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Oral Delivery of Protein Nanocapsule for Diabetes Therapeutics

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Oral Delivery of Protein Nanocapsule for Diabetes Therapeutics

A thesis submitted in partial satisfaction

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in Chemical Engineering

by

Kang Peng

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ABSTRACT OF THE THESIS

Oral Delivery of Protein Nanocapsule

for Diabetes Therapeutics

by

Kang Peng

Master of Science in Chemical Engineering

University of California, Los Angeles, 2017

Professor Yunfeng Lu, Chair

Oral delivery of protein drugs like insulin can be a direct treatment for protein-deficiency diseases and relieve patients from traditional painful injections, but is limited by poor stability and low bioavailability of proteins throughout oral delivery route. In this thesis, a single-protein nanocapsule platform, based on polymerization of positive and mucoadhesive monomers, has been applied to encapsulate orally administrated insulin and its formation is confirmed by various characterization techniques. *In vitro* diffusion and *in vivo* distribution studies both exhibit great superiority of this nanocapsule platform in bioavailability and residence time over native protein, showing its potential application for the therapy of diabetes.

The thesis of Kang Peng is approved.

Harold G. Monbouquette

Tatiana Segura

Yunfeng Lu, Committee Chair

University of California, Los Angeles

2017

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1 Introduction and Background

1.1 Therapeutic Protein and Nanocarrier

Life on earth cannot exist without proteins. These macromolecules perform a huge series of functions in living creatures, such as construction of basic biological structures, cellular signal transduction and catalysis of abundant biochemical reactions, helping our bodies maintain regular physiological activities[1]; while malfunctioning of some specific proteins could be harmful to human bodies and lead to severe diseases. Compared with traditional gene therapy which is promising but lacks safety and long-term expression control,[2] it would be better to directly administrate normal exogenous proteins into bodies and replace those bad ones, featuring high efficiency and selectivity.[3] However, proteolytic degradation in gastrointestinal fluids and blood, short circulating time and high immunogenicity will interfere with or neutralize the medical effects of therapeutic proteins.[4-6]

To circumvent these limitations, nanocarriers such as liposomes, polymer-conjugated nanoparticles and nanocapsules were extensively developed to assist protein delivery with improved efficacy and reduced side effects.[7-10] Liposomes are microscopic vesicles made from natural or synthetic phospholipids, where proteins can be stabilized in the aqueous core or within the bilayers, but their rapid clearance by the reticuloendothelial system remains a problem when administrated intravenously.[11-12] Polymer-conjugated nanoparticles like enzymes attached by poly(ethylene glycol) (PEG) can also protect enzymes from unspecific interactions with other macromolecules and thus prolong the circulation time in human bodies, yet strong immune response induced by these conjugates has been increasingly reported.[13] Therefore, nanocapsule

platform, characteristic of a denser shell of dendritic polymers which encapsulates and shields the inner protein better than polymer-conjugated nanoparticles, has been widely explored as a promising nanocarrier for protein drug delivery.[7] Herein, this thesis will focus on the development of the nanocapsule platform and expand its application in the field of oral delivery of therapeutic protein drugs, especially based on the single-protein nanocapsule system previously constructed by Lu Group.[14]

1.2 Diabetes Mellitus and Insulin Therapeutics

Diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism.[15] It occurs either when the pancreas does not produce enough insulin (Type 1 diabetes) or when the body cannot effectively respond to the insulin it produces (Type 2 diabetes) , while the latter one comprises the majority (90%) of people with diabetes around the world.[15] The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs, such as retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers.[15] According to the report from World Health Organization (WHO), an estimated 422 million adults were living with diabetes in 2014, compared to 108 million in 1980.[16] That is to say, about one out of eleven people were struggling with this disease. In 2012, diabetes caused 1.5 million deaths all over the world.[16] It is becoming an enormous threat and burden to people's lives (**Figure 1**).

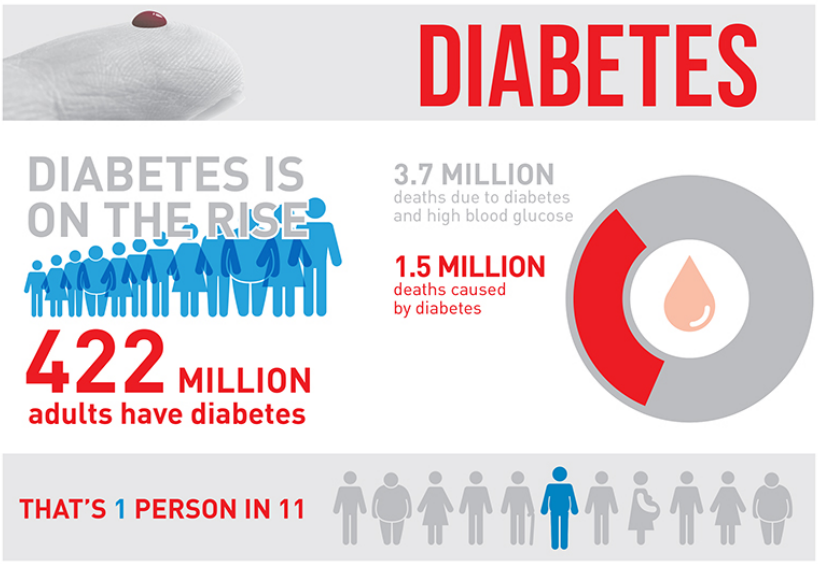


Figure 1. Global report on diabetes from WHO. Adopted from Ref. 16.

To resolve the issue, insulin therapy, which is necessary for both types of diabetes, has received a great deal of attention nowadays. As seen from the pathogeny of diabetes, insulin plays a critical role in the regulation of glucose metabolism and homeostasis.[17] It is a kind of tiny protein secreted by beta cells of pancreatic islets from the pancreas, consisting of 51 amino acids with a molecular weight of 5.8 kDa (**Figure 2**). It possesses a significant effect on the metabolism of carbohydrates through a feedback mechanism. When the concentration of blood glucose is at a high level (>200 mg/dL), this signal can be sensed by beta cells which at once accelerate the secretion of insulin, followed by binding of insulin with relevant receptors on the surfaces of fat, liver and muscle cells. Next the breakdown of glycogen in the liver will be inhibited and transport of glucose from blood into fat or muscle cells will be stimulated, resulting in a quick drop of blood glucose which could inversely impedes the release of insulin from

pancreatic islets.[18] Due to this accurate regulation, blood glucose level can be kept in a normal scale of 80-130 mg/dL.[19]

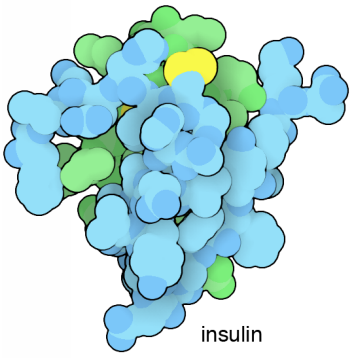


Figure 2. Structures of insulin and its storable hexameric complex (left), where green and blue parts represent A-chain and B-chain respectively, with disulfide linkages in yellow. Figure is adopted from PDB-101.

