DESIGN OF SELF-ASSEMBLED ZEIN-BASED NANOPARTICLES

AS AN EFFECTIVE DRUG CARRIER AND TRANSPORTER

by

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ABSTRACT OF THE DISSERTATION

Design of Self-assembled zein-based nanoparticles as an effective drug carrier and transporter

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Cancer is the second leading cause of death in the world. General characteristics of tumors include abnormal blood vessels and lack of lymphatic system. Due to large sizes, drug-loaded delivery systems cannot diffuse out the tumor blood vessels, as a result of progressive accumulation: the EPR effect. That’s the main reason to utilize nanoparticles in cancer therapy. Besides, the size, shape, surface chemistry and targeting groups of nanoparticles are important in controlling biodistribution and cellular internalization of engineered nanoparticles.
Zein specifically acts as reservoirs for lipophilic bioactive substances or drugs due to the strong hydrogen bond and hydrophobic interaction between nutraceutical and proteins. Zein nanoparticles (NPs) are commonly prepared via phase separation method. Although it is a relatively easy technique, the resultant zein colloidal dispersion usually form poorly redispersable aggregates and sediments after drying. Also, individual zein NPs exhibited a burst drug release profile at physiological conditions due to the swelling of the zein NPs and the drug cannot tightly bind to zein particles. Therefore, we aim to design novel zein-based NPs to enhance the stability and sustained release of individual zein NPs. At the meantime, the cellular uptake and bioefficacy of the encapsulated drug were also discussed in this work.

In the first part of this work, hydrophilic carboxymethyl konjac glucomannan and calcium ion crosslinking were used to modify the surface of zein nanoparticles, therefore zein-carboxymethyl konjac glucomannan-calcium (ZCC) NPs were assembled. As a result, ZCC NPs showed good stabilities in cell culture medium at 37 °C, and enhanced pH stability at a range from 5.0 to 8.5 and lower surface hydrophobicity. The endocytic pathway of ZCC NPs is cell-line dependent, and ZCC NPs enhanced the cellular uptake through dynamin-mediated endocytosis can be assigned to clathrin-mediated endocytosis in HT29 cells. And the stability and cellular uptake were enhanced compared to individual zein NPs.

Because of the anti-inflammation and anti-cancer properties, andrographolide (AG) and its derivatives, which are labdane diterpenoid compounds extracted from Andrographis paniculata Nees, are often used as herbal drugs in Asian countries. AG sparingly soluble in water (3.29μg/ml at 25 °C), which restricts its therapeutic use due to
low bioavailability by oral administration. ZCC NPs was used as a drug carrier for AG to enhance the water solubility and the bioefficacy. Importantly, ZCC NPs loaded AG were shown effectively decreasing the cancer cell population and resulting cell death. The efficacy of AG to regulate the in vitro NF-κB expression was notably enhanced by the ZCC nanoparticle delivery system due to enhanced dose efficiency resulting from better solubility, transportability and reduced toxicity. In these preliminary studies, ZCC NPs were proved to be appealing delivery systems for hydrophobic bioactive compounds.

Efficient pH-sensitive delivery systems sensitively response to subtle pH variations in the tumor microenvironment. In this paper, we utilized the acid-sensitive bonds between metal ions and dihydromyricetin (DMY) and designed a pH-sensitive zein-based delivery system. The self-assembled zein NPs were coated with a DMY-Zn$^{2+}$ coordinated bonding layer. Z-DMY/Zn$^{II}$ NPs not only acted as an efficient drug carrier, but also a highly pH-responsive drug release system. Therefore, Z-DMY/Zn$^{II}$ NPs had a better sustained release compared with individual zein NPs. Moreover, Z-DMY/Zn$^{II}$ NPs demonstrated excellent cellular uptake by HeLa cells. Z-DMY/Zn$^{II}$ NPs effectively inhibited the cancer cell growth and exhibited an efficient anti-cancer activity toward HeLa cells. As a result, Z-DMY/Zn$^{II}$ NPs acts as a promising pH-responsive food-grade colloidal delivery system encapsulating hydrophobic nutraceuticals in cancer therapy.
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CHAPTER 1. INTRODUCTION

1.1 Cancer

Cancer is the second leading cause of death in the world. The World Health Organization says that 8.8 million deaths were due to cancer in 2015, and cancer was responsible for 1 in 6 deaths globally. According to the cancer Statistics published by the National Cancer Institute, there will be approximate 1,685,210 new cases diagnosed as cancer in the United States and 595,690 cancer patients are going to die in 2016. However, owing to research efforts to tumor biology, diagnostic devices and treatments, the patient survival has increased in the past years. Current cancer therapy involves surgery, radiation and chemotherapy, which could also kill normal cells and cause side effect to the patient. Overall, the efficacy of the treatment is believed to be directly related to the targeting ability of the treatment to cancer cells and the minimum affecting effects to healthy cells. Therefore, it would be promising to develop chemotherapeutic drugs that can target tumorous tissues either passively or actively. The advances of cancer treatment in both new chemotherapeutic drugs and new ways of delivering chemotherapeutic drugs are rapidly progressing [1]. Hopefully this development can help us with lowering the toxic of the drugs to healthy cells and targeting the cancer cells. This thesis will primarily address new methods for delivering chemotherapies with a focus on novel biodegradable nanoparticles.

1.1.1 The biology of a tumor and therapeutic barriers
Cancer usually begins with single cell dysfunction by either up-regulating cancer growth-promoting genes or down-regulating cancer growth-suppressing genes [2]. In both cases, the mutation results in replication of cancerous cells at a rate high than healthy cells. As long as a small tumor mass has formed, tumor cells will displace healthy cells because of the insufficient supply of nutrients to the healthy tissues. When the tumor reaches 2 mm³, oxygen and nutrients on their microenvironment can simply diffuse to the center of the tumor [3].

At the outer edge of the tumor mass, cancer cells will continue dividing without regard to nutrient supply. However, many tumor cells on the inside will apoptosis and create a necrotic core within tumors because of the insufficient nutrients. Basically, a steady state tumor size of 2 mm³ forms when the cell proliferation rate is equal to the cell apoptotic rate. To grow beyond this size, cellular hypoxia initiates, which is lack of oxygen, followed by angiogenesis [4]. The progress of tumors from a non-angiogenic phenotype to an angiogenic phenotype is essential for cancer progression and spread of tumor cells throughout the body, as a result of metastasis [5][6]. In particular, tumor blood vessels are generally differentiated from normal vessels by imbalance of angiogenic regulators, proliferating of endothelium cells, abnormal basement membrane formation and pericyte deficiency [7]. Typical tumor angiogenesis is characterized by quickly and disorganized creating branched, short-circuited and rippled vessels, with poorly constructed leaky walls (Figure 1) [4]. These features leads to an enhanced vascular permeability and enlarged gap junctions between endothelium cells and lymphatic drainage, which referred as the enhanced permeability and retention (EPR) effect [8]. The enlarged gap junctions can have sizes ranging from 10 to 1000 nm
depending on tumor type, while the endothelial pores in healthy vessels is only 5 to 10nm [1]. The enhanced permeability in tumor is caused by the leaky vessels, and the non-functional or absent lymphatic system leads to the retention effect in tumor [4]. As a result, nanocarriers (20–200 nm) can often enter and accumulate in the tumor efficiently, while the lymphatic system in normal tissues would drainage nanocarriers from the extracellular compartment [9]. Therefore, the abnormal vascular feature is crucial to the EPR effect in tumor and macromolecular drug selectively targeting at tissue level.

The fast-growing cancer cells with high metabolic rate evolve under an insufficient supply of oxygen and nutrients. Therefore, cancer cells use high glycolysis to obtain extra energy. Glycolysis results in the production of lactic acid which leads to lower pH in tumors [10]. While the intracellular pH of normal tissues and tumors are similar, tumors showed a lower extracellular pH (5.6-7.0) than normal tissues (7.4). The pH-sensitive liposomes are fabricated to be stable at a physiologic pH, but dissembled in acidic environment of targeted tumor cells [11].

In cancer therapy, the unique tumor properties including angiogenesis, leaky blood vessels and acidic environments could be utilized for drug delivery [12]. However, there are also properties making targeted delivery challenging. A low blood flow in the immature vessels makes it difficult to obtain a sufficient number of drugs. Besides, due to the poor vascularization, tumor cells may survive on marginal conditions which are away from any blood vessels [13]. It is hard to treat these cancer cells with ordinary drug delivery and new methods are demanded. Due to lack of lymphatic system in tumors, the interstitial pressure at tumor center is usually higher than the surrounding tissues, which makes it challenge to deliver drugs to the central area of tumors [14]. Another limiting
obstacle is the immune system composed by complement protein. The immune system is the body's built-in defense system which is able to attack anything foreign and of a certain size. The process of protein opsonization leading to phagocytosis by monocytes and macrophage cells is referred as reticuloendothelial system (RES) [15].

![Image](image_url)

**Figure 1** Differences between normal and tumor tissues. A. Normal tissues contain linear blood vessels with pericytes. Fibroblasts, collagen fibers and macrophages, lymph vessels are present in the extracellular matrix. B. Tumor tissues contain abnormal blood vessels with gaps and sac-like formations. The extracellular matrix presents more collagen fibers, fibroblasts and macrophages. Lymph vessels are lacking. (Reprinted from Ref. 4)

1.1.2 Cancer and inflammation

The close relationship between cancer and inflammation could be proved by many clinical and epidemiological evidence. Cancers caused by infections are also expected to increase between 2010 and 2020. Although some of the infection agents directly favor tumor progression (e.g., EBV, HPV, HTLV-I), others (e.g., hepatitis
viruses) induce carcinogenesis by promoting chronic inflammation in the host [16]. Inflammatory bowel disease (IBD) patients have an increased susceptibility to colorectal cancer, hepatocarcinoma, leukemias and other tumors, indicating that the intestinal inflammation could cause both local and systemic protumor effects [16].

**Inflammation can cause cancer**

Promoters initiates tumor progression by inducing cell proliferation, inflammatory cells recruiting and DNA replication. Inflammation, particular chronic inflammation is one of the important promotors that drives cell transformation and tumor initiation. Normal inflammation is self-limiting, while chronic inflammation with persistent infections induce DNA damage by leukocytes. Inflammatory cells have great influence on neoplastic processes by producing suitable environment for tumour development, assisting genomic instability and inducing angiogenesis. The inflammatory cells, producing chemokines and cytokines, regulate the development and migration of tumor, as well as the differentiation of cells in the tumour microenvironment, such as fibroblasts, neoplastic cells and endothelial cells. The inflammatory mediator nitric oxide has the complex role in cancer by mediating protumor or antitumor effects. The important factor is the status of the p53 gene. When the p53 is mutant in cancer cells, NO contributes to the protumor effects including inducing DNA damage, increasing angiogenesis and tumor cell proliferation, and suppressing antitumor immunity. Other inflammatory mediators like NF-κB also has important effects in tumor initiation and promotion. NF-κB induces NOS2, COX2 and many genes that regulate the angiogenesis, cell cycle and cell survival [17].
Cancer can cause inflammation

Oncogenes are often mutated or overexpressed in tumor cells, they also affect epithelial cells and stromal cells. The overexpressed of the oncogenic form of RAS, RET, BRAF, and MYC in epithelial cells can induce oncogene-driven inflammation, including inflammatory cytokines, chemokines, and angiogenic and growth factors. For cancer-associated inflammation, tumor-associated macrophages (TAMs) derived from monocytes selectively present at the tumor site. TAMs have a dual function in tumor. Although they may kill tumor cells, TAMs produce tumor progression mediators, such as cytokines, proteases, angiogenic factors (i.e., VEGF and PDGF) and lymphangiogenic growth factors [18].

The reduced cancer risk of long-term using aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) is another evidence for the importance of inflammation during tumour progression. The colon cancer risk of users reduced by 40-50%, and could be potentially preventative for oesophagus, stomach and lung cancer. The of chemopreventative mechanisms of NSAIDs are inhibiting COX-1 and COX-2 and regulating the pro-inflammatory cytokine, i.e., TNF-α. Anti-inflammatory therapy is effective towards early tumor progression and malignant conversion. Tumour cells promote cytokines and chemokines attracting leukocytes including macrophages. In late tumor progression, there are surplus inflammatory cells in tumor. Therefore, the overall innate immunity is weakened. The future challenge of cancer therapy is to regain a normal inflammatory network and host response by decreasing tumour-promoting properties (i.e., pro-inflammatory cytokines) and increasing tumour-suppressing properties (i.e., anti-inflammatory cytokines) [19].
1.2 Nanoparticles as an emerging platform for cancer therapy

The chemotherapeutic drugs often can kill healthy cells and increase the systematic toxicity to the patients. It will be desirable to create chemotherapeutic drug delivery systems can either passively or actively target cancer cells. The first clinical trials of passive targeting nanocarriers were in the mid-1980s, and first market products, liposomal drugs and polymer-protein conjugates, were in the mid-1990s. The doxorubicin in long-circulating PEG-liposomes (Doxil/Caelyx) was proved to enhance the survival of patients with breast cancer, ovarian cancer and refractory Kaposal’s sarcoma. As a protective layer over the surface, PEG slow down the recognition and clearance of liposome by opsonins [20]. The polymer-protein conjugates (SMANCS) was used in clinical application for hepatocellular carcinoma. It was synthesized by covalently linking anticancer protein neocarzinostatin (NCS) to styrene maleic anhydride (SMA) polymers. SMANCS was defined as passive targeting, attributing to leaky tumor vasculature and abnormal lymphatic drainage [21]. In contrast to local administrated SMANCS, PEGylated protein were administrated parenterally. For example, PEG-l-asparaginase (Oncaspar) was approved by FDA in1994 for treatment of acute lymphoblastic leukemia. PEGylated granulocyte colony-stimulating factor (GCSF, Neulasta) has been used for the prevention of chemotherapy-associated neutropaenia. PEGylated protein is a useful tool to increase solubility and stability of protein, reduce protein immunogenicity and reticuloendothelial clearance, and prolong the plasma half-life of protein [22].

Nanocarriers possess many advantages over free drugs. First, the nanocarriers act as protect coating against from degradation and interaction with the biological environment. Secondly, nanocarriers control the pharmacokinetic and drug
biodistribution and enhance the drug absorption into solid tumors. Thirdly, nanocarriers improve the cellular uptake and intracellular penetration. In order to translate nanocarriers to clinical application, the nanocarriers should be long circulating, made from biocompatible and functionalized materials, soluble and colloidal stable under physiological conditions, and exhibiting high uptake in the target cells compared to normal cells [23].

1.2.1 Passive and active targeting

Various drug delivery systems have been studied to reduce side effects of bioactives and improve their biological efficacy in cancer therapy. Among these delivery systems, it is generally accepted that particles in a size of less than 500 nm is able to avoid the effect of RES, leading to a longer circulation time, and therefore have greater chance to target the site of interest [24]. Studies also pointed out that in order to reduce the RES effect by macrophages, particles need to be less than 100 nm in diameter and modified with a hydrophilic surface. The regular method to protect carriers from the RES is coating nanoparticles with hydrophilic polyethylene glycol (PEG) chains [5]. As a result, the half-life of blood circulation has increased by several orders of magnitude.

Nanoparticles with different surface hydrophobic and hydrophilic performance will also be cleared by other parts of the RES, such as the liver and spleen. Nanoparticles surfaces with more hydrophobicity will be preferentially cleared by the liver and spleen, followed by lungs [25]. However, nanoparticles prepared from hydrophilic poly(vinyl pyrrolidone) (PNVP) showed no more than 1% uptake by the liver and spleen and showed 5–10% remained in circulation after 8 hours of injection [26]. However,
nanoparticles prepared by 50% PNVP and 50% N-isopropyl acrylamide showed preferentially taken up by the liver [27]. At the surface of the nanoparticles, hydrophilic polymers could form a cloud of chains to repel opsonin proteins by the steric repulsion forces, thereby delaying and blocking the opsonization process [28].

**Passive targeting**

The enhanced permeability of the blood vessels and the abnormal lymphatic system in tumors allows the nanoparticles to accumulate and release drugs into the tumor cells. While free drugs non-specifically diffusing, passive targeting of nanoparticles to cancer tissue via the leaky vessels relies on the EPR effect [23]. Because of small sizes, molecules diffuse freedom in and out the tumor blood vessels and thus their effective and accumulated concentrations in the tumor drop rapidly. However, due to large sizes, drug-loaded delivery systems cannot diffuse out the tumor blood vessels, as a result of progressive accumulation: the EPR effect (Figure 2) [29]. The enhanced permeability of the tumor blood vessels has the rapid and defective angiogenic properties. In addition, the abnormal lymphatic drainage in tumors allows the nanocarriers accumulate and release drugs nearby the cancer cells. It has been evaluated that the pore size of leaky tumor blood vessels ranging from 200 to 600 nm [30]. Thus, small nanoparticles can extravasate into tumor blood vessels and retain in the cancer tissue through passive targeting, however they are rejected by intact normal vasculature [24]. Studies using different mean size of liposomes concluded that the threshold size for vesicles extravasating into tumors is ~400 nm, while other studies showed that particles with diameters less than 200 nm are more effective [31].
Nevertheless, some limitations exist to passively reach the tumor. (1) The passive targeting depends on the degree of angiogenesis and tumor vascularization which varying with tumor types and sites [32]. (2) the high interstitial pressure of solid tumors has adversely effects on efficiently uptake and even distribution of drugs in the tumor [5]. (3) Some drugs cannot diffuse efficiently because targeting cells is not feasible. The random nature and lack of control could induce multiple drug resistance (MRD).

![Diagram of passive and active targeting](image)

**Figure 2** Passive targeting (1) Nanocarriers selectively extravasate into tumors blood vessels through the leaky vasculature. (2) The influence of the size and EPR effect. Smaller molecules easily diffuse, whereas long-circulating nanocarriers are retained in the tumor. (Reprinted from Ref. 22)

**Active targeting**

In active targeting, the particles selectively bind to specific cells by attaching targeting receptors on the target cell surface to the surface ligands of the nanoparticles by various conjugation chemistries[33]. After binding through ligand–receptor interactions, bound nanoparticles are internalized by target cells, followed by releasing the drug inside the cells. Generally, when using active targeting nanocarriers, it is significant that the surface ligands of the nanoparticles binds with receptors that are uniquely expressed on the target cell surface [34]. To maximize selectivity, it is necessary that the receptor is
overexpressed on target cells than normal cells. Researchers have successfully delivered liposomes to B-cells which contains the anti-CD19 monoclonal antibody (mAb) in the range of $10^4$–$10^5$ copies per cell, while lower density receptors cannot be effectively targeted by B-cells [35] [36].

**Triggered drug delivery**

Triggered drug delivery can change drug biodistribution in response to either exogenous stimuli (e.g. temperature, light, magnetic field, ultrasound or electric pulses) or endogenous stimuli (e.g. pH, enzyme or redox potential) [37]. This article will focus on pH-responsive systems, which are predominantly studied.

