Zwitterionic Polymer For Drug Delivery Applications

Yang Lu
Wayne State University, fj4608@wayne.edu

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ZWITTERIONIC POLYMER FOR DRUG DELIVERY APPLICATIONS

by

YANG LU

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

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CHAPTER 1. INTRODUCTION

Zwitterionic polymers are a unique type of polymers having an equivalent number of homogenously distributed anionic and cationic groups on their polymer chains\cite{1}. Common zwitterionic polymers include poly(betaine) or poly(choline) carrying a positive and a negative charge on the same monomer unit such as 2-methacryloyloxylethyl phosphorylcholine (MPC)\cite{2}, sulfobetaine methacrylate (SBMA)\cite{3}, and carboxybetaine methacrylate (CBMA)\cite{4}. There are also a series of zwitterionic-like polymers prepared by copolymerization of monomers with positive and negative charges at the ratio of 1:1 (For example, the monomers can be selected from charged natural amino acids\cite{5}), showing a similar property of zwitterionic materials\cite{6}. The highly soluble and neutral zwitterionic materials are known to provide strong hydration through ionic solvation, forming a molecular level water layer to resist nonspecific binding and attachment from proteins, blood, cells, and bacteria\cite{7}. Such distinctive resistance is known as non-fouling property, which is desirable in many scientific areas\cite{8}.

Based on the super-hydrophilic and non-fouling property of zwitterionic materials, they have been widely involved to render excellent stealth properties to a variety of substrates of different geometries, including decorating nano-sized carbon dots\cite{9}, and coating a flat macroscopic surface, to resist protein binding and bacteria attachment\cite{10}. The use of zwitterionic materials for biological and medical applications has been considered promising. Recent studies indicated that there was negligible foreign body reaction after implanting a zwitterionic poly(carboxybetaine methacrylate) (PCBMA) hydrogel into the mice subcutaneously\cite{11}. PCBMA hydrogel surface was also demonstrated to retain the stem cell phenotype and multipotency, regardless the existence of differentiation-promoting media and cytoskeletal-
manipulation agents\textsuperscript{12}. This remarkable performance in interacting with host tissues or cells has been attributed to the non-fouling nature of the zwitterionic materials.

The focus of this thesis is to explore two additional properties of these super-hydrophilic zwitterionic polymers and take advantage of these properties in solving several challenges in the drug delivery field.

(1) Capability to stabilize micelles constructed with zwitterionic polymers.

In the last decades, polymeric micelles have attracted considerable interest as drug delivery carrier for a series of drugs, including low molecular mass hydrophobic drugs, proteins and genes\textsuperscript{13}. Typically, micelles would self-assemble into the macromolecular structure in an aqueous environment and protect insoluble hydrophobic payloads in its hydrophobic core region. Compared with other drug delivery strategies, such as liposomes, polymer or protein-drug conjugates, polymeric nanoparticles, or pathogens\textsuperscript{14}, polymeric micelles show two significant advantages: 1) Micelles have relatively small size. Hydrodynamic sizes of a micelle are usually less than 50 nm\textsuperscript{15}. Considering the dimensions of physiological pores in the body’s vasculatures (e.g., kidney glomerulus pores, inter-endothelial junctions for healthy tissues, tumors, and cancerous tissues), nanomedicine of smaller sizes (e.g., < 100 nm) is favored in blood circulation, tissue penetration, and cellular uptake\textsuperscript{16}. Recent evidence shows a sub-50 nm drug formulation is desirable to achieve the collective outcome of deeper tumor tissue penetration and more efficient cancer cell internalization, as well as a cellular response\textsuperscript{14, 17}. Therefore, in vivo circulation performance of encapsulated drugs could be drastically improved, leading to high accumulation in the targeted site (e.g., tumor tissue) due to the enhanced permeability and retention (EPR) effect\textsuperscript{18}. 2) Feasibility to manufacture in large scale. Typically, the micelle is the simplest assembled entity, with well-defined molecular structures and assembling behaviors.
Such simple structure enables simple drug formulation and ease of manufacturing. Such feasibility is vital since other advanced particle systems are complicated to prepare and have built up obstacles for production in a stable and consistent manner. One notable example is the PEGylated liposome with encapsulated doxorubicin (DOXIL®), which used to have a periodic global shortage due to manufacturing issues\(^{[19]}\). In contrast, a micelle formulation is simple to manufacture, such as Taxotere® or Taxol®, containing only the drug (docetaxel), the micelle (polysorbate 80), and the solvent (alcohol);\(^{[20]}\) can obtain fast FDA-approval; and be easily manufactured on a large scale. With these appealing features, micelles are well accepted as a first line technology to formulate drugs\(^{[13a]}\). Nevertheless, a fundamental limitation for micelles as drug delivery carriers is their low stability to environmental changes, especially their spontaneous dissociation at relatively low concentration. Once the concentration is below their critical micelle concentration (CMC), micelle entities would disassociate into free surfactant and cause a burst release of previously encapsulated drugs into the bloodstream. For example, micellar drug Taxotere® was quickly removed from blood circulation once intravenously (i.v.) injected\(^{[21]}\). This quick clearance can reduce the therapeutic performance of encapsulated drugs and even cause toxicity concern\(^{[22]}\). Therefore, how to stabilize micelles, especially in physiological conditions, is a challenge for drug delivery application.

In this dissertation, it is reported that zwitterionic Poly(carboxybetaine) (PCB) polymer could conjugate with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) lipid to form a micelle with ultra-low CMC. A novel method was proposed to verify its ultra-low CMC and the high stability in different media were also studied. The mechanism for zwitterionic PCB to achieve ultra-low CMC property has been preliminarily explored. Details are described in Chapter 2. Using melanoma as a tumor model, the antitumor performance of docetaxel
encapsulated in DSPE-PCB micelle is described in Chapter 3 where enhanced antitumor performance compared with control formulations, including the commercial Taxotere®, was observed.

(2) Capability to improve the pharmacokinetics of proteins while mitigating the commonly seen bioactivity loss.

Therapeutic protein drugs, such as insulin, have been used clinically for a long time. Using polymers to modify a protein drug could effectively increase its circulating half-life in the bloodstream, reduce the dosage required, and improved the therapeutic performance—a significant advantage for patients. Polymer-protein conjugation has been extensively explored in the past decades and has shown the enhanced stability of the protein under storage, transport, and other stresses[23]. For example, protein stability under lyophilization is significantly increased after modification with polymers.[24] The most widely used conjugation strategy is called PEGylation, by using polyethylene glycol (PEG) to conjugate proteins.[25] However, a major problem with PEGylation, as well as conjugating with other common polymers, is the sacrificed bioactivity of the conjugated protein.[26] Significant results indicate that conjugating PEG, and other polymers as well, on proteins through conventional covalent coupling chemistry resulted in polymer chains non-selectively attached on the protein surface groups (e.g., amines), and potential attachment near to the protein’s active site significantly inhibited the protein from performing designated biological function (e.g., amine group on Gly A1 of insulin)[27]. A much higher dosage was required to reach the same therapeutic effect for the protein-drug with reduced bioactivity and significantly compromised pharmacological activity. Compared with unmodified native form, polymers modified protein drugs may demand high production costs and cause long-term toxicity concerns due to the drastically increased working dose. We discovered that
zwitterionic PCB-polymer-modified insulin did not show a significant reduction (student t-test) of \textit{in vitro} bioactivity compared with its native form. By improving the pharmacokinetics of insulin while maintaining its bioactivity, PCB-insulin was found to show even higher blood glucose lowering capability compared with native insulin in an animal study. PCB-insulin was obtained via a simple conventional coupling chemistry and was expected to solve the bioactivity loss issue in large. Details are described in Chapter 4.

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CHAPTER 2. DISCOVERY OF ULTRA-LOW CMC ZWITTERIONIC MICELLES

A fundamental challenge for conventional micellar carriers is micelle systems disassemble into free surfactants when diluted at concentrations below the critical micelle concentration (CMC). Such premature disassemble limits the bioavailability in vivo of injected hydrophobic drugs encapsulated in micellar systems. In this chapter, a novel micelle comprising a superhydrophilic zwitterionic polymer domain and a superhydrophobic lipid domain is introduced. Such a micelle was demonstrated to own an undetectable CMC below $10^{-6}$ mM, a value that is orders of magnitude lower than the CMC ($> 10^{-3}$ mM) of typical micellar systems. In addition, when zwitterionic moieties or zwitterionic polymers were added into a micelle solution, conventional micelle could be stabilized even at a concentration below their inherent CMC.

2.1 Introduction

Micelles play a pivotal role in drug delivery\cite{15, 22a, 28}. They are small sub-50 nm entities physically assembled by water-soluble amphiphilic surfactants and can solubilize hydrophobic drug molecules that are not well dissolved in water. Compared with other drug stabilizing or delivery strategies (including from liposomes, polymer- or protein-drug conjugates and polymeric nanospheres to cells or pathogens)\cite{14a-k, 14m, 14n, 29}, micelles have been received as the first-line technology.\cite{22a} Their molecular structures and assembling behaviors are well defined, and a drug solution stabilized by micelles can be easily manufactured. These features enables large scale production and stable clinical performance of the formulations. However, the main challenge with micelles is that all conventional micelles, unless chemically crosslinked,\cite{13b, 15, 22a, 28a} will disassemble into free surfactants under diluted conditions (e.g., below critical micelle concentrations, CMC, which is typical when infusing the micelle/drug formulation into the body).
Drugs previously stabilized in the hydrophobic region of a micelle core will become insoluble due to the loss of micelle structure. Such precipitation will severally reduce its bioavailability and deteriorate its therapeutic performance.\textsuperscript{[22, 30]} This challenge remained insurmountable since all micelles have an apparent CMC and cannot keep stabilizing hydrophobic cargo when diluted at concentrations below the CMC.\textsuperscript{[28b]}

