SELF-ASSEMBLY POLYMERIC NANOPARTICLES COMPOSED OF POLYMERS CROSSLINKED WITH TRANSITION METALS FOR USE IN DRUG DELIVERY

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A major drawback of chemotherapy is the lack of selectivity leading to damage in healthy tissue, which results in severe acute side effects to cancer patients. The use of nanoparticles as a drug delivery system has emerged as a novel strategy to overcome the barriers of immunogenic response, controlled release of therapeutic, and targeting the toxicity only to cancerous cells. In this study, polymeric nanoparticles composed of transition metals and particles derived from natural biopolymers have been generated via self-assembly. For example, nanoparticles composed of cobalt crosslinked with albumin (Co-alb NPs) via Co-amine coordination chemistry of lysine residue were synthesized in various sizes. The method to generate Co-alb NPs involves no thermal heat, organic solvent or any surfactants, which is ideal for the production of large amounts in a timely manner. The Co-alb NPs displayed exceptional stability under physiological conditions (pH 7.4) for several days with minor changes in size; however, degradation could be triggered by reductant (reduced glutathione (GSH), 10 mM) with complete disappearance of particles in less than 2 hours. Numerous therapeutics that are highly effective toward cancer cells have been developed; however, many cannot be administered to patients due to poor solubility in water and pH dependent properties. We have successfully encapsulated 7-ethyl-10-hydroxycamptothecin (SN-38) into Co-alb NPs with encapsulation efficiency as high as 94% and loading capacities greater than 30%. We employed an emulsion-solvent evaporation method to incorporate SN-38 into Co-alb (SN38 Co-Alb NPs). Release of the drug from SN38 Co-Alb NPs was determined for particles incubated in PBS or PBS-GSH. SN38 Co-Alb NPs were exceptionally stable under physiological condition (PBS pH 7.4), but
exhibited sustained release of SN-38 over time in the presence of GSH. Uptake and toxicity of the particles were also investigated in a gastric carcinoma cell line (SNU-5) where high degrees of macropinocytic uptake were observed. The particles displayed significant toxicity making them a prime candidate for further testing in animal models.
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1.1 Overview

Nanotechnology has been one of the most studied research areas in both academia and industry over the last two decades. Applying nanotechnology in medicine and drug delivery has emerged as a promising tool to enable the development new class of therapeutics. According to National Nanotechnology Initiative, nanoparticles are defined to be in the range of 1-100nm (https://www.nsf.gov/crssprgm/nano/reports/omb_nifty50.jsp). However, many published reports detail the use of nanoparticles in the higher size range of 100-500nm. While the therapeutics developed over the last 50 years have been effective toward killing highly cancer cells, the classical therapies for treating cancer induce severe cytotoxic effects. However, these drugs also kill healthy cells in bone marrow and GI tract (stomach and intestines), which leads to a reduction of leukocytes, red blood cells, platelets, inflammation, and hair loss. Utilizing nanoparticles in drug delivery can overcome some of these barriers by improving the solubility of poorly insoluble drugs, enabling the delivery of drugs to intracellular target sites, co-delivery of two or more drugs simultaneously for combination effects, and enhancing circulation time of therapeutics in vivo. The properties of nanoparticles enable them to prolong circulation of the drug in blood stream. Nanoparticles can be modified biologically and chemically to deliver therapeutics drugs to target organs and facilitate controlled released in response to pH, temperature or biodegradability of the nanoparticles. The encapsulating of therapeutics in nanoparticles enables the protection of the biological activity of the drug and reduces the toxic effects of therapeutics on healthy cells while reaching the target location.
1.2 Passive Targeting vs. Active Targeting

Nanoparticles rely on three primary routes of targeting to deliver therapeutics to the site of action (Figure 1.1). Passive targeting is based on enhanced permeability and retention (EPR) effect, which was first discovered by Maeda and coworkers.\textsuperscript{5} Tumors support their growth by recruiting blood vessels, which creates a complex of array extra blood vessels at the tumor. These vessels are often highly complex, heterogeneous, and irregular in length and structure and constitute a defective, leaky vasculature system. Due to this phenomenon, macromolecules (greater than 40kDa) can readily accumulate at the tumor interstitium. However, in order to maximize therapeutic effect, these nanoparticles must avoid rapid clearance by the mononuclear phagocyte system (MPS), the body’s natural mechanism for clearing the foreign objects.\textsuperscript{6}

Figure 1.1: Active targeting vs Passive targeting adapted from Farokhzad et al \textsuperscript{1}

Figure 1.1 depicts particles that can be engineered with specific ligand that binds to targeted receptor on the surface of tissue to facilitate receptor-mediated endocytosis. This enhances the
cellular uptake and accumulation of particles in the targeted tissue. It also depicts passive targeting, which particles tend to accumulate in inflammatory tissue via EPR effect as mentioned above. Active targeting involves the utilization of appropriate ligands that bind to receptors over expressed on the surface of tumor cells, but not on healthy cells. These ligands could be peptides, aptamers, antibodies, vitamins or carbohydrates that guide the particles to selective binding sites. Upon binding to these overexpressed receptors, the nanoparticle-drug conjugates can be engulfed or diffused across the cell membrane via receptor-mediated endocytosis. There are number of factors contributed to the success of active targeting such as the binding affinity of ligand to receptors and the number of receptors at the tumor site. 

1.3 Polymer used in drug delivery

Natural polymers such as chitosan or proteins have been studied extensively in drug delivery because of their distinct properties of biodegradability, biocompatibility, non-toxicity, non-immunogenicity and low-cost. Natural polymers are ideal for biomedical application due to their physical structures and properties similar to biological components in the human body. The three most common types of polymers utilized in targeted drug delivery include polysaccharides, protein and polyesters, which are found in many of the products that we use in our daily lives. Polysaccharides, for example, can be obtained from various sources of plants (alginate, pectin), microbes (dextran, xanthan) and animals (chitosan). Polysaccharides can exist as cationic forms (chitosan) or anionic forms (alginate, heparin, hyaluronic acid), which are used widely in the synthesis of nanoparticles via ionic crosslinking with counter ion species. Polysaccharides are also well known for their bioadhesive properties due to non-covalent bonding between hydrophilic groups of hydroxyl, carboxyl, amino groups and biological tissues (mucous
membrane or epithelial) resulting in extending residence time in vivo and higher drug payload loading.\(^9\)

Table 1.1: Polymers derived from natural resources used in fabrication of drug delivery material\(^{10}\)

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<td>HA, Alginic acid, pectin, carrageenan, chondroitin sulfate, dextrane sulfate</td>
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<td>Amphipathic polymers</td>
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Chitosan is one of the most popular polysaccharide used in research regarding drug delivery. Formation of chitosan nanoparticles relies on three primary mechanisms: covalent bonding, ionic gelation, and self-assembly of hydrophobic molecules.\(^{8a}\) The common method of preparation of covalently crosslinked chitosan nanoparticles has been to use an aldehyde (such as glutaraldehyde) to crosslink amine residues. However, these aldehydes are fairly toxic to cells, which limit its use in drug delivery. Ionic crosslinking is another method that has been studied extensively to form nanoparticles by crosslinking cationic chitosan with an anionic crosslinker, such as tripolyphosphate (TPP) molecules. The size of nanoparticles and surface charge density could be controlled by altering the conditions of the synthesis including chitosan concentration, solution pH or chitosan to TPP weight ratio.\(^{11}\) Particles from 110-180 nm synthesized using this method exhibited enhanced cellular uptake in addition to high encapsulation efficiency (>90%) of bovine serum albumin (BSA), a model protein in drug delivery. Calvo and coworkers
demonstrated the release of BSA from nanoparticles over several days, which mainly resulted from the desorption of proteins from nanoparticles.\textsuperscript{12}

Self-assembly of nanoparticles can be driven when the polymer of interest contains both a hydrophilic and hydrophobic fragments (an amphiphilic copolymer), which spontaneously form micelles or micelle aggregates in aqueous solution. Such polymeric micelles are promising drug carriers due to their unique characteristics. The hydrophobic core of micelles helps to increase the solubility of hydrophobic drugs and protect the drugs from degradation while the hydrophilic shells extends the circulation time of micelle-drug conjugates. Formation of micelles from chitosan has been widely studied in drug delivery. For example, Lee and coworkers successfully generated nanoparticles composed of chitosan covalently conjugated with deoxycholic acid via carbodiimide-mediated reaction.\textsuperscript{13} However, most chitosan derivatives are insoluble at physiological pH 7.4, which has created problems in harnessing the material for use in clinical applications.

Nanoparticles generated from proteins have been seen as promising delivery vehicles to reduce toxicity and improve antitumor activity. Albumin has been particularly attractive in this respect. The first protein-based nanoparticle chemotherapeutic was Abraxane®, approved by FDA in 2005 for the treatment of metastatic breast cancer in patients who failed traditional chemotherapy or relapsed within 6 months.\textsuperscript{14} Abraxane consists of paclitaxel and human albumin generated via a patented freeze drying process that forms particles with average size of 130 nm when reconstituted in solution. Abraxane does not contain Cremophor EL (CrEL)- the solublizing agent traditionally used to administer paclitaxel, which therefore avoids the hypersensitivity reactions upon intravenous administration, which has been the source of many of adverse reaction in patients. Albumin is known to transport many hydrophobic molecules (vitamin,
hormones, etc.) with reversible binding sites, which allows for transportation and release of molecules at the cell surface.\textsuperscript{15} Albumin binds to Albondin, 60-kDa glycoprotein expressed on the surface of a variety of cell types. Albumin is taken into the cell via caveolae-mediated endocytosis.\textsuperscript{16}

The success of cisplatin as chemotherapeutics has brought significant attention to the potential use of transition metal complexes as therapeutics. Cisplatin, a platinum (II) complex, forms adducts with DNA strands through inter and intra crosslinking, which leads to disruption of replication and ultimately cell death. Cisplatin has been widely used as anticancer agents and it enters cells mainly through diffusion. Like many other traditional chemotherapeutics, it exhibits a number of unwanted toxic effects due to a lack of selectively which targets healthy tissues. There has been growing interest in studying other transition metals (Co, Re, Ru, Cu) for targeting hypoxic environments by utilizing the oxidation state of metal core as a trigger for masking/unmasking toxicity (Figure 1.2).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image1.png}
\caption{Activation of metal complex altered by redox state, adapted from Graf et al.\textsuperscript{17}}
\end{figure}
This approach has been exploited to deliver cytotoxins for cancer therapy. In order to proliferate, tumors develop a blood vessel system, which rapidly grows to support the large amount of oxygen needed to enable rapid proliferation. The environment surrounding tumors is often described as hypoxic because the supply of oxygen to the tumor is not sufficient to meet the demands of rapid growth. The hypoxia tumor environment exhibits lower oxygen concentration, which can aid in resistance of chemotherapy. However, this anaerobic condition offers the possibility of using the redox state of metal complexes as a chemical trigger to target tumor selectively since the environment can be differentiated from normal tissue. Recent research has shown promising results in developing bioreducible metal complexes that carry cytotoxic ligands (aromatic N-oxides, nitrogen mustard ligand, etc.,) that are active only in a hypoxic environment. Cobalt$^{3+}$ complexes are typically inert to ligand exchange; however they become exchange labile upon reduction to Co$^{2+}$. Denny and coworkers reported the synthesis of Co$^{3+}$ complexes containing nitrogen mustard ligands, which masks the activity of nitrogen mustard due to coordination to an inert metal core. Once delivered to the tumor tissue, the complex can be reduced to labile Co$^{2+}$ complex thereby releasing the bound mustard to attack DNA. The acidic environment of tumor cells can also be used to achieve the activation of cobalt complex due to the production of free radicals. Incorporating of Schiff base ligands to cobalt has been shown to form highly efficient chemo-sensitizers that can be used in conjunction with radiation. Since the 1970-1980s, there has been growing interest in investigating ruthenium (Ru) complexes as potential candidates for antitumor agents. Ruthenium complexes such as NAMI-A and KP1019 have entered clinical trials with NAMI-A showing strong cytotoxic effects in vivo while KP1019 mainly exhibited antitumor activity toward cancer cells in vitro.
It is postulated that the biological activity of ruthenium complexes depends on the redox state of the complex in tumor environment; however the detailed mechanism of action has not yet been identified. In contrast to other metal complexes, Ru was shown to accumulate in high concentrations (about 50% of ruthenium) in the nucleus where it forms adducts with DNA upon reduction from Ru$^{3+}$ to Ru$^{2+}$. The redox potential of Ru can be altered by variation of ligands, for example, the redox potential of NAMI-A and KP-1019 are 25mV and 30mV, respectively. Both of these drugs are reduced in solutions containing glutathione or ascorbate. Other transition metals, such as iron (Fe) and copper (Cu) have been investigated for therapeutics potential. Because of unwanted side effects, there has been increasing effort in the development of delivery systems (nanoparticles) to encapsulate and deliver metal-based therapeutics to desired locations. For example, a Pt$^{4+}$ complex was tethered to single-walled carbon nanotubes and was shown to be efficiently delivered to cells via endocytosis with higher payload (one carbon nanotubes could carry up to 65 Pt$^{4+}$ molecules). Gold nanoparticles have also been used to deliver antisense oligonucleotides and Pt$^{4+}$ complexes (Pt(succ)). This approach resulted in increased cellular uptake and therapeutic effect compared to Pt(succ) alone. Metal complexes already play a significant role in cancer treatment where it is estimated that approximately half of all patients who undergo chemotherapy will receive cisplatin or one of its derivatives. Even though cisplatin plays such a major role in the clinic, there has not been a thorough investigation of metal complexes for use in drug delivery. The hypoxic, or reducing, environment of tumors can activate these metal complexes to unmask cytotoxic drugs. In recent years, significant efforts have focused on developing nanoparticulate systems that are stable in buffered solution but respond to intracellular signals such as changes in pH or redox potential. Each segment of human body exhibits its own characteristic pH such as
gastrointestinal tract (pH 1-3), blood vessel (pH 7.4), tumor (pH 5.7-7.8), which can be exploited to stimulate drug release.26