Diabetes may be treated by replacing the missing insulin in the blood so that the most direct and conventional way is to administrate exogenous insulin to human bodies, especially by subcutaneous injections which can mimic the insulin secretion of healthy pancreas and assist the patients to recover normal regulation activities.[20] Although the subcutaneous route has been the mainstay of insulin delivery until now, it may also cause some side effects such as peripheral hyperinsulinemia, stimulation of smooth muscle cell proliferation, or incorporation of glucose into lipids of arterial walls followed by diabetic micro- and macroangiopathy.[20-21] Moreover, pain and trauma from the burden of too many daily injections also lead to low patient compliance and safety issues.[22] Thus the development of strategies to achieve oral administration of therapeutic proteins has become a primary goal in biopharmaceutical field, due to significant advantages which the oral routes would have over other parenteral ones.[23] For example, orally delivered

insulin can simulate the physiological fate of insulin in normal bodies, providing better glucose homeostasis while causing less pain and inconvenience owing to its non-invasive characteristic.[20]

1.3 Oral Delivery Route and Barriers

Although oral administration of protein drugs stands out as a promising method for the treatment of diabetes, these macromolecules still need to overcome several common barriers during their delivery process. First of all, they will transit along the gastrointestinal (GI) tract and arrive at small intestine to get in touch with the intestinal villi (**Figure 3a**), where most nutrients, electrolytes and fluids are absorbed. Lots of proteins are sensitive to the pH variation in the GI tract, typically from highly acidic environment in the stomach (pH 1.2-3.0) to slightly basic condition in the intestine (pH 6.5-8.0), leading to conformation changes and the following loss of activities.[24-26] Moreover, most proteins are vulnerable to enzymatic degradation by the proteases in GI tract, like pepsin and trypsin. These proteases can break down the protein drugs into smaller peptides or amino acids, also leading to their inactivity.[26] Hence GI tract contains the first chemical barrier and the second enzymatic barrier.

Next the proteins will adhere and infiltrate through the viscous mucus layer on the surface of villi, which functions as the third diffusion barrier full of negatively charged mucin glycoproteins. This layer is a semipermeable layer secreted by the epithelial surface below (**Figure 3b**), allowing nutrients, water and small molecules to permeate through but prevents bacteria and other pathogens.[27-28] Thus, an orally administered drug must infiltrate through the unstirred mucus layer before approaching the layer of intestinal epithelium. Furthermore, the proteins have to traverse across intestinal epithelial cells through either the transcellular or

paracellular routes. Absorptive enterocytes, with a dense brush-border of organized microvilli at their apical surfaces, are the most abundant type of cells in this layer.[26] Permeabilities of protein drugs through enterocytes are much affected by the sizes, weights, lipophilicities and other surface properties of those molecules. For instance, absorption through transcellular pathway by passive diffusion is limited to lipophilic drugs with a molecular weight (MW) below 700 Da, which is much smaller than that of normal protein.[26] Therefore the layers of mucus and intestinal epithelium can be two major absorption barriers for protein drugs to overcome, resulting in low permeability and oral bioavailability (<1%).[22] Finally, proteins will enter into the portal vein, reach peripheral circulation and be transported to other tissues or organs. Specifically for insulin, though it is stable in acidic environment and not so much influenced by the chemical barrier,[29] enzymatic degradation and insufficient permeation still restrict its application for oral administration. Thus it can be concluded that particulate carriers are necessary to shield protein drugs and prevent them from enzymatic degradation, while achieving the control of drug release and increasing their absorption in small intestine.[26]

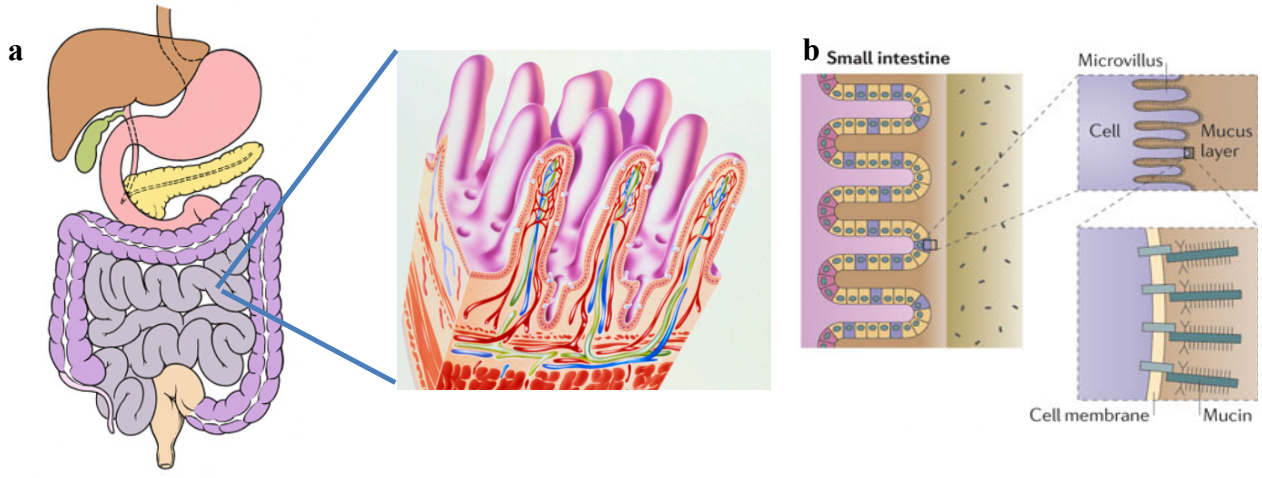


Figure 3. a, Diagram of the gastrointestinal tract (left) and structure of intestinal villi (right). **b**, Fine structure of mucus layer and intestinal epithelium. Adopted from Ref. 30.

1.4 Single-Protein Nanocapsule Platform

The commencement and advance of nanotechnology and nanomedicine in the past few years has opened a new perspective for oral delivery of biomolecules.[22] As mentioned in previous chapter, a novel encapsulation method has been developed by Lu Group. It is a type of nanocapsule platform where a thin polymer network is formed synthetically *in situ* around a single protein or protein complex. Synthesis of the nanocapsule involves two steps (**Figure 4a**): protein molecules are first conjugated with amine-reactive acrylate molecules to attach polymerizable groups onto the proteins; then *in situ* polymerization is initiated in aqueous solution, yielding a thin polymer shell around each protein molecule.[7] For some specific proteins, since modification of proteins before encapsulation may damage the conformation of active sites and decrease their activities, a simplified technique is brought forward by enriching monomers and crosslinkers around protein molecules through electrostatic or hydrogen-bonding interactions, instead of conjugating acrylate groups.[7] Such nanocapsule platform has significant advantages over other platforms, like enhanced stability against heat or proteolysis (**Figure 4b, 4c**) and tunable surface properties by changing monomers with desired charge or hydrophilicity.[14,31]

To summarize for insulin, this established single-protein nanocapsule is applied to develop an oral delivery platform for treatment of diabetes. Nanocapsules encapsulating insulin molecules will firstly be synthesized and engineered to adapt to those delivery barriers; then their diffusion behaviors in those biological membranes will be evaluated *in vitro* by changing the

surface properties; finally *in vivo* biodistribution and pharmacokinetics of nanocapsules will also be investigated to test their therapeutic benefits.