Efficient pH-sensitive delivery systems sensitively respond to subtle pH variations in the tumor microenvironment. pH variations have been utilized to control the delivery and release of drugs in specific organs (e.g. gastrointestinal tract [38], colon [39]), intracellular compartments (e.g. endosomes or lysosomes [40]), and also subtle environment whose changes are related to pathological situations (e.g. cancer or inflammation). For example, at the cellular level, pH-responsive systems can release the loaded drug into late endosomes (pH ~5–6) or lysosomes (pH ~4–5), and the delivery systems will escape from lysosomes to the cell cytoplasm [41]. Two main strategies exist to design a pH-responsive system in response to pH variation, we can either take advantage of conformational [42] and/or solubility changes of ionizable groups in polyacids or polybases [43], or the cleavage of acid-sensitive bonds or acid-degradable crosslinking between anchored molecules and polymer backbones [44]. pH-sensitive conjugates also mediate the release of drugs covalently bonded to polymer backbones, protein scaffolds, and mesoporous silica nanoparticles (MSNPs) pores. For example,
MSNPs with β-cyclodextrin nanovalves are responsive to acidic endosomal microenvironment [45].

pH-sensitive delivery systems have utilized the slight pH variation between normal tissues (~7.4) and the extracellular tumorous environment (6.5–7.2). Due to abnormal angiogenesis in fast-growing tumors with high glycolysis, the lower pH in the tumor is caused by a high production of acidic metabolites. For example, amino-group protonation (pKa ~6.3) caused by chitosan swelling results in the release of loadings in the local acidic tumorous environment [46]. PEG–Poly (β-amino ester) micelles sudden disassembling at pH 6.4–6.8 triggers the leakage of entrapped camptothecin [47].

1.2.2 Degradable nanoparticles, protein nanoparticles

Nanoparticles are solid colloidal particles with size ranging from 10nm-1000 nm. The major goal of designing nanoparticles is drug targeting and controlling the body distribution of loaded drugs. Nanoparticles with modified particle size and surface properties offer distinct advantages for drug delivery and drug targeting [48]. First of all, the particle size [49], surface charge [50] and particle morphology [51] can be controlled. Secondly, nanoparticles can carry a variety of active agents and release molecules in a controlled manner, such as hydrophilic [52] or hydrophobic bioactive compounds [53], peptides [54] and proteins [55]. The encapsulated bioactive compounds can be released from nanoparticles in a precise and sustained manner to maintain drug concentrations over time, or the drug release can be responsive to stimuli unique to the delivery environment, such as pH [56], temperature [57], light [58] and ionic strength [59]. Thirdly, nanoparticles not only enhance the solubility, stability and bioaccessibility of
drugs [60], but reduce the undesired toxic side effects to the rest of the body [61]. Lastly, with their enhanced accessibility in the body and enhanced cellular uptake, nanoparticles have a prolonged blood circulation time and more chance to be transported to different body sites via the circulation [62].

Nanoparticles are versatile carriers because of the variety of materials that have been used for encapsulation [63]. Nanoparticles have also been fabricated from a variety of food-grade materials, including proteins, polysaccharides, lipids, phospholipids, and surfactants. Moreover, these ingredients can be combined to form composite nanoparticles; For instance, nanoemulsions (lipid droplet stabilized by a protein or surfactant) [64], Maillard reaction conjugates (covalently linked proteins and carbohydrates) [65] and complex coacervates (electrostatically linked two oppositely charged proteins and polysaccharides) [66]. The choice of materials depends on many factors including (a) particle sizes, (b) intrinsic properties of drugs such as stability and aqueous solubility, (c) desired drug loading and release profile, (d) surface properties of nanoparticles including surface charge and hydrophobicity, (e) biodegradability and biocompatibility, and (f) cytotoxicity and antigenicity [67]. Nanoparticles have been proved to be able to encapsulate, protect, and release bioactive components, thereby improving their stability and bio-efficacy.

Proteins have a variety of amino acid composition capable of chemical modification for drug encapsulation. Nanoparticles have been successfully prepared by both water-soluble proteins (e.g., whey protein [68], β-lactoglobulin [69], gelatin [70], casein [71], albumin [72] and lactoferrin [73]) and insoluble proteins (e.g., corn [74] and wheat [75]). However, water-soluble proteins have limitations due to the restricted ability
to sustain drug release. Because of the simple and low cost phase separation to produce nanoparticles, a lot of research has been done to utilize the hydrophobic cores of water-insoluble protein to form nanoparticles. Therefore, the application of hydrophobic zein, which are specifically act as reservoirs for lipophilic bioactive substances or drugs due to the strong hydrogen bond and hydrophobic interaction between nutraceutical and proteins, will be covered in this paper.

1.2.3 Zein-based nano/microparticles preparation

Zein, an alcohol-soluble prolamins isolated from mazine, was approved by the US Food and Drug Administration as GRAS (generally-recognized-as-safe) excipient since 1985. On the basis of solubility and sequence similarity, zein is classified into 4 groups [76]: 19 and 22 kDa α-zein, 14 kDa β-zein, 16 and 27 kDa γ-zein and 10 kDa δ-zein. Among these, α-zein and γ-zein include about 80% and 10-20% of total zein mass, respectively. α-Zein consists of eminently homologous repeat units with a high α-helix content[77]. Zein is primarily composed of neutral and hydrophobic amino acid residues (e.g., alanine, proline and leucine) but also comprises some polar amino acids (e.g., glutamine) [78]. Thus, zein is restricted to limited solvents including aqueous alcohols (up to 70% ethanol), alkaline solution (pH > 11), acetone and acetic acid. Zein behaves like polymer and polyelectrolytes in aqueous alcohol solution and acetic acid, respectively.

Phase separation, also known as anti-solvent precipitation or liquid-liquid dispersion, has been generally used to prepare zein-based nano/microparticles (Figure 3) [79]. Drug molecules or nutraceuticals could be covalently conjugated to zein or
physically dispersed in zein-based nano/microparticles [80]. Briefly, zein and drug are dissolved in an 70%-90% aqueous alcohol solution, followed by adding to the bulk deionized water with or without other ingredients. Many factors may influence the resultant particle properties, such as drug loading and release profiles. A reduced final zein concentration, an increased mixing shear rate or an enhanced initial alcohol concentration could all reflect on smaller nanoparticles.

![Fabricate zein-based micro/nanoparticles using phase separation method.](Reprinted from Ref. 86)

Because the isoelectric point of zein is 6.2, individual zein nanoparticles usually lose colloid stability and demonstrate a strong aggregation tendency in neutral pH both at product conditions and at physiological pH in the intestine [81]. Studies have showed that maintaining the pH of the water phase slightly higher than the isoelectric point of zein can result in a smaller particle size.

The application of zein delivery systems in food and pharmaceuticals has been extensively studied due to its biodegradability, biocompatibility, and low toxicity. Besides, applications of zein-based delivery system would add extra value to the byproduct of corn starch and ethanol-producing. However, the practical applications of
drug loaded zein nanoparticles are restricted by two challenges. First, due to the strong hydrophobicity, zein nanoparticles are commonly prepared via phase separation method. Although it is a relatively easy technique, the resultant zein colloidal dispersion usually form poorly redispersable aggregates and sediments after drying [81]. Second, high energy technique, including high speed homogenization and supercritical carbon dioxide, are commonly used when preparing zein nanoparticles, and therefore the obstacles of large-scale industrial production limit the application of zein nanoparticles [77].

To overcome the above obstacles, many researchers are making efforts to prepare stable, homogeneous, redispersible and desired drug release profiles of zein nanoparticles by low energy methods. Recently, formation of complex zein nanoparticles with other biopolymers has been investigated as a promising approach to tailor the surface properties of particles. Introducing interactive biopolymers into the formulation can produce zein- biopolymer composite colloidal particles resulting in improved colloidal stability, functionality and more sustained drug release. For example, anionic polymers modified zein nanoparticles, such as gum Arabic, alginate and pectin, have been proved to have better physical stability than pure zein nanoparticles by reducing aggregation and sedimentation in aqueous solutions [82]. Other coating materials, such as sodium caseinate [81], carboxymethyl chitosan [83] and polyethylene glycol (PEG) [84], are proved to improve the membrane permeability and cellular uptake of zein nanoparticles and result in enhanced bioavailability of loaded drugs. Moreover, because more and more consumers demand for clean labels, we limit ourselves to using natural ingredients to stabilize the zein nanoparticles, such as proteins and polysaccharides.
1.2.4 Zein-based polymeric complex delivery systems

Bare zein nanoparticles are relatively unstable and tend to aggregate in aqueous solutions due to their high surface hydrophobicity. Therefore, hydrophilic or amphiphilic biopolymers are investigated to stabilize and improve the water dispersibility of zein nanoparticle. The polymer–polymer complexes-based drug delivery systems could take advantages of multiple composed polymers in the complexes, such as, delayed digestion [76], sustained release [85] and improved mucoadhesive property [86], etc. As a weak polyelectrolyte, zein interacts with polysaccharides or proteins to form complexes through hydrophobic interactions and hydrogen bonds.

Amphiphilic proteins (i.e. sodium caseinate [81], lactoferrin [87] and β-lactoglobulin [88]) could adsorb to the surfaces of the exposed neutral and hydrophobic amino acid residues of zein nanoparticles, and thus reduce the surface hydrophobic attraction between individual zein particles. Studies of zein-casein nanoparticles showed a sustained drug release and therefore the retention for systemic drug absorption in the rat gastrointestinal tract was as long as 24 h. Besides, zein-lactoferrin nanoparticles has been proved to increase the cellular uptake through receptor mediated endocytosis and can be recognized by intestinal lactoferrin receptors. Lactoferrin, an iron-binding glycoprotein, has high nutritional value as an iron transporter, and as well high biological value including antioxidant, antibacterial, antiviral, and anticancer properties Lactoferrin has also been used as targeting ligand to improve the drug delivery across intestinal epithelial barrier. However, protein-coated zein nanoparticles (i.e. sodium casein) still shows the limitations of overcoming particle aggregation because they are highly sensitive to the pH
values that are near the isoelectric point of the coated protein layer. At the pH equals to this point, the electrostatic repulsion between particles is not enough to overcome the attractive interactions between each particle (i.e. van der Waals and hydrophobic). Researches has previously addressed this problem by coating zein with caseinate-maltodextrin conjugates formed by Maillard reaction [89]. Also, electrostatic complexes coating of alginate and gelatin has also been used to enhance the antioxidant activity and bioaccessibility of curcumin under simulated gastrointestinal conditions [90]. The attachment of carbohydrate and protein could successfully improve the stability of zein nanoparticles by increasing the steric repulsion between zein particles.

Polysaccharides also have been used to improve the aggregation stability of zein nanoparticles by generating strong steric hinderance and electrostatic repulsion between particles. Among zein-polysaccharides complex delivery systems, chitosan coated zein nanoparticles is the most investigated one. Chitosan coated zein nanoparticles can be tailored through ionic gelation or phase separation for hydrophilic and hydrophobic compound encapsulation, respectively. For example, selenite loaded zein-chitosan nanoparticles [86] provided significant higher encapsuation efficiency and controlled release profile in gastrointestinal tract compared with zein nanoparticles without coating. However, zein-chitosan nanoparticles had several limitations. First, the improvement of release profile is limited due to the fast dissolution of chitosan in acidic condition, unless high polymer concentrations were used. Second, the freeze dried zein-chitosan nanoparticles have a poor water redispersibility due to the water-insoluble property of chitosan. To overcome this obstacle, the water soluble carboxymethyl chitosan (CMC) as an alternative coating on zein surface encapsulating vitamin D3 [91] and indole
compounds [83]. CMC forming gels at acidic pH makes it a better barrier to protect zein against enzymatic degradation. The strong negative surface charged CMC increases the repulsive forces among nanoparticles and forms well-separated zein nanoparticles with small energy input.

Zein-pectin nanoparticles [92] is another popular zein-polysaccharides complex delivery systems. Pectin is a natural and negative charged polysaccharide. Zein-pectin nanoparticles has drawn the attention because of the unique digestibility of pectin. It has been widely used in colonic targeting drug delivery because pectin stays intact in gastrointestinal tract while be broke down by pectinolytic enzymes and microflora in colon. However, the high water-soluble pectin negatively affected its drug delivery efficiency due to rapid dissolution, hydrophobic zein is a promising candidate to form complex with pectin to sustain the drug release. Zein-pectin hydrogel beads have been fabricated and observed under fluorescence microscopy. It indicated that zein was located throughout the beads and largely distributed in the periphery of the matrix, which not only sustained the swelling of water-soluble pectin in gastrointestinal tract but also shielded the drug loaded zein from protease digestion. In conclusion, zein-pectin hydrogel beads provided minimal drug release in gastrointestinal tract and sustained release in the colon, which has been proved with piroxicam though both in vitro dissolution test and ex vivo model with rat cecum.

Polyethylene glycol (PEG) is approved by FDA and widely used as water-soluble excipient in delivery systems. Besides, PEG has also been utilized to modify the surface hydrophobicity of nanoparticles and proteins. It has been reported the formation of PEGylated zein and the hydrophobic zein could form self-assembled micelles with the
modification of hydrophilic PEG chains [93]. Zein-PEG micelles can especially enhance the bioavailability of drugs that are hypersensitive to P-gp efflux. Zein-PEG micelles has been proved to improve the chemical stability and water solubility of curcumin. Moreover, compared to free curcumin, the curcumin-loaded zein-PEG micelles demonstrated more efficient cellular uptake and transepithelial permeability in drug resistant cancer cells due to the hydrophilic property of PEG [79].

1.2.5 Zein-based pH-sensitive delivery systems

A usual release stimulus for targeted release in drug delivery is the pH gradient of the extracellular regions between diseased tissue (pH 5.6-7.0) and healthy tissues (7.4). pH-responsive zein nanoparticles are stable self-assemblies that meet the requirements of biocompatible and biodegradability. The breakdown of pH-responsive bonds initiates the degradation of the pH-responsive zein nanoparticles and the controlled release of loadings at low pH. Thus, design pH-sensitive zein nanoparticles can selectively deliver loadings into cancer cells by endocytosis is promising in cancer therapy.

Hongshan et al. [94] have described novel pH-responsive zein-based nanoparticles based on the coordination bonding between metal ions and tannic acid (TA). TA, a polyphenol coupled to glucose through ester bonds, contains a large number of hydroxy groups with water soluble and biodegradable properties. The spontaneous complexation of metal ions and the TA catecholic unit, together with the adhesion of TA on protein particles were utilized to form coating on zein nanoparticles to control pH-dependent process and intracellular drug delivery based on prolonged retention in stimulated physiological conditions and enhanced cellular uptake in drug resistant cancer
cells. After crosslinking of metal ion-TA, the PTX-loaded zein nanoparticles were intact at physiological pH where one metal ion forms stable films with three TA molecules (Figure 4). At pH < 7 PTX was released from the core because the acetal groups were hydrolyzed and metal ion forms mono- or bis- complexes with TA [95].

![Figure 4 pH-dependent coordinated bonding between catecholic moieties and iron(III). (Reprinted from Ref. 102)](image)

**1.3 Cellular uptake**

**1.3.1 Uptake mechanisms**

The primary consideration of nanoparticle-based therapy is the precise release of drugs in target organs, tissues, and cells. In order to reach their target, drug-loaded nanoparticles must get over numerous transport barriers [96]. For example, crossing plasma membrane barrier is the precondition for effective translocation of nanoparticles in an intracellular level. Thus, the detailed background of the internalization mechanisms of nanoparticles is significant. The plasma membrane is a highly complex environment, which is necessary for different types of cells developing the normal function. Ions and small molecules (e.g. glucose or amino acids) are transferred through the plasma membrane by specialized transporters, while larger molecules such as NPs are
internalized through endocytosis. Endocytosis occurs as vesicular active transport. Intracellular membrane-enclosed vesicles are generated from the plasma membrane, and vesicles are enclosing and transporting lipids, proteins, and extracellular fluid into the cell. On the contrary, where inner vesicles are fused with the plasma membrane to release the molecules extracellularly is termed exocytosis. The highly dynamic and well-regulated endocytosis and exocytosis are crucial to regulate cellular adhesion, communication, and division. Cells can approximately endocytose fluid up to five-times of their volume within one hour [96].

The number of nanoparticles are remained inside the cells as long as nanoparticles are existing in the external medium. Once the outer concentration gradient is disappeared, the exocytosis begins and leads to a loss about 65% of the initial level in 30 min. In the meantime, at least 15% of uptake could be maintained after 6 h. Interestingly, this nanoparticle exocytosis was only found in medium containing serum, which was the reason of inducing nanoparticle exocytosis. Albumin in serum is likely adsorbed onto nanoparticles and transferred along with nanoparticles, which result in an increased exocytosis of nanoparticles [97].

While, the drop of in vivo extracellular nanoparticle concentration is unlikely so rapid. It has been previously confirmed that drug levels in the vascular tissue could maintain for at least 7 – 14 days once nanoparticles are translocated into the tissue. Therefore, there could be a constant sustaining of nanoparticles near the tissue, which might cause mass transport equilibrium and in turn higher intracellular nanoparticle concentrations. After administration into the body, nanoparticles confront with many different types of cells. Even though the in vivo dynamics of endocytosis and exocytosis
are quite different from that of *in vitro*, it is still crucial to understand the endocytosis mechanisms, intracellular route and the factors affecting the internalization of nanoparticles [97]. Also, it is important to learn the endocytosis mechanisms to further explore novel drug delivery systems in treatment of complex diseases.

Phagocytosis and pinocytosis are the two main subgroup of endocytosis. Phagocytosis is limited to professional phagocytes: macrophages, monocytes, neutrophils and dendritic cells. Phagocytosis is actin-dependent, and it happens when phagocytes internalize foreign materials in a size of larger than 0.5μm, which is also part of the natural immune system [98]. Whole bacteria and cells with tens of micrometers in size could be phagocytosed by phagocytes after the foreign materials has been opsonized and recognized by the phagocytes.

Macropinocytosis occurs for non-specific internalization of solute more than 0.2 μm diameter, while micropinocytosis, including clathrin/caveolae-independent, clathrin-mediated and caveolin/lipid raft-mediated involves smaller solute in all cell types. Given that the size range of NPs usually used for cancer therapeutics is 10–200 nm, cancer cells would internalize nanoparticles through micropinocytosis. Both the five different endocytic pathways and phagocytosis are demonstrated in **Figure 5** [99].

In order to study the endocytic pathways, the different inhibitors could be used to selectively inhibit and block one of the critical steps of the pathways. For example, chlorpromazine selectively inhibits clathrin-mediated endocytosis by blocking the supplement of clathrin, therefore the assembling of clathrin coated vesicles are limited. All of the endocytic pathways can be inhibited by culturing at 4°C.
1.3.2 Intracellular route

*Clathrin-mediated endocytosis pathway*

The most discussed endocytosis for eukaryotic cells internalizing materials is Clathrin-mediated endocytosis [100]. Clathrin-mediated endocytosis involves uptake of nutrients, intercellular signaling and membrane recycling. Vesicle formation starts with creating a curvature in the membrane, extensive protein machinery participates this process, such as the FCHo2 F-BAR domain, epsin, amphiphysin, endophilin. Binding vesicle with receptors are important for creating the spherical clathrin-coated pit, such as adaptor protein complexes (e.g., the AP-2 heterotetrameric complex, AP180), and clathrin assembly lymphoid myeloid leukemia protein. GTPase dynamin formed as a ring surrounding the neck of clathrin pit can release the vesicle from the plasma membrane. Then the clathrin pit will disassemble by heat shock cognate 70 proteins and auxilin. Nanoparticles endocytosed via clathrin-mediated endocytosis enter early endosomes with a low pH and eventually metabolized in lysosomes. Clathrin-mediated endocytosis is the major receptor-mediated cellular uptake for nanoparticles. Although the mechanisms of the internalization of non-targeted nanoparticles are not completely understood, it seems that nanoparticles in a size of ~100 nm and particularly positively charged are more likely internalized by cells via clathrin-mediated endocytosis under normal conditions. Nanoparticles with sizes up to a diameter of ~150 nm was found via Clathrin-mediated endocytosis, while larger particles were reported to utilize caveolin-mediated internalization [96].
Caveolin-mediated endocytosis pathway

Caveolin-mediated endocytosis forms flask-shaped vesicles by caveolin-oligomerization in the plasma membrane. The vesicles are cut off from the plasma membrane by dynamin 2. The material internalized through caveolin-mediated pathway is first localized in vesicles called caveosomes, which can unite with early endosomes or directly fuse with lysosomes after the internalization.

