybe the driven forces for the complexes formation. Therefore, according to the number of complexes formation under different $R_{\text{min}}$ we can know which force dominate the association.

3.4 Results and Discussions

3.4.1 Rationality Analysis of Energy and Accepted Ratios

a.

[Graph showing energy (in $k_B T$) vs. MCS (Monte Carlo Steps)]

b.
Figure 3-8. The evolution of energy with simulation time. (a) Total energy, (b) energies caused by Van Der Waals, (c) energies caused by Electrostatic Interaction, (d) energies caused by Hydrophobic interaction. The curves were collected from the simulation of samples with molar ratio of BLG to NAR equaled to 1:2.

Figure 3 - 8 showed the evolution of energy with Monte Carlo Steps (MCS) in the whole simulation process. The total energy equaled to the combination of van der waals interaction, electrostatic interaction and hydrophobic interaction. In every 4000 MCS, there would be a replica swap. In each swap we will record all the kind of energies of current state. These four figures illustrated that at very first 225 (90,000 MCS) states, the lines have strong fluctuation due to the swap between high temperature and low temperature replicas. After 225 times swap, the increase energies tended to be stable until the end of simulation which can be regarded as the thermodynamic equilibrium. These energy figures are the effective evidences to prove that
the optimization of simulation parameters are reasonable. The energies were continuously and there was no gap between two swap indicated that we have selected a suitable temperature range. Additionally, the lines were almost leveling off at the end of simulation which indicated that the optimized simulation time was long enough to achieve the thermodynamic equilibrium.

![Energy Contributions](image)

**Figure 3-9.** The fraction of energy contributions from BLG/BLG, BLG/NAR and NAR/NAR complexes formation in Van Der Waals, Electrostatic and Hydrophobic interaction, respectively.

From the three component energies Figure 3 - 8 (b) (c) (d), we can calculate that it was nearly 83% of the total energy were contributed from hydrophobic interaction. The following were Van Der Waals (14%), then electrostatic interaction (3%). Therefore, the hydrophobic interaction was the main driven force in the simulation process. In the three component energies, each of them was the summation of the protein/protein interaction, BLG/NAR interaction and NAR/NAR interaction. We can also see the detailed energy contribution from these three interactions according to Figure 3 - 9, which extracted the values from the previous figures,
calculated and summarized the fraction of energy contributions from BLG/BLG, BLG/NAR and NAR/NAR complexes formation in Van Der Waals, Electrostatic and Hydrophobic interaction respectively which showed as Figure 3 - 9.
d.

![Graph showing accepted ratio against replica temperature factor with two lines representing Pswap and Paccept. The graph is labeled 1:10.]
Figure 3-10. The accepted ratios of configuration update (Paccept) and replica swap (Pswap) against REMC factor under different BLG/NAR ratios (a) 1:1 (b) 1:2 (c) 1:5 (d) 1:10 (f) 1:15. And the average accepted ratios for six motions (spin & translation applied to BLG/BLG, BLG/NAR, NAR/NAR complexes) with BLG to NAR ratio of 1:2.
Figure 3 - 10 (a)-(e) showed the accepted ratios for configuration update $P_{\text{accept}}$ and replica swap $P_{\text{swap}}$ under different REMC factors 0.4 - 0.8. They followed the Metropolis rule and Metropolis-Hastings rule respectively to avoid local minima trap in order to reach the global minima location. We have set two elementary motions - translation & spin at the beginning of simulation process, these two motions applied to BLG/BLG, NAR/NAR BLG/NAR complexes, therefore there would be six motions occur in the whole simulation process. Figure 3 - 11 (f) showed the accepted ratios for all the six motions under ratio BLG to NAR 1:2.

Our anticipated optimized results are that we can differentiate the accepted ratios. The replicas with high REMC factors had higher accepted possibilities and vice versa. We can see Figure 3 - 11 (a)-(e) showed under all ratios, the accepted possibilities for configuration update went up with REMC factor increased, that is exactly what we expected. Besides, for all the figures, the accepted ratios for replica swap were stable at the given REMC factor range. These accepted ratios were strong evidences to prove the sampling efficiency optimization were reasonable and we had selected an appropriate REMC factor range and increase.
3.4.2 Visualization of the trajectories of BLG/NAR complexes formation

Figure 3-11. The simulation process snapshots of BLG/NAR complexes at the molar ratio of BLG to NAR was 1:10. (a) Athermal simulation as the initial configurations for REMC thermal process. (b) 10\textsuperscript{th} snapshot of REMC thermal process. (c) 80\textsuperscript{th} snapshot of REMC thermal process. (d) 150\textsuperscript{th} snapshot of REMC thermal process.

The simulation process was divided into two steps - athermal process and thermal process. As shown in Figure 3 - 11, we set 220 molecules (20 BLG molecules, 200 NAR molecules, BLG : NAR = 1:10) in a cubic box with the boundary size of 360 Å. In order to eliminate the artificial affect, all the CG modeled BLG and NAR molecules were evenly dispersed in this box, then pretreated these particles in the simulation of 4000 MCS x 200 swap under only Van Der Waals force field. Selected the final configurations of athermal process as the initial configurations
for all the replicas of thermal process. Figure 3 - 11 (a) showed the initial state configurations.

In the REMC thermal process, the whole process was in the simulation of 4000 MCS x 600 swaps under the force fields of Van Der Waals, electrostatic and hydrophobic interaction. In each 4 swaps, we recorded one snapshot for the current state. Therefore, we had 150 snapshots in total. Figure 3 - 11 (b)-(d) showed that we selected 10th (begin stage), 80th (middle stage) and 150th (final stage) snapshots to visualize the whole progress. At first, the molecules were relatively out of order which was close to the final configuration of athermal process. When the number of states was 80, we could see the prototypes of three kinds of complexes (BLG/BLG, BLG/NAR, NAR/NAR). The final stage snapshot represented the final configurations under thermodynamic equilibrium, the BLG molecules were surrounded by NAR molecules. We will extract the coordinates of all the particles in the following data analysis part.
3.4.3 Data analysis of simulation

3.4.3.1 Association of particles

![Diagram showing PCF vs molecular separation distance for different ratios](image)
b. \textbf{Figure 3-12.} Molecular pairwise correlation function (PCF) with the different NAR to BLG molar ratios. (a) BLG/BLG interaction (b) BLG/NAR interaction

The PCF was used to describe the distributions of particles around a certain particle in simulation system. It was calculated through

\[
g(r)_{XY} = \frac{\sum_i \sum_j \delta \left( r - |\vec{r}_{X,i} - \vec{r}_{Y,j}| \right)}{4\pi r^2 C_{XY}}
\]

In which X, Y represented BLG or NAR particles. \(r_{X,i}\) meant the location of the \(i_{th}\) X particle, \(C_{XY}\) was the constant to normalize the correlation to the unit value. \(\delta(t)\) was the Dirac delta function which is commonly used to represent the density distribution of charged particles. The value of this function is 0 if \(t\) not equal to 0, and its integral over the whole domain is equal to 1.\textsuperscript{120}
In the Figure 3 - 12, the PCF was used to study the effect of molar ratios on the complexes aggregation. BLG/NAR correlations showed two prominent enrichment peaks, and these two prominent regions were in the uniform region for protein in Figure 3 - 12(b) which indicated that NAR addition could lead to the two specific binding. But with the ratio went up until 1:10, there were only one peak suggested that such kind of selectivity has vanished. The strength of PCF corresponded to the aggregation of two particles. Therefore, when there was little amount of NAR in the complexes, it had a stronger aggregation. Until the molecular separation distance was larger than 30 Å, the value of PCF returned to 1 which means evenly distribution. These results suggested that the addition of NAR could lead to two specific binding and orientated BLG arrangement, while a large amount of NAR could inhibit such selectivity and suppressed the self-aggregation of proteins.
3.4.3.2 Binding behaviors

![Graph showing binding behaviors](image)

- The weight of the complex (kDa) is plotted against the ratio.
- Two lines represent different ratios: 6 Å and 12 Å.
- The graph illustrates how the weight decreases as the ratio increases.
b. 

Figure 3-13. The effects of (a) The size of complexes (b) The number of complexes under different molar ratios of NAR to BLG 1, 2, 5, 10, 15 at Rmin cutoffs with 6 Å (short range force) and 12 Å (long range force), respectively.

From the previous introduction part, we know that $R_{\text{min}}$ equals to 6 Å means short range force which includes hydrogen and hydrophobic interaction. $R_{\text{min}}$ equals to 12 Å means long range force which includes electrostatic interaction. Therefore, in this Figure 3 - 13, 6 Å means 0 - 6 Å, which contained all hydrophobic interaction and partially electrostatic interaction (0 - 6 Å). 12 Å contained both hydrophobic interaction and electrostatic interaction.

We can see that with the ratio went up from 1 to 2, the size of complexes decreased abruptly from 54 kDa to 40 kDa and then went slowly until approached gentle (33kDa). The number of
complex increased first and then went to gently from 6 to 10. Therefore, when the ratios went up, the complexes changed from 'big and less' to 'small and much'.

The BLG molecule is a strongly self-assembled protein, so at low ratio, when there was very little naringenin in the complex, the protein would interact with each other to form complexes with large sizes, and small molecules NAR acted as bridge that facilitates this kind of binding. With the ratio went up, more naringenin would compete with BLG to bind with protein. Some binding sites which have bound with BLG would be replaced by naringenin. Because the molecular weight of BLG is much larger than that of naringenin, so the size of complexes decreased with more naringenin involved. More BLG would be released from the former self-assembled complexes which led to the number of complex increased.

As for the number of complexes, we know that the curve of 12 Å represented as a combination of two forces. It meant that scanning the length of 12 Å around the central protein and regarded it as a whole complex. Obviously the longer length it scanned, the less number of complexes it would get. So scanning the length of 6 Å had a larger number of complexes. Additionally, most sizes of the complexes could be ascribed to the hydrophobic interaction (especially 71% for ratio 1:10 complexes).
The average number of NAR per BLG under different molar ratios of NAR to BLG 1, 2, 5, 10, 15 at $R_{\text{min}}$ cutoffs with 6 Å (short range force) and 12 Å (long range force), respectively.

The plots in Figure 3-14 were calculated by using the number of NAR totally released in the simulation box divided the number of BLG in complexes. We can see that with the ratio went up, the number of NAR per BLG in the complexes was also increased in 12 Å sample. At low molar ratios (1, 2, 5), the number of NAR surrounded by a certain protein was larger than the exact molar ratio between NAR to BLG. According to Figure 3 - 13(a) the size of complexes, at these points, very large size complexes were formed which contained many proteins with more NAR. Therefore, we can estimate that part of BLG molecules interact with most of NAR to form BLG/NAR complexes, so the number of NAR per BLG molecule was larger than the
exact ratio we released in the simulation box. And the else proteins were self-aggregation to form BLG/BLG complexes. When the ratios went to 10 and 15, the number of NAR in the complexes almost equaled to 10 and 15 which represented the interactions were in saturated.

3.4.3.3 Top ranked enrichment regions
Figure 3- 15. Illustration of (a) top ranked prominent regions structure models. BLG is shown in cartoon with gray color, and (b) top 1, (c) top 2, (d) top 3 most enriched regions of NAR are represented as yellow, purple and green, respectively.

Depending on the distance from the central protein, the top ranked prominent regions are shown as Figure 11, the detailed amino acids are colored as yellow, purple and blue. Figure 11 (a) exhibited the whole NAR enrichment regions around protein. We zoomed in these three regions and the top 1 binding site was in the pocket of a large binding region and it was at the vicinity of a series amino acids (AAs) - ILE(147) ARG(148) LEU(149) SER(150) PHE(151) ASN(152), SER(30) LEU(31) LEU(32) ASP(33) ALA(34) GLN(35), these binding AAs were not found in molecular docking (MD) study which we did before. The neighboring amino acids series of top 2 ranked region showed in Figure 11 (b) was Leu(117) Glu(108) Asn(90) Leu(39) Val(41) Ile(71) Ile(84), they were partially in consistence with the results of MD. Top 3 ranked regions were Thr(4) Leu(1) Gln(5) Ile(2), we can also find such binding sites in MD study.
3.5 Conclusions

In this work, the interaction between BLG and NAR was investigated by using Replica Exchange Monte Carlo (REMC) simulation. Firstly, we set up the coarse grained (CG) model which on the basis of all-atom model, represented the residues of protein and rings of NAR by using CG particles and the orientation of forces by using vector. The size, partial charge and hydrophobic coefficient were extracted from CG model which would be used in the following experiment. Next was the simulation part. Before starting the real process, we did athermal process which started under Van Der Waals in the simulation of 4000 MCS x 200 swaps, and selected the final configurations of particles as the initial state to eliminate the artificial effect. Then, we set 8 parallel replicas with different REMC factors to do the thermal REMC simulation. In each MCS, the configuration would be updated in an accepted ratio which followed the Metropolis rule. In every 4000 MCS, the replica would be swapped with the neighboring replica in an accepted ratio which followed the Metropolis-Hastings rule. The optimization of energy (Van Der Waals, Electrostatic interaction, Hydrophobic interaction) and accepted ratios (Paccept for configuration update and Pswap replica swap) was the significant step in the whole simulation process. They were optimized by adjusting the range of REMC factor, increase speed and simulation time to keep them in a reasonable range which can achieve the thermodynamic equilibrium.

Paccept went up with REMC factor increased and Pswap was fixed at a certain range of possibilities. There was no gap between replicas and the energy was in equilibrium at the end of simulation time. These optimization results were effective evidences to prove the simulation
was reasonable. The contribution to the total energy was $E_{\text{hydro}} > E_{\text{vdw}} > E_{\text{ele}}$, the hydrophobic interaction contributed the most fraction of energy in the simulation process. The visualization of simulation by Pymol could be allowed to see the dynamic BLG and NAR binding process. The addition of NAR could lead to two specific bindings and orientated BLG arrangement, while increasing the ratios of NAR to BLG could inhibit such selectivity and suppressed the self-aggregation of proteins. Our data also showed when increased the ratio, the BLG/NAR complexes changed from "big (54kDa) and less (6)" to "small (33kDa) and much (10)", and most size of complexes formation could be ascribed to the hydrophobic interaction. The number of NAR per central protein also went up with ratios increased, but at low ratios, the number of NAR per BLG molecule was larger than the exact ratio we released in the simulation box due to only part of BLG molecules interacted with NAR, while at high ratios, the interactions were in saturated.

The significant meaning of this approach is that providing many detailed information and dynamic visualization about the binding behaviors of bio-macromolecules and small bioactive molecules. As a computer based simulation method, the research connects the macroscale phase to the perspective of atomic level.
4. Chapter IV. Fabrication and characterization of β-lactoglobulin (BLG) and oligochitosan (OCS) nanocomplexes.