![Figure 1.3: Nanoparticle-drug conjugate responses to pH change](image)

A common approach is to design particles that exhibit pH-dependent swelling nanoparticles. For example, acrylate polymers such as polymethacrylic acid (PMAA) are used to formulate nanoparticles that change rapidly in the stomach due to pH-dependent behavior of carboxyl groups.28 Combining polyethylene glycol (PEG) with PMAA in different ratios can be used to achieve mass swelling ratios up to 40-90 fold as calculated by the mass of swollen polymer/mass of dry polymer. These polymer are ideal to deliver insulin due to their rapid release of 90% within 2 hours at pH 7.4 while only a small fraction of insulin is released at pH 1.2.29 Another example is the development of self-aggregated nanoparticles of modified carboxymethyl chitosan (a natural polymer) conjugated with deoxycholic acid followed by incorporation of doxorubicin, an anticancer agent. The drug-nanoparticle conjugate was stable at pH 7.4, but released drug rapidly at lower pH, such as those found in tumor environments, due to protonation of carboxymethyl and amine group of modified chitosan.30 This method is an excellent example of utilizing a pH sensitive nanoparticle for the delivery of therapeutics.
Besides pH dependent stimuli, redox responsive nanoparticles have been used to deliver peptides/nucleic acids. The difference in reduced GSH concentration has been reported to be 100-1000 times greater in intracellular space compared to extracellular compartments.\textsuperscript{31} Many anticancer agents, either small molecules drugs or biologics (peptides, proteins) only exert their therapeutics effect inside a specific intracellular compartment such as cytosol, the nucleus, or mitochondria. Utilizing biopolymers to develop redox responsive drug delivery systems therefore has gained significant attention. For example, polyphosphate has been studied extensively in drug delivery and tissue engineering due to its good biocompatibility and biodegradation properties. The polyphosphates break down to harmless byproducts via enzymatic mechanism or hydrolysis of phosphate linkage. Liu and coworkers have successfully formulated a self-assembling micelle of amphiphilic hyperbranched multiarm copolyphosphates (HPHSEP-star-PEPx) with disulfide bonds in the backbone. They encapsulated doxorubicin, a widely utilized chemotherapeutic, and showed that in a reducing environment of 10 mM DTT the micelle rapidly reduces in size over 48 hours due to breakage of disulfide bonds in the micelle core.\textsuperscript{31}
Figure 1.4 Nanoparticle in response to redox environment of glutathione 10mM adapted from Graf et al. 17

Cytosol is known to contain a high concentration of reduced glutathione (2-10mM), which is 100-1000 times higher in comparison with extracellular compartments (2-20μM). Glutathione (GSH) is therefore an ideal internal stimulus to disassemble nanovehicle-drug conjugates to achieve efficient release of targeted therapeutics. Glutathione’s common role in biological systems is normally to prevent damage to cells by scavenging excess reactive oxygen species, free radicals and peroxides. Glutathione can easily be oxidized to glutathione disulfide (GSSG) and reduced back to glutathione by NADPH/NAD+. The relative concentrations of GSH and GSSG are important in determining the level of effect in cells. A study from Hu and coworkers 32 reported the formation of redox-responsive nanoparticles comprised of chitosan to form glycolipid-like nanocarriers (CSO-ss-SA) that responded to a reductive environment like that found in tumors. The particles were used to deliver the hydrophobic anticancer agent paclitaxel along with a Nile fluorescence marker. Elevated drug released was observed in the presence of glutathione (10 mM) due to rapid cleavage of disulfide bonds into free thiols, thus disassembling the nanocarrier-drug conjugate.
In Chapter 2, the synthesis of hydrogel nanoparticles via ionotropic gelation of phosphonate modified chitosan by copper sulfate (CuCs) is discussed. The size of CuCs nanoparticles could be tuned via changes in pH or the concentrations of copper and chitosan. Bovine serum albumin (Alb) was used as model protein for encapsulation to demonstrate the efficiency of encapsulation and release from CuCs nanoparticles.

In Chapter 3, we investigated the prodrug strategy utilizing the redox chemistry of cobalt complexes for targeting tumor associated hypoxia. Hypoxic tissues are known to be oxygen deficient, and there are elevated level of glutathione in cytosol. These environments could be exploited to trigger the release of therapeutics via cleavage or degradation of drug-nanoparticle conjugate. We utilized bovine serum albumin crosslinked with cobalt (II) chloride hexahydrate (CoCl$_2$.6H$_2$O) to generate bioresponsive nanoparticles that show exceptional stability under physiological conditions (PBS or cell media serum) but degrade quickly in reducing environments (presence of glutathione 10mM). In addition, we demonstrated the PEGylation of proteins via cobalt coordination chemistry, which would be expected to lead to extended circulation time, reduced immunogenicity and increased the stability of protein therapeutics. By exploiting the coordination chemistry of cobalt, we expect this strategy will facilitate the masking/unmasking of the biological activity of proteins. PEG was grafted to transferrin (Tf) via cobalt crosslinking to form PEG-Tf-Co. Un-oxidized and oxidized PEG-Tf-Co were then exposed to ethanolamine (50 mM) as a competing ligand, which resulted in the un-oxidized form being completely degrade through ligand exchange while the oxidized compound was stable.

In Chapter 4, we investigated the encapsulation and release of a chemotherapeutic, 7-ethyl-10-hydroxyl camptothecin (SN-38), into cobalt-albumin (Co-Alb) nanoparticles. SN-38 is the active metabolite of irinotecan, a chemotherapeutic commonly used in cancer treatments. The
mechanism of action of SN-38 is inhibition of topoisomerase I, which induces DNA damage and eventually cell death. In order to be active, the parent compound, irinotecan, has to be converted to SN-38 after administration; however only a small amount of SN-38 is generated prior to excretion of the drug. SN-38 itself cannot be administered to patients directly because of its poor solubility in water and pH dependent characteristics. SN-38 was successfully encapsulated into Co-Alb nanoparticle with high encapsulation efficiencies and loading capacities. In addition, the drug-nanoparticle conjugate was stable in PBS (pH 7.4), but degraded rapidly in a reducing environment (glutathione 10 mM).

Chapter 5 is focused on the future directions of these projects. We seek to demonstrate that the crosslinking strategy is completely reversible and releases the protein in its native state. Lysozyme, an enzyme commonly found in immune system, was crosslinked with cobalt via lysine residues to generate particles (Co-Lyso NPs). Lysozyme exhibits lytic activity toward the bacteria *Micrococcus Lysodeikitus*. We measured the activity of Co-Lyso NPs incubated in PBS and in PBS-GSH (10 mM) toward the bacteria lysis via UV/Vis. We found that the Co-Lyso NPs incubated PBS-GSH exhibited similar lytic activity in comparison to the native enzyme.

1.4 References


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CHAPTER 2
PREPARATION OF WATER SOLUBLE COPPER-CHITOSAN NANOPARTICLE FOR PROTEIN CARRIER

2.1 Abstract
The purpose of this study was to demonstrate a new method for synthesizing a controlled-release drug delivery system composed of the biopolymer chitosan crosslinked with copper. Water-soluble n-methylene phosphonic chitosan was crosslinked with copper sulfate at pH 5 and pH 6 in presence of TRIS buffer to generate nanoparticles. Dynamic Light Scattering (DLS) and FTIR were employed to confirm the presence of nanoparticles. The size range of the nanoparticles was between 13 nm-120 nm, which could be tuned by altering the pH of the solution and amount of n-methylene phosphonic chitosan and copper sulfate. This study also investigated the encapsulation efficiency of bovine serum albumin (Alb) within copper-chitosan nanoparticles. Results showed that BSA was efficiently encapsulated within nanoparticles with high loading capacity (36% - 92%). In addition, sustained release of BSA from nanoparticles was observed for over 48 hours indicating the potential use of copper-chitosan nanoparticles as a protein delivery vector.

2.2. Introduction
The use of biodegradable hydrophilic polymers to deliver peptides, proteins, or bioactive agents has progressed rapidly. Encapsulation of these cargos in nanoparticles was expected to overcome some barriers in drug delivery and targeting such as rapid elimination from circulation, enzyme degradation, and accumulation in non-selected organs or tissues. We were therefore interested in developing chitosan hydrogel nanoparticles for the delivery of such cargos. Chitosan, a natural polysaccharide comprised of chitosan β-(1-4)-linked D-glucosamine
(deacetylated unit) and multiple acetylated groups, has been used widely in the pharmaceutical industry.³ Chitosan is produced from the deacetylation of chitin, the principle component of from crustaneans shells.⁴ Chitosan, a linear cationic polyelectrolyte, is known for its favorable properties, which include biodegradability, biocompatibility, mucoadhesiveness, and lack of toxicity.⁵ Chitosan nanoparticles have exhibited great potential for protecting peptides/proteins from enzymatic degradation and to enhance the bioavailability of these molecules.⁶ Despite its excellent properties, all but very low molecular weight chitosan is insoluble in neutral aqueous solutions, which has limited its therapeutic application in drug delivery.⁷ Recent research focuses primarily on chitosan-tripolyphosphate nanoparticles, which can be synthesized by crosslinking the cationic amino groups of chitosan with the anionic phosphate groups of TPP.⁸ Heras and coworkers reported the modification of chitosan structure by converting the NH₂ groups into NH₂-CH₂-PO₃²⁻ which renders the polymer negatively charged at neutral pH and thus enhanced the solubility of chitosan in neutral aqueous solutions.⁹ In addition, phosphonates are known to have high affinities for transition metals.

We studied a slightly different approach for forming nanoparticles of modified chitosan by electrostatic interaction of anionic n-methylene-phosphonic chitosan (modified chitosan) crosslinked with cationic transition metals via ionic gelation. Various factors were applied to modify the size of nanoparticles. For example, the pH of the solution influenced the size of particles as did the concentrations of modified chitosan and copper sulfate.

We successfully formulated nanoparticles with sizes ranging from 13-120 nm, which were expected to increase accumulation in target sites via the enhanced permeability of retention (EPR) effect.¹⁰ The aim of this study was to demonstrate a new method to formulate chitosan
nanoparticles via electrostatic interaction and to examine the efficiency of loading and release of a model protein, bovine serum albumin (Alb), incorporated within nanoparticles.

2.3. Materials and Methods

2.3.1. Materials

Chitosan (high molecular weight 100,000-300,000) was obtained from Acros Organic. Formaldehyde 37% by weight, cupric sulfate and tris(hydroxymethyl)aminomethane were supplied by Fischer Scientific. Bovine serum albumin (95-99% purity) was from Sigma Aldrich. Phosphorous acid and glacial acetic acid were obtained from EM Science. Ultrapure water (Millipore, Thermo Scientific) was used in all experiments. All other chemicals were reagent grade.

2.3.2 Synthesis of N-methylene phosphonic chitosan

The synthesis and characterization of n-methylene phosphoric chitosan was previously demonstrated by Heras et al.9 Chitosan solution 2% (w/v) was dissolved in glacial acetic acid 1% (v/v). One part of phosphorous acid (by weight) was dissolved in water and added to the solution drop wise with stirring for one hour. Then, the temperature of the reaction vessel was raised to 70 °C and one part of formaldehyde 36.5% (by weight) was added drop-wise over 1 h with reflux. Heating was continued for 6 h at 70 °C.

The clear pale yellow solution was dialyzed (12KDa MWCO) in ultrapure water for 48 hours or until the pH of water increased to 6.8. Finally, the solution was freeze-dried for 3 days. The final product obtained was analyzed by 1H NMR, and then used for the formation of copper-chitosan nanoparticles.
2.3.3 Preparation of Copper-chitosan (CuCs) nanoparticles

Chitosan solution (2mg/ml) was prepared by dissolving n-methylene phosphonic chitosan in ultrapure water. The solution was left stirring for few hours until solution was transparent. Copper sulfate (1mg/ml) and TRIS buffer (2mg/ml) were also prepared by dissolving in ultrapure water.

The nanoparticles were prepared at selected ratio of chitosan to CuSO₄ volume ratios of 4:1, 4:2, 4:3 and 4:4. TRIS solution was used to adjust the pH in the range of 5-6. The nanoparticles suspensions were sonicated for 5 minutes at room temperature and then dialyzed in ultrapure water overnight (12KDa MWCO).

2.3.4 Measurement of size and zeta potential of Copper-chitosan (CuCs) nanoparticles

Measurement of particle size was performed by dynamic light scattering [Nanotrac Ultra, Microtrac USA]. The zeta potential of the nanoparticles was measured using a Delsa™Nano C [Beckman Coulter, USA]. All samples were in aqueous solution.

2.3.5 FT-IR

N-methylene phosphonic chitosan and copper-chitosan nanoparticles were freeze-dried for at least 48 hours before analysis. FT-IR analysis of all samples was obtained using an FT-IR Nicolet 6700 (Thermo Fishcher) with Small Orbit diamond fitted for attenuated total reflectance (ATR) accessory. The samples were placed on a diamond plate and subjected to light within mid infrared spectrum. All samples were analyzed at 4cm resolution and collected after 256 scans.

2.3.6 Preparation and evaluation of BSA-loaded Nanoparticles

CuCs nanoparticles at the ratio of 4:3 v/v modified chitosan: copper sulfate were selected for further testing and loading of BSA. CuCs synthesized at pH 5 and 6 were collected after
dialysis. BSA-loaded nanoparticles were obtained by incorporating 4 mL of CuCs nanoparticles to a BSA-containing solution until the final concentration of BSA achieved at 2, 1, or 0.5 mg/mL. The final volume of all solution was 8 mL. Solutions were gently stirred at room temperature for 1 hour. The BSA-loaded nanoparticles were purified by centrifugation at 14000 rpm at 4°C for 30min. The supernatant was collected and analyzed for BSA content.