In this chapter, it is demonstrated that such stability challenge could be potentially solved by a so-called “sharp polarity contrast” micelle system (Fig. 2-1). In this sharp contrast system, each surfactant molecule comprises a super-hydrophilic zwitterionic polymer domain and a superhydrophobic lipid domain. The polarity contrast between the two domains is drastically “sharper” than most conventional micelle molecules. With an appropriate molecular weight (MW) of zwitterionic polymers, such sharp-contrast micelles have an undetectable ultra-low CMC below $2.7 \times 10^{-6}$ mM. Such ultralow CMC for micelle is at least six orders of magnitude lower than that for sodium dodecyl sulfate (SDS, CMC = 8.2 mM; commonly found in cleaning and hygiene products) and four orders of magnitude lower than Polysorbate 80 (CMC = 0.012 mM; widely used to formulate drugs, e.g., chemo drug docetaxel under the trade name of TAXOTERE\textsuperscript{®[31]}). Even when a zwitterionic moiety or polymer was added into a micelle solution (not chemically modifying the micelle), the existing micelle molecules in the new solution were stabilized at concentrations below their inherent CMC. It is demonstrated that the ultra-low CMC micelle could stabilize hydrophobic cargoes (without micelle dissociation) in extremely diluted conditions (for example, $< 10^{-3}$ mM), including clinically relevant dilution conditions in serum in which conventional micelles failed to stabilize hydrophobic cargoes.
2.2 Materials and Methods

2.2.1 Material

DSPE-PCB 2K, 5K, and 10K were synthesized and characterized following a previously established method.\[32\] The molecular weight of N-hydroxysuccinimide ester (NHS)-PCB was characterized via Waters Breeze 2 system gel permeation chromatography (GPC) with Waters 2414 reflex detector. The mobile phase was phosphate buffered saline (PBS) at a flow rate of 1 mL/min. Polysorbate 80, SDS, CTAB, H\text{AuCl}_4, \text{NaBH}_4,\text{ styrene, divinylbenzene, trifluoroethanol were obtained from Sigma-Aldrich. (St. Louis, USA). DSPE-PEG 5K was obtained from Laysan Bio, Inc. (Arab, USA). Hexadecyltrimethylammonium bromide (CTAB), (dodecyl dimethylglycine) CB12C, n-Tetradecyl-N, N-Dimethylglycine (CB14C), 3-(decyldimethylammonio) propane sulfonate (SB10C) and 3-(N, N-Dimethylpalmitylammonio) propane sulfonate (SB16C) were obtained from Anatrace Product. LLC (Maumee, USA).

2.2.2 Preparation of gold nanoparticles (NPs) encapsulated by DSPE-PCB 5K

CMC for DSPE-PCB 5K (a zwitterionic carboxy betaine polymer (PCB) of 5000 Dalton molecular weight (MW) conjugated to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) lipid) was determined via an established pyrene method.\[33\] During a typical “Diluting-concentrating” method for CMC determination, a probe (< 5 nm) is first loaded into a micelle core at high micelle concentration (> CMC). To load gold nanoparticle (NP) probe, micelles and H\text{AuCl}_4 are dissolved in 10 mL deionized water and vigorously stirred at room temperature, followed by dropwise addition of 1 mL \text{NaBH}_4 aqueous solution (micelle: Au: \text{NaBH}_4 (molar ratio) is typically 1:1:6). The solution turns red, and the stirring is kept for another 5 min. To load cross-linked Polystyrene NP probe, micelles, photo-initiator 2959 (2-hydroxy-4'-(2-hydroxyethoxy)-2-methyl-propiophe), styrene and divinyl benzene (molar ratio is typically
1:10:1.5:1) are sonicated in deionized water to form nano-emulsion, followed by 20 min reaction under UV light (365 nm). Micelles with either gold NP or PS NP loaded are further purified through 10K MWCO ultrafiltration to remove rough materials forming the probes, and 0.2 μm filter to remove unexpected large particles. The size of the probes can be determined by DLS (Zetasizer Nano-ZS, Malvern Instruments) by taking the size difference between empty micelles and loaded micelles. For gold NPs probe, its size can be additionally measured by the maximum absorbance wavelength using a UV-visible spectrometer (Thermo Scientific Multiscan Go). During the diluting-concentrating procedure for CMC determination, the liquid sample was not further treated with filtration.

Aggregation Numbers ($N_{agg}$) of DSPE-PCB 5K micelles were calculated from the ratio of an apparent molecular mass of the micelles which was measured via static light scattering (SLS), and $M_w$ for a single DSPE-PCB 5K chain. Apparent molecular masses of DSPE-PCB 5K micelles was obtained by measuring a series of samples with different concentrations (from 1.2 g/L to 4.8 mg/L) using a Malvern Zetasizer Nano-ZS.

**2.3 Results and Discussion**

Sharp-contrast micelle-forming molecule DSPE-PCB 5K was synthesized following a previous method,$^{[32]}$ and its structure is shown in **Fig. 2-1** DSPE-PCB 5K powder is extremely water-soluble, and its resulting micelles were imaged by transmission electron microscopy (TEM) (**Fig. 2-2a**). The size of this micelle was measured by dynamic light scattering (DLS) (**Fig. 2-2b**) to have a hydrodynamic diameter of 20±3.5 nm (mean ± stand deviation, N=3) in phosphate-buffered saline (PBS). The hydrophobic core domain of DSPE-PCB 5K micelle was detected using pyrene as reflected by the decrease in the intensity ratio of the first to the third highest energy bands ($I_1/I_3$) in the emission spectra of pyrene from 1.7 in water to 1.1 in micelle solution.
(Fig. 2-3), following a previous method. It should be noted that PCB such as 5K Dalton molecule weight is extremely water soluble without forming particles or aggregates (hydrodynamic diameter =3.7±1.0 nm). The aggregation number ($N_{agg}$) of DSPE-PCB 5K micelle in water was 44.

The CMC for DSPE-PCB 5K was determined to be $1.3 \times 10^{-3}$ mM using the pyrene probe method (Fig. 2-4). The pyrene method is one of the most sensitive and precise methods of CMC determination. However, it does not have sufficient sensitivity to measure a CMC below $10^{-3}$ mM because the final concentration for pyrene is maintained at $6 \times 10^{-4}$ mM (in our previous protocol as well as others) to give good quality fluorescent spectra. A micelle concentration approaching or below $10^{-4}$ mM cannot significantly change the aggregation behavior of pyrene, and potential micelle formation cannot be detected (as reflected by pyrene spectrum change). As a consequence, $1.3 \times 10^{-3}$ mM as measured by the pyrene method is an upper CMC estimation for DSPE-PCB 5K. There are other fluorescent dyes such as 2-p-toluidinynaphthalene-6-sulfonate (TNS) and 1-anilinonaphthalene-8-sulfonate (ANS), or other ways to determine CMC including light and X-ray scattering, surface tension study, and isothermal titration calorimetry. None of those methods can precisely detect a CMC < $10^{-4}$ mM. Limited success in determining a CMC < $10^{-4}$ mM has been achieved by extrapolation of CMC values of structurally-similar molecules and through filtration of radioactively labeled surfactants. However, these approaches are not readily available.

To explore a potential CMC below $10^{-3}$ mM, a “diluting-concentrating” method was developed. An initial stable colloid was prepared by reducing soluble Au$^{3+}$ ions to insoluble gold nanoparticles (NPs) in the presence of micelles at concentrations far above their CMCs. Small gold NPs (< 5 nm) were formed as non-soluble probes and partitioned into the hydrophobic cores.
of stabilized micelles. The gold probe size was estimated from maximum absorbance peak of gold NPs, and also from the size increase from empty micelle to probe-loaded micelle (measured by DLS). The micelle-protected gold NPs were subject to serial dilutions in water, and extremely diluted micelle concentrations (10^{-6} mM) can be reached. Potential aggregation of gold NPs resulted in an increase in both the hydrodynamic size of the colloid and the maximum absorbance wavelength for gold NPs, as measured by DLS and UV-spectroscopy, respectively. Considering that the samples may be too dilute for these measurements, they were concentrated using a rotary evaporator at 35 °C under reduced pressure and then re-measured for these parameters. The aggregation of hydrophobic gold NPs cannot be reversed by this concentrating process and was detected from their corresponding concentrated samples. Generally, once hydrophobic gold NPs aggregated, they cannot be re-dispersed to their original size and size-distribution, even with sonication. There is a possibility that DSPE-PCB 5K may be unique from all the other surfactants in that it is capable of re-stabilizing the aggregated gold NPs upon concentration. This possibility is excluded based on the following experiment: A polysorbate 80 protected gold NPs solution at micelle concentration of 2.1 mM had an initial hydrodynamic size of 20.5 ± 3.2 nm (size of gold NPs). Then the solution was diluted with water to reach micelle concentration of 0.0042 mM. To this solution, DSPE-PCB 5K was added to reach the concentration of 0.0042 mM. The mixture was then condensed to the polysorbate 80 concentration of 2.1 mM which is above its CMC. If DSPE-PCB 5K could re-stabilizing the aggregated gold NPs upon concentration, the size of gold nanoparticle after concentration should be similar to the size before dilution. However, size of gold nanoparticle in this concentrated mixture was measured to be 68.3 ± 7.5 nm, indicating that the previously aggregated gold NPs
did not re-disperse. So DSPE-PCB 5K is not unique to re-stabilizing the aggregated gold NPs upon concentration.

Since cargo aggregation is attributed to the dissociation of protecting micelles, this method indirectly detects CMC values. A series of common micelles commercially available with known CMCs labeled by the manufacturers were used to verify this method, including non-ionic micelles (polysorbate 80, DSPE-PEG 5K and 2K), cationic micelles (hexadecyltrimethylammonium bromide (CTAB)), anionic micelles (sodium dodecyl sulfate (SDS)), zwitterionic carboxy betaine and sulfobetaine micelles with varied hydrophobic chain lengths (dodecyl dimethylglycine (CB12C), N-Tetradecyl-N-N-Dimethylglycine (CB14C), 3-(deyldimethylammonio) propane sulfonate (SB10C), 3-(N,N-Dimethylpalmitylammonio)propane sulfonate (SB16C)). Representative measuring curves are shown in Fig.2-5. Measuring curves for all samples tested are shown in Fig. 2-6. Without exception, CMC values for all micelles are right positioned at a concentration, below which both the hydrodynamic size of the colloid and the maximum absorbance wavelength for gold NPs start to increase significantly (Student’s $t$-test, $p < 0.05$). Similar to common probes for CMC determination (for example, pyrene and other dye molecules and so on.), the presence of gold NPs in the hydrophobic micelle core does not seem to influence the dissociation behavior of micelles. If it did, an artefact signifying that the colloid system does not aggregate below the CMC would appear.