Caveolin-mediated endocytosis generally captures smaller volumes and particles of a size of around 60-80nm, and the main cellular processes includes cholesterol homeostasis, internalization of proteins and signal transduction. However, caveolin involves in the uptake superior to clathrin-mediated endocytosis for particles above 200nm and some pathogens. Also, it has been found that negatively charged nanoparticles primarily trigger cellular internalization via caveolins [101].
**Clathrin-independent endocytosis pathway**

Clathrin-independent endocytosis pathway participates the entry for bacterial toxins, plasma membrane repair, cellular spreading, cellular polarization, and intercellular signaling. Instead of coat proteins, the actin and actin-binding proteins are required for vesicle formation and internalization during clathrin-independent endocytosis. Cargos internalized through clathrin-independent endocytosis are either transferred to endosomes/lysosomes pathway, or recycled back to forming the plasma membrane. For example, clathrin-independent endocytosis is the preferential internalization pathway for polyplexes of chitosan oligomer nanoparticles for DNA delivery [102].

**Macropinocytosis**

Macropinocytosis is an actin-dependent non-specific endocytosis. Cells could internalize large volumes of extracellular fluid by macropinosomes (diameter of 0.5–10 μm) via macropinocytosis. Micron-size particles are usually internalized by cells via macropinocytosis. Macropinocytosis also participates the uptake of apoptotic cell fragments, bacteria and viruses. Macropinocytosis is not directly regulated by receptors or cargo molecules. Therefore, the start of a tyrosine kinase receptor results in actin polymerization, followed by actin-mediated ruffling, and vesicle (macropinosome) formation. Moreover, macropinosomes and other endocytic routes share some same proteins, such as Cdc42, Arf6, and Rab5, suggesting a connection between macropinocytosis and other endocytic processes. The macropinosomes are highly
sensitive to cytoplasmic pH and undergo a fate similar to endosomes before fusing with lysosomes [96].

1.3.3 Cellular uptake of nanoparticles

The composition of the engineered nanoparticles must be carefully selected based on the biocompatibility and immunotoxicity, which means that it should not be toxic to normal cells or taken up by phagocytic cells in the immune system [103]. Biocompatible also suggests that the composition of nanocarriers are either degradable or able to be removed by the kidneys to avoid accumulation. Recent researches have revealed that sizes, shapes, and surface chemistries of nanoparticles govern the endocytosis and subsequent cytosolic access of nanoparticles in different types of cells [98] and in vivo biodistribution [104][105]. For example, cationic nanoparticles, which could form ionic interactions with tissues and cells, is more efficient in cell membrane penetration than anionic nanoparticles and are internalized by live cells via different pinocytosis pathways [97]. Likewise, particles with diameters less than 150 nm were more likely through clathrin-coated pits, whereas caveolin-dependent endocytosis became predominant with increasing particle size. Also, surface functionality of nanoparticles also plays a crucial role on endocytosis pathways. Recently, studies have reported that quantum dots with non-specific ligands on the particle surface were primarily internalized by human kidney and liver cells via caveolin-mediated endocytosis [99].

Size
The uptake mechanism of nanoparticles is dependent on their size. For example, the gold nanoparticles with a size of 50 nm showed the most efficient endocytosis compared with other sizes, whereas the cellular uptake of 100 nm polystyrene (PS) nanoparticles into human colon adenocarcinoma cells was more efficiently than those with other sizes. The internalization efficiency of the 50 nm PS nanoparticles was the lowest of all sizes [106]. Thus, these two studies show that the endocytosis pathway could vary according to nanoparticle size, as well as nanoparticle materials.

Particle size is known to impacts the mechanism of cellular uptake, which includes phagocytosis, macropinocytosis (>1 μm), clathrin-dependent endocytosis (~120 nm), caveolar-dependent endocytosis (~60 nm) or clathrin-independent endocytosis and caveolin-independent endocytosis (~90 nm) [107][108]. In the meantime, particle size also influences the biodistribution profile of spherical particles and non-spherical particles [109]. The same general principles govern these particles: uptake by the liver and spleen should be avoided to prolong blood circulating of particles [110]. Rigid, spherical particles with sizes in 100–200 nm have the biggest potential for long circulation. While for non-spherical particles, engineering particles >300 nm or maintaining two sides of length on a length scale <200 nm and at least one sides of length >100 nm can prolong the blood circulation of particles [111].

In biological fluids, such as blood, plasma, saliva, and cell culture medium, most nanoparticles tend to aggregate and increase their overall size. Aggregates (nanoparticles strongly bonded) and agglomerates (nanoparticles loosely bonded under weak forces, e.g. van der Waals force) behave differently within biological conditions compared with nanoparticles in their individual form [98]. Therefore, size uniformity of nanoparticles
before they enter the cell should be taken into consideration when the size-dependent endocytosis of nanoparticles in biological systems is examined.

If the nanoparticles are already forms aggregates or agglomerates before binding to the cell membrane, their endocytosis pathway would be different from the endocytosis pathway of individual nanoparticles. Pre-coating of the nanoparticle surface with secondary layer such as polyethylene glycol (PEG) or albumin could reduce ionic strength, enhance nanoparticle stability and prevent nanoparticles from agglomeration or aggregation in the biological solutions. Therefore, coating nanoparticle with secondary stabilized molecules is very necessary for us to learn how single nanoparticles interact with different types of cells.

**Surface chemistry**

Surface characteristics of particles play three key roles in the functionalities of formulated nanoparticles. First of all, surface chemistry is related the process of opsonization and the RES response of particles. The main recognition of foreign particles for RES are immunoglobulin and complement proteins. The strategy is to adsorb a hydrophilic polymeric coating on particle surface, such as PEG, which acts as a steric brush that provides resistance to protein adsorption [112]. Secondly, ligands bind target cell surface receptors to achieve cellular targeting in the nanoparticles design [113]. Thirdly, ligands also required in organelle targeting [114]. Thirdly, The efficiency and pathway of endocytosis could also be influenced by the surface charge of nanoparticles, because biological systems contain various charged biomolecules. Therefore, the
endocytosis pathway of nanoparticles could be highly impacted by the charge of biomolecules covering the nanoparticle surface.

Because of negatively charged cell surface, positively charged particles would expect to be more efficiently endocytosed by cells compared to negatively charged particles. For example, a study in HeLa cells suggested that the positively charged particles showed a 2-fold higher uptake compared with negatively charged nanoparticles with the same particle size (80 nm). However, a higher endosytosis of negatively charged nanoparticles has been demonstrated in HEK cells [115]. Further, due to different surface charges, the uptake of negatively charged carboxymethyl chitosan-grafted nanoparticles and positively charged chitosan hydrochloride-grafted nanoparticles by macrophages were significant different. It was revealed that negatively charged nanoparticles with a size of 100 nm were more easily phagocytized by macrophages compared to positively charged nanoparticles [98].

Recently, more studies have been focusing on utilizing biological nanomaterials to modify the surface functionalization of drug delivery systems, because of their biocompatibility and inherent cell-binding properties. For example, apoferritin was used as a drug carrier to enter the cells through ferritin receptor-mediated endocytosis [116]. Additionally, PEG and poloxamine polymers not only reduce ionic strength and promote particle dispersion, but also prevented nanoparticles phagocytosis by macrophages [117].

Three different zein-polymer complexes including zein-PEG (ZPEG) micelles, zein-lactoferrin (ZLF) nanoparticles and zein-casein (ZC) nanoparticles were fabricated to study the effect of surface materials on the endocytosis pathways using different
endocytosis inhibitors [63]. Phenylarsine oxide, an inhibitor of clathrin-dependent endocytosis, reduced the endocytosis of ZLF and ZPEG by 50% and 36%, while ZC showed only a small decrease in the uptake. The cell uptake of the three formulations decreased by minimal amount (12–25%) without significant difference when filipin was used to determine lipid raft mediated endocytosis. In the present of cytochalasin-D, an inhibitor for a nonspecific process—macropinocytosis, there were 64% and 42% decrease of internalization of ZPEG and ZC respectively, on the other hand, the reduced internalization of ZLF was only 20%. Besides, the involvement of lactoferrin receptor mediated endocytosis of ZLF were proved by competitive inhibition studies with the treatment of free lactoferrin. In addition, in the presence of calcein which is a P-glycoprotein substrate, ZPEG showed the strongest concentration dependent P-glycoprotein inhibitory effect among the three formulations. In conclusion, the results indicated that the surface composition influences the endocytosis mechanism of transcellular transport of zein nanoparticles. Nevertheless, further studies are required to understand the mechanism of the intracellular and transcellular transport of zein-based nanoparticles.

Shape

Current findings suggest that particle shape is as crucial as particle size in controlling biodistribution and cellular internalization of engineered nanoparticles. For example, the circulation half-live of filamentous micelles with a monofilament size of up to 18 μm were reported around 5 days [118], while micrometre-sized rigid spherical particles are cleared from the blood circulation almost immediately [62]. What’s more, it was reported that the elliptical disk-shaped polystyrene microparticles were rapidly
internalized within 6 minutes when the macrophage first contacted particles along the major axis [119]. However, when the minor axis first contacted by macrophage, the particles were still not taken up after 12 hours. For symmetry spheroids, they were rapidly and uniformly internalized by macrophage [120].

Particle geometry is also important for particle internalization. The most efficient uptake in cervical cancer cells was first nanoscale rods and then spheres, cylinders and cubes [121]. The endocytosis of cylindrical particles strongly depends on their aspect ratio, whereas the receptor-dependent endocytosis of gold nanorods immensely reduced with increasing of aspect ratio. Recent experiments have showed a shape effect of nanoparticles on uptake efficiency in many types of cells. As a result, it was found that macrophages internalize rod-shaped nanoparticles more efficiently compare to spherical nanoparticles. On the contrary, the spherical nanoparticles were more efficiently endocytosed by human cervical cancer cells or lung epithelial cells compared to rod-shaped nanoparticles. Although preliminary data demonstrated the significance of particle shape, the further studies of optimum parameters for drug delivery nanoparticles are still required.

In all, the size, shape, surface chemistry and targeting groups of nanoparticles control the properties of nanoparticles. The nanoparticles fabricated in this subject falls under the category degradable protein nanoparticles and will be illustrated in detail in the following section.
1.4 Carboxymethyl konjac glucomannan (CKGM)

Konjac glucomannan (KGM) is derived from tubers of *Amorphophallus konjac* C. Koch with high molecular weight, water-soluble, and non-ionic properties. Linear KGM is formed from β-D-glucose and β-D-mannose residues with 1: 1.6 molar ratio. Every 9–19 sugar units has one acetyl group located at the C-6 position of KGM backbone. Carboxymethyl konjac glucomannan (CKGM) is formed from carboxymethyl modified KGM (Figure 6) [122]. CKGM and its derivatives is believed to be a promising new biodegradable material for fabricating eatable films, drug delivery systems, enzyme encapsulation, and adsorbing heavy metal ions. This hydrophilic ionic polysaccharide (CKGM) is a promising coating material for Z NPs to balance the hydrophobic/hydrophilic property of zein. Moreover, the carboxyl groups could form coordination bonding and crosslink with metal ions, etc. Ca$^{2+}$ [123]. Because of the relatively high negative charge density, CKGM could potentially stabilize zein nanoparticles over a wider pH range with a lower usage compared to other polysaccharides. The aim of this study is to utilize CKGM to enhance the stability of zein nanoparticles, and further increase cellular uptake in cancer cells.

![Carboxymethylation reaction of KGM to CMKGM](image)

Figure 6 The carboxymethylation reaction of KGM to CMKGM. (Reprinted from Ref.
1.5 Andrographolide (AG)

Because of the anti-inflammation and anti-cancer properties, andrographolide (AG) and its derivatives, which are labdane diterpenoid compounds extracted from Andrographis paniculata Nees, are often used as herbal drugs in Asian countries. Indeed, andrographolide belongs to the electrophilic natural products in the Michael acceptor system category. Indeed, the $\alpha$, $\beta$-unsaturated $\gamma$-lactone moiety structure of AG enables its forming covalent bonds with nucleophilic centers of proteins, such as NF-$\kappa$B. Also, studies have demonstrated the $\alpha$, $\beta$-unsaturated lactone moiety of andrographolide reacts with free and reduced glutathione (GSH) via a Michael addition at the $\Delta$ exocyclic double bond and then dehydrating of the adduct [124]. Andrographolide is a colorless and crystalline bicyclic compound with a very bitter taste. AG sparingly soluble in water (3.29$\mu$g/ml at 25 °C), which restricts its therapeutic use due to low bioavailability by oral administration. The bioavailability of andrographolide determined in rabbits suggested that the $C_{\text{max}}$ value of andrographolide was 0.09 ± 0.02$\mu$g/mL and the $\text{AUC}_{0-12h}$ value was 109.75 ± 4.07 ng h/ml. Recently, there are several delivery systems improving the solubility and bioavailability of andrographolide, such as hydroxypropyl-beta-cyclodextrin complex, solid dispersion with polyvinylpyrrolidone, and self-microemulsifying drug delivery system (SMEDDS) with Cremophor RH 40, Capryol 90 and Labrasol [125]. In this study, zein nanoparticles is a promising delivery system with decreased side effects of drugs to healthy cells and enhanced tumor delivery.
1.6 Dihydromyricetin

Dihydromyricetin (2,3-dihydroflavonol, DMY, Figure 7) extracted from *Ampelopsis grossedentata* found in South China, has been effective in treatment of cough, liver diseases, hypertension and high blood sugar. Additionally, DMY has antioxidant, antibacterial, antiviral, and antitumor properties. Moreover, due to its high degree of superdelocalizability, appropriate spatial configuration, strong conjugated large π bond and coordinated oxygen atoms, DMY acts as a good metal ion-chelating ligand [126]. Some DMY-metal complex shows better anticancer activity and antioxidant than free DMY. However, the in vivo activities and mechanisms of different DMY-metal complex still remain uncertain, and the related transport, bioavailability and metabolism are still unanswered [127]. However, the biological properties of DMY has been restricted by its low oral bioavailability (no more than 10% in rats), which is probably resulted from the combined effects of poor permeability (Peff = (1.84 ± 0.37) × 10⁻⁶ cm/s) and low solubility (0.2 mg/mL at 25 °C) [128]. For Class IV compounds belonging to the biopharmaceutics classification system (BCS), it is very important for DMY to improve either solubility or permeability to enhance their clinical performance.
Figure 7 Backbone structures of flavonol and dihydromyricetin.

The relative low bioavailability and oral absorption of phenolic compounds are mainly attributed to their low solubility, low permeability, poor stability and active efflux by P-glycoprotein process, and fast metabolisms in the gastrointestinal tract. However, the aqueous solubility of phenolic phytochemicals may be enhanced by nanoparticles encapsulation. Nanoparticles could also prevent phenolic compounds against the oxidation and degradation. What’s more, nanoparticles could possibly enhance the absorption and permeability of phenolic compounds by disrupting tight junctions and cellular uptake by epithelial cells though endocytosis [129].

Common routes for pH-sensitive systems involve protonation of copolymers or pH-sensitive bonds. First, pH-responsive systems can be developed from copolymers that switch between swollen (hydrophilic state) and collapsed (hydrophobic state) based on acidic or alkaline blocks, which result in protonation and deprotonation of reactive functional groups, such as amine and carboxylic acid groups [40]. Changing from a hydrophobic state to a hydrophilic state results in the polymer solubilization and the disassembling the architecture of the nanocarriers. Secondly, pH changes affect the
forming and breaking down of metal ion–ligand coordination bonds because at lower pH metal ions could compete with protons in acids for binding with ligand [94].

Figure 8 The proposed chelation of Fe$^{2+}$ by dihydromyricetin (DMY). (Reprinted from Ref. 123)

The Fe$^{2+}$-chelating property of DMY was proposed to result from carbonyl groups and hydroxyl groups at ortho position, such as the 4-C=O group and the 5,3′,4′,5′-OH groups (Figure 8) [130]. The planar configuration of sp2 carbons of DMY can easily form a stable five-member ring or six-member ring in Fe$^{2+}$ chelation. However, because the C-3 is sp3 hybridized, which is an R-configuration, the 3-OH group and the ortho carbonyl group (4-C=O) does not share a planar, making it impossible to form a stable ring through the chelation of Fe$^{2+}$. Also, the 2,3-double bond in DMY was confirmed being important for stable copper chelation. The chelation ability of DMY and metal ions can be utilized to form robust pH-sensitive zein NPs to control release DMY.
CHAPTER 2: PREPARATION AND CHARACTERIZATION OF ZEIN-CARBOXYMETHYL KONJAC GLUCOMANNAN NANOPARTICLES FOR ANDROGRAPHOLIDE ENCAPSULATION

2.1 Introduction

Because of the anti-inflammation and anti-cancer properties, andrographolide (AG) and its derivatives, which are labdane diterpenoid compounds extracted from Andrographis paniculata Nees, are often used as herbal drugs in Asian countries. Indeed, andrographolide belongs to the electrophilic natural products in the Michael acceptor system category. Indeed, the $\alpha, \beta$-unsaturated $\gamma$-lactone moiety structure of AG enables its forming covalent bonds with nucleophilic centers of proteins, such as NF-$\kappa$B. Also, studies have demonstrated the $\alpha, \beta$-unsaturated lactone moiety of andrographolide reacts with free and reduced glutathione (GSH) via a Michael addition at the $\Delta$ exocyclic double bond and then dehydrating of the adduct. Andrographolide is a colorless and crystalline bicyclic compound with a very bitter taste. AG sparingly soluble in water (3.29 $\mu$g/ml at 25 °C), which restricts its therapeutic use due to low bioavailability by oral administration. The bioavailability of andrographolide determined in rabbits suggested that the $C_{\text{max}}$ value of andrographolidethe was 0.09 ± 0.02 $\mu$g/mL and the $AUC_{0-12h}$ value was 109.75 ± 4.07 ng h/ml. Recently, there are several delivery systems improving the solubility and bioavailability of andrographolide, such as hydroxypropyl-beta-cyclodextrin complex, solid dispersion with polyvinylpyrrolidone, and self-
microemulsifying drug delivery system (SMEDDS) with Cremophor RH 40, Capryol 90 and Labrasol [125]. In this study, zein nanoparticles is a promising delivery system with decreased side effects of drugs to healthy cells and enhanced tumor delivery.

Zein particles were widely applied as delivery systems for drugs and nutrients including preserving the stability of labile nutraceuticals [131], enhancing the bioavailability of food bioactives [132], improving antimicrobial properties of essential oils [133]. As reported, the polymer–polymer complexes combined the advantages of different polymers, such as delayed digestion, controlled release [134], and improved mucoadhesive property [135], etc. Because of polyelectrolyte nature, zein is able to form complexes with proteins or polysaccharides through hydrogen bonds and hydrophobic interactions. Konjac glucomannan (KGM) is derived from tubers of *Amorphophallus konjac* C. Koch with high molecular weight, water-soluble, and non-ionic properties. Carboxymethyl konjac glucomannan (CKGM), a kind of KGM derivatives, is formed from carboxymethyl modified KGM, and is believed to be a promising new biodegradable material for fabricating eatable films [136], drug delivery systems [137], enzyme encapsulation [138], and adsorbing heavy metal ions [123]. This hydrophilic ionic polysaccharide (CKGM) is a promising coating material for Z NPs to balance the hydrophobic/hydrophilic property of zein. Moreover, the carboxyl groups could form coordination bonding and crosslink with metal ions, etc. Ca$^{2+}$. In this section, a AG-loaded zein/CKGM/Ca$^{2+}$ NPs was developed and the optimized preparation and characterization were conducted.