Figure 2.1: Synthesis scheme of n-methylene chitosan and copper-chitosan (CuCs) nanoparticle

The absorbance of the supernatant, which contained free BSA, was determined by UV/Vis spectrophotometry (Synergy Microplate Reader, Biotek). We employed a Pierce™ BCA Protein Assay Kit (Pierce, Thermo Fisher), which had the wide range of protein detection from 20-2000 µg/mL, to quantify the amount of protein present. The protein dye complex formed using the kit had maximum absorbance at 562 nm.

The BSA encapsulation efficiency (AE) of the nanoparticles was calculated as below:

\[
AE = \left( \frac{\text{Total amount BSA} - \text{Free amount BSA}}{\text{Total amount BSA}} \right) \times 100
\]

2.3.7 Determination of BSA release

To study BSA release, the pellet of nanoparticles was collected, dried under vacuum, and then transferred to clean vial containing 5 mL of PBS pH7.4. The sealed vial was placed in warm
water bath at 37°C and agitated at 100 rpm. At predetermined times, 500 uL of sample was taken from the vial and centrifuged at 14000 rpm for 4 minute. The original sample in vial was replenished with 500 uL of fresh PBS medium after the removal of each aliquot for analysis. The supernatant was analyzed by BCA Protein Assay Kit (Pierce, Thermo Fisher). A calibration curve of known protein concentration in PBS was made for each experiment. Each sample was run in triplicate.

2.4. Results and Discussion

2.4.1 Characterization of N-methylene phoshonous chitosan (modified chitosan) and Copper chitosan nanoparticles (CuCs)

a. FTIR
Chitosan nanoparticles (CuCs-NP) were prepared by crosslinking N-methylene phosphonic chitosan with copper sulfate in TRIS buffer at pH 5 and 6. Figure 2.2 shows characterization by FTIR of N-methylene phosphonic chitosan, copper-chitosan nanoparticles at pH 5 (CuCs-pH5) and copper-chitosan nanoparticles at pH 6 (CuCs-pH6-NP). A broad band was observed at 2650-3500 cm⁻¹ attributed to P-OH group stretching vibration of n-methylene chitosan. A shift in this band was observed from 3170 cm⁻¹ to 3210 cm⁻¹ for CuCs-pH5-NP and to 3250 cm⁻¹ for CuCs-pH6-NP. The sharp peak at 1164 cm⁻¹ and 1068 cm⁻¹ contributed to P=O and P-OH group stretching vibration of n-methylene chitosan. The shift to a new peak of 1170 cm⁻¹ of CuCs-pH5-NP and 1210 cm⁻¹ of CuCs-pH6-NP and diminishing of the peak at 1068 cm⁻¹ was attributed to the ionic interaction between PO₃H₂ and Cu²⁺. The intensity of two distinct peaks of 1657 cm⁻¹ and 1598 cm⁻¹ decreased dramatically and new peaks appeared in both CuCs-NP indicating the involvement of Cu(2+) in the redistribution of vibration frequency of the –NH groups. Therefore, we concluded that Cu(2+) bound to n-methylene phosphonic chitosan predominately
at the PO$_3^{2-}$ groups. It was also postulated that Cu(2+) could interact with the ammonium groups of chitosan, which would enhance the inter-and intramolecular interactions of the chitosan nanoparticles, which has been reported by others.$^{12}$

Figure 2.2: FTIR of n-methylene phosphonic chitosan, Copper-chitosan nanoparticles pH5 (CuCs-pH5) and Copper-chitosan nanoparticles pH6 (CuCs-pH6)

b. Size and zeta potential of CuCs nanoparticles

CuCs-NPs were stable after dialysis at room temperature for several days. The formation of nanoparticles was observed within the range of pH 5-6. At higher pH, aggregation occurred. Figure 2.3 depicts the size of nanoparticles, which could be tuned by changing the volume ratio of copper sulfate while keeping the volume of modified chitosan the same. Nanoparticles size ranged from 44 nm to 121 nm at pH5 and 14 nm to 60 nm at pH6.
Higher concentrations of copper sulfate used in CuCs-NP formation resulted in larger sizes of nanoparticles; which supports the main role of copper(2+) as crosslinking agent in formation of nanoparticles via ionic bonding. Cu(2+) crosslinked PO$_3^{2-}$ groups via electrostatic interactions. TRIS solution (2mg/mL) was used to adjust the pH of the nanoparticle solutions, which also played a significant role in size distribution where an increase in pH resulted in a decrease in size. The length of time sonication was chosen to be exactly 5 minutes. We observed the size of CuCs-NP to decrease upon extended periods of sonication. Baxter et al., concluded that by extension of ultrasound intensities and time, chitosan decreases in molecular weight and intrinsic viscosity.$^{13}$

Figure 2.3 Effect of pH and volume ratio between n-methylene chitosan and copper sulfate on the mean particle size (n=3). Volume ratio between n-methylene phosphonic chitosan: copper sulfate ratio are 4:1, 4:2, 4:3 and 4:4. Results were taken after overnight dialysis.
Nanoparticles were obtained spontaneously under mild conditions with short sonication times. The size of CuCs-NP was determined by DLS before and after dialysis, which showed consistent sizes over this period. Zeta potential, the surface charge of nanoparticles, is an important factor in the design of nanoparticles because of its impact on the stability of nanoparticles in suspension and interaction with cellular membranes.\textsuperscript{14} The zeta potential of CuCs-pH5 and CuCs-pH6 were within the range of $+18\text{mV}$-$+25\text{mV}$, which was appropriate for drug delivery\textsuperscript{15}

2.4.2 Encapsulation of BSA within nanoparticles

![Size distribution by intensity](image)
We chose bovine serum albumin (BSA) as a model protein to study the encapsulation and release of protein within nanoparticles. BSA was incorporated into CuCs nanoparticles via simple mixing of solutions containing BSA and CuCs nanoparticles with stirring at RT to achieve final concentrations of 2, 1, and 0.5 mg/mL of BSA in solution. BSA was encapsulated within the nanoparticle via ionic interaction as described by Calvo and coworkers.\textsuperscript{16} As Figure 2.5 illustrates, the efficiency of encapsulation depends on the initial concentration of BSA and the acidity of nanoparticle solution. In this study, BSA encapsulation efficiency ranged from 36-68\% for CuCs-pH5 and 60-92\% for CuCs-pH6 which indicates that larger amounts of BSA were incorporated at higher pH. We observed that the higher the pH of the nanoparticle solution and concentration of BSA, the more opaque the CuCs-BSA solution became, which is consistent with reports by Calvo.\textsuperscript{16}
2.4.3 Release of bovine serum albumin (BSA)

We chose to employ phosphate buffered saline PBS (pH 7.4) as the release medium because of its similarity and compatibility to body fluids.\textsuperscript{17} We observed sustained released of BSA from nanoparticles as shown in Figure 2.6. A rapid release from 22% to 49% was observed after 1 hour, which was followed by slower release up to 8 hour. The initial burst release of protein was likely to be associated with the protein adsorbed on the particle surface. According to a report from Xu,\textsuperscript{18} large molecules, such as BSA (compared to the size of nanoparticles), were easily desorbed from the surface of nanoparticles in a short period of time, rather than diffusing out of the particle interior during incubation. A higher release rate of protein from CuCs-pH6 nanoparticles was observed (Figure 2.7), which was correlated with a greater amount of protein encapsulated within nanoparticles. The different rates of release were hypothesized to be associated with differing rates of degradation of the nanoparticles.\textsuperscript{19}

![Figure 2.6: Cumulative release of BSA from CuCs-pH5 nanoparticles in vitro at pH 7.4 (n=3)]
2.5. Conclusion

In summary, modified chitosan-copper(2+) nanoparticles (CuCs-pH5 and CuCs-pH6) were successfully prepared. The size of the particles could be controlled by modifying the acidity of the particle-forming solutions as well as the concentration ratio between n-methylene phosphonic chitosan and copper sulfate. The nanoparticle size distribution was in range from 13-120 nm, which was optimal for drug delivery and capitalizing on the EPR effect. The stability of the nanoparticles was quite good with particles persisting for several days at room temperature. The nanoparticles showed promising protein encapsulation and release.

2.6. References


3.1 Abstract

Alfred Werner received the Nobel Prize in 1913 for his pioneering work in developing coordination theory for metal complexes. A crucial experiment leading to the widespread acceptance of Werner’s theory involved the resolution of a set of chiral cobalt complexes, specifically \( \textit{cis} - [\text{Co(en)}_2(\text{NH}_3)X]X_2 \) (\( X = \text{Cl, Br} \)). The kinetic inertness of the Co complexes studied was likely a major contributor to the success of this endeavor by providing a robust system to probe ligand geometry. Here, we revisit this classic coordination chemistry and show how it can be utilized in the field of targeted drug delivery for the bioconjugation of amine-containing (bio)molecules. The chemistry proved to be quite robust, and could be used to crosslink albumin into nanoparticles (20-500 nm in diameter) that exhibited exceptional stability under physiological conditions. The chemistry was pursued as a “traceless” linker that would degrade under reducing conditions, which was observed experimentally through the degradation of our cobalt crosslinked particles upon exposure to reduced glutathione. Bioconjugation of poly(ethylene glycol) (PEG) chains to a prototypical protein (transferrin) was also possible making this chemistry attractive for a host of applications in nanoparticle therapeutics.
3.2 Introduction

This article overviews ongoing research by the author’s group related to the classical coordination chemistry of Alfred Werner.\textsuperscript{1-3} A manifestation of the 21st Century version of Werner’s inorganic chemistry is provided in regard to bioinorganic chemistry and polymer nanoparticle therapeutics.\textsuperscript{4}

Bioconjugation reactions occupy a central role in the field of targeted drug delivery. Such reactions can be used to covalently tether two molecules together allowing the favorable attributes of one species to be imparted to the other.\textsuperscript{5} The chemistry of the linker can also be designed to facilitate a (bio)chemical reaction in a predefined environment\textsuperscript{6} thereby allowing for separation of the two species at the target location. Polymer-drug conjugates\textsuperscript{7} provide an excellent example of both principles. In the former, the residence time in circulation, which is much longer for a macromolecular polymer due to higher molecular weight, can be transferred to low molecular weight therapeutics that would otherwise be expected to exit rapidly from circulation. In the latter, an enzymatic cleavage site can be incorporated as part of the tether so that the drug is released upon exposure to an active enzyme localized at the site of interest.

There are numerous other examples including the attachment of targeting moieties and PEG chains to delivery vectors, and utilizing stimuli-responsive chemistries\textsuperscript{6} to facilitate particle degradation in certain biological environments.

A vast number of chemistries have been explored in bioconjugation reactions and in drug delivery in general; however, only a few have translated to clinical use.\textsuperscript{8} A striking feature of all of the nanoparticle therapeutics that have made it to the clinic is the absence of “chemistry” during particle synthesis. Liposomes,\textsuperscript{9} such as Doxil, protein particles, such as Abraxane, poly(lactic acid) particles, such as BIND-14, and cyclodextrin particles, such as CALAA-01, all
rely on a self assembly process for particle formation that is based predominately on intermolecular interactions, such as hydrophobic/hydrophilic, and not on covalent bonding. As a result, particle degradation does not require the breaking of covalent bonds, which compromises the stability of some of the aforementioned therapeutics. Polymer-drug conjugates, which are generally not considered nanoparticle therapeutics, on the other hand rely solely on chemical conjugation highlighting the differences in approaches between the two disciplines and foreshadowing an area ripe for exploitation in nanoparticle therapeutics. The cobalt chemistry introduced here bridges this gap and can theoretically be utilized under a variety of conditions to synthesize polymer-drug conjugates or nanoparticle therapeutics.

3.2.1 Synthesis of Therapeutic Nanoparticles

There has been significant interest in generating particles derived from proteins for the delivery of therapeutics. Albumin-based nanoparticles have been viewed as particularly attractive because of their biocompatibility and lack of immunogenicity. Albumin, the most abundant serum protein, binds a variety of hydrophobic small molecules for transport in circulation making it somewhat of an ideal carrier in terms of drug loading. Indeed, albumin has been shown to bind and solubilize a variety of otherwise poorly water-soluble drugs. In addition, a receptor for albumin, 60-kDa glycoprotein (gp60) receptor (albondin), is known to be over-expressed by a variety of cancer cell types, which engenders a natural propensity for albumin-based nanoparticles to target cancer cells. Recent studies have revealed that certain tumor cell types take up large amounts of serum proteins to utilize as a source of essential amino acids needed for their rapid growth. Glutamine, which is used in nucleotide synthesis, was found to be significantly depleted in pancreatic tumors and may therefore represent a limiting factor in the growth of pancreatic tumors. In glutamine-sensitive tumor cell lines, the growth inhibition
induced by glutamine deprivation could be reversed through the addition of albumin to the cell culture media. Ras-transformed cancer cells in particular were shown to take up serum proteins via macropinocytosis and then proteolytically degrade them into amino acids that were then fed into central carbon metabolic pathways for bioenergetics and macromolecular synthesis. This phenomenon will likely be recapitulated in a host of other tumor types, which will further increase interest in utilizing serum proteins in the synthesis of therapeutic nanoparticles.

