**Improving Decorporation Efficacy of DTPA and HOPO Using
Chitosan/TPP Nanoparticles: Preparation, Characterization and
Release Testing**

by

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Abstract

Chitosan (CS) is a non-toxic, biodegradable and biocompatible polymer. It is hydrophilic and has adhesion to biological mucosa. Its amino groups can link with the chelating agents that have been identified for decorporating internalized radionuclides such as plutonium and americium. These chelating agents include diethylene triaminepentaacetic acid (DTPA) and 1,5,10,14-tetra(1-hydroxy-2-pyridon-6-oyl)-1,5,10,14-tetraazatetradecane (3,4,3-LI(1,2-HOPO)). The purpose of this research is to synthesize chitosan nanoparticles with encapsulated DTPA or 3,4,3-LI(1,2-HOPO), in a hope to improve the efficacy of the decorporation agents by taking advantage of the mucoadhesion property of chitosan.

Chitosan nanoparticles encapsulated with decorporation agents (DTPA and HOPO) were synthesized by ionic gelation method, in the presence of a cross-linker sodium tripolyphosphate (TPP). Reversibility of ionic gelation makes it feasible to unfold the chitosan nanoparticles and release the encapsulated DTPA or HOPO.

The resultant CS-DTPA/TPP and CS-HOPO/TPP nanoparticles were characterized by transmission electron microscopy, Fourier transform infrared spectroscopy and dynamic light scattering, which can provide information about morphology, chemical structure and size distribution, respectively. The CS/TPP, CS-DTPA/TPP and CS-HOPO/TPP nanoparticles show a mono-morphology which is full and dense spherical structure. CS-DTPA/TPP and CS-HOPO/TPP nanoparticles have a similar

size distribution, which indicates that the majority of the particles are smaller than 100nm.

The release profiles of CS-DTPA/TPP and CS-HOPO/TPP nanoparticles were obtained via *in vitro* release studies. The release process includes an initial burst period, a slow release in the later stage, and a relatively stable level in the final stage. Within the initial burst period, the cumulative amounts of DTPA and HOPO account for 70-80% and 60-70% of the final cumulative amounts, respectively.

Lysozyme has no effect on the drug release from the CS-DTPA/TPP and CS-HOPO/TPP nanoparticles, no matter what weight ratio of the decorporation agent (DTPA or HOPO) to chitosan is. Lysozyme, existing in respiratory tract secretions, cannot influence the 3-stage release process. This cationic protein will not greatly change the final cumulative amount of the decorporation agents released from the CS-DTPA/TPP or CS-HOPO/TPP nanoparticles.

Keywords: chitosan, decorporation agents, DTPA, 3,4,3-LI(1,2-HOPO), ionic gelation, nanoparticles, *in vitro* release studies

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List of Abbreviations and Acronyms

<i>Abbreviation or Acronym</i>	Full name
ACN	acetonitrile
CS	chitosan
DLS	dynamic light scattering
DTPA	diethylenetriaminepentaacetic acid (pentetic acid)
ESI	electrospray ionization
FTIR	Fourier transform infrared spectroscopy
HPLC	high-performance liquid chromatography
HOPO	1,5,10,14-tetra(1-hydroxy-2-pyridon-6-oyl)-1,5,10,14-tetraazatetradecane
3,4,3-LI(1,2-HOPO)	1,5,10,14-tetra(1-hydroxy-2-pyridon-6-oyl)-1,5,10,14-tetraazatetradecane
IR	immediate release
LC-MS	liquid chromatography-mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
MWCO	molecular weight cut off
NPs	nanoparticles

SIM	single ion monitor
SLF	synthesizing simulated lung fluid
SR	sustained release
TEM	transmission electron microscopy
TPP	sodium tripolyphosphate
w/w	weight ratio
ZOCR	zero-order controlled release

Chapter 1 Introduction

1.1 Nuclear Accidents and Internal Contamination

Nuclear energy is considered as a clean and cost-effective power resource, that has gained world-wide popularity over the past 5 decades. However, globally there have been recorded nuclear power plant accidents (defined as incidents that either resulted in the loss of human life or more than 50,000 US dollars of property damage), including ten accidents in Canada from 1952 to 2011^[1].

Radionuclides can be released into the environment during nuclear accidents, such as the Chernobyl (1986) and the Fukushima (2011), with the potential to pose health risks to human through external exposure or internal contamination (inhalation, ingestion or wound). Internalized radionuclides can be transported by the blood and deposit in the target organs (e.g. bone, kidney, liver)^[2]. Effective treatments vary for different radionuclides and are related to intake pathways, the level of contamination, the chemical form of each radionuclide being exposed to, as well as the time interval between treatment and contamination^[3-5]. For internal contamination with actinides, such as plutonium and americium, timely administration of decorporation agents is crucial for effective treatment; these agents chelate with actinides and form soluble stable complexes that can be excreted from the body. The U.S. Project BioShield Act of 2004 authorized the Department of Health and Human Services (DHHS) to treat, identify or prevent harm from any biological, chemical, radiological, or nuclear agent that may cause a public health emergency affecting national security^[6]. Consistent with

this authority, the National Institute of Allergy and Infectious Diseases (NIAID) focused on the development of novel radionuclide chelation and decorporation agents.

1.2 Decorporation Agent: DTPA

Pentetic acid (diethylenetriaminepentaacetic acid, DTPA) is an effective decorporation agent to remove actinides^[4,7,8]. However, more than 90% of the administered DTPA is excreted within 24 hours; repeated doses are often necessary to achieve required decorporation goal^[8]. Frequent administration of DTPA causes adverse health effects to human as it can also chelate with essential elements and deplete them from the human body.

Figure 1 Structure of DTPA

Diethylenetriamine-pentaacetate salts (Ca-DTPA and Zn-DTPA) are the most common forms used for radionuclides decorporation. Ca-DTPA chelates transuranic metals (americium, californium, curium, neptunium, plutonium), rare earth metals (cerium, lanthanum, yttrium) and transition metals (niobium, zirconium)^[10]. Zinc diethylenetriamine-pentaacetate (Zn-DTPA) is regarded as an effective decorporation agent for the treatment of internal contamination by Pu and Am with less toxic effect than Ca-DTPA^[9]. These two DTPA compounds are most effective if the actinides to be

chelated are in soluble forms^[11].

1.3 Decorporation Agent: 3,4,3-LI(1,2-HOPO)

The synthetic octadentate ligand 3,4,3-LI(1,2-HOPO) has a spermine structure that is formed by four 1-hydroxy-pyridine-2-one (1,2-HOPO) subunits. 3,4,3-LI(1,2-HOPO) is a wider spectrum actinide decorporation agent and can chelate with plutonium (Pu), americium (Am), curium(Cm), uranium (U) or neptunium (Np) ions to form stable complexes at physiological pH^[12-18]. This novel decorporation agent is currently undergoing pre-clinical development^[18]. Moreover, this promising compound has higher potency and can chelate more internally deposited radionuclides than the commonly used actinide decorporation agent DTPA^[14-16,18]. Based on the limited preclinical report, 3,4,3-LI(1,2-HOPO) also shows relatively low toxicity *in vivo*^[14,15,18,19].

Figure 2 Structure of 3,4,3-LI(1,2-HOPO)

1.4 Drug Carrier: Chitosan

Chitosan, a linear polymer of β -(1 $\rightarrow$ 4)-linked 2-amino-2-deoxy-D-glucose residues, is a deacetylated form of chitin. Chitosan can be prepared from chitin by chemical N-deacetylation. Both chitin and chitosan have been considered as promising biofunctional polymers for industrial applications, as both an abundant resource in nature and an environmentally friendly polymeric material^[20,23].

1.4.1 Chitin: Original Natural Polymer

Chitin (poly β -(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine) is regarded as a significant natural polysaccharide and is the most abundant polymer after cellulose. Shells of marine crustaceans, such as crabs and shrimps, are the main commercial sources of the chitin. In industrial chitin extraction from crustaceans, acid treatment is used to dissolve metal salts, primarily calcium carbonate. Alkaline extraction is used to decompose proteins and pigments due to the stability of chitin under mild acid and basic conditions^[21].

Figure 3 Structure of chitin

1.4.2 Chemical Properties of Chitosan

The deacetylated form of chitin is called chitosan when the deacetylation degree of chitin reaches about 50% (depending on the origin of chitin polymer)^[21]. Chitosan, as an amino polysaccharide, can be regarded as a weak base. Acid treatment can make chitosan uncoil and form positively charged water-soluble polymer; this process is protonating the glucosamine units. Therefore, chitosan is soluble in aqueous acid media. Chitosan molecule will lose the charge and precipitates in neutral solution^[22].

Figure 4 Structure of chitosan

1.4.3 Biological Properties of Chitosan

Chitosan is a non-toxic, biodegradable and biocompatible polymer. It shows biological, physiological and pharmacological properties that are different from cellulose. Deacetylation of chitin enhances the biological activity of chitosan by improving its solubility in the acidic environment of the stomach. These biological properties include antimicrobial activity, eliciting biological responses, mucoadhesion, hypolipidemic activity, immune enhancement, hemostatic activity, and promotion of wound healing^[20]. Chitosan is a dietary fiber supplement that promotes a feeling of fullness but has no

calories. It is a non-digestible aminopolysaccharide product being promoted for weight loss although there is little research to back the claims that it helps people lose weight. Lysozyme existing in serum can degrade chitosan^[23]. The degree of N-deacetylation and molecular weight of chitosan can affect lysozyme degradation behavior^[24]. The degradation process is very slow for the chitosan with a relatively higher deacetylation degree while those with a lower deacetylation degree experience faster degradation process^[25]. The chitosan with lower molecular weight will be degraded faster in lysozyme than the chitosan with higher molecular weight^[24]. The *in vitro* lysozyme degradation under the physiological conditions (pH=7.4, 37°C) indicates that the cumulative mass loss reaches approximately 46% for the porous chitosan (deacetylation degree= 89%) membrane after 7 days^[24].