### 2.2 Materials and Methods
2.2.1 Materials

Zein sample with a 97% protein content was obtained from Showa Sangyo (Tokyo, Japan), Carboxymethyl konjac glucomannan (CKGM) with degree of substitution of 0.01 was synthesized in our lab. Andrographolide (AG) of 98% purity was provided by Xi’an tongzi biotech (Xi’an, China). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), 100× penicillin and streptomycin and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). 8-anilino-1-naphthalenesulfonate (ANS) were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO).

2.2.2 Preparation of nanoparticle

5 mg/ml zein was prepared in 70% (v/v) aqueous ethanol solutions. The CKGM solution was dissolved by stirring weighted CKGM in water for 6 hours. Then, 1 ml of the zein solution was quickly added into 6 mL of CKGM solution with different concentrations. The ZC NPs was formed with mass ration of zein–CKGM at 3:1, 2:1, 1:1. For calcium coating, 10 μl, 20 μl, 40 μl of 10 mg/ml calcium solution was added into the above solution to obtain ZCC NPs. A single phase was formed after stirring vigorously. For AG loading, AG was fully dissolved in zein solution (5 mg/ml), the weight ratios of zein to AG was 3:1. The final obtained AG concentration was 0.238 mg/ml. Figure 9 illustrates the process for the synthesis of AG-loaded Z NPs.

2.2.3 Particle size and zeta potential measurements
The average particle size of NPs was obtained by dynamic light scattering (DLS) using a BIC 90 Plus particle size analyzer (Brookhaven Instrument Corp., New York, NY) at 25.0°C and a solid-state laser at 658 nm with a scattering angle of 90°, and it was calculated from Cumulant analysis. Zeta-potential was measured by a particle electrophoreses instrument using Zetasizer Nano-ZS, Malvern Instruments, Worcs, UK). Zeta potential values was then calculated from electrophoretic mobility using the Smoluchowski theory and averaged over for three replicates.

2.2.4 Atomic force microscopy (AFM) Measurements

Images of NPs were obtained using a Nanoscope IIIa Multi-Mode AFM (Veeco Instruments, CA), which was using silicon cantilever to operate in tapping mode. Before morphological measurements, 20 μl diluted nanoparticle suspensions were dripping onto a newly cleaved mica surface, and then samples for AFM image were dried with nitrogen gas.

2.2.5 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of dry KGM, blank Z NPs, ZC NPs and ZCC NPs prepared at the mass ratio of zein–CKGM at 2:1 were measured by Thermal Nicolet Nexus 670 FTIR spectrometer (Thermo Nicolet Corp., Madison, WI). Each FTIR spectra were collected at the wave number range from 3800 to 600 cm\(^{-1}\) with 4 cm\(^{-1}\) resolutions. The original FTIR spectra were then smoothed using OMNIC 7.2 software.

2.2.6 pH stability
The pH of the NPs was adjusted in a range of 4-8.5 using HCl or NaOH. And the particle size, PDI and zeta potential were determined after the pH of NPs was adjusted.

2.2.7 Cell culture media stability

To study the stability in cell culture media, NPs were diluted 10 times in DMEM with 10% FBS. The diluted NPs were then incubated at 37 °C, and the particle size and PDI were determined within 24 hours.

2.2.8 Contact angle

To verify the wettability of NPs, the water-in-air contact angle of NPs films were measured according to the literature [139]. Nanoparticle solutions evaporating overnight on glass at room temperature formed homogeneous nanoparticle film. The water contact angles $q_{aw}$ of water droplets deposited onto casting films were calculated using VCA optima (AST Products Inc. Billerica, MA).

2.2.9 Spectrofluorometer analysis

The surface hydrophobicity was measured using ANS probe [140]. Briefly, the NPs were diluted with water (adjust to pH 7.4) to obtain zein concentrations in a range from 20 to 100 μg/ml. An equal volume of 50 μg/mL ANS dissolved in pH 7.4 water was then added into each diluted sample. The mixture was then incubated at 37 °C for 30 min. The fluorescence intensity was collected using Fluoromax-3 spectrofluorometer (Horiba Scientific Inc., Edison, NJ) with excitation/ emission wavelengths at 355 nm/460 nm. Samples were measured in 1 cm light path quartz cuvettes (NSG Precision Cells, Farmingdale, NY) at 37 °C. The slope of fluorescent intensity versus zein concentration
represented the index of surface hydrophobicity, which was calculated by linear regression analysis. The regression line with $R^2 > 0.994$ was obtained.

2.2.10 In vitro release study

In vitro release study was carried out using dialysis method in 0.01M PBS at pH 7.4. Briefly, AG loaded zein nanoparticles, zein/CKGM nanoparticles or zein/CKGM/Ca$^{2+}$ nanoparticles was placed in dialysis bags (3500 MWCO), then immersed in 50 mL 0.01 M PBS (pH 7.4), followed by gently shaken at 100 rpm in 37.0 °C. At predetermined intervals, 1 mL dissolution sample was collected from PBS solutions and the concentration of AG was measured by HPLC method, using a UltiMate 3000 HPLC system with UV-absorption detector. The analyze was determined at room temperature on a reverse-phase C18 column (250 mm × 4.6 mm, particle size 5μm, Thermo, Germany). Elution was isocratic, using a mobile phase consisting of 40% HPLC-grade acetonitrile and 60% HPLC-grade water (v/v). The analysis was carried out at a flow rate of 0.6 mL/min with UV detection at 223 nm.
Figure 9 Illustration of the synthesis and structures of AG-loaded zein NPs coated by CKGM and Ca\(^{2+}\) and the proposed model for intracellular delivery processing of AG-loaded zein/CKGM/ Ca\(^{2+}\) NPs in tumor cells.

2.2.1 Statistical analysis

All results were expressed as means ± standard deviation. Statistical analysis was analyzed by Student’s t-test or one-way ANOVA to determine the differences between the datasets. Significant difference was defined at \(p < 0.05\).

2.3 Results and discussion

2.3.1 Effect of molar ratio of zein to CKGM on the nanoparticles properties

Table 1 showed the influence of molar ratios on the formation of zein-CKGM NPs (ZC NPs). For CKGM coated zein NPs without crosslinked by calcium ions, the variation of particle size and zeta potential depended on the molar ratios of zein to
CKGM, which were ranged from 114.9 nm to 188.5 nm and -43.7 to -52.0 mV, respectively. After coating calcium ions on ZC NPs, the particle size and zeta potential also varied in a concentration-dependent manner. With the addition of more calcium ions, the surface charge of zein-CKGM-Ca$^{2+}$ (ZCC NPs) became less negative. It seems that calcium probably crosslinked with CKGM through electronic interaction, higher calcium content lead to lower absolute value of surface charge, thus resulted in the particle aggregation and larger particle size [141]. For the NPs with a molar ratio of zein to CKGM at 2:1 with or without calcium ions, the particle sizes were 135.1 and 177.0 nm with relative small PDI and large absolute surface charge value. It was suggested that with a size of 100–200 nm, polymeric NPs have the best properties for cellular uptake [142]. Therefore, the optimum molar ratio of zein to CKGM at 2:1 in ZC NPs and ZCC NPs was used accordingly in the following experiments.

Table 1 Characterization of Nanoparticles (pH 7.4) (Results are Displayed As Mean ± Standard Deviation (n = 3))

<table>
<thead>
<tr>
<th>Volume of Calcium solution (μl)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zein:CKGM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:1</td>
<td>114.9±3.4</td>
<td>0.234±0.003</td>
<td>-43.7±2.7</td>
<td>125.9±2.3</td>
</tr>
<tr>
<td>2:1</td>
<td>135.1±1.3</td>
<td>0.244±0.002</td>
<td>-49.6±1.5</td>
<td>148.6±3.2</td>
</tr>
<tr>
<td>1:1</td>
<td>188.5±2.2</td>
<td>0.234±0.012</td>
<td>-52.0±3.2</td>
<td>220.2±3.9</td>
</tr>
</tbody>
</table>
2.3.2 Effect of CKGM coating on the nanoparticles morphology

As shown in Figure 10A, Z NPs have spherical and smooth surface features with a uniform particle size distribution around 100 nm. However, most of the particles were accumulated and clumped in large aggregation formats and almost no individual particles can be seen. This may be contributed to the drying process of making the AFM samples, which also may adversely affect the redispersion ability of Z NPs in practical use [143]. After coating CKGM, it still cannot clearly see individual particles from ZC NPs. Interestingly, the addition of calcium allows the separation of ZCC NPs and ZCC NPs to be more clearly dispersed into individual NPs with a uniform distribution and well-defined spherical shape, showing the effect of crosslinking between CKGM and calcium ions.

The individual particle sizes of Z NPs, ZC NPs and ZCC NPs were approximately in a range of 60–80 nm from AFM images (Figures 10A). Figures 10B showed the particle size distribution obtained by DLS, which were larger than the results from AFM. Similarly, J. Li et al. (2012) [144] reported that the sizes of chitosan-tripolyphosphate particles measured by AFM (20-30 nm) were much smaller than those from DLS (170 nm). The main reason was due to sampling differences. AFM provided an image of the particles in the dry state while DLS gave the average particle size in solution. The particle size calculations from DLS involve a hydration layer around the NPs that is excluded from the dry NPs in AFM.
Figure 10 A. Atomic Force Microscopy (AFM) of zein NPs, zein-CKGM NPs and zein-CKGM-Ca\(^{2+}\) NPs. B. Particle size distribution of zein NPs, zein-CKGM NPs and zein-CKGM-Ca\(^{2+}\) NPs.

2.3.3 Effect of CKGM coating on the nanoparticles interaction force

Figure 11 showed the representative FTIR spectra of zein, CKGM, and their corresponding NPs with or without Ca\(^{2+}\). In the infrared spectra, peaks in a range from 3200 to 3400 cm\(^{-1}\) indicated the hydrogen bonding. The hydrogen bonding characteristic peaks of zein and CKGM were observed at 3292 and 3221 cm\(^{-1}\), respectively, which were shifted to 3298 and 3299 cm\(^{-1}\) after ZC NPs fabricated, indicating hydrogen bonding interactions [145]. The amide I band and the amide II band of zein showed at 1654 and
1541 cm\(^{-1}\), demonstrating C = O stretching and C–N stretching, respectively. The spectral peaks in ZC NPs demonstrated shifts to 1632 cm\(^{-1}\) and 1540 cm\(^{-1}\), and ZCC NPs demonstrated shifts to 1627 cm\(^{-1}\) and 1535 cm\(^{-1}\), which indicated electrostatic interactions between zein and CKGM [146]. The peak at 1412 cm\(^{-1}\) in CKGM can be attributed to the symmetric stretching vibration of the carboxyl group, and it shifted to 1418 cm\(^{-1}\) in ZCC NPs, indicating the existence of ionic crosslinking between the carboxyl groups and the calcium ions [141].

Figure 11 Fourier transform infrared spectroscopy (FTIR) spectra of different samples. Z, zein powder; CKGM, carboxymethyl konjac glucomannan powder; ZC, zein-CKGM NPs; ZCC, zein-CKGM-Ca\(^{2+}\) NPs.
2.3.4 Effect of CKGM coating on the nanoparticles stability

In order to accurately conduct the experiments based on cellular level, it is significant to measure the stabilities of NPs in cell culture media at 37 °C. The NPs coated with different concentrations of calcium ions were prepared with zein–CKGM mass ratio at 2:1. NPs were diluted 10-fold in DMEM, which is the medium for most mammalian cell lines. The particle size of NPs need to be stable during the period of cell experiments at 37 °C. The stabilities results are shown in Figure 12. For Z NPs, particle size increased from 92.2 nm to about 168.4 nm after 24 h of incubation (Figure 12a). And similar phenomena occurred in ZC NPs changing from 135.1 nm to 266.1 nm (Figure 12b). The particle size and PDI were also compared with ZC NPs added by 10 μl, 20 μl and 40 μl volumes of calcium solution (data not shown). And the minimum volume of calcium solution to stabilize NPs was 20 μl. After adding 20 μl of 10 mg/ml calcium solution, only a slight increase in particle size (5-10 nm) was observed on ZCC NPs (Figure 12c). The augment particle size of Z NPs and ZC NPs may be due to the interaction of protein NPs with the high glucose content in DMEM to form larger particles [140], while the CKGM-Ca²⁺ coating could avoid such circumstances. Thus, the optimal volume of 10 mg/ml calcium solution used accordingly in the following experiments was 20 μl.

Both Z NPs and coated Z NPs are stable when increasing pH, while Z NPs have tendency to aggregate at pH 6 because the isoelectric pH of zein protein equals to 6.2 (Figure 13a), while these phenomenon was avoided in the coated Z NPs and the stability of Z NPs was improved. Coating Z NPs with CKGM could form a CKGM gel layer. The
response of NPs to different pH was related to the molecular conformation of CKGM at different pH values. At high pH, the carboxyl groups (pKa value at pH ≈ 4.6) in the CKGM were gradually changed to –COO⁻ and electrostatic repulsion between the –COO⁻ groups was stronger and thus had a higher affinity with water molecules [147][148], which makes NPs more negative charged. Therefore, the particle size of the NPs decreased and tent to be constant. At low pH, the carboxyl groups were partially protonated in the –COOH form, which may result in larger particle size and lower surface charge. The ZCC NPs have a particle size ranging from 220.2 nm to 177.7 nm (pH 5.0- pH 8.5) (Figure 13c). This may be due to the electrostatic interactions between calcium and CKGM, resulting in a denser and thicker coating of Z NPs, and therefore better pH stability.
Figure 12 Particle size and PDI in cell culture media as a function of time: zein NPs(a), zein-CKGM NPs(b) and zein-CKGM-Ca$^{2+}$ NPs(c). Control referred to the NPs without adding to the culture media. Data displayed as mean ± SD (n=3).
Figure 13 Influence of the pH on particle size, PDI and zeta potential of zein NPs(a), zein-CKGM NPs(b) and zein-CKGM-Ca^{2+} NPs(c).
2.3.5 Effect of CKGM coating on the nanoparticles hydrophobicity

ZC NPs formulated with or without calcium (Figure 14a) were found to be more hydrophilic compared with Z NPs (Figure 14b and 14c) as seen from the smaller water contact angle $q_{wa}$. The hydrophilic nature of Zein/CKGM nanoparticles was manifested by water contact angle $q_{wa}=38.8^\circ$C (Figure 14b), which is larger than zein/CKGM/Ca$^{2+}$ nanoparticles ($q_{wa}=44.3^\circ$C) (Figure 14c). The reason probably because the electronic interaction between carboxyl group and calcium ions decreased the electrostatic repulsion between ionized carboxyl group in CKGM and thus had a lower affinity with water molecules [149]. Although the absolute value of surface hydrophobicity differs depending on the measurement system and the calculation method, the hydrophobicity measured using ANS probe (Figure 14d) showed the tendency well-correlated to that obtained by contact angle. The slope of fluorescent intensity versus zein concentration was calculated by linear regression analysis and used as an index for surface hydrophobicity [74]. Z NPs had a larger affinity to hydrophobicity fluorescence probe ANS at low concentration, which indicated a high surface hydrophobicity. ZC NPs and ZCC NPs showed smaller attachment to ANS which indicated lower surface hydrophobicity. As a result, the hydrophilic polymer coating such as CKGM caused the surface modification of hydrophobic Z NPs.
2.3.6 Effect of CKGM coating on the nanoparticles release profile

The in vitro release of AG from nanoparticles was monitored in pH of 7.4 0.01 M PBS at 37°C. The free drugs were rapid released within 4 hours, while all nanoparticles released AG in a sustained manner, which may provide an enduring capacity in fighting against cancer cells. It was showed a rapid initial burst at first 4 hours, followed by a slow release in all samples. The controlled and sustained behavior of AG release possibly because the hydrophobic interactions of AG and zein delay the penetration of AG in water, thereby inhibiting the diffusion of AG into the release medium. On the other hand,
when the delivery system first becomes immersed in the release medium, a rapid and short release of drug is usually observed in controlled-release polymeric drug delivery systems, followed by a stable “plateau” profile (Figure 15). The rapid release is usually referred to as the initial “burst release”, and low-MW drugs, peptides, and proteins usually have higher tendencies for burst release due to osmotic pressures. The rapid release of AG from zein nanoparticles can be attributed to two factors. First, the size was highly important in the first release of AG, as the small nanoparticles had a relatively larger total surface area and great fraction of AG near the surface was rapidly released. Second, the swelling of the nanoparticles induced the release of any AG that was not tightly binding to the nanoparticles. In the late incubation time, a slow release stage represents the equilibrium swelling of nanoparticles. The presence of CKGM or CKGM-calcium facilitated a second layer, providing a good barrier against the diffusion of AG and decreasing the release rate. Accumulative release at 4 hours was reduced from 79.7% to 77.6% and 73.8% by adding CKGM and CKGM-Ca\(^{2+}\) coatings, respectively. These results indicated that the greater cross-linking density and less swelling ability of zein nanoparticles.
2.4 Conclusion

As an effective plant-driven anti-inflammation and antitumor agent, the therapeutic use of andrographolide (AG) is restricted by its poor water solubility. Zein nanoparticles (NPs) are well-known delivery systems for hydrophobic nutrients or drugs. In order to enhance the stability and cellular uptake of zein NPs, novel NPs were developed as zein-carboxymethyl konjac glucomannan (ZC) NPs and zein-carboxymethyl konjac glucomannan-calcium (ZCC) NPs through self-assembly, which could be used as nanocarriers for AG. The encapsulated AG concentration maintained at 238 μg ml⁻¹. The ZCC NPs exhibited spherical with diameters about 177.0 ± 2.8 nm and narrow particle size distribution, as well as negative surface charge. The morphology image revealed ZCC NPs, unlike zein NPs and ZC NPs, were more dispersed as
individual NPs with homogeneous distribution. Both hydrophobicity fluorescence probe and contact angle results showed carboxymethyl konjac glucomannan coated zein NPs have a lower surface hydrophobicity than zein NPs. Compared with zein NPs and ZC NPs, ZCC NPs demonstrated good stabilities to maintain particle size (160–180 nm) in cell culture medium at 37°C, and enhanced pH stability over a broad range from 5.0 to 8.5. The AG release profile of ZCC NPs was in a sustained manner in pH 7.4 at 37°C. The presence of CKGM or CKGM-calcium facilitated a second layer, providing a good barrier against the diffusion of AG and decreasing the release rate.
CHAPTER 3 IN VITRO DETERMINATION OF DELIVERY VEHICLE AFFECTING THE BIOLOGICAL FUNCTION OF ANDROGRAPHOLOIDE ON CANCER CELLS

3.1 Introduction

Self-assembled biodegradable NPs including protein-based NPs are considered to be promising anti-cancer drug delivery systems because of the good compatibility and low toxicity. NPs with particle size ranging from 100 to 200 nm mostly prolong retention in tumors through enhanced permeability and retention (EPR) effect caused by the leaky vessels, and the non-functional or absent lymphatic system leads to the retention effect in tumor [23]. NPs internalize through the plasma membrane of cancer cells through endocytosis. Current findings suggest that particle size, particle shape and surface chemistry play key roles in controlling biodistribution and cellular internalization of engineered NPs [150]. Especially, the NPs functionalized with hydrophilic polymer imparts a character against the immune system and prolongs their systemic circulation [151].