4.1 Introduction

In order to design the BLG/OCS nanocomplexes, we systematically investigated the interaction between BLG and OCS by measuring the phase behavior, particle size and microstructure as a function of pH, mass ratios and ionic strengths. The formation and dissociation properties have been studied by turbidimetric titration at different pH values, BLG/OCS mass ratios, and ionic strengths. Dynamic light scattering (DLS) was used to monitor the particle size, polydispersity Index (PDI) of the complexes. The morphologies of the nanoparticles were characterized by atomic force microscopy (AFM). The thermos-characteristics of BLG after binding with different amount of OCS were calculated by Differential Scanning Calorimetry (DSC). The circular dichroism (CD) was used to measure the secondary structure change of BLG after binding with different amount of OCS.

4.2 Materials and Methods

4.2.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B. It was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with
the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments. Oligochitosan was purchased from Kunpoong Bio. Co., Ltd. (South Korea, Batch No KPH 1007GB) without further treatment. Milli-Q water was used in all experiments.

4.2.2 Methods

4.2.2.1 Preparation of Nanocomplexes

OCS was dissolved in 0.01 M NaCl solution and BLG was dissolved in 0.01 M NaCl solution as stock solutions. Both of them were under moderate stirring and after fully dissolved they were adjusted to pH 3. The BLG/OCS complexes were generated by adding the certain amount of BLG and OCS solutions at ambient temperature with magnetic stirring. The nanocomplexes were generated spontaneously under the function of electrostatic interaction between BLG and OCS. The pure BLG and the mass ratios of BLG to OCS equal to 1:2, 1:5 and 1:10, part of them were freeze dried to powders and further used in the experiment of differential scanning calorimetric (DSC).

4.2.2.2 Characterization of BLG/OCS nanocomplexes

4.2.2.2.1 Turbidimetric Titration

The pH-dependent turbidity was monitored by Brinkmann PC910 colorimeter which equipped with a 1 cm path length probe at a light wavelength of 420 nm. The colorimeter was calibrated by using Milli-Q water as 100% transmittance as T. Turbidity was defined as 100 -
Both BLG and OCS solutions were dissolved in 0.01 M NaCl to exclude the interference of other ions. Also, both solutions were filtered with 25 mm 0.45-um Whatman GD/X syringe filters before the turbidimetric titration experiments. 0.5 M HCl and 0.5 M NaOH solutions were used to adjust the pH of the mixed protein/polysaccharide solutions. The pH meter used was Thomas Scientific pH meter (Model 8025) which was calibrated with three buffers of pH 4, 7, 10. All titration were carried out with moderate magnetic stirring and the time interval between each measurement was fixed at 1 min. All measurements were conducted in triplicate at ambient temperature.

In order to study the effect of mass ratios (BLG/OCS) on the interaction between protein and polysaccharide, BLG solution (0.01 M NaCl) and OCS (0.01 M NaCl) solution mixed together at various concentrations (BLG concentration fixed at 1 mg/mL, varies OCS concentration was applied with the mass ratios of BLG/OCS at 10, 5, 2, 1, 0.5, 0.2) at ambient temperature with magnetic stirring and the pH was increased from 3 to 9 gradually. The pH was controlled by pH-meter, and the turbidity of solution was monitored by turbidimetric meter. For investigating the effect of ionic strength on the interaction between protein and polysaccharide, using the same experiment conditions with that of mass ratios at different salt concentrations of 10 mM, 50 mM, 100 mM. One thing needs to be noticed is that all the above experiments were conducted in three times and the error bars were omitted for the tidy of diagrams).

4.2.2.2.2 Dynamic light scattering measurements
The particle size and PDI of BLG/OCS nanocomplexes with different mass ratios (BLG:OCS = 20:1, 15:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:15 and 1:20 with the concentration of BLG fixed at 1 mg/mL, the preparation method followed the description above) was measured by the 90 Plus Particle Size Analyzer that equipped with Brookhaven BI-9000AT digital correlator (Brookhaven Instruments Corporation, NY, USA). All the measurements were conducted at a fixed scattering angle of 90° and in triplicate. The particle sizes of nanocomplexes were obtained from the function of intensity-intensity autocorrelation.

\[ G(q, t) = a + b \times \left( e^{-\left( \frac{t}{\tau} \right)^d} \right)^2 \]

Using Sigert relation to deduce the normalized autocorrelation function \( g(q, t) \):

\[ g(q, t) = \left[ G(q, t) - 1 \right]^{1/2} \]

The stretched exponential function - William-Watts (WW) was used to fit \( g(q, t) \):

\[ g(q, t) = \exp\left[ -\left( \frac{t}{\tau} \right)^{\beta} \right] \]

In which \( \beta \) means the distribution parameter which ranges between 0-1.

The mean relaxation times were obtained from the function of:

\[ < \tau > = \tau \frac{\Gamma(1/\beta)}{\beta} \]

\( D \) – which is the diffusion coefficient was calculated from the function of:

\[ D = < \tau >^{-1} q^{-2} \]

\( q \) – which is the amplitude of scattering vector obtained from the function of:
\[ q = \frac{4\pi n}{\lambda} \sin \left( \frac{\theta}{2} \right) \]

In which \( n \) represents the refractive index, \( \lambda \) represents laser wavelength and \( \theta \) means the scattering angle (90°).

From all those parameters, we can obtain the hydrodynamics diameter by using Stokes-Einstein equation:

\[ d = \frac{kT}{3\pi \eta D} \]

That \( k \) means the Boltzmann constant, \( T \) represents the absolute temperature and \( \eta \) means solvent viscosity.

### 4.2.2.2.3 Atomic Force Microscopy (AFM) Measurements

The morphologies of BLG/OCS nanocomplexes were measured by a Nanoscope IIIa Multi-Mode Atomic Force Microscope (AFM, Veeco Instruments Inc., CA, USA) with the tapping mode. The operation was that fresh made BLG/OCS complexes solutions (BLG:OCS mass ratios = 1:2 and 1:5 with the concentration of BLG fixed at 1 mg/mL) were dripped one droplet (estimated volume = 0.5 mL) onto the surface of pre-cleaned mica slides and rested for 1h. The surface was rinsed with Milli-Q water and then using nitrogen gas with moderate flow rate blow the mica slides carefully until fully dry for measurement.

### 4.2.2.4 Differential Scanning Calorimetry (DSC) Measurements

The thermal characteristics of protein/polysaccharide complexes were studied by DSC. All these experiments were performed in a TA Q100-DSC thermal analyzer (TA instruments,
New Castle, ED, USA). Peak denaturation temperature (Td, °C) and the enthalpy of
denaturation (Hd, J/g) of BLG, BLG/OCS complexes powders with different ratios
(BLG/OCS = 1:2, 1:5, 1:10) were calculated using the associated software. The process of the
experiment was weighing about 10 mg of each sample powder in an aluminum pan and then
sealed well. The reference was an empty sealed pan. Samples were heated from 25°C to
200°C with a heating rate of 10°C/min. The DSC profiles can be plotted as a curve of heat
flow (W/g) versus temperature. The exothermic reactions will occur in the sample that shown
by a negative peak. This curve can be used to determine peak denaturation temperature (Td)
and denaturation enthalpy (Hd) of transitions. The enthalpy change can be expressed by the
following equation:

$$\Delta H = KA$$

Where ΔH means the enthalpy of transition and K is the calorimetric constant, A means the
area under the curve.

4.2.2.2.5 Circular Dichroism (CD)

The changes of secondary structure of BLG after binding with OCS was studied by using
Jasco J-1500 circular dichroism spectrum (Jasco Corporation, Rochester, New York USA) in
a quartz cell with an optical path length of 1 mm. BLG and OCS were dissolved in 10 mM
NaCl solutions at pH 7.4 before mixing. The concentration of BLG stock solution was 0.1
mg/mL and that of OCS was 1 mg/mL. Mixed different amounts of OCS solutions
(concentrations go from 2.5x10^-3 to 25x10^-3 mg/mL) to BLG solution respectively and
measured with a scanning rate of 100 nm/min at 25°C. The CD spectra were calculated as the
mean values of three times’ scanning in the far-UV regions (190 – 250 nm). The spectra obtained were converted by the Jasco Standard Analysis software. The percentages of different components of the secondary structure of BLG were also calculated by the associated software.

4.3 Results and Discussions

4.3.1 Turbidimetric Titration

It is well known that the primary force existing in the complexes of protein and oppositely charged polysaccharide is electrostatic interaction. The net charge of BLG depends on the charge state of amino acids side chains that are on the surface of this globular protein. The charge state of amino acids side chains can be modulated greatly by the pH, different protein to polysaccharide ratios and ionic strength. In this system, there will be no interactions under the condition of both BLG and OCS take the same charge due to the electrostatic repulsion, in other word, it means that the complexes cannot be formed when the pH less than 5.2 (the isoelectric point of BLG). However, when the pH of solution is near to 5.2, some soluble complexes will be formed because of the locality pH change resulting the reduction of the electrostatic repulsion which will enhance the interaction between protein and polysaccharide. Further increasing pH until above 5.2, the charge of BLG will be reversed to take negative charge. At this point, the interaction will be enhanced abruptly due to the strong electrostatic attractive interaction. Moreover, the protein/polysaccharide will dissociate into soluble complexes again if keep increasing the pH of solution. This is because the pH is above the
pKa (~ 6.3 - 6.4) of OCS, the amine group of OCS side chains will be deprotonated which lead to the electrostatic repulsion between protein and polysaccharide.

Figure 4-1. Turbidities of different BLG:OCS mass ratios as a function of pH ranging from 3 to 9 in 10 mM NaCl. BLG:OCS = 10, 5, 2, 1, 0.5, 0.2 with the concentration of BLG fixed at 1 mg/mL.

Figure 4 - 1 showed that the turbidimetric titration curves of complexes of BLG/OCS as a function of different mass ratios under different pH values. One thing needs to be mentioned was that the turbidimetric titration of purely BLG with the concentration of 1 mg/mL and purely OCS with concentrations of 0.1, 0.2, 0.5, 1, 2, 5 mg/mL respectively had been made under the same conditions with that of the preparation of complexes, the turbidity of all of them vibrated between 0 - 10% so that the data were not shown in the Figure 4 - 1. According
to the diagram, we can see that all of the curves with different mass ratios had the similar trend. Below pH 5.0, the turbidities remained very low for all the BLG/OCS mass ratios, which indicated that there were no complexes formed between protein and polysaccharide. Starting from pH 5.0, turbidities of the curves that mass ratios were 10 and 5 increased abruptly while the other ratios did not. This was probably due to the saturation of BLG with OCS that can lead to the overlapped curves of ratios of 10 and 5. At pH equal to 5.2, 5.4, 5.5, 5.7 and 5.9, the complexes had been formed for other five ratios. These pH values defined as pH$_{\phi 1}$ that indicated the starting pH point of insoluble complexes formation. Then all the curves reached a maximum value (larger than 80%) at various pH values which indicated that more insoluble complexes would be formed with the pH increasing. These pH values had been defined as pH$_{\max}$. Also we can find out that both pH$_{\phi 1}$ and pH$_{\max}$ shifted to larger values with higher concentrations of OCS contained in the system as a result of the charge neutralization at a higher pH as more BLG bind around the chain of OCS. When the pH approached to pH$_{\phi 2}$ (pH value which the insoluble complexes start to dissociate), the turbidity decreased and then leveled off because of the amine group on the OCS deprotonated as we have discussed above.
Figure 4-2. Turbidities as a function of pH ranging from 3 to 9 for BLG:OCS = 1 at different concentrations of NaCl with 10 mM, 50 mM and 100 mM with the concentration of BLG fixed at 1 mg/mL.

The strength of electrostatic interaction is not only affected by pH and mass ratios but also influenced by ionic strength. Therefore, the effect of different ionic strength (10, 50 and 100 mM NaCl) was studied at BLG/OCS equal to 1 under the condition of that pH gradually increased from 3 to 9. Actually, the effect of the salt concentration larger than 100 mM on phase behavior had also been investigated and found that the turbidities kept low so that the data for higher salt concentration hadn’t been shown in Figure 4 - 2. From the diagram, we can see that pH\textsubscript{φ1} shifted to larger while pH\textsubscript{φ2} shifted to smaller with pH increasing. Moreover, the turbidities corresponded to the pH\textsubscript{max} of each curve kept going down with ionic...
strength increasing. We can also conclude that this system was very sensitive to the change of ionic strength, and there was almost no turbidity when the salt concentration larger than 100 mM. This is because that the added ions can screen the charges of protein and polysaccharide, resulting weaker tendency to form complexes and they were easier to be dissociated. Further increasing the ionic strength (> 100 mM) led to a completely inhibition effect of interactions between BLG and OCS.

4.3.2 Particle Size and PDI Measurements

In this study, we selected pH 7.4 for nanoparticles assembly and then investigated the particle size and PDI by using Dynamic Scattering Light (DLS) for different mass ratios of BLG to OCS (BLG:OCS = 20:1, 15:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:15, 1:20 with the concentration of BLG fixed at 1 mg/mL). According to the results of turbidimetric titration, one significant outcome was that nanoparticles can be formed at pH equal to 7.4 (physiological pH of human body). This unique property enables this system has a potential ability to keep its own structure under the pH value of human body.
Table 4-1. Particle size, PDI of different BLG:OCS nanocomplexes at pH 7.4. The concentration of BLG fixed at 1 mg/mL, and the concentrations of OCS varies with different mass ratios.

<table>
<thead>
<tr>
<th>BLG:OCS</th>
<th>Particle Size (diameter, nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:1</td>
<td>973.3±22.2</td>
<td>0.347±0.021</td>
</tr>
<tr>
<td>15:1</td>
<td>915.6±49.4</td>
<td>0.328±0.007</td>
</tr>
<tr>
<td>10:1</td>
<td>981.8±54.2</td>
<td>0.283±0.010</td>
</tr>
<tr>
<td>5:1</td>
<td>1261.1±48.1</td>
<td>0.309±0.020</td>
</tr>
<tr>
<td>2:1</td>
<td>1697±37.8</td>
<td>0.356±0.020</td>
</tr>
<tr>
<td>1:1</td>
<td>1686.3±16.5</td>
<td>0.300±0.021</td>
</tr>
<tr>
<td>1:2</td>
<td>604.3±1.7</td>
<td>0.225±0.017</td>
</tr>
<tr>
<td>1:5</td>
<td>205.2±1.0</td>
<td>0.240±0.021</td>
</tr>
<tr>
<td>1:10</td>
<td>234.9±5.0</td>
<td>0.293±0.005</td>
</tr>
<tr>
<td>1:15</td>
<td>346.2±4.1</td>
<td>0.290±0.004</td>
</tr>
<tr>
<td>1:20</td>
<td>415.2±9.4</td>
<td>0.271±0.011</td>
</tr>
</tbody>
</table>

The physical properties including particle size and PDI of BLG/OCS complexes assembled at
pH equal to 7.4 which is the physiological pH of human body with different mass ratios were listed as Table 4-1. From this study, we can see that there were three stages of the particle size variation. First when the ratio of protein to polysaccharide went from 20 to 2, the particle size increased from 1 mm to near 1.7 mm. Further addition of polysaccharide, the particle size went down until the ratio of OCS to BLG is 1 to 5, the smallest particles around 200 nm had obtained at 5. Continue adding polysaccharide, the size would increase again. The phenomenon could be explained as at the first stage, if there was little amount of OCS in the solution, with the addition of polysaccharide, the bridging effect would be stronger, and resulting the aggregation of protein. When the ratio was equal to 1 the largest aggregation would be yielded. For the second stage, with the ratio of polysaccharide to protein went up, more OCS absorbed onto the surface of protein, the electrostatic repulsive force got stronger and stronger to avoid the aggregation. When the ratio of OCS to BLG was 5, the repulsion force would be the strongest and the particle size was the smallest. At the third stage, kept adding OCS, the particle size increased again because the more polysaccharide absorbed, the larger shell it would occur. But at this condition, the PDI was less than 0.3 that indicated the stability was much better from that of first stage.