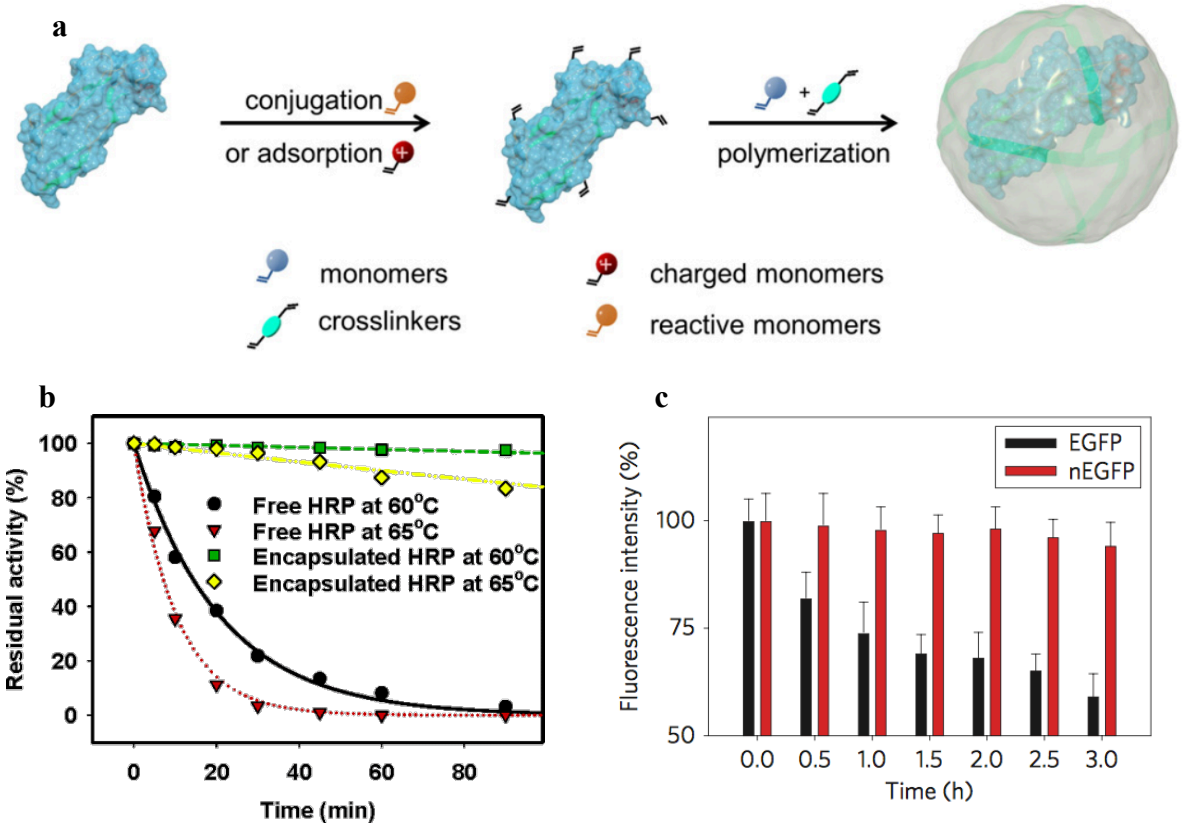
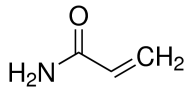
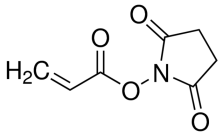
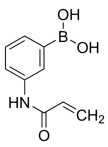


Figure 4. a, Schematic representing the preparation of single protein nanocapsule via *in situ* synthesis of crosslinked polymer shell on the enzyme surface. Adopted from Ref. 7. **b**, Thermal inactivation kinetics of free horseradish peroxidase (HRP) and encapsulated HRP at 60 °C and 65 °C indicating that the encapsulated HRP possesses significantly enhanced thermal stability. Adopted from Ref. 31. **c**, Fluorescence intensity of native enhanced green fluorescent protein (EGFP) and encapsulated EGFP after exposure to 1 mg/mL trypsin and α-chymostrypsin at 50 °C. Fluorescence intensities are normalized to native EGFP before exposure to protease. Adopted from Ref. 14.

2 Methods and Materials

2.1 Design and Synthesis of Protein Nanocapsule

To establish the oral delivery platform, bovine serum albumin (BSA), a very common and stable protein was used as an example to synthesize its nanocapsules and conduct preliminary experiments, since the diffusion behaviors of nanocapsules are mainly based on the characteristics of capsules themselves, not the type of proteins. As mentioned above, synthesis of protein nanocapsules involves a first step of absorbing and enriching monomers and crosslinkers around protein molecules, and a second step of *in situ* polymerization along the protein surface to form a crosslinked network. Both processes are carried out in buffer solutions, at neutral pH and low temperature in order to mimic the normal physiological environment and keep the protein stable. In addition, the properties of nanocapsules can be highly tunable to achieve different purposes by changing the types of monomers involved in the polymerization step. Major types of monomers used in this project are shown below (**Table 1**).

Name	Chemical Structures	Properties
Acrylamide (AAm)		Normally used hydrophobic monomers for the polymerization of nanocapsules
N-Acryloxysuccinimide (NAS)		Conjugating vinyl groups to the surface of protein
3-(Acrylamido) phenylboronic acid (A-PBA)		PBA monomers that provide mucoadhesivity

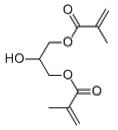
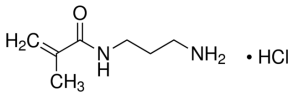
Name	Chemical Structures	Properties
Glycerol dimethacrylate (GDMA)		Degradable crosslinker
N-(3-Aminopropyl) methacrylamide (APm)		Positively charged monomers for intracellular delivery

Table 1. Major types of monomers used in protein nanocapsule synthesis.

Since achieving oral delivery of nanocapsules has to overcome a series of natural barriers in order to make drugs carried into effect, monomers in the polymerization reaction should have relevant functions to provide necessary characteristics. For example, in order to overcome chemical and enzymatic barrier, polymer network synthesized from AAm monomers is the basic foundation of nanocapsule shells, preventing proteins from degradation of proteases and any conformational changes induced by pH variation. Hydrophilicity difference between various monomers can provide choices of the surface hydrophilicity of nanocapsules.