In the last section, a liquid–liquid phase separation method was used to fabricate AG-loaded zein-CKGM NPs (ZC NPs) and zein-CKGM-Ca2+ NPs (ZCC NPs). Optimized preparation and characterization were conducted. Here, the intracellular transport of NPs was qualitative and quantitative study by fluorescence microscopy and flow cytometry using HT-29 colon cancer cells and A549 lung cancer cells. As the fluorescence probe,
NPs was labeled with coumarin-6 to study the effects of incubation time, NPs concentrations, endocytosis inhibitors on cellular uptake. The subcellular localization of NPs was visualized by confocal laser scanning microscopy (CLSM). Besides, MTT and apoptosis assay were conducted to evaluate the anti-cancer activity of AG-loaded ZCC NPs.

3.2 Materials and Methods

3.2.1 Materials

Zein sample with a 97% protein content was obtained from Showa Sangyo (Tokyo, Japan), Carboxymethyl konjac glucomannan (CKGM) with degree of substitution of 0.01 was synthesized in our lab. Andrographolide (AG) of 98% purity was provided by Xi’antongzebiotech (Xi’an, China). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), 100× penicillin and streptomycin and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). 8-anilino-1-naphthalenesulfonate (ANS) were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO). Human lung carcinoma A549 cells and human colorectal carcinoma HT29 cells were obtained from Dr. Kiron M. Das of Department of Medicine, Robert Wood Johnson University Hospital. Coumarin-6, Coumar-6 (3-(2-Benzothiazolyl)-N, N-diethylumbelliferylamine, 3-(2-Benzothiazolyl)-7-(diethylamino)coumarin, ≥98%), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dynasore and chlorpromazine were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO). DAPI (4’,6-diamidino-2-phenylindole), CellMask Deep Red Plasma membrane Stain and
LysoTracker Red were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). All other reagents were of analytical grade and obtained from commercial sources.

3.2.2 Preparation of coumarin-6 labeled nanoparticles

5 mg/ml zein containing 0.02% coumarin-6 was prepared in 70% (v/v) aqueous ethanol solutions. All other conditions used in the preparation of fluorescent nanoparticles remained the same. The CKGM solution was dissolved by stirring weighted CKGM in water for 6 hours. Then, 1 ml of the zein solution was quickly added into 6 mL of CKGM solution with different concentrations. The ZC NPs was formed with mass ratio of zein–CKGM at 2:1. For calcium coating, 20 μl of 10 mg/ml calcium solution was added into the above solution to obtain ZCC NPs. A single phase was formed after stirring vigorously. For AG loading, AG was fully dissolved in zein solution (5 mg/ml), the weight ratios of zein to AG was 3:1. The final obtained AG concentration was 0.238 mg/ml.

3.2.3 Cell culture

A549 lung cancer cells and HT29 colon cancer cells were cultured at 37 °C in DMEM medium supplemented by 10% (v/v) FBS, 100 U ml⁻¹ penicillin, and 100 g/ml streptomycin. Cells were cultured in incubators at 37 °C under 95% relative humidity and 5% CO₂.

3.2.4 Cytotoxicity assay

A549 and HT29 cells were seeded at a density of 1.0 × 10⁴ cells/well in 96-well plates. After 24 h incubation, the cells were incubated with DMSO-dissolved AG or AG-
loaded ZCC NPs at different doses for 24 h. 200 μL of 0.5 mg/mL MTT solution was then added to each well for 4 h incubation. The medium was then replaced with 150ml DMSO to dissolve the blue formazan crystals. The optical density of each well was measured at a wavelength of 490 nm using a Model 680 microplate reader (BIO-RAD, Hercules, USA). The cell viability was a percentage of absorbance of cells treated with sample compared to the absorbance of the untreated cells. Data were presented as average ± SD (n = 6). The IC₅₀ (the concentration to cause 50% inhibition) values were calculated using OriginPro 8.

3.2.5 Cellular uptake experiments

The qualitative study of cellular uptake was conducted by a Nikon TE 2000 inverted optical microscopy (Nikon Corporation, Japan). HT29 cells and A549 cells were seeded at a density of 1×10⁵ cells per well in a 12-well plate. After 24 h incubation, the cells were treated with coumarin-6 labeled NPs and incubated for 1, 2, 4 or 6 h (37°C, 5% CO₂), respectively. The mixture of zein and coumarin-6 dissolved in 70% ethanol solution was used to prepare the fluorescent NPs, and the smallest amount of coumarin-6 (5 μg coumarin-6/ 1mg zein) was used to label zein. The zein and coumarin-6 mixtures were followed by ultracentrifuging at 4000 × g for 30 min, and then passed through a 10 kDa MWCO Amicon filter. As a result, less than 3% coumarin-6 in the filtering medium was measured by a fluorescence spectrophotometer at 505 nm. SimplePCI imaging software (Compix Inc., Japan) were used to processed the images. The treated cells were harvested and then centrifuged, the cell pellets were resuspended in DPBS. The quantitative study of cellular uptake was analyzed by Accuri C6 Flow Cytometer (BD
Biosciences, New Jersey, USA) for green fluorescence. To compare the cellular uptake results, cells were treated identically in microscopy imaging and flow cytometry quantification studies. There were 10,000 cells measured in each sample. For uptake inhibition studies, the cells were firstly treated with a serum-free DMEM medium with either 50 μM chlorpromazine or 80 μM dynasore for 30 min. Afterward, the cells were incubated with coumarin-6 labeled NPs containing the same concentration inhibitors and further incubated for 30 min. Then the cells were washed 3 times with PBS. The cells were either observed using fluorescence microscopy or trypsinized and processed for flow cytometry. To ensure the results were comparable, cells were treated identically in microscopy imaging and flow cytometry quantification studies.

### 3.2.6 Cellular localization image

HT29 cells and A549 cells were seeded in a 35-mm² glass bottom cell culture dish at a density of 1×10⁵ cells per dish. After 24 h incubation, cells were washed 3 times with PBS, then the cells were incubated with PBS containing 0.25 μg/mL CellMask deep red for 5 min to stain cell membranes. Coumarin-6 labeled NPs in serum-free DMEM solution were applied to the cells 5, 10, 15, 30 and 40 min before the measurement was started. To further subcellular localization of ZCC NPs, the cells were treated with coumarin-6 labeled ZCC NPs for 4 h. During the last 10 min of NPs treatment, cells were exposed to 50 nM LysoTracker Red to stain lysosomes and also DAPI to stain nuclei. The cells were fixed with 4% formaldehyde for 20 min and washed 3 times with PBS before observation. Cellular uptake was visualized using a Zeiss LSM 710 confocal laser scanning microscopy (Zeiss, Germany).
3.2.7 Cell apoptosis and growth morphology observation

A549 cells were seeded at a density of $5 \times 10^5$ cells per well in a 6-well plate. After 24 h incubation, the cells were treated with blank NPs, free AG and AG-loaded ZCC NPs (equivalent AG concentration for 24 h. Afterwards, cells were washed 3 times with PBS, then collected by trypsinization and centrifugation. The cells viability was conducted using a live/dead assay kit (Invitrogen, Carlsbad, CA) and proformed using flow cytometry. The data were analyzed by FlowJo software. Cells were also observed using a Nikon TE 2000 inverted optical microscopy (Nikon Corporation, Japan) with 200 times magnification.

3.2.8 Statistical analysis

All results were expressed as means ± standard deviation. Statistical analysis was analyzed by Student’s t-test or one-way ANOVA to determine the differences between the datasets. Significant difference was defined at $p < 0.05$.

3.3 Results and discussion

3.3.1 Effective of nanoparticles concentration on the internalization of nanoparticles

Obviously, the therapeutic effect of drug-loaded NPs depends on the internalization of diseased cells and the sustained retention of NPs. In this study, HT-29 cells and A549 cells were cell models used to study the advantages of the AG-loaded NPs relative to free drugs in therapeutic applications. Fluorescent dye-labeled particles are commonly used to study cellular uptake. Other researchers had demonstrated that less
than 0.6% of dyes permeated from NPs for more than 48 h under sink condition, and coumarin-6 is an ideal marker for studying the uptake behavior of NPs [117]. Zein was labeled with coumarin-6 and the corresponding cellular uptake was measured by fluorescence microscopy and flow cytometry.

**Figure 16a and c** showed the typical microscopic images of HT29 cells and A549 cells incubation for 4 hours with different concentration of coumarin-6 loaded Z NPs, ZC NPs and ZCC NPs at 37 °C. **Figure 16b and 16d** showed the correlated quantitative fluorescence intensity. NPs showed cell line-dependent uptake, and the specific endocytosis may due to the different cell surface properties [152]. The HT29 and A549 had a different behavior with different concentration of NPs. For HT29, the uptake rate was more constant, while the A549 had a significant fast uptake, and the uptake began to saturate after low concentration. However, for both HT29 cells and A549 cells, the amount of cellular uptake was in the same order ZCC NPs >ZC NPs >Z NPs.

Cellular uptake depends on many factors including particle size, zeta potential, particle compositions, surface hydrophobicity and cell lines and cell densities [151]. Surface hydrophilicity and particle size were the key features determining cellular uptake efficiency in this study. As shown in hydrophobicity study, Z NPs was much hydrophobic compared with ZC NPs and ZCC NPs. Nanoparticle absorption can be considered as an adhesion process prior to the internalization process. Due to its biological adhesive nature, the polysaccharide (KGM) coating of NPs will promote the adhesion of particles to cell membranes. Therefore, ZC NPs and ZCC NPs have higher affinity to the cell membrane compared with Z NPs. From DMEM stability data, the particle size of Z NPs and ZC NPs was unstable and changed from 92.2nm to 177.2nm.
and 135.1nm to 242.5nm, respectively. ZCC was stable in DMEM (177nm-180nm). Besides, even though Z NPs and ZCC NPs had similar particle size in DMEM medium, the amount of cellular uptake of ZCC NPs is higher than Z NPs probably due to its more suitable hydrophobicity- hydrophilicity balance for cellular internalization [152]. Similarly, Zhang et al. [153] found that poly(lactide)-tocopheryl polyethylene glycol succinate (PLA–TPGS) copolymer had higher cell uptake efficiency with higher levels of hydrophilic TPGS.

Although they had similar hydrophobicity, ZC NPs had less cellular uptake than ZCC NPs. The probable reason is that the internalization process of large-size NPs requires stronger driving force and more energy. Besides, particle size is a key feature that affects the final biodistribution and blood clearance. For particles with hydrodynamic radius over 200 nm usually exhibit faster clearance [154]. In other words, 242.5 nm ZC NPs would be more likely to be cleared from the blood stream than 177 nm ZCC NPs. In addition to the blood clearance rate, the final biodistribution is also affected by particle size. For example, 150 nm PEGylated NPs showed much more bone marrow uptake in rabbits compared with 250 nm NPs [28].
Figure 16 ac. Fluorescence microscope image of Z NPs, ZC NPs and ZCC NPs on the effect of concentrations; bd. Fluorescence intensity of Z NPs, ZC NPs and ZCC NPs on the effect of concentrations. It should be noted that the concentrations of blank NPs equal to AG loaded NPs in the cytotoxicity study. (a)(b) HT29 and (c)(d) A549. Nanoparticles were stained with coumarin-6. The scale bars represent 100 μm. Data are expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 versus the Z NPs group.

3.3.2 Effective of incubation time on the internalization of nanoparticles

Figure 17a and 17c showed the typical microscopic images of HT29 cells and A549 cells after 1 h, 2 h, 4 h and 6 h treatment with coumarin-6 loaded Z NPs, ZC NPs
and ZCC NPs at 37 °C. **Figure 17b and 17d** showed the correlated quantitative fluorescence intensity. To ensure comparability of the results, the concentration of zein in Z NPs, ZC NPs and ZCC NPs were identical in these studies. In both cells, the maximum cellular uptake of Z NPs was at 2 h and it began to decrease after 4 hours, while the peak green fluorescence appeared at 4 h for ZC and ZCC in both cells, which means that the hydrophilic coating enhanced florescence intensity and the fading time was prolonged. The fading fluorescence could also partially due to the fluorescence quenching of coumarin-6 in the lysosomes [152].

Figure 17 ac. Fluroesence microscope image of zein NPs, zein-CKGM NPs and zein-
CKGM-Ca\textsuperscript{2+} NPs on the effect of times; bd. Fluorescence intensity of zein NPs, zein-CKGM NPs and zein-CKGM-Ca\textsuperscript{2+} NPs on the effect of times. (a)(b) HT29 and (c)(d) A549. Nanoparticles were stained with coumarin-6. The scale bars represent 100 μm. Data are expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 versus the Z NPs group.

3.3.3 Effective of inhibitors on the internalization of nanoparticles

**Figure 18a and 18c** showed the typical microscopic images of HT29 cells and A549 cells treated with inhibitors when incubating with coumarin-6 loaded Z NPs, ZCNPs and ZCC NPs at 37 °C. **Figure 18b and 18d** showed the correlated quantitative fluorescence intensity. Cellular uptake of NPs was dramatically decreased at 4 °C, indicating that 90% of NPs uptake were energy-dependent endocytic process while the remaining 10% may be physical diffusion or adhesion [28].

Dynasore is a specific inhibitor of dynamin. Therefore, all dynamin-dependent endocytosis pathways including clathrin- and caveolin- mediated endocytosis are inhibited by dynasore. To further elucidate the internalization mechanism, chlorpromazine was applied to the cells. In the presence of chlorpromazine, the clathrin assembly at the plasma membrane is suppressed, therefore, the clathrin- mediated endocytosis is inhibited by chlorpromazine [155][156]. In HT29 cells, dynasore with Z and ZCC NPs being inhibited by 13% compared with 48% (**Figure 18a and 18b**). Similarly, in A549 cells, dynasore with the Z NPs and ZCC NPs being inhibited by 16% compared with 38% (**Figure 18c and 18d**). Because dynasore efficiently inhibits dynamin, the marked uptake of ZCC NPs by HT29 cells and A549 cells indicated that CKGM coating Z NPs involved with dynamin-mediated endocytosis. Apparently, CKGM
on the nanoparticle surface appears to be significant in activating the dynamin-mediated endocytosis pathway. By contrary, dynasore had less effect on Z NPs uptake in both HT29 cells and A549 cells. Due to the unstable particle size of ZC NPs, dynasore did not inhibit cellular uptake of ZC NPs as significant as ZCC NPs.

In HT29 cells, chlorpromazine suppressed Z NPs and ZCC NPs about 55% compared with 64%, (Figure 18a and 18b). Similarly, in A549 cells, chlorpromazine inhibited Z NPs and ZCC NPs about 15% compared with 20% (Figure 18c and 18d). Chlorpromazine suppressed Z NPs internalization to approximately the same or larger degree than dynasore in both cell lines, which indicated that clathrin-mediated endocytosis was the main pathway contributed to dynamin-mediated pathways; other dynamin-mediated processes may have no apparent contribution [157]. These also happened for ZC NPs and ZCC NPs in HT29 cell lines. While in A549 cell lines, the inhibition of chlorpromazine was much lower than dynasore, especially for ZCC NPs. Thus, it is not sufficient to simply attribute the enhanced cellular uptake of ZCC NPs associated with dynamin-mediated pathways to clathrin-mediated endocytosis. We conclude that ZCC NPs enhanced the cellular uptake through dynamin-mediated endocytosis is cell line dependent. In HT29, ZCC NPs enhanced the cellular uptake through dynamin-mediated endocytosis can be assigned to clathrin-mediated endocytosis.
Figure 18 ac. Fluorescence microscope image of zein NPs, zein-CKGM NPs and zein-CKGM-Ca^{2+} NPs on the effect of inhibitors.; bd. Fluorescence intensity of zein NPs, zein-CKGM NPs and zein-CKGM-Ca^{2+} NPs on the effect of inhibitors. (a)(b) HT29 and (c)(d) A549. Nanoparticles were stained with coumarin-6. The scale bars represent 100 μm. Data are expressed as mean ± SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 versus the Z NPs group.

3.3.4 Cellular localization of nanoparticles

Effective cellular internalization is a prerequisite for NPs to efficiently deliver loadings to targeted cells or tissues, especially the drugs with therapeutic targets in the cytoplasm or nucleus [158]. After 5-40mins incubation with coumarin 6-loaded ZCC NPs,
the confocal microscopic images of HT29 cells and A549 cells were shown in Figure 19. It can be seen that after 30mins large amount of coumarin 6-loaded ZCC NPs (green) penetrated into cell membrane (red, stained by CellMask Deep Red), which suggested that the NPs were internalized by the cells. At 40min the majority of the scattered green dots were inside the membrane. It can therefore draw the conclusion that the fluorescent NPs were located inside the cells rather than within the cell membrane [159].

![Figure 19 CLSM images of intracellular uptake of zein-CKGM-Ca\textsuperscript{2+} NPs. Cells were counter-stained with CellMask Deep Red for membrane. Nanoparticles were stained with coumarin-6. The scale bars represent 20 \(\mu\text{m}.\)]
3.3.5 Transportation of NPs to lysosomes

**Figure 20** showed the subcellular localization images of ZCC NPs in living HT29 cells and A549 cells treated for 2 hours. ZCC NPs were effectively internalized by cells and were mainly located in the cytoplasm and perinuclear region. An intensive green fluorescence near the nucleus of HT29 cells or A549 cells could be observed at this time point. Thus, ZCC NPs were taken up by tumor cells and stayed at the perinuclear area. To explore the intracellular mechanism of ZCC NPs in more detail, the distribution of ZCC NPs in the vesicular compartment was characterized using a lysosomal marker, Lysotracker Red (**Figure 20**). As highlighted by white arrows, the overlapping of green and red signals represented the internalization of ZCC NPs in the lysosome. Thus, ZCC NPs could be efficiently delivered from plasma membrane to the lysosome for degradation, which is essential for the drug delivery and cellular function.

![Figure 20 CLSM images of intracellular uptake of zein-CKGM-Ca\(^{2+}\) NPs. Cells were counter-stained with DAPI with nucleus and LysoTracker Red with lysosome.](image-url)
Nanoparticles were stained with coumarin-6. The scale bars represent 20 μm.

3.3.6 In vitro proliferation of nanoparticles on cancer cells

After demonstrated that ZCC NPs can be effectively endocytosed by tumor cells, the in vitro cytotoxicity efficacy of AG-loaded ZCC NPs was conducted using MTT assay. The plant-derived drug, andrographolide (AG), was used as drug model. Figure 21 apparently showed that blank NPs did not show cytotoxicity to HT29 cells and A549 cells for 24 h incubation. For both AG-DMSO and AG-ZCC NPs samples, it showed dose-dependent toxicity to HT29 cells and A549 cells, and the toxicity increased with increasing of drug concentration. However, compared to DMSO-dissolved AG, ZCC NPs encapsulated AG showed no significant drug efficacy enhancement. For HT29 cells, the concentration to inhibition of biological process and cause a cell population by half in comparison with the control (IC$_{50}$) were achieved as 39.73±0.10 μM and 45.55±0.11 μM of AG equiv/ml for free drug and ZCC NPs, respectively. For A549 cells, IC$_{50}$ were 46.03±0.06 and 55.56±0.06 μM of AG equiv/ml for free drug and ZCC NPs, respectively. This may be due to the fact that higher concentration gradients under in vitro conditions lead to the rapid transport of free drugs into the cells through passive diffusion, which immediately affects the growth of cells despite drug release processes [160]. However, AG-loaded ZCC NPs are delivered into cells by endocytic routes.
3.3.7 Cell apoptosis of lung cancer cells and morphology observations

Since the blockade of mitotic progression and inhibition of cell population may eventually result in cell apoptosis, we conducted the apoptosis study on A549 cancer cells treated with different formulations [158]. Because of the distinct cytological morphology of apoptotic cell death, the images showed changes in number, shape, and size of the AG treated cells compared with the controls (Figure 22 a). The abnormal cell morphology and the shrinkage of some cells in AG and AG-loaded ZCC NPs treated images were caused by cell death. The cell death of A549 cells caused by AG formulations was further measured using an apoptosis assay with flow cytometry. Figure 22 b shows the apoptotic diagrams and the proportion of apoptotic and necrotic cells after treatment with the blank
NPs, DMSO-dissolved AG and AG-loaded ZCC NPs for 24 h. The proportion of A549 cells undergoing apoptosis (early apoptosis and late apoptosis) increased from 4.535% (control) to 42.25% and 38.48% after incubation with DMSO-dissolved AG and AG-loaded ZCC NPs, respectively (Figure 22 c).

