3-Helix Micelles as Drug Nanocarriers

By

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Engineering - Materials Science and Engineering in the Graduate Division of the University of California, Berkeley

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Abstract

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Control over particle size, cargo loading, encapsulation stability, and drug release is critical to optimize the safety and efficacy of drug formulations. This dissertation is focused on evaluation, optimization, and validation of 3-helix micelles as drug nanocarriers. The fundamental understanding of molecular parameters than can be used to tune loading content, drug retention stability, and release of drug from 3-helix micelles is important for their clinical translation as a viable platform for drug formulation and delivery. It is equally critical to characterize the structure and stability of drug loaded 3-helix micelles in biological conditions relevant to different applications, which in turn would dictate the pharmacokinetics, biodistribution, efficacy and clearance of drug formulations based on 3-helix micelles. The knowledge gained from these studies serves as guideline to identify the suitable design features to facilitate the development of 3-helix micelle based therapeutic nanocarriers.

3-helix micelles are formed by the self-assembly of a new family of amphiphilic peptide-polymer conjugates. Studies aimed at understanding intermolecular interactions that dictate the structure of the amphiphilic conjugates allowed to identify the building block that can be used to generate 3-helix micelles with requisite structure and stability. 3-helix micelles assembled from different amphiphiles were investigated for their biological stability and blood circulation time, and this set of studies resulted in a set of guidelines that could be used to tailor the stability of 3-helix micelles for different biological applications.

Systematic studies on encapsulation of drugs with different molecular structure underscored the significance of molecular geometry of drugs on their loading content in 3-helix micelles. These studies were critical in understanding the parameters that could be used to tune cargo loading content in 3-helix micelles. Drugs loadings of 7-8 wt% could be achieved depending on the cargo and micelle composition. This loading content
is close to the theoretical limit imposed by the small core size of 15 nm 3-helix micelles, and carriers with similar loadings have shown enhanced therapeutic effect.

Doxorubicin (DOX) loaded 3-helix micelles exhibited efficient cellular internalization, elicited cytotoxicity for a range of cancer cell lines, and were sensitive to proteolytic degradation to facilitate drug release. Intravenous administration of DOX loaded 3-helix micelles showed selective accumulation in tumor tissue for an extended period with reduced off-target toxicity, relative to free drug.

Lastly, in vivo studies with 3-helix micelles demonstrated that they could be administered to brain by two different techniques that have been developed for anatomical targeting of severely restricted brain tissue. The results demonstrated that 3-helix micelles could penetrate into deep regions in the brain, not commonly accessible to conventional nanocarriers, which is very encouraging for their potential as nanocarriers for drug delivery to brain.
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## Chapter 1

### Nanocarrier Design: Promise of 3-Helix Micelles

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This chapter begins with the motivation for the work undertaken in this dissertation, which focuses on the design of a unique drug nanocarrier. It is followed by an introduction to evolution of successful nanocarriers that are routinely used in clinical diagnosis and treatment. Critical challenges associated with carrier design are discussed to identify the key features for nanocarriers that need to be optimized and controlled to meet critical requirements for drug formulation and delivery. This is followed by an introduction to recently developed 3-helix micelles based on a new family of amphiphilic peptide-polymer conjugates. The promise of 3-helix micelles as a unique drug formulation platform is discussed. Lastly, the questions that must be addressed to facilitate the development of 3-helix micelles as safe and effective drug nanocarriers are identified and the chapter concludes with the outline of the work presented in this dissertation.

1.1 Motivation
Various nanoparticles have been studied as nanocarriers to improve solubility, stability, pharmacokinetics and biodistribution of hydrophobic drugs. Liposomes and polymeric micelles have been investigated extensively as drug nanocarriers, but have shown modest therapeutic benefits due to cargo leakage and poor penetration in tumor tissue. There is a great need to develop stable drug nanocarriers, 10-30 nm in size, with deep tumor penetration and enhanced therapeutic efficacy. Recently developed 3-helix micelles, 15 nm in size, exhibit high in vivo kinetic stability and favorable biodistribution with minimal accumulation in liver and spleen and appear highly promising as nanocarriers. Detailed understanding of molecular parameters that dictate cargo loading and encapsulation stability in 3-helix micelles is critical in order to realize their potential as effective drug nanocarriers. It is equally important to establish the effect of drug loading on the structural and biological stability of micelles, which in turn, determines the pharmacokinetics, efficacy and clearance profile of drug formulated 3-helix micelles. Micellar subunit based on protein tertiary structure combined with small size and exceptional stability of 3-helix micelles present unique challenges for drug formulation. It is critical to develop an understanding of the parameters that can be used to tune micelle stability, cargo loading content, release kinetics, and in vivo circulation time. The knowledge gained through these studies would facilitate the design of nanocarriers based on 3-helix micelles for different biological applications with strict requirements on safety and efficacy.

1.2 Evolution of Nanocarriers
Nanotechnology has proved to be highly promising for the design of diagnostic and therapeutic platforms with superior properties that allow many benefits including early detection, active targeting, and safe and effective treatment for different diseases. Nanoscale materials with sizes similar to naturally occurring biomolecules such as protein receptors, antibodies, and nucleic acids are better suited to interact effectively with the complex biological environment in the body. Nanoparticles, by virtue of their size, are highly effective in navigating systemic and cellular barriers that allows them to reach their target and exert intended biological effect.
Several distinct platforms based on lipids, albumin, polymers, dendrimers and inorganic/metalllic materials have been approved or have entered clinical development as diagnostic and therapeutic nanocarriers (Figure 1.1). Liposomes were the first therapeutic nanomedicine to reach commercialization with the FDA approval of Doxil (liposome encapsulated doxorubicin) in 1995. The encapsulation of doxorubicin changes the pharmacokinetics and biodistribution, allowing for longer circulation half-life, higher tumor concentration, and reduced cardiotoxicity of formulated drug compared to free drug. Albumin bound paclitaxel nanoparticles, Abraxane, became the second class of therapeutic nanomedicine to be commercialized in 2005, for the treatment of metastatic breast cancer. Polymeric micelles based on amphiphilic block copolymer self assembly were approved for paclitaxel delivery in Korea in 2007, and are in clinical trials in United States. Targeted polymeric nanoparticles whose surface is modified with cancer cell specific ligands have entered clinical development in United States for treatment of lung and prostate cancer. Iron oxide nanoparticles have shown high potential as diagnostic agents that are in clinical use as contrast agents for magnetic resonance imaging. Most of the nanocarriers that have advanced to clinical development are in the size range of 50-150 nm, which indicates a need for smaller nanocarriers, 10-30 nm in size.