Several techniques have been reported for synthesizing protein-based particles with a large number of these including chemical crosslinking with formaldehyde or gluteraldehyde to increase particle stability under dilute conditions. While this does lead to enhanced stability, biomaterials synthesized using this technique have been shown to leach residual aldehyde, which is highly toxic. Methods based on desolvation, coacervation, and emulsion have also been reported; however, the particles in general lack stability when reconstituted in an aqueous solution in the absence of the aforementioned protein-protein crosslinking. One promising example was reported recently where a combination of desolvation with intramolecular thiol crosslinking led to the formation of stably crosslinked particles that could be loaded with curcumin. The method involves cleavage of intramolecular albumin disulfide bonds using reduced glutathione followed by desolvation to form particles. As the particles were assembling, some number of intermolecular disulfide bonds were formed leading to protein-protein crosslinking and thus stabilization. While the method is appealing due to its simplicity, one concern is the impact the process has on albumin’s tertiary structure that could result in loss of some of its favorable properties, such as low immunogenicity and/or the ability to bind to the gp60 receptor.
One of the most recent nanoparticle therapeutics to receive approval, Abraxane, utilizes albumin to solubilize paclitaxel.\textsuperscript{11} Abraxane is supplied as a solid that must be reconstituted shortly before administration and extended storage (>8h) of the formulation in solution is not recommended. This has been attributed to the transiently stable paclitaxel-loaded albumin particles that are formed upon reconstitution, which has led to significant debate over the ultimate fate of these particles once they come in contact with a patient’s serum. However, even if the particles disassemble completely upon administration and cannot therefore fully capitalize on the EPR effect, a significant therapeutic benefit can still be realized if paclitaxel remains bound to albumin given recent findings on the uptake of serum proteins by cancer cells.\textsuperscript{17}

A self-assembly process is the most attractive for synthesizing nanoparticles for drug delivery because the building blocks used in particle formation can be rigorously characterized via standard methodologies to ensure quality control.\textsuperscript{21} Ultimate approval by regulatory agencies will require stringent characterization of all components,\textsuperscript{22} which becomes excessively complicated for in situ polymerization methods (such as radical induced polymerization of acrylate monomers) with respect to molecular weight, polydispersity, crosslink density, etc. An excellent example of a self-assembly process was reported recently by Davis and co-workers.\textsuperscript{23-26} Polycationic cyclodextrin polymers were used to condense polyanionic siRNA to form therapeutic nanoparticles. A major advance in their work involved the conjugation of PEG chains and targeting ligands to the particle surface through host/guest interactions between adamantane-labeled PEG and transferrin with the cyclodextrin groups of the polymer. The surface ligands greatly increased the serum stability of the nanoparticles facilitating the targeted delivery of siRNA to tumors. The most attractive feature of this synthetic method is the overall simplicity of the process. The contents of two vials (one containing siRNA and the other
containing targeting ligands, PEG, and cyclodextrin polymer) were simply mixed resulting in particle formation. Furthermore, the siRNA-containing cyclodextrin particles were shown to self assemble in vivo even when the individual components were injected separately making concerns regarding the serum stability of the particles a non-issue.

Another popular method for synthesizing nanoparticles is the use of ionotropic gelation to form hydrogel networks from biocompatible biopolymers. This method has been commonly used to crosslink chitosan and alginate, among other polymers, into nano- and microparticles. A polyanion, such as tripolyphosphate (TPP), is typically used to crosslink positively charged chitosan and a polycation, such as Ca\(^{2+}\), for negatively charged alginate. A major concern with particles synthesized via this method is stability under physiologically relevant conditions, such as in serum. The ionic interactions between the biopolymer and crosslinker can be severely weakened in such environments leading to rapid disassembly. In one report, chitosan nanoparticles formed via ionotrophic gellation using tripolyphosphate (TPP) aggregated within 2 hours upon exposure to cell culture media containing serum. Thus, while self assembly is attractive for particle formation, a method for locking the conformation of the construct once formed via covalent bonding remains a significant challenge.

3.2.2 Common Prodrug Strategies

Prodrug strategies that rely on both internal and external stimuli to induce bond breaking have been reported. The three most common strategies capitalizing on internal stimuli are predicated on linkers that are cleavable at reduced pH, by enzymatic activity, or in a reducing environment. With respect to acid-labile linkages, acetal and hydrazones have been designed that are stable at pH 7.4 but labile at pH 5, which relies on the known acidity of tumor regions as well as acidification observed in some modes of endosomal trafficking to facilitate
degradation. The major drawback of acid-labile prodrug linkages continues to be the difficulty in designing substitution patterns that allow hydrolysis rates to achieve on/off selectivity. In addition to the sometimes complex organic chemistry required for their synthesis, these linkages often exhibit undesired hydrolysis of the prodrug linkage in circulation. Enzymatic cleavage sites offer better on/off selectivity in terms of degradability and have shown great promise for generating stimuli-responsive polymer-drug conjugates. However, when applying this strategy to the synthesis of nanoparticles (i.e., crosslinked chitosan), physical access of the cleavage site to the enzyme active site remains a challenge, which could significantly impair enzymatic degradation.

Finally, disulfides have been studied extensively as reductively labile bioconjugation reagents. Both the hypoxic nature of tumor regions and cytosol, which contains significantly elevated levels of reduced glutathione, can be used to trigger thiol-exchange reactions and the breaking of prodrug bonds. More recently, thioesters were introduced in this vein; however, the utilization of both disulfides and thioesters as prodrug linkages often requires a great deal of synthetic effort, and other approaches requiring fewer synthetic steps are desirable. Redox prodrug strategies have been the subject of intense study in the field of metallopharmaceuticals, but the translation of this work to nanoparticle therapeutics remains an underexplored area. Transition metals are uniquely positioned to capitalize on a change in redox environment and the first example applying this concept in drug delivery appeared recently. The basis for this strategy is to capitalize on transition metal bonding motifs that are inert to ligand exchange when the metal is in a higher oxidation state, but labile in a reduced oxidation state. The reduction potential of the chosen metal complex can often be fine tuned through subtle changes in the bonding environment of the ligated metal.
3.3 Results and Discussion

3.3.1 Early Work from Our Laboratory

In spite of the success of cisplatin, the use of transition metals in drug delivery has been greatly hampered by what seems to be a general skepticism toward the use of metals in the treatment of disease. Prof. Trevor Hambley, one of the leaders in the field of metal-based therapeutics, offered an interesting perspective on the current thinking by many researchers in pharmaceutical development, “Medicinal inorganic chemists will confront the same barriers as other medicinal chemists, but will also have to overcome the distrust of metals that is borne out of the ignorance that still exists in most areas of pharmaceutical industry and may be exacerbated by the heightened concerns over toxicity.” Indeed, this perception has resulted in missed opportunities for capitalizing on the unique bonding motifs of transition metals in drug delivery. Our research is focused on expanding the role of transition metals in drug delivery. In our initial work, we utilized readily accessible carboxylate-functionalized, acrylate-based nanoparticles (CNPs) to demonstrate proof-of-principle for the encapsulation and release of a prototypical transition metal, Cu. Cu is involved in a number of cellular redox processes, so it was postulated that we could capitalize on this propensity in order to induce lethal oxidative stress in cancer cells where the normal redox buffering capacity of the cell is known to be near saturation. CNPs from 50 to 500 nm in diameter could be trivially synthesized and loaded with up to 16 wt% Cu. The dependence of Cu release from CNPs was shown to be significantly increased as solution pH was lowered. However, we also observed significant competing ligand effects due to the facile ligand exchange in Cu$^{2+}$ complexes. Release was rapid in phosphate buffered saline at neutral pH, which we later rationalized based on the high affinity of phosphate for transition metals in general. The particles were shown to be toxic to cancer cells, however, the instability under
physiologically relevant conditions was a significant concern and led us to explore other polymer/metal combinations. Attempts at binding other metals to CNPs were unsuccessful, which prompted us to explore other functional groups for the encapsulation of a wider variety of metals.

Particles containing phosphate groups branching from the polymer backbone were synthesized to exploit the inherent affinity of phosphate for metals highlighted above. This was achieved by replacing the acrylic acid monomer used in the synthesis of CNPs with a commercially available monomer containing phosphate. Our phosphate-functionalized nanoparticles (PNPs) indeed bound a variety of metals. Every transition metal surveyed thus far has bound to PNPs making them attractive as a delivery vector for a host of metal complexes.43

In all cases examined, the metal was retained (>80%) under all release conditions investigated; however, we expect the metal to eventually be extracted in the biological milieu of the cell where a wider variety of metal-binding ligands are present or by degradation of the particle itself. Because of the promising anti-metastatic properties observed for several Ru complexes,44-47 we have bound Ru to PNPs and are currently conducting preclinical investigations.

3.3.2 Development of Prodrug “Werner-Complexes”

Prodrug complexes of Co have previously been studied as masking agents for the toxicity of nitrogen mustard complexes,48,49 and as enzyme inhibitors.50 Co chemistry has also been used in other areas tangentially related to the applications in targeted drug delivery described here.51-54 For the coordinated mustard complex, little toxicity was observed for the Co3+ coordination complex; however, upon reduction to Co2+ the toxic mustard complex was released due to facile ligand exchange in the latter. The synthesis of the Co3+ prodrug mustard complex relied on ligand exchange by a somewhat labile Co3+ complex, which was inherently less favorable than
exchange in typical Co$^{2+}$ complexes. Our initial attempts at capitalizing on this Co-prodrug chemistry in nanoparticle therapeutics focused on synthesizing the Co$^{3+}$ precursor previously reported, which we then attempted to use as a crosslinker for lysine residues on proteins. However, all attempts at bioconjugation using this precursor in the synthesis of particles were unsuccessful. This may be the result of the requirement for the presence of charcoal during the reaction, which was reported to provide a surface for the reaction to occur. We postulated a more attractive approach would be to carry out ligand exchange using Co$^{2+}$, which is known to be highly labile, followed by oxidation to Co$^{3+}$ to “lock” the Co ligand environment. This was particularly attractive because it would allow for the synthesis of particles by self assembly that could then be locked into confirmation via stable dative bonds.

This chemistry is quite well known for Co complexes dating back to Alfred Werner, who was awarded the Nobel Prize in 1913 for his pioneering work on coordination theory of transition metal compounds that was predicated on his study of cobalt-amine compounds specifically.$^{1, 2}$ CoCl$_2$, which exists as a hexa-aquo complex in aqueous solutions, undergoes facile exchange of all six waters with ammonia to form hexa-amine Co$^{2+}$. The Co$^{2+}$ complex then spontaneously oxidizes in air to the corresponding Co$^{3+}$ complex. We therefore set out to demonstrate that CoCl$_2$ could be used as a prodrug bioconjugation reagent for crosslinking amine-containing (bio)molecules (Figure 3.1).
Figure 3.1: Generalized method for bioconjugation of two amine-containing molecules using Co as a traceless prodrug crosslinker

This strategy possesses several significant advantages over other bioconjugation reagents commonly used in drug delivery. The reagent itself is inexpensive and requires no synthesis unlike its organic counterparts. Furthermore, there is the potential to conjugate up to six amine-containing molecules per prodrug complex whereas conventional bioconjugation reagents are typically limited to two. Perhaps most importantly, the chemistry of the conjugation reaction involves only a lone pair of electrons on the nitrogen of the amine-containing species forming a dative bond with Co (i.e., not even a proton transfer reaction is required). When the Co is reduced, the complex could again undergo facile ligand exchange releasing the coordinated amine in an unaltered or native state. Finally, the bioconjugation reaction is impervious to water, which is a major obstacle for many standard bioconjugation reagents, such as those based on N-hydroxysuccinimide, that undergo unwanted hydrolysis reducing overall coupling efficiency.
3.3.3 Synthesis of Cobalt Crosslinked Albumin Nanoparticles

Our first attempt at a bioconjugation reaction utilizing this novel cobalt chemistry was in the synthesis of crosslinked albumin nanoparticles. We postulated that we could very simply and reversibly form intermolecular crosslinks via lysine residues in the protein (Figure 3.2), which are the target of many commercially available bioconjugation reagents.

Addition of CoCl₂ to an aqueous solution containing albumin did not induce any observable changes. The solution was a reddish-pink color due to the presence of the hexaaquo complex [Co(H₂O)₆][Cl⁻]. The lack of reactivity was due to the slightly acidic (pH~6) nature of the solution where virtually all the lysine residues of the protein would be expected to be in the
protonated form. Immediately upon addition of base (pH of 9-10) the solution became turbid with a faint blue color (Figure 3.3). Dynamic Light Scattering (DLS) was used to confirm the presence of nanoparticles (Figure 3.4). We found that by adjusting the relative proportions of the starting materials we could trivially synthesize Co$^{2+}$ crosslinked albumin nanoparticles (Co$^{2+}$-Alb NPs) ranging from 10 to 500 nm in diameter (Figure 3.5, Table 3.1).

Figure 3.3: Synthesis of Co$^{3+}$-Alb NPs

Figure 3.4: Particle size analysis of Co-Alb NPs
Figure 3.5: Effect of Co (A), albumin (B), and NaOH (C) concentrations on nanoparticle size

Table 3.1: Nanoparticle size as a function of reaction conditions.