**DSPE-PCB 5K has an undetectable ultra-low CMC.** Using the validated “diluting-concentrating” method, DSPE-PCB 5K micelle protected gold NPs do not show signs of cargo aggregation at a concentration as low as to $2.7 \times 10^{-6}$ mM (Fig. 2-5; no significant signal increase based on Student’s $t$-test). Their CMC is considered ultra-low and below our current detection
limit of 2.7 x 10^{-6} \text{ mM}. The detection limit can potentially be lowered by one order of magnitude by further diluting the micelles (for example, into a 10 L container), then concentrating 1.0 - 1.5 L each time (using the current 2 L evaporation flask). This approach is laborsome and has little room to further lower the CMC detection limit. In contrast, DSPE-PEG 5K micelles, widely used to construct nanomedicines, have an apparent CMC about 1 x 10^{-3} \text{ mM}, consistent with literature values\cite{35, 44}, and dissociate at concentrations, at least, three orders of magnitude higher than DSPE-PCB 5K. Compared with DSPE-PCB 5K, which has multiple zwitterionic CB moieties as the polar group, CB14C contains only one zwitterionic carboxy betaine and has higher CMC at 3.4 x 10^{-2} \text{ mM}. To confirm that DSPE-PCB 5K has an ultra-low CMC, following experiments were performed: 1) Gold NP colloids were formed in the presence of PCB 5K replacing DSPE-PCB 5K and were found to be unstable (aggregate). Note that DSPE-PCB 5K/gold colloid was stable at room temperature for weeks without precipitation. This control experiments excludes the possibility of PCB tightly interacting with gold NPs, preventing dissociation of DSPE-PCB 5K micelles (an artifact of lowered CMC). 2) Gold NP probes were replaced by cross-linked polystyrene (PS) NP probes. Following the same “diluting-concentrating” method, where colloid aggregation was detected by DLS, CMC results were obtained similarly to Fig. 2-5a, regardless of the probe used (Fig. 2-6). It should be noted that both gold and crosslinked PS NPs are not soluble under extremely diluted conditions (10^{-6} \text{ mM}). This insoluble property is the key to obtaining reliable CMC results. Soluble salt NPs (e.g., CaCO_3) were not found to work as probes since they are released from the micelle at a diluted concentration below their water solubility and aggregated when concentrated to the concentration above their water solubility (Fig. 2-7). 3) DSPE-PCB 5K micelles at an extremely low concentration (2.7 x 10^{-6} \text{ mM}) have been observed using TEM (Fig 2-8).
Zwitterionic PCB and its amount are critical to achieving ultra-low CMC property.

To explore the unique role of zwitterionic PCB in contributing to ultra-low CMC property, conventional micelles (i.e., SDS and Polysorbate 80) were incubated either the free form of the CB moiety or PCB 5K (with multiple CB) at high concentration (500 mg/mL for free form carboxybetaine moiety and 80 mg/mL PCB) and found that conventional micelles were stabilized in both cases (Fig. 2-9a, b and Fig. 2-10a, b). It should be noted that similar stabilizing effects have been found in both inorganic and organic salt solutions.\[45\] We attribute this effect to the super-hydrophilicity of CB and PCB (similar to salts), which increases the polarity of the aqueous phase,\[32, 46\] strengthens the hydrophobic-hydrophobic interactions and drives micelle assembly at concentrations far below inherent CMC. The presence of ethylene glycol oligomer (OEG) or polymer (PEG), in contrast, increased the CMC for conventional micelles (Fig. 2-9a, b and Fig. 2-10a, b), which is consistent with the literature.\[47\] OEG and PEG are known for their hydrophobic nature and tend to decrease the polarity of the aqueous environment,\[32, 46\] behaving the opposite to super hydrophilic CB and PCB. OEG and PEG are typical water-soluble materials, and in some cases, their hydrophobic nature has been overlooked. They should be strictly considered as amphiphilic materials since they can be dissolved in both water and organic solvents such as dichloromethane. Zwitterionic polymers, by contrast, can be dissolved in water but can be hardly dissolved in most organic solvents and are generally considered to be superhydrophilic. Because of the polarity difference, PEG and zwitterionic polymers have shown opposite effects such as in stabilizing lipid membrane\[48\], and avoiding interaction with protein active center so as to reduce bioactivity loss of polymer-protein conjugates\[49\]. The micelle stabilizing effect of CB or PCB 5K at a series of concentrations were further examined. It was found that a minimum of roughly 200 mg/mL CB and 40 mg/mL PCB
5K were required to prevent the dissociation of conventional micelles, e.g., SDS and Polysorbate 80 (Fig. 2-10c, d, e, f). Thus, the presence of zwitterionic CB moieties as polar groups for DSPE-PCB 5K appears to be the key to the ultra-low CMC property.

In addition, it was found that an appropriate molecular weight (MW) of PCB (amount of CB moieties per polymer) is critical for DSPE-PCB to achieve the lowest possible CMC (Fig. 2-9c, d). PCB MW is expected to be as high as possible to ensure the CMC lowering effect. However, PCB MW should not be too large since micelles with longer hydrophilic polymer chains typically have high CMCs (e.g., shown by hydrophilic polyvinylpyrrollidone (PVP)-hydrophobic alkyl chain micelles[35]). It appears that DSPE-PCB 5K has a the balanced PCB MW and achieves the lowest possible CMC (< 10^-6 mM (undetectable)) compared with DSPE-PCB 2K (CMC = 1.2 - 6 x 10^-5 mM) and 10K (CMC = 0.9 - 1.8 x 10^-5 mM), and other reported micelles containing zwitterionic polymer blocks.[50]

One major challenge with drug delivery is the dissociation of carrier entities (e.g., micelles) as they are infused into the blood stream since the drug carrier is typically diluted below its CMC. Blood serum comprises a large number of proteins (albumin) that decompose micelles and bind with this surfactants.[51] Compared with water, dilution in serum is even more challenging for assembled carrier systems. Here the stability of ultra-low CMC and conventional micelles in fetal bovine serum (FBS) using the gold NP probes at 0.055 mM micelle concentration was tested. (Fig. 2-11). It was found that DSPE-PCB 5K (ultra-low CMC) showed excellent stability over 72 h. Conventional Polysorbate 80 and DSPE-PEG 5K micelles were not stable; for DSPE-PEG 5K, it dissociated at this concentration even above its CMC. The diluted micelle concentration tested (10^-2 mM) is chosen by considering the situation of a 60 kg adult patient having 4.5 L blood receiving 75 mg/m^2 TAXOTERE® to treat breast cancer.[52] The hypothetical
maximum micelle concentration (i.e. Polysorbate 80) in the blood is calculated to be $5.5 \times 10^{-2}$ mM, assuming all drug was injected simultaneously. The reality is TAXOTERE® has to be mixed with infusion saline and be slowly infused (typically complete in one hour).\cite{53} As drugs gradually enter the blood, they are immediately diluted, with micelle concentration lower than $10^{-2}$ mM. The serum stability test at $10^{-2}$ mM indicates that ultra-low-CMC micelles are more stable than conventional ones as drug carriers at clinic-relevant concentrations.
Figure 2-1 Ultra-low-CMC micelles and their unusual ability to stabilize cargoes in extremely diluted conditions with micelle concentrations far below CMCs of common micelles. Conventional micelles dissociate at a concentration below CMC, and thus cannot stabilize hydrophobic cargo. Structure for ultra-low-CMC micelles is shown. For conventional micelles, polar groups are either non-ionic (e.g., polysorbate 80, having hydrophobic nature) or only contained one ionic group (e.g., sodium dodecyl sulfate). Sharp-contrast micelles with multiple polar zwitterionic (polyzwitterionic) groups were recently reported[32].
Figure 2-2 TEM and DLS data of DSPE-PCB 5K micelle. a, Representative TEM image for DSPE-PCB 5K micelle (5 mM) in water taken by JEOL 2010 Transmission electron microscopy. Stained with 2% uranyl acetate. The scale bar in the figure is 50 nm. b, DLS data for DSPE-PCB 5K in PBS (0.5 mM), showing an average diameter of 20 ± 3.5 nm (mean ± SD, N = 3) with a polydispersity index of 0.13.

Figure 2-3. The hydrophobic core domains of DSPE-PCB 5K micelles were detected using pyrene as reflected by the decrease in the intensity ratio of the first to the third highest energy bands (I1/I3) in the emission spectra of pyrene from 1.7 in water (a) to 1.16 in 0.17 mg/mL micelle in water (b). The excitation wavelength was 334 nm.
Figure 2-4. The CMC of DSPE-PCB 5K was determined to be $1.3 \times 10^{-3}$ mM using the pyrene probe method (mean ± SD, N=3).
**Figure 2-5** | “Diluting-concentrating” method to probe CMCs for micelles. Aggregation of gold probe at different micelle concentrations was reflected by a, hydrodynamic size of the colloid and b, gold maximum absorbance wavelength (mean ± SD, N=3). Dashed arrows in both figures indicate the CMC values. CMC data for DSPE-PEG are from reference.\[^{[35, 44]}\] CMC data for commercial available micelles are provided by the manufacturers.