Figure 1.1: Development timeline for different nanocarrier platforms.
Despite their enormous potential, clinical development of nanocarriers has faced considerable challenges. Control over the structure, stability and other physicochemical property of nanocarriers is required to optimize their performance in complex biological conditions relevant to clinical use. Detailed understanding of the effect of particle design parameters on their biophysicochemical properties must be established to facilitate the development of nanocarriers that can be synthesized reproducibly with consistent quality, and can be tailored for different biological applications. Fundamental studies focused on understanding the effect of size, shape, stability, and surface chemistry of nanoparticles on their pharmacokinetics, biodistribution, and clearance profile in the body are required to facilitate the design of carrier platforms with high potential of clinical success in the field of nanomedicine.

1.3 Design Considerations for Drug Nanocarriers

Nanoparticles have shown tremendous potential for different biomedical applications including prevention, diagnosis, and treatment of a number of critical diseases. The scope of nanocarriers in the field of medicine is vast, and it is critical to address the fundamental challenges involved in the design of nanocarriers intended for effective use in different biological applications. This section discusses some of the key features that need careful attention for nanocarrier design for drug formulation and delivery which is the focus of the work described in this dissertation.

Size: Nanoparticle size has a critical effect on their transport behavior at systemic, tissue and cellular levels. Currently nanoparticles in the size range of 20-200 nm are being developed as drug delivery vehicles for cancer therapeutics. The lower bound on size is based on the measurement of sieving coefficients for the glomerular capillary walls in kidney, which suggests that threshold for first-pass elimination by the kidneys is 10 nm. The upper limit on carrier size is primarily dictated by the ability of particles below 200 nm to preferentially accumulate in tumor tissues through leaky vasculature mediated by enhanced permeability and retention (EPR) effect. Current FDA-approved nanocarriers, 100-130 nm in size, can extravasate into tumor via EPR effect, but fail to penetrate deep into the tumor tissues. This results in inefficient transport of the chemotherapeutic drug into the tumor with modest survival benefits. Studies have shown that following extravasation into tumor interstitium, a drug or drug encapsulated vehicle should be capable of travelling up to 100 μm away from the tumor vasculature in order to reach all cells within the tumor. Nanocarriers need to be below a certain size to achieve significant tumor penetration. Recent studies have established that smaller particles, 10-30 nm in size, can more effectively penetrate the physiological barriers imposed by abnormal tumor vasculature and dense interstitial matrix in tumor tissues and thus result in deeper and more homogeneous tumor penetration with higher intratumor drug concentration. Smaller nanoparticles in size range of 15-40 nm have also been shown to exhibit greater extent of cellular accumulation and faster rate of internalization in cancer cells. Based on these considerations on favorable characteristics exhibited by smaller particles, there is a clear need to design and synthesize drug nanocarriers in the size range of 10-30 nm with potentially enhanced therapeutic efficacy.
Stability: The kinetic stability of nanocarriers has critical implications for their in vivo circulation time, biodistribution, cargo release, and clearance pathway. Liposomes have been extensively used as drug nanocarriers, however their limited stability presents a serious challenge for their translation as safe and effective drug nanocarriers. A number of stable polymeric micelles with hydrodynamic diameters of 40-150 nm have been developed, where engineering the intermolecular interactions between micelle forming block copolymers has been used as an effective approach to increase carrier stability. However, smaller micelles, 10-20 nm in size, generally are not as stable, exhibiting typical exchange half-lives of a few hours, and this dramatically compromises their potential as nanocarriers. In order to utilize the favorable transport and biodistribution profile offered by smaller nanocarriers less than 20 nm in size, control over their in vivo stability and blood circulation time is required. Dendrimers represent the predominant class of smaller 10-15 nm nanocarriers that can achieve extended blood circulation half-life required for increased accumulation at tumor site mediated by EPR effect. However, the complex chemistries involved in synthesis of biodegradable dendrimers with controlled size that can be cleared from the body, limits their use as an effective nanocarrier platform. We believe that the design of smaller nanocarriers, 10-20 nm, with high circulation time and tunable stability, is important to address the current gaps in the field of effective nanocarriers intended for drug delivery.

Cargo Loading and Release Kinetics: Reproducible loadings of drugs at therapeutically effective concentrations are required for nanocarrier based drug formulations. Liposomes and polymeric micelles have been used extensively for drug formulation, and loadings over a broad range of 2-25 wt% have been achieved in these systems. Systematic studies focused on understanding the effect of molecular parameters on cargo loading capacity have established the significance of intermolecular interactions between drug and carrier material and the physical space available in nanocarrier for drug localization in tuning drug loading content in these systems. Engineering non-covalent interactions such as hydrophobic, ionic, hydrogen bonding interactions between drug and carriers is routinely used to tune loading content in nanocarrier formulations. Higher loadings in the range of 15-25 wt% have been achieved in larger nanocarriers, 100-150 nm in size. There have been limited reports on encapsulation of drugs in smaller nanocarriers which makes the studies aimed at understanding the molecular parameters that control cargo loading content in 10-20 nm nanocarriers absolutely essential.