<table>
<thead>
<tr>
<th>[Albumin] (mg/ml)</th>
<th>[Co] (μM)</th>
<th>[NaOH] (equiv.)</th>
<th>Particle Size (nm)</th>
<th>Standard Deviation (nm)</th>
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<td>2.0</td>
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</tbody>
</table>
Next, we oxidized Co$^{2+}$-Alb NPs to Co$^{3+}$-Alb NPs using H$_2$O$_2$, which induced a color change from faint blue to a more intense yellow without significantly altering the turbidity of the solution (Figure 3.3). Cobalt undergoes a transition from a high spin $d^7$ Co$^{2+}$ complex with weak field ligands to low spin $d^6$ Co$^{3+}$ with strong field ligands, which results in changes to the adsorption bands and thus the color changes observed. The same chemistry could be used to crosslink solutions of chitosan (MW = 5,000), as well as mixtures of the chitosan and albumin (data not shown).
Based on literature precedent,\textsuperscript{38, 39} we expected Co\textsuperscript{3+}-crosslinked species to be stable under physiologically relevant conditions whereas their Co\textsuperscript{2+} analogues would not. Indeed, incubation of Co\textsuperscript{2+}-Alb NPs in phosphate-buffered saline (PBS) or cell culture media containing fetal bovine serum (FBS) led to rapid degradation of the particles and the formation of a precipitate (Figure 3.6). Conversely, Co\textsuperscript{3+}-Alb NPs exhibited exceptional stability under these conditions where solutions incubated without agitation at 37°C for greater than 14 days remained well dispersed (Figure 3.7). This is in stark contrast to particles formed upon reconstitution of Abraxane, albumin-bound paclitaxel, which also degrade rapidly under physiologically relevant conditions. While stability under physiologically relevant conditions was demonstrated, Co\textsuperscript{3+}-Alb NPs were expected to be degraded in a reducing environment. As expected, Co\textsuperscript{3+}-Alb NPs incubated at 37°C in 10 mM reduced glutathione (GSH) were rapidly degraded with complete disappearance of particles in less than 5 h according to DLS (Figure 3.8). Efforts are currently underway to load therapeutics into our albumin nanoparticles. \textsuperscript{10}

![Figure 3.6: Stability of Co\textsuperscript{2+}-Alb NPs and Co\textsuperscript{3+}-Alb NPs under physiologically relevant conditions.](image)
Figure 3.7: Particle size as a function of incubation time for Co$^{2+}$-Alb NPs and Co$^{3+}$-Alb NPs as monitored by DLS

Figure 3.8: Degradation of Co$^{3+}$-Alb NPs under reducing conditions (GSH – reduced glutathione)
3.3.4 Utilizing Cobalt for Protein PEGylation.

Attaching PEG chains to proteins has been pursued in an effort to increase the circulation time of protein therapeutics, to reduce their immunogenicity, and increase stability.\textsuperscript{37, 55-57} A number of PEGylated proteins are already in the clinic including enzymes, cytokines, antibodies, and growth factors. More recently, PEGylation has been introduced as a method for masking/unmasking the biological activity of proteins.\textsuperscript{37, 57} For example, the enzymatic activities of trypsin\textsuperscript{57} and lysozyme\textsuperscript{37} can be greatly ameliorated through PEGylation, which is restored upon cleavage of the PEG chains. Reversible PEGylation could also be used to modulate the toxicity of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in vitro in Jurkat T cells.\textsuperscript{37} These strategies were based on introducing sites for PEG chain cleavage by enzymatic action or a reducing environment, but a number of other prodrug and non-cleavable linkages have been employed as well.

In an effort to determine the feasibility of using cobalt chemistry as an alternative strategy, we investigated the PEGylation of transferrin (Tf, Figure 3.9). For this reaction, CoCl\textsubscript{2} and H\textsubscript{2}N-PEG-COOH (MW = 5,000) were premixed (1:1) in borate buffer (pH = 9) followed by the addition of an aqueous solution of human transferrin. The coupling reaction was monitored by DLS where an increase from \~7 to \~14 nm was observed (Figure 3.10) indicating successful conjugation of the PEG chains to Tf generating PEG-Co\textsuperscript{2+}-Tf. The PEGylated protein was then oxidized using H\textsubscript{2}O\textsubscript{2} leading to the formation of PEG-Co\textsuperscript{3+}-Tf. Both oxidized and un-oxidized PEGylated transferrins were then exposed to ethanolamine (50 mM) as a competing ligand where the un-oxidized transferrin was expected to degrade via ligand exchange. Indeed, added ethanolamine induced rapid shedding of the PEG chains from transferrin for the Co\textsuperscript{2+} derivative, which rapidly returned it to the size of native Tf by DLS. The Co\textsuperscript{3+} complex was unaffected by
the addition of competing ligand (Figure 3.10). Current efforts are focused on demonstrating that the protein is released in its native form.

Figure 3.9: PEGylation of transferrin using Co coordination chemistry

Figure 3.10: PEGylation of transferrin (Tf) via cobalt bioconjugation chemistry as analyzed by DLS
3.4 Future Directions

The use of Werner complexes as widespread bioconjugation reagents opens a vast new space for exploration in nanoparticle therapeutics. Their ease of synthesis and stability undoubtedly contributed to Werner’s ability to elucidate their structures given the limited set of experimental tools at his disposal in the late 19th Century. This chemistry possesses a number of favorable characteristics beyond these that make it highly attractive as a prodrug strategy. Amines are ubiquitous in biology providing the potential for a variety of bioconjugation substrates. Here we demonstrated protein-protein and protein-polymer conjugations, but others including oligonucleotide-oligonucleotide remain to be explored. Oligonucleotide bioconjugation represents a significant opportunity because the greatest barriers to the realization of the full potential of micro- and si-RNA therapeutics remain protecting them from degradation in circulation and delivery to the target cell. This bioconjugation method could also be applied to delivering amine-containing therapeutics, such as gemcitibine. The conjugation of such drugs to albumin or other proteins will greatly extend their residence time in circulation and accumulation in tumor cells where the active drug can be released upon reduction of Co. The octahedral bonding environment of Co also represents a relatively unique structural feature in nanoparticle therapeutics, which is based predominately on organic materials. Only two of the Co coordination sites are required for a bioconjugation reaction, which leaves four sites available for modification to improve various attributes of the delivery system, such as tuning the Co reduction potential. The results reported here are just the beginnings of a great deal of work that will be required before the full potential of Werner complexes in targeted drug delivery is known.
3.5 Materials and Methods

3.5.1 Synthesis of Co(III)-Crosslinked Albumin Nanoparticles

An aqueous solution (1.125 mL) containing bovine serum albumin (10 mg) and cobalt(II) chloride hexahydrate (11.1 mM) was prepared in a glass vial. While sonicating (Branson, model #2510), NaOH (50 μL, 0.25 M in water) was added at which time the solution became turbid and faintly blue. The solution was allowed to stand undisturbed for 15 min. Dynamic light scattering (DLS, Microtrac Nanotrac Ultra) was used to measure particle size where the volume average particle size was 332 nm (SD = 31 nm). The nanoparticles were centrifuged at ~21,000 x g (Eppendorf, model #5810R) for 5 s and the supernatant removed. The particles were re-dispersed in 1.0 mL of DI water and centrifuged again at ~21,000 x g for 5 s. The supernatant was removed and the particles were re-dispersed in 1.0 mL of DI water. Hydrogen peroxide (20 μL of a 30% solution) was added while sonicating and the solution was thoroughly mixed by pipetting. The turbidity of the solution did not change, but the color changed from faint blue to a more intense yellow-brown. The solution was allowed to stand undisturbed for 30 min. The particles were centrifuged at ~21,000 x g for 5 s and the supernatant removed. The particles were re-dispersed in 1.0 mL of DI water and centrifuged again at ~21,000 x g for 5 s. The supernatant was removed and the particles were re-dispersed in 1.0 mL of DI water. DLS was used to measure particle size where a diameter of 262 nm (SD = 39.5 nm) was obtained. Similar results were obtained upon scaling the reactants by a factor of 7x. Particles 20-500 nm in diameter were synthesized by changing the amounts of cobalt chloride and NaOH used in the reaction.
3.5.2 Nanoparticle Stability

Four samples containing 1 mL of the nanoparticle solutions obtained above both before (2 samples) and after (2 samples) addition of H₂O₂ were centrifuged at ~21,000 x g for 5 s and the supernatants removed. The resulting solids were re-dispersed in 1.0 mL of either 10 mM PBS or RPMI-1640 (ATCC, cat. #30-2001) containing 10 % fetal bovine serum (ATCC, cat. #30-2020). The solutions were incubated at 37°C with 5% CO₂ without agitation and particle size was measured periodically by DLS.

3.5.3 Cytotoxicity

SNU-5 cells (ATCC, cat. # CRL-5973) were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, cat. # 30-2005) with 10% FBS (Fisherbrand Research Grade Fetal Bovine Serum, cat. # 03-600-511). Cells (50,000/well) were seeded on 96-well plates and the desired particle amounts were added to the wells. The plates were incubated for an additional 24 h at 37°C with 5% CO₂. After incubation, cell viability was evaluated using MTT. MTT (Sigma-Aldrich, cat. #M2128-16) dissolved in culture media (5 mg/mL) was added to each well (25 μL/well). The cells were incubated for 4 h at 37°C with 5% CO₂ after which time 0.08 M HCl in 2-propanol (100 μL/well) was added. Light absorption was measured on a Synergy 2 multimode microplate reader (BioTek). The viability of the cells exposed to particles was expressed as a percentage of the viability of cells grown in the absence of particles on the same plate.

3.5.4 Transferrin PEGylation

A borate buffered solution (47.6 mM, 1.05 mL) containing cobalt(II)chloride hexahydrate (0.95 mM) and H₂N-PEG-COOH·HCl (5 mg, Rapp Polymere, cat.#1350002032) was prepared in a glass vial. While sonicating, human transferrin (0.5 mL of a 1 mg/mL aqueous solution, Sigma-
Aldrich, cat. #T0665-50MG) was added. The solution had no discernible color and was allowed to stand undisturbed for 15 min. Dynamic light scattering (DLS, Microtrac Nanotrac Ultra) was used to measure particle size where the volume average particle size was 13.6 nm (SD = 4.2 nm). Hydrogen peroxide (20 μL of a 30% solution) was added while sonicating and the solution was thoroughly mixed by pipetting. The solution became faintly yellow but with no turbidity. The solution was allowed to stand undisturbed for 30 min.

3.6 References


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CHAPTER 4
PREPARATION AND EVALUATION ENCAPSULATION OF ANTICANCER AGENT SN38 INTO COBALT-ALBUMIN (ALB) NANOPARTICLES

4.1 Abstract

SN-38, a hydrophobic therapeutic with great antitumor potential in cancer therapy, was successfully encapsulated in Co-albumin nanoparticles (Co-Alb NPs) with high loading capacities (34%-79%) and excellent encapsulation efficiencies (96%-99%) via an emulsion/solvent evaporation method. The size of the Co-Alb NPs played an important role in the efficiency of the drug loading process. Smaller Co-Alb NPs could be loaded with higher concentrations of SN-38. UV/Vis, fluorescence, dynamic light scattering, zeta potential were employed to characterize SN-38 Co-Alb NPs. The drug loaded particles retained the therapeutic for days in the absence of reductant. Release studies revealed an initial burst release of SN-38 from Co-alb-SN38 upon exposure to a reducing environment (glutathione, 10mM) and gradual release up to 84% of encapsulated therapeutic over three days. Fluorescently labeled Co-Alb NPs were utilized to examine uptake of the particles in a gastric carcinoma cell line (SNU-5) where significant uptake was observed via macropinocytosis. The drug loaded particles exhibited significant toxicity to SNU-5 cells with an IC$_{50}$ value (less than 50 nM) rivaling many of the nanoparticle therapeutic formulations currently undergoing clinical trials.

4.2. Introduction

7-ethyl-10-hydroxycamptothecin (SN-38) and irinotecan both belonged to the class of camptothecin derivatives that inhibit topoisomerase I, an enzyme that plays a vital role in DNA replication and transcription, leading to DNA damage in cells and eventual cell death.
Irinotecan has been approved for treatment of metastatic colorectal cancer and is commercially available as Camptosar (Pfizer Inc, New York). SN-38 is the active metabolite of irinotecan, which exerts a much stronger therapeutic effect (100-1000 times in comparison with irinotecan) and longer half-life in many cancer cell lines. Recent studies have shown that only a small amount of irinotecan is converted to SN-38 (2-8%) via carboxylesterase metabolism in liver and tumor cells; therefore, a higher dose of irinotecan must be administered to achieve maximum antitumor efficacy. However, despite the promising antitumor efficacy toward a wide range of experimental tumor models, SN-38 has been limited in application due to its poor solubility in water and inactivity at neutral pH. SN-38 only exerts its therapeutic effect when present in the lactone form under slightly acidic conditions whereas the open-chain carboxylate form does not exhibit any therapeutic effect. Hydrolysis of the lactone ester can easily occur in SN-38 to convert from the lactone form to the carboxylate form depending on the pH of the solution. Since SN-38 was known to lose its therapeutic effect under basic conditions, developing an innovative nanoparticulate system that facilitates high drug loading and better stability and encapsulation of the active form of SN-38 remains a challenge. In an attempt to enhance the solubility and stability of SN-38, much effort has been made to encapsulate the therapeutic within a nanoparticle carrier to protect the biological activity of the drug until delivery to the desired location.
Targeted drug delivery systems derived from albumin have emerged as promising tools in oncology and drug delivery in general because of the protein's low toxicity, lack of immunogenicity, biocompatibility and biodegradability. Albumin, the most abundant protein in blood, is distributed at approximately 1/2 kg widely within the circulatory and lymphatic system, intra and extra cellular compartments. Albumin is highly soluble in water, stable during long-term storage, and is easy to purify. Albumin contains a variety of binding sites for the transport of small molecules, which is highly favorable in terms of drug loading. Due to its unique primary structure, either negative (oligonucleotide) or positive (ganciclovir) species could be bound to albumin. Recent research has shown that tumors consume large amounts of albumin as nutrient source to support for their rapid growth. Additionally, the receptor for albumin- 60kDa glycoprotein (gp60) (albondin)- is over expressed in many cancer cell types. Recent studies
have now clearly demonstrated that a host of cancer cell types utilize serum proteins as a major source of the required nutrients and building blocks, which was found to be associated with a Ras mutation present in ~20% of all cancers. Such cells are capable of engulfing large amounts of protein via macropinocytosis, which is a non-specific process that lacks the requirement for engaging a particular receptor on the cell surface and can form vesicles up to 5 μm in diameter.

These findings will likely have important implications in the field of nanoparticle therapeutics given the efforts currently devoted to elucidating cellular targeting/internalization strategies and the ongoing debate over their effectiveness. Particles are already known to accumulate at tumor sites due to the enhanced permeability and retention effect, so an important question arises as to whether a targeting/internalization strategy will be necessary for tumors exhibiting this behavior. While Davis et al. found no increase in accumulation of particles in tumors based on the presence of targeting ligands, an increase in internalization was observed for targeted particles. However neuroblastoma cells like the ones used in those tumor models do not typically possess mutated Ras, so it is unclear whether differential uptake would have been observed in the presence of mutated Ras.