1.4.4 Mucoadhesion of Chitosan

The attachment of natural or synthetic macromolecule to a biological tissue is defined as bioadhesion. In pharmaceutical science, bioadhesion to a mucus membrane is described as mucoadhesion^[26].

Mucoadhesion has promising potential application to optimize localized drug delivery and system delivery. The drug dosage can be directly released on the target site (e.g. within the gastrointestinal or respiratory tract). Systemic delivery can be achieved by contacting intimately with the absorption site (e.g. the nasal cavity)^[27,28]. The mucoadhesive materials usually have hydroxyl, carboxyl or amine groups that lead to the hydrophilic property of these materials^[29-31].

Chitosan has been proposed to be applied in mucosal drug delivery owing to its mucoadhesion activity^[32]. Chitosan can form a hydrogen bond because there are the -OH and -NH₂ groups existing in chitosan molecule.

Mucoadhesion property of the chitosan is related to chain flexibility due to the linear molecule structure. In addition, the cationic polyelectrolyte property of chitosan allows interaction with a negatively charged mucosal surface^[33]. Besides, the mechanism of chitosan mucoadhesion depends on producing a non-covalent bond, such as hydrogen bond, van der Waals force, and ionic interaction, between chitosan and mucosa^[35]. More specifically, chitosan has been used as a drug carrier for peptide drugs in the nasal and peroral delivery, in order to improve drug absorption^[33,34].

1.5 The Relationship between Drug Carrier Particle Size and Biodistribution

Biodistribution is related to the physicochemical properties of particles, especially size. Earlier studies showed that there is an enhanced ability for small particles to reach the target site^[36]. Small particles (<20-30 nm) are eliminated by renal excretion^[37]. Mononuclear phagocytic system (MPS) cells (existing in liver, spleen, and bone marrow) can take up larger particles. Bone marrow, the heart, the kidney and the stomach are target sites for larger particles (30-150 nm)^[38]. Nanoparticles of 150-300 nm are mainly distributed in liver and spleen^[39]. Table 1 shows the relationship between particle size and target site; it indicates that particle size does matter in the performance of drug delivery (and drug release)^[36].

Target site	Particle size	Animal model
Bone marrow	85-150 nm	Guinea-pig, rabbit, rat
Inflamed organs Liver	80 nm-1.4 µm	Hamster Mice
Kidney	20-30 nm	Guinea-pig, rabbit, rat
Liver Spleen	150nm	Mice
Lung	1-400 nm	Dog
Spleen	150 nm	Mice

Table 1 Target sites and particle size distribution in the body

1.6 Preparation of Chitosan Micro- and Nanoparticles

1.6.1 Emulsion Cross-linking Method

The key step of this method (Figure 5) is the reactive amine group of chitosan cross-linking with the aldehyde group of the cross-linking agent, such as glutaraldehyde^[39]. The chitosan aqueous solution (stabilized by a surfactant) is dispersed in the oil phase to form a water-in-oil (w/o) emulsion^[39]. The extent of cross-linking and the speed of stirring in the process of emulsion formation determine the size of final microspheres^[39]. However, the drawbacks of this method are that the cross-linking agent might possibly induce chemical reactions with the surfactant and it is difficult to remove the un-reacted cross-linking agent^[39].

Figure 5 Schematic of emulsion cross-linking method

1.6.2 Precipitation Method

This method (Figure 6) utilizes the insolubility of chitosan in alkaline medium. A chitosan solution is blown into an alkali solution (e.g. sodium hydroxide, NaOH-methanol, and ethanediamine), then a compressed air nozzle is applied to form coacervate droplets. The particle size depends on the compressed air pressure and the spray-nozzle diameter^[39].

Figure 6 Schematic of precipitation method

1.6.3 Ionic Gelation Method

Ionic gelation method relies on the electrostatic attraction, instead of the formation of chemical bonds. Thus, this method is mild and simple. The process of this method

(Figure 7) is about a reversible physical cross-linking process^[39]. Unlike the chemical cross-linking method, the possible toxicity of cross-linking reagents can be avoided via ionic gelation method. Sodium tripolyphosphate (TPP) is a polyanion reagent that can attract cationic chitosan by electrostatic forces. The first step of ionic gelation method is to obtain cationic chitosan by dissolving chitosan in an aqueous acidic solution. The next step is adding TPP solution to the chitosan solution under constant stirring at a high speed^[39].

Figure 7 Schematic of ionic gelation method

1.6.4 Spray Drying Method

The key step in this method is using a stream of hot air to dry atomized droplets. During the spray drying process, acetic acid treatment is used to form a chitosan solution, followed by adding a cross-linking agent. The last step is atomization in a stream of hot

air to form small droplets. Evaporation of solvents produces the free flowing particles. The size of particles obtained by this method is influenced by various process parameters, such as the hot air nozzle size, spray flow rate, the pressure atomization, inlet air temperature and extent of cross-linking^[39]. There are many other available methods used in the preparation of chitosan microspheres, such as emulsion-droplet coalescence method, sieving method and reverse micellar method^[39].

Figure 8 Schematic of spray drying method

1.7 Drug delivery

1.7.1 Controlled Release System

Drugs can be delivered by a controlled release system, at a constant or predetermined rate for a relatively long time (e.g. 12 hours)^[40]. The conventional immediate release

(IR) system usually present such limitations as: (a) frequent administration is required due to the short retention time of drug; (b) the profile of drug plasma concentration shows the typical peak-valley tendency, which is difficult to keep stable; (c) drug concentration fluctuation as the unavoidable result of frequent administration, can lead to over medication (drug plasma concentration reaching toxic level) or under medication (drug plasma concentration beyond the minimum effective level)^[41,42]. An idealized zero-order controlled release (ZOCR) system can keep the release rate at an expected level for a relatively long period; this helps maintain drug plasma level^[41]. The sustained release (SR) system achieves prolonged drug release and reduces the frequency of drug administration^[41].

The rates of drug release from a nanoparticle-based controlled drug release system depends on (a) release from the nanoparticle surface that adsorbs or binds with the drugs; (b) diffusion of the drug through the nanoparticle matrix; (c) erosion or degradation of nanoparticle matrix; and (d) a combination of these three processes^[45].

In vitro release testing can determine drug release profiles and quantify the amount of drug released under the release conditions. *In vitro* release study involves different methods: (a) cells diffusion using biological or artificial membranes; (b) dialysis in reverse sac technique; (c) diffusion in dialysis bag/tubes technique; (d) ultra-filtration technique; (e) ultra-centrifugation technique; (f) centrifugal ultra-filtration technique^[44,45]. Dialysis tubing technique is easy and convenient to operate. It can also be used in separating the released drug and the nanoparticles^[45]. In addition, the tubing can separate nanoparticles to prevent clogging up the LC-MS/MS that will be used to

analyze the dialysate samples.

1.7.2 Mucosal Routes of Drug Delivery

Administration of drugs via oral route may be the most convenient route among various routes of drug delivery. However, it has disadvantages which often result in lowering the treatment efficacy^[46]. Typical disadvantages include enzymatic degradation of the drug within the gastrointestinal (GI) tract and metabolism of drug within the liver (hepatic first pass metabolism), decomposition of the drug in the liver or the GI tract that leads to the reduce of drugs dosage. Thus, some types of drugs, especially peptides and proteins, are prohibited to be administrated via oral route^[28,46].

Compared with oral route of drug administration, drug delivery via mucosal routes (i.e., the mucosa of the nasal, oral, rectal and vaginal cavities) is much better, especially in avoiding pre-systemic elimination within the GI tract and enhancing the drug absorption^[28,46].

1.7.2.1 Oral Mucosal Route

Oral mucosa includes several parts which are the labial, palatal, sublingual, buccal, gingival mucosa. Drug administration via the oral mucosa route primarily depends on the buccal and sublingual tissues due to their better permeation^[47]. Therefore, drug administration in oral mucosal cavity is classified into 3 categories : (1) sublingual delivery, which can achieve systemic delivery through the mucosal membranes lining the floor of the mouth; (2) buccal delivery, which depends on the mucosal membranes of the cheeks; and (3) local delivery, which delivers the drug directly into the oral

cavity^[28,46].

The limitation of the oral mucosal route is the relatively small surface area (200 cm²) of oral mucosa, compared to the surface area of the gastrointestinal tract (350 000 cm²) or skin (20 000 cm²)^[47,49]. The small surface area has a negative effect on improving drug absorption.

Besides, there is a significant challenge for the administration of drugs via the oral mucosal route. Before passing through the oral mucosa, the drug needs to overcome both hydrophilic and hydrophobic barriers on oral mucosa. First of all, the drug diffuses through the lipophilic cell membrane, followed by passing through the hydrophilic interior of the oral epithelium cells. Also, enzymatic degradation in mucosa leads to rapid decomposition of drugs, especially for the type of peptides and proteins drugs^[49]. Thus, oral mucosa route is not an excellent for drug delivery.

1.7.2.2 Nasal Route

Nasal route is one of the mucosal routes for drug delivery and it can achieve systemic delivery. The total surface area available in the nasal mucosa is estimated to be about 180 cm², of which 10 cm² is olfactory mucosa and 170 cm² is the richly vascularized respiratory mucosa^[78]. The large surface area of the nose can improve the drug absorption because the epithelial surface is covered by numerous microvilli. Also, the subepithelial layer is highly vascularized and the administered drug can be delivered directly into the systemic circulation through the venous blood from the nose. Thus, it can avoid drug decomposition in the liver or GI tract due to first-pass metabolism or

enzymatic degradation. Vestibular, olfactory, and respiratory areas are three different functional zones in the nasal cavity and have different functions, as shown in table 3^[50].