Cargo release kinetics from nanocarriers is an equally critical design consideration and requires careful optimization based on the stage and intended use of drug formulated carrier. During storage, reformulation, and blood circulation after administration, high encapsulation stability with minimal cargo leakage from carrier is desirable to minimize changes in administered doses and accumulation of drug at off-target sites in the body. Drug nanocarriers intended for intravenous administration are confronted with highly concentrated protein environment, which can lead to fast drug release. Premature drug release from the formulation may lead to systemic side effects due to distribution of drug to nontarget tissues and inefficient drug accumulation at the target site. Evaluation of the stability of drug association with nanocarrier in plasma relevant conditions is
critical for accurate prediction of circulation stability and biodistribution of drug loaded carriers, which would be important for establishing the viability of nanocarriers as \textit{in vivo} therapeutic platforms. The release of the drug at disease site is an equally critical requirement for effective nanocarriers. Nanocarriers have been designed to release drugs in response to disease specific environmental triggers such as pH and proteases.\textsuperscript{50-53} Combining all these considerations, an ideal drug nanocarrier should exhibit small drug release during storage and circulation, but should be sensitive to controlled or triggered drug release at the disease site for desired therapeutic benefits.

1.4 Promise of Peptide-Polymer Conjugates for Nanocarrier Design

Hybrid materials based on combination of proteins, polymers, lipids are highly promising to combine the unique properties offered by each class of materials to result in a new family of materials with potentially novel structure and superior properties.\textsuperscript{54-56} Covalent conjugation of peptide/proteins and synthetic polymers is a promising strategy to combine the structural and functional complexity offered by peptide/proteins and chemical diversity of polymers. Peptide-polymer conjugates have shown the potential of synergistically combining the advantages of the constituent materials to result in hierarchical nanostructures.\textsuperscript{57-59} Amphiphilic materials based on peptide-polymer conjugates are promising for their self assembly and structures obtained could have potential applications as nanocarriers. Conjugation of hydrophobic polymers to enzymes and proteins has led to the development of giant amphiphiles containing large protein head group and hydrophobic polymer tails.\textsuperscript{60-63} Giant amphiphiles have been studied for the fundamental understanding of their assembly into micelles and vesicles; however their potential as drug nanocarriers has not been explored. Peptide amphiphiles based on covalent conjugation of short lipid chains to a peptide headgroup represent the most significant family of hybrid materials that have been studied for drug delivery applications. Peptide amphiphiles that assemble into high aspect ratio nanofibers have been used for encapsulation of a number of anti-cancer and anti-inflammatory drugs.\textsuperscript{64, 65} The biological studies with these constructs have shown the promise of peptide based nanostructures as vehicles for sustained drug release with applications in regenerative medicine and cancer therapeutics.\textsuperscript{66, 67}

We are interested in development of a new family of hybrid materials based on peptide/proteins and polymers for the design of small nanoparticles, 10-20 nm in size, that meet critical requirements for safe and effective nanomedicine as discussed in the last section. Our group recently reported the design of small spherical micelles from self-assembly of a new family of amphiphiles based on coiled-coil 3-helix bundle forming peptide-poly(ethylene-glycol) conjugates.\textsuperscript{11} Figure 1.2 shows the design of the amphiphile based on a 3-helix peptide-polymer conjugate. It consists of a short peptide helix with a PEG chain covalently attached to the middle of the helix and two hydrophobic alkyl tails attached to the N-terminus. The peptides self-associate into 3-helix bundles, forming a trimeric subunit where three PEG chains are anchored to the exterior of 3-helix bundles. The designed amphiphiles form stable 3-helix micelles, 15 nm in size. The 3-helix micelles demonstrated significantly enhanced \textit{in vivo} kinetic stability in comparison to existing micelles in similar size range. The superior stability of
3-helix micelles has its origin in the molecular structure of micellar headgroup. Directional entropic repulsions between PEG chains mediated by protein tertiary structures in the shell of 3-helix micelles slow down the subunit exchange and stabilize individual micelles.68

Figure 1.2: Schematic drawing of the designed amphiphile containing a short peptide helix with a polymer chain covalently linked to the side chain of the helix, hydrophobic tails attached to the N-terminus. Three amphiphiles associate to form a subunit, where the headgroup contains a 3-helix bundle with polymers covalently attached to the exterior. This subunit self assembles into 3-helix micelles, where the shell is composed of 3-helix bundles and the core is composed of aliphatic chains.

1.5 Questions Investigated and Synopsis of Subsequent Chapters
The development of a nanocarrier platform that meets requirements for a safe and effective drug delivery vehicle is the fundamental motivation for the work described in this dissertation. From drug delivery point of view, the critical requirements for the success of nanocarriers intended for intravenous administration are reproducible drug loadings in significant quantity, aqueous solubility of the formulation, stability in blood circulation with high retention of encapsulated agent, accumulation of the carrier at the disease site, controlled drug release from the carrier and minimal toxicity to healthy tissues. There has been limited progress in the design of micellar nanocarriers in size range of 10-30 nm that meet above mentioned requirements for effective drug formulation and delivery. The work described in this dissertation addresses the challenges associated with development of micellar drug formulations, 15-20 nm in size. Systematic studies described here provide critical insights into the molecular parameters that control biological stability, cargo loading content, release kinetics, and biodistribution of smaller drug nanocarriers. These studies have important implications for clinical translation of drug formulations based on nanoparticles much smaller than conventional nanocarriers.