Figure 22 The microscopic images of A549 cells after treatment with the NPs, AG and AG-loaded NPs for 24 h (a). The apoptosis assay on A549 cells after treatment with the NPs, AG and AG-loaded NPs for 24 h (b). The proportion of apoptotic and necrotic cells after treatment with NPs, AG and AG-loaded NPs. AG concentration was 24 mg/ml. (c). Data displayed as the mean SD (n = 3).

3.4 Conclusion
Fluorescence microscopy and flow cytometry studies demonstrated that ZCC NPs could effectively endocytosed by A549 lung cancer cells and HT29 colon cancer cells. To study endocytic pathways involved in cellular uptake of nanoparticles, the pharmacological inhibitors dynasore, chlorpromazine and 4 were used. The endocytic pathway of ZCC NPs is cell-line dependent, and ZCC NPs enhanced the cellular uptake through dynamin-mediated endocytosis which can be assigned to clathrin-mediated endocytosis in HT29 cells. Cellular localization of nanoparticles concluded that the fluorescent NPs are located inside the cells but not within the cell membrane. ZCC could be efficiently endocytosed by various tumor cells and transported through the endosome/lysosome pathway. Importantly, ZCC NPs loaded AG were shown effectively decreasing the cancer cell population and resulting cell death. In these preliminary studies, ZCC NPs were proved to be appealing delivery systems for hydrophobic bioactive compounds.

To study endocytic pathways involved in cellular uptake of nanoparticles, the pharmacological inhibitors dynasore, chlorpromazine and 4 °C were used. Energy-dependent endocytic process might be responsible for up to 90% of NPs uptake. In both HT29 cells and A549 cells, ZCC enhanced the cellular uptake through dynamin-mediated endocytosis which can be assigned to clathrin-mediated. Cellular localization of nanoparticles concluded that the fluorescent NPs are located inside the cells but not within the cell membrane. ZCC could be efficiently endocytosed by various tumor cells and transported through the endosome/lysosome pathway. Importantly, ZCC NPs showed low toxicity while AG-ZCC NPs were shown to effectively kill cancer cells in both MTT
assay and Annexin V-FITC/PI assay. Zein-CKGM-calcium NPs are believed to be promising nanocarriers for the supplementation or treatment of hydrophobic nutrients.
CHAPTER 4 IN VITRO DETERMINATION OF DELIVERY VEHICLE AFFECTING THE BIOLOGICAL FUNCTION OF ANDROGRAPHOLIDE ON RAW 264.7 MACROPHAGE CELLS

4.1 Introduction

Endogenous or exogenous stimuli cause normal defensive response in infected or injured host cells is so called inflammation. Prolonged and excessive inflammatory responses are usually related to many chronic inflammations and some other diseases, such as bacterial and virus infections, arthritis, chronic hepatitis, periodontitis, autoimmune diseases and atherosclerosis [161]. In macrophage RAW 264.7 cells, lipopolysaccharide (LPS) as an endotoxin generates the productions of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukins-1 (IL-1), prostaglandin E2 (PGE2), nitric oxide (NO). The production of PGE2 and NO are induced by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively. In addition, multiple intracellular signaling transduction pathways are triggered by LPS, including nuclear factor-κB (NF-κB) pathway and the mitogen-activated protein kinase (MAPK) pathway such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK1/2) and p38 MARK [162]. The anti-inflammatory potential of andrographolide (AG) had been proved by many researches. The IκBα -independent inhibition of NF-κB activation occurs through the JNK-Akt signaling to regulate the activation of p65.
Using nanoparticles for modification of active compounds aims to improve their biocompatibility and minimize their toxicity. We aimed to study the anti-inflammatory effect of AG and AG loaded zein/CKGM/Ca\textsuperscript{2+} nanoparticles in LPS-stimulated macrophage RAW 264.7 cells. In vitro assessment of cellular uptake, cytotoxicity, and protein knockdown efficiency of AG and AG loaded zein/CKGM/Ca\textsuperscript{2+} nanoparticles will be carried out in LPS-stimulated macrophage RAW 264.7 cells. Several pro-inflammatory cytokines and mediators such as NO, phospho-ERK and phospho-NF-κB will be tested.

4.2 Materials and Methods

4.2.1 Materials

Zein sample with a 97% protein content was obtained from Showa Sangyo (Tokyo, Japan), Carboxymethyl konjac glucomannan (CKGM) with degree of substitution of 0.01 was synthesized in our lab. Andrographolide (AG) of 98% purity was provided by Xi’an antongzebiotech (Xi’an, China). Murine macrophages RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Lipopolysaccharides (LPS), coumari-6 (3-(2-Benzothiazolyl))-N, N-diethylumbelliferylamine, 3-(2-Benzothiazolyl)-7-(diethylamino)coumarin, ≥98%), and (3-4,5- dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) were obtained from Sigma- Aldrich (St. Louis, MO, USA). The antibodies against phospho-ERK, phospho-NF-κB and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), 100× penicillin and streptomycin and phosphate buffer saline (PBS) were purchased from
4.2.2 Preparation of coumarin-6 labeled nanoparticles

5 mg/ml zein containing 0.02% coumarin-6 was prepared in 70% (v/v) aqueous ethanol solutions. All other conditions used in the preparation of fluorescent nanoparticles remained the same. The CKGM solution was dissolved by stirring weighted CKGM in water for 6 hours. Then, 1 ml of the zein solution was quickly added into 6 mL of CKGM solution with different concentrations. The ZC NPs was formed with mass ration of zein–CKGM at 2:1. For calcium coating, 20 μl of 10 mg/ml calcium solution was added into the above solution to obtain ZCCNPs. A single phase was formed after stirring vigorously. For AG loading, AG was fully dissolved in zein solution (5 mg/ml), the weight ratios of zein to AG was 3:1. The final obtained AG concentration was 0.238 mg/ml.

4.2.3 Cell culture

Macrophage RAW 264.7 cells were cultured at 37 °C in DMEM containing 10% inactivated FBS in a humidified incubator with 5% CO₂.

4.2.4 Confocal laser scanning microscopy (CLSM)

Macrophage RAW 264.7 cells (1×10⁵ viable cells per dish) were cultured in 35-mm² glass bottom dishes for 24h in 1 mL of growth medium. After 24 h incubation, the culture medium was replaced with 1 mL of fresh medium containing coumarin-6 labeled nanoparticles and incubated for 1, 2, 4 and 6 h (37°C, 5% CO₂). The cells were then fixed
with 4% formaldehyde for 20 min and washed with PBS for 3 times. The qualitative characterization of fluorescence intensity was imaged using model LSM 710 confocal laser scanning microscopy (Zeiss, Germany).

4.2.5 MTT cytotoxicity assay

Macrophage RAW 264.7 cells (3.0 × 10^4 viable cells per well) were cultured in 96-well plates for 24h, followed by removing the culture medium, and the cells were incubated with DMSO-dissolved AG or AG loaded zein/CKGM/Ca^{2+} nanoparticles at different doses for 24h. The cell viability was determined by treating cells with 200 μL of MTT (0.5 mg/mL) solution for 4 h. The culture medium was removed and the formed blue formazan crystals was dissolved in 150 ml DMSO and read at 490 nm using Model 680 Microplate Reader (BIO-RAD, USA). The 50% inhibitory concentration values (IC50) of DMY and DMY loaded NPs were also calculated using OriginPro 8.

4.2.6 Nitric oxide assay

Macrophage RAW 264.7 cells (3.0 × 10^4 viable cells per well) were seeded in 96-well plates. After 24 h incubation, then cells were stimulated with medium alone or medium containing 1 μg/ml lipopolysaccharide (LPS) alone or the same concentration of LPS in the presence of DMSO-dissolved AG or AG loaded zein/CKGM/Ca^{2+} nanoparticles at different doses. After a 24h incubation period, 100 μL of conditional supernatant were mixed with 50 μL of 1% sulphanalamide (in 5% phosphoric acid) and 50 μL 0.1% N-1- naphtylethlenediamine dihydrochloride (in distilled water) at room temperature for 10 min. The absorbance of nitrite at 550nm was measured and the nitrite concentration was calculated from a sodium nitrite serial dilution standard curve.
4.2.7 Western blotting

RAW 264.7 cells were treated with the same conditions as nitric oxide assay and cell pellets were collected after washing with phosphate-buffered saline (PBS), followed by suspending and incubating for 20 min at 4 °C in the ice-cold pH 7.4 lysis buffer (50 mM Tris–HCl, 1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 5 g/mL leupeptin, and 5 g/mL aprotinin). For extraction the total protein, cells were centrifuged 10 min at 15000 rpm, 4 °C. The protein concentration was measured at 750nm with a spectrophotometer using the BCA kit (Sigma-Aldrich). 30 μg of protein were denatured and electrophoresed through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to methanol-activated PVDF membranes. The membrane was blocked with 5% milk powder in PBS for 1 h at room temperature, followed by incubation overnight at 4 °C with primary antibodies specific for p-ERK1/2, NF-κB and GAPDH at dilutions of 1:1000 (v/v) in 5% milk powder in PBS. Blots were washed three times with Tween 20/Tris-buffered saline (TBST) and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody at a 1:2000 (v/v) dilution in 5% milk powder in PBS for 1h at room temperature. GAPDH was utilized to normalize for protein loading. After washing three times with TBST, blots were detected using LI-COR Odyssey imaging system. All determinations were performed in triplicate.

4.3 Results and discussion

4.3.1 Cellular uptake of nanoparticles in RAW 264.7 macrophage cells
To visualize the cellular uptake of zein/CKGM/Ca\textsuperscript{2+} NPs at 1, 2, 4 and 6 h incubation with RAW 264.7 macrophages cells, the intracellular localization of coumarin-6-labeled zein/CKGM/Ca\textsuperscript{2+} NPs was observed by CLSM (Figure 23). Zein/CKGM/Ca\textsuperscript{2+} NPs labeled by coumarin-6 showed green fluorescence dots, indicating the presence of zein/CKGM/Ca\textsuperscript{2+} NPs in RAW 264.7 macrophages cells. At six hour of incubation, the green spots obviously increased with the accumulation of incubation time, indicating that more zein/CKGM/Ca\textsuperscript{2+} NPs were continuously internalized by RAW 264.7 macrophages cells with extension of incubation time and more NPs were endocytosed via endosomal pathway.

![CLSM images](image)

Figure 23 CLSM images of intracellular uptake of zein/CKGM/Ca\textsuperscript{2+} nanoparticles NPs by RAW 264.7 macrophages cells. The scale bars represent 20 \( \mu \text{m} \).

4.3.2 Effect of AG and AG loaded nanoparticles on cell viability of RAW 264.7 macrophages
Chronic inflammation is a slow process mediated by exposure to a low level of irritants for a long time and has been shown to result from autoimmune disorders in inflammatory or immune cells. Macrophages play a big part in managing inflammatory responses including the overproduction of inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines [163]. Using nanoparticles for modification of active compounds aims to improve their biocompatibility and minimize their toxicity. To select proper andrographolide (AG) concentrations to investigate the anti-inflammatory properties, murine macrophage RAW264.7 cells were incubated with DMSO-dissolved AG or AG loaded zein/CKGM/Ca\(^{2+}\) nanoparticles at different doses for 24 h, and cell viability was determined using MTT assay. To examine the cytotoxicity by MTT assay, we incubated RAW264.7 macrophages with the test zein–carboxymethyl konjac glucomannan nanoparticles (with or without loading of AG) for 24 h. In this study (Figure 20), the viability of RAW 264.7 macrophages treated by free-form andrographolide was high at 3\(\mu\)M (>94.9%), thus, implementing that 3\(\mu\)M AG did not simulate cytotoxicity, and the NO suppression of AG at 3\(\mu\)M was not caused by cytotoxicity effect. Also, 4 \(\mu\)M DMSO dissolved AG has a low cell viability which was 71.75\%. Interestingly, the viability of RAW 264.7 cells treated with AG loaded nanoparticles was as high as 97% at 6\(\mu\)M. No significant cytotoxicity was observed for
groups receiving the nanoparticles without loading of andrographolide. These results indicated the low cytotoxicity of AG loaded zein/CKGM/Ca$^{2+}$ nanoparticles, which was also shown in Figure 24. Conclusively, using zein–carboxymethyl konjac glucomannan nanoparticles for modification of andrographolide can improve the biocompatibility and minimize the toxicity of the drug.

4.3.3 Effects of AG and AG loaded nanoparticles on NO production in LPS-stimulated RAW 264.7 macrophage cells

Figure 24 Cytotoxicity of DMSO-dissolved AG or AG loaded zein/CKGM/Ca$^{2+}$ nanoparticles on RAW264.7 cells.

using LPS-stimulated RAW 264.7 macrophage cells. After LPS stimulation, the NO
production was measured by Griess reagent. AG and AG loaded nanoparticles exhibited promising NO inhibitory properties as shown in Figure 25. From the MTT result, the highest concentration for AG and AG loaded zein/CKGM/Ca\textsuperscript{2+} nanoparticles to suppress NO production is 3 μM and 6 μM respectively. The NO inhibition of 3 μM AG and 3 μM nanoparticle encapsulated AG was found to be comparable in LPS-induced murine macrophage. 6 μM nanoparticle encapsulated AG was potent in NO inhibition in LPS-simulated RAW 264.7 macrophage cells. The respective blank zein/CKGM/Ca\textsuperscript{2+} nanoparticles did not show any inhibitory effects on NO production.

Figure 25 Effect of DMSO-dissolved AG or AG loaded zein/CKGM/Ca\textsuperscript{2+} nanoparticles on NO production in RAW 264.7 cells. LPS (1 μg/mL) was added and the cells were incubated 24 h.
4.3.4 Effects of AG and AG loaded nanoparticles on the phosphorylation of NF-κB in LPS-stimulated RAW 264.7 macrophage cells

As an intrinsic membrane component of Gram-negative bacteria, lipopolysaccharide (LPS) is an endotoxin that triggers the strongest microbial initiators of inflammatory response. The nuclear factor-κB (NF-κB) family comprise NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel. The functionally active NF-κB exists mostly as heterodimers constitutive of a p50 or p52 subunit and p65. NF-κB regulates various genes associated with inflammatory response, immune response and cell survival [164].

LPS induces NF-κB activation and promotes the degradation of IκB via several signal transduction pathways. The resulting liberated NF-κB heterodimer is then transferred to the nucleus, where it activates the transcription of iNOS, COX-2 and pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-8 [165]. For this reason, the LPS stimulated RAW 264.7 macrophage cells is a superior model for the screening anti-inflammatory drugs and the subsequent evaluation of inflammatory pathways.

Mitogen-activated protein kinases (MAPKs) mediated by ERK, JNK, and p38 response to mitogens and relate to a great variety of cellular activities such as gene expression, growth/differentiation, and survival/apoptosis. LPS also activates all three types of MAPKs in macrophage RAW 264.7 cells. Amongst, ERK activation is believed to be involved in increasing the production of iNOS and pro-inflammatory cytokines in macrophage RAW 264.7 cells [166]. In addition, several studies have illustrated that MAPKs play critical roles in the modulation of NF-κB activity. Because, in response to
inflammatory agonists, the MAPK pathways (Erk and p38) are activated concurrently with NF-κB, since they are required for the induction of NF-κB–dependent genes.

To investigate whether the NF-κB signaling pathway and the MAPKs signaling pathway are involved in the anti-inflammatory mechanism of AG and AG loaded nanoparticles, the phosphorylation and total protein levels of NF-κB (p65) and ERK1/2 in LPS-stimulated RAW 264.7 macrophage cells were determined using western blot.

In this study, we evaluated the potent anti-inflammatory effects of AG through down-regulating NF-κB and up-regulating MAPKs (ERK1/2) signaling pathways in the LPS-induced macrophages, and examined that AG dose-dependently suppressed the phosphorylation of p65 (Figure 22). Phosphorylation of ERK1/2 and p65 was increased only in cells treated with LPS. We found that AG and AG loaded zein/CKGM/Ca\(^{2+}\) nanoparticles markedly attenuated LPS-induced phosphorylation of p65, while blank nanoparticles did not show any suppression effect. Also, AG loaded nanoparticles suppress p65 in a dose-dependent manner. The previous studies that explore the molecular mechanisms of NF-κB pathway of AG are consistent with present results we found. The results showed in Figure 26 demonstrated that phosphorylations of p65 may be responsible for the anti-inflammatory effects of AG in LPS-stimulated macrophage RAW 264.7 cells. The efficacy of AG to regulate the in vitro NF-κB expression was notably enhanced by the nanoparticle delivery system due to enhanced dose efficiency resulting from better solubility, transportability and reduced toxicity. Interestingly, 3 μM AG and 3 μM or 6 μM AG loaded nanoparticles appreciably increased phosphorylated (activated) ERK1/2, suggesting the involvement of other signaling molecules and pathways. These results indicate that suppression of phosphorylation of ERK1/2 might
not contribute to the inhibitory effect of AG on LPS-induced NF-κB activation in macrophage RAW 264.7 cells [165]. More studies about the signaling kinases mechanism of AG is needed in the future.

<table>
<thead>
<tr>
<th>LPS (1µg/ml)</th>
<th>AG3-NPs</th>
<th>AG6-NPs</th>
<th>AG3</th>
<th>NPs</th>
<th>-</th>
<th>Control</th>
<th>KDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK1/2</td>
<td>1.65</td>
<td>1.49</td>
<td>1.64</td>
<td>1.44</td>
<td>1.35</td>
<td>1.00</td>
<td>44/42</td>
</tr>
<tr>
<td>NF-κB</td>
<td>3.55</td>
<td>2.24</td>
<td>3.30</td>
<td>6.01</td>
<td>5.98</td>
<td>1.00</td>
<td>65</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37</td>
</tr>
</tbody>
</table>

Figure 26 Inhibitory effects of AG and AG loaded zein/CKGM/Ca2+ nanoparticles on phosphorylation of ERK1/2 and and NF-κB (P65) in LPS-induced RAW 264.7 cells. The data are representative example of three experiments.

4.4 Conclusion

In conclusion, our observations suggest that AG possesses promising anti-inflammatory effects by inhibiting LPS-simulated NF-κB activation in macrophage RAW 264.7 cells. AG could thus serve as a potential anti-inflammatory food supplement or drug candidate. The efficacy of AG to regulate the in vitro NF-κB expression was notably enhanced by the zein/CKGM/Ca2+ nanoparticle delivery system due to enhanced dose efficiency resulting from better solubility, transportability and reduced toxicity. Therefore, zein/CKGM/Ca2+ nanoparticle is a promising delivery system for anti-inflammation drug, i.e. AG.
CHAPTER 5 FLUORESCENCE QUENCHING STUDY OF DIHYDROMYRICETIN BINDING TO ZEIN

5.1 Introduction

In this study, in order to improve the solubility and permeability of DMY, we encapsulate DMY in zein nanoparticles. To quantify the binding of DMY and zein, quenching of protein fluorescence is the method. Quenching of protein fluorescence is an important technique to gain understanding of the complexation of small molecules and proteins. Because of the overlapping of the fluorophore (i.e., proteins) emission and quencher (i.e., polyphenols) absorption bands, fluorescence quenching measures the decrease of fluorescence intensity when a fluorophore interacts with a quencher molecule [167]. Tryptophan and tyrosine, which are typically excited at 280 nm, are the most significant fluorophores of proteins. The emission maximum of tryptophan and tyrosine are typically at around 350 and 303 nm in the polar environment. Conversely, the emission maximum shows a blue-shift when in the less polar environment [168]. Dynamic and static quenching are the two most common quenching mechanisms. (1) Dynamic quenching involves a small molecule deactivates the excited-state fluorophore via a random noninteractive collision, and (2) static quenching involves a ground state complex formation between the small molecule and the fluorophore, which makes the fluorophore nonfluorescent [126]. In this study, both static and dynamic quenching will be considered, and mathematical models will be used to interpret the fluorescence quenching results and quantify the binding of the fluorophore and the quencher.
Zein is the most abundant prolamine-type proteins found in maize which have been widely used for the lipophilic functional compound encapsulation, such as curcumin, quercetin, essential oils, vitamin E, grape seed extract and all-trans-retinoic acid [77]. Binding affinity is one of the major factors affects the ability of zein to encapsulate hydrophobic compounds. Therefore, the aim of this work is to explore the nature and magnitude of the interaction of DMY and zein using fluorescence spectroscopy.