Nasal cavity	Function
Vestibular zone	Airborne particles filter
Olfactory zone	Drugs metabolize
Respiratory zone	Optimal target site for drug absorption

Table 2 Functions related drug absorption of three zones in the nasal cavity

However, there are some drawbacks of the nasal route. These drawbacks include potential irritation to nasal cavity when nasal dosage forms are delivered to the action sites, and nasal mucus secretion affecting the drug absorption on the action site^[46]. Apart from these disadvantages, nasal mucociliary clearance is the primary factors that affect drug absorption. Thus, the drug absorption via nasal route is relatively low, which is the greatest limitation of the nasal route^[51].

1.7.2.3 Pulmonary Route

The pulmonary route of drug delivery is better than other non-parenteral routes (e.g. oral, nasal or rectal routes) because the favorable physiological features of the lungs have a positive effect on improving drug absorption^[52,53]. The large surface area ($\sim 75 \text{ m}^2$), the low thickness of epithelial barrier ($0.1\text{--}0.5 \text{ }\mu\text{m}$ in alveoli), extensive vascularization, slow mucociliary clearance, and relatively low proteolytic or

enzymatic activity can significantly enhance drug absorption^[54-57].

Before reaching the deep lungs, inhaled particles need to overcome the lung defense mechanisms (especially the airway structure and the mucus layer) which play an important role in protecting the epithelium in the tracheobronchial region^[55]. In the upper airways, cilia in the mucous layer clear particles rapidly. The thickness of the pulmonary epithelium in the trachea is 50 to 60 μ m, which is regarded as a barrier to drug absorption^[59].

The particles should be small enough because the particles have to pass through the mouth, throat and conducting airways to finally reach the deep lung. However, when the size is too small, the particles cannot deposit and will be breathed out again^[55-57,59]. Nanoparticles with different size will deposit in the different region. In general, large particles (diameter > 5 μ m) deposit by inertial impaction in the mouth and upper airways, and smaller particles (diameter = 1-5 μ m) deposit deeper in the lungs by inertial impaction and sedimentation. Very small particles (diameter < 1 μ m) are driven by diffusion, mostly suspended and exhaled^[59].

However, the particles can deposit by diffusion in the alveolar region of the lungs, when the diameter of particles is less than 100nm^[59]. Inhaled particles of different sizes show different deposition efficiencies in different deposition region^[79]. For example, about 90% of inhaled particles with the size about 1nm can deposit in the nasopharyngeal region, approximately 10% of particles with the same size in the tracheobronchial region, and essentially none in the alveolar region. More than 50% of the inhaled particles with the size about 20 nm can deposit in the alveolar region, while about 15%

of the inhaled particles can locate in the nasopharyngeal and tracheobronchial region. In the alveolar region, small nanoparticles with the diameter less than 100 nm show a relatively higher deposition efficiency than larger nanoparticles (diameter > 100 nm)^[79].

Drug delivery via the pulmonary route can achieve local treatment of respiratory diseases (e.g. asthma or cystic fibrosis) in the lungs. Moreover, the benefits of this delivery route are that the administrated dose can be decreased and side effects can be avoided^[59,60]. Alternatively, the pulmonary route can also achieve systemic drug delivery by targeting deposit in the alveolar region where the drugs can be absorbed through the thin layer of epithelial cells into the systemic circulation^[53,54,59]. Besides, a rapid onset of action, the avoidance of the first-pass metabolism, or the delivery of biotherapeutics (i.e. peptides and proteins) can be achieved by the pulmonary route^[61,62].

Dry powder inhaler (DPI) systems are highly efficient in pulmonary drug delivery; it can be applied in treating respiratory diseases, such as asthma, and systemic diseases^[63].

Dry powder inhaler systems have the advantages of long-term stability and sterility because dried powders are prepared by freeze drying technique that is a preservation method to keep particles stable in a dry state^[63]. Besides, dry powder inhalation systems can help to disperse powders by using compressed air or electric motor^[59].

1.7.3 Advantages of Nanoparticles in Drug Delivery

In general, nanoparticles are defined as solid colloidal particles ranging in size from 1

to 1000 nm^[59]. Nanoparticles can be used therapeutically as drug carriers by entrapping, encapsulating, absorbing or attaching drugs or biologically active materials. These drug carrier nanoparticles are composed of biodegradable materials, such as PLGA (lactide/glycolide copolymer), which can be degraded to biocompatible complexes that are resorbable in the body^[59].

Nanoparticles are better than microparticles in drug delivery owing to the size and surface characteristics. The diameter of the smallest capillaries is about 5-6 μm . The particles being distributed in the bloodstream must be smaller than 5 μm . Nanoparticles can pass through capillaries and can be delivered to lungs, liver, spleen or the systemic circulation^[80,81]. In addition to the advantage of size, the surface characteristics of nanoparticles determine their life span and are related to the capture by macrophages. Hydrophilic surfaces of nanoparticles can escape macrophages capture, which avoids depletion of nanoparticles before reaching to the target sites^[81]. Based on the size and surface characteristics of nanoparticles, it is feasible for nanoparticles to avoid clearance, prolong the residence time at the action site and increase circulation time, as transport across the physical (e.g. mucosal) barriers^[59]. Therefore, medical applications of nanoparticles can be achieved by reducing the frequency of dosage administration and improving the therapeutic efficiency.

Kawashima *et al* successfully prepared PLGA (lactide/glycolide copolymer) nanoparticles with insulin and delivered the particles to guinea pig lungs, which resulted in significant reduction of glucose level in blood owing to the prolonged effect over 48h as compared to insulin solution^[64]. In addition, Zhang *et al.* used a different

drug carrier [poly(butyl cyanoacrylate)] to deliver insulin to the lungs of rats and achieved a prolonged duration of a hypoglycemic effect over 20h; meanwhile, glucose level decreased to less than 80% of the original level^[65].

1.8 Objectives of This Project

Based on the literature, it is clear that DTPA and 3,4,3-LI(1,2-HOPO) are effective for actinides decorporation and can chelate with actinides to form stable complexes being excreted in urine. However, the majority of these administrated decorporation agents will be excreted before chelating with actinides, which leads to reducing the treatment effectiveness. Thus, the frequent administration of decorporation agents is required, though side effects (e.g. depletion of essential elements) are inevitable for humans.

The purpose of this research is to combine the advantages of chitosan and decorporation agents to improve the efficacy of decorporation therapy. Ionic gelation method can be applied in preparing chitosan nanoparticles with encapsulated decorporation agents. Characterization methods will be applied in obtaining further information about the synthesized nanoparticles. *In vitro* release studies are useful in determining the release profiles of CS-DTPA/TPP and CS-HOPO/TPP nanoparticles. Lysozyme is assessed for its effect on the drug release behavior due to its degradation on chitosan.

Chapter 2 Methodology

2.1 Materials, Methods and Instruments

2.1.1 Materials

Chitosan (CS, low molecular weight) was purchased from SIGMA-ALDRICH (product of Iceland) and soluble in dilute aqueous acid. Diethylenetriamine-pentaacetic acid pentasodium salt solution (concentration, 40% in H₂O, MW 503.26) was purchased from SIGMA-ALDRICH (product of Germany). 3,4,3-LI(1,2-HOPO) was provided by Lawrence Berkeley National Laboratory, Berkeley, CA, USA. Sodium triphosphate pentabasic (TPP, MW 367.86 g/mol) was purchased from SIGMA-ALDRICH (product of Germany). D-Mannitol (Bioultra, ≥99.0%, MW 182.17) was purchased from SIGMA-ALDRICH (product of France). Sodium hydroxide was purchased from SIGMA-ALDRICH (product of Sweden). Lysozyme from chicken egg white (lyophilized powder, protein ≥90%, ≥40,000 unites/mg protein) was purchased from SIGMA-ALDRICH (product of Canada). Acetic acid, glacial (GR, ACS, FW 60.05) was purchased from EMD Chemicals Inc. (Germany, product of U.S.A.). Sodium acetate (NaC₂H₃O₂·3H₂O, FW 136.08) was purchased from ANACHEMIA (Canada). Milli-Q water is greater than 16 MΩ.cm, at 25 °C (0.22 μm filter). Iron(III) chloride hexahydrate (FeCl₃·6H₂O) is the product of SIGMA-ALDRICH. Magnesium chloride hexahydrate (MgCl₂·6H₂O), sodium citrate tribasic dihydrate (Na₃C₆H₅O₇·2H₂O), sodium acetate trihydrate (C₂H₃NaO₃·3H₂O), calcium chloride dihydrate (CaCl₂·2H₂O), potassium chloride, sodium sulfate, sodium

hydrogen carbonate (NaHCO_3), both of them were obtained from SIGMA-ALDRICH.

Sodium chloride crystal was purchased from J.T.Baker, and disodium hydrogen orthophosphate (Na_2HPO_4) was purchased from BDH chemicals.

2.1.2 Instruments

Instrument	Company
Magnetic stirrer	Fisher Scientific
pH meter	Fisher Scientific
Electronic balance	Mettler Toledo(model AT201)
All stainless steel water bath	Precision(model 186)
Fourier transform infrared spectroscopy	ABB Bomen (MB Series)
Freeze dry systems	Labconco(model7753020)
Nano-DLS (Dynamic light scattering)	Brookhaven Instruments
LC-MS with ultraviolet (UV) detector	Agilent Technologies Triple Quad LC/MS (model 6460)
Transmission electron microscopy	FEI Tecnai (G2 F20)

Table 3 Instruments and apparatuses

2.2 Synthesis of CS/TPP Dry Powders

Chitosan was dissolved in 1% acetic acid in order to obtain positively charged chitosan acid solution with the concentration of 1 mg/mL (weight/volume). Then, 2M NaOH solution was added into chitosan acetic acid solution to adjust the pH value to about 4.5. Sodium tripolyphosphate (TPP) was dissolved in ultrapure water to obtain a concentration of 0.5 mg/mL (weight/volume). Under mild magnetic stirring at room temperature, the TPP solution was added dropwise to chitosan acetic acid solution to reach CS/TPP ratio of 4:1. The formation of CS/TPP nanoparticles can be affected by the pH value of the solution and the weight ratio of chitosan to TPP. The pH value of the chitosan solution should be controlled in the range of 4 to 6 before adding TPP solution. Otherwise, nanoparticles suspension cannot be formed. Besides, the CS/TPP nanoparticles will be relatively more stable when the weight ratio of chitosan to TPP is in the range of 3:1 to 6:1^[82]. With the increase of chitosan to TPP weight ratio in the range, there is the increase in the particle size^[83].