4.3.3 Morphology of Nanoparticles Measurements
Figure 4-3. Morphologies of BLG/OCS complexes at pH = 7.4 (a) BLG:OCS = 1:2 (b) height morphology of BLG:OCS = 1:2 (c) BLG:OCS = 1:5 (d) height morphology of BLG:OCS = 1:5.

Figure 4-3 had shown the study about the morphologies of BLG/OCS complexes with different ratios and the AFM was utilized with tapping mode, the scanning size was 2*2 um. From the diagram we can see that the nanoparticles were sphere-like and dispersed homogeneously. The particle size could be also estimated by the height map as shown in Figure 4-3 (b) & (d). The 1:2 nanoparticles were about 150 nm and 1:5 were around 40 nm. By compared with 600 nm and 200 nm measured by DLS, notably the particle sizes were much smaller. It is because the AFM gave images of the particles in dry state, while DLS measured the hydrated particle sizes of samples in solution. Also, from these two pictures as Figure 4-3 (a) & (c), the particles of 1:2 were more likely to aggregate while 1:5 were more homogeneously. Therefore, the changes of nanoparticles morphology were in consistent with the change of particle sizes determined by DLS.
According to the results of these experiments, we can conclude that the evenly dispersed 200 nm and sphere liked nanoparticles could be formed when the pH equal to 7.4 with ratio of OCS to BLG equal to 5. These findings showed that BLG/OCS nanoparticles could be a promising encapsulation system.
4.3.4 Thermal Characteristics of BLG/OCS Complexes

Figure 4-4. Differential scanning calometry (DSC) profiles of BLG, BLG/OCS complexes powders with mass ratios of 1:2, 1:5, 1:10.

Table 4-2. Peak denaturation temperature ($T_d$) and denaturation enthalpy ($H_d$) of BLG, BLG/OCS complexes powders with mass ratios of 1:2, 1:5, 1:10.

<table>
<thead>
<tr>
<th>Materials</th>
<th>$T_d$ (℃)</th>
<th>$H_d$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLG</td>
<td>91.77</td>
<td>230.2</td>
</tr>
<tr>
<td>BLG/OCS = 1:2</td>
<td>107.91</td>
<td>477.1</td>
</tr>
<tr>
<td>BLG/OCS = 1:5</td>
<td>109.52</td>
<td>489.9</td>
</tr>
<tr>
<td>BLG/OCS = 1:10</td>
<td>114.70</td>
<td>502.2</td>
</tr>
</tbody>
</table>
Figure 4 - 4 showed the DSC profiles of BLG, BLG/OCS complexes powders with different mass ratios. The peak denaturation temperature ($T_d$) and denaturation enthalpy ($H_d$) of different complexes were determined by DSC profiles, then calculated through associated software and tabulated in Table 4 - 2. We can see that the peak denaturation temperature ($T_d$) and denaturation enthalpy ($H_d$) of pure BLG ($T_d = 91.77^\circ$C, $H_d = 230.2$ J/g) were much lower than that of protein in complexes forms. With the increasing of mass ratios of BLG/OCS complexes, $T_d$ and $H_d$ were also increased. We know that the higher peak denaturation temperature ($T_d$) and denaturation enthalpy ($H_d$) means a more thermal stability which indicated that the complexes state of BLG/OCS possessed a more thermal stability than the free form of BLG, and with the mass ratios of BLG to OCS went up, the complexes would be more thermally stable. This finding showed that BLG/OCS complexes were more suitable to encapsulate the thermally sensitive nutraceuticals. Moreover, in the DSC profiles there were only one peak of different mass ratios complexes curves indicated that BLG and OCS have formed coacervates through intermolecular forces rather than mixtures through mechanical forces. This was in agreement with the findings of the experiments above.
4.3.5 Circular Dichroism (CD) Measurements

Figure 4-5. Far-UV CD spectra of 0.1 mg/mL BLG interacting with different concentrations of OCS (from $2.5 \times 10^{-3}$ mg/mL to $25 \times 10^{-3}$ mg/mL) at pH 7.4.

Table 4-3. The components percentages of secondary structure changes of BLG treated with gradient concentrations of OCS.

<table>
<thead>
<tr>
<th>Samples</th>
<th>α-helix %</th>
<th>β-sheet %</th>
<th>β-turn %</th>
<th>random coil %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLG</td>
<td>18.7</td>
<td>38</td>
<td>19.8</td>
<td>23.5</td>
</tr>
<tr>
<td>BLG +10ul OCS</td>
<td>14.5</td>
<td>37.2</td>
<td>24.6</td>
<td>23.7</td>
</tr>
<tr>
<td>BLG +20ul OCS</td>
<td>16.7</td>
<td>39.7</td>
<td>18.8</td>
<td>24.8</td>
</tr>
<tr>
<td>BLG +30ul OCS</td>
<td>15.3</td>
<td>39.2</td>
<td>19.9</td>
<td>25.6</td>
</tr>
<tr>
<td>BLG +40ul OCS</td>
<td>18</td>
<td>40.2</td>
<td>15.3</td>
<td>26.5</td>
</tr>
<tr>
<td>BLG +50ul OCS</td>
<td>14.1</td>
<td>40.5</td>
<td>21.6</td>
<td>23.8</td>
</tr>
</tbody>
</table>

Figure 4-5 showed the CD spectra of BLG interacting with different concentrations of OCS.
(from $2.5 \times 10^{-3}$ mg/mL to $30 \times 10^{-3}$ mg/mL) at pH 7.4 and then the data was tabulated as Table 4 - 3 calculating through the associated software. The results gave almost similar trends of all curves that indicated maybe there was no significant change in the secondary structure of BLG. According to the protein conformational analysis, even there was a little increase of β-sheet and slightly reduction of α-helix we can also concluded that no major changes of protein conformation happened after the formation of BLG/OCS complexes. Therefore, the OCS complexation showed minor stabilization of protein secondary structure.

4.4 Conclusions

The result of turbidimetric titration indicated that with the more amount of OCS binding with BLG, both pH$_{\phi 1}$ and pH$_{max}$ shifted to larger values as a result of the charge neutralization at a higher pH due to the more BLG bind around the chain of OCS. With the higher salt concentration contained in the system (up to 100 mM NaCl), the pH$_{\phi 1}$ shifted to larger, pH$_{\phi 2}$ shifted to smaller values and pH$_{max}$ kept going down indicated that high salt concentration led to a weaker tendency of interaction between BLG and OCS due to the screening effect. The results of DLS showed the smallest particles could be obtained at the ratio of protein to polysaccharide was 1:5 with the size around 200 nm under pH = 7.4. The morphologies of nanocomplexes were sphere-like and dispersed homogeneously. DSC results showed that after binding with OCS, both the peak denaturation temperature ($T_d$) and denaturation enthalpy ($H_d$) were increased indicated that the thermal stability was enhanced, and with more OCS binding, the better thermal stability the system would be. The results of CD showed that there
was no significant change in the secondary structure of BLG after binding with OCS.
5. CHAPTER V. Encapsulating Naringenin with Betalactoglobulin/Oligochitosan Nanoparticles and Evaluation of Its Effectiveness on the Bioefficacy of Naringenin

5.1 Introduction

The BLG/OCS complexes have been utilized in the encapsulation of NAR. DLS was used to characterize the particle size, PDI. The encapsulation efficiency (EE) of NAR in the nanoparticles was measured by high performance liquid chromatography (HPLC) after ultracentrifugation. Scanning electron microscope (SEM) was used to observe the morphology of NAR-encapsulated nanoparticles. The effect of pure NAR, NAR in encapsulated form and BLG/OCS nanoparticles on the cell viability was evaluated by MTT assay against human liver cancer HepG-2 cell line. Confocal laser scanning microscopy (CLSM) was used to observe the gradient transportation process of NAR encapsulated nanoparticles into HepG-2 cells.

5.2 Materials and Methods

5.2.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B66. It was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments.
Oligochitosan was purchased from Kunpoong Bio. Co., Ltd. (South Korea, Batch No KPH 1007GB) without further treatment. 95% ethyl alcohol, ACS/USP/NF grade acetone, HPLC grade acetonitrile and water were purchased from Pharmco-AAPER (CT, USA). HPLC grade acetic acid was purchased from EMD Chemicals Inc. Ultracentrifuge tubes were purchased from EMD Millipore Co. (MA, USA). The model of SEM was Joel Neoscope and Supra 55VP. Caco-2 and HepG-2 cell line were purchased from American Type Culture Collection (ATCC) website and the cells we used were in ten passages. Dulbecco’s modified Eagle’s medium (DMEM), non-essential amino acid, and phosphate buffered saline (PBS) were purchased from Life Technologies (Gibco®, NY, USA) as well as Fetal bovine serum (FBS). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA) and prepared with the concentration of stock solution of 5 mg/mL, stored in dark then used in the following experiments after diluted in ten times. The protein staining reagent - fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich.

5.2.2 Methods

5.2.2.1 Preparation of NAR encapsulated nanoparticles

The stocking solution of BLG and OCS were prepared by dissolving certain amount of BLG freeze dried powder and OCS powder in 0.01 M NaCl solution, repectively. NAR stocking solution was prepared by adding 10 mg NAR white powder into 4 mL PEG-200 first, until fully dissolved and then adding 6 mL DI water to reach a final concentration of 1 mg/mL (40 % w/w
PEG-200). The process of production of the NAR encapsulated nanoparticles was mixing NAR solution into BLG followed by adding OCS and then adjusting the pH equal to 7.4 with moderate stirring at ambient temperature. The NAR/BLG nanocomplexes formed first through hydrophobic and hydrogen interactions, then NAR encapsulated by protein/polysaccharide generated by electrostatic interactions between BLG and OCS.

5.2.2.2 Characterization of Nanoparticles

Particle size and PDI of BLG/OCS nanoparticles encapsulated with various concentrations of NAR were measured by DLS and the measurements were conducted in triplicate. The morphology of the complexes was observed by SEM. The fresh made NAR loaded nanoparticles solution was transferred to the ultracentrifuge tube with the molecular weight cut-off (MWCO) of 10kDa and after centrifugation at 11,000 g for 30 min, transferring the supernatant to HPLC vials and applied to HPLC to determine the concentration in the supernatant. The encapsulation efficiency that was calculated by using the following equation:

$$EE\ (%)=\frac{\text{Amount of NAR Loaded in Nanocomplexes}}{\text{Total Amount of NAR Added}} \times 100\%$$

5.2.2.3 Evaluation of the cell viability of NAR with and without BLG/OCS Nanoparticles Encapsulated

The cytotoxicity effects of NAR with encapsulation of BLG/OCS nanoparticles or without encapsulation were examined through MTT assay by using human colon carcinoma Caco-2 cell line and human liver cancer HepG-2 cell line, respectively. The process was that seeding both
Caco-2 cell and HepG-2 cell in 96 wells plate with the density of 10,000 cells/well and cultured in 100 ul DMEM (containing 10% FBS and 1% non-essential amino acid; especially for Caco-2 cell, extra adding 1% Penicillin-Streptomycin P/S) at 37°C and 5% CO2 for 24 h. Medium was then replaced by 200 ul BLG/OCS complexes (OCS:BLG = 0, 2, 3, 4, 5, 8, 10, 15, 20), NAR (18.4 – 91.8 uM) and BLG/OCS nanocomplex (OCS:BLG = 5) encapsulated with NAR (18.4 – 91.8 uM) and cultured at 37°C with 5% CO2. After 24h treatment, the sample solutions were then disposed and used PBS rinsed all wells. 100 ul MTT solution (0.5 mg/mL) was then replaced with PBS and incubated at 37°C for 4 h. After that, the MTT solution was disposed and 100 ul DMSO was added in each well to dissolve the insoluble formazan in purple color. Using microplate reader (BioTek Instruments Inc., VT, USA) to measure the absorbance of the solution in each well at 570 nm. All experiments were performed in triplicate.

5.2.2.4 In vitro Cellular Uptake of Nanoparticles Encapsulated with NAR

To observe the cellular uptake of the NAR encapsulated nanoparticles, BLG was labeled by FITC according to a literature procedure. In short, 1% w/v BLG solutions were treated with 4 mM CaCl2 and then using NaOH to adjusted pH to 9.5. In the meantime, FITC staining solutions were made by adding 0.1 mL FITC solution (40 mg/mL in ethanol) to 10 mL BLG solutions prepared as above in 0.1 M carbonate buffer at pH 9.0. The FITC/BLG mixtures were stored in a dark room for 2 h with moderate stirring. To remove the free FITC that were unreacted, the mixtures were then dialyzed against distilled water for 48 h by using a dialysis membrane (10 kDa MWCO, Thermo Scientific, Rockford, USA) in a dark room. Then the
solutions were freeze dried to obtain FITC-conjugated BLG powder and stored in dark environment for further use. HepG-2 were seeded in a 24-well plate at a density of 100,000 cells/well in 1 mL of DMEM medium (containing 10% FBS + 1% P/S) and then incubated the cells at 37°C and 5% CO2 for 24 h to allow cell attachment. DMEM medium was decanted and replaced with 1 mL pure DMEM containing FITC labeled NAR-encapsulated nanoparticles and then incubated for 0, 0.5, 3, 6, 9, 12 h, respectively. The preparation method was same as before and in this study, the final NAR concentration was 49.5 µM in which concentration exhibited 80% cell viability, and the ratio of BLG to OCS was 1:5 with a fixed BLG concentration of 0.1 mg/mL. Before observation, the cells were rinsed with PBS for 3 times and stained with 4,6-diamidino-2-phenylindole (DAPI) and then using 4% (w/v) paraformaldehyde solutions to fix the samples. Confocal laser scanning microscopy (CLSM) (Zeiss LSM 710, Germany) was applied to observe the cellular uptake of the nanoparticles.

5.3 Results and Discussions

5.3.1 Preparation and characterization of NAR encapsulated BLG/OCS Nanoparticles

According to the results of pH-turbidimetric and DLS of BLG/OCS complexes in the above part, we know that when the ratio of protein to polysaccharide equals to 1:5, the nano-size particles can be obtained at pH = 7.4. Thus, in the present study, we focused on various concentrations of NAR encapsulated by the certain nanoparticles that the BLG/OCS = 1:5.