5.2 Materials and Methods

5.2.1 Materials

Zein sample with a 97% protein content was obtained from Showa Sangyo (Tokyo, Japan), Dihydromyricetin (DMY) of 98% purity was purified in our lab. All other reagents were of analytical grade and obtained from commercial sources.

5.2.2 Preparation of stock solutions

Zein fluorescence was measured in an 85% ethanol solution with constant concentration (1 mg/ml) in the presence of different concentrations of DMY at 298K, 303K and 308K. About 3600 μL of zein solution was placed in the 1 cm quartz cell, followed by adding different volumes (0 to 30 μl) of DMY solution (2 mg/ml) to the cell. The zein-DMY complex was continuously and vigorously stirred before measurement.

5.2.3 Fluorescence spectroscopy

Fluorescence intensity was conducted at 298K on a Fluoromax-3 spectrophotometer (Horiba Scientific Inc., Edison, NJ, USA) equipped with a 1 cm quartz
cell. The excitation wavelength was set at 280 nm and the emission spectra were collected in a range of 290-350nm.

5.3 Results and discussion

5.3.1 Fluorescence characteristic of zein

The fluorescence spectra of zein were obtained in 85% (v/v) aqueous alcohol solution. The emission maximum of zein (304 nm) exhibited a blue-shift (Figure 27), which is a shorter wavelengths compared to the emission maximum reported for water soluble protein lactoferrin (around 334nm). Besides, the significant shorter emission maximum of zein is attributed to its approximate 5.0% w/w tyrosine amino acid residues, which have a representative emission maximum around 304 nm [169].

![Figure 27 Emission spectra of the zein obtained with the increase of the DMY concentration (T=298K; λ=280nm). [Zein]=1mg/ml; [dihydromyricetin] is increased from 1.64μM to 49.2μM.](image)
The zein solution concentration was set at 1 mg/ml with serial concentrations of DMY. DMY solution (2 mg/ml) from 0 to 30 μl was added to 3600 μL of zein solution. As shown in Figure 27, the fluorescence emission spectra of zein were measured as a function of increasing concentration of DMY. The fluorescence intensity of zein gradually decreased with the increase of DMY concentration at 298 K and the emission maximum of zein was gradually red-shifted. These spectral responses indicated the interactions between zein and DMY and could be used to calculate the binding affinity between zein and DMY. The fluorescence of zein was clearly quenched by DMY addition as a result of decreasing fluorescence intensity. The quenching process causes the decrease of fluorescence intensity. Dynamic quenching involves the collisions between the quencher and the fluorophore, and static quenching is due to the non-fluorescent complex formation between the fluorophore and a quencher. The investigation of effects of temperature can distinguish these two mechanisms. Temperature increase results in a larger diffusion rate and hence enhancement of dynamic quenching, while complex formation force is inversely proportional to the temperature due to the dissociation of weak binding. The Stern-Volmer equation could interpret the static and dynamic quenching as follows [170]:

\[
\frac{F_0}{F} = 1 + K_Q\tau_0[Q] = 1 + K_{SV}[Q]
\]

In this equation \( F_0 \) represents the fluorescence intensity of the sample in the absence of quencher at peak emission (i.e., 304 nm); \( F \) is the fluorescence intensity of the same sample in the presence of a quenching agent (304 nm); \([R]\) is the total quenching agent (i.e., DMY) concentration; \( K_{SV} \) is the Stern–Volmer quenching constant, which is calculated as the slope of a plot of \( \frac{F_0}{F} \) versus \([R]\); \( kq \) is the bimolecular quenching
constant; $\tau_0$ is the average lifetime of the fluorescence molecules without the quenching agent.

Figure 28 Stern-Volmer plots for the fluorescence quenching of zein by DMY at 298, 303 and 308 K.

Figure 29 The plot of $\ln \left( \frac{F_0 - F}{F} \right)$ versus $\ln [Q]$ for the interaction of DMY and zein.
The fluorescence quenching results at 298, 303 and 308 K obviously adhere to the Stern-Volmer equation (Figure 24). The Stern-Volmer quenching constant ($K_{SV}$) is inversely related to temperature. These results indicated that the quenching mechanism of zein-DMY is probably static quenching. The quenching constants of zein-DMY complexes are summarized in Table 2.

Table 2 Stern-Volmer quenching constants ($K_{SV}$) of zein-DMY complexes at 298, 303 and 308 K.

<table>
<thead>
<tr>
<th>pH</th>
<th>T (K)</th>
<th>$K_{SV}$ (×10^4 M⁻¹)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>298</td>
<td>3.47</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>3.44</td>
<td>0.9869</td>
</tr>
<tr>
<td></td>
<td>308</td>
<td>3.39</td>
<td>0.9865</td>
</tr>
</tbody>
</table>

5.3.2 Analysis of binding equilibria

The equation describes the binding equilibrium between non-bound and bound fluorescence molecules as follows [169]:

$$\ln \left[ \frac{(F_0 - F)}{F} \right] = \ln K_b + n \ln [Q]$$

Here, the binding constant for one site $K_b$ and the number of substantive binding sites $n$ at 298, 303 and 308 K are shown in Table 3. The binding constant value $K_b$ decreased with increasing temperature. This indicated that complex formation between DMY and zein was less favorable and partially disassembled at higher temperatures. To
study the interaction between DMY and zein at three temperatures, Figure 29 shows the plot of \( \ln[(F_0-F)/F] \) versus \( \ln[Q] \) yields \( \ln K_b \) as the intercept and \( n \) as the slope. The number of binding sites (\( n \)) is roughly equal to 1.0, illustrate that DMY binds at a 1:1 stoichiometry to zein, which suggested that there is probably one DMY molecule associated to each zein molecule.

Table 3 Apparent binding constant \( K \) and number of binding sites (\( n \)) at different temperatures. Enthalpy (\( \Delta H \)), entropy (\( \Delta S \)) and free energy (\( \Delta G \)) change based on the van’t Hoff equation.

<table>
<thead>
<tr>
<th>pH</th>
<th>T(K)</th>
<th>( K_b ) (x10^5 M(^{-1}))</th>
<th>n</th>
<th>( R^2 )</th>
<th>H (kJ mol(^{-1}))</th>
<th>S (J mol(^{-1})K(^{-1}))</th>
<th>( \Delta G ) (KJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>298</td>
<td>2.21</td>
<td>1.1826</td>
<td>0.9994</td>
<td>-154.62</td>
<td>-417.60</td>
<td>-30.18</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>0.42</td>
<td>1.0297</td>
<td>0.9957</td>
<td></td>
<td>-417.60</td>
<td>-28.09</td>
</tr>
<tr>
<td></td>
<td>308</td>
<td>0.29</td>
<td>0.99576</td>
<td>0.9957</td>
<td></td>
<td></td>
<td>-26.00</td>
</tr>
</tbody>
</table>

5.3.3 Thermodynamic analysis

In general, the possible interaction forces between DMY and zein include hydrogen bonds, hydrophobic attraction, van der Waals forces, electrostatic interactions, and even covalent binding. The thermodynamic parameters calculated by binding constants could partially unveil the nature of interaction between DMY and zein. The enthalpy change (\( \Delta H \)) and entropy change (\( \Delta S \)) are considered as constant if temperature doesn’t change significant, and the Gibbs free energy change (\( \Delta G \)) upon addition of DMY can be modelled by the van't Hoff plot [169]:
\[ \ln K_b = - \frac{\Delta H}{RT} + \frac{\Delta S}{R} \]

Here, \( K_b \) is the binding constants at certain temperatures (T) and R is the universal gas constant. The values of \( \Delta H \) and \( \Delta S \) could be calculated from the van't Hoff plot of \( \ln K_b \) versus \( 1/T \), which yields \( \Delta H \) as the intercept and \( \Delta S \) as the slope (Figure 26). The values of \( \Delta H \), \( \Delta S \) and \( \Delta G \) are demonstrated in Table 3. \( \Delta G \) could be obtained as follows [169]:

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

The thermodynamic parameters are significant to confirm the interactions between binding ligands and fluorescence molecules. The negative Gibbs free energy change (\( \Delta G \)) for interaction of DMY and zein revealed the binding reaction occurred spontaneously. The negative enthalpy (\( \Delta H \)) suggested the significance of hydrogen bonds and van der Waals in the DMY-zein interaction and the interaction was exothermic. This along with the negative entropy (\( \Delta S \)) indicated that the interaction could arrange solvent molecules surrounding both DMY and zein molecules in a more ordered configuration. Actually, the interaction between DMY and zein is usually a combination of the different interaction types, the other kind of interaction forces is not significant compared with van der Waals and hydrogen bonds. Other complementary techniques are necessary to further fully investigate the interaction types and strengths in DMY-zein complexes.
5.4 Conclusion

Fluorescence quenching studies showed that DMY-zein complexes mainly bind through van der Waals forces and hydrogen bonds. This result is advantageous for the formulation of zein nanoparticles or nanocomplex to encapsulate DMY. However, the DMY–zein binding constant decreased with temperature, which suggested the significance of utilized the retention or release of encapsulated bioactive compounds at increased temperatures in delivery systems design. In conclusion, this study provides valuable information for designing zein nanoparticles to encapsulate flavonoid compounds such as DMY.
CHAPTER 6 GREEN-STEP ASSEMBLY OF ZEIN NANOPARTICLES FOR DIHYDROMYRICETIN LOADING AND pH-RESPONSIVE DELIVERY

6.1 Introduction

Current cancer therapy involves surgery, radiation and chemotherapy, which could also kill normal cells and cause side effect to the patient. Overall, the efficacy of the treatment is believed to be directly related to the targeting ability of the treatment to cancer cells and the minimum affecting effects to healthy cells [1]. Therefore, it would be promising to develop chemotherapeutic drugs that can target tumorous tissues either passively or actively. The advances of cancer treatment in both new chemotherapeutic drugs and new ways of delivering chemotherapeutic drugs are rapidly progressing. Hopefully this development can help us with lowering the toxic of the drugs to healthy cells and targeting the cancer cells. This thesis will primarily address new methods for delivering chemotherapies with a focus on novel biodegradable nanoparticles.

Due to rapid and unorganized angiogenesis with high glycolysis and acidic metabolites, the extracellular pH of fast-growing tumors is slightly lower than normal tissues [171]. pH-responsive delivery systems have utilized the extracellular pH variations between healthy tissues (~7.4) and the tumor tissues (5.6–7.2). To design a pH-responsive system, we can either take advantage of conformational and/or solubility changes of ionizable groups in polyacids or polybases, or the cleavage of acid-sensitive
bonds between anchored molecules and polymer backbones [172]. In this paper, we utilized the acid-sensitive bonds between metal ions and dihydromyricetin (DMY).

Dihydromyricetin (2,3-dihydroflavonol, DMY), extracted from *Ampelopsis grossedentata* found in South China, has been effective in treatment of cough, liver diseases, hypertension and high blood sugar [126]. The antioxidant, antitumor, antibacterial and antiviral properties of DMY has been restricted by its low oral bioavailability (no more than 10% in rats), which is probably resulted from the combined effects of poor permeability ($\text{P}_{\text{eff}} = (1.84 \pm 0.37) \times 10^{-6} \text{ cm/s}$) and low solubility (0.2 mg/mL at 25 °C) [173]. Moreover, due to its high degree of superdelocalizability, appropriate spatial configuration, strong conjugated large $\pi$ bond and coordinated oxygen atoms, DMY acts as a good metal ion-chelating ligand [127]. pH changes affect the forming and breaking down of metal ion–DMY coordination bonds because at lower pH metal ions could compete with protons in acids for binding with ligand (DMY). Zinc has a great variety of physiological functions in human body, such as cell metabolism regulation [174], DNA rewinding [175]. Zinc is the active medical ingredients to treat skin injuries and deadly diarrhea [176]. Additionally, Zinc complexes have shown promising properties in anti-cancer [177], anti-inflammation [178], anti-microbial [179], anti-oxidation [170], anti-convulsion [180], anti-diabetes [181] and Alzheimer disease treatment [182]. In this study, Zn$^{II}$ forms complexes with zein protein and DMY acting as pH-sensitive delivery systems.

In this paper, the chelation ability of DMY and Zinc(II) was utilized to form pH-sensitive zein NPs to control release DMY. We fabricated a DMY-Zn$^{II}$ coated zein NPs to enhance the sustained release, cellular uptake and the anti-cancer activity of DMY.
Stable metal-DMY coated zein nanoparticles were fabricated via self-assembly of zein, metals and DMY through electrostatic interaction and hydrogen bonds. The preparation process was optimized in terms of the particle size, polydispersity index (PDI), fluorescence quenching, circular dichroism (CD) spectra and in vitro release profile of the nanoparticles. DMY-loaded nanoparticles were evaluated for in vitro cytotoxicity and cellular uptake. This pH-responsive zein NPs had great potential in dihydromyricetin loading and anti-cancer drug delivery. This study provided valuable information for the future design of more effective and safer food grade delivery systems.

6.2 Materials and Methods

6.2.1 Materials

Zein sample with a 97% protein content was obtained from Showa Sangyo (Tokyo, Japan), Dihydromyricetin (DMY) of 98% purity was purified in our lab. Zinc chloride (ZnCl₂·2H₂O) were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO). Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), 100× penicillin and streptomycin, trypsin-EDTA and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Human cervix carcinoma HeLa cells was purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO). Coumari-6 (3-(2-Benzothiazolyl)-N,N-diethylumbelliferylamine, 3-(2-Benzothiazolyl)-7-(diethylamino)coumarin, ≥98%) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide) were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO). DAPI (4’,6-diamidino-2-phenylindole), CellMask Deep Red Plasma membrane Stain and LysoTracker Red were purchased from Thermo Fisher Scientific Inc.
(Waltham, MA). All other reagents were of analytical grade and obtained from commercial sources.

6.2.2 Fluorescence spectroscopy

Fluorescence intensity was measured at 298 K on a Fluoromax-3 spectrophotometer (Horiba Scientific Inc., NJ, USA) equipped with a 1 cm quartz cell. In the presence of different volumes (0 to 30 μl) of DMY solution (2 mg/ml), the fluorescence of zein (1 mg/ml) was measured at 298 K. The excitation wavelength was set at 280 nm and the emission spectra were collected in a range of 290-350 nm.

6.2.3 Circular Dichroism (CD) spectroscopy

CD spectra were recorded at 190-260 nm wavelength using an AVIV circular dichroism spectrometer (model 400, Lakewood, NJ, USA) in a 1 mm path length quartz cuvette. CD spectra was used for determining the secondary structure of zein (0.1 mg/mL) with different zein-DMY ratios in 85% (v/v) aqueous ethanol at pH 7.0. The fraction of the secondary structure of zein–DMY complex were processed by curve fitting using SELCON3 program in CD Pro software.

6.2.4 Preparation of nanoparticles

NPs were prepared using anti-solvent precipitation method. Briefly, 10 mg/mL zein and 4 mg/ml DMY was fully dissolved in 85% (v/v) aqueous ethanol solution. Then, 1 mL of zein solution was quickly added into 9 mL of MOPS buffer (10 mM, pH 7.4). Immediately following this, 10, 20 or 30 μl of metal solution (100 mM ZnCl₂ ·2H₂O solutions) was poured and a single phase was formed after vigorously stirring.
6.2.5 Particle size and zeta potential measurements

The average particle size of NPs was obtained by dynamic light scattering (DLS) using a BIC 90 Plus particle size analyzer (Brookhaven Instrument Corp., NY, US) at 25.0 °C and a solid-state laser at 658 nm with a scattering angle of 90°, and it was calculated from Cumulant analysis. Zeta-potential was obtained by a particle electrophoreses instrument using Zetasizer Nano-ZS (Malvern Instruments, Worcs, UK). Zeta potential values was then calculated from electrophoretic mobility using the Smoluchowski theory and averaged over for three replicates.

6.2.6 In vitro release study

In vitro release profile of Z-DMY NPs and Z-DMY/ ZnII NPs was determined using dialysis method in PBS solution (0.01M, pH 6.8, 6.2, 5.0 or 4.0). Briefly, under 100 rpm gently shaking in 37.0 °C, an aliquot of NPs was placed in 3500 MWCO dialysis bags immersed in 50 mL PBS solution. 1 mL dissolution sample was collected from PBS solutions at predetermined intervals, and the concentration of DMY was measured at room temperature using UltiMate 3000 HPLC system with wavelength scan from 200 to 400 nm. The analyze was conducted on a reverse-phase C18 column (column size: 250 mm × 4.6 mm, particle size: 5 μm, Thermo, Germany). The mobile phase was a gradient of water/acetonitrile at a flow rate of 0.8 mL/min. The gradient consisted of 80 to 88% water in 5 min, followed by maintaining 8 min, and a decrease to 65% water in 7 min, followed by 20 min holding time [173].

6.2.7 Cell culture
Human cervix carcinoma HeLa cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 100 U ml⁻¹ penicillin, and 100 g/ml streptomycin at 37 °C, 95% relative humidity and 5% CO₂.

6.2.8 MTT cell proliferation assay

HeLa cells were seeded in 96-well plates at a density of 1.0 × 10⁴ cells/well and the cell viability with DMSO-dissolved DMY, Z-DMY NPs, Z-DMY/ Zn²⁺ NPs and drug free NPs at different doses were determined using tetrazolium dye, MTT. After 24 h incubation at 37°C in growth medium, cells were treated with 200 L of MTT (0.5 mg/mL) solution for 4 h, the formed blue formazan crystals was dissolved in 150 ml DMSO and read at 490 nm using Model 680 Microplate Reader (BIO-RAD, USA). The 50% inhibitory concentration values (IC50) of DMY and DMY loaded NPs were also calculated using SPSS 18.0.

6.2.9 Confocal laser scanning microscopy (CLSM)

To trace the cellular uptake of NPs, NPs were labeled with coumarin-6. When preparing fluorescent NPs, Z-DMY/ Zn²⁺ NPs were prepared under all the same conditions except that coumarin-6 was added to 85% ethanol solution after dissolving zein and DMY, the mass ratio of zein and coumarin-6 was 200 to 1. HeLa cells (1×10⁵ cells per well) were cultured in a 12-well plate for 24h, followed by removing the culture medium, and the cells were incubated with coumarin-6 labeled Z-DMY/ Zn²⁺ NPs for 1, 2, 4 or 6 h (37°C, 5% CO₂). During the last 10 min of NPs treatment, cells lysosomes were stained with 50 nM LysoTracker Red and cell nuclei were stain with DAPI (blue). Then the NPs were removed and the cells were washed 3 times with PBS before fixing with 4%
formaldehyde for 20 min. The qualitative characterization of fluorescence intensity was imaged using model LSM 710 confocal laser scanning microscopy (Zeiss, Germany). The quantitative characterization of fluorescence intensity was determined by Accuri C6 Flow Cytometer (BD Biosciences, New Jersey, USA). To obtain comparable results, cells were treated in the same conditions with microscopy imaging studies. The treated cells were harvested and centrifuged to get the cell pellets. The cell pellets were washed and re-suspended in PBS solution before measurement.