The system was kept stirring and reacting for 1 hour, followed by adding 5% (weight/volume) mannitol acting as the dispersant. The CS/TPP mannitol mixture was pre-frozen. The dry powders were obtained in a freeze dryer. The synthesis mechanism of CS/TPP nanoparticles is as follows^[76]:

Scheme 1 Chemical equation for formation of CS/TPP NPs

2.3 Synthesis of CS-DTPA/TPP and CS-HOPO/TPP Dry Powders

2.3.1 Preparation of CS-DTPA Complex

According to the research of Liu *et. al*, CS-DTPA complex can be synthesized in the acid medium^[58]. First of all, a certain amount of chitosan was dissolved in 1% acetic acid in order to obtain positively charged chitosan solution and the concentration of the chitosan-acetic acid solution was 1 mg/mL (weight/volume). Next, 40% DTPA-Na salt solution was added into the chitosan-acetic acid solution. The amount of 40% DTPA-Na salt solution varied in different samples in order to obtain different weight ratio between chitosan and DTPA. Negatively charged DTPA reacted with positively charged chitosan for overnight to obtain CS-DTPA complex. The assumed synthesis reaction equation as follows:

Scheme 2 Chemical equation for synthesis of CS-DTPA complex

2.3.2 Preparation of CS-HOPO Complex

The synthesise process of CS-HOPO complex is similar with the preparation of CS-DTPA complex. The first step is also to obtain positively charged chitosan by dissolving chitosan in 1% acetic acid. The obtained chitosan acid aqueous solution was 1 mg/mL (weight/volume). Afterward, 3,4,3-LI(1,2-HOPO) particles were added to ultrapure water to form the stable solution.

The obtained 3,4,3-LI(1,2-HOPO) solution was added dropwise to the chitosan acetic acid solution and reacted continuously for overnight to form chitosan-3,4,3-LI(1,2-HOPO)complex. The samples with different weight ratios of chitosan to HOPO, can be obtained by changing the amount of 3,4,3-LI(1,2-HOPO).

The formation of CS-HOPO complex is assumed by this chemical equation:

Scheme 3 Chemical equation for formation of CS-HOPO complex

2.3.3 Preparation of CS-DTPA/TPP and CS-HOPO/TPP NPs

The nanoparticles were prepared by ionic gelation method. Sodium tripolyphosphate (TPP) was dissolved in ultrapure water to obtain the concentration of 0.5 mg/mL (weight/volume). Before adding TPP solution to the CS-DTPA or CS-HOPO complex, the pH value of the system was adjusted to 4.5. 2M NaOH solution was added to the CS-DTPA complex to control the pH value of the system while adjusted the pH value of CS-HOPO system by adding 98% acetic acid. Under mild magnetic stirring at room temperature, the TPP solution was added dropwise to CS-DTPA or CS-HOPO complex to reach CS/TPP weight ratio of 4:1. The system was kept stirring and reacting for 1 hour.

2.3.4 Preparation of Dry Powders

Aqueous solutions of mannitol in a concentration of 5% (weight/volume) were prepared. 5% mannitol solution acted as dispersant that can avoid aggregation of

nanoparticles during the freeze drying process. Nanoparticles suspension was added to the mannitol solution. The final CS-DTPA/TPP and CS-HOPO/TPP mannitol complex were pre-frozen overnight. Dry powders were obtained in a freeze dryer. The temperature in the freeze dryer was -53°C.

2.4 Characterization Using Fourier Transform Infrared Spectroscopy

The structure of CS-DTPA/TPP (with different chitosan to DTPA weight ratio) and CS-HOPO/TPP (with different weight ratio of chitosan HOPO) dry powders were characterized by Fourier transform infrared Spectroscopy (FTIR). A small amount of sample (1.7 mg) was mixed with KBr and compressed to form tablets. The IR spectrum was obtained in the spectral region of 600-4000 cm^{-1} .

2.5 Characterization Using Dynamic Light Scattering

Dynamic light scattering (DLS) was used to measure the size distribution of formed dry powders. A certain amount of CS-DTPA/TPP or CS-HOPO/TPP dry powders (20 mg) were dissolved in the buffer solution (2 mL). The buffer solution for CS-DTPA/TPP system was prepared by dissolving 1mmol sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2$) in 1% (v/v) acetic acid solution. 10 mM KNO_3 was chosen as the buffer solution for CS-HOPO/TPP system. Dispersion in ultrasonic instrument was required before DLS analysis.

2.6 *In vitro* Release Studies of Decorporation Agents from Nanoparticles

The physiological conditions of drug administration via pulmonary route were

considered. Simulated lung fluids (SLF) have several formulas, which have different compositions, ion concentrations, pH values. For the release studies, the SLF solution was prepared based on the method described by Marques *et al*^[77]. Gamble's solution was chosen as the simulated lung fluid used in the *in vitro* release test because Gamble's solution represents the interstitial fluid deep within the lungs^[77]. The further application of the synthesized nanoparticles in this project is delivering the drug to the deep lung and reach to the alveoli region. Thus, the environment of *in vitro* release testing should be consistent as much as possible with the environment of the deep lung.

After the administration of the dry powders to the body, the dry powders will dissolve in the fluid to recover the nanoparticles. Mannitol cannot affect the therapy efficacy of the nanoparticles due to its solubility and non-toxicity in the body. The first step of *in vitro* release testing was to obtain recovered nanoparticles. A certain amount of CS-DTPA/TPP or CS-HOPO/TPP dry powders were dissolved in the SLF to reach a concentration of 10 mg/mL. Then, the recovered CS-DTPA/TPP or CS-HOPO/TPP nanoparticles suspension was placed in the dialysis tube (MWCO 3500).

The dialysis tube was immersed in 50mL of SLF (pH=7.4) and kept in a 37±1 °C water bath under stirring. At appropriate time intervals, individual dialysate was withdrawn. Meanwhile, the same volume of fresh SLF solution was added to the dialysis system to keep the volume of SLF solution at constant. Finally, the collected samples were analyzed by LC-MS/MS to obtain the concentrations of decorporation agent (DTPA or HOPO) in the dialysate .

2.7 *In vitro* Release Studies in the Presence of Lysozyme

Based on the literature, lysozyme can degrade drug carrier chitosan and decompose the chain structure of chitosan^[23]. To investigate whether lysozyme can affect DTPA release, *in vitro* release testing in the presence of lysozyme was carried out.

A certain amount of CS-DTPA/TPP or CS-HOPO/TPP dry powders were dissolved in simulated lung fluid (SLF) to reach a concentration of 10 mg/mL. The next step was dissolving lysozyme in simulated lung fluid to reach a concentration of 10 mg/mL. Dialysis membrane with MWCO 3500 was applied in dialysis process. The CS-DTPA/TPP or CS-HOPO/TPP nanoparticles suspension and lysozyme solution were placed in a dialysis tube. The dialysis tube was immersed in 50mL SLF (pH=7.4) and kept in a 37±1 °C water bath under stirring. At appropriate time intervals, individual dialysate was withdrawn. Meanwhile, the same volume of SLF was added to the dialysis system to keep the total volume of SLF at constant. The concentration of decorporation agent (DTPA or HOPO) in different samples was determined using LC-MS/MS.

2.8 Determination of DTPA Concentration by LC-MS/MS

The dialysate samples collected from the releasing tests were determined by LC-ESI-MS/MS. The Fe(III)-DTPA standards and samples were dissolved in the diluent of 0.1% formic acid before injection. Chromatographic separation was achieved on a C18 column (50mm * 2.1mm) maintained at room temperature with two mobile phases: 0.1% formic acid in Milli-Q water (A) and 100% ACN in 0.1% formic acid

(B). The mobile phases were prepared by mixing 98% A (0.1% formic acid in Milli-Q water) and 2% B (100% ACN in 0.1% formic acid). Formic acid has an effect on increasing the formation of protonated ions and sensitivity. The flow rate was 0.400 mL/min and the injection volume was 1 μ L.

Mass spectrometry analysis was performed using an ESI-MS/MS system which was equipped with an electrospray ionization source operating in negative mode. Fe(III) ions were added into dialysate and DTPA chelated with Fe(III) ions to form stable $[M - 4H^+ + Fe^{3+}]^-$ cluster ions. The Fe(III)-DTPA cluster ions are negative, which is the reason why electrospray ionization source operated in negative mode. The operating parameters were as follows: the gas flow rate was set to 9.8 L/min with the gas temperature of 300 °C, nebulizer was 15 psi, capillary voltage was 4000 V, fragmentor was 135 V, and cell accelerator voltage was 7 V. Single Ion Monitor (SIM) was chosen to record the peak when m/z is 445.

2.9 Determination of 3,4,3-LI(1,2-HOPO) Concentration by LC-MS/MS

Liquid chromatography (LC) in combination with mass spectrometry (MS) has the ability to obtain an accurate characterization, structural elucidation, and determination of unknown trace impurities in bulk drug substance batches^[68,69]. Moreover, liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) shows high sensitivity and specificity on the characterization of impurities and degradation products^[69,70]. However, there is a challenge for liquid chromatography to analyze metal chelators because they can chelate with different metals resulting in the

formation of multiple complexes^[71]. Based on the observations by Liu *et al* in 2015, iron (III) can be used to chelate 3,4,3-LI(1,2-HOPO), which is an efficient way to suppress complex formation with any other metals^[71].