a) **Polysorbate 80.** Non-ionic surfactant. CMC (provided by manufacture) = 0.012 mM

\[
\begin{array}{c}
\text{Polysorbate 80} \\
\text{Average Size (nm)}  \\
\text{Concentration (mM)}
\end{array}
\]

\[
\begin{array}{c}
\text{Max. Absorbance Peak (nm)}  \\
\text{Concentration (mM)}
\end{array}
\]

b) **DSPE-PEG 5K.** Non-ionic surfactant. CMC (from ref\[^{[44a]}\]) = 1-1.5 x10\(^{-3}\) mM

\[
\begin{array}{c}
\text{DSPE-PEG 5K} \\
\text{Average Size (nm)}  \\
\text{Concentration (mM)}
\end{array}
\]

\[
\begin{array}{c}
\text{Max. Absorbance Peak (nm)}  \\
\text{Concentration (mM)}
\end{array}
\]
c) DSPE-PEG 2K. Non-ionic surfactant. CMC (from ref.\cite{44a}) = 0.5 - 1 \times 10^{-3} \text{ mM}

d) CTAB. Cationic surfactant. CMC (provided by manufacturer) = 0.92 \text{ mM}
e) SDS. Anionic surfactant. CMC (provided by manufacturer) = 8.2 mM

f) CB12C. A zwitterionic surfactant with single carboxy betaine as a hydrophilic group and 12 carbon chain as a hydrophobic group. CMC (provided by manufacturer) = 1.5 mM
g) CB14C. A zwitterionic surfactant with single carboxy betaine as a hydrophilic group and 14 carbon chain as a hydrophobic group. CMC (provided by manufacturer) = 0.034 mM

h) SB10C. A zwitterionic surfactant with single sulfobetaine as a hydrophilic group and 10 carbon chain as a hydrophobic group. CMC (provided by manufacturer) = 24–40 mM
i) SB16C. A zwitterionic surfactant with single sulfobetaine as a hydrophilic group and 16 carbon chain as a hydrophobic group. CMC (provided by manufacturer) = 0.01~0.06 mM

j) DSPE-PCB 5K. A zwitterionic surfactant with poly carboxy betaine as a hydrophilic group and DSPE chain as a hydrophobic group. CMC is undetectable < 2.7 x 10^-6 mM

Figure 2-6. “Diluting-concentrating” method to determine micelle CMCs using gold NPs as a probe. Measuring curves for all samples tested are shown (a-j), including non-ionic, cationic, anionic, and
zwitterionic surfactants. Aggregation of the gold probe at different micelle concentrations was reflected by the hydrodynamic size of the colloid and gold maximum absorbance wavelength (mean ± SD, N=3). Known CMC values for micelles were listed and indicated by dashed arrows. The current “diluting-concentrating” method is able to detect a CMC as low as $2.7 \times 10^{-6}$ mM.

**Figure 2-7.** CaCO$_3$ probes were encapsulated in DSPE-PCB 5K micelle, by dissolving micelles and CaCl$_2$ in 10 ml deionized water followed by dropwise addition of 1 mL Na$_2$CO$_3$ aqueous solution under vigorous stirring at room temperature for 15 min (micelle:CaCl$_2$:Na$_2$CO$_3$ (molar ratio) = 1:1:1). (a) The size of DSPE-PCB 5K/CaCO$_3$ nanoparticles before dilution. Micelle concentration was 3 mg/mL (0.5 mM), and CaCO$_3$ concentration was 0.05 mg/mL (0.5 mM). (b) The size of DSPE-PCB 5K/CaCO$_3$ nanoparticles after 1000-fold dilution to micelle concentration of $5 \times 10^{-7}$ M, and CaCO$_3$ concentration of 0.05 mg/L, and then concentrated back to the original concentration at (a). The solubility of CaCO$_3$ in water is 0.013 mg/mL or 13 mg/L. The concentration of CaCO$_3$ encapsulated in micelle before dilution
(0.05 mg/mL) was above its solubility (0.013 mg/mL), and the concentration of CaCO$_3$ encapsulated in micelle after dilution (0.05 mg/L) was below its solubility (13 mg/L). CaCO$_3$ was found to be released at diluted concentration and aggregated into large particles after condensation as shown at (b).

**Figure 2-8.** TEM figures of DSPE-PCB 5K micelle at an extremely low concentration ($2.7 \times 10^{-6}$ mM). Stained with 2% uranyl acetate. The scale bar in the figure is 20 nm.
Figure 2-9 Effect of supplying zwitterionic moieties on the CMCs of conventional micelles, and impact of the zwitterionic PCB MW on the CMC of DSPE-PCB. a, b, Gold NPs were loaded in SDS (a) and Polysorbate 80 (b), and upon water dilution, their aggregation behavior (due to micelle dissociation) was measured by the increase in maximum absorbance peak of gold (mean ± SD, N=3). When 500 mg/ml CB was used as dilution solvent, no significant gold aggregation was observed in the dilution range, indicating a stabilized micelle (despite a concentration below its inherent CMC). In contrast, when 500 mg/mL OEG was used as dilution solvent, there was gold NP aggregation at high micelle concentrations, showing an increase of micelle CMC. CB and OEG alone (without the aid of micelles) cannot stabilize gold NPs, leading to aggregation. It should be noted that DLS data are not reliable in this experiment since the large concentration of small-sized CB or OEG moieties interferes with the hydrodynamic size readings for aggregated gold NPs. c, d, “Diluting-concentrating” method to probe CMCs for DSPE-PCB 2K, 5K, 10K micelles. Aggregation of the gold probe at different micelle concentrations was reflected by the gold maximum absorbance wavelength (c) (mean ± SD, N=3) and the hydrodynamic size of the colloid (d) (mean ± SD, N=3). CMC values were determined for DSPE-PCB 2K and 10K and are indicated by dashed arrows.
Figure 2-10. Gold NPs were loaded in SDS (a) and Polysorbate 80 (b), and upon water dilution, their aggregation behavior (due to micelle disassociation) was measured by the increase in
maximum absorbance peak of gold (mean ± SD, N=3). When 100 mg/mL PCB polymer (MW= 5K) was used as dilution solvent, no gold nanoparticle aggregation was observed in the dilution range, indicating micelles were stabilized even at a concentration below its inherent CMC. In contrast, when 100 mg/mL PEG polymer (MW= 5K) was used as dilution solvent, there was gold NP aggregation at the concentrations above the inherent CMC of micelles. PCB and PEG alone (without the aid of micelles) cannot stabilize gold NPs, leading to severe aggregation. It should be noted that DLS data are not reliable in this experiment since the presence of a large concentration of small size PCB or PEG interferes with the hydrodynamic size reading for the aggregated gold NPs. Different concentration of PCB solutions was used as dilution solvent, and within the concentration series, 40 mg/mL PCB was the minimum required to stabilize (c) SDS and (d) Polysorbate 80 upon dilution (mean ± SD, N=3). Different concentrations of CB solutions were used as dilution solvents, and within the concentration series, 200 mg/mL CB was the minimum required to stabilize (e) SDS and (f) Polysorbate 80 upon dilution (mean ± SD, N=3).

Figure 2-11 Stability of ultra-low-CMC micelle and conventional micelles with gold NP probes encapsulated at $5.5 \times 10^{-2}$ mM micelle concentration in 100% fetal bovine serum (FBS) over 72 h at room
temperature. Potential micelle disassociation resulted in gold NP aggregation, which was measured by gold maximum absorbance peak (mean ± SD, N=3). Known CMC values for each micelle are indicated inside the graph.
CHAPTER 3 THERAPEUTIC PERFORMANCE OF DRUG/DSPE-PCB 5K FORMULATION

Commercially available micelle drug formulations, such as Taxotere®, usually have stability issue and prematurely release drug when infused into the blood stream due to the high CMC values of the micelles being used. The previous Chapter described a novel zwitterionic micelle DSPE-PCB 5K with ultra-low CMC feature and demonstrated its stability in FBS over 72 hours at a clinically relevant condition. Therefore, it is of interest to evaluate the therapeutic performance of such ultra-low CMC micelles as drug delivery carriers. In this chapter, a docetaxel/DSPE-PCB 5K formulation was prepared via thin film hydration method using the ultralow CMC micelle introduced in chapter 2. This new formulation was expected to show better performance due to the high stability of ultralow CMC zwitterionic micelle. To evaluate the anti-tumor performance of such new formulation, a melanoma tumor model on mice was developed. Ultralow CMC micelles encapsulating docetaxel showed full elimination of tumors, whereas conventional docetaxel micellar formulations did not reverse tumor growth. Ultralow CMC micelles might become next-generation carriers for drug delivery.

3.1 Introduction

A well-known micelle based drug formulation is Taxotere®, a chemotherapy medication used to treat a number of types of cancers. One single-dose vial of Taxotere® contains the active ingredient docetaxl, polysorbate 80 micelle molecule and anhydrous ethanol. Clinically, this formulation required dilution in 250 mL infusion bag containing 0.9% sodium chloride or 5% dextrose and was then slowly infused into the blood stream which lasted for 1 hour. There are two limitations for this formulation: 1) Fully prepared Taxotere infusion solution, the diluted form, should be used within 4 hours including the 1 hour i.v. administration due to the low stability of the micelle formualtion. 2) When infusing into the blood, the concentration of polysorbate 80
(<< 5.5 × 10⁻² mM) is much lower than its CMC value (0.012 mM). At such low concentration, polysorbate 80 would disassemble into free surfactants and cannot stabilize and carry docetaxel for blood circulation. In Chapter 2, zwitterionic micelle DSPE-PCB 5K was demonstrated to have an ultralow CMC value and showed high stability in clinical-relevant environment. Therefore, a new docetaxel formulation was prepared using zwitterionic micelle, and its therapeutic outcome was examined by intravenously injecting to mice inoculated with melanoma tumor (B16F10). Melanoma is a challenging disease to treat due to the well-documented chemoresistance\textsuperscript{[54]}. Docetaxel was not clinically used to treat melanoma, but had been explored to treat melanoma in animal studies through nanoparticle mediated delivery, though with marginal improvement of the therapeutic outcome\textsuperscript{[55]}. Considering the advantage of the ultralow CMC zwitterionic micelle, it is curious to know if the new micelle formulation of the old drug can enhance therapeutic treatment of melanoma in animal studies.

3.2 Materials and Methods

3.2.1 Materials

Docetaxel was obtained from LC Laboratories (Woburn, USA). B16F10 cells were obtained from ATCC. Trifluoroethanol, Phosphate-Buffered Saline tablets, and acetonitrile (LC grade) were purchased from Sigma-Aldrich (St. Louis, USA). RPMI 1640 medium was purchased from Gibco (Dublin, Ireland).