As for the next absorption barrier of mucus layer, A-PBA molecules are introduced to the polymerization system, since those phenylboronic groups have high binding affinities to the glycoproteins in mucus layer, which increase the mucoadhesivity of nanocapsules.[32-33] Therefore, when transiting along the GI tract and interact with mucus layer, due to the function of PBA, protein nanocapsules can be more trapped in the mucus layer. They would have a greater opportunity to pass through the following enterocyte layer owing to a larger nanocapsule concentration gradient between mucus and enterocyte layer.

For the enterocyte layer, the use of positively charged monomer APm can facilitate the transcellular delivery process.[34] Moreover, APm can provide reactive amino groups which can be exploited for further modifications, like conjugation of antibodies and other targeting ligands.

Finally for further modification of nanocapsules by choosing suitable crosslinker, it is necessary to emphasize that most functional proteins need to be released from the polymer coating in order to come into effect after administration. Here in this nanocapsule platform, GDMA, a type of degradable crosslinker which remains stable at normal physiological pH but will break down at acidic or enzymatic conditions, was selected to assist the release of nanocapsules in the blood.

Monomers are chosen relating to the requirements of nanocapsules, and different sets of monomers can produce various surface properties. Thus three groups of nanocapsules with AAm shells was set and synthesized, including one with only AAm/APm, another one with only AAm/ PBA and the last one with all the three monomers, for the purpose of evaluating functions of these monomers separately. Synthesis of each protein nanocapsule is easy and direct. Taking AAm/APm/PBA nanocapsule as an example, BSA was first dialyzed in potassium phosphate buffer to remove residual ammonium salts in the products. Then monomers of AAm, APM, PBA and GDMA were added and enriched around BSA molecules in the buffer solution. Next *in situ* polymerization was initiated by ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). After polymerization, nanocapsules were dialyzed against potassium phosphate buffer to remove excess molecules. Other groups of nanocapsules were synthesized according to similar procedures. For visualization or quantification of the nanocapsules in the following

characterization steps, fluorescent dye molecules, like 5/6-carboxy-fluorescein succinimidyl ester (NHS-fluorescein) or rhodamine B isothiocyanate, was conjugated to the proteins as fluorescent labels, creating a pre-step before polymerization. Excess monomers and dyes were removed by desalting or hydrophobic interaction column to purify the nanocapsules.

2.2 Characterization of Nanocapsule

The encapsulation of nanocapsules can be observed by agarose gel electrophoresis, which can differentiate proteins or nanoparticles on the basis of their size and charge. Usually the change in size and surface properties before and after polymerization of proteins will result in different migration behaviors on the agarose gel. This method is rapid and effective, and results are simple to analyze. Specifically, 8 μL of each type of protein nanocapsules (0.5 g/L) labeled with fluorescent dyes were separately added to one well of agarose gel, with native protein as the control group. Then the gel was immersed in gel buffer and run at 110 V for 15 minutes. Finally the gel was taken out of the buffer and inspected over ultraviolet lamp with the excitation wavelength of 365 nm.

Another technique, called as dynamic light scattering (DLS) measures the size and zeta potential distribution of protein nanoparticles. After polymerization, there expected to be an increase in size of the protein due to the coating of polymer, as well as a change in surface charge exhibited by the newly-formed shell. These experiments were carried out as belows. Nanocapsule samples were firstly diluted with 0.01 M phosphate buffer into around 0.1 g/L, then transferred into clean DTS 1070 folded capillary cells for size and zeta potential measurements in a Zetasizer Nano ZS instrument (Malvern Co.).

Formation of the nanocapsules can be observed under transmission electron microscope as well. A 10-μL droplet of the nanocapsule samples was contacted with carbon-coated copper grids for 1 minute, then the droplet was removed and the grids were stained by 1% sodium phosphotungstate for 15 seconds. After getting dried out in the air, the grids were inspected under TEM.

2.3 Proteolytic Stability and Cell Viability

When orally delivered into the GI tract, nanocapsules will instantly face the problem of hydrolysis by protease, so it is important to test the ability of nanocapsules to protect proteins from decomposition. Here we use trypsin as a model protease. Proteolytic stability was conducted using the following procedures: solutions of native protein and protein nanocapsules (AAm/APm/PBA), mixed with 1 mg/mL trypsin and 0.01 M Ca^{2+}, were incubated at 37˚C for 12 hours, followed by agarose gel electrophoresis together with the group of BSA and BSA nanocapsule solutions without trypsin treatment as the control.

Positively charged monomer APm is applied in the polymerization and endows the nanocapsule with positive surface that is easier for cell internalization. However, such positive surface may bring cytotoxicity to the cells through which they will penetrate. Thus cell viability seems to be required and was evaluated with Caco-2 cell lines. Cells were seeded in 96-well plates one day before the viability assay. Specific amounts of native BSA and BSA nanocapsules (AAm//PBA/APm) were incubated with the cells at 37˚ C for 12 hours. After incubation, CellTiter-Blue was then added to each well and incubated for 4 hours before measuring the fluorescence with a plate reader (Ex:560nm, Em: 590nm).

2.4 *In Vitro* Release of Nanocapsule

When achieving the oral delivery of insulin nanocapsules, insulin peptides are expected to release from the nanocapsule shells in physiological condition such as bloodstream, since they needs to bind with the receptors on surfaces of specific cells to induce the following signal transduction and regulation processes. Based on previous researches about degradable nanocapsules,[14] several types of crosslinkers with different degradation properties were selected to synthesize the nanocapsules and their release curves were investigated by dialysis method at PBS buffer (pH 7.4). Native insulin curve serves as the control since it can freely pass through the dialysis film (molar weight cut-off = 10 kDa).

2.5 Diffusion of Nanocapsule through Mucus Layer

Along the oral delivery route discussed above, small intestine is believed to be the most important part where nutrients are absorbed from GI tract to bloodstream, and where the mucus layer is the thinnest and easiest to break though.[35] So it is of great significance to evaluate the diffusion process and permeation rate of the nanocapsule in the mucus and enterocyte layers.

The single mucus layer will firstly focused on and *in vitro* experiments were conducted to evaluate diffusion behaviors of nanocapsules through that layer. The apparatus here was the combination of transwell and its insert (**Figure 5**). The insert divides the whole well into two parts, the upper and lower compartments. The only passage connecting the two spaces is the supportive microporous membrane filter at the bottom of insert, where the membrane with the pore size of 0.4 μm permits the transport of small molecules but inhibits the cell migration from

the upper to the lower compartment. During the experiments, mucus is added to the insert and forms the layer supported by the membrane.

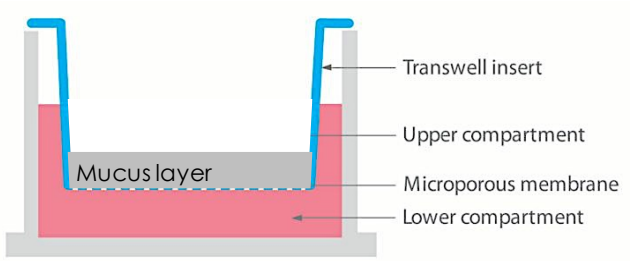


Figure 5. Structure of the apparatus of transwell insert used for the study of diffusion behavior.