Iron (III) at the concentration of 5mg/mL was added into 3,4,3-LI(1,2-HOPO) solution and dialysate samples to form Fe(III)-3,4,3-LI(1,2-HOPO) complex. And then the sample solutions coupled with iron (III) were needed to be centrifuged to obtain the supernatant. Assay standards and supernatant solutions were dissolved in the 0.1% formic acid.

Chromatographic separation was achieved on a C18 column (50 mm * 2.1 mm) maintained at room temperature with two different mobile phase: 0.1% formic acid in Milli-Q water (A) and 100% ACN in 0.1% formic acid (B). And the mobile phases were prepared by mixing 70% A (0.1% formic acid in Milli-Q water) and 30% B (100% ACN in 0.1% formic acid). The flow rate was 0.400 mL/min and the injection volume was 1 μ L.

Mass spectrometry analysis was performed using an ESI-MS/MS system which was equipped with an electrospray ionization source. Electrospray (ESI) is one kind of soft ionization MS method, which can provide molecular mass and fragments information^[72-75]. Furthermore, identification of impurities and degradation products bases on the fragments information. The parameter settings were: gas temperature 300°C, gas flow 9.8 L/min, nebulizer 15 psi, capillary voltage 4000 V, fragmentor 135 V, and cell accelerator voltage 7 V. According to the conclusion of Liu *et al* in 2015, the sharp peak in chromatogram at $m/z = 875$ corresponded to the complex fragment in the

form of $[3,4,3\text{-LI}(1,2\text{-HOPO})^4\text{-Fe}^{3+}_2\text{OH}]^{+71}$. So electrospray ionization source operated in the positive single ion monitor(SIM) mode was chosen to record the peak.

Chapter 3 Results and Discussion: DTPA as Decorporation

Agent

3.1 Characterization of Morphology and Size Distribution

Chitosan-based nanoparticles were synthesized by ionic gelation method, in the presence of cross-linker TPP. A number of characterization methods were used to determine the morphology, size distribution and structure of CS/TPP and CS-DTPA/TPP nanoparticles made in this project.

Transmission electron microscope was applied to determine the morphology of the nanoparticles. CS/TPP and CS-DTPA/TPP dry powders were first suspended in ultrapure water to remove the large amount of dispersant mannitol added to CS-TPP and CS-DTPA/TPP for more effective freeze drying. Figure 11 shows TEM images of CS/TPP nanoparticles. Fig.9 [a] indicates the morphology of fresh CS/TPP nanoparticles before freeze drying while Fig.9 [b] indicates the morphology of CS /TPP nanoparticles obtained from dry powders being suspended in ultrapure water. Both TEM images exhibit spherical morphology for the CS/TPP nanoparticles.

Figure 9 Transmission electron microscopy images of CS/TPP NPs [a] fresh CS/TPP NPs before freeze drying. [b] CS /TPP dry powders suspended in ultrapure water.

After encapsulating DTPA, CS-DTPA/TPP nanoparticles with two different weight ratio of chitosan to DTPA were investigated by transmission electron microscope. Figure 10 [a], [b] and [c] correspond to CS-DTPA/TPP nanoparticles with the lower DTPA to chitosan weight ratio of 2.5:1. The morphology of CS-DTPA/TPP nanoparticles with the higher weight ratio of DTPA to chitosan (25:1) can be observed from Figure 10 [d], [e] and [f]. The CS-DTPA/TPP nanoparticles are morphologically uniform and spherical. Although the weight ratio of chitosan to DTPA is different, the CS-DTPA/TPP NPs show a mono-morphology. Figure 10 [a] and [d] show the morphology of fresh CS-DTPA/TPP nanoparticles. It can be observed that the nanoparticles disperse well in the system which means that there is no aggregation. After freeze drying, the morphology of the CS-DTPA/TPP nanoparticles still presents spherical structure. However, there are some nanoparticles in the form of aggregation

(Figure 10 [c] and [f]). The formation aggregation makes the nanoparticles larger, which probably have negative effects on drug delivery to the target site. The prepared CS-DTPA/TPP nanoparticles are expected to reach the alveolar region in the deep lungs and achieve systemic drug delivery by circulation. If the nanoparticles are too larger than the size requirement, the drug cannot be delivered to the alveolar region.

Figure 10 Transmission electron microscopy images of CS-DTPA/TPP nanoparticles with different CS/DTPA weight ratio. [a] fresh CS-DTPA/TPP (CS/DTPA=1:2.5) nanoparticles. [b][c] CS-DTPA/TPP (CS/DTPA=1:2.5) dry powders suspended in ultrapure water. [d] CS-DTPA/TPP (CS/DTPA=1:25) fresh NPs. [e][f] CS-DTPA/TPP (CS/DTPA=1:25) dry powders suspended in ultrapure water.

The size distribution of the synthetic nanoparticles was analyzed by using dynamic light scattering (DLS). Figure 11 [a] presents the particle size distribution of CS-DTPA/TPP NPs with a weight ratio of chitosan to DTPA of 1:1. Most of the CS-DTPA/TPP NPs are in the range of 0-50 nm while some particles are larger than 100 nm, indicating that they are in the form of aggregation states. According to the distribution frequency, aggregation state accounts for the minority. Figure 11 [b] provides further details for particle size distribution in the range of 0-50 nm. Particle size in the range of 0-15 nm accounts for about 40%.

Figure 11 Particle size distribution of CS-DTPA/TPP (CS:DTPA=1:1) NPs

Figure 12 [a] shows that more than 75% of CS-DTPA/TPP nanoparticles (CS:DTPA=1:2.5(w/w)) are smaller than 100 nm. There are a small number of the CS-DTPA/TPP nanoparticles in the form of aggregation that results in their size being larger than 100 nm. Figure 12 [b] provides further details for particle size distribution in the range of 0-50 nm. Particle size in the range of 0-15 nm accounts for about 20%.

There are more than 10% of the CS-DTPA/TPP nanoparticles in the range of 15-30 nm. Besides, the particle size in the range of 30-50 nm accounts for about 13%. The size distribution in the range of 0-50 nm is wide.

Figure 12 Particle size distribution of CS-DTPA/TPP (CS:DTPA=1:2.5) NPs

The size distribution of CS-DTPA/TPP nanoparticles with a much higher weight ratio of chitosan to DTPA (reaching to 1:25) is shown in Figure 13. Most of the CS-DTPA/TPP NPs are in the range of 0-50 nm while some particles are larger than 100 nm indicating the formation of aggregation state. Figure 13 [b] provides further details for particle size distribution in the range of 0-50 nm. Particle size in the range of 0-15 nm accounts for about 72% of all of the nanoparticles.

Figure 13 Particle size distribution of CS-DTPA/TPP (CS:DTPA=1:25) NPs

Based on the DLS results, the size distribution of CS-DTPA/TPP NPs with different weight ratios of chitosan to DTPA can be obtained and these CS-DTPA/TPP NPs show similar particle size distribution. Nearly all CS-DTPA/TPP NPs are less than 100 nm, and the majority fall within the range of 0-50 nm. In comparison to large biomolecules (e.g. proteins and peptides), DTPA is a small molecule. Thus, increasing the weight ratio of DTPA to chitosan cannot greatly change the size of the CS-DTPA/TPP nanoparticles.

Both TEM and DLS results indicate that aggregation state of the CS-DTPA/TPP nanoparticles cannot be completely suppressed. As mentioned above, small nanoparticles (less than 100 nm) can reach the alveolar region in the deep lungs and achieve systemic drug delivery by circulation. Therefore, the formed CS-DTPA/TPP nanoparticles can satisfy the size requirement of drug delivery.

3.2 Characterization of Chemical Structure

In the synthesis procedures, the purpose of dissolving chitosan in an acidic aqueous medium was making positively charged chitosan. The -NH_2 functional group of chitosan was protonated to form -NH_3^+ that provided the possibility to react with the -COO^- functional group of DTPA-Na by N-acetylation reaction. To further determine the chemical bond between chitosan and DTPA, FTIR was applied in analyzing the chemical structure of CS-DTPA/TPP dried powders. The FTIR spectrum of CS-DTPA/TPP (CS:DTPA=1:25, w/w) dried powders is shown in Figure 14. O-H and C-H stretching vibrations are in the range between 3700 and 2500 cm^{-1} ^[23]. The presence of $\text{-CH}_2\text{-}$ and -CH- at the frequency of 2939.3 cm^{-1} and 2910.4 cm^{-1} , respectively can be seen. The amide absorption peaks at 1633.6, 1571.9, and 1417.6 cm^{-1} correspond to stretching vibration of C=O bond (amide I), bending vibration of N-H bond (amide II), and stretching vibration of C-N bond (amide III), respectively^[23]. Moreover, absorption peaks at 1282.6 and 1207.4 cm^{-1} involve in stretching vibration of C-O bond. As for the peaks of 1083.9 and 1020.3 cm^{-1} , they are assigned to mannitol dispersant^[87].

Figure 14 FTIR spectrum of CS-DTPA/TPP dried powders (CS:DTPA=1:25)

As shown in Figure 15, CS-DTPA/TPP nanoparticles with different weight ratios of chitosan to DTPA were investigated by FTIR. By increasing the weight ratio of DTPA to chitosan, the intensity of amide peaks can be strengthened because more the -NH_2 functional groups of chitosan react with the -COOH functional groups of DTPA to form -CONH- . In the CS-DTPA/TPP dried powders, the existence of amide bond demonstrates that the chitosan-based nanoparticles have successfully encapsulated DTPA.