6.3 Results and discussion

6.3.1 The effect of dihydromyricetin (DMY) concentrations on the fluorescence intensity of zein

The fluorescence spectra of zein were obtained in 85% (v/v) aqueous alcohol solution. The emission maximum of zein (304 nm) exhibited a blue-shift (Figure 1), which is a shorter wavelengths compared to the emission maximum reported for water soluble protein lactoferrin (around 334 nm) [167]. Besides, the significant shorter emission maximum of zein is attributed to its approximate 5.0% w/w tyrosine amino acid residues, which have a representative emission maximum around 304 nm [169].

The zein solution concentration was set at 1 mg/ml with serial concentrations of
DMY. DMY solution (2 mg/ml) from 0 to 30 μl was added to 3600 μL of zein solution. As shown in Figure 31, the fluorescence emission spectra of zein were measured as a function of increasing concentration of DMY. The fluorescence intensity of zein gradually decreased with the increase of DMY concentration at 298 K and the emission maximum of zein was gradually red-shifted. These spectral responses indicated the interactions between zein and DMY and could be used to calculate the binding affinity between zein and DMY [126]. The fluorescence of zein was clearly quenched by DMY addition as a result of decreasing fluorescence intensity.

Figure 31 Emission spectra of the zein obtained with the increase of the DMY concentration (T=298K; λ=280nm). [Zein]=1mg/ml; [dihydromyricetin] is increased from 1.64μM to 49.2μM.

6.3.2 The effect of dihydromyricetin (DMY) concentrations on the secondary structure of zein

The CD spectra of zein with different concentrations of DMY was exhibited in Figure 32. As shown in the inserted table in Figure 32, the quantitative analysis of secondary structure (α-helix, β-sheet, β- turn, and random coil) of zein were estimated by SELCON3. The CD spectra of zein showed the characteristics of α-helix-rich secondary structure with two negative peaks at around 209 and 223 nm [183]. The alteration of CD bands intensity in Figure 32 clearly exhibited the evidence for the secondary structure change of zein in 85% aqueous ethanol that was caused by DMY treatment. The fractional contents of the secondary structure of native zein was 43.5% α-helix, 15.0% β-sheet, 20.2% β-turn and 19.1% random coil, respectively. It can be seen that the addition
of DMY to zein solution causes an obvious change of α-helix and β-sheet configurations at pH 7.0, which was highly dependent on DMY concentrations. At the zein-DMY mass ratios of 1:0.05, 1:0.1 and 1:0.2, the contents of α-helix of zein were increased to 50.5%, 49.0% and 49.3%, respectively. At the same time, β-sheet contents were decreased to 11.5%, 11.6% and 10.8%, respectively. In the presence of DMY, the conformation change of zein might be caused by the interaction between zein and DMY. Because the structure of α-helix was supported by hydrogen bond, an increase in α-helical content indicated that more hydrogen bonds were formed between zein and DMY with a suitable ratio of 1:0.05–1:0.2 [184]. Many other researchers also pointed out that protein secondary structure changes due to hydrophobic interactions and hydrogen bond between protein and polyphenol [168]. Kanakis et al. (2011) also reported that the α-helix content of β-lactoglobulin was increased and a stronger structural stabilization was formed due to binding of EGCG [185]. When the zein-DMY mass ratios were increased to 1:0.3–1:0.4, the CD bands intensity was restored to the pure zein state, suggesting that the binding pattern of zein and DMY might be changed. The finding interpreted that the aggregation might be formed between zein molecules with high DMY concentration since increased β-sheets were usually showed in aggregated proteins [186]. Recent finding also revealed that polyphenol with low concentration caused the secondary structure change of the native unfolded protein by covering the polyphenol, while the complex precipitation caused by bridging effect could occur due to increasing the polyphenol concentration [187].
Figure 32 CD spectra of zein in 85% (v/v) aqueous ethanol with different zein/DMY ratios at pH 7.0. (Insets) Fractional contents of the secondary structure in zein after heat treatment.

Figure 33 Illustration of the synthesis and structures of Z-DMY NPs and Z-DMY/ZnII
NPs under physiological pH 7.4 or tumor environment pH 5.0.

6.3.3 The effect of metal- DMY coating on particle size and zeta potential of zein nanoparticles

Here, we reported a simple, fast and green approach to fabricate a novel core-shell drug delivery system by applying one-step assembly of metal-DMY coordination complexes around zein NPs surface (Figure 33). The supramolecular shell was generated onto the zein NPs surface by mixing DMY and metal within the as-prepared zein NPs solution. DMY acted as organic ligand while metal ions were used as inorganic cross-linker [7,8]. The spontaneous complexation of metal ion with the DMY catecholic unit or adjacent hydroxyl groups and a carbonyl group was utilized to form cross-linking on zein NPs [9].

Table 4 showed the effect of Zn\textsuperscript{ii} on the particle size, polydispersity index (PDI) and zeta potential of zein-DMY NPs. Before the addition of metal ions, the particle size and zeta potential of zein-DMY NPs was 62.5 nm and −24.7 mV, respectively. The particle size of zein-DMY NPs increased to varying degrees with the rise of metal ions, which was highly related to the certain concentration of metal ions. The particle size was larger with higher metal ion concentrations. After the addition of Zn\textsuperscript{2+}, the particle size of Z-DMY/ Zn\textsuperscript{ii} NPs was between 138.2nm and 161.4nm, and the zeta-potential was also obviously decreased to high negative charges, which was between -26.1±0.3 mV and -31.4±0.9 mV. These findings suggested the existence of electrostatic shielding and cross-linking among zein molecular, DMY molecule and zinc ions, which leading to a thicker
coating structure and larger absolute value of the zeta-potential [160]. These finding may suggest that Z-DMY/ Zn\textsuperscript{II} NPs may more likely have a more homogenous distribution because of the larger absolute value of the zeta-potential.

Table 4 Particle size, polydispersity index (PDI), zeta potential of NPs in different formulations\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Particle size</th>
<th>PDI</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-DMY</td>
<td>62.5±0.4</td>
<td>0.07±0.04</td>
<td>-24.7±0.2</td>
</tr>
<tr>
<td>Z-DMY/ Zn\textsuperscript{II}\textsubscript{1}</td>
<td>138.2±0.6</td>
<td>0.05±0.05</td>
<td>-26.1±0.3</td>
</tr>
<tr>
<td>Z-DMY/ Zn\textsuperscript{II}\textsubscript{2}</td>
<td>142.7±2.3</td>
<td>0.13±0.06</td>
<td>-29.1±0.3</td>
</tr>
<tr>
<td>Z-DMY/ Zn\textsuperscript{II}\textsubscript{3}</td>
<td>161.4±1.3</td>
<td>0.14±0.03</td>
<td>-31.4±0.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Z, zein; DMY, dihydromyricetin; All NPs were prepared at a zein: DMY ratio of 1:0.4. Z-DMY/ Zn\textsuperscript{II}\textsubscript{1}, Z-DMY/ Zn\textsuperscript{II}\textsubscript{2} and Z-DMY/ Zn\textsuperscript{II}\textsubscript{3} represented an initially used 10, 20 or 30 μL of 100 mM Zn\textsuperscript{II}.

6.3.4 The effect of metal- DMY coating on in vitro drug release of zein nanoparticles

To better understand the controlled DMY release profile from DMY-loaded Z NPs, the in vitro release was conducted under pH 7.4 and 5.0 to simulated physiological conditions and endosomal/lysosomal environment, respectively [188]. Scheme 1. Illustration of the synthesis and structures of Z-DMY NPs and Z-DMY/ Zn\textsuperscript{II} NPs under physiological pH 7.4 or tumor environment pH 5.0. Figure 34a showed that individual zein NPs exhibited a burst release profile at pH 7.4 and 5.0, i.e., 73%-85% within 8h (Figure 34a). These findings probably due to the hydrophobic interactions and hydrogen
bond between zein molecule and DMY molecule were broken down under acidic environment [81][189]. In contrast, DMY-Zn\textsuperscript{II} coated Z NPs showed a relatively slow release profile at pH 7.4, and the release profile of Z-DMY/ Zn\textsuperscript{II} NPs was concentration-dependent as a function of Zn\textsuperscript{II} concentration. At the volumes of 100mM Zn\textsuperscript{II} was 10, 20, 30 µl, the contents of DMY released from Z-DMY/ Zn\textsuperscript{II} NPs at pH 7.4 within 8h were around 45%, 38% and 26%, respectively. At the same time, 80%, 78% and 62% DMY were released at pH 5 with 8h, respectively (Figure 34b, 34c and 34d). These findings suggested that DMY-Zn\textsuperscript{II} coordinated bonding effectively retarded the diffusion of DMY from NPs and assisted the structure stability of Z-DMY/ Zn\textsuperscript{II} NPs at physiological conditions. This variation of release profile at different pH suggested the highly pH-responsive release properties of Z-DMY/ Zn\textsuperscript{II} NPs. In addition, Z-DMY/ Zn\textsuperscript{II} NPs were relatively stable under physiological conditions in the absence of stimuli whereas sensitively responding to acidic conditions equivalent to the endosomal/lysosomal environment, which could markedly reduce the side effects of anti-cancer drugs in cancer therapy [190]. Here, Z-DMY/ Zn\textsuperscript{II}\textsubscript{2} NPs with 20 µl 100mM Zn\textsuperscript{II} was chose to do the biological efficiency test due to its relatively low release at pH 7.4 but relatively fast release at pH 5.
Figure 34 In vitro release profiles of DMY from (a) Z-DMY, (b) Z-DMY/ ZnII1, (c) Z-DMY/ ZnII2, (d) Z-DMY/ ZnII3 in PBS under different pH conditions. Z-DMY represented DMY-loaded zein NPs. Z-DMY/ ZnII1, Z-DMY/ ZnII2 and Z-DMY/ ZnII3 represented DMY-loaded zein/ ZnII NPs with initially used 100 mM ZnII 10 μl, 20 μl and 30 μl, respectively. All the NPs were prepared at zein: DMY ratio of 1:0.4.

6.3.5 In vitro proliferation of nanoparticles on cancer cells

The in vitro cytotoxicity of DMY free zein NPs, DMY, Z-DMY NPs and Z-DMY/ ZnII NPs against HeLa cells was assessed by MTT assay. Figure 35 exhibited the dose-dependent effects DMY in DMSO, DMY-loaded zein NPs and Z-DMY/ ZnII NPs on the cell viability. In addition, DMY free zein NPs did not show any cytotoxicity on HeLa cells, indicating that the blank zein NPs were nontoxic to HeLa cells. The cytotoxicity of DMY and DMY loaded NPs were calculated using the half maximal inhibitory
concentration (IC$_{50}$) [94]. For hela cells, the IC$_{50}$ of DMY in DMSO, Z-DMY NPs and Z-DMY/ Zn$^{II}$ NPs were 44.4 ± 0.02 μM, 46.7 ± 0.02 μM and 52.6 ± 0.02 μM, respectively. Noticeably, the cytotoxicity of Z-DMY/ Zn$^{II}$ NPs was lower than that of free DMY and Z-DMY NPs, which attributed mainly to the sustained drug release profile of the Z-DMY/ Zn$^{II}$ NPs. Free DMY can freely enter cancer cells through plasma membrane and kill the cells [84]. According to the release profiles, 22% DMY release from Z-DMY/ Zn$^{II}$ NPs before entering cancer cells and cancer cells internalized Z-DMY/ Zn$^{II}$ NPs through endocytosis, and the cumulative released amount of DMY from Z-DMY/ Zn$^{II}$ NPs was around 70% at pH 5 equivalent to the endosomal/lysosomal environment. The sustain release characteristic probably explained why Z-DMY/ Zn$^{II}$ NPs had a higher IC$_{50}$. In addition, according to the release profiles, Z-DMY NPs exhibited faster release of DMY compared to Z-DMY/ Zn$^{II}$ NPs at pH 7.4 or 5. As a result, the cytotoxicity of Z-DMY NPs was similar to free DMY in Figure 35.
Figure 35 In vitro cytotoxicity of the Blank NPs, DMY dissolved in DMSO, Z-DMY NPs and Z-DMY/ Zn$^{II}$ NPs against hela cells incubated for 24 h. The indicated concentrations are DOX doses. It should be noted that for evaluating Z-DMY NPs and Z-DMY/ Zn$^{II}$ NPs, equal concentrations of blank NPs were employed to eliminate the effect of vehicles in MTT assay. Data displayed as mean ± SD (n = 6).

6.3.6 Intracellular Uptake Study

To visualize the cellular uptake of Z-DMY/ Zn$^{II}$ NPs at 1, 2, 4 and 6 h incubation with HeLa cells, the intracellular localization of coumarin-6-labeled Z-DMY/ Zn$^{II}$ NPs was observed by CLSM (Figure 36). The green, blue and red fluorescence dots indicated Z-DMY/ Zn$^{II}$ NPs, nuclei and endosomes/lysosomes. Z-DMY/ Zn$^{II}$ NPs labeled by coumarin-6 showed green fluorescence dots, indicating the presence of Z-DMY/ Zn$^{II}$ NPs in HeLa cells. The yellow pixels resulting from the combination of green and red pixels
represented the co-localization of Z-DMY/ Zn\textsuperscript{II} NPs with endosome/lysosome. At first hour of incubation, the merged image displayed green dots. However, the yellow spots obviously increased as well as the green dots after 2h incubation of Z-DMY/ Zn\textsuperscript{II} NPs in HeLa cells, indicating that more Z-DMY/ Zn\textsuperscript{II} NPs were continuously internalized by HeLa cells with extension of incubation time and more NPs were endocytosed via endosomal pathway. At acidic endosomal/lysosomal environment, pH-responsive Z-DMY/ Zn\textsuperscript{II} NPs destabilized and released DMY from endosomes/lysosomes to cell cytoplasm [190].

The quantitative analysis of Z-DMY/ Zn\textsuperscript{II} NPs was further measured using flow cytometry. The fluorescence intensity represented the average number of NPs internalized by each cell (10000 cells were analyzed in each measurement). As shown in Figure 37, the fluorescence intensity measured using flow cytometry increased to higher level with the extension of incubation time, which was corresponding to the CLSM images results. In conclusion, the Z-DMY/ Zn\textsuperscript{II} NPs were effectively internalized by the HeLa cells.
Figure 36 CLSM images of intracellular uptake of Z-DMY/ ZnII NPs by Hela cells. Cells were counter-stained with DAPI for nuclei, Lyso Tracker Red for lysosomes. The scale bars represent 20 μm.
Figure 37 Fluorescence intensity of Z-DMY/ Zn\textsuperscript{II} NPs by Hela cells on the effect of time. Nanoparticles were stained with coumarin-6.

6.4 Conclusion

The self-assembled zein NPs were coated with a DMY- Zn\textsuperscript{II} coordinated bonding layer. The Z-DMY/ Zn\textsuperscript{II} NPs not only acted as an efficient drug carrier, but also a highly pH-responsive delivery system. Z-DMY/ Zn\textsuperscript{II} NPs demonstrated excellent cellular uptake by HeLa cells. Z-DMY/ Zn\textsuperscript{II} NPs effectively inhibited the cancer cell growth and exhibited an efficient anti-cancer activity toward HeLa cells. Z-DMY/ Zn\textsuperscript{II} NPs acts as a promising pH-responsive food-grade colloidal system encapsulating hydrophobic nutraceuticals.
Chapter 7: Summary and Future work

7.1 Summary of the dissertation

Hydrophilic carboxymethyl konjac glucomannan and calcium ion crosslinking were used to modify the surface of zein nanoparticles. The stability and cellular uptake were successfully enhanced compared to individual zein NPs. The effective of pH and cell culture medium on stability of nanoparticles were studied using dynamic light scattering. The effects of concentration of fluorescently labeled nanoparticles, incubating time, and inhibitors on qualitative and quantitative aspects of cellular uptake of nanoparticles were carefully investigated and elucidated using confocal laser scanning microscopy and flow cytometry. Modified zein NPs could be effectively used as a drug carrier for AG (andrographolide) to enhance the water solubility and the bioefficacy. It is promising to utilize modified zein NPs as carriers for anti-cancer and anti-inflammation food supplements. NPs loaded AG were shown effectively decreasing the cancer cell population and resulting cell death, evidenced by MTT assay and apoptosis assay. The efficacy of AG to regulate the in vitro NF-κB expression was notably enhanced by the Modified zein NPs delivery system due to enhanced dose efficiency resulting from better solubility, transportability and reduced toxicity. Therefore, hydrophilic carboxymethyl konjac glucomannan modified zein NPs were proved to be appealing delivery systems for hydrophobic bioactive compounds. Further investigation was on design a pH-sensitive zein-based delivery system, and it was utilizing the acid-sensitive bonds between metal ions and dihydromyricetin (DMY). Fluorescence quenching study of dihydromyricetin
binding to zein provided valuable information for designing zein nanoparticles to encapsulate DMY. The DMY-$\text{Zn}^{2+}$ coordinated bonding layer can effectively affect the control release profile of zein NPs when $\text{Zn}^{II}$ concentration was optimized. The cellular uptake and anti-cancer activity toward HeLa cells were investigated as well. Z-DMY/$\text{Zn}^{II}$ NPs acts as a promising pH-responsive food-grade colloidal delivery system encapsulating hydrophobic nutraceuticals in cancer therapy. In summary, novel zein NPs generated in this research could provide valuable information for food industry to develop nutraceuticals fortified functional food.

7.2 Future work

Due to the limitation of experimental design, time and resources, many further work could be done. Since the cell culture study in this thesis has testify the decreased toxicity and improved biocompatibility of the formulated nanoparticles in vitro, the improved therapeutic efficacy of formulated nanoparticles could be established in animal models of cancer. Although in vitro proofs of concept have been reported for a number of pH-responsive systems, only a few have been tested in in vivo preclinical models, and very few (thermosensitive liposomes and iron oxide nanoparticles) have reached the clinical stage. Specially, we can assess the ability of drug-loaded nanoparticles in a rapidly growing subcutaneous tumor model compared to empty nanoparticles, drug alone. In the experiments, tumor cells plus drug-loaded nanoparticles are injected subcutaneously into the flank of a mice. The control group will be injected empty nanoparticles or drug alone. The volume of the growing tumor will be evaluated within 14 days. It is meaningful to try the in vivo efficacy of Zein-DMY/ $\text{Zn}^{II}$NPs as a food supplement to prevent cancers.
Besides, the cellular internalization routes and intracellular localization of the nanoparticles has been determined, however more detailed investigations are needed to assess the impact and relevance of subcellular targeting for future clinical applications. Compared to study the monolayer of cultured living cells, optical imaging thicker tissue or multicellular organism in living animal models are challenging due to poor visible light transmission through biological tissue. A near- infrared optical window is the key to deep-tissue optical imaging the biodistribution of nanoparticles in various organs. It is important to study the biodistribution of zein nanoparticles in detail, so far there are no researches that directly evaluate blood or immune system biocompatibility of zein nanoparticles in vivo or ex vivo. Considering the effect of size, shape, charge, hydrophobicity of nanoparticles, it will be very interesting to study the important parameters affect the biodistribution of zein nanoparticles, especially zein nanoparticle coating with different concentration of carboxymethyl konjac glucomannan (CKGM).
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