The focus of the work described in this dissertation is on evaluation, optimization, and validation of 3-helix micelles as a nanocarrier platform for drug delivery. 3-helix micelles are formed by self-assembly of hybrid materials based on amphiphilic peptide-polymer
conjugates. Fundamental understanding of the structure of different amphiphiles that could be potentially used for the generation of 3-helix micelles is critical to establish the choice of building block that would result in consistent and reproducible micellar assembly with desired size and stability. Chapter 2 discusses the conjugation of hydrophobic polymers to the side of coiled-coil helix bundle which results in amphiphilic materials that can be used to generate hierarchical assemblies. The results from these studies were crucial for understanding the intermolecular interactions that dictate the peptide structure and function in amphiphilic conjugates. The designed conjugates were found to retain peptide structure in organic medium, but were not suitable for use as an amphiphilic building block for generating micellar nanocarriers in biologically relevant aqueous environments. Amphiphiles consisting of a headgroup based on a hydrophilic peptide-polymer conjugate and tail composed of hydrophobic alkyl chains were identified as the optimal building blocks for generating 15 nm 3-helix micelles as described in earlier work. Chapter 3 is focused on the characterization of cargo encapsulation stability, pharmacokinetics, and biodistribution of 3-helix micelles as a function of length and hydrophobicity of tail forming alkyl chains in this new family of amphiphilic peptide-polymer conjugates. The results from this study established the effect of a key molecular parameter on biological stability of 3-helix micelles and were important for the identification of the construct most suitable for use as a stable nanocarrier in complex biological environments. Chapter 4 discusses the detailed structural and biological characterization of doxorubicin loaded 3-helix micelles to validate the potential of 3-helix micelles as a viable drug nanocarrier platform for intravenous administration. Chapter 5 describes the loading of a range of drugs with different molecular structure in 3-helix micelles to explore their versatility as a drug formulation platform. These studies provide critical insights into the molecular parameters that dictate cargo loading content in 3-helix micelles that are significantly smaller than conventional nanocarriers. The results from this systematic study serve as guideline to tune loading content and encapsulation stability for a range of structurally diverse drugs in 3-helix micelles. Lastly, Chapter 6 presents an evaluation of the feasibility of the administration of 3-helix micelles by two different techniques that allow anatomic targeting of brain for therapeutic delivery.
Chapter 2

Amphiphilic Peptide-Polymer Conjugates with Side-Conjugation

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Polymers conjugated to the exterior of a protein mediate its interactions with surroundings, enhance its processability and can be used to direct its macroscopic assemblies. Most studies to date have focused on peptide-polymer conjugates based on hydrophilic polymers. Engineering amphiphility into protein motifs by covalently linking hydrophobic polymers has the potential to interface peptides and proteins with synthetic polymers, organic solvents and lipids to fabricate functional hybrid materials. Here, we synthesized amphiphilic peptide-polymer conjugates in which a hydrophobic polymer is conjugated to the exterior of a heme-binding 4-helix bundle and systematically investigated the effects of the hydrophobicity of the conjugated polymer on the peptide structure and the integrity of the heme-binding pocket. In aqueous solution with surfactants present, the side-conjugated hydrophobic polymers unfold peptides and may induce an \( \alpha \)-helix to \( \beta \)-sheet conformational transition. These effects decrease as the polymer becomes less hydrophobic and directly correlate with the polymer hydrophobicity. Upon adding organic solvent to solubilize the hydrophobic polymers, however, the deleterious effects of hydrophobic polymers on the peptide structures can be eliminated. Present studies demonstrate that protein structure is sensitive to the local environment. It is feasible to dissolve amphiphilic peptide-polymer conjugates in organic solvents to enhance their solution processability while maintaining the protein structures.

2.1 Introduction

As an emerging family of soft matter, hybrid materials based on peptide/proteins and polymers have the potential to achieve structural control over multiple length scales, down to the molecular level, and exhibit enriched chemical heterogeneity and functionalities inherent to peptides/proteins.\(^1\)-\(^{10}\) The chemical nature of conjugated polymers and the architecture of the protein-polymer conjugate have profound effects on the protein structure and functionalities and the self-assembly of peptide-polymer conjugates.\(^{11}\)-\(^{20}\) Hydrophilic peptide-polymer conjugates with different architectures have been prepared and studied.\(^{11}\)-\(^{15}\) Conjugating poly(ethylene glycol) (PEG) to the exterior of the helix bundle enhances peptide helicity, stabilizes helix bundle formation and does not interfere with the binding pocket in the interior of the helix bundle.\(^{14}\) The conjugated PEG also mediates the peptides’ interactions with the local environment, assisting protein solubilization in organic solvent and interfacing with synthetic polymers.\(^{21}\)

Amphiphilic peptide-polymer conjugates prepared from chemical linking of hydrophobic polymers to peptides appear promising to expand the diversity of nanostructures and built-in functionalities of hybrid biomaterials. Conjugated hydrophobic polymers will significantly enhance the processability of the peptide/protein-polymer conjugates and expand the library of matrices in which to embed the biological motifs.\(^{22}\)-\(^{24}\) Most of the studies on amphiphilic conjugates to date have focused on peptide and polymers conjugated as hybrid diblock copolymers. Self-assembly of conjugates containing helical polypeptides have been extensively investigated in solution and in solid state. In solution, peptide amphiphilies assemble into different morphologies such as spherical micelles, worm like micelles and vesicles.\(^{25}\)-\(^{28}\) The dependence of secondary structure of peptide on environmental parameters such as pH, temperature, ionic strength has been utilized to modulate the shape and size of nanostructures. In solid phase, hierarchical assemblies
such as hexagon-in-lamellae and hexagon-in-hexagon have been observed.\textsuperscript{29-32} Amphiphilic conjugates containing peptides forming β-sheets and nanotubes have also been investigated.\textsuperscript{33-37} Aggregation of peptides into β-sheets results in fibrillar and tape-like helical superstructures.\textsuperscript{33-35} Conjugated polymers can be used to control the peptide self-organization, such as to tailor the aspect ratio of polymer-covered nanotube.\textsuperscript{36, 37}