Table 5-1. Particle size, PDI and encapsulation efficiency of different NAR:BLG for the nanocomplexes of BLG:OCS = 1:5 with a fixed BLG concentration of 1 mg/mL, pH = 7.4.
## Table 5-1

<table>
<thead>
<tr>
<th>NAR:BLG:OCS w/w/w</th>
<th>Particle Size (nm)</th>
<th>PDI</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05:1:5</td>
<td>203 ± 1.2</td>
<td>0.198 ± 0.030</td>
<td>98.4 ± 0.087</td>
</tr>
<tr>
<td>0.075:1:5</td>
<td>209 ± 2.1</td>
<td>0.192 ± 0.009</td>
<td>99.0 ± 0.098</td>
</tr>
<tr>
<td>0.1:1:5</td>
<td>229 ± 1.5</td>
<td>0.201 ± 0.017</td>
<td>96.8 ± 0.112</td>
</tr>
<tr>
<td>0.15:1:5</td>
<td>235 ± 2.3</td>
<td>0.214 ± 0.022</td>
<td>94.5 ± 0.354</td>
</tr>
<tr>
<td>0.2:1:5</td>
<td>253 ± 4.8</td>
<td>0.226 ± 0.011</td>
<td>92.3 ± 0.213</td>
</tr>
<tr>
<td>0.25:1:5</td>
<td>348 ± 8.9</td>
<td>0.301 ± 0.043</td>
<td>89.9 ± 0.438</td>
</tr>
<tr>
<td>0.3:1:5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

**Figure 5-1.** The picture of the nanoparticles encapsulated of various concentrations of NAR (from left to right, 0.05, 0.075, 0.1, 0.15, 0.2, 0.25 and 0.3 mg/mL NAR). The concentration of BLG fixed at 1 mg/mL and the ratio of BLG to OCS was 1:5, pH = 7.4.

From Table 5-1, we can see that with adding more amount of NAR to the BLG/OCS solutions, the particle size went from 200 nm to 350 nm with all the PDI less than 0.3 that means the results were reliable. Until the concentration of NAR reached to 0.3 mg/mL, there were some
precipitates at the bottom of small vials so that the values of that were not shown in the table.

In additional, the more amount of NAE added to solutions, the less encapsulation efficiencies they were. However, the EEs of all the samples were about or larger than 90% indicated that the system has good encapsulation ability.

![Figure 5-2. SEM images of NAR encapsulated BLG/OCS nanoparticles and with the ratio is BLG:NAR:OCS = 1:0.05:5 and 1:0.25:5, respectively. The scale bars of A,C were 20 um and those of B, D were 50 um.](image)

To study the morphologies of the NAR encapsulated BLG/OCS nanoparticles, the SEM was used. According to Figure 5 - 2, we can see that the nanoparticles were in sphere shape. When the concentration of NAR is 0.05 mg/mL, the particle size measured by SEM seemed smaller than that of 0.25 mg/mL and as well as the distribution of nanoparticles was more evenly than that of 0.25 mg/mL.
5.3.2 In vitro cytotoxicity study

The cytotoxicity of BLG/OCS Nanoparticles (NPs) encapsulated with NAR was evaluated by MTT assay against both human colon carcinoma Caco-2 cell line and human liver cancer HepG-2 cell line.
Figure 5-3. The cell viability of CaCO-2 cell line after treating with A. The BLG/OCS nanoparticles with different ratios; B. The different concentrations of pure NAR; C. The different concentrations of NAR (18.4, 27.5, 36.7, 45.9, 55.1, 73.5 and 91.8 μM) encapsulated with BLG/OCS nanoparticles (BLG:OCS = 1:5).
BLG/OCS NPs for HepG-2 Cell Line

A

Cell Viability (%)

control 2 3 4 5 6 7 8 9 10 15 20

Pure NAR for HepG-2 Cell Line

B

Cell Viability (%)

control 18.4 27.5 36.7 45.9 55.1 73.5 0.08% PEG

Concentration of NAR (uM)
Figure 5-4. The cell viability of HepG-2 cell line after treating with A. The BLG/OCS nanoparticles with different ratios; B. The different concentrations of pure NAR; C. The different concentrations of NAR (18.4, 27.5, 36.7, 45.9, 55.1 and 73.5 μM) encapsulated with BLG/OCS nanoparticles (BLG:OCS = 1:5).
CaCO-2 cells are often used to stimulate the small intestinal wall cells of human body. In our research, this cell line was used in the in vitro cytotoxicity study in order to find an appropriate concentration of NPs that can be applied in the further study like cellular uptake and transport. The complexes assembled by BLG and OCS at different ratios were diluted 10 times to yield a final concentration of BLG was 0.1 mg/mL. After 24 h treatment of the complexes, a correlation between the ratios and cell viability was observed as Figure 5 – 3 A. We can see that the cell cytotoxicity had increased with the ratio of OCS to BLG went up. When the ratio was less than 5, the cell cytotoxicity was relatively high (larger than 80%). The effect of pure NAR was also investigated and as shown in Figure 5 – 3 B. Additionally, we also test 1% PEG - 200 due to it was the solvent of NAR and there was almost no toxicity at all. We can see that when the concentration of pure NAR went up from 18.4 to 73.5 uM, the cell viability remained constant around 90% until the concentration reached 91.8 uM, the viability dropped abruptly. According to the results of these two experiments, we designed the test of cell viability of NAR encapsulated NPs with the ratio of 5 (OCS/BLG) and shown as Figure 5 – 3 C. The viability of NPs encapsulated 18.4 to 73.5 uM NAR seemed to be constant that around 80%, which had the similar trend to that of pure NAR. It was also dropped abruptly when the concentration of NAR reached 91.8 uM.

HepG-2 cells are very often used in the cytotoxicity study to test the bioefficacy of anti-tumor. The complexes assembled by BLG and OCS at different ratios were diluted 10 times to yield a final concentration of BLG was 0.1 mg/mL. After 24 h treatment of the complexes, a correlation
between the ratios and cell viability was observed as Figure 5–4 A. We can see that the cell cytotoxicity had increased with the ratio of OCS to BLG went up. When the ratio was less than 8, the cell cytotoxicity was relatively high (larger than 80%). The effect of pure NAR was also investigated and as shown in Figure 5–4 B. Additionally, we also test 0.8% PEG-200 due to it was the solvent of NAR and there was almost no toxicity at all. We can see that when the concentration of pure NAR went up from 18.4 to 45.9 uM, the cell viability remained constant around 90% and further increasing the concentration will lead to a slightly reduction of viability.

According to the results of these two experiments, we designed the test of cell viability of NAR encapsulated NPs with the ratio of 5 (OCS/BLG) and shown as Figure 5–4 C. The viability of NPs encapsulated 18.4 to 36.7 uM NAR seemed to be constant that around 80%, which had the similar trend to that of pure NAR. It was also dropped down slowly with the concentration of NAR increasing.

According to the results of MTT assays above, we can conclude that nanoparticles with or without NAR exhibited a concentration-dependent cytotoxicity in both CaCO-2 and HepG-2 cell lines.

5.3.3 Intracellular Uptake of NAR loaded BLG/OCS Nanoparticles
The cellular uptake of NAR-loaded BLG/OCS NPs through HepG-2 cells was observed by
CLSM at different time intervals and shown as Figure 5- 5. The HepG-2 cells were treated
with FITC-labeled BLG/OCS NPs and then stained with DAPI to identify the location of cell
nucleus. Therefore, we can see the whole process of cellular uptake of NAR-loaded
BLG/OCS NPs to HepG-2 cells within 12 hours. There were three layers of this pictures, the
first layer of images exhibited with only red color that means the location of BLG/OCS NPs,
the second layer of images showed with only blue color that means the location of HepG-2
cell nucleus, and the third layer was the overlay of both fluorescence colors, which indicated
the amount of NPs endocytosis by HepG-2 cells. We can see that in the HepG-2 cells, minor
red color could be observed at 30 min indicating that there was limited endocytosis at this
time point. After three hours incubated with NPs, there was little light intensity of red color
around the border of nucleus indicating that only some NPs got into the cells at this time
point. When the time intervals were six hours and nine hours, a marked increase in the red

Figure 5- 5. Cellular uptake of NAR encapsulated BLG/OCS NPs: CLSM images of the
intracellular uptake of FITC-labeled NAR encapsulated BLG/OCS NPs by HepG-2 cells
(labeled by DAPI) in the time interval of 0, 0.5, 3, 6, 9, 12 h, respectively.
fluorescence intensity could be observed in the cells, which means that there was relatively large amount of NPs endocytosis by HepG-2 cells. Additionally, the fluorescence quenching happened when the time interval was twelve hours. In conclusion, the CLSM studies showed that the NAR loaded NPs had good cellular uptake ability by HepG-2 cells. Moreover, at the time intervals of 6 and 9 hours, there was more NPs endocytosis by the cells.

5.4 Conclusions

In this part, NAR was successfully encapsulated with BLG/OCS NPs with the particle size between 200 to 350 nm and PDI less than 0.3. The encapsulation efficiencies of all the NAR loaded NPs were larger than 90% that exhibited they had a good encapsulation ability. The morphologies of NPs showed that they were sphere like and evenly distributed. But with the added amount of NAR increased, the morphology changed from spherical particle to partially aggregates. The results of cytotoxicity studied showed that the cell viability after treated with NAR loaded NPs were concentration dependent. The more NAR, the lower cell viability it was. The cellular uptake study showed NPs endocytosis were time dependent and at the time intervals of 6 h and 9 h, there were large amount of NPs getting into the HepG-2 cells. Therefore, the BLG/OCS NPs were a good candidate encapsulation system for NAR and further realizing the bioefficacies of NAR.
6. CHAPTER VI. Preparation and Characterization of Pickering Emulsion Stabilized by The Beta-lactoglobulin/Oligochitosan Nanoparticles

6.1 Abstract

As I have mentioned in the previous chapter, the loading capacity of NAR by using only BLG/OCS nanoparticles (NPs) is low so that cannot reach its limit of the pharmaceutical effect. In this part, the NPs assembled by BLG and OCS were applied to stabilize the Pickering emulsions (PE) with medium chain triglyceride (MCT) as the oil internal phase. According to the former experiment, after optimization, at pH equal to 7.4 which is close to the microenvironment of human body, when the ratio of BLG to OCS is 1:5, the stable NPs can be self-assembled with the particle size as low as 200 nm. The choice of NPs was also in terms of contact angle and interfacial tension. I have investigated the effect of different oil fraction (\(\Phi = 30 - 70\%\)), different protein concentration (0.1 - 0.5\%) and different pH values (2 – 7.4) on the stability of PE. The abilities of NPs to stabilize the PE were measured by the observation, creaming index calculation during the storage time. The microstructures of PE could be observed by optical microscope and fluorescence microscope. The samples at different oil fraction and pH values were also applied to the rheometer to measure the viscoplastic properties of PE. We can conclude that all the NPs in the protein range of 0.1 – 0.5\% could stabilize the PE with different oil fraction. Especially, fully emulsions could be formed when \(\Phi = 70\%\) while others formed partially emulsions. These emulsions were stable against different pH values after a period storage time up to 1 month but when the pH equal to 2 the emulsions coalescence and oiling off. The rheological characterizations indicated that the BLG/OCS NPs stabilized
PE showed gel-like behavior and became more stiffness with the pH values went up.

6.2 Introduction

As introduced in the chapter I, the loading capacity of nutraceutical by only using nanoparticles encapsulation is low so that cannot reach the certain therapeutic effect. In order to increase the loading, pickering emulsion (PE) will be applied as the delivery vehicle. Pickering emulsion which is stabilized by particles have attracted more and more attention due to its excellent stability against the coalescence and Ostwald ripening which reported by a previous literature\(^74\). What’s more, by compared with conventional emulsion, the internal oil phase of PE is extreme high which can up to 70\%, resulting the high loading of hydrophobic nutraceuticals. In addition, PE has the feature of surfactants-free that many other conventional emulsions are emulsified by the chemical surfactants such as Tween, Span which will cause the adverse effects such as irritancy, colitis and some other metabolic syndromes\(^{75, 128}\). Extensive work has been published about the PE stabilized by non-food grade particles\(^75, 129-131\). Nowadays, more and more researchers start to focus on the food grade particles such as proteins or polysaccharides due to they are biocompatible, nontoxic and degradable. However, it is hard for food grade materials to reach the conditions of pickering emulsion fabrication. Because many macromolecules are very easy to be affected by the microenvironments such as pH, temperature \textit{etc}. Additionally, most of protein or polysaccharide cannot preserve a suitable contact angle which is a key parameter of pickering emulsion stabilization. Xiao et. al used kafirin nanoparticles to stabilize PE, then investigated its microstructures and rheological properties at different conditions\(^132\). Mwangi \textit{et. al} reported that chitosan particles can stabilize PE and stable against many different environmental factors\(^133\).
However, utilizing protein/polysaccharide particles as pickering stabilizers were relatively less published.

In this chapter, I will use BLG/OCS NPs as the stabilizer to stabilize PE with MCT as the internal oil phase. Many kinds of NPs stabilized PE were compared with conditions of different oil fractions and protein concentrations. The stabilities of the PE were characterized against pH values changes. The rheological properties of these samples were also investigated.

6.3 Materials and Methods

6.3.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B64. It was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments. Oligochitosan was purchased from Kunpoong Bio. Co., Ltd. (South Korea, Batch No KPH 1007GB) without further treatment. F. Nile red was purchased from VWR International (PA, USA). fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich (MO, USA). Milli-Q water was used in all the experiments.
6.3.2 Methods

6.3.2.1 Preparation and Characterization of the BLG/OCS NPs

OCS and BLG were dissolved in DI water containing 10mM NaCl, respectively. The pH of both solutions was adjusted to 7.4 by using 0.1M HCl and 0.5M NaOH. Under the magnetic stirring, the OCS solution was added dropwise into BLG solution to generate the self-assemble nanocomplexes.

The FITC labeled BLG/OCS nanocomplexes were prepared in the similar way with little modification that BLG was FITC labeled. The preparation process of FITC labeled BLG has been described as above chapter which was followed by a literature with little modification. In brief, 1% w/v BLG solutions were treated with 4 mM CaCl$_2$ and then using NaOH to adjusted pH to 9.5. In the meantime, FITC staining solutions were made by adding 0.1 mL FITC solution (40 mg/mL in ethanol) to 10 mL BLG solutions prepared as above in 0.1 M carbonate buffer at pH 9.0. The FITC/BLG mixtures were stored in a dark room for 2 h with moderate stirring. To remove the free FITC that were unreacted, the mixtures were then dialyzed against distilled water for 48 h by using a dialysis membrane (10 kDa MWCO, Thermo Scientific, Rockford, USA) in a dark room. Then the solutions were freeze dried to obtain FITC-conjugated BLG powder and stored in dark environment for further use.

6.3.2.2 Contact Angle Measurements

The contact angle was measured by using VCA-Optima XE Dynamic Contact Angle Analyzer (AST Products, Inc., MA, USA). The BLG/OCS nanocomplexes (c(BLG) = 2.5 mg/ml)
solution were dripped onto the pre-cleaned microscope glass slide and dried until forming a film before measuring. Then using a CCD camera to capture images immediately after a drop of mili-Q water (2 ul) was deposited onto the surface of nanocomplexes film. The contact angle was determined by the associated software with the analyzer. The results were recorded on average and repeated ten times at least.