3.2.2 Preparation of docetaxel/DSPE-PCB 5K formulation

To prepare docetaxel/DSPE-PCB 5K formulation, 150 mg of DSPE-PCB 5K and 6 mg docetaxel were co-dissolved in 5 mL trifluoroethanol in a round-bottom flask. The solvent was evaporated by rotary evaporation to obtain a thin film. The film was kept in a vacuum for 2 hours
at room temperature to remove the residual trifluoroethanol. After the addition of 3 mL saline and stirring at 800 rpm for 30 min, a transparent micelle solution with docetaxel loaded was obtained. Docetaxel/DSPE-PEG 5K and docetaxel/Polysorbate 80 formulations were prepared following the same procedure. Taxotere was prepared following a reference procedure\textsuperscript{52-53, 56}. The weight ratios of the drug over surfactant for all formulations were kept at 4%, which is the drug loading level for TAXOTERE\textregistered\textsuperscript{57}.

Drug release profiles were determined by placing 100 μL different formulations into Slide-A-Lyzer MINI dialysis microtubes (3500 Da MW cutoff) and dialyzed against PBS at 37 °C with gentle stirring. At different time intervals, docetaxel remained in the dialyzer was quantified through HPLC by a Waters Breeze 2 System equipped with a Waters 2998 photodiode array detector at 230 nm and a Waters Symmetry C\textsubscript{18} column (4.6 × 7.5 mm). The mobile phase was a mixture of acetonitrile/water (50:50 volume/volume) at a flow rate of 1 mL/min. The docetaxel showed a single elution peak at 5.47 min.

### 3.2.3 Animal Experiments

The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University, and performed in compliance with relevant laws and institutional guidelines. B16F10 cells (ATCC, CRL-6475) were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C under a 5% CO\textsubscript{2} atmosphere. For \textit{in vivo} tumor therapeutic studies, healthy C57BL/6 mice (7 weeks old, 20-25 g) received a subcutaneous injection of 100 μL B16F10 cells (5 × 10\textsuperscript{6} cells/mL) in the right flank. After 8 days of inoculation, the tumor became visible, and different formulations were administered by intravenous injection via tail vein 5 times every other day at the dosage of 10 mg docetaxel/kg body weight. The tumor
volume of the mice was measured day by day and calculated with an equation of \((L \times W^2)/2\) where \(L\) is the long length, and \(W\) is the short length of the tumor. Mice were euthanized if their weight loss exceeded 15% or if their tumor grew to a volume greater than 1000 mm\(^3\). For maximum tolerated dose (MTD) study, healthy C57BL/6 mice received one intravenous injection of different docetaxel formulations through the tail vein at the dose of 25 mg/kg, 40 mg/kg, or 55 mg/kg. Treated mice were subsequently observed day by day and checked for mortality, body weight, and signs of toxicity. To measure docetaxel concentration in plasma, healthy C57BL/6 mice (7 weeks old, 20-25g) were treated with intravenous injection via tail vein at the dosage of 10 mg/kg. At different time intervals, 50 µL blood samples were collected and transferred to sodium heparin treated tubes. Docetaxel was extracted from the plasma supernatant with ethyl acetate, and the extracted solution was evaporated under a gentle stream of nitrogen. The extraction residues were reconstituted in the mixture of acetonitrile/water (50:50 volume/volume). The docetaxel concentration was measured using HPLC as mentioned above.

To test bio-distribution, healthy C57BL/6 mice (7 weeks old, 20-25 g) received a subcutaneous injection of 100 µL B16F10 cells \((5 \times 10^6 \text{ cells/mL})\) in the right flank. After 8 days of inoculation when tumors became visible, different formulations were administered by intravenous injection via the tail vein. Mice were euthanized at the certain time point. Organs (liver, heart, kidney, spleen, lung and tumor tissues) were collected, thoroughly washed with saline \((0.9\% \text{ NaCl})\), weighted, and stored for further analysis. Organs or tissues were homogenized with Mini-beadbeater-24 (Biospec Products, Inc., USA) in acetonitrile/water (50:50 volume/volume). The resulting 200 µL samples of homogenized tissue were mixed with 1 mL ethyl acetate. Well-mixed samples were then centrifuged at 4000 g for 5 minutes. The organic layer was transferred to a clean tube and evaporated under a gentle stream of nitrogen.
The extraction residue was reconstituted in 1 mL mixture of acetonitrile/water (50:50 volume/volume). The docetaxel concentration was measured using HPLC.

3.3 Results and Discussion

To study the applicability of using ultra-low CMC micelles to deliver real drugs, antitumor drug docetaxel was chosen and docetaxel/DSP-E-PCB 5K formulation was prepared using a simple thin-film hydration method (see Experimental Section). Control formulations include docetaxel/Polysorbate 80 and docetaxel/DSPE-PEG 5K, similarly made through thin-film hydration method, and Taxotere® formulated based on literature reports. The weight ratios of the drug over surfactant for all formulations were kept at 4%, which is the drug loading level for Taxotere®. The hydrodynamic diameters for all tested samples were around 22 nm as determined by DLS. However, after diluting these samples in FBS to reach micelle concentration of $5.5 \times 10^{-2}$ mM (the hypothetical maximum micelle concentration in the blood), only docetaxel/DSPE-PCB 5K can maintain its hydrodynamic size, while all other control formulations appeared to be unstable over the tested time period. Drug release profiles were determined by placing the formulations into Slide-A-Lyzer MINI dialysis microtubes (3500Da MW cutoff) and dialyzed against PBS at 37 °C with gentle stirring. Docetaxel remained inside the dialysis tubes at different time points was quantified using HPLC to calculate drug release. DSPE-PCB 5K showed the highest retention of docetaxel compared with other control micelles, with 50% drug released in 3.7 h. The drug release mechanism in docetaxel/DSPE-PCB 5K formulation is expected to be based on drug dissolution or drug leaching. This has been indicated by the control study showing the zwitterionic micelles can stabilize non-dissolvable gold NPs and crosslinked PS NPs but not soluble CaCO$_3$ NPs at low concentrations ($5 \times 10^{-4}$ mM). The stability of different docetaxel formulations after
lyophilization was further checked. Results indicated that after lyophilization docetaxel/DSPE-PCB 5K maintained its original hydrodynamic size, while all control micelle formulations aggregated (Fig. 3-3).

The maximum tolerated dose (MTD) of docetaxel/DSPE-PCB 5K was determined using healthy C57BL/6 mice to be 55 mg docetaxel/kg body weight, significantly higher than control formulations docetaxel/DSPE-PEG 5K (40 mg/kg), docetaxel/Polysorbate 80 (40 mg/kg), and Taxotere® (25 mg/kg) (Fig. 3-4). To examine the antitumor activity of different docetaxel/micelle formulations, C57BL/6 mice were inoculated with B16F10 cells in the right flank. On day 8 after inoculation, they received an intravenous injection of one of the drug formulations, followed by 4 more every other day. The dose for all drug/micelle formulations was 10 mg docetaxel/kg body weight. Docetaxel/DSPE-PCB 5K showed significantly better anti-tumor activity compared with all other micellar docetaxel formulations, including Taxotere® (Fig. 3-5a). All tumors treated with this formulation were eliminated by day 33 after the first treatment, without recurrence over the 70 days observation period after tumor inoculation (Fig. 3-5b and Fig. 3-5c). None of the control formulations based on docetaxel were able to fully inhibit the melanoma tumor. In fact, it is rare for any chemotherapy drugs to completely eliminate melanoma.[31c, 59]

To further investigate the in vivo circulation profile of docetaxel/DSPE-PCB 5K, different docetaxel formulations were injected intravenously to healthy C57/BL6 mice. At different time points, blood samples were collected, and the concentration of docetaxel in plasma was measured via HPLC. It was found that docetaxel/DSPE-PCB 5K showed longer blood circulation than all other micellar docetaxel formulations (Fig. 3-6). The α and β half-life of docetaxel/DSPE-PCB 5K were calculated to be 0.6 hours and 5.9 hours, while for
TAXOTERE® these values were 4 min and 36 min, respectively. For bio-distribution, healthy C57/BL6 mice were treated with intravenous injection of different docetaxel formulations after inoculation of B16F10 cells. Bio-distribution of docetaxel at different time points has been further studied. Significantly higher tumor accumulation of docetaxel/DSPE-PCB 5K was observed at 24 hours post-injection compared with control formulations (Fig. 3-7). Such enhanced drug accumulation to tumor site has been observed at as early as 2 hours after administration (Fig. 3-8). The antitumor performance of docetaxel/DSPE-PCB 5K formulation was attributed to the stabilization ability of the ultra-low CMC micelle which improved the blood circulation and drug accumulation in tumor.

In summary, the ultra-low CMC zwitterionic micelle was used to prepare a new formulation of docetaxel. Therapeutic performance was evaluated on a melanoma model and compared with Taxotere and other controls. Results showed that the docetaxel/ ultra-low CMC micelle formulation can completely eliminate the melanoma tumor on mice, while the control formulations, including TAXOTERE®, was unable to do so. Excellent therapeutic performance was attributed to the ultra-stability in protecting cargoes without dissociation. Therefore, it is expected the ultra-low CMC micelle can be utilized to enhance the delivery and therapeutic effects of other drugs in general.
Figure 3-1. Stability of different docetaxel formulations diluted in FBS (mean ± SD, N=3).