Figure 15 FTIR spectrum of CS-DTPA/TPP dried powders with different weight ratio of chitosan to DTPA (1.25:1, 1:1, 1:2.5, 1:25)

3.3 *In vitro* Release Behavior of DTPA

In vitro release of DTPA from CS-DTPA/TPP nanoparticles was conducted under simulated physiological conditions (simulated lung fluid pH 7.4, 37°C) to evaluate the drug release ability. Encapsulated DTPA in the nanoparticles can release from the chitosan nanoparticles system after a period of time.

The concentration of DTPA in the samples can be determined using LC-MS/MS analysis with reference to the standard curve of the Fe(III)-DTPA solution shown in Fig.

16 [b]. DTPA is a Fe(III) chelating agent that can form stable complexes with metal ions, such as Ca(II), Cd(II), Co(II), Cu(II), Mn(II) and Zn(II)^[84]. The formation of multiple related metal complexes is a challenge for LC-MS/MS determination of DTPA. It is necessary to convert DTPA to a single metal complex.

The stability constants of metal-DTPA interaction are in the following order Fe > Cu > Al > Zn^[85]. The stability constants are 28.6 and 21.5, respectively Fe(III)-DTPA and Cu(II)-DTPA. Among these essential metals, Fe(III) has the highest binding affinity with DTPA^[88].

Therefore, Fe(III) is added into DTPA standard solutions and samples before LC-MS/MS analysis. This can avoid the formation of DTPA complexes with other metal ions existing in the simulated lung fluid, thus improving the accuracy of DTPA determination by LC-MS/MS.

Figure 16 [a] was a chromatogram obtained by LC-MS/MS showing the characteristic peak of Fe(III)-DTPA at m/z=445. The Fe(III)-DTPA peak area was used to construct the standard curve shown in Fig.16 [b]. Based on the linear trend line equation, the concentration of DTPA in each unknown dialysate sample could be calculated.

Figure 16 Mass spectra of the characteristic peak of Fe-DTPA at m/z of 445;[b]

Fe-DTPA standard curve.

To obtain the cumulative amount of DTPA released (A_n), it can be calculated based on the equation as follows:

$$A_n = V_e \sum_{i=1}^{n-1} C_i + V_0 C_n$$

where V_e is the volume of each withdrawn dialysate sample (3mL), V_0 is the whole volume of the release media (simulated lung fluid outside of dialysis membrane, 50mL) and C_n represents the concentration of DTPA in the nth sample.

In vitro release of DTPA from CS-DTPA/TPP nanoparticles is conducted under simulated lung fluid in the absence of lysozyme. The cumulative amounts of DTPA released from CS-DTPA/TPP nanoparticles with different weight ratios of chitosan to DTPA are shown in Figure 17. For all of the CS-DTPA/TPP nanoparticles (CS/DTPA=1:25, 1:2.5 and 1:1) prepared in this project, the release profiles present an initial burst, a slow release in the later stage and a relatively stable level in the final

stage.

Figure 17 [a] Cumulative amount of DTPA released from CS-DTPA/TPP in the absence of lysozyme; [b] Magnification of DTPA release profiles for CS/DTPA=1:2.5 and CS/DTPA=1:1

In the initial 6 hours, the cumulative amounts of DTPA released rapidly reach 4.68, 0.64 and 0.57mg, respectively. In the next 92 hours, the cumulative DTPA amounts slowly increase to 6.59, 0.82 and 0.73 mg, respectively. After 98 h until 168 h, there is no significant increase in the cumulative DTPA amounts. After 7 days (168 hours), the final cumulative amounts of DTPA are 6.70, 0.85 and 0.77 mg, respectively. During the initial burst period, CS-DTPA/TPP nanoparticles have rapidly released 70-75% of the final cumulative DTPA amounts.

3.4 Effect of Lysozyme on DTPA Release Behavior

As previously mentioned, the drug carrier chitosan is a biodegradable polymer and its chain structure can be broken by lysozyme that is an enzyme existing *in vivo*^[23]. To further study the lysozyme effect on DTPA release, *in vitro* release testing was conducted using simulated lung fluid in the presence of lysozyme. The release profiles of CS-DTPA/TPP nanoparticles with various weight ratios of chitosan to DTPA (CS/DTPA=1:25, 1:2.5 and 1:1) are shown in Figure 18.

Figure 18 [a] Cumulative amount of DTPA released from CS-DTPA/TPP in the presence of lysozyme; [b] Magnification of DTPA concentration profiles for CS/DTPA=1:2.5 and CS/DTPA=1:1

In the presence of lysozyme, the release profiles of CS-DTPA/TPP nanoparticles also present three release stages which are an initial burst period, a slow release in the later stage and a fairly stable level in the final stage. Within the initial 6 hours, the

cumulative amounts of DTPA are 4.90, 0.66 and 0.59 mg, respectively. After 7 days (168 hours), the final cumulative amounts of DTPA reach 7.03, 0.87 and 0.74 mg, respectively. During the initial burst period, the cumulative amounts of DTPA account for 70-80% of the final cumulative amount of DTPA released from the CS-DTPA/TPP nanoparticles.

As shown in Figure 19, the release profiles of CS-DTPA/TPP nanoparticles with lysozyme and without lysozyme are compared. 50 mg of CS-DTPA/TPP dried powders suspended in simulated lung fluid were used in the release testing. After 7 days (168 hours), the cumulative amounts of DTPA released from the CS-DTPA/TPP nanoparticles reach a stable level (with or without the lysozyme). For the CS-DTPA/TPP nanoparticles with the highest weight ratio of DTPA to chitosan (DTPA/CS=25:1), the final cumulative amounts of DTPA are 7.03 mg with lysozyme and 6.70 mg without lysozyme. The final cumulative amounts of DTPA released from the CS-DTPA/TPP nanoparticles (DTPA/CS=2.5:1, w/w) reach 0.87 mg and 0.85 mg, respectively. In comparison, the cumulative amounts of DTPA released from the CS-DTPA/TPP nanoparticles with the lowest weight ratio of DTPA to chitosan (DTPA/CS=1:1) are 0.74 mg and 0.77 mg, respectively. The final cumulative amounts of DTPA are similar for these two relatively low weight ratios of DTPA to chitosan.

Although the weight ratios of DTPA to chitosan are different, the release profiles present the same release process that includes an initial burst period, a slow release in the later stage and a relatively stable level in the final stage. The presence of lysozyme in the release environment does not greatly change the cumulative amount of DTPA

released from the CS-DTPA/TPP nanoparticles.

Figure 19 Comparison of two releasing environments (with lysozyme and without lysozyme) for CS-DTPA/TPP nanoparticles

3.5 Hypothesis of DTPA Release Mechanism

Agnihotri *et al*^[57] pointed out that many factors, such as the extent of cross-linking, morphology, density and size of the particles, physicochemical properties of the drug, as well as the presence of adjuvants, can affect the profile of drug release from chitosan-based particles. Furthermore, *in vitro* release can also be affected by the pH, ionic strength, and presence of enzymes in the release medium. Three different mechanisms may involve in drug release from chitosan-based particles: (a) release from particle surface, (b) diffusion through the swollen matrix or (c) polymer degradation resulting in the release.

Generally, more than one type of mechanism is involved in any drug release system^[57]. Chitosan is a hydrophilic polymer, so the mechanisms (a) and (b) are the main ones that control drug release from the nanoparticles in seven days.

As for CS-DTPA/TPP particles, simulated lung fluid can penetrate the particles once the dried powders are dispersed in the aqueous medium, resulting in swelling of the chitosan matrix and converting the glassy polymer to a rubbery matrix^[57], which has effects on the diffusion of DTPA encapsulated in the nanoparticles. Although the chain structure of chitosan can be broken by lysozyme and lead to polymer erosion, the mechanism of DTPA release from the CS-DTPA/TPP nanoparticles follows the simple diffusion and surface release. The degradation of polymer chain has little function on DTPA release.

Chapter 4 Results and Discussion: 3,4,3-LI(1,2-HOPO) as Decorporation Agent

4.1 Characterization of Morphology and Size Distribution

Chitosan nanoparticles successfully encapsulated 3,4,3-LI(1,2-HOPO) via the ionic gelation method, in the presence of cross-linker TPP. The CS-HOPO/TPP nanoparticles so formed were characterized by TEM and DLS.

Figure 20 Transmission electron microscopy images of CS-HOPO/TPP NPs (CS/HOPO=1:25, w/w) [a] fresh CS-HOPO/TPP NPs before freeze drying. [b] and [c] CS-HOPO /TPP dry powders suspended in ultrapure water.

It can be seen in Figure 20 that the CS-HOPO/TPP nanoparticles (CS/HOPO=1:25, w/w) present a spherical-particle morphology. Figure 20 [a] shows the morphology of the fresh CS-HOPO/TPP nanoparticles before freeze drying. It is clear that the nanoparticles disperse well in the liquid system. The morphology of the

CS-HOPO/TPP dry powders suspended in the aqueous medium can be seen in Figure 20 [b] and [c]. After freeze drying, the CS-HOPO/TPP nanoparticles still present a uniformly spherical morphology. However, it is hard to completely avoid the formation of aggregation. Figure 20 [c] indicates that single small particles tend to stick together to appear in the aggregation state.

Figure 21 Transmission electron microscopy images of CS-3,4,3-LI(1,2-HOPO)/TPP NPs with different CS/HOPO weight ratios. [a][b] CS/HOPO=1:10 dry powders suspended in ultrapure water, [c][d] CS/HOPO=1:25 dry powders suspended in ultrapure water.

The morphologies of the CS-HOPO/TPP nanoparticles with different weight ratios are shown in Figure 21. The TEM images of CS-HOPO/TPP nanoparticles reveal a mono-morphology that is a spherical shape. The morphology of the CS-HOPO/TPP nanoparticles is independent of the two weight ratios of chitosan to 3,4,3-LI(1,2-HOPO) studied.