Conjugation of hydrophobic polymers to enzymes and proteins has led to the development of giant amphiphiles containing large protein head group and hydrophobic polymer tails.\textsuperscript{18, 19, 38, 39} In solution, giant amphiphiles self-assemble into vesicles, micelles, and fibers of micellar rods similar to those formed by conventional amphiphilic block copolymers. Peptide-polymers based on protein sequences forming tertiary structures, such as coiled-coil helix bundles, are of particular interest as they strike a good balance between structural complexity and richness in functionalities.\textsuperscript{1, 10} Protein tertiary motifs often have predictable protein folding and can be used as model systems to develop fundamental understanding of the thermodynamics governing the co-assembly of peptides and synthetic polymers.\textsuperscript{40, 41} Some protein tertiary motifs can mimic basic functionalities of complex proteins and carry out catalytic reactions, transport, light-induced charge separation and sensing.\textsuperscript{42-45} Directed assemblies of their conjugates may directly lead to functional biomolecular materials. Amphiphilic peptide-polymer conjugates derived from or designed on transmembrane proteins are particularly interesting as this may potentially lead to hybrid membranes with unique transport properties with high integrity in aqueous solution. The amphiphilic peptide-polymer conjugates may also form vesicles with helix bundles embedded at the interfaces and lead to man-made reactors mimicking those seen in cells.\textsuperscript{46, 47} However, studies of linear type peptide-polymer conjugates showed that the hydrophobicity of the conjugated polymer affects the integrity of the coiled-coil helix bundle. A strong distance dependence was observed, and simple blending of helix bundle peptides in hydrophobic polymer thin films led to smaller structural perturbations than covalent conjugation of the polymer to the peptides.\textsuperscript{20} Thus, it is requisite to quantify the effects of the side conjugation of a hydrophobic polymer and identify conditions under which protein structures can be retained.

Here, amphiphilic peptide-polymer conjugates based on a heme-binding 4-helix bundle were synthesized where polymers are site-selectively conjugated to the exterior of the helix bundle. The effects of polymer hydrophobicity on the peptide structures were systematically investigated. A direct correlation between the polymer hydrophobicity and peptide structure in aqueous solution with surfactants present is observed. The presence of the hydrophobic polymer unfolds the peptide helices and induces an α-helix to β-sheet conformational transition. Both lead to deleterious effects on the binding pocket of the heme-binding helix bundle. However, by adding organic solvents that solubilize the hydrophobic polymers, the peptide helical structure is retained. These results show that the protein structure in a peptide-polymer conjugate reflects a competition between peptide-polymer interactions and polymer-solvent interactions. Present studies provide useful guidelines to design amphiphilic peptide-polymer conjugates and to direct their macroscopic assemblies.
2.2 Results and Discussion

2.2.1 Synthesis of Side Conjugates

A coiled-coil 4-helix bundle forming peptide based on the diheme Cytochrome b subunit of cytochrome bc1 was used and referred to as H10H24 (Ac-GGGEIWKLHEEFLKKFEELLKLHELKKM-NH2). Though the histidine residues in this reported sequence fall at positions 9 and 23 due to deletion of a previously employed N-terminal cysteine residue, they will still be referred to as H10 and H24 for consistency with previous reports. The overall scheme for the synthesis of amphiphilic peptide-polymer conjugates is depicted in Figure 2.1.

![Figure 2.1: Schematic of the synthesis of peptide-polymer conjugates. (a) Solid phase synthesis of H10H24 with Lys(Alloc) at position 15. (b) Orthogonal deprotection of Lys(Alloc) on solid phase. (c) Solid phase conjugation of carboxy terminated polystyrene (n = 6-15) to the peptide. (d) Cleavage and deprotection of H10H24-PS. H10H24-PMMA and H10H24-PNIPAAM were synthesized using a similar approach. Black circle denotes the resin.](image)

H10H24 was synthesized using solid phase synthesis, incorporating Lys(Alloc) at the 15th position. The Alloc protecting group was selectively removed on the resin and used to site-selectively conjugate the hydrophobic polymers. Alloc removal was monitored by MALDI-TOF MS as shown in Appendix (A.1.1). The shift of the peak position by 85 units from 3820 to 3735 Da is consistent with the removal of the Alloc group. Carboxy-terminated PS was subsequently conjugated to Lys15 before cleaving the conjugates from the resin. The details of purification and characterization of H10H24-PS are given in Appendix (A.1.2). Other conjugates, H10H24-PMMA and H10H24-PNIPAAM, were also synthesized using a similar approach as detailed in Appendix.
2.2.2 Structural Characterization of PS Conjugates

n-Octyl-β-D-glucopyranoside (OG), a nonionic detergent with a critical micelle concentration of 0.73 wt % was used to solubilize the amphiphilic conjugates. Based on previous studies of the linear H10H24-PS conjugate, where a PS chain is attached to the peptide N-terminus, buffer solution containing 0.45 wt % OG was used.\textsuperscript{20} Upon dissolving in 25 mM potassium phosphate, pH 8.0, containing 0.45 wt % OG (referred to hereafter as phosphate/OG buffer), H10H24-PS forms nanoparticles ~5.1 nm in diameter as observed by DLS (Figure 2.2). This is smaller than the micellar nanoparticles, ~8.8 nm in diameter, formed by its linear analog L-H10H24-PS.\textsuperscript{20}

Figure 2.2: DLS of H10H24-PS ($D_h = 5.1$ nm) in phosphate/OG buffer, at concentration of $60 \mu$M.

Figure 2.3 shows the CD spectrum of 60 μM H10H24-PS conjugates dissolved in phosphate/OG buffer solution. Peaks at 208 nm and 222 nm, a characteristic feature of α-helical conformation, can be clearly seen. In comparison to the CD spectrum of pristine H10H24, a plateau around 216 nm was also seen, suggesting the presence of β-sheets. The relative fractions of three peptide conformations for H10H24-PS were determined by fitting the experimental CD spectrum with a linear combination of α-helix, β-sheet, and random coil basis spectra. The basis CD spectrum used for α-helix was that of pure H10H24, and the basis spectra used for β-sheet and random coil were based on data collected for poly(L-lysine) as described previously.\textsuperscript{48} The fitting yielded ~54% α-helical, ~19% β-sheet, and ~27% random coil content (A.1.4). The helicity of H10H24-PS conjugate is lower than that of H10H24 (~75%). The presence of PS unfolds the peptide helix and induces an α-helix to β-sheet transition. The effects of side-conjugated PS on the heme-binding pockets of H10H24 were also evaluated. The heme titration
experiments showed that H10H24-PS binds only two hemes per bundle, rather than four hemes per bundle as observed for unmodified H10H24 (A.1.5).