6.3.2.3 Preparation and Characterization of the Pickering Emulsions

The BLG/OCS NPs stabilized PE was generated by adding medium chain triglyceride (MCT) into the NPs solution with a total volume 8 ml. Different oil fraction (0.3, 0.5, 0.7) were applied with various protein concentrations (0.1, 0.25 and 0.5%). Then mixing them together under high-speed homogenizer (model IKA-ULTRATURRAX T25 digital, IKA 190 Works, Inc., Wilmington, NC, USA) with a 10 mm dispersion probe under the condition of 10,000 rpm for 2 min, stored at room temperature. The fluorescence labeled PE were prepared also in this way using 1 uM stained MCT and FITC labeled BLG/OCS NPs.

The pH stability of PE was determined by diluting the upper cream layer of PE for 5 times with Milli-Q water at pH equal to 2, 4.5, 6, 7.4, respectively. The microscopic images were then observed after a storage period time of 24 h.

6.3.2.4 Observation and Measurements of the Pickering Emulsion

Several parameters would be introduced. After PE were formed and stored for a period of time (3 h and 7 days respectively), The emulsified phase volume fraction was recorded as
\[
creaming \text{ index} = \frac{H_e}{H_t} \times 100\%
\]

In this formula, \(H_e\) means the heights of emulsion phase and \(H_t\) means the total heights of emulsions after specific storage time. The stability index (SI) was calculated\textsuperscript{134} as internal phase fraction after 7 days’ storage divided by the fraction after 3 h storage. Mean and standard deviations of three replicates were reported. Visual characteristics of the upper and bottom layers of emulsions were also summarized.

### 6.3.2.5 Visualization of Pickering Emulsions Microstructures

The optical microscopic images (PE samples with different oil fractions and different BLG concentrations) were monitored by using a Nikon TE-2000-U inverted fluorescence microscope equipped with a CCD camera (Retiga Exi, Q-imaging). According to the method from one literature\textsuperscript{135} that one droplet of sample was pipetted and deposited onto the surface of microscope slide gently before observation. The photos were taken Confocal laser scanning microscopy (CLSM) (Zeiss LSM 710, Germany) was applied to observe the fluorescence of PE which prepared with nile red stained MCT and FITC labeled NPs. The light was filtered through the bandpass filters and then yielded excitation wavelengths of 488 nm for FITC, 550 nm for nile red.

### 6.3.2.6 Rheological Measurements

The viscoelastic properties of PE were measured on a Discovery HR-2 Hybrid Rheometer (TA Instruments, DE, USA) with 25 mm stainless-steel parallel plate geometry. Samples with
different oil fractions and different BLG concentrations were applied to both steady shear rate experiments and dynamic frequency sweep model. Dynamic strain sweep model was tested before to determine the linear viscoelastic region and the results confirmed 1.5% strain values. The conditions for dynamic frequency sweep test were 0.1-100 rad/s, and the curves of storage modulus $G'$ and loss modulus $G''$ were recorded. The conditions for steady shear experiments were that the shear rate was increased from 0.1 to 100 s$^{-1}$, and the viscosities ($\eta$) were recorded. All of the measurements were carried out with 20 data points per decade at room temperature.

6.4 Results and Discussions

6.4.1 Characteristics of BLG/OCS NPs

The preparation of BLG/OCS NPs was based on the self-assemble method. At pH 7.4 which was close to the pH value of the microenvironment of human body, adding OCS solution into BLG solution to form the BLG/OCS nanocomplexes with the ratio of protein to polysaccharide was 1:5 (w/w) which was shown as Figure 6 - 1(a). The particle size was 205.2±1.0 nm with PDI values equal to 0.240±0.021. Both height image and 3D image of AFM tapping mode confirmed that the nanocomplexes formed by BLG and OCS were spherical particle liked shown as Figure 6 - 1(b).
Figure 6-1. (a) Visual image of BLG/OCS solution in 10 mM NaCl. (b) Height image (bottom left) and 3D image (bottom right) of AFM tapping mode of BLG/OCS NPs (BLG:OCS = 1:5 w/w, c(BLG) = 1mg/ml).
The wettability property of BLG/OCS NPs at the air-water interface was investigated by measuring contact angle of water droplet onto the BLG/OCS surface film at BLG concentration equal to 2.5 mg/ml. This method was followed by de Folter\textsuperscript{80}. The contact angle was shown as Figure 6 - 2. Its contact angle at the interface of water-air was $40.3 \pm 2.7^\circ$. Recall that the energy required for detaching the colloidal particles from interface is strongly related to contact angle. If the contact angle is between 30 to 50°, the particles at interface can be regarded as irreversible adsorbed.

![Figure 6-2](image)

**Figure 6-2.** Water in air contact angle measured by dropping water droplet onto the surface film of BLG/OCS, c(BLG) = 2.5 mg/ml.

### 6.4.2 Storage stability of BLG/OCS NPs stabilized Pickering Emulsions

Here are some photographs to show the BLG/OCS stabilized PE with BLG concentrations c(0 – 0.5%, w/v) and oil fraction $\Phi$ (0.3 – 0.7) as variables after storage for a period time of 3 h and 7 days respectively shown as Figure 6 - 3.
Figure 6-3. Photographs taken at 3 h and 7 days with different BLG concentrations (0 – 0.5%, w/v) and different oil fraction (0.3 – 0.7). Format A-B-C-D-E (A: storage time, B: BLG concentration, C, D, E: oil fraction, from left to right of each photo).
We can see that the cream volume fraction of all the emulsions underwent a fast shrink at 1h and followed by a slow shrink up to 3h storage time, thus the samples were observed at 3 h because at that time, it tended to be stable. If no NPs added, the coalescence occurred right after the preparation and we can see a clear boundary between water and oil. When there was NPs added to the oil, the samples remarkably avoided coalescence among oil droplets even as low as 0.1% protein concentration. Both 0.1% and 0.25% BLG concentration samples could form partially emulsions (upper emulsified phase and lower water phase) no matter how many oil fraction occupied the total emulsions. When the oil fraction was 70%, the fully emulsified phase could be formed and it is gel like. In general, increasing the oil fraction could lead to a progressive increase of the emulsified cream volume. Increasing the BLG concentration could also resulted in a higher emulsified volume.

The measurements of internal phase fraction, stability index and the phenomena of both upper phase and bottom phase were summarized as Table 6 - 1. Obviously, the increase of both BLG concentration and oil fraction could lead to the increase of cream layer. But oil fraction played a major role for increasing up to more than 50% of internal oil phase fraction. For all the samples, the stability index (SI) were in the range of 93 – 100% which was relatively stable. Similarly, increasing the BLG concentrations involved in the complexes solution could also result in the increase of cream layer. For the samples with 0.5 and 0.7 oil fractions except low BLG concentration (0.1%), we can see that the internal oil phase fraction occupied approached or even exceed 88% which was a typically close packed emulsion system. All of these indicated
that the BLG/OCS NPs stabilized PE were relatively stable against coalescence.
Table 6 - 1. Summary of the characteristics of BLG/OCS NPs stabilized emulsions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Internal oil phase fraction (H_e/H_t) * 100%</th>
<th>Stability Index (SI) %</th>
<th>Emulsions characterization after 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>c(blg)</td>
<td>3 h</td>
<td>7 days</td>
<td>Upper phase</td>
</tr>
<tr>
<td>Φ(oil)</td>
<td></td>
<td></td>
<td>Bottom phase</td>
</tr>
<tr>
<td>0.1-0.3</td>
<td>45</td>
<td>42</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1-0.5</td>
<td>80</td>
<td>77</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1-0.7</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25-0.3</td>
<td>57</td>
<td>55</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25-0.5</td>
<td>82</td>
<td>81</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25-0.7</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-0.3</td>
<td>60</td>
<td>57</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-0.5</td>
<td>85</td>
<td>83</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-0.7</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.4.3 Microstructures of BLG/OCS NPs stabilized PE influenced by particle concentrations and oil phase fractions

The optical microscopic images of BLG/OCS stabilized PE with different blg concentration at fixed 50% oil fraction was shown as Figure 6-4.

Figure 6-4. Pickering emulsions stabilized by BLG/OCS NPs with oil fraction = 0.5, scale bar = 200 um. (a) c(BLG) = 0.1% (b) c(BLG) = 0.25% (c) c(BLG) = 0.5%

We can see that the emulsion droplet sizes decreased as BLG concentrations increased from 0.1% to 0.5%. Taking into consideration that the emulsified phase volume increased from 77% to 83% as the concentrations increased, the reduction of droplets sizes was because of that when the concentration of BLG increased, more oil droplets surface would be covered by the NPs. This indicated that higher density of BLG/OCS particles would anchor more particles onto the interface, thus contributed to higher interfacial area during the coalescence process.

The influence of different oil fractions on the stabilization of BLG/OCS NPs based PE were also investigated at \( \Phi = 30 \text{ - } 70\% \) and shown as Figure 6 - 5.
We can see that the droplets sizes of PE increased gradually with the increase of oil phase fraction from 30% to 70%. The large droplets were formed due to the protein particles available for the stabilization of interface decreased. In another word, the number of NPs covered oil droplets water interface was becoming less with the increase of oil fraction involved in the PE systems. In conclude, the change of oil droplets sizes was a combined result of the interface covered by the particles and the particles migrate onto the surface which was in correspondence with a literature\textsuperscript{132}. Taking the former visual observation into consideration, the change of oil fraction played a dominated role on the change of PE droplets sizes.

6.4.4 The effect of different BLG concentrations and oil fractions on the rheological properties of BLG/OCS NPs stabilized PE

The emulsions’ rheological properties played a guiding significance role in the utilization process. Thus, we looked into the effect of different formation conditions (protein concentrations and oil fractions) on the rheological properties of PE. Both steady shear and oscillating frequency sweep experiments would be applied to test the viscosity and rheological properties. The dynamic sweep tests were carried out before each oscillating frequency sweep...
test from 0.1% to 100% strain with the frequency fixed at 0.1Hz in order to obtain the linear viscoelastic region. The result showed that when the strain value equal to 1.25%, it was in the linear viscoelastic region and then this value was applied to all the tested samples.
Figure 6-6. Rheological properties of PE (oil fraction = 50%) with various BLG concentrations (from 0.1% to 0.5%) (a) Oscillating frequency sweep curves at fixed strain (1.25%) with frequency ranging from 0.1 to 100 rad/s. (b) Viscosity versus shear rate from 0.5 to 100 s\(^{-1}\).

As shown in Figure 6-6 (a), we can see that the storage modulus G' were higher than the loss modulus G'' of all the tested samples over the whole range of frequency 0.1 – 100 Hz. The result indicated that the pickering emulsions stabilized by BLG/OCS NPs showed gel-like property and such property could be attributed to the three dimensional network of particles formation. Besides, both G' and G'' were not dependent much on the angular frequency especially
for the middle range frequency about 0.5 – 60 Hz which was a typical property of a highly flocculated elastic structure\textsuperscript{38}. While when the frequency was from 60 to 100 Hz, there was a transition point of the line and both $G'$ and $G''$ were enhanced in consistently. The increased at high angular frequency showed that the viscous behavior of PE were dominated\textsuperscript{136}. By compared with different particles concentrations composition, we found that the storage modulus $G'$ were almost identical but the loss modulus $G''$ were increased with the particles concentration increased which indicated that the PE with higher particles concentration exhibited more viscous property and higher gel strength. Because the volume of cream layer didn’t change much between these samples, the increased gel strength could be attributed to the decrease of PE droplet sizes and a denser pack of droplets were formed. Figure 6 - 6 (b) showed that the viscosities of all the samples decreased with the increase of shear rate that reflected the high shear rate could breakdown of the network within PE.
Figure 6-7. Rheological properties of PE (BLG concentration = 0.25% w/w) with various oil fractions (from 30% to 70% w/w) (a) Oscillating frequency sweep curves at fixed strain (1.25%) with frequency ranging from 0.1 to 100 rad/s. (b) Viscosity versus shear rate from 0.5 to 100 s⁻¹.
As shown in Figure 6 - 7(a), we can see that in the samples of PE at all oil fraction, the storage modulus $G'$ were higher than the loss modulus $G''$ over the whole range of frequency. This indicated that the pickering emulsions stabilized by BLG/OCS NPs showed gel-like property which is similar to the former different ratios samples. Additionally, with oil fraction increased, both storage and loss storage modulus increased in correspondence. Recall that emulsion with higher oil fraction resulted in a higher cream volume, this was probably resulted from emulsion droplets were in close proximity with each other and a tighter packing density. Remember that the values of both $G'$ and $G''$ were similar in the samples of Figure 6 - 6(a) no matter how much protein concentration involved. By compared with these samples, the sample with 70% oil fraction has a higher modulus so that we know that the oil fraction involved in the PE has a more significant influence in the property of gel-like structure. The viscosity of samples shown in Figure 6 - 7(b) was also increased with a went up of oil fraction, especially for 70%. We can also conclude that the rheological property was dominated by the oil fraction.

### 6.4.5 The effect of pH on the stability of BLG/OCS NPs stabilized PE

The pH stability is a significant index for emulsions. In the current study, the pH stability of BLG/OCS NPs stabilized PE was characterized by diluting the PE cream layers with 0.01M NaCl solutions under different pH (2, 4.5, 6 and 7.4) which stimulates the digestion process of food and covered all the pH of human stomach, small intestine and most food system.
Figure 6-8. pH stability of BLG/OCS NPs based PE, c(protein) = 0.1% w/w, Φ= 50% w/w. The scale bar is 200 um. (a) pH = 2 (b) pH = 4.5 (c) pH = 6 (d) pH = 7.4
Figure 6 - 8 showed the microstructures of PE under different pH values after 24h storage time. We can see that the emulsion droplets sizes increased with the pH increased. At pH 2, the droplets were the smallest and there was a phenomenon that the samples can be easily spread on the glass slides. At this pH, there is no interaction between protein and polysaccharide due to electrostatic repulsion. And the emulsify ability is mainly ascribed to the amphiphilic property of protein molecules rather than particles. The droplet sizes did not change too much at pH 4.5 and 6. The emulsion droplet size was close to 200 um when the pH equal to 7.4. Thus, we can conclude that low pH could affect the stability of PE.

6.4.6 The effect of storage time on the stability of BLG/OCS NPs based PE
Figure 6-9. The photographs of PE stabilized by BLG/OCS NPs under different pH with a period of storage time (3 hours, 1 week and 1 month, respectively).
Table 6 - 2. Summary of the characteristics of BLG/OCS NPs stabilized pickering emulsions under different pH with a period of storage time (3 hours, 1 week and 1 month, respectively).