Our own efforts to target c-Met, which is a receptor tyrosine kinase highly over-expressed by SNU-5 cells as well as a number of other cancers, have been hampered by significant non-specific uptake irrespective of surface targeting ligands. These results prompted us to not only investigate uptake and toxicity of our new drug delivery platform in SNU-5 cells, but also to examine the macropinocytic propensity of SNU-5 cells.

Nanoparticles containing anticancer agents have shown promising results in tumor models due to the enhanced permeation and retention (EPR) effect. Particles within a size range from 10-500 nm accumulate into interstitial space in tumors because of defective lymphatic drainage that
allows for greater retention of large particles. Exploiting the EPR effect can greatly increase the amount of drug delivered to the tumor and reduce undesired effects of targeting normal cells.\textsuperscript{13} In one example, liposomal formulation SN-38 showed significant antitumor activity toward cancer cell lines and xenograft mouse models in comparison with irinotecan.\textsuperscript{7} Polymeric micelles have also been utilized to encapsulate and deliver SN-38 to HT-29 cancer cells and showed promising results in terms of therapeutics efficacy.\textsuperscript{14} However, our goal here was not only to overcome the extracellular barriers (long circulation time, accumulation to target locations, selectively binding to diseased cells, etc..) but also to overcome intracellular barriers (cellular internalization, endosomal escape, drug release, etc..).\textsuperscript{15} There is strong interest in developing novel bioresponsive drug-nanoparticle conjugates that exhibit triggered release of therapeutic in response to changes in pH or reducing environment.\textsuperscript{16} Many anticancer agents exhibit their therapeutic efficacy within specific intracellular compartments such as cytosol and the nucleus, which are known to contain high concentration of glutathione (GSH) (2-20mM) while extracellular compartments contain a much smaller concentration (2-20uM). Glutathione is an ideal stimulus to destabilize the drug-nanoparticle conjugate to triggered release of therapeutics. Combining these factors, the encapsulation of SN-38 into Cobalt-BSA nanoparticles is presented here along with an evaluation of the stability of the drug-conjugate under various conditions. Uptake and toxicity of the drug loaded particles was also investigated.

4.3. Material and Method

4.3.1 Materials

Bovine serum albumin (Lot # 041M1816V), reduced glutathione (Lot # SLBH7927V) and cobalt (II) chloride hexahydrate were obtained from Sigma Aldrich (St Louis, MO). Phosphate buffered saline 10X solution was purchased from Thermo Fisher (Fisher BioReagents\textsuperscript{TM}).
Acetonitrile (ACS grade) was obtained from Merck (Kenilworth, NJ). Sodium phosphate monobasic monohydrate USP was purchased from Mallinckrodt (St Louis, MO). Albumin–fluorescein isothiocyanate conjugate (cat. #A9771), bovine serum albumin (cat. #A2153), o-(2-amoineethyl)polyethylene glycol (MW 5K, cat. #672130), and thiazolyl blue tetrazolium bromide, MTT (cat. #M5655) were from Sigma-Aldrich, fetal bovine serum (cat. #03-600-511), 5-(N-ethyl-N-isopropyl)amiloride, EIPA (cat. #37-781-0) were from Fisher Scientific, SNU-5 cells (cat. #ATCC® CRL-1420) Jurkat cells (cat. #ATCC® TIB-152), RPMI-1640 medium (cat. #ATCC® 30-2001), IMDM medium (cat. #ATCC® 30-2005) were from ATCC, Met (L6E7) Mouse mAb (cat. #8741), and anti-mouse IgG (H+L), F(ab’)2 Fragment (Alexa Fluor® 488 Conjugate) (cat. #4408) were from Cell Signaling Technology, and micromer®-redF (cat. #30-02-501) was from Micromod. Ultrapure water (Millipore, Thermofisher) was used throughout all experiments. All other chemical used were in analytical grade unless notified. Flow cytometry was performed using either an Amnis FlowSight (EMD Millipore; Seattle, WA) or Guava EasyCyte 6.2L (EMD Millipore; Seattle, WA) instrument. The Amnis FlowSight image-based flow cytometer was equipped with a 488 nm (60 mW) and Side Scatter (7.85 mW). The FlowSight uses a CCD camera system to simultaneously collect both quantitative fluorescence and image data.1,2 For the FlowSight, spectral compensation was completed after analysis using an automated wizard and single color control samples in the IDEAS software. Prior to collecting samples, the performance of the FlowSight was validated using FlowSight calibration beads (EMD Millipore).

4.3.2. Preparation of Co3+-albumin NP (Co-alb NP) and loading of SN-38 into Co-alb NP

Bovine serum albumin (Alb) was dissolved in ultrapure water at a concentration of 10mg/mL. Cobalt chloride hexahydrate CoCl2·6H2O (100 mM) and NaOH (25 mM) solutions were also
prepared in ultrapure water. In a small glass vial, 200 µL of bovine serum albumin (10mg/ml) was mixed with 50 µL of NaOH 25 mM and the solution yielded no color or turbidity changes upon sonication (Branson, model #2510) for 5 second. Then, 50 µL of cobalt chloride hexahydrate was added to the mixture and the solution immediately turned turbid and blue. The solution was sonicated for another 5s and left undisturbed for 15 minute at room temperature. Hydrogen peroxide (5 µL of 30%) was added to the solution followed by 1 mL of ultrapure water. The solution immediately turned an intense yellow without any observable changes in turbidity. The nanoparticle solution was dialyzed (12 KDa MWCO, Sigma Aldrich, USA). The nanoparticle were approximately 150 nm in diameter according to DLS. Following the same methodology, by modulating the amount of cobalt chloride, NaOH and bovine serum albumin, Co-alb NPs of 50 nm and 100 nm was also prepared.

SN-38 (7-ethyl-10-hydroxycampothecin) was purchased from TCI Development Co (Shanghai, China). SN-38 (2.5 mg) was added to 0.5 mL of ethanol. Sonication was applied for few seconds, which the solution immediately became turbid, followed by the addition of 0.5mL of dichloromethane (DCM) to the vial. After few seconds of sonication, SN-38 was completely dissolved in ethanol:DCM (1:1v/v) yielding a clear light yellow solution. The lactone form of SN-38 was maintained by adjusting the pH of solution to 3.0 using 0.1M HCl.

Emulsification/solvent evaporation was used to incorporate SN-38 into Co-Alb NP. In a small glass vial, 1 mL of Co-Alb NPs solution prepared above was added to 0.5 mL of the SN-38 solution (2.5mg/ml in ethanol:DCM 1:1v/v). The solution immediately became turbid upon sonication, followed by gently stirring for at least one hour at room temperature until organic phase evaporated. The pale yellow solution was then centrifuged at 21,000 x g for 10 minutes. The supernatant containing free SN-38 was removed and kept for HPLC analysis. The pellet
was washed with ultrapure water and centrifuged at 21,000 x g for 10 minutes to eliminate free SN38. Finally, the pellet was lyophilized and collected as dry brown powder (Co-Alb-SN38 conjugate). The solid powder was kept in a desiccator for long term storage.

4.3.3. Encapsulation efficiency and characterization of Co-Alb-SN38 conjugate

To determine SN-38 loading, the supernatant collected after centrifugation (before the wash-step) was used to measure the free drug using HPLC (UltiMate 3000, Thermo Scientific, USA) with reference to a calibration curve of SN-38 concentration at absorbance of 265nm. Drug loading capacity and encapsulation efficiency were calculated as shown below:

Drug loading capacity =
\[
\frac{\text{Total amount of feeding drug} - \text{Free amount drug}}{\text{Total weight of nanoparticle}} \times 100
\]

Encapsulation Efficiency =
\[
\frac{\text{Total amount of feeding drug} - \text{Free amount drug}}{\text{Total amount feeding drug}} \times 100
\]
In addition to using HPLC for confirmation of loading, we employed fluorescence spectroscopy to obtain spectra of Co-alb-SN38 in comparison with free SN38. A predetermined amount of dried Co-alb, Co-alb-SN38 and SN-38 were dissolved in water/ethanol (1:1 v/v) with final concentrations of 1 mg/mL. Sonication was applied for 1 min to fully disperse the samples. Samples were excited at 380 nm and monitored for emission in the range of 450-680 nm (Photon Technology International, NJ, USA).

Size distribution and mean diameter of Co-alb-SN38 were determined using dynamic light scattering (NanoTrac, Microtrac, USA). The zeta potential of nanoparticle solutions were measured by Zetasizer (Malvern, USA). Nanoparticles were prepared in ultrapure water at a concentration of 1 mg/mL for zeta potential measurements.

Co-Alb NPs were examined under scanning electron microscopy (SEM) in order to observe morphology. One drop (5-15 μL) of a 1 mg/mL solution was placed on a clean silicon wafer, which had been pre-coated with a thin layer of gold. The samples were dried under vacuum and coated with a thin layer of gold using Spin Coater Model #108 (Cressington Sputter Coater).

To examine the stability of Co-alb-SN38 NPs, dried powder of Co-alb-SN38 NP was dissolved in either PBS (pH 7.4) and PBS-GSH 10 mM in small glass vials. Sonication was employed in order to achieve the maximum dispersion of SN-38 NPs in solution. Both solutions were incubated at 37 °C with 5% CO₂ to simulate physiological conditions. The size of nanoparticles was monitored via dynamic light scattering (DLS) at predetermined times of 2, 24, 48, 72 hours. Co-Alb-SN38 NP appeared to be consistently stable in PBS while the nanoparticles disappeared in reducing environment of PBS-GSH 10 mM within 2 hour.
4.3.4. Drug release in PBS and PBS-GSH (10 mM)

Solutions of Co-Alb-SN-38 (0.5 mL of a 5 mg/mL solution) in PBS and in PBS-GSH 10 mM were prepared and injected into a dialysis cassette (MWCO 7000KDa, Slide-A-Lyzer Dialysis Cassette, Thermo Scientific). The bag was then immersed floating in 100 mL of PBS or PBS-GSH in a covered beaker and kept at 37 °C with 5% CO₂. At predetermined times of 1 hr, 2 hr, 4hr, 8hr, 24 hour, 48 hour, 72 hour, 2 mL of the solution was withdrawn and replaced with 2 mL of a fresh solution of PBS or PBS-GSH 10 mM.

4.3.5 HPLC analysis

A reversed-phase high performance liquid chromatography method with UV detection was developed and validated for quantitation of SN-38 encapsulation and release in Co-Alb-SN38 NPs. We employed an UltiMate 3000 Standard LC system (Thermo Scientific, USA) that was equipped with a pump HPG-32000 SD, analytical autosampler WPS-3000SL and UV Detector MWD-3000 (Thermo Fisher, USA). A reversed phase C18 column (4μm, 250mm×4.60mm) was used. The mobile phase consisted of buffer containing acetonitrile and Na₂H₂PO₄ (60:40 v/v) with the pH of the mobile phase being adjusted to 3.1 with H₃PO₄ to ensure the molecular structure of SN-38 was maintained as the closed lactone ring. To quantify the amount of SN-38 encapsulated in Co-Alb-SN38, the supernatant collected after centrifugation (0.5 mL) was extracted with 1 mL of the mobile phase (acetonitrile: Na₂H₂PO₄ 60:40 v/v), filtered, and injected to the HPLC system for analysis. To determine the amount of SN38 released in PBS or PBS-GSH 10 mM, 0.5 mL of sample collected was extracted with 1 mL of mobile phase and filtered before injection for analysis. An aliquot of 10 μL was injected in triplicate with a flow rate of 1 mL/minute. The column temperature was maintained at 30°C. The UV detector was set at 265nm according to the method of Xuan et al. The retention time of SN38 was found to be
5.12 minute. A calibration curve of SN-38 was prepared in the range from 0.001 μg/mL to 0.1 μg/mL with a linear regression equation of \( y = 0.7639x + 0.4931 \) (the linear coefficient \( R^2 = 0.998 \)). The concentration of unknown SN38 samples was calculated according to the calibration plot.

4.3.6 Degradation of Co-Alb NPs as a function of glutathione concentration

Co-Alb NPs were incubated with varying concentrations of reduced glutathione. The absorbance of the solution was monitored by UV/Vis (350 nm) over time.

4.3.7 Synthesis of Co-Alb-FITC NPs

All solutions used in the synthesis of nanoparticles were freshly prepared and used within one week. A 1 mL aqueous solution containing 7.5 mg of bovine serum albumin-FITC conjugate and 2.5 mg of was prepared followed by the sequential addition of CoCl₂ 6H₂O (200 μL, 0.1 M in H₂O) and NaOH (50 μL, 0.25 M in H₂O) while sonicating. The solution was allowed to stand undisturbed for 10 min at room temperature and then centrifuged at 21,000 xg for 30 s. The supernatant was removed and the particles were washed once with 1 mL ultra-pure water. After washing, the particles were re-dispersed in 1 mL of ultra-pure water and analyzed by dynamic light scattering (DLS). DLS results indicated a monodisperse population of particles with a number average diameter of 498 nm (Figure S2). Hydrogen peroxide (0.2 μL, 30% v/v in H₂O) was added and the solution allowed to stand undisturbed for 10 min. The particles were then centrifuged at 21,000 xg for 1 min, the pellet washed once with ultra-pure water, and re-dispersed in 1 mL of ultra-pure water. An average diameter of 493 nm was observed by DLS (Figure S2), indicating no change in particle size upon oxidation of Co²⁺ to Co³⁺. Co-Alb-FITC NP solutions were used immediately or stored for up to 1 week in the dark at 4°C.