The physical quantity to determine the diffusion of nanocapsules in mucus layer is the percentage of particles that can be trapped in this layer when flowing over the intestinal mucosa with other foods, suggesting their distribution between layers. This term is important for the oral delivery of nanocapsules, especially in the early stage when nanocapsules begin to contact with mucus layer. As already known, orally administrated nanocapsules will quickly pass through the surface of mucus layer and simultaneously the mucus itself is renewed.[26] If nanocapsules cannot be trapped in the mucus efficiently, they will be easily removed and not be able to pass through the following layers. The fraction of particles in the mucus layer could be measured by fluorescence spectrometer. Specific amounts of solutions of Rhodamine B labeled nanocapsules were added to mucin solution (1 g/L) in the transwell insert. The bottom of inserts were contacted with PBS buffer in lower well plate for 12 hours to reach a balance for nanocapsule distribution. After that, concentration of nanocapsules in the buffer was determined by fluorescence intensity of Rhodamine B. Fraction of nanocapsules in mucus layer was calculated

by the ratio of $N_1/N=(N-N_2)/N$, where N_1 and N_2 are moles of nanocapsules in the inserts and wells, respectively, and N is the original total mole of nanocapsules added to the transwell.

2.6 Penetration of Nanocapsule through Cell Layer

After traversing the mucus layer, nanocapsules will face the second absorption barrier of intestinal epithelium, or enterocyte layer. Enterocytes are closely packed with each other through tight junctions (TJs), forming the structure which allows selective absorption of nutrients, electrolytes and fluids but at the same time prevents the host from environmental pathogens. For *in vitro* study of this transcellular penetration of nanocapsules, transwell insert was still used as the apparatus but the mucus layer in the insert was replaced by cell layer.

Caco-2 cell line, a type of colon epithelial cells universally was applied to mimic the structure of intestinal epithelium. Caco-2 cells formed an intact monolayer after being seeded to the transwell insert at 1×10^5 cells/insert and grew for 21 days, while both the upper and lower compartments were filled with Dulbecco's modified Eagle's Medium (DMEM) with 20% fetal bovine serum (FBS) to cultivate the cells. Nanocapsules of horseradish peroxidase (HRP) or native HRP enzymes were added to the cells in the insert and penetrated through the cell layer. Here HRP nanocapsules instead of dye-labeled BSA nanocapsules were chosen, for the purpose of a higher measurement sensitivity by HRP activity assay to determine the concentration of nanocapsules in each compartment. After 12-hour diffusion, concentrations of HRP nanocapsules or native HRP were measured by UV spectrometer.

2.7 *In Vivo* Study of Nanocapsule in Mice

After researches on diffusion behaviors of nanocapsules *in vitro* with mucus and cells, it is necessary to have a preliminary understanding of their performance and distribution with time *in vivo*, in order to test if they really stay in the body for a long time without being cleared quickly after oral administration. Native protein and nanocapsules, stained by Cyanine5.5, a type of near-infrared (NIR) fluorescence-emitting dye, were fed separately by gavage into two mice, then NIR imaging of their bodies were determined 2 hours and 24 hours later after gavage.

3 Results and Discussion

3.1 Characterization of BSA and Insulin Nanocapsules

Characterization of BSA nanocapsules are shown below (**Figure 6**). From electrophoresis result, after polymerization, nanocapsules approximately kept static (neutral surface charge) or moved towards the anode (positive surface charge), in contrast to the negatively charged native proteins moving to the cathode. Especially for APm-involved nanocapsules, they had significantly positive charge and longer bands as expected. This result could be confirmed by the zeta potential distribution, suggesting that formation of polymer shells could alter the surface charge of original proteins. An increase in size distribution could also be observed in size distribution and TEM image, approximately from 5 nm native BSA to 10-20 nm nanocapsules, proving that synthesis of this nanocapsule platform is reasonable.

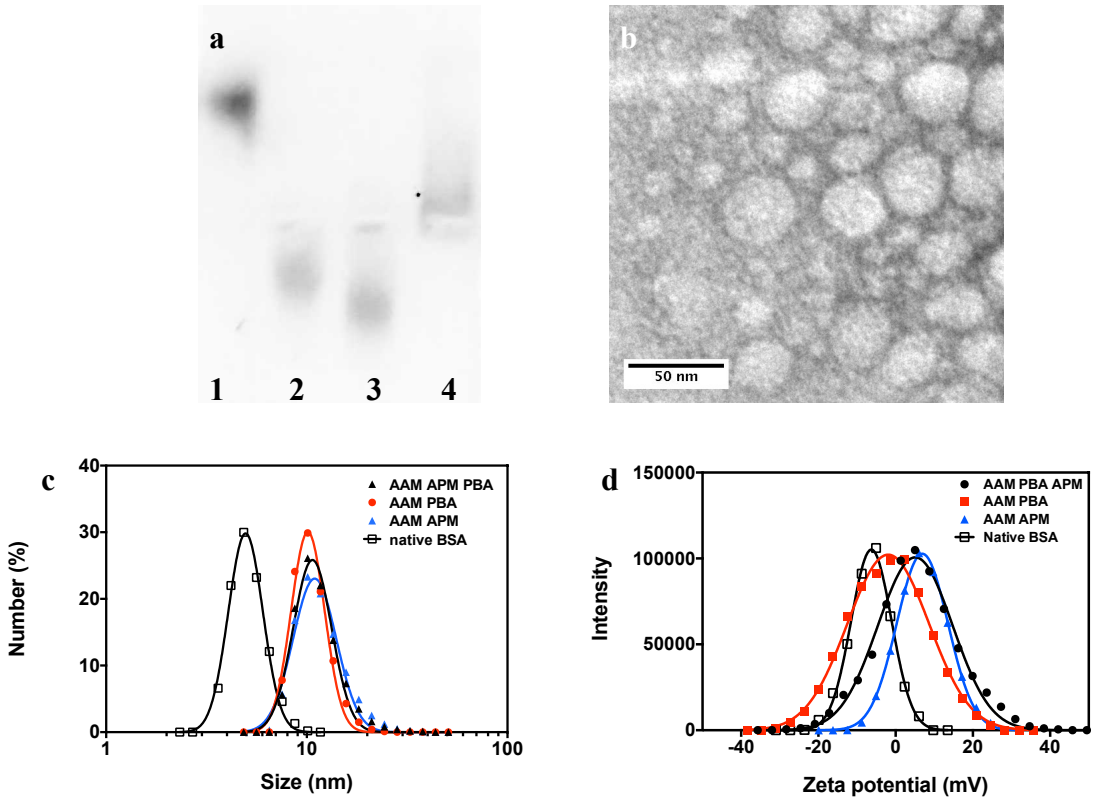


Figure 6. a, Agarose gel electrophoresis of different types of BSA nanocapsules. **1**, native BSA; **2**, AAM/APM/PBA; **3**, AAM/APM; **4**, AAM/PBA. **b,** TEM images of AAM/APM/PBA nanocapsules. **c, d,** Size (**c**) and zeta potential (**d**) distribution of BSA/AAM nanocapsules.