<table>
<thead>
<tr>
<th>Samples 0.1-0.5</th>
<th>Internal oil phase fraction (H_e/H_t)*100%</th>
<th>Stability index (SI) %</th>
<th>Emulsions characterization after 1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>71</td>
<td>-</td>
<td>Oil off</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>73</td>
<td>Phase separation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>Slightly opaque</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>77</td>
<td>73</td>
<td>gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>Slightly opaque</td>
</tr>
<tr>
<td>pH 6</td>
<td>82</td>
<td>78</td>
<td>gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>Slightly opaque</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>87</td>
<td>83</td>
<td>gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>Transparent</td>
</tr>
</tbody>
</table>

Figure 6 - 9 and Table 6 - 2 are the photographs and table of PE stabilized by BLG/OCS particles with different pH after storage periods of 3 hours and 7 days, respectively. We can see that the internal oil phase increased with the pH value increased. The stability indexes of all the samples were close to 95%. After 3h storage, except sample with pH 7.4, the lower phase of other samples was turbid. This is due to the interaction between protein and polysaccharide is driven by electrostatic force which is mainly influenced by pH value, they take the same charge when the pH was as low as 2. And after 7 days’ storage time, the macroscopic phase separation still occurred of the pH 2 sample. Thus, we can conclude that the stability of samples was not good after storage for a period of time, especially for pH 2 sample.
6.5 Conclusions

In this part, the BLG/OCS NPs can stabilize pickering emulsion with MCT as the oil phase. By compared with encapsulating NAR only with NPs, the loading capacity by using BLG/OCS NPs based PE encapsulation has enhanced greatly. All the NPs in the protein range of 0.1 – 0.5% could stabilize the PE with different oil fraction. Especially, fully emulsions could be formed when \( \Phi = 70\% \) while others formed partially emulsions. These emulsions were stable against different pH values after a period storage time from 3 hours up to 1 month but when the pH equal to 2 the emulsions coalescence and oiling off which means it is unstable with the time increased. The BLG/OCS NPs stabilized PE showed gel-like behavior and became more stiffness with the pH values went up. The oil fraction has a higher contribution to the gel structure than that of protein concentrations.
Chapter VII. Assembling and characterizing genipin crosslinked BLG/OCS nanoparticles based pickering emulsions

7.1 Abstract

The research of the last chapter mentioned that BLG/OCS NPs stabilized PE has a poor pH stability, especially for pH 2. In this part, a natural crosslinked – genipin was used to enhance the stability of pickering emulsion. The effect of genipin concentration and crosslinking reaction time on the size and contact angle of particles was investigated. The result showed that the more genipin added, the smaller size can be reached before crosslinking for up to 10 hours (~250 nm), after that the size of particles would get larger even larger than the original size. The morphologies of particles were sphere-lied. The contact angle of the particles between water and air ranging from 40° to 25° with different reaction time (0 – 24 hours).

Additionally, the effect of different oil fraction (Φ = 30 – 70%), different protein concentration (0.1 - 0.5%) and different pH values (2 – 7.4) on the stability of PE were also studied and found that after crosslinking, the pH stability of particles had greatly enhanced. The sizes were kept between 140 to 170 um in these pH values ranges. The samples at different oil fraction and pH values were also applied to the rheometer to measure the viscoplastic properties of PE. Genipin didn’t change too much of this property of PE, the result indicated that the genipin crosslinked BLG/OCS NPs stabilized PE showed gel-like behavior and became more stiffness with the pH values went up.

7.2 Introduction
The BLG/OCS nanoparticle was designed to stabilize the NAR contained MCT to form the pickering emulsion in order to enhance the loading capacity of NAR which was discussed detailed in the last chapter. We know that the protein/polysaccharide complexes are assembled mainly through the electrostatic interaction so that they are sensitive to the environmental factors, such as pH, ionic strength or temperature. Therefore, we used the pickering emulsion to enhance the stability especially for the pH stability to enable such kind of system as oral delivery vehicle, no matter which pH value from very acidic pH in the stomach to weak basic environment in the small intestine when go through the human GI tract\textsuperscript{137}. Additionally, the advantage of using PE can greatly increase the loading amount of NAR to reach the bioefficacy. A problem came across in the research of last chapter was that only using BLG/OCS NPs stabilized PE was not stable when the pH is low (especially pH = 2). To overcome this problem, we used genipin as crosslinker to crosslink protein and polysaccharide.

Genipin is an aglucone geniposide\textsuperscript{138} which was isolated from Genipa Americana during a wide investigation of extracts\textsuperscript{138}. It is a traditional herbal medicine which has been used in the food and fabric industries as a natural colorant\textsuperscript{139}. The reason we can use it as covalent crosslinker is that many researches have reported genipin can bind with biopolymers which contain primary amine groups such as chitosan, resulting covalent coupling\textsuperscript{140-142}. The mechanism of the reaction as followed\textsuperscript{143}: 
Figure 7-1. The crosslinking reaction between genipin and the primary amine groups of chitosan.

Figure 7 - 1 showed chitosan interacts with genipin and yielding two main crosslinking reactions. We can conclude that genipin can crosslink the biopolymers that the main reaction to occur was a nucleophilic attack on genipin by primary amine groups that led to formation of a heterocyclic compound of genipin link to the glucosamine residue in OCS and the amino acids in BLG.

Additionally, many synthetic crosslinkers have been reported a high risk of potential cytotoxic effects. Liang et al. claimed that the LD50 of genipin in mice is 382 mg/kg, which is almost 5000 - 10000 times less cytotoxicity than the commonly used crosslinkers such as glutaraldehyde. Genipin is also responsible for the pharmacological properties of gardenia including anti-carcinogenic, anti-inflammatory, neuroprotective, etc. In the meantime, it can be biotransformed through demethylation, ring-opening, cysteine-conjugation and glucuronadation. Based on all these advantages of genipin as crosslinker, we used it in our study to crosslink BLG and OCS in the process of nanoparticles preparation.
7.3 Materials and Methods

7.3.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B64. It was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments. Oligochitosan was purchased from Kunpoong Bio. Co., Ltd. (South Korea, Batch No KPH 1007GB) without further treatment. Neobee 1053 MCT was provided by Stephan Company (IL, USA). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (VA, USA) with purity > 98%.

7.3.2 Methods

7.3.2.1 Preparation of genipin crosslinked nanoparticles (GNPs) and genipin crosslinked BLG/OCS NPs stabilized pickering emulsion

OCS and BLG were dissolved in DI water containing 10 mM NaCl, respectively. The pH of both solutions was adjusted to 7.4 by using 0.1M HCl and 0.5M NaOH. Mixing genipin solution with OCS solution for 5 min by addition of BLG solution dropwise during the magnetic stirring to generate genipin coslinked BLG/OCS particles (GNPs). In order to prepare GNPs encapsulated NAR, first mixing protein solutions with NAR, then adding into
genipin crosslinked OCS solution under magnetic stirring to form self-assembled particles. The reaction was carried under room temperature.

For the samples of genipin crosslinked PE, they were generated by adding MCT into the GNPs solution with a total volume 8 ml. Different oil fraction (0.3, 0.5, 0.7) were applied with various protein concentrations (0.1, 0.25 and 0.5%). Then mixing them together under high-speed homogenizer (model IKA-ULTRATURRAX T25 digital, IKA 190 Works, Inc., Wilmington, NC, USA) with a 10 mm dispersion probe under the condition of 10,000 rpm for 2 min, stored at room temperature.

7.3.2.2 The characterization of GNPs

A 90Plus Particle Size Analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instruments Corporation, NY, USA) was used to measure the particle size of GNPs. All the measurements were conducted at 90° scattering angle. Each sample was measured in triplicate at room temperature.

The morphologies of genipin crosslinked BLG/OCS nanoparticles were measured by a Nanoscope IIIa Multi-Mode Atomic Force Microscope (AFM, Veeco Instruments Inc., CA, USA) with the tapping mode. The operation was that fresh made GNPs solutions with different crosslinking time were dripped one droplet (estimated volume = 0.5 mL) onto the surface of pre-cleaned mica slides and rested for 1h. The surface was rinsed with Milli-Q
water and then using nitrogen gas with moderate flow rate blow the mica slides carefully until fully dry for measurement.

The contact angle was measured by using VCA-Optima XE Dynamic Contact Angle Analyzer (AST Products, Inc., MA, USA). The BLG/OCS nanocomplexes (c(BLG) = 2.5 mg/ml, BLG:OCS = 1:5 wt/wt) solution were dripped onto the pre-cleaned microscope glass slide and dried until forming a film before measuring. Then using a CCD camera to capture images immediately after a drop of mili-Q water (2 ul) was deposited onto the surface of nanocomplexes film. The contact angle was determined by the associated software with the analyzer. The results were recorded on average and repeated ten times at least.

7.3.2.3 The characterization of genipin crosslinked PE

The optical microscopic images (PE samples with different oil fractions and different BLG concentrations) were monitored by using a Nikon TE-2000-U inverted fluorescence microscope equipped with a CCD camera (Retiga Exi, Q-imaging). The method was that one droplet of diluted sample was pipetted and deposited onto the surface of microscope slide gently before observation.

The viscoelastic properties of PE were measured on a Discovery HR-2 Hybrid Rheometer (TA Instruments, DE, USA) with 25 mm stainless-steel parallel plate geometry. Samples with different oil fractions and different BLG concentrations were applied to both steady shear rate experiments and dynamic frequency sweep model. Dynamic strain sweep model was tested
before to determine the linear viscoelastic region and the results confirmed 1.5% strain values. The conditions for dynamic frequency sweep test were 0.1-100 rad/s, and the curves of storage modulus $G'$ and loss modulus $G''$ were recorded. The conditions for steady shear experiments were that the shear rate was increased from 0.1 to 100 s$^{-1}$, and the viscosities ($\eta$) were recorded. All of the measurements were carried out with 20 data points per decade at room temperature.

7.4 Results and Discussions

7.4.1 The particle size of genipin crosslinked BLG/OCS nanoparticles

Figure 7-2. Photos of genipin crosslinked BLG/OCS nanoparticles with different genipin concentration (0, 0.1, 0.25, 0.375 and 0.5 ml/ml respectively) when the crosslinking time was 24h.

Figure 7-2 showed that after crosslinking 24h, the photos of GNPs solutions. With the more genipin involved in the solution, the deeper color they occurred. When the genipin
concentration was over 0.5 mg/ml, the flocculent precipitate would happen at the bottom of vials which led to unstable system, so we didn’t show the unstable samples.

Figure 7-3. Particle sizes with different genipin concentration (0, 0.1, 0.25, 0.375 and 0.5 ml/ml respectively) and different crosslinking time (0, 2, 4, 6, 8, 10, 16 and 24 hours).

Figure 7-3 showed that the effect of particle sizes changes on different genipin concentrations and crosslinking time. We can see that all the samples had the same trend, except the control group, the particle size were first gradually decreased until the crosslinking time up to about 10 h, and then increased again. When the reaction time reached 24 h, the particle sizes were slightly larger than those of initial status. The increase of particle size was due to dimerization, oligomerization and esterization between the heterocyclic structures of GNPs. These structures contribute to the blue pigments during the reaction between genipin with methylamine. As for the curves with different genipin concentrations, the more added,
the smaller particles they would be. When the genipin concentration reached 0.5 mg/ml, the particle size can be as low as 250 nm with the crosslinking time was at 10 hours.

### 7.4.2 Morphologies of genipin crosslinked BLG/OCS particles

![AFM images of genipin crosslinked BLG/OCS nanoparticles](image)

Figure 7-4. AFM images (upper: height images, lower: 3D images) of genipin crosslinked BLG/OCS nanoparticles (BLG:OCS = 1:5, wt/wt, c(genipin) = 0.5 mg/ml) with crosslinking time of 4h, 10h and 24h respectively.

Figure 7-4 showed that the AFM tapping mode height image and 3D image confirmed that the genipin crosslinked particles were sphere liked. The shapes were not change too much when crosslinking time were 4 and 10 hours, they were sphere-liked and evenly dispersed. For 24h, GNPs have a trend of holding together and the size got larger which were in consistent with the result of DLS. With more crosslinking time, the particles would get together, resulting a larger particle size.
7.4.3 The contact angle of genipin crosslinked BLG/OCS nanoparticles with different
crosslinking time

In order to form a stable pickering emulsion, we should know the surface property which is
critical parameter in determining the stability of pickering emulsion. According to the
detachment energy we have discussed in the introduction, we know that the stabilization is
strongly related to the contact angle of the interface.
Figure 7-5. The contact angles of BLG/OCS genipin crosslinked particles (BLG:OCS = 1:5, wt/wt, c(genipin) = 0.5 mg/ml) under 0, 4, 8, 16 and 24h reaction time.
Figure 7 - 5 showed the contact angles of GNPs at particle-air-water interface with different crosslinking time. The results were summarized and we can see that the contact angles were all the way decrease but didn’t change too much at the first 16 hours which ranged from 40° to 35°. From 16 to 24 hours, it became smaller as low as 25°, indicating that GNPs were more hydrophilicity after 16 hours reaction.

7.4.4 Microstructure of GNPs based PE with different protein concentrations, crosslinking time and oil fraction
Figure 7-6. Optical microscopic images of GNP-based PE with different BLG concentrations (0.1, 0.25 and 0.5%) under different crosslinking time, the oil fraction fixed at 50%, scale bar = 200 μm.
From Figure 7 - 6, We can see that the emulsion droplet sizes decreased as the protein concentration increased from 0.1% to 0.5%. This is because higher density of particles would anchor more particles onto the interface, thus contributed to higher interfacial area during the coalescence process. Additionally, the droplet sizes increased with the extending crosslinking time.
**Figure 7-7.** Optical microscopic images of GNPs based PE with different oil fraction (0.3, 0.5 and 0.7) under different crosslinking time, the protein concentration fixed at 0.25%, scale bar = 200 um.
From Figure 7-7, we can see that the droplets sizes increased gradually with the increase of oil phase fraction, this is due to fewer amount of GNPs were available to cover the oil-water interface, thus only large droplets were formed. Also, the droplet sizes increased with the extending crosslinking time.

7.4.5 Emulsion sizes of GNPs based PE under different pH values

Figure 7-8. The droplet sizes of GNPs based PE under different pH values (2, 4.5, 6, 7.4), BLG/OCS = 1:5, wt/wt, the crosslinking time = 8h.

As I have mentioned in the last chapter, the NPs stabilized PE were not stable with the pH change, especially pH 2. Thus, the droplet sizes of both NPs based PE and GNPs based PE
were measured and analyzed by ImageJ software. We can see that the size of GNPs PE changed from 140 um to 170 um, they did not change too much when the pH went from 2 to 7.4 by compared with the uncrosslinked samples. Therefore, after crosslinked with genipin, the nanoparticles based PE has greatly enhanced the pH stability.

7.4.6 The rheological properties of GNPs based PE

![Graph](image)

**Figure 7-9.** Rheological properties of GNPs based PE (c(BLG) = 0.25% w/w, BLG:OCS = 1:5, c(genipin) = 0.5 mg/ml) with varies crosslinking time (4, 8, 16 and 24h). (a) Oscillating frequency sweep curves at fixed strain (1.25%) with frequency ranging from 0.1 to 100 rad/s. (b) Viscosity vs. shear rate graph.
(b) viscosity versus shear rate from 0.5 to 100 s$^{-1}$.

Figure 7 - 9 showed that all the samples showed gel like structure as the storage modulus larger than loss modulus. The gel like behavior could be attributed to the formation of three dimensional particle network. Moreover, the longer crosslinking time samples had lower $G'$ and $G''$, indicating weaker gel-like emulsion. Increasing the angular frequency will break down the network, reflected by decreasing the complex viscosity.