Figure 3-2. The release profile of different docetaxel formulations in dialysis against PBS. (mean ± SD, N=3)
Figure 3-3. Stability of different docetaxel formulations as reflected by the hydrodynamic size of the formulation before and after lyophilization (mean ± SD, N = 3). The circles in each bar represent an original data point.
Body Weight (% of initial) vs. Time post-administration (days)

- **PBS**
- **docetaxel/Polysorbate 80 25mg/kg**
- **docetaxel/Polysorbate 80 40mg/kg**
- **docetaxel/Polysorbate 80 55mg/kg**

Biological Activity (maximal vs. control) for two different formulations:

**PBS**

**docetaxel/DSPE-PEG 25 mg/kg**

**docetaxel/DSPE-PEG 40 mg/kg**

**docetaxel/DSPE-PEG 55 mg/kg**
Figure 3-4. Dose escalation in healthy mice. Solutions of different formulations a, Taxotere; b, docetaxel/DSPE-PCB; c, docetaxel/DSPE-PEG; d, docetaxel/Polysorbate 80 were administered through intravenous tail vein injection. (mean ± SD, N=3).
Figure 3-5. (a) Anti-tumor performance of docetaxel/DSPE-PCB 5K formulation (mean ± SD, N = 7) compared with other formulations (mean ± SD, N = 7). On day 10 after treatment (*), a Mann-Whitney Test indicated that docetaxel/DSPE-PCB 5K delayed the tumor growth greater than all other four controls at $p = 0.005$. (b), Survival rate curves of mice treated with the different formulations. (c), Probability of cure. In mice bearing invisible tumors < 1mm$^3$ in size or a flattened and darkened scar tissue developed, it was considered that the tumor had been completely eradicated. Lines representing docetaxel/DSPE-PEG 5K, docetaxel/polysorbate 80, taxotere and PBS are overlapped.
Figure 3-6. The concentration of docetaxel in blood plasma of different formulations (mean ± SD, N = 4).
Figure 3-7. Bio-distribution of different docetaxel formulations at 24 hours post i.v. injection (mean ± SD, N= 4). One-Way ANOVA analysis with Bonferroni multi-comparison: significant difference between docetaxel/DSPE-PCB 5K and docetaxel/DSPE-PEG 5K, docetaxel/DSPE-PCB 5K and docetaxel/Polysorbate 80, docetaxel/DSPE-PCB 5K and Taxotere, at p < 0.001 (**), p < 0.0001 (****), and p < 0.0001 (****), respectively.

Figure 3-8. Bio-distribution of different docetaxel formulations. a, bio-distribution of docetaxel from different formulations at 2 hours post i.v. injection (mean ± SD, N= 4). One-Way ANOVA analysis with Bonferroni multi-comparison: significant difference between docetaxel/DSPE-PCB 5K and docetaxel/Polysorbate 80, docetaxel/DSPE-PCB 5K and Taxotere, at p < 0.01 and p < 0.05, respectively.
No significant difference between docetaxel/DSPE-PCB 5K and docetaxel/DSPE-PEG 5K. b, bio-distribution of docetaxel from different formulations at 8 hours post i.v. injection (mean ± SD, N= 4). One-Way ANOVA analysis with Bonferroni multi-comparison: significant difference between docetaxel/DSPE-PCB 5K and docetaxel/DSPE-PEG 5K, docetaxel/DSPE-PCB 5K and docetaxel/Polysorbate 80, docetaxel/DSPE-PCB 5K and Taxotere, at p < 0.01, p < 0.0001, and p < 0.001, respectively.
CHAPTER 4. SIMPLE PROTEIN MODIFICATION USING ZWITTERIONIC POLYMER

In this chapter, it is demonstrated that when the zwitterionic carboxybetaine polymer (PCB) was conjugated to insulin through simple conventional coupling chemistry, the resulting PCB-insulin did not show a significant reduction of in vitro bioactivity. The reported simple strategy of using PCB for protein conjugation is expected to solve the bioactivity loss issue for most protein-polymer conjugations. The obtained PCB-insulin shows two significant advantages as a novel pharmaceutical agent. First, for PCB-insulin, in vivo pharmacological activity of lowering blood glucose was increased compared with native insulin. Such increase is expected to be due to both the improved pharmacokinetics and retained bioactivity of PCB-insulin. Second, its production is simple from manufacturing standpoints. The conjugation procedure involves only one-step coupling reaction and does not require a site-specific linkage technique. The synthesized PCB-insulin conjugates do not need chromatographic separation to purify. These two advantages are expected to be translated to other types of therapeutic proteins as well.

4.1 Introduction

The major purpose of polymer-protein conjugation is to improve pharmacokinetics (PK) and retain bioactivity in order to improve the pharmacological activity (the inherent capacity of drug to alter chemical or physiological functions of a cell, tissue organ or organism) of a modified protein. The most widely used conjugation strategy is PEGylation that uses polyethylene glycol (PEG) to conjugate proteins. Despite improved PK, PEGylation is known for its sacrificing the conjugated protein activity. Results indicate that conjugating PEG, and other commonly seen polymers as well, on proteins through conventional covalent coupling chemistry resulting in polymer chains
non-selectively attached on the protein surface groups (e.g., amines), and potential attachment near a protein’s active site inhibit the protein from performing designated biological function (e.g., amine group on Gly A1 of insulin)\(^{[27]}\). There are alternative methods allowing for site-specific conjugation to proteins, e.g., involving re-engineering the proteins, or laborsome separation, but those procedures are complex.\(^{[61]}\) When PEG is conjugated to proteins in a site-selective manner, however, the resulting PEG-proteins are still subject to bioactivity loss. Eli Lily has developed a PEGylated insulin Lispro (LY2605541) where a 20 kDa PEG was conjugated at the amino group of Lys B28 of insulin Lispro. For LY2605541, a high molar concentration is still required to achieve comparable glucose lowering effect to the unconjugated insulin as observed in the \textit{in vitro} insulin receptor binding studies.\(^{[62]}\)

In this chapter, it is reported that the conjugation of zwitterionic carboxybetaine polymer (PCB) on insulin through conventional coupling chemistry improves the PK of the protein, but does not compromise the insulin \textit{in vitro} bioactivity. Remarkably, this conjugation strategy increases the \textit{in vivo} glucose lowering activity for \(~24\%\) compared with native insulin at equal molar dose when intravenously (i.v.) injected. In addition, the synthesis of PCB-insulin does not require complicated site-specific linkage technique and can be implemented through a simple one-step coupling reaction. The resulting PCB-insulin conjugates do not require chromatographic separations to obtain purified isoforms, potentially enabling a simplified scaling-up, and manufacturing procedure.

\textbf{4.2 Materials and Methods}

\textbf{4.2.1 Materials}
Human recombinant insulin was purchased from Life Technologies Corporation. Methoxypolyethylene glycol 5000 Da acetic acid N-succinimidyl ester (PEG-NHS), streptozotocin (STZ), Cu(I)Br, and all necessary solvents were purchased from Sigma-Aldrich. HEPES buffer was purchased from Hyclone.

4.2.2 Synthesis of PCB-(N-hydroxysuccinimide) (NHS)

PCB-NHS was synthesized based on a previous procedure.[32] Briefly, NHS-PCB-tBu was firstly synthesized through atom transfer radical polymerization (ATRP) of 2-tert-Butoxy-(2-(methacryloyloxy)ethyl)-dimethyl-2-oxoethanaminium (CB-tBu), which was carried out in anhydrous dimethylformamide (DMF), initiated by N-hydroxysuccinimide 2-bromopropanoate and catalyzed by Cu(I)Br/1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA). The resulting NHS-PCB-tBu was purified through precipitation in acetone, and later treated with trifluoroacetic acid to obtain PCB-NHS, which was further precipitated in ethyl ether, and dried under vacuum condition.

4.2.3 Synthesis of PCB-insulin and PEG-insulin

Conjugates of PCB-insulin and PEG-insulin were synthesized by reacting NHS ester groups of the polymer with available amine groups on the protein. Typically, insulin (1 mg/mL) was dissolved in 0.1 M Na₂CO₃ buffer. The insulin stock solution was homogeneously divided into three portions, with one as free insulin sample, and the other two for conjugation with PCB and PEG, respectively. For the conjugation reaction, pre-dissolved PCB-NHS 5K or PEG-NHS 5K was added into the insulin solution at polymer: insulin molar ratio = 1.2:1, and the reaction mixture was stirred at 700 round/min rate at 0 °C, for one hour to obtain the PCB-insulin or PEG-insulin rough products. After the reaction, the buffer was adjusted to pH 7.4 by HCl. To obtain PEG-insulin and PCB-insulin where most of the three amines of insulin were conjugated
with PEG or PCB, pre-dissolved PCB-NHS 5K or PEG-NHS 5K was added into the insulin solution at polymer: insulin molar ratio = 20:1 in 0.2 M HEPES buffer, and the reaction mixture was stirred at 400 round/min rate for 40 hours.

4.2.4 Purification of PCB-insulin and PEG-insulin

To purify PCB-insulin and PEG-insulin, the rough conjugation products were placed in a dialysis kit (6-8 kDa MWCO, Sigma-Aldrich). The MW cut off retains the polymer-insulin conjugates, but allows both free insulin and unconjugated PCB-NHS or PEG-NHS to go through the membrane. Completion of purification was indicated by observing no unconjugated insulin or polymer partitioned to the solvent phase outside of the dialysis tubing. Unconjugated insulin can be detected using UV-vis spectrometer (at 276 nm). Also, both unconjugated insulin and polymer can be sensitively detected by DLS (Zetasizer Nano-ZS, Malvern); a dialysate solution with the count rate (the number of photons detected per second) as low as that of pure water indicating that the solution is free of insulin or polymer. The purified polymer-insulin samples were desalted using a PD-10 column and freeze-dried for storage. To quantify the molar concentration of purified PCB-insulin and PEG-insulin solution sample, both methods of UV-vis absorbance (276 nm) and a Mercodia human insulin ELISA assay were used.

4.2.5 Other Characterization

The hydrodynamic sizes of the insulin and its conjugate samples were measured by a DLS method using a Malvern Nano-ZS Zetasizer at 25 °C. The samples of free insulin, PEG-insulin and PCB-insulin were dissolved in 0.2M HEPES buffer and analyzed on an Autoflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker
Daltonics). The mass spectra were collected with a repetition rate of 100 Hz, accelerating voltage of 19 kV and an average of 700 shots. The data were analyzed with Biotools program (Bruker).

CD spectra were collected by using a Chirascan spectrophotometer from Applied Photophysics at 25 °C. Insulin samples were dissolved in PBS buffer at 0.2 mg/mL. The CD spectra were measured from 200 to 250 nm with the light-path length of 1 mm. The scanning speed of CD spectra measurement was set at 200 nm/min. Each spectrum was the average of three scans to increase the signal-to-noise ratio of the measurements. The secondary structure proportions for insulin and its conjugates were analyzed using CDSSTR software.