Previous work showed that the conjugation of PEG to the same position stabilizes the peptide helical conformation and does not interfere with the integrity of the binding pocket in the interior of H10H24 helix bundle. Thus, the observed protein structural changes can be attributed to the hydrophobicity of PS. The fact that H10H24-PS binds two heme molecules suggests that the assembled peptide bundle is partially maintained and the structural transformation observed here occurs at the immediate vicinity of the site of PS conjugation. To reduce the unfavorable interactions between the PS block with water, the PS has to be capped by either OG surfactant and/or amino acids. The hydrophobic interactions between the PS and the amino acid residues in the vicinity of the conjugation site lead to perturbations in the local peptide structure. The fitting of the CD spectrum indicated ~19% β-sheet content in H10H24-PS. We speculate that amino acids (~6 residues) in the vicinity of the polymer conjugation site have transformed to a β-strand, while the rest of the peptide still retains ~54% helicity. Hartgerink et al showed that insertion of a hydrophobic amino acid at the f position of the helix heptad repeat may lead to an α-helix to β-sheet transition. A hydrophobic patch or cluster consisting of residues at the d, f, and a positions leads to an alternating hydrophilic/hydrophilic pattern that stabilizes β-sheet conformation.49

Figure 2.3: CD spectra of 60 µM H10H24-PS and H10H24 solutions in phosphate/OG buffer, at concentration of 60 µM.
Based on DLS, CD and heme titration results, we conclude that H10H24-PS conjugate forms a 5.1-nm nanoparticle in phosphate/OG buffer, consisting of one 4-helix bundle with PS capped by the OG surfactants as schematically shown in Figure 2.4.

Figure 2.4: Schematic drawing of H10H24-PS, 4-helix bundles in phosphate/OG buffer. Thick lines show the random coil portion of unfolded peptide, while the thin rod segment is the β-strand portion. Polystyrene chains shown as thin curved lines, interact with the hydrophobic tail of surfactant.

A closer look at H10H24 sequence in the vicinity of Lys15 suggests that two phenylalanine residues, Phe13 and Phe17, at the $d$ and $a$ heptad positions, respectively, could interact with the phenyl side chains of PS at 15th position and form a β-sheet. The β-sheets on two parallel adjacent peptides of the anti-parallel 4-helix bundle of H10H24-PS can satisfy the local hydrogen bonding between the peptide chains. PS chains conjugated to the exterior of the 4-helix bundle are accessible to solution and can be solubilized by OG surfactants. The architecture of the amphiphilic peptide-polymer conjugate and the local polymer-peptide interactions play an important role in the protein structure and its phase behavior in solution.

2.2.3 Structural Characterization of PMMA Conjugates
To evaluate the effect of polymer hydrophobicity, H10H24-PMMA was synthesized by attaching PMMA to Lys15. PS and PMMA have similar backbones, but different side chains that lead to differences in hydrophobicity. The water contact angle of PS is ~90°, indicating that PS is more hydrophobic than PMMA which has a water contact angle of ~75°. DLS shows H10H24-PMMA forms nanoparticles with diameters ~6 nm in phosphate/OG buffer. The heme titration experiments show similar results for H10H24-PMMA and H10H24-PS conjugates, showing that the helix bundle formation was partially retained upon PMMA side-conjugation. All together, these results suggest
H10H24-PMMA conjugates form nanoparticles containing one 4-helix bundle capped by OG, similar to that shown in Figure 2.4.

Figure 2.5 shows the CD spectra of H10H24-PMMA conjugates with a peptide concentration of 60 µM dissolved in phosphate/OG buffer. CD spectra of H10H24-PS and H10H24 are also shown for comparison. Minima at 208 and 222 nm confirmed α-helical conformation. In comparison to H10H24-PS, the sharp, well-defined minima for H10H24-PMMA suggest that the β-sheet content is reduced significantly. The fitting of the experimental CD spectrum of H10H24-PMMA with a linear combination of basis spectra for α-helix, β-sheet, and random coil conformations as discussed earlier yields ~60% helix and ~40% random coil content. The helical content determined by fitting is close to the percent helicity calculated based on the ellipticity value at 222 nm (~58%). The helical content for H10H24-PMMA (~60%) is slightly higher than that for H10H24-PS (~54%). More interestingly, the majority of the unfolded fraction conforms to β-sheet in the case of H10H24-PS, whereas the unfolded fraction adopts a random coil conformation in H10H24-PMMA conjugate. As discussed above, hydrophobic amino acid residues, particularly Phe13, Leu14, and Phe17, present near the polymer conjugation site (15th position) interact with the attached polymer. The hydrophobic interaction of amino acid side chains of H10H24 is greater with the phenyl groups of PS than with the more polar methyl esters in PMMA. The maintenance of individual 4-helix bundles in the grafted H10H24-PS conjugates is advantageous for their use as building blocks for the generation of functional materials. The strength of peptide-polymer interactions in the case of H10H24-PMMA is not sufficient to induce β-sheet structure and the amino acids adopt a random coil conformation.

Figure 2.5: CD spectra of 60 µM H10H24-PMMA solution in phosphate/OG buffer.
These observations provide very interesting insights into the nature of polymer-peptide interactions. Both PS and PMMA are considered highly hydrophobic polymers; however the presence of ester groups in PMMA renders it less hydrophobic than the completely non-polar PS. The difference in the degree of hydrophobicity of PS and PMMA plays a significant role in determining the interaction between the polymers and the neighboring amino acids and leads to differences in the conformation and self-assemblies of the two conjugates. The peptide’s local secondary and tertiary structure and functionality of helix bundles is largely dictated by the hydrophobicity of the conjugated polymer.