4.3.6 Synthesis of cMet-Targeted NPs

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Ligands utilized for cMet targeting experiments included an antibody to cMet, an aptamer reported from the literature, and poly(ethylene glycol) (PEG, 5K MW) as a negative control. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 8 mg) and N-hydroxysuccinimide (NHS, 1.5 mg) were dissolved in 50 μL 0.5M MES buffer at pH 6.5. Micromer Red NPs (50 μL, 10 mg/mL) were added and the solution incubated for 20 min while vortexing. The particles were washed once with water and then re-suspended in 80 μL of PBS. The desired targeting ligand (20 μL) was then added and the solution incubated at room temperature for 3-6 h while vortexing. Ethanolamine (20 μL, 50 mM in PBS) was added and the solution was incubated at room temperature for 30 min while vortexing. PBS (0.5 mL) was added and solution was centrifuged at ~21,000×g for 3 min. The particles were washed once with PBS and re-suspended in 100 μL of PBS. The particle solutions were either used immediately or stored at 4°C.

4.3.8 Uptake of Co-Alb-FITC NPs by gastric carcinoma cells

SNU-5 cells were cultured at 37°C and 5% CO₂ in IMDM medium supplemented with 20% fetal bovine serum at cell densities between 1x10⁵ and 1x10⁶ cells/mL, and Jurkat cells were cultured at 37°C and 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum at cell densities between 1x10⁵ and 1x10⁶ cells/mL. For nanoparticle uptake experiments, cells were transferred to serum-starved IMDM or RPMI-1640 medium (0.1% FBS) and incubated at 37°C and 5% CO₂ for 10-16 h prior to dosing with nanoparticles. Cells (2 mL, 500,000 cells/mL) were incubated with the desired nanoparticle or control particle solution (100 μg/mL) with or without added EIPA for the times indicated. Cells were then washed twice with PBS, fixed for 12 min in 3.7% formaldehyde in PBS, washed twice with PBS, stained with NucBlue® Fixed Cell ReadyProbes® Reagent, and then washed twice more with PBS. Samples were either analyzed immediately or stored at 4°C in the dark until analysis.
4.4. Results

During the preparation of SN38 loaded NPs, three different sizes of Co-Alb NP were generated (50nm, 100nm and 150nm) before drug incorporation. Fluorescence spectroscopy confirmed the presence of SN38 in Co-Alb-SN38. At the excitation wavelength of 380 nm and emission wavelength of 560 nm, there was strong broad peak in the Co-Alb-SN38 spectrum similar to that observed for SN-38 itself. There was no corresponding peak in spectra of Co-Alb NPs only (Figure 4.3). Because of its poor solubility in water, a water/ethanol solution was used to dissolve the compound. The encapsulation efficiency and loading capacity of the Co-Alb-SN38 NPs are summarized in Table 4.1. As the size of the particle decreased, encapsulation efficiency and loading capacity increased. This emulsion/evaporation method is attractive for encapsulating hydrophobic drugs because of its simplicity, high yield, and does not require heating. The average zeta potential of Co-Alb NP and Co-Alb-SN38 was found to be at -19.1mV and -20.2mV. Surface charge helps prevent the aggregation of nanoparticles in solution due to strong repelling forces.
Figure 4.2: Size measurement of Co-alb NP and Co-alb-SN38 via DLS
Figure 4.3: Fluorescence spectra of SN-38, Co-albumin and Co-albumin-SN38 at emission wavelength 560nm, excitation wavelength 380nm.

Table 4.1 Encapsulation efficiency and loading capacity for Co-Alb SN38 NPs of various sizes

<table>
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<tr>
<th>Size of Co-Alb NP</th>
<th>Encapsulation efficiency (e.e)</th>
<th>Loading capacity (l.c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nm</td>
<td>99.91 %</td>
<td>79.93 %</td>
</tr>
<tr>
<td>100 nm</td>
<td>99.76 %</td>
<td>59.86 %</td>
</tr>
<tr>
<td>150 nm</td>
<td>96.20 %</td>
<td>34.91 %</td>
</tr>
</tbody>
</table>

Co-Alb NPs displayed exceptional stability for days in PBS at pH 7.4, therefore it was expected that the same stability for Co-Alb-SN38 NPs would be observed. Indeed, Co-Alb-SN38 NPs were stable for several days in PBS; however the particles rapidly degraded with no particles.
detected after incubating in PBS-GSH 10 mM for 2 hours (Figure 4.4). Similar results could be seen visually in samples incubated under the two different reaction conditions (Figure 4.5). SEM images showed the morphology of Co-Alb particle, which appeared to be roughly spherical in shape (Figure 4.6). The release profile of Co-Alb-SN38 in PBS pH 7.4 and in PBS-GSH 10 mM over 72 hours is shown in Figure 4.7. An initial burst release of SN-38 was observed in PBS-GSH 10 mM with ~40% being released after 1 hour, which then gradually increased up 84% after 72 hours. In contrast, there was no particle degradation or drug release detected in PBS. Co-Alb NP degradation as a function of [GSH] was also investigated. As the particles degrade, the turbidity of the solution also decreases. Co-Alb NPs were incubated in 0.5, 1, 2, 5, or 10 mM GSH and the absorbance of the solution was monitored. A gradual increase in particle degradation was observed with increasing glutathione concentration (Figure 4.8).

![Figure 4.4: Stability of Co-alb-SN38 in PBS (pH7.4) and in PBS-GSH 10 mM monitored by DLS](image)

Figure 4.4: Stability of Co-alb-SN38 in PBS (pH7.4) and in PBS-GSH 10 mM monitored by DLS
Figure 4.5: Release of SN-38 in PBS pH 7.4 (the left beaker) and in PBS-GSH 10 mM (the right beaker) after 48 hour
Figure 4.6: SEM image of Co-Alb NPs.

Figure 4.7: Release profile of SN-38 in PBS and in PBS-GSH 10 mM
In order to examine particle uptake by image-based flow cytometry, fluorescein isothiocyanate labeled particles (Co-Alb-FITC NPs) were prepared. The average diameter of Co-Alb-FITC NPs utilized in uptake experiments was ~500 nm. SNU-5 cells were incubated with Co-Alb-FITC NPs in the presence or absence of a commonly used inhibitor of macropinocytosis, 5-(N-ethyl-N-isopropyl)amiloride (EIPA). Rapid particle uptake was observed in the absence of EIPA with virtually all cells exhibiting an increase in FITC emission after just 30 min of exposure (Figure 4.9A). Representative images of events from the dot plots (Figure 4.9B) visually confirmed these results. Uptake was observed to increase steadily during the first ~9 h at which point it
appeared to reach saturation and no further uptake was observed upon prolonging exposure to particles (Figure 4.9C). Cells pre-treated with EIPA for 5 min prior to incubation with Co-Alb-FITC NPs displayed significantly reduced uptake (Figure 4.10A and 4.10B) supporting the assertion that uptake was via macropinocytosis. The relative proportion of cells exhibiting high uptake was reduced from 73% in the absence of EIPA to just 16% in the presence of 75 μM EIPA. EIPA was also found to reduce uptake in a dose-dependent manner. Cells incubated in the absence of inhibitor exhibited >8 times more macropinocytic uptake relative to cells pre-incubated with 100 μM EIPA (Figure 4.10C). The inhibitory effect of EIPA remained relatively constant over 8h, but became greatly diminished at 24h. Staining patterns similar to those for Co-Alb-FITC NPs were observed for SNU-5 cells incubated with free Alb-FITC, albeit less intense, that also showed a reduction in uptake in the presence of EIPA (Figure 4.11A). These collective results clearly demonstrated a high degree of macropinocytic activity by SNU-5 cells. Another cell line utilized in our laboratory, Jurkat T lymphocyte cells, was also examined for particle uptake. Jurkat cells displayed very little binding/uptake of particles even at prolonged exposure (less than 2% exhibiting high uptake at 9 h) and the small amount observed was largely un-affected by the presence of inhibitor (Figure 4.11B). Non-specific uptake in SNU-5 cells was also observed for several other fluorescent particle formulations containing various c-Met targeting ligands covalently attached to the particle surface (Figure 4.12). The uptake patterns observed made it difficult to conclusively demonstrate c-Met selective targeting in SNU-5 cells even though these cells are known to express very high levels of the receptor. While some selectivity could be observed in the form of competitive inhibition by free targeting ligand, high degrees of staining were uniformly observed.
The inherent toxicity of Co-Alb NPs was measured in SNU-5 cells via a standard assay based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cells were incubated with Co-Alb NPs for 48 h prior to conducting the MTT viability assay. No significant toxicity was observed up to the highest dosing of nanoparticles (Figure 4.13), which indicates a high degree of biocompatibility of the particles and no significant toxicity resulting from Co. Co-Alb SN38 NPs displayed significant toxicity with an IC50 of ~50 nM (Figure 4.14).

Figure 4.9: (A) Dot plots of side scatter vs. albumin-FITC fluorescence of SNU-5 cells incubated for the specified with Co-Alb-FITC NPs (100 μg/mL), (B) representative FlowSight images of SNU-5 cells from dot plots in part A, and (C) percentages of cells displaying no, low, or high uptake of Co-Alb-FITC NPs from part A.
Figure 4.10: (A) Dot plots of side scatter vs albumin-FITC FITC fluorescence for SNU-5 cells dosed with various concentrations of EIPA 5 min prior to incubation (2 h) with Co-Alb-FITC NPs (100 μg/mL), (B) representative FlowSight images of SNU-5 cells from dot plots in part A, (C) relative macropinocytic uptake of Co-Alb-FITC NPs in the presence of varying amounts of an EIPA (4h incubation time). *uptake values reported are relative to uptake at 100 μM EIPA.

**a slight decrease in cell viability was observed at 100 μM EIPA.
Figure 4.11: (A) Dot plots of side scatter vs. albumin-FITC fluorescence for SNU-5 cells incubated with free FITC-albumin (100 μg/mL) both with and without EIPA, (B) Dot plots of side scatter vs. albumin-FITC fluorescence for Jurkat cells incubated with Co-Alb-FITC NPs (100 μg/mL) both with and without EIPA.
Figure 4.12: Dot plots of side scatter vs. micromer®-redF fluorescence for SNU-5 cells incubated with cMet-targeted particles (Apt NPs, Met Ab NPs) or un-targeted (PEG NPs) indicating small increases in fluorescence for the targeted particles, but significant non-specific uptake as evidenced by the uptake of PEGylated NPs. A slight reduction in uptake of Met Ab NPs was observed after pre-incubation of the cells with free Met Ab.
Figure 4.13: Cell viability studies for exposure of SNU-5 cells to Co-Alb NPs (48 h incubation with particles prior to conducting MTT assay).
4.5. Discussion

Albumin is known to be taken up by cancer cells as a nutrient source. One of the most common mechanisms for particle uptake in targeted drug delivery relies on receptor-mediated endocytosis. Albumin is an excellent candidate in this respect because its receptor (albodon or gp60) is known to be overexpressed in a variety of cancer cells. Several research groups have reported the synthesis of bovine serum albumin nanoparticles for targeted drug delivery. These include a co-evacation method, and using high concentrations of glutaraldehyde as a
crosslinking agent to form albumin nanoparticles. Our strategy for generating albumin nanoparticles does not involve any stabilizing agents or thermal heating. The size and zeta potential of Co-alb-SN38 fell within the optimal range for cellular uptake via endocytosis. The high encapsulation efficiency and loading capacity was attributed to the van der Waals interaction between hydrophobic drug molecules (SN-38) and hydrophobic interior of the carrier (Albumin).

There was no release of SN-38 in PBS up to 84 hours. In contrast, an initial burst release of 40% after 1 hour in PBS-GSH 10 mM from Co-Alb-SN38 was observed, followed by gradual release of SN-38 up to 84% after three days in reducing environment containing glutathione (10 mM).

The Co-Alb-SN38 were found to exhibit exceptional stability in buffer pH 7.4, which holds great promising in protecting the therapeutic effect of SN-38 in circulation and in reducing the dose-related toxicity of SN-38. Initial burst release of SN-38 from NPs was ascribed to the amount of SN-38 loaded on or near the surface of NP.\textsuperscript{25} Glutathione (GSH) can be easily oxidized to GSSG in cellular environments with redox potential ranging from -240mV to -170mV,\textsuperscript{26} which matches the redox potential of cobalt (approximately -200mV).\textsuperscript{27} The inert cobalt $^{+3}$ complex is converted to a labile cobalt $^{+2}$ complex, which can then undergo facile ligand exchange. The release of SN-38 in sustained manner over long periods of time was ideal to increase the therapeutic effect of SN-38.

Free drug was reported to be pumped out of the cell by the P-glycoprotein pump.\textsuperscript{28} By utilizing a nanoparticle to deliver SN-38, we could overcome this obstacle by entrapping the active lactone form of SN-38 in Co-Alb NPs, which behaved as an intracellular drug depot to protect active SN-38 from unwanted cytotoxic side effect and to release therapeutic drug in sustained manner. The drug-NP conjugates will also likely avoid the P-glycoprotein pump by entering the
cell via receptor-mediated endocytosis, therefore, enhancing the cellular uptake of nanoparticle-drug complex, which has been observed by others.\textsuperscript{29}

Several important conclusions can be drawn from the uptake results reported here. First, Co-Alb NPs are efficiently internalized by cancer cells that display high levels of macropinocytic uptake. Second, no toxicity was observed for Co-Alb NPs thereby establishing their biocompatibility and foreshadowing their potential as a drug delivery vector. Third, because SNU-5 cells are not known to harbor Ras mutations, our results could indicate that some non-Ras mutated tumor types also rely on macropinocytosis as a mechanism of cell survival. Tumors derived from such cells could conceivably take up nanoparticles from circulation even in the absence of surface-bound targeting ligands, which would be significant benefit in the field of nanoparticle therapeutics. It must be noted, however, that it is also possible the cell line utilized in our laboratory has acquired a Ras mutation, which will be the subject of future studies. In either case, with such a large proportion of cancers potentially exhibiting macropinocytic behavior, capitalizing on this phenomenon remains an attractive area for further exploration. Finally, it is important to be aware that macropinocytosis could be occurring in a significant proportion of cancer cell lines used for routine research purposes, which can complicate the evaluation of the selectivity for many cellular targeting strategies.