Based on BSA nanocapsules, encapsulation of insulin can be achieved by conducting similar synthesis methods. Since the biological activity of insulin is concerned, conjugation with vinyl groups onto the protein surface is not required for insulin nanocapsules. Characterization results are shown below (**Figure 7**), not only confirming the formation of insulin nanocapsules with transitions of size and surface charge, but also suggesting that this platform is versatile for various proteins.

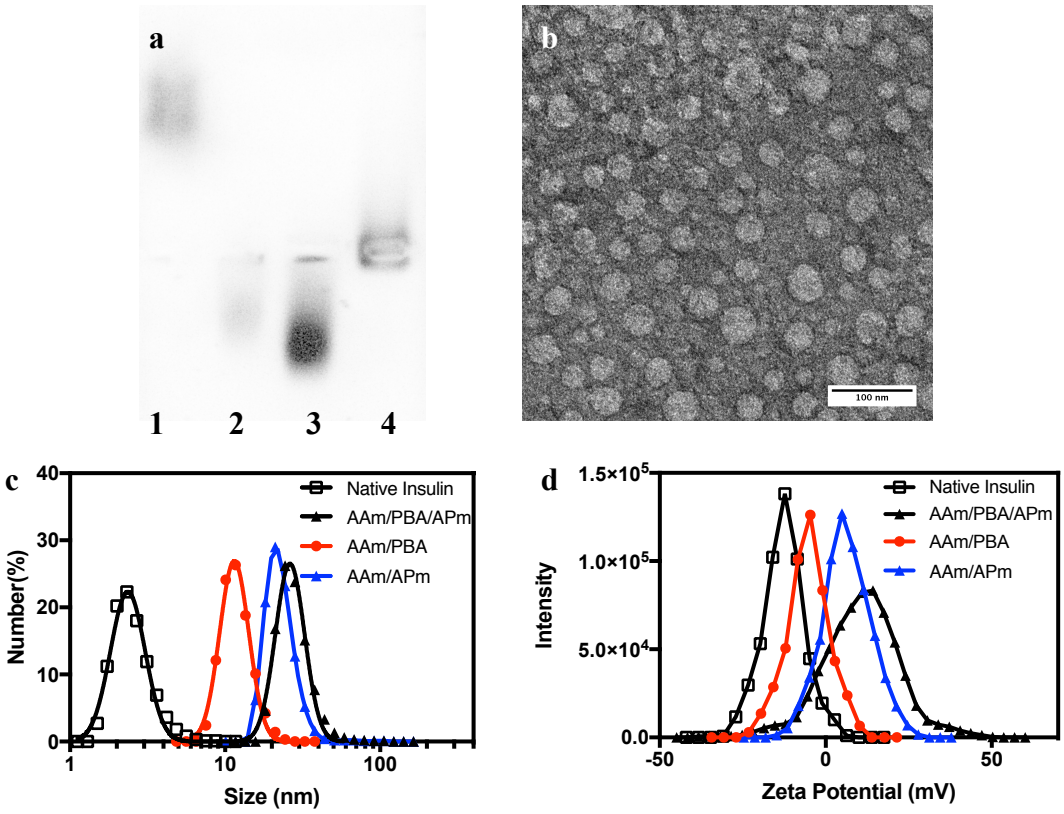


Figure 7. a, Agarose gel electrophoresis of different types of insulin nanocapsules. **1**, native insulin; **2**, AAM/APM/PBA; **3**, AAM/APM; **4**, AAM/PBA. **b,** TEM images of AAM/APM/PBA nanocapsules. **c, d,** Size (**c**) and zeta potential (**d**) distribution of insulin/AAM nanocapsules.

3.2 Proteolytic Stability of BSA and Insulin Nanocapsules

From the electrophoresis result (**Figure 8**), native BSA was significantly hydrolyzed into small pieces after trypsin treatment, but the bands belonging to nanocapsules still remained the same, without any band from native BSA or its residues, showing good proteolytic stability for this nanocapsule platform. For insulin nanocapsules, they still have the same property to keep the stability of encapsulated protein from hydrolysis. Proteolytic stability was carried out using almost the same procedure as BSA. The only difference is that after incubation with trypsin, SDS-PAGE gel was run instead to specifically investigate the stability of nanocapsules at

sequential time points. According to the result (**Figure 9**), after being incubated with trypsin for a period of time as long as 8 hours, insulin nanocapsules still kept the same band shape without any band belonging to native insulin, showing similar proteolytic stability as BSA nanocapsules.

Figure 8. Agarose gel electrophoresis of **1,** native BSA; **2**, native BSA after trypsin treatment for 12 hours; **3**, AAm/APm/PBA nanocapsules; **4**, nanocapsules after trypsin treatment for 12 hours.

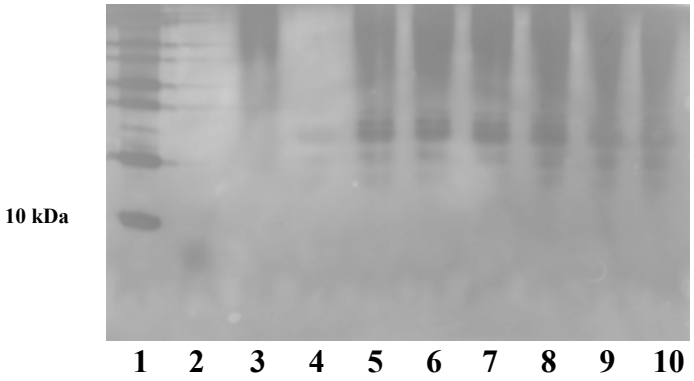


Figure 9. Agarose gel electrophoresis of **1,** ladder; **2**, native insulin; **3**, insulin AAm/APm/PBA nanocapsule without trypsin treatment; **4**, trypsin; **5-10**, nanocapsules after trypsin treatment for 1, 2, 3, 4, 6, 8 hours respectively.

3.3 Cell Viability of BSA Nanocapsule

Cell viability assay below (**Figure 10**) suggested that for a large scale of concentration (20-1600 nM), AAm/PBA/APm nanocapsules did not show significant cytotoxicity compared to native BSA, confirming their safety for cell penetration.