7.5 Conclusions

In this part, genipin was used to crosslink between BLG and OCS molecules to form nanoparticles. We optimized the genipin concentration and crosslinking time. The result showed that the more genipin added, the smaller size can be reached before crosslinking for up to 10 hours, after that the size of particles would get larger. The morphologies of particles were sphere-like. The contact angle of the particles between water and air ranging from 40° to 25° with different reaction time. All the NPs in the protein range of 0.1 – 0.5% could stabilize the PE with different oil fraction. Especially, they have much better pH stability than NPs without genipin crosslink, pH 2 particularly. The genipin crosslinked BLG/OCS NPs stabilized PE also showed gel-like behavior and became more stiffness with the pH values went up. The oil fraction has a higher contribution to the gel structure than that of protein concentrations.
8 CHAPTER VIII. Improving oral bioaccessibility of naringenin using genipin crosslinked pickering emulsion: in vitro studies

8.1 Abstract

According to the previous chapter, we have designed the genipin crosslinked BLG/OCS nanoparticles based pickering emulsion and we tested its pH stability from pH 2 to 7, concluded that it could maintain its own structure even under low pH value. In this study, we use two in vitro gastrointestinal digestion methods – pH-stat lipolysis and dynamic TNO’s gastrointestinal (Tim-1) model. We compared the results derived from these two methods and found some same and differences. The results showed that the bioaccessibility of NAR of the formulated samples were almost three times higher than that of pure NAR suspension in both methods. For the genipin crosslinked samples, they were 49% determined by pH-stat lipolysis and 42% measured by Tim-1. As for NAR oil suspension, they were 14% determined by pH-stat lipolysis and 13% measured by Tim-1. Tim-1 has a relatively complicated design and a more comprehensive simulation, the measured bioaccessibilities would be a little decrease than that measured by lipolysis model. The result of Tim-1 model also revealed that jejunum is the major organ for the absorption of NAR, and there would be the largest amount of available NAR during the time of 120-180 min. The bioaccessibility data obtained from the in vitro method can provide a valuable guidance for the further in vivo study to investigate the final oral bioavailability of NAR.

8.2 Introduction
In the chapter one, we have described many bioefficacies of nutraceutical, especially for naringenin. In summary, naringenin possesses a significant antioxidant property and has an ability to prevent low density cholesterol (LDL) generation which can avoid cardiovascular diseases effectively. Additionally, it has a wide spectrum of biological activities, such as antibacterial, anti-inflammatory and anti-carcinogenic properties. Naringenin, as a member of hydroxylated flavonoid family, the hydrophobic nature resulting low aqueous solubility which may impact their bioavailability. Also, we have also analyzed the reasons for low bioavailability of nutraceuticals in the chapter one. It can be summarized as the complicated physiological environment of human body will destroy the structure of certain bioactive compounds which lead to insufficient concentration of nutraceuticals will get into the target site of action that cannot deliver the health promoting effects they possessed. Thus, the bioavailability means the amount of unaltered bioactive compounds reached the systematic circulation. Normally, the intravenous injection is defined as 100%, while the oral administration method presents a much lower bioavailability due to incomplete structure or absorption.

After consuming food through the oral administration route, the food will go through stomach, some parts of them will destroy by the low pH and enzymatic degradation. Then reaching GI tract for the intestinal absorption, and there are still parts of them will be damaged because of pH change, ionic interaction, enzymatic degradation or rapid GI elimination rate. Even though some can be absorbed into through intestinal enterocytes, they are subjected to the structural transformation due to first pass effect or liver metabolism.
Taking into accounts of all the situations discussed above, the oral availability can be defined as the following equation\textsuperscript{20, 153}:

\[
Foral = FB \times FT \times FM
\]

$FB$ means the faction of ingested nutraceuticals which can be accessible for intestinal absorption. $FT$ means the fraction of unaltered compound which has been transported through the intestinal portal. $FM$ describes the fraction which has not been metabolized by various organs, such as liver, lung and heart\textsuperscript{152}. Thus, the bioavailability of nutraceuticals strongly depends on the survival of pre-absorption degradation ($FB$) and post-absorption metabolism ($FT$ & $FM$). Using an appropriate delivery vehicle can greatly protect the intact structure of bioactive compounds from pH instability, ionic strength change and enzymatic degradation in the microenvironment of stomach or small intestinal. So in this study, we will focus on the pre-absorption part, that is, bioaccessibility, which means the amount of nutraceuticals reaching the intestinal absorption site without structure destroy.

The tests of bioaccessibility can be divided by in vivo model and in vitro model. In vivo model will involve animal or human subjects which costs lots of time or money, also lead to the problems of ethical and practical issues\textsuperscript{154}. Thus, in this study, we will use in vitro digestion model to investigate the bioaccessibilities of the naringenin suspension, naringenin encapsulated by BLG/OCS pickering emulsion and genipin crosslinked pickering emulsion respectively to verify it can be improved by the optimized delivery system.
The pH-stat lipolysis model has been widely used in the lipid digestion process of small intestinal which is suitable for the pickering emulsion digestion due to containing a high fraction of oil phase. It simulates the environment of small intestinal, including bile salts, lipases, trypsin and mineral ions, and it also controls the temperature which fixed at 37°C. This method is based on measuring the amount of free fatty acids (FFAs) in the digestion of lipid which mainly digested by lipase. That is, triglycerides (TAGs) which is the main component of lipid molecule can be digested by lipase to produce one monoacylglycerol and two FFAs as a result. In order to keep the pH value of the whole system at neutral pH, NaOH will be titrated into the mixture and its volume versus time will be recorded. When the lipolysis process has finished, after ultracentrifuging, the amount of hydrophobic compounds maintained in the middle micelle layer will be tested and regarded it as the amount which can be available for the intestinal absorption. pH-stat lipolysis model is a suitable measure method to evaluate the bioaccessibility of hydrophobic compounds encapsulated in the oil based emulsion\textsuperscript{155-156}. However, it is a sort of static model which cannot simulate the GI emptying and motility, it also cannot replicate the secretion and elimination processes\textsuperscript{152}. So the bioaccessibility predicted by pH-stat in vitro model can be usually overestimated\textsuperscript{152}.

While the TNO Gastro-Intestinal model (TIM-1) is a dynamic multi-compartment model which can simulate GI digestion controlled by computer\textsuperscript{157}. Here is the schematic presentation of TIM-1\textsuperscript{154}. 
As we can see from Figure 8-1, TIM-1 is composed of mainly four compartments, they are gastric, duodenal, jejunal and ileal respectively. There is a glass capsule with a flexible inner silicon jacket inside in each compartment. The water is heated to 37℃ and is pumped through the interlayer between glass and silicon to simulate the body temperature. In the working process, the silicon jacket will be compressed and release in cycles to imitate the gastrointestinal peristalsis. The secretion of pepsin, gastric juice, trypsin, bile, pancreatin, intestinal juice will be pumped to the GI compartments. Hydrochloric acid will be pumped to the gastric compartment and sodium bicarbonate will be pumped to the intestinal
compartment to regulate the pH values. All of the secretion processes are controlled by computer. Two hollow fiber filtrate systems are connected to the jejunal and ileal compartments which composed of semi-permeable membranes to simulate the passive absorption of small molecule compounds. Samples are collected at set intervals from jejunal and ileal parts, the accumulated amount of bioactive compounds from these two parts can be regarded as the bioaccessibility. The fluids pass through the ileocaecal valve would be transferred to colon in theoretical\cite{159}.

By compared with single factor model – pH-stat lipolysis, the TIM-1 model has taken more factors into account. Such as peristalsis movement, GI residence time, passive absorption and elimination. It would be better to use this model to simulate the digestion process of food. By compared with in vivo model, TIM-1 has exhibited some advantages, such as low cost, easy manipulation and it is available for us to collect samples at any stage of digestion and any time\cite{152}. Additionally, some studies had showed that the results of TIM-1 model had a good consistency with in vivo experiments\cite{157,160}.

In our previous studies, we have successfully encapsulated naringenin by using genipin crosslinked BLG/OCS nanoparticles based pickering emulsion. It exhibited a good pH stability which enable it as a good candidate for the oral delivery vehicle of hydrophobic compounds. The purpose of research in this chapter is to measure the bioaccessibility of naringenin to verify whether this encapsulation system can protect the bioactive compounds from degradation. In order to reach this purpose, we will use both pH-stat lipolysis model and
TIM-1 model to simulate the digestion process to test the bioaccessibilities of naringenin in the use of suspension, pickering emulsion and genipin crosslinked pickering emulsion.

8.3 Materials and Methods

8.3.1 Materials

The raw beta-lactoglobulin sample powder (lot JE003-3-922) was purchased from Davisco Foods International, Inc. which composed of 5.2% moisture, 92.0% protein, 0.3% fat and 2.5% ash in every 100 g of powder with almost equal amount of genetic variants A and B64. It was further purified through centrifugation at 10,000 g for 5 min on 5 wt% BLG solution with the pH equal to 5.2. Then the supernatant was lyophilized and used in all following experiments. Oligochitosan was purchased from Kunpoong Bio. Co., Ltd. (South Korea, Batch No KPH 1007GB) without further treatment. Neobee 1053 MCT was provided by Stephan Company (IL, USA). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (VA, USA) with purity > 98%. Naringenin was purchased from Shanxi Huike Botanical Development Co., LTD (Xi’an, P.R. China). It was analytical grade and used it directly. Polyethylene glycol with average molecular weight 200 (PEG - 200) was purchased from Sigma-Aldrich (CAS Number 25322-68-3). Pepsin A from porcine stomach mucosa (2500-3500 units/mg, P-7012), pancreatin with 8× USP specification, bile salt, sodium chloride, calcium chloride, potassium dihydrogen phosphate, tris maleate, and sodium taurodeoxycholate (NaTDC), and trypsin from bovine pancreas (7500 N-α-benzoyl-L-arginine ethyl ester (BAEE) units per mg, T9201) were purchased from Sigma-Aldrich Co.
Fresh pig bile was purchased from local NJ farm. Rhizopus lipase (150,000 units/mg F-AP-15) was from Amano Enzyme Inc. (Nagoya, Japan).

8.3.2 Methods

8.3.2.1 Preparation of naringenin bulk suspension

Naringenin was fully dissolved in PEG-200 first and then adding the same volume of water to dilute it. Then adding MCT to make the water phase / oil phase = 1:1 (v/v) with the naringenin concentration fixed at 5mg/ml. Using an IKA Ultra-Turrax T25 homogenizer under 12 000 rpm for 2 min, stored at room temperature.

8.3.2.2 Preparation of naringenin encapsulated with BLG/OCS nanoparticles pickering emulsion

OCS and BLG were dissolved in DI water containing 10mM NaCl, respectively. The pH of both solutions was adjusted to 7.4 by using 0.1M HCl and 0.5M NaOH. Naringenin was fully dissolved in PEG-200 first and then adding the same volume of protein solution to dilute it, following adding OCS solutions to make naringenin contained BLG/OCS nanoparticles. The pickering emulsion samples were generated by adding MCT into the nanoparticles solution. The oil fraction was fixed at 0.5 with protein concentration fixed at 0.25%, BLG/OCS = 1:5 (w/w) and naringenin concentration fixed at 5mg/ml. Then mixing them together under high-speed homogenizer (model IKA-ULTRATURRAX T25 digital, IKA 190 Works, Inc., Wilmington, NC, USA) with a 10 mm dispersion probe under the condition of 12,000 rpm for 2 min, stored at room temperature.
8.3.2.3 Preparation of naringenin loaded BLG/OCS nanoparticle based genipin
crosslinked pickering emulsion

OCS and BLG were dissolved in DI water containing 10 mM NaCl, respectively. The pH of
both solutions was adjusted to 7.4 by using 0.1M HCl and 0.5M NaOH. Mixing genipin
solution with OCS solution for 5 min by addition of BLG solution dropwise during the
magnetic stirring to generate genipin cosslinked BLG/OCS particles. In order to prepare
GNPs encapsulated NAR, first mixing protein solutions with NAR, then adding into genipin
crosslinked OCS solution under magnetic stirring to from self-assembled particles. For the
samples of genipin crosslinked PE, they were generated by adding MCT into the GNPs
solution. The oil fraction was fixed at 0.5 with protein concentration fixed at 0.25%,
BLG/OCS = 1:5 (w/w) and naringenin concentration fixed at 5mg/ml. Then mixing them
together under high-speed homogenizer (model IKA-ULTRATURRAX T25 digital, IKA 190
Works, Inc., Wilmington, NC, USA) with a 10 mm dispersion probe under the condition of
12,000 rpm for 2 min, stored at room temperature.

8.3.2.4 Determination of bioaccessibility by using in vitro pH-stat lipolysis model

The principle of using pH-stat lipolysis model to determine the bioaccessibility of
nutraceuticals has been described in the introduction. According to the previous in vitro
lipolysis experiment made by Yu et al\textsuperscript{161}, the formulation of fed state buffer is showed as
below:
**Table 8-1.** Formulation of fed state buffer in 1000 mL

<table>
<thead>
<tr>
<th>Components of fed state buffer</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris maleate</td>
<td>11.8600</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.7664</td>
</tr>
<tr>
<td>CaCl2·2H2O</td>
<td>0.7351</td>
</tr>
<tr>
<td>NaTDC</td>
<td>10.4336</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>3.8004</td>
</tr>
</tbody>
</table>

For the pancreatin enzyme solution, adding 1g pancreatin powder to 5 mL fed state buffer, then stirring in moderate for 15 min, and then using centrifuge with a speed of 2,000 rpm to get rid of the sediments, the supernatant was collected and kept on ice until use. 0.25g sample was mixing with 9 mL fed state buffer in the glass vial, then putting it into an oil bath and the temperature was fixed at 37°C. The reaction solution was stirred at a speed of 200 rpm and using a pH meter to monitor pH value, adjusting the value to 7.4 by titrating the 0.25M NaOH solution. The reaction time was set at 2h to simulate the digestion process of small intestinal. The volume of NaOH was recorded which was supposed equal to the amount of FFAs released through the whole digestion process. Digestion of the bulk oil, naringenin loaded pickering emulsion and genipin crosslinked pickering emulsion samples were conducted as the same procedure. The extent of lipolysis was calculated by using the following equation:

\[
\text{Extent of lipolysis} = \frac{(V_{\text{NaOH}} - V_{\text{blank}}) \times c_{\text{NaOH}} \times M_w(MCT) \times 100}{2m_{\text{MCT}}}
\]
In which mMCT is the mass of lipid used in the samples. Mw(MCT) is the molecular weight of the lipid in unit of g/mol. VNaOH is the volume we recorded during the experiment. cNaOH is the concentration used to titrate and in this experiment, is 0.25M. Vblank is the NaOH volume titrated into the blank sample. In order to use this equation correctly, there is an assumption that one molecule triacylglycerol can be converted to two molecules of FFAs during titration so that the amount of NaOH added equals to the amount of FFAs released.