2.2.4 Structural Characterization of PNIPAAM Conjugates
The effect of the hydrophobicity of the conjugated polymer on the protein structures was further studied by conjugating the most widely studied thermo-responsive polymer, poly(N-isopropylacrylamide) (PNIPAAM). Figure 2.6 shows the CD spectrum of H10H24-PNIPAAM with peptide concentration of 60 µM dissolved in phosphate/OG buffer at 25°C. Peptide secondary structure for H10H24-PNIPAAM was preserved at room temperature, with a helical content similar to the unmodified peptide. Heme titrations further showed that the hydrophobic pocket in the interior of helix bundle for cofactor binding is completely intact after the PNIPAAM conjugation (A.1.6).

Figure 2.6: CD spectra of 60 µM H10H24-PNIPAAM solution in phosphate/OG buffer containing phosphate buffer.
PNIPAAm exhibits a coil-to-globule transition above its lower critical solution
temperature (LCST) at 32°C in aqueous solution. Below the LCST, it is hydrophilic,
and above the LCST, it is hydrophobic. The secondary structure of H10H24-PNIPAAm
conjugates was investigated as a function of temperature. CD spectra were recorded as
the temperature was increased from 5°C to 95°C at a rate of 1°C/min. Figure 2.7 shows
the temperature-dependant CD spectra of H10H24-PNIPAAm conjugates dissolved in
phosphate/OG buffer. The gradual disappearance of the well-defined minimum at 222 nm
with increasing temperature is evident and is consistent with the disruption of the peptide
helical conformation with temperature. The original helicity was recovered when
H10H24-PNIPAAm was cooled back to room temperature, indicating that thermal
unfolding of the conjugates was reversible. The inset plots the helicity of H10H24 and
H10H24-PNIPAAm as a function of temperature. The helicity is normalized to the value
at the starting temperature (5°C). The melting curve of H10H24-PNIPAAm is similar in
shape to that of H10H24. Conjugating PNIPAAm does not affect the cooperativity of the
unfolding processes. The helical content of both H10H24 and H10H24-PNIPAAm
decreases with increasing temperature.

Figure 2.7: CD spectra of 60 µM H10H24-PNIPAAm solution in phosphate/OG buffer
with increasing temperature from 10°C to 90°C. Inset plots the change in helicity
normalized to the initial value of helicity at 5°C for 60 µM H10H24-PNIPAAm and
H10H24 solution in phosphate/OG buffer as a function of temperature.
To decouple this inherent temperature-dependent change in the peptide structure from the changes due to thermally-induced differences in PNIPAAm hydrophobicity, Figure 2.8 shows the difference between the helicity of H10H24-PNIPAAm and H10H24 as a function of temperature. This difference increases at temperatures higher than 35°C, close to the LCST of PNIPAAm. We attribute the larger helicity losses for H10H24-PNIPAAm to the increase in polymer hydrophobicity above the LCST, which leads to unfolding of α-helices. These observations agree well with those seen for H10H24-PS and H10H24-PMMA conjugates.

Figure 2.8: Difference in the normalized helicity between H10H24-PNIPAAm and H10H24 as a function of temperature. The dashed line corresponds to the temperature (~35°C), where the decrease in helicity of the conjugate compared to the unmodified peptide starts to increase.

### 2.2.5 Effect of Organic Solvent on Peptide Structure in Conjugates

The results to this point suggest that peptides unfold and act as surfactants to mediate non-favorable interactions between the hydrophobic polymer and water molecules. Studies of these three different conjugates clearly demonstrate that the peptide structures depend on the hydrophobicity of the polymer. The local protein structure reflects a competition between polymer-solvent interactions and polymer-peptide interactions. The presence of the surfactant OG in the aqueous solution is not sufficient to mediate the non-favorable interactions between PS and aqueous media. We hypothesized that changing the polarity of the solvent media may solubilize the hydrophobic polymer domain more effectively, and eliminate the need to unfold the peptide to shield the PS-water interactions, and minimize the effects of hydrophobic polymers on the local peptide
folding. Two solvent systems were investigated, methanol and a THF/water mixture. H10H24 maintains its secondary structure in methanol solution. PS alone is not soluble in methanol. However, the differences in the solubility parameters between methanol and PS are much smaller than that between water and PS, 5.4 vs. 14.3 (cal/cm$^3$)$^{1/2}$. This may lead to a weaker driving force to unfold the protein to shield the PS-methanol interactions. The energy lost by forming the helices may be sufficient to overcome unfavorable interactions of PS with methanol molecules. The second solvent system used was THF/water co-solvent containing good solvents for both the PS block and the peptide. H10H24-PS conjugates were readily solubilized in the THF/water mixture at a 4:6 volume ratio.

As shown in Figure 2.9, helical structures with high percent helicity were found for both the H10H24-PS and H10H24-PMMA conjugates dissolved in methanol. Similar effects were seen for the co-solvent of THF and water at a 4:6 volume ratio. By balancing the interactions between hydrophobic polymers with local media, the peptide secondary structure can be maintained.

Figure 2.9: CD spectra of 60 µM H10H24-PS and H10H24-PMMA solutions in different organic solvents, namely methanol and tetrahydrofuran. CD spectrum of unmodified H10H24 in phosphate buffer at similar concentration is also shown.
Figure 2.10 shows the schematic drawing describing the conformation of the amphiphilic peptide-polymer conjugate dissolved in organic solvents. Many coiled-coils are stabilized by the hydrophobic interactions where hydrophobic residues at \(a\) and \(d\) positions are buried in the interior of the helix bundle. Organic solvents also solubilize these hydrophobic residues and disrupt the helix bundle formation. This leads to the loss of binding pockets as well as other built-in functionalities in the interior of the bundle. This requires further optimization to balance energetic contributions from helix bundle formation and polymer-solvent interactions. In addition to hydrophobic interactions, other driving forces such as hydrogen bonding, salt-bridges and disulfide bonds can be used to stabilize the protein tertiary structures. Our recent studies showed that upon incorporating water-soluble peptide-polymer conjugates such as H10H24-PEG into a polymer thin film, specific intermolecular interactions lead individual helices to associate into helix bundles with the heme-binding pocket remaining intact. It is unclear whether this is case specific for H10H24 alone and requires further investigation. However, it does present a promising lead to process amphiphilic peptide-polymer conjugates based on coiled-coil helix bundles without interfering with protein structure and functionality.