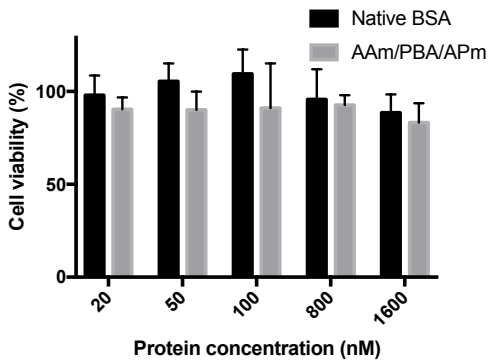


Figure 10. Cell viability assay by CellTiter-Blue with BSA and AAm/PBA/APm nanocapsules. Caco-2 cells were used as a model cell line.

3.4 *In Vitro* Release of Insulin from Nanocapsules

As shown and calculated from fluorescent data (**Figure 11**), nanocapsules with non-degradable bis-acrylamide (BIS) crosslinker and acid-degradable GDMA crosslinker showed only 10% insulin release at pH 7.4, while degradable PLA-PEG-PLA diacrylate crosslinker had a 2-fold release than the other crosslinkers, which was a promising crosslinker for the synthesis of degradable nanocapsule in blood. The release curve also suggested that the degradation of nanocapsules could be adjusted and optimized by choosing various crosslinkers to accommodate relevant release requirements.

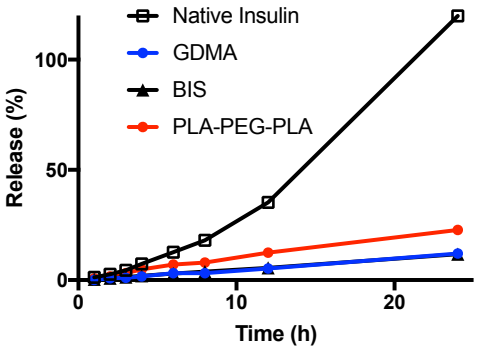


Figure 11. Release curve of insulin at PBS buffer (pH 7.4) from AAm/APm/PBA nanocapsules with non-degradable (BIS), acid-degradable (GDMA) and degradable (PLA-PEG-PLA diacrylate) crosslinkers respectively.

3.5 Diffusion of BSA Nanocapsule through Mucus and Cell Layers

For the diffusion process through mucus layer, the fraction of each type of nanocapsules trapped in mucus layer had been collected and calculated (**Figure 12**). Compared with native BSA, AAm/PBA/APm nanocapsules showed larger distribution in mucus layer, and were also kept more in mucus layer than the other two nanocapsules. This phenomenon was mainly due to two factors. On the one hand, APm monomers endowed the nanocapsules with positively charged shells, which facilitated their combinations with negatively charged glycoproteins in mucus through electrostatic interactions; on the other hand, PBA groups on the surfaces had high binding affinities to those glycan chains of the mucus glycoproteins.[33] Therefore, the synergistic effect of two factors would enforce APm/PBA nanocapsules to interact with mucin and diffuse more in this layer than native BSA. In addition, since AAm/APm/PBA nanocapsules were trapped more in mucus layer, the concentration gradient of nanocapsules in and out of epithelial cells would become larger as well, which contributed to the penetration of these particles through the following cell layer.

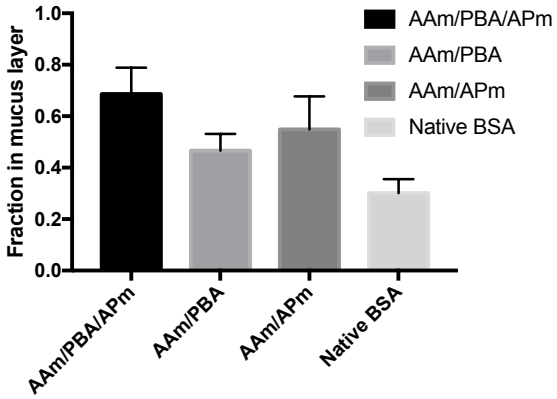


Figure 12. Fractions of all types of nanocapsules and native BSA in the mucus layer.

For the penetration process through cell layer, according to results of permeate fractions (**Figure 13**), AAm nanocapsules penetrated more than native protein as expected. It could also be found that APm-involved nanocapsules diffused more across the cell layer than those non-APm ones, indicating the contribution positive surface charge had made to assist transcellular transport or endocytosis during this diffusion process. Thus in general, it can be concluded that enterocyte penetration could be such a process that can be affected by surface charges of the penetrating nanocapsules.

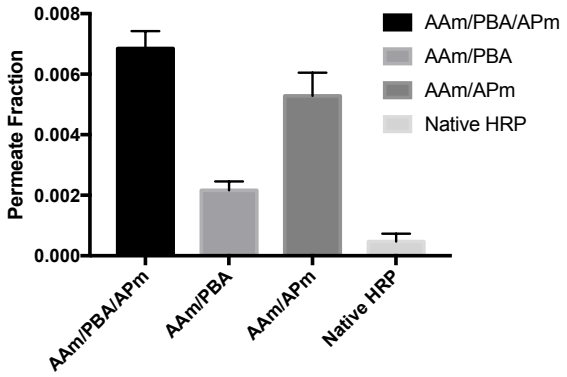


Figure 13. Permeate fractions of all types of HRP nanocapsules and native HRP through Caco-2 cell monolayer.

3.6 *In Vivo* Distribution of BSA Nanocapsule

Imaging photos are shown below (**Figure 14**). As seen from the result, after 24 hours native BSA proteins in mouse body were almost cleared and could be determined at a negligible level, while AAm nanocapsules still kept their significant fluorescence signals and distribution, especially in the part of intestine and liver. Compared with native proteins in the control group, AAm nanocapsules truly showed more stability and longer resident time *in vivo* than native proteins which could easily be digested early in the GI tract.

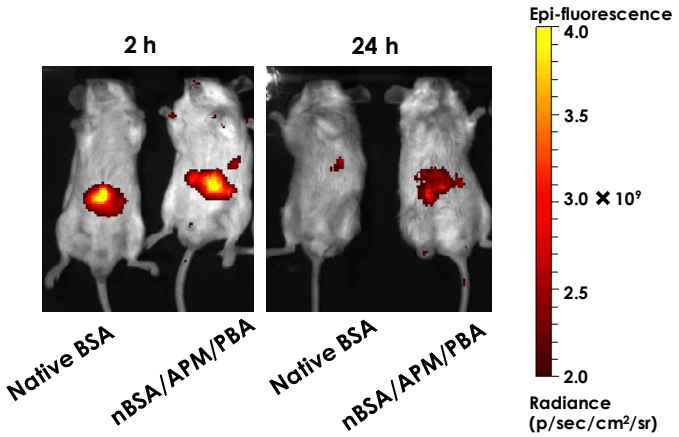


Figure 14. NIR imaging photos of the mouse bodies fed separately with native BSA and BSA/AAm nanocapsules stained by Cy5.5, 2 hours (left) and 24 hours (right) after gavage. Mice were fasting for 12 hours before gavage.