For insulin bioactivity test, about 25,000 CHO-M1 cells/well (ATCC) were seeded in a 96-well tissue culture treated plate and incubate for 24 h before being cultured in serum-free medium. These cells were then treated with 10 μg/mL native insulin, PEG-insulin, and PCB-insulin samples, respectively, for 45 minutes. Then cells were lysed, and a commercially available kit (Cisbio) was used to assay phosphorylated AKT at Ser473. The fluorescence emission at 665 and 620 nm was measured on an HTRF reader (BioTek, USA).

4.2.6 Animal Experiments

All animal experiments were performed according to the National Institute of Health (NIH) guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University. To establish streptozotocin (STZ)-induced diabetic mice model, healthy mice (C57BL/6J, male, 6-8 weeks of age, Jackson Lab) received a daily i.p. injection of 5 mg/mL STZ at 50 mg/kg for 5 consecutive days. 17 days after the first injection, body weight and blood glucose were measured to confirm the diabetic status. Only mice whose nonfasted blood glucose levels are above 300 mg/dL for two consecutive days were
considered diabetic for further in vivo testing. To examine the blood glucose lowering effect of insulin after conjugation, diabetic mice received 10 nmol/kg body weight of free insulin, crude products and purified forms of PCB-insulin and PEG-insulin through tail vein injection (mouse group size = 6). 5-10 µL blood samples were collected from the tail for glucose testing using a clarity plus blood-glucose meter at predetermined time intervals. The mice fasted during the glucose testing period. To examine blood circulation profile of insulin after conjugation, diabetic mice received 10 nmol/kg body weight of free insulin, purified PCB-insulin and purified PEG-insulin through the tail vein (mouse group size = 6). At predetermined time intervals, 25 µL blood samples were collected from the tail vein. The insulin concentration in the blood serum was determined using a Mercodia human insulin ELISA assay. The mice fasted during the blood insulin testing period.

4.3 Results and Discussion

Zwitterionic polymer materials have attracted increasing attention as novel non-fouling materials.\cite{63} Particles with surfaces modified with zwitterionic polymers, such as PCB, have been found to efficiently avoid interactions with plasma proteins and uptake by the reticuloendothelial system (RES),\cite{63a, 64} show significantly prolonged blood circulation half-life (improved PK),\cite{32, 64} and demonstrated reduced immunogenicity.\cite{65} Here, the general strategy in obtaining PCB-insulin conjugate is by reacting an N-hydroxysuccinimide (NHS) ester terminated PCB with the available amine groups of human recombinant insulin (Figure 4-1a). To prepare PCB-insulin conjugate, NHS ester terminated PCB (PCB-NHS) was synthesized following established procedures.\cite{32} The MW for PCB-NHS is ~5000 Da as previously reported: Mₙ = 4890 Da determined by \(^1\)H
NMR, Mn = 5410 Da, polydispersity = 1.03 characterized by gel permeation chromatography (GPC).\cite{32} Then PCB-NHS was reacted with the amine group of insulin at PCB-NHS: insulin molar ratio of 1.2:1 to synthesize PCB-insulin conjugate. The reaction was conducted in a Na$_2$CO$_3$ solution (pH > 10) at which condition the acylation may occur at any of the three amines (GlyA1, PheB1, or LysB29), but is prioritized at epsilon amine of LysB29.\cite{66} The LysB29 epsilon amine is the most basic with a pKa of approximately 10. Hence at pH = 10, LysB29 is preferred for most nucleophilic agents.

In a similar way, PEG-insulin control was obtained by reacting a commercially available PEG-NHS (5000 Da, MW) with insulin. A purification method was developed using a 6-8 kDa MWCO dialysis kit to remove unreacted insulin (~5800 Da) and unconjugated PCB-NHS or PEG-NHS (about 5000 Da). The purified PCB-insulin and PEG-insulin were analyzed by MALDI-TOF MS. (Figure 4-1b). The obtained PCB-insulin and PEG-insulin were NOT further separated to obtain their respective purified isoforms (i.e., a polymer conjugated to LysB29 of insulin) before evaluating their PK and bioactivity.

The obtained PCB-insulin was first evaluated for its improved blood circulating potential or PK. The hydrodynamic size of conjugated proteins is a major factor determining theirs in vivo blood circulation behavior.\cite{14n, 62a, 67} An increase in hydrodynamic size typically supersedes the renal ultrafiltration cut-off and reduces the glomerular filtration of modified proteins. A prolonged blood circulation half-life has been extensively reported for PEGylated proteins.\cite{68} We measured the hydrodynamic size of conjugated insulin with dynamic light scattering (DLS) method by using a Malvern Nano-ZS Zetasizer (Figure 4-2a). The hydrodynamic sizes of insulin, PEG-insulin, and PCB-insulin in pH 8.4 HEPES buffer are 1.4±0.2 nm, 2.8±0.7 nm, and 2.2±0.6 nm,
respectively (average ± standard deviation, n=3). Similar to PEG-insulin, there is a significant hydrodynamic size increase for PCB-insulin over native insulin, implying potentially protracted blood circulating lifetime for PCB-insulin.

The PK of PCB-insulin was examined in a diabetic mouse model. All animal experiments were performed according to the National Institutes of Health (NIH) guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University. C57BL/6 mice received a daily i.p. injection of 5 mg/mL streptozocin (STZ) at 50 mg/kg for 5 consecutive days. STZ-mice were placed on a nonfasted regular diet. 17 days after the first injection, weight and blood glucose were measured to confirm diabetic status. Only mice whose nonfasted blood glucose levels are above 300 mg/dL for two consecutive days were considered diabetic. Insulin and purified polymer-insulin conjugates were injected into three groups of diabetic mice through the tail vein at an equal molar dose of 10 nmol/kg. Blood samples were collected at various time intervals, and the concentration of serum insulin was quantified with human insulin ELISA assay (Figure 4-2b). The relative area under the curve (AUC) was calculated, and the blood circulation half-life ($t_{1/2}$) was obtained to fit a one-compartment model$^{[32]}$ (Table 4-1). The AUCs for both PCB-insulin and PEG-insulin are larger than that of native insulin. The relative AUC for PCB-insulin is almost 1.43 times of that of native insulin. $t_{1/2}$ for PCB-insulin (4.2 h) and PEG-insulin (3.6 h) are significantly longer than free insulin (2 h). Typically, elimination half-life indicates the time necessary for the amount or the concentration of drug in serum or plasma, to reduce by 50%. Half-life is usually regarded as the most reliable indicator of the rate of drug removal by the body$^{[69]}$. The rate of removal is expected to be slow when the half-life is
long, which implies a long effective duration of the drug in the blood circulation\textsuperscript{[70]}. The data here indicate that the modified insulin using both PCB and PEG showed prolonged blood circulation effect compared with naked insulin.

The obtained PCB-insulin was secondly evaluated for potential bioactivity change. the effect of polymer conjugation on the secondary structure of insulin was studied, since proteins generally require stabilized structures to contain their particular bioactivity.\textsuperscript{[71]} Circular Dichroism (CD) Spectroscopy has been widely involved in characterizing the secondary conformation of insulin and its derivatives,\textsuperscript{[68a, 72]} and was used here to evaluate free insulin, PCB-insulin, and PEG-insulin (spectra in Figure 4-3a; quantified results of secondary structures in Table 2). We found that the percentage of $\alpha$-helix structure for PEG-insulin is 46%, and is slightly less than that for free insulin which is 50%. The random coil structure proportion for PEG-insulin is 30% and is more than 24% for free insulin. A similar trend has been reported in the literature for PEG conjugated insulin,\textsuperscript{[68a]} indicating a slightly destabilizing effect on the protein structure. For the CD spectrum of PCB-insulin (Figure 4-3a), there is an obvious decrease of the negative peak at 222 nm, indicating the increase of $\alpha$-helix structure. Quantified results (Table 2) show that the $\alpha$-helix structure proportion of PCB-insulin is 53% (slightly more than 50% of insulin), and the random coil structure proportion is 23% (a little less than 24% of insulin). This suggests that the conjugation of PCB has a moderate stabilizing effect on the secondary structure of insulin, and implies the potential retention of insulin bioactivity after PCB conjugation.

\textit{In vitro} activity of PCB-insulin was tested using a cell-based protein kinase B phosphorylation assay. Briefly, 25,000 CHO-M1 cells/well (ATCC) were seeded in a 96-well tissue culture treated plate and incubated for 24 h before being cultured in serum-free
medium overnight (12 hours). These cells were then treated with 50 µL of native insulin, rough products of PEG-insulin and PCB-insulin without any purification (containing both conjugated and unconjugated insulin), or purified products of PEG-insulin and PCB-insulin (containing solely of conjugated insulin) for 45 minutes. The effective insulin concentration in each of the sample was maintained at 2 nmol/mL. For rough conjugate products (unpurified), the effective insulin concentration can be easily controlled by the feeding molar content of insulin for the conjugation reaction. For purified conjugate products, we used both UV-vis absorbance (276 nm), and a Mercodia human insulin ELISA assay to quantify effective insulin concentration. Then treated cells were lysed, and a commercially available kit (Cisbio) was used to assay phosphorylated AKT at Ser473. Figure 4-3B indicates that there was no significant decrease of in vitro bioactivity of both the rough and purified zwitterionic PCB-insulin, but decrease on the PEG-insulin was observed. The purified PEG-insulin lost ~27% activity compared with native insulin.

The obtained PCB-insulin was lastly evaluated for its in vivo pharmacological activity. STZ induced diabetic was treated with i.v. injection of PCB-insulin and their blood glucose concentration at various time points following the injection were monitored. Diabetic mice received free insulin, as well as PCB-insulin and PEG-insulin rough products without any purification (containing both conjugated and unconjugated insulin), to the mice, at the dose of 10 nmol insulin or its conjugate /Kg (for native insulin dose, 10 nmol/kg = 1.5 IU/kg). The in vivo pharmacological activity of insulin samples was evaluated by quantifying the area above the curve (AAC) of the relative intensity of blood glucose concentration. In Figure 4-3C, there was no significant difference of blood
glucose lowering abilities between PEG-insulin and native insulin (student t-test). However, PCB-insulin shows significantly higher blood glucose lowering ability than native insulin at p < 0.05 in a student t-test (Figure 4-3C). We further injected free insulin, as well as purified PCB-insulin and PEG-insulin samples to the mice at the dose of 10 nmol/Kg. The purified PEG-insulin did not show any better glucose lowering effect than native insulin (Figure 4-3D). But the purified PCB-insulin sample had even higher blood glucose lowering ability than native insulin (Figure 4-3D), and then its non-purified form as well (by comparing Figure 4-3C and 4-3D). The pharmacological activity of PEG-insulin is about 97.5% which showed no significant difference comparing with that of native insulin (100%). For PCB-insulin, pharmacological activity is about 124.3% which showed significance at p < 0.005 by comparing the AACs for PCB-insulin and free insulin in a student t-test.