4.6. Conclusion

The present study demonstrated that SN38 could be encapsulated within Co-Alb nanoparticles via emulsion/solvent evaporation method. The encapsulation efficiency and loading capacity could be modulated by changing the size of Co-Alb NP. The release profile indicated an initial burst release followed by sustained release up to three days in reducing environment while the Co-Alb-SN38 showed exceptional stability in PBS for several days. Co-Alb NPs were
efficiently taken up by SNU-5 cells and found to be non-toxic. Co-Alb SN38 NPs exhibited a high level of toxicity, which warrants further studies in animal models.

4.7. References


CHAPTER 5

FUTURE DIRECTION

5.1 Bioconjugation strategy in delivery of therapeutic proteins for cancer targeting

Since Stayton and Hoffman first introduced the development of responsive polymer-protein conjugates in 1980, research toward therapeutic proteins has grown rapidly with various bioresponsive polymer-protein conjugates being developed relying on both covalent and non-covalent crosslinking strategies. A variety of triggers to release the native protein have been engineered to respond to endogenous environments such as pH, temperature, enzymatic activity, reductive environment of cytoplasm. Chen and coworkers have successfully demonstrated a method based on thioester chemistry for modulating protein activity. By combining a PEGylation strategy with thioester chemistry, they demonstrated that the biological activity of proteins could be modulated depending on the conjugate’s environment. Recently, glutathione responsive nanoparticles have been developed by a number of groups. Thayumanavan and coworkers demonstrated the formation of GSH-sensitive micelles utilizing an amphiphilic copolymer crosslinked by disulfide bond. However, the stability of the micelles was compromised under large volume dilution and interacting with cellular compartments making them unlikely to move forward to clinical trials. Conjugating polymers or other modifying groups to peptides/proteins is usually targeted toward amine functional groups (lysine residues). The most common method has been to form stable amide linkages, which was demonstrated in the case of copolymerization of N-isopropylacrylamide with N-acryloxysuccinimide to generate a copolymer containing esters functional group via RAFT polymerization. This copolymer was then grafted with proteins (lysozyme, myoglobin, albumin) using lysine residues. The traditional methods of constructing bioresponsive conjugates involve external stimuli such as heat or metal
catalysts, which can affect the stability of the product as well as lead to low yield and side reactions. We have developed a self-assembly assembly approach similar to our cobalt nanoparticles that we aim to employ for the delivery of protein therapeutics. In chapter 3, we successfully demonstrated the PEGylation of transferrin protein (Tf) via cobalt coordination chemistry. Since the approval of the first protein therapeutic, human insulin for treatment of type I and type II diabetes, there has been more than 130 protein therapeutics approved by FDA (80% of these protein were generated by DNA recombinant technology). Therapeutic proteins have a number of advantages in comparison to small molecules due to their specificity and the complexity of their tertiary structures. TRAIL (tumor necrosis factor TNF-related apoptosis inducing ligand) has been studied with promising results for a cancer therapy. It induces apoptosis in human cancer tissue (spleen, prostate, lung), but not in healthy cells in vitro. However, the therapeutic potential of TRAIL has been hampered by severe acute toxicity when systemic administration in animals. This and other therapeutic proteins would benefit tremendously from a delivery vector that could guide the protein to the site of action and release it in its native form.

We have attempted to demonstrate the reversible nature of the cobalt crosslinking chemistry with respect to protein function. In order to confirm the release of native protein, we have exploited the lytic abilities of lysozyme toward bacteria Micrococcus lysodeikticus. Preliminary results confirm the presence of particles comprised of lysozyme crosslinked by cobalt. Upon reduction by glutathione (PBS-GSH 10 mM, pH7.4), the particles degraded rapidly similar to results obtained for Co-Alb NPs.
5.2 Material and Methodology

Lysozyme from chicken egg white (lyophilized, ≥ 40,000 units/mg protein) and L-glutathione (reduced) were obtained from Sigma Aldrich. PBS 10x buffer solution (purified) was from Fisher. Micrococcus lysodeikticus ATCC (No. 4698) was obtained from Sigma Aldrich (USA). Ultrapure water was used throughout all experiments. All other chemicals were regents grade unless otherwise noted.

Stock solutions of NaOH (0.25 M), lysozyme in borate buffer 1x (10 mg/mL) and CoCl₂.6H₂O (0.1 M) were prepared in ultrapure water. In a glass vial, 100 μL of NaOH and 300 μL of lysozyme solution were mixed. The mixture was sonicated for 5 s. The solution was colorless. Upon addition of cobalt chloride (100 μL) was then added, the color immediate changed to light purple. Sonication was applied for 5 s followed by the addition of 1 mL of ultrapure water. Next, 10 μL of 30% H₂O₂ was added to oxidize Co²⁺ to Co³⁺, which resulted in a color change from purple to light yellow. Dynamic light scattering was used to measure the size of Co-lysozyme NP, which was ~500nm. The solution was allowed to sit undisturbed for 15 minutes, followed by centrifugation for 2 minutes at 10,000 rpm. The supernatant was discarded and the pellet was washed with water. The pellet was then suspended in 2 mL of PBS or 2 mL of PBS-GSH 10 mM. Meanwhile, we prepared lysozyme in PBS (0.1 mg/mL) and in PBS-GSH (10 mM). All four solutions were incubated at 37°C for at least 1 hr. To a 96 well-microtiter plate, solutions of Co-lysozyme solution in PBS, Co-lysozyme in PBS-GSH, lysozyme in PBS and lysozyme in PBS-GSH (20 μL per well) followed by Micrococcus lysodeikticus working suspension in PBS (1 mg/ml, 150 μL per well). The turbidity of the solutions were then monitored using a microplate reader (Synergy 2, Biotek) at 450 nm every 45 seconds for 25 minutes at 25°C per manufacturer protocol.
5.3 Preliminary Results

Cobalt-lysozyme (Co-Lys) NPs were successfully synthesized utilizing cobalt coordination chemistry. Co-Lys NPs synthesized for enzymatic studies had a diameter of ~500 nm (Figure 5.1). The particles displayed excellent stability in PBS at pH 7.4. Lysozyme breaks down the cell walls of bacteria via hydrolysis of 1,4-beta linkages of N-acetyl-D-glucosamine. A convenient assay for its activity is to use the turbidity of a solution of bacteria to monitor cell wall degradation. As the enzyme exerts its activity, the bacteria are destroyed, which reduces the turbidity of the solution. Higher enzymatic activity of lysozyme corresponds to faster decrease in the absorbance at 450nm. Co-Lys NPs incubated in PBS greatly attenuated the activity of the enzyme (Figure 5.2) while incubation in PBS-GSH restores its native activity in comparison to control solutions (lysozyme incubated in PBS and in PBS-GSH 10 mM). Therefore, we can conclude that the protein is released in its native state upon reduction by glutathione.

The next step is to translate these findings to a therapeutically relevant protein. We have chosen hyaluronidase as an initial candidate because of the role of hyaluron in pancreatic cancer. Pancreatic tumors develop a thick fibrous coating that makes the penetration of drugs difficult, which greatly limits therapeutic action at the tumor. A number of researchers have examined the effects of directly injecting the enzyme into circulation and have achieved some positive benefits. However, proteins in general are not stable in circulation, so encapsulating the enzyme in the form of a nanoparticle would not only increase its stability, but would also lead to greater tumor accumulation due to the EPR effect.
Figure 5.1: Size measurement of Co-lysozyme particles using dynamic light scattering
5.4 Phage Display

The goal of cancer therapy is to eradicate cancerous tumors while sparing healthy tissues. Traditional methods of targeting involve the utilization of monoclonal antibodies to target biomarkers in order to differentiate tumor cells from healthy ones and to achieve enhanced cellular uptake. However, monoclonal antibodies have disadvantages due to their high molecular weight and low tissue penetration in vivo. Small peptides have emerged as ideal candidates to overcome some of these barriers. Peptides possess great potential as therapeutics and diagnostics for cancer treatment due to their unique pharmacokinetic properties including high affinity, specificity, and minimal immunogenicity. Investigators have expended significant effort in utilizing combinatorial chemistry to identify peptides that bind to specific tumor or cancerous cell markers. The combinatorial chemistry of
phage display technology includes billions of random peptides that can be screened for affinity to a specific target. The history of phage display technology dates back to 1985 when George Smith fused foreign genetic materials resulting in the display of foreign peptides on the surface of phage virion. Typical commercial phage libraries can contain $10^9$ to $10^{12}$ random peptides sequences. Phage display technology has become a powerful technique for screening for protein-protein and peptide-protein interaction.

To achieve affinity selection, a phage library is introduced to a specific target (receptor, protein, peptide), which will result in binding of only certain phages virion. The next step is to simply wash away all unbound phages and recover the bound phages. The recovered phage can then be propagated and cloned by infection in host bacteria. The peptide sequence expressed on the surface of the phage can then be determined by typical sequencing methods. Over the last 10 years, there have been hundreds of reports published that utilized phage display technology to isolate peptides that bind to specific targets such as antibodies, enzymes, receptors, and carbohydrates. For example, phage display was used in vitro to identify peptides that bind to antigen associated with tumor cell receptors. Epidermal growth factor receptor (ErbB-2) is known to be associated with tumor growth and metastases when overexpressed in cancer cells, predominantly in breast and prostate cancers. By using the phage display method, Karasseva and coworkers reported a peptide sequence KCCYSL that specifically bound to ErbB-2 receptor in prostate and breast cancer cell lines, but not healthy cells. Noda and coworkers have employed phage display to select peptides for cell surface carbohydrate antigens that were associated with cancer metastasis. They reported the peptides sequences of RVVKESR, YSALEEG and MMGVGTS that bind to different sites on to lipopolysaccharide molecules of Salmonella.
Selecting peptides *in vitro* posed hidden problems due to the fact that it might not be effective against tumors in live animals due to the stability, and the low expression of antigen *in vivo*. In an effort to overcome these problems, identifying targeting peptides directly in live animal has been conducted. Pasqualini and coworkers reported using this approach to identify peptide sequences that bind to organs and tumor vasculature. These peptides sequences appear to be in RGD or NGR motifs, which were then used in conjunction with doxorubicin to target tumor growth while inducing no effects in healthy cells. Recent literature has shown the remarkably vital role of micro RNA (miRNA) in regulation of tumor growth. miRNA are non-protein coding and single stranded RNA that regulates the expression of mRNA by binding to certain regions of mRNA. In addition, mRNAs are also known to be involved in regulation of oncogenes and tumor suppressor genes. For example, mRNA-221 is over expressed in multiple types of cancer including color cancer and melanoma. Solid evidence has shown that higher concentrations of miRNA-221 are found in cancer patients in comparison to healthy individuals. miRNA-221 is suspected to be associated with tumor progression and prognosis because the levels of miRNA-221 could be correlated with tumor thickness and prognosis. To the best of our knowledge, there has been no effort to apply phage display technology to identify peptides for targeting miRNA-221. A phage library could be screened against miRNA-221 to identify peptides that have the most affinity to this most important target. Selected peptides could be labeled with molecular imaging probes (radionuclei, quantum dots, fluorophore) for diagnostics, or conjugated with therapeutics to achieve a synergistic effect. With this strategy, we could develop a “theranostic” capable of simultaneously monitoring the progression of tumor cells and delivering therapeutics.
5.5 Conclusion

Since the introduction of the first stimuli-responsive drug delivery system in 1978, a thermoresponsive liposome for local delivery, significant progress has been made in nanomedicine applications. It is critical to design and formulate efficient drug carriers that target diseased tissues specifically to minimize non-specific toxicity, and to increase the relatively short half-lives of drugs in circulation, and to reduce immunological responses. The advancement of field of materials science has aided the rapid development of stimuli-responsive nanoparticles, such as liposomes, dendrimers, micelles, and quantum dots in various sizes. Selectively targeting tumors by exploiting the enhanced permeability and retention (EPR) effect and/or ligand-receptor interactions has gained tremendous interest from the scientific community; however, there is still much work to be done in understanding the complex interaction between particle structure and mechanism of action \textit{in vivo} owing to the difficulties in controlling release of the drug from the delivery vector. There is great interest in creating a stimuli responsive system that can achieve on/off selectivity, which would facilitate the controlled release of the therapeutic at the exact target location with better dosage control. In the last few decades, there have been many reports on various types of stimuli-response delivery systems, which rely on either external stimuli (temperature change, ultrasound, electric and magnetic field) or internal stimuli (pH changes, redox potential and enzymatic activity). There are great challenges in the translation of such stimuli-responsive drug delivery systems from the lab bench to clinical application owing to complexity in architecture of these nanocarriers, which leads to challenges in manufacturing scale-up and quality control. Controlled release of drugs from bioresponsive nanoparticles is further complicated in translational medicine because one scenario does not apply to all patients (pH of tumors varies in individual patients, enzymatic levels or
concentrations of reducing agents in the blood stream). There have been reports of two stimuli-responsive candidates that have reached clinical trials: the thermosensitive liposome (Thermodox) for treating breast cancer and the magnetic iron oxide (magnetic carrier for doxorubicin MTX-DOX) for the treatment of glioblastoma.\(^ {23}\) This is just the beginning for the use of novel materials in solving problems in the controlled release of therapeutics, which can minimize the undesired systemic toxicity of therapeutics drugs. There are multiple reports detailing stimuli-responsive nanoparticles that perform excellently \textit{in vitro}\(^ {24}\) (including our Co-Alb NPs), which are ready for \textit{in vivo} experiments in animal models.

5.6 References