The size distribution of the synthetic CS-HOPO/TPP nanoparticles was analyzed by using dynamic light scattering (DLS). The size distribution of CS-HOPO/TPP nanoparticles with a CS/HOPO weight ratio of 1:10 is shown in Figure 22. The CS-HOPO/TPP nanoparticles in the size range of 0-100 nm accounts for about 90% of all the nanoparticles. Approximately 75% of all the nanoparticles are smaller than 50 nm. However, some nanoparticles form aggregates larger than 100 nm. Figure 22 [b] provides further details for particle size distribution in the range of 0-50 nm. Particle in the size range of 0-10 nm account for about 50%.

Increasing the weight ratio of HOPO to chitosan in the synthesis of CS-HOPO/TPP nanoparticles does not increase the size of resultant nanoparticles. Figure 23 indicates the size distribution of CS-HOPO/TPP nanoparticles with a higher weight ratio HOPO/CS=25:1. Almost all of the resultant nanoparticles are smaller than 100nm. Nanoparticles in the size range of 0-100nm account for 90%. Particles larger than 100 nm indicate the formation of aggregation.

Although the weight ratios of HOPO to chitosan are varied, the CS-HOPO/TPP nanoparticles have similar particle size distribution. Almost all nanoparticles are smaller than 100 nm, and the nanoparticles in the size range of 0-50 nm account for the

majority.

For the drug administration, the CS-HOPO/TPP nanoparticles could be delivered to the lungs via inhalation. As previously mentioned, small nanoparticles (less than 100 nm) can reach the alveolar region deep within the lungs to achieve systemic drug delivery by circulation. Based on the size distribution, the CS-HOPO/TPP nanoparticles can satisfy the size requirement for drug delivery.

Figure 22 Particle size distribution of CS-HOPO/TPP NPs (CS:HOPO =1:10)

Figure 23 Particle size distribution of CS-HOPO/TPP NPs (CS:HOPO=1:25(w/w))

4.2 Characterization of Chemical Structure

Fourier Transform infrared spectroscopy was applied in analyzing the chemical structure of CS-HOPO/TPP dried powders. Figure 24 shows the FTIR spectrum of 3,4,3-LI(1,2-HOPO). O-H, N-H, and C-H stretching vibrations are in the range between 3700 and 2500 cm^{-1} . The absorption peaks at 3411 and 3271 cm^{-1} correspond to O-H and N-H stretching vibrations, respectively. The wide absorption peak at 1650.9 cm^{-1} corresponds to stretching vibration of C=O bond (amide I). The absorption peak at 1301.8 cm^{-1} is related to stretching vibration of C-N bond (amide III) in the secondary amide.

Figure 24 FTIR spectrum of 3,4,3-LI(1,2-HOPO)

Before encapsulating HOPO, CS/TPP dried powders were analyzed by using FTIR. Figure 25 indicates the FTIR spectrum of CS/TPP dried powder in the presence of mannitol as a dispersant. The presence of O-H, N-H and C-H bond stretching vibrations at the frequency of 3396.4, 3301.6 and 2937cm⁻¹, respectively, can be observed. The absorption peaks at 1566.4 and 1195.4 cm⁻¹ are respectively assigned to bending vibration of N-H bond and stretching vibration of C-N bond in the aliphatic amine. In addition, the frequency at 1084.3 and 1021.1 cm⁻¹ represent characteristic absorption peaks of dispersant mannitol^[87].

Figure 25 FTIR spectrum of CS/TPP dried powder in the presence of mannitol

As shown in Figure 26, it can be observed the FTIR spectrum of CS-HOPO/TPP (CS:HOPO=1:25, w/w) dried powder in the presence of mannitol. The frequency at 3389.2, 3269.1, and 2938.6 cm^{-1} respectively corresponds to O-H, N-H, and C-H stretching vibration. The sharp absorption peak at 1637.5 cm^{-1} represents stretching vibration of C=O bond (amide I) while the wide absorption peak at 1521.7 cm^{-1} corresponds to bending vibration of N-H bond. Besides, the absorption peaks of 1321.6 and 1196 cm^{-1} are assigned to stretching vibration of C-N bond. The difference between these two absorption peaks is that the wavenumber at 1321.6 cm^{-1} is related to amide III in the secondary amide while the wavenumber at 1196 cm^{-1} is related to C-N bond in the aliphatic amine. The frequency at 1085 and 1021 cm^{-1} represent characteristic absorption peaks of dispersant mannitol^[87].

Figure 26 FTIR spectrum of CS-HOPO/TPP (CS:HOPO=1:25, w/w) dried powder in the presence of mannitol as dispersant

Figure 27 Comparison of FTIR spectrum

After encapsulating HOPO, there are many differences in absorption peaks between the FTIR spectrum of 3,4,3-LI(1,2-HOPO) and CS/TPP dried powders. It can be seen that the absorption peak of C=O bond (amide I) of CS-HOPO/TPP (CS:HOPO=1:25) dried powders becomes sharper than the absorption peak of HOPO. This can be assumed that there are intramolecular hydrogen bonds existing in HOPO. The intramolecular hydrogen bond is formed between the oxygen atom of the carbonyl group and the hydrogen atom of the hydroxyl group (as shown in Figure 27), which results in the peak being broadened. After forming the CS-HOPO/TPP nanoparticles, the intramolecular hydrogen bonds are broken, and intermolecular hydrogen bond is formed between the hydroxyl group of HOPO and the amino group of chitosan. Figure 27 indicates that the characteristic peak shape of the N-H bond is obviously different between the spectrum of CS-HOPO/TPP (CS:HOPO=1:25) dried powders and the spectrum of CS/TPP dried powders.

As shown in Figure 28, increasing the weight ratio of HOPO to chitosan can strengthen the intensity of the characteristic absorption peaks. In addition, the absorption peak of stretching vibration of C=O bond (amide I) shift to the higher frequency (wavenumber) from 1625.9 to 1637.5 cm^{-1} as a consequence of decomposition of the intramolecular hydrogen bond.

Figure 28 FTIR spectrum of CS-HOPO/TPP dried powders with different weight ratio of chitosan to HOPO (respectively, 1:10, 1:25)

4.3 *In vitro* Release Behavior of 3,4,3-LI(1,2-HOPO)

Chitosan is chosen as the drug carrier owing to its adhesion to biological membrane. The functional drug HOPO can be encapsulated in chitosan nanoparticles to achieve drug delivery. To evaluate the ability of the CS-HOPO/TPP nanoparticles to effectively deliver HOPO, the relationship between release time and the cumulative amount of HOPO released from the nanoparticles was studied.

LC-MS/MS analysis was applied to determining the HOPO concentration in the collected dialysate samples, with reference to the standard curve for Fe (III)-HOPO shown in Figure 29[b]. According to the similar biochemical properties of iron (III) and

plutonium (IV), 3,4,3-LI(1,2-HOPO) was evaluated as a decorporation agent for chelation with plutonium (IV). HOPO shows a high affinity for plutonium (IV) as well as for iron (III)^[71]. Before analyzing each dialysate sample, iron (III) was added into the sample to chelate with HOPO so as to suppress or eliminate the formation of HOPO complexes with any other metal ions.

Figure 29 [a] TIC chromatograms reconstructed from the characteristic MS peak of Fe(III)-HOPO at m/z of 875 [b] Fe-HOPO standard curve.

Figure 29 [a] shows the TIC chromatograms reconstructed from the characteristic MS peak of Fe(III)-HOPO at m/z=875. The standard curve of Fe(III)-HOPO is shown in Figure 29[b]. The concentration of HOPO in each unknown dialysate can be calculated, based on the linear trend line equation. It is feasible to obtain the cumulative amount of HOPO (A_n) released from the nanoparticles, based on the equation as follows:

$$A_n = V_e \sum_{i=1}^{n-1} C_i + V_0 C_n$$

where V_e is the volume of the withdrawn dialysate sample (1mL), V_0 is the whole volume of the release media (simulated lung fluid outside of dialysis membrane, 50mL) and C_n represents the concentration of HOPO in the nth sample.

In vitro release of HOPO from CS-HOPO/TPP nanoparticles is first conducted with simulated lung fluid in the absence of lysozyme. Figure 30 shows the release profiles of HOPO from the CS-HOPO/TPP nanoparticles that were prepared using different weight ratios of chitosan to HOPO (CS/HOPO=1:50, 1:25, 1:10). For these nanoparticles with higher HOPO/CS weight ratio (HOPO/CS=50:1 and 25:1), the release profiles present an initial burst, a slow release in the later stage and a relatively stable level in the final stage. In comparison, the CS-HOPO/TPP nanoparticles with the lowest HOPO/CS weight ratio (HOPO/CS=10:1) present a relatively shorter release process.

Figure 30 Release profiles of HOPO from the CS-HOPO/TPP nanoparticles in the absence of lysozyme

In the initial 7 hours, the cumulative amounts of HOPO released from the nanoparticles with the higher HOPO/CS weight ratios (50:1 and 25:1) rapidly increase to 0.69 mg and 0.43 mg, respectively. In the next 41 hours, the cumulative HOPO amounts slowly increase to 1.00 mg and 0.63 mg, respectively. After 48 hours until 72 hours, there is no significant increase in the cumulative amounts of HOPO. After 72 hours, the final cumulative amounts of HOPO released from the CS-HOPO/TPP nanoparticles reach 1.01 mg and 0.64 mg, respectively. During the initial burst period, the CS-HOPO/TPP nanoparticles with the higher HOPO/CS weight ratio (HOPO/CS= 50:1 and 25:1) have released about 68% of final cumulative HOPO amount. However, for the nanoparticles with the lowest HOPO/CS weight ratio (HOPO/CS=10:1), the cumulative HOPO amount reaches a stable level after 7 hours. The cumulative amount

of HOPO released from the nanoparticles with the lowest weight ratio is only 0.13 mg after 7 hours.