It should be noted that a typical rough product of PCB-insulin contains unconjugated insulin, PCB-conjugated insulin and unconjugated PCB polymer. If PCB polymers were proved to be non-toxic (in a future work), such rough product can potentially be directly used as a medicine to minimize the cost by not wasting the unconjugated insulin in a purification process.

Despite many possible factors proposed, long half-life in blood circulation has been considered to be the most important reason responsive to the high pharmacological activity of proteins. The increased in vivo pharmacological activity of PCB-insulin was attributed to its prolonged blood circulation lifetime plus the retained in vitro bioactivity. PEG-insulin also showed a longer blood circulation character, but because of
the loss of \textit{in vitro} bioactivity, its overall \textit{in vivo} pharmacological activity did not show improvement over native insulin.

The reason for PCB conjugation to retain \textit{in vitro} bioactivity of the modified insulin might share a similar mechanism to a previously reported PCB conjugated enzyme system. PCB conjugation to \(\alpha\)-chymotrypsin did not sacrifice the protein’s catalytic activity, which has been attributed to the superhydrophilic nature of PCB polymer that did not interfere with the hydrophobic-hydrophobic, substrate-binding site interaction.\cite{26a} By contrast, PEG weakened such interaction with its amphiphilic nature and imposed steric hindrance.\cite{26a} Retained bioactivity of PCB-insulin can be similarly explained by the unimpeded insulin ligand-receptor interaction.

It should be noted that our coupling condition was controlled to have one PCB polymer chain to be conjugated preferentially at LysB29 of insulin to produce PCB-insulin of even higher \textit{in vivo} pharmacological activity than native insulin. Nevertheless, when PCB was conjugated to three amines of insulin (\textit{Figure 4-4}), there was a drastic loss of \textit{in vitro} bioactivity (\textit{Figure 4-5A}) and the \textit{in vivo} pharmacological activity as well (\textit{Figure 4-5B}), potentially due to the occupation of Gly A1 near to the insulin’s active site.

To summarize, results in this project indicate that one-PCB-chain modification per insulin obtained from conjugation chemistry shows improved PK, retained \textit{in vitro} bioactivity and increased \textit{in vivo} pharmacological activity of lowering blood glucose compared with native insulin.
Figure 4-1. (a) The schematic illustration of PCB conjugation with native recombinant human insulin. There are three amino acids with free amino groups (GlyA1, PheB1, and Lys B29) that are available for PCB conjugation. The disulfide bonds in insulin were labeled with yellow sticks. (b) MALDI-TOF Mass spectra of native insulin, PEG-insulin, and PCB-insulin.
Fig. 4-2 (a) Hydrodynamic sizes for insulin, PEG-insulin, and PCB-insulin, respectively, as measured by DLS. Samples were dissolved in 0.2 M pH8.4 HEPES buffer. (b) The serum insulin concentration as a function of time after the tail vein injection of free insulin, PEG-insulin, and PCB-insulin, respectively. The data are presented as the percentage of the initial values of serum insulin concentration over time ± standard deviation (n=6).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Relative area under the curve (AUC)</th>
<th>Half-life elimination time (t(_{1/2})) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>341 ± 24</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>PEG-insulin</td>
<td>447 ± 30</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>PCB-insulin</td>
<td>466 ± 34</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

Table 4-1: The relative area under the curve (AUC) and elimination half-life (t\(_{1/2}\)) of native insulin and its conjugates in blood serum. Data are illustrated by average ± standard deviation, n=6.
Fig. 4-3. (A) Circular dichroism (CD) spectra of native insulin, PEG-insulin, and PCB-insulin (0.2 mg/mL insulin or conjugates at 0.2 M pH8.2 PBS buffer). (B) The bioactivity of native insulin, rough PEG-insulin, purified PEG-insulin, rough PCB-insulin, and purified PCB-insulin, as measured by phosphorylation of the insulin receptor in vitro. The relative activity was presented as the percentage relative to native insulin ± standard deviation (n=6). *p < 0.05, **p < 0.01, n.s. = not significant. The relative blood glucose concentration as a function of time after the tail vein injection of free insulin, PEG-insulin, or PCB-insulin (C) before and (D) after purifying insulin conjugate products. The data are presented as the percentage of the initial values of blood glucose concentration ± standard deviation (n=6).
Table 4-2: The secondary structure proportion of insulin samples measured from Circular Dichroism (CD) Spectroscopy

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha$-helix</th>
<th>$\beta$-structure ($\beta$-turn and $\beta$-sheet)</th>
<th>Random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free insulin</td>
<td>50%</td>
<td>26%</td>
<td>24%</td>
</tr>
<tr>
<td>PEG-insulin</td>
<td>46%</td>
<td>24%</td>
<td>30%</td>
</tr>
<tr>
<td>PCB-insulin</td>
<td>53%</td>
<td>24%</td>
<td>23%</td>
</tr>
</tbody>
</table>

**Figure 4-4:** MALDI-TOF Mass spectra of native insulin, purified PEG-insulin and purified PCB-insulin where most of the three amines of insulin were conjugated with PEG or PCB.
Figure 4-5: (A) The bioactivity of native insulin, rough PEG-insulin, purified PEG-insulin, rough PCB-insulin and purified PCB-insulin, respectively, as measured by phosphorylation of the insulin receptor in vitro. For the polymer-insulin tested, most of the three amines of insulin were conjugated with PEG or PCB. The relative activity was presented as the percentage of native insulin (n=6). *** p < 0.001. (B) The relative blood glucose concentration as a function of time after the tail vein injection of free insulin, rough PEG-insulin, and rough PCB-insulin (before purifying insulin conjugate products). The data are presented as the percentage of the initial values of blood glucose concentration ± standard deviation (n=6).
CHAPTER 5. CONCLUSION

The focus of this thesis is the potential of the zwitterionic PCBs for drug delivery applications, including constructing micelles for chemo drug delivery and modifying glucose regulating proteins. Specifically, this thesis involves the discovery of the ultra-low CMC property of DSPE-PCB 5K micelle and the enhanced therapeutic performance of docetaxel/DSPE-PCB 5K formulation. In addition, zwitterionic PCB was used to conjugate with insulin and was found to be able to mitigate the long-lasting bioactivity loss problem when proteins were modified with current polymers.

Compared with common conventional micelles, DSPE-PCB 5K showed ultra-low CMC property and high colloid stability in clinically relevant conditions. Such stabilizing effect appeared to be powerful; It was found that even when a zwitterionic moiety or polymer was added into a micelle solution (not chemically modifying the micelle), the existing micelle molecules in the new solution was stabilized at a concentration below its inherent CMC. It is further demonstrated that the ultra-low CMC micelle could stabilize hydrophobic cargo (without micelle dissociation) in diluted conditions and the findings reported here are expected to help the design of other micelle-based formulations to improve their stability. Using a melanoma tumor model, docetaxel/DSPE-PCB 5K formulation showed excellent anti-tumor performance by eliminating tumors in mice without a sign of recurrence. It is expected that the novel micelle system demonstrated here can encapsulate other commercially available chemo drugs and further promoted their therapeutic performance compared with their current micellar formulations.

Polymer-protein conjugation suffered from the bioactivity loss of protein since it was developed. It was found that PCB-insulin conjugation via simple one-step coupling chemistry does not compromise the bioactivity of insulin as confirmed in the in vitro test. In vivo study
indicated enhanced blood circulation time of the PCB-insulin conjugate. Combined with the advantage of improved pharmacokinetics and the prevention of bioactivity loss, PCB-insulin showed better glucose regulation effects in vivo compared with PEGylated insulin and unmodified natural insulin. This finding is likely to inspired future highly effective polymer-protein conjugates to be developed.

In the future, it is of interest to further explore the application of zwitterionic polymer in the drug delivery field based on these two projects. For the ultralow CMC micelle work, it will be of interest to test the encapsulation of other hydrophobic drugs and study anti-tumor performance in other types of cancer models. Further, the scale-up issue of producing ultralow zwitterionic micelles requires investigation for potential future commercialization and clinical trials. For the polymer-protein conjugation work, we are eager to know the mitigation of bioactivity loss of the modified protein can work on other types of therapeutic proteins. This will involve similar studies to this dissertation by conjugating PCB to a few protein drugs of high therapeutic value and measuring the bioactivity of the protein after conjugation.
REFERENCES


ABSTRACT

ZWITTERIONIC POLYMERIC FOR DRUG DELIVERY APPLICATIONS

by

YANG LU

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Advisor: Dr. Zhiqiang Cao

Major: Chemical Engineering

Degree: Doctor of Philosophy

This dissertation focuses on the applications of zwitterionic polymer materials in drug delivery. In one project, zwitterionic polymers were used to construct micelle surfactants. The zwitterionic micelles were found to have an ultralow (< \(2.7 \times 10^{-6}\) mM) critical micelle concentration (CMC) and a potential mechanism for zwitterionic micelles achieving the ultralow CMC property has been studied. Based on the ultralow CMC feature, zwitterionic micelles were used to prepare a formulation of commercial antitumor drug docetaxel, and the resulting formulation showed excellent antitumor performance. In another project, zwitterionic polymers were used to modify the protein drug insulin via conjugation reaction. Compared with current protein modification methods, such as PEGylation, zwitterionic polymer conjugation increased 24% pharmacological activity compared with native insulin without compromising bioactivity.
AUTOBIOGRAPHICAL STATEMENT

Education

B.Sc. in Applied Chemistry, Tianjin University -2013

Ph.D. in Chemical Engineering, Wayne State University –expected 2018

Publications