To determine the bioaccessibility of naringenin, the media after digestion completed was untracentrifuged for 40 min at 4°C with a speed of 4,000 rpm immediately. There will be three phases of the vial, the upper is the oil phase, the middle one is the micelle phase contained with naringenin is what we want and the lower phase is water with sediments. The concentration of naringenin in the micelle phase was measured using HPLC. The bioaccessibility tested by in vitro lipolysis model was calculated by the following equation:

\[
\text{Bioaccessibility (\%) } = \frac{\text{amount of naringenin in micelle \times 100\%}}{\text{amount of naringenin total added in the formulation}}
\]

8.3.2.5 Determination of bioaccessibility by using in vitro TIM-1 model

The flowchart of using TIM-1 model to determine the bioaccessibility of nutraceuticals has been described in the Table 8 - 2. The formulation for fed state prepared for TIM-1 digestion process is as below:
<table>
<thead>
<tr>
<th>components</th>
<th>Total amount (g)</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
<th>Component 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatin solution</td>
<td>267.5</td>
<td>Pancreatin</td>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>powder</td>
<td>250 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.5 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric electrolyte solution (GES)</td>
<td>500</td>
<td>Sodium</td>
<td>Sodium</td>
<td>Potassium</td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chloride</td>
<td>chloride</td>
<td>chloride</td>
<td>chloride di-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.2 g/L</td>
<td>2.2 g/L</td>
<td>hydrate</td>
<td>hydrate 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>g/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>g/L</td>
</tr>
<tr>
<td>Gastric enzyme solution</td>
<td>151.5</td>
<td>GES 150 g</td>
<td>Sodium</td>
<td>Lipase</td>
<td>Pepsin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>acetate</td>
<td>37.5 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>buffer 1M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric water</td>
<td>150</td>
<td>Water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>150 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestinal electrolyte</td>
<td>2000</td>
<td>Sodium</td>
<td>Potassium</td>
<td>Calcium</td>
<td>Water</td>
</tr>
<tr>
<td>solution (SIES)</td>
<td></td>
<td>chloride</td>
<td>chloride 0.6</td>
<td>chloride di-</td>
<td>2000 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 g/L</td>
<td>g/L</td>
<td>hydrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3 g/L</td>
<td></td>
</tr>
<tr>
<td>Jejunal secretion</td>
<td>1500</td>
<td>SIES 25x</td>
<td>Water</td>
<td>Bile</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 g</td>
<td>1300 g</td>
<td></td>
<td>150 g</td>
</tr>
<tr>
<td>Ileum secretion</td>
<td>1500</td>
<td>SIES</td>
<td>1500 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum electrolyte secretion</td>
<td>450</td>
<td>SIES</td>
<td>450 g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The pH values of the four compartments – gastric, duodenum, jejunum and ileum, and the secretion rates were controlled by computer automatically. The samples which can be regarded as the ‘meal’ (300 g) which is composed of 20 g samples (naringenin contain bulk oil, naringenin pickering emulsion and naringenin loaded genipin crosslinked pickering emulsion), 95 g GES, 5 g gastric enzyme solution and 180 g water, were poured into the stomach compartment through the food inlet and tested for 6 hours to simulate the digestion process of human body. To determine the bioaccessibility of naringenin, dialysates were collected at the interval of 30, 60, 90, 120, 150, 180, 210, 240, 300, 330 and 360 min from jejunum and ileum respectively. And the volume from these two compartments were measured carefully then recorded. In the meanwhile, the effluxes were also collected from the outlet of ileal compartment. The collected samples were stored on the ice until they were analyzed by HPLC. We can calculate the bioaccessibility of naringenin from jejunum and ileum respectively. Based on this result, we can infer which compartment is the main naringenin absorption site. While the total bioaccessibility of naringenin was calculated through the accumulated amount of it in both jejunum and ileum compartments.
8.4 Results and Discussions

8.4.1 Bioaccessibility of naringenin using the pH-stat lipolysis model

For hydrophobic nutraceuticals, the low bioavailability is strongly related to the low aqueous solubility and stability in the GI tract. By using a certain encapsulation system can protect them from degrading in the microenvironment of human body. In particular, the pickering emulsion contains a high fraction of oil phase which can greatly improve the solubility of naringenin, and the genipin crosslinked vehicle can maintain the structure stability even though in low pH which had been validated in the previous study. The whole process can be summarized as the lipid and the outer protein shell are hydrolyzed by lipase, pepsin, pancreatin and trypsin. Then mixing with bile salts and phospholipids to form micelle, and the naringenin are released to participate into the lipid formulation, some of them will be incorporated into the micelle core which will be transported to the absorption site of the epithelial cells. The digestion profiles of naringenin contained bulk oil, naringenin loaded pickering emulsion and naringenin loaded genipin crosslinked pickering emulsion were monitored using the pH-stat lipolysis model, the extent of lipolysis and the bioaccessibility tested were showed in Figure 8 - 2 and Figure 8 - 3.
From Figure 8-2, we can see that during the 2h digestion process, it was about 20% of the MCT lipids in the bulk oil were hydrolyzed from triglycerides to free fatty acids, while naringenin contained pickering emulsion digested lipids about 35%, what’s more, there was about 40% MCT of genipin crosslinked samples can be digested and it was two times than that of bulk oil sample. In addition, the release rate of free fatty acids at initial was fastest for genipin crosslinked sample, followed by pickering emulsion sample was faster by compared with bulk oil sample. This increased digestion rate could be attributed to BLG/OCS particles which led to larger oil-water interface area of emulsion droplets by compared with oil only sample. The same phenomenon has also been observed in other literatures\textsuperscript{162}. 

\textbf{Figure 8-2.} The extent of lipolysis using pH-stat lipolysis model of bulk oil, pickering emulsion and genipin crosslinked pickering emulsion samples.
According to Figure 8-3, the change of release kinetics profile of these samples led to the increase of bioaccessibility of naringenin from 14% (bulk oil) to 49% when encapsulated by genipin crosslinked pickering emulsion. The bioaccessibility of BLG/OCS nanoparticle based pickering emulsion was between these two samples which was 42%. In the pickering emulsion, BLG/OCS nanoparticles were located between the water and oil phase, these particles can keep their own structure under pH 7.4 which can be slowly digested by enzyme, thus protecting naringenin from degradation. The genipin crosslinked samples didn’t increase bioaccessibility too much, because the main function of crosslinking is to enhance the stability under low pH value, while this lipolysis process just simulated the condition of small intestine.
8.4.2 Bioaccessibility of naringenin using the TIM-1 model

The bioaccessibilities of naringenin for both pure NAR suspension and genipin crosslinked BLG/OCS nanoparticles based pickering emulsion were studied by using Tim-1 system. After the analysis of HPLC, the percentages of input of naringenin came from jejunum dialysate, ileum dialysate were calculated respectively and were shown in Figure 8 - 4.
Figure 8-4. Bioaccessible NAR (% of input) was measured each 30 min at the first 2 hours, and then was measured each 60 min from 2 to 6 hours for both pure NAR suspension and genipin crosslinked PE samples from A. jejunum dialysate; B. ileum dialysate; C. total bioaccessible NAR in both jejunum dialysate and ileum dialysate.

According to Figure 8-4A, for the pure sample, the bioaccessible amount of NAR increased gradually during the first 2 hours, the concentration reached to a peak value until the time was between 90-120 min. Then the concentration of NAR started to decrease all the time. While for the genipin crosslinked PE samples, the concentration for NAR increased abruptly when the time was 30-60 min, then it reached a maximum with a plateau when the time was 2 to 3 hours which indicated that this formulation can prolong the release time. This because the genipin crosslinked BLG/OCS nanoparticles can sustain the structure of the system even under the change of pH from 2 to 7.4. Also the protein polysaccharide complexes can protect the bioactive compound from degradation by environment. BLG has a relatively rigid
structure which can be hydrolyzed by various enzymes slowly. After 3 hours, the content of bioaccessible NAR decreased until the end of digestion. As for the available NAR in ileum shown in Figure 8 - 4B, it has a similar trend with that of jejunum while the bioaccessible amount was much lower than that in jejunum dialysate which indicated that the jejunum was the major organ for NAR absorption. Besides, Figure 8 - 4C showed the overall bioaccessibility was defined as the summation of the available content of NAR in both jejunum and ileum. We can see clearly that the genipin crosslinked PE samples has higher available amount of NAR in every time intervals. The formulated samples had greatly enhanced the bioaccessibility of NAR by compared with pure NAR suspension samples.
Figure 8. Cumulative bioaccessibility profile of NAR (% of input) accumulated in every 30 min at the very first 2 hours and every 60 min during the rest 4 hours from A. Jejunum dialysate B. Ileum dialysate C. both jejunum part and ileum part for the samples of pure NAR suspension and genipin crosslinked PE.

Besides, the cumulative bioaccessibility of pure NAR suspension and genipin crosslinked PE samples were showed in Figure 8-5. At last, the bioaccessibility of NAR derived from the formulated samples was more than three times than that of pure NAR samples after 6 hours’ digestion of Tim-1, it was 42% vs 13%. Same trend was observed in both jejunum and ileum dialysate. And jejunum is the major absorption organ because it accumulated the most of NAR. Therefore, we can conclude that the genipin crosslinked BLG/OCS nanoparticles based pickering emulsion has increased the bioaccessibility of NAR greatly through Tim-1 model.

8.5 Conclusions
In both static and dynamic simulated in vitro gastrointestinal digestion systems, the formulated samples exhibited higher bioaccessibility of NAR than that of oil suspension samples. For the genipin crosslinked samples, they were 49% determined by pH-stat lipolysis and 42% measured by Tim-1. As for NAR oil suspension, they were 14% determined by pH-stat lipolysis and 13% measured by Tim-1. The bioaccessibility of NAR has enhanced greatly by using the genipin crosslinked formulation. The differences of bioaccessibilities between these two methods could be attributed to that Tim-1 has a more complicated, more accurate and longer time simulation, it also took the condition of stomach into consideration. Therefore, the measured bioaccessibilities would be a little decrease than that measured by lipolysis model. According to the result of Tim-1 model, we know that jejunum is the major organ for the absorption of NAR, and there would be the largest amount of available NAR during the time of 120-180 min.
SUMMARY AND FUTURE WORK

In this work, a novel protein/polysaccharide delivery vehicle – BLG/OCS complexes had been assembled. Through optimizing the pH and ratio, BLG/OCS complexes could form stable nanoparticles (size ~ 200 nm) at pH 7.4 which is close to the physiological environment of human body, such property provides a foundation as a delivery system that the BLG/OCS NPs may maintain their structure in the intestinal tract after oral administration. A typical hydrophobic flavonoid – NAR was introduced to be encapsulated by BLG/OCS NPs. utilizing the bioactivities of this kind of flavonoid is still hard due to the oral bioavailability of it is very low, mainly as a result of low water solubility. Encapsulation provides a potential route to solve this problem. NAR could be successfully encapsulated by BLG/OCS NPs with high encapsulation efficiency (> 90%) which can protect it from the change of outer environment and also enhance the solubility.

But the driven force between protein and polysaccharide is the electrostatic interaction so that the NPs cannot endure the acidic environment such as the stomach. Genipin had been applied as a natural crosslinker to crosslink BLG and OCS, the pH stability experiments showed that after optimization the time and concentration of genipin crosslinked, the system possessed a good stability under pH 2. The loading capacity of NAR by using NPs was not enough to exhibit bioefficacy, therefore the pickering emulsion stabilized by genipin crosslinked BLG/OCS NPs was introduced to enhance the loading capacity and stability due to it had a high oil fraction. Using two in vitro method – pH lipolysis and TIM-1 to test whether the
bioaccessibility had been improved or not. The results showed that genipin crosslinked samples had enhanced 3.5 folds by compared with NAR oil suspension.

The most significant step for the encapsulation is the interaction between BLG and NAR and we have done lots of chemical experiments. The computer simulation is also an important method to explore the unknown area. Protein and polysaccharide are biomacromolecules which have complicated structure. The theoretical approach cannot handle such sophisticated situations so that it cannot cover all the possibilities to provide an accurate prediction or description. The Replica Exchange Monte Carlos was applied to investigate the effect of different ratios on the interaction between BLG and NAR. After simulation, the information about change of the complexes’ structure during binding process can be obtained, and the snapshots of binding can also be available.

Through this study, the bioaccessibility of NAR had been greatly enhanced by the encapsulation of genipin crosslinked BLG/OCS NPs based pickering emulsion due to increasing the solubility and permeability of NAR. The in vitro bioaccessibility cannot determine the bioavailability very well due to many complicated bioactive compound associated with absorption, uptake, transport, distribution and metabolism. In the future, the pharmakinetics of NAR after oral administration of the developed formulation still need to be investigated by using in vivo animal model.
To sum up, my Ph. D study provides a potential delivery system to solve the low bioaccessibility of nutraceuticals via oral route. This strategy can also be applied in utilizing other kinds of hydrophobic nutraceuticals. This study also provides a computer based approach – REMC which can exhibit many detailed information and dynamic visualization about the binding behaviors of bio-macromolecules and small bioactive molecules. The significant of this research is that connecting the macroscale phase to the perspective of atomic level.
REFERENCES


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### APPENDIX: LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>FULL NAME</th>
</tr>
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<tbody>
<tr>
<td>BLG</td>
<td>beta-lactoglobulin</td>
</tr>
<tr>
<td>OCS</td>
<td>oligochitosan</td>
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<tr>
<td>NAR</td>
<td>naringenin (5,7,4’-trihydroxyflavonone)</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscope</td>
</tr>
<tr>
<td>FQ</td>
<td>fluorescence quenching</td>
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<tr>
<td>MD</td>
<td>molecular docking</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>PE</td>
<td>pickering emulsion</td>
</tr>
<tr>
<td>TIM-1</td>
<td>TNO Gastro-Intestinal Model</td>
</tr>
<tr>
<td>REMC</td>
<td>Replica Exchange Monte Carlo</td>
</tr>
<tr>
<td>CG</td>
<td>Coarse Grained</td>
</tr>
<tr>
<td>PI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PDI</td>
<td>polydispersity Index</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>EE</td>
<td>Encapsulation efficiency</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>DAPI</td>
<td>40,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>MCT</td>
<td>medium chain triglyceride</td>
</tr>
<tr>
<td>SI</td>
<td>stability index</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>GNP s</td>
<td>genipin coslinked BLG/OCS particles</td>
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<tr>
<td>LDL</td>
<td>low density cholesterol</td>
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<tr>
<td>FFAs</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>TAGs</td>
<td>triglycerides</td>
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<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
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<tr>
<td>GES</td>
<td>Gastric electrolyte solution</td>
</tr>
<tr>
<td>SIES</td>
<td>Small intestinal electrolyte solution</td>
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<td>REMC</td>
<td>Replica exchange Monte Carlo</td>
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<td>MCMC</td>
<td>Markov Chain Monte Carlo</td>
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<td>CG</td>
<td>Coarse grained</td>
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<tr>
<td>PDB</td>
<td>Protein database</td>
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<tr>
<td>VCCLAB</td>
<td>Virtual Computational Chemistry Laboratory</td>
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<tr>
<td>MCS</td>
<td>Monte Carlo Step</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small angle X-ray Scattering</td>
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