4.4 Effect of Lysozyme on 3,4,3-LI(1,2-HOPO) Release Behavior

As mentioned above, lysozyme can degrade drug carrier chitosan and break its chain structure^[23]. To further study the lysozyme effect on HOPO release, *in vitro* release testing was conducted under simulated physiological conditions (simulated lung fluid, 37 °C), in the presence of lysozyme. The release profiles of CS-HOPO/TPP nanoparticles with various weight ratios of chitosan to HOPO (CS/HOPO=1:50, 1:25 and 1:10) are shown in Figure 31.

Figure 31 Release profiles of HOPO from the CS-HOPO/TPP nanoparticles in the presence of lysozyme

In the presence of lysozyme, there is no significant difference in the release profiles. Three release stages that still are an initial burst period, a slow release in the later stage and a fairly stable level in the final stage, are shown in the release profiles of the nanoparticles with these higher HOPO/CS weight ratios. As for the lowest weight ratio of HOPO to chitosan, the cumulative release amount reaches a stable level within 7 hours. In comparison, within the initial 7 hours, the cumulative amounts of HOPO released from these higher HOPO/CS weight ratios, rapidly increase to 0.68 mg and 0.40 mg, respectively. After 72 hours, the final cumulative amounts of HOPO slowly reach 0.98 mg and 0.67 mg, respectively. During the initial burst period, the cumulative amounts of HOPO account for 60-70% of the final cumulative amounts of HOPO released from the CS-HOPO/TPP nanoparticles.

As shown in Figure 32, the release profiles of CS-HOPO/TPP nanoparticles with lysozyme and without lysozyme are compared. 50 mg of CS-HOPO/TPP dried powders suspended in simulated lung fluid were applied in the release testing. For the CS-HOPO/TPP nanoparticles with the highest HOPO/CS weight ratio, the final cumulative amounts of HOPO are 0.98 mg with lysozyme and 1.01 mg without lysozyme. The final cumulative amounts of HOPO released from the nanoparticles (HOPO/CS=25:1, w/w) are 0.67 mg and 0.64 mg, respectively. In comparison, the cumulative amounts of HOPO released from the nanoparticles with the lowest HOPO/CS weight ratio reach a stable level after the initial 7 hours, in both of these two release conditions. The final cumulative amounts of HOPO are 0.09 mg and 0.08 mg, respectively. These results are consistent with the common knowledge that enzymatic

depolymerization is nonrandom and bond specific –lysozyme may attack only the β -1,4 linkage and cannot be used to degrade chitosan completely^[86]. Therefore, depending on how HOPO was initially bound with chitosan in our work, the presence of lysozyme may not have any direct impact on its release from the CS-HOPO/TPP nanoparticles. Although the weight ratios of HOPO to chitosan are different, the presence of lysozyme in the release media cannot greatly change the release profiles and the final cumulative amount of HOPO released from the CS-HOPO/TPP nanoparticles.

Figure 32 Comparison of two release environments (with lysozyme and without lysozyme) for the nanoparticles with different weight ratios of HOPO/CS.

4.5 Hypothesis of 3,4,3-LI(1,2-HOPO) Release Mechanism

As previously mentioned, three mechanisms can affect drug release from chitosan-based particle: (a) release from the surface; (b) diffusion; (c) polymer degradation leading to the release.

Drug carrier chitosan is swellable matrix due to its hydrophilicity, which is related to HOPO release mechanism. Simulated lung fluid can penetrate the particles once the CS-HOPO/TPP dried powders are suspended in the aqueous medium, resulting in swelling of the chitosan matrix, which contributes to the diffusion of HOPO in the nanoparticles. Although lysozyme can degrade the chitosan polymer and break its chain structure, there is no obvious change in release profiles and the final cumulative amounts of HOPO. Thus, the mechanism of HOPO release from the CS-HOPO/TPP nanoparticles follows the simple diffusion and surface release. The degradation of chitosan by lysozyme plays an insignificant role in the release of HOPO from the CS-HOPO/TPP nanoparticles.

Chapter 5 Conclusions and Future Work

5.1 Conclusions

Ionic gelation method can be applied in synthesizing chitosan-based nanoparticles that encapsulate the decorporation agents DTPA and HOPO, in the presence of TPP acting as a cross-linker. To preserve in a better way, freeze drying technique is applied to prepare the CS-DTPA/TPP and CS-HOPO/TPP dried powders, in the presence of 5% (w/v) mannitol solution. It is important to point out that the mannitol solution acts as the dispersant to suppress the aggregation of nanoparticles.

To determine the morphology of the formed chitosan-based nanoparticles, transmission electron microscope was used. The formed CS/TPP, CS-DTPA/TPP and CS-HOPO/TPP nanoparticles present mono-morphology that is a full and dense spherical structure. Additionally, the weight ratio of chitosan to the decorporation agents (DTPA or HOPO) cannot affect the morphology in the range of weight ratios studied.

Dynamic light scattering was applied in obtaining the size distribution of the formed nanoparticles. Almost all of the CS-DTPA/TPP nanoparticles are smaller than 100 nm, no matter what the weight ratio of DTPA to chitosan is. The CS-DTPA/TPP nanoparticles larger than 100 nm suggest the occurrence of aggregation, which accounts for the minority. As for the CS-HOPO/TPP nanoparticles, there is a similarity in the size distribution compared to the CS-DTPA/TPP nanoparticles. Although the weight ratio of HOPO to chitosan is varied, greater than 90% of all the CS-HOPO/TPP

nanoparticles are smaller than 100 nm. Moreover, the size of CS-HOPO/TPP nanoparticles in the range of 0 to 50 nm accounts for the majority. It is difficult to completely avoid the formation of aggregates (the size larger than 100 nm) where a few CS-HOPO/TPP nanoparticles tend to stick together. Based on the DLS results, the weight ratio of chitosan to the decorporation agent (DTPA or HOPO) and the type of decorporation agents has no effect on the size distribution of the formed nanoparticles. It is assumed that DTPA (molecular weight = 393) and HOPO (molecular weight = 750) are small molecules compared with chitosan.

Fourier transform infrared spectroscopy was applied in determining the chemical structure of the formed nanoparticles. N-acetylation has occurred between the $-NH_2$ functional groups of chitosan and the $-COOH$ functional groups of DTPA. According to the FTIR results, the intensity of amide absorption peaks varies when the weight ratio of chitosan to DTPA is changed. Increasing the weight ratio of DTPA to chitosan can result in strengthening the intensity of amide absorption peaks at 1633.6, 1571.9, and 1417.6 cm^{-1} because chitosan linking with DTPA depends on the amide bond. As for the CS-HOPO/TPP nanoparticles, chitosan linking with HOPO depends on the intermolecular hydrogen bond. By comparing the FTIR spectra of HOPO, CS/TPP, and CS-HOPO/TPP particles, the oxygen atom of the hydroxyl groups of HOPO and the amino groups of chitosan are involved in forming the intermolecular hydrogen bonds. The *in vitro* release studies have revealed that there are 3 release stages which are the initial burst period, the slow release stage and the relatively stable level in the final stage. During the initial burst period, CS-DTPA/TPP nanoparticles can rapidly release

70-80% of the final cumulative DTPA amount. After 98 hours, the cumulative amount of DTPA released from the CS-DTPA/TPP nanoparticles reaches a relatively level and there is no obvious increase in the cumulative amount. As for the CS-HOPO/TPP nanoparticles, the cumulative amount of HOPO released within the initial burst period accounts for 60-70% of the final cumulative HOPO amount. After 48 hours, there is no significant increase in the cumulative amount of HOPO released from the CS-HOPO/TPP nanoparticles. However, the CS-HOPO/TPP nanoparticles cannot release more HOPO after 7 hours, when the weight ratio is HOPO/CS=10:1.

To obtain the effect of lysozyme on the drug release, the *in vitro* release testing in the presence of lysozyme was carried out. The release profiles still present 3 release stages that are the initial burst period, the slow release stage and the relatively stable level in the final stage. In addition, there is no great difference in the final cumulative amounts between the release media with lysozyme and without lysozyme. The mechanism of the decorporation agents released from the CS-DTPA/TPP and CS-HOPO/TPP nanoparticles follows the simple diffusion and surface release.

5.2 Future Work

Future studies will be carried out to determine whether CS-DTPA/TPP and CS-HOPO/TPP nanoparticles can achieve controlled drug release, using an animal model. As previously mentioned, chitosan can be applied in mucosal drug delivery owing to the mucoadhesive activity. Pulmonary route is chosen for drug administration

because the mucus inside the lungs can enhance the deposition of nanoparticles. Therefore, the synthesized CS-DTPA/TPP and CS-HOPO/TPP nanoparticles will likely be delivered to the deep lungs of the animals. In the animal testing, DTPA and HOPO decorporation agents without chitosan will be used as the control. At appropriate time intervals, urine samples will be collected from the animals and measured for the concentration of the decorporation agents using LC-MS/MS. Further experiments will involve animals contaminated with actinides to validate the improved decorporation efficacy achieved by the modified nanoparticles containing the decorporation agents.

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Appendices

Appendix - Spectroscopic Characterization

Figure 14 FTIR spectra of CS-DTPA/TPP dried powders (CS:DTPA=1:25)

Figure 15 FTIR spectra of CS-DTPA/TPP dried powders (CS/DTPA= 1:2.5)

Figure 15 FTIR spectra of CS-DTPA/TPP dried powders (CS/DTPA= 1:1)

Figure 24 FTIR spectra of 3,4,3-LI(1,2-HOPO)

Figure 25 FTIR spectra of CS/TPP dried powder

Figure 26 FTIR spectra of CS-3,4,3-LI(1,2-HOPO)/TPP (CS:HOPO=1:25) dried powder

Figure 28 FTIR spectra of CS-3,4,3-LI(1,2-HOPO)/TPP (CS:HOPO=1:10) dried powder