4 Future Work

4.1 *In Vitro* Diffusion Behaviors of Insulin Nanocapsule

From previous chapters, formation of insulin nanocapsules with various characterization methods has been demonstrated, while their diffusion behaviors are still required for further investigation and confirmation. For mucus layer, the same series of diffusion experiments can be carried out with insulin nanocapsules by means of transwells, verifying the previous results about the effects of surface properties on diffusion behaviors of nanocapsules. In addition, diffusion coefficients of all types of insulin nanocapsules can be detected when they pass through the mucus layer, to make sure if those surface properties could truly improve the diffusion efficiency.

As for enterocyte layer, similar penetration process can still be conducted, while the penetrated insulin nanocapsules labeled with dyes can be measured by fluorescence. Moreover, the amount of insulin penetrating through cell layers can also be determined by using enzyme-

linked immunosorbent assay (ELISA), to achieve high measuring sensitivity like HRP activity assay. Surface properties of nanocapsules, namely the ratios and types of monomers for the polymerization, can be changed to optimize the penetration efficiency in both layers.

4.2 Transport Mechanism through the Cell Layer

Nanocapsules permeation may occur across the enterocyte layer, through either transcellular or paracellular routes.[26,36] In this part the delivery mechanism of nanocapsules will be focused on and it will be specified by which method the nanocapsules will penetrate through the cell layer.

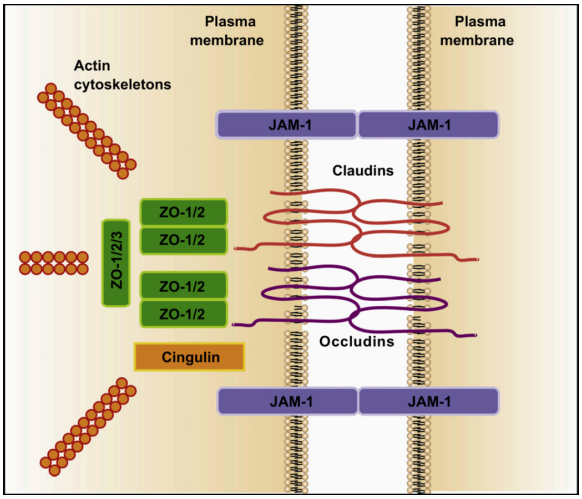


Figure 15. Schematic illustrations of the tight junctions (TJs) between contiguous epithelial cells. TJs located at the apical domains of epithelial cells consist of a complex of transmembrane [claudins, occludins and junctional adhesion molecules (JAMs)] and cytoplasmic (ZO-1, ZO-2, ZO-3, cingulin) proteins. Adopted from Ref. 26.

Transport across the intestinal epithelium via paracellular route is restricted by tight junctions (TJs) between neighboring cells. TJs are composed of a complex combination of transmembrane integral proteins and junctional adhesion molecules (JAMs) along with several intracellular plaque proteins and a variety of regulatory proteins (**Figure 15**).[26] They all

contribute to the physical integrity of intestinal epithelium. Due to this structure, transport across the epithelium via the paracellular route is minimal, and only small hydrophilic drugs with a MW of less than 200 Da can transport through this way.[26] However, TJs can be opened by using enhancers such as cationic and anionic polymers and calcium chelators for the purpose of paracellular transport.[26] In order to confirm whether nanocapsules would induce the open of TJs during penetration process, trans-epithelial electrical resistance (TEER) of the Caco-2 cells can be determined between the upper and lower compartments of transwells which are applied to conduct penetration experiments (The value of TEER is normally 500 $\Omega \cdot cm^2$ for intact Caco-2 cells).[22] TEER reflects the integrity of cell monolayers. As TEER decreases, tight junctions between the cells become weaker and the permeability of the monolayer increases. Thus the TEER values of Caco-2 monolayers before and after the penetration process of nanocapsules could be compared together, so that the open of TJs will be confirmed if a decrease in TEER is observed.

As for transcellular pathway, protein nanocapsules can probably be taken up by active transport of enterocytes. The active transcellular transport begins with an endocytic process that occurs at the apical cell membrane, then the particles are transported through the cells and released at the basolateral pole of cells.[26] Three major mechanisms for this active transport are macropinocytosis, clathrin-mediated endocytosis and caveolin-mediated endocytosis. In order to investigate which mechanism dominates the transport of nanocapsules, three endocytosis inhibitors, amiloride, chlorpromazine (CPZ) and β-cyclodextrin, which can inhibit the above three endocytosis respectively, will be used for the experiments.[14] For example, nanocapsules can be incubated with cells in the presence of one inhibitor CPZ. If the cellular uptake and

penetration of nanocapsules becomes significantly less than those cells without CPZ, the mechanism of clathrin-mediated endocytosis proves to be predominant. Characterization of cellular uptake can be observed by fluorescence microscopy and quantified by fluorescence intensity from the dye-labeled protein nanocapsules. In addition, if the transport mechanism could be confirmed as transcellular pathway instead of paracellular pathway, the toxicity of nanocapsules coming from opening the tight junctions of enterocytes could be minimized, which can become another advantage of nanocapsule platform.

4.3 *In Vivo* Distribution of Insulin Nanocapsules

The *in vivo* experiments of insulin nanocapsules are critical for this project, considering about the complicated digestive environments in living creatures. This research can be divided into two parts. The first one is just the same as the experiment conducted with BSA nanocapsules in the previous chapter, in order to specify the biodistribution of insulin nanocapsules with time in mouse body after oral administration. Moreover, organs such as intestine and liver can be collected from mice several hours later after gavage, and visualized by fluorescence to specify the accurate distribution between organs. Taking tissue slices from the intestine also seems to be an alternative choice to directly observe the distribution of nanocapsules between mucus layer and enterocytes in the small intestine .

The second part is to evaluate the therapeutic effects of insulin nanocapsules on the blood glucose level. Mice will be fed with solutions of insulin nanocapsules and after that blood samples will be taken periodically and detected by glucose-meter. Variation of glucose level in the bloodstream can reflect the influence of nanocapsules. Insulin levels of the blood from mice

could also be monitored by ELISA kit. It is expected that the orally administrated nanocapsules have more moderate regulation on the glucose level compared to normal subcutaneous injection, without causing severe hypoglycemia to the mice.

5 Conclusion

This thesis is aimed to fabricate positively charged, mucoadhesive and degradable protein nanocapsules for oral delivery platform to overcome diffusion barriers and treat diabetes. Comprehensive characterization methods like gel electrophoresis, size/zeta distribution and TEM imaging have been carried out to demonstrate the formation of BSA and insulin nanocapsules, which could prove them to be an adaptable nanocarrier. Moreover, *in vitro* diffusion researches in mucus and cell layers show their advantages over native proteins in overcoming diffusion barriers through oral delivery route. *In vivo* study of the nanocapsule distribution in mice also suggest that they have a longer resident time than native proteins. With further researches on diffusion behaviors, biodistribution and pharmacokinetics of insulin nanocapsules, this nanocapsule platform would show more potential applications in the field of oral delivery for diabetes therapeutics.

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