Figure 2.10: Schematic of H10H24-PS depicting peptide as single helix with polymer chains solubilized in organic solvent. Polymer chain is shown as curved line and organic solvent molecules are shown as dots.

2.3 Conclusion
A new family of amphiphilic peptide-polymer conjugates was synthesized by site-selectively conjugating hydrophobic polymers to the exterior of a de novo designed 4-
helix bundle. The secondary structure of the conjugate was found to be highly sensitive to its microenvironment. The resulting protein structure was found to depend on the competing interactions between the amino acids near the conjugation sites, polymer and the solvent. The use of organic co-solvents presents an effective approach to maintain protein structures while enhancing their solution processability. Present studies provide useful guidelines to design amphiphilic peptide-polymer conjugates and to direct their macroscopic assemblies.

2.4 Experimental

2.4.1 Materials
Amino acid side chains were protected as: Glu(OtBu), Trp(Boc), His(Trt), and Arg(Pbf). Allyloxy carbonyl protected lysine (Lys(Alloc)) was used for Lys15 for site-specific conjugation of polymers. Lys(Boc) was used otherwise. Amino acids, HCTU, and Wang resin were purchased from Novabiochem Corp. N,N-Dimethylformamide (DMF), piperidine, acetic anhydride, dichloromethane (DCM), trifluoroacetic acid (TFA), triisopropylsilane (TIS), phenylsilane (PhSiH₃), diethyl ether, diisopropyl ethanamine (DIPEA), dimethyl sulfoxide (DMSO), acetonitrile were purchased from Sigma-Aldrich. Tetrakis (triphenylphosphine) palladium (0) (Pd(PPh₃)₄) was purchased from Strem Chemicals Inc. Hemin was purchased from Fluka, and n-octyl-β-D-glucopyranoside (OG) was purchased from Fisher Scientific. Carboxy-terminated polymers (PS-COOH (1100 Da), PMMA-COOH (1700 Da), and PNIPAAM-COOH (2800 Da)) were purchased from Polymer Source.

2.4.2 Synthesis of Peptides
Peptides were synthesized on an automated solid phase peptide synthesizer (Prelude, Protein Technologies Inc.) using standard 9-fluorenylmethyl carbamate (Fmoc) chemistry and HCTU/DIPEA coupling on Wang resin. The loading of the resin was 0.27 mmol/g and the peptide was synthesized on a scale of 50 μmole. The N terminus was acetylated using a 5 ml mixture of DMF, acetic anhydride and pyridine in a 3:1:1 volume ratio. The resin was washed with DCM for three times and air dried.

2.4.3 Synthesis of Peptide-Polymer Conjugates
To selectively remove the Alloc group from Lys15, resin-bound peptide was treated with Pd(PPh₃)₄ catalyst (0.2 eq) and radical trapping agent PhSiH₃ (24 eq) in 2 ml of DCM for 30 minutes. The polypropylene reaction vessel was placed on a rotator shaker to facilitate complete mixing. After 30 minutes, resin was thoroughly washed with DCM, and the reaction was repeated two more times. Quantitative Alloc removal was observed. The orthogonal deprotection resulted in a free ε-amine at Lys15 while the rest of the amino acids were fully protected. The conjugation procedures were the same for the preparation of conjugates with different carboxy-terminated polymers (PS-COOH, PMMA-COOH, and PNIPAAM-COOH). Polymers were activated with HCTU (4 eq) and DIPEA (8 eq) in 2 ml of DMF for 30 minutes, and the activated polymer mixture was then added to the reaction vial containing peptide-loaded resin. The reaction was performed at room
temperature for 96 hours. Then, the reaction mixture was filtered off the resin and the resin was thoroughly washed with DMF to remove unreacted polymer and other reagents. Finally, the peptide-polymer conjugates were cleaved off the resin and deprotected in 5 ml of 95:2.5:2.5 TFA:water:TIS for 3 hours. The cleavage solution was evaporated under a stream of nitrogen prior to precipitation in cold diethyl ether and collection via centrifugation. The yield for the three conjugates was ~35%.

2.4.4 Matrix Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)
Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectra were recorded on an Applied BioSystems Voyager-DE Pro spectrometer. The matrix used for sample analysis consisted of α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 1:1 acetonitrile:water containing 0.1% TFA. Samples were dissolved in 1:1 acetonitrile:water solution and mixed with matrix, spotted on a stainless steel sample plate, and allowed to dry.

2.4.5 Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC)
The crude peptide-polymer conjugates were dissolved in 1:1 acetonitrile:water and purified using RP-HPLC (Beckman Coulter) on a preparative-scale C18 column (Vydac). Samples were eluted with a linear AB gradient, where solvent A was water containing 0.1% (v/v) TFA and solvent B was acetonitrile containing 0.1% (v/v) TFA. Elution was monitored with a diode array detector at wavelengths of 220 and 280 nm. The flow rate was 10 mL/min, and peptides were injected at a concentration of 10-15 mg/mL. The gradient used for PS and PMMA conjugates was 30% to 100%B over 30 min followed by 100%B for 15 minutes. The conjugates started eluting at ~65%B. The gradient used for PNIPAAM conjugates was 30% to 60%B over 20 minutes followed by 60% to 100%B over 30 minutes. PNIPAAM conjugates eluted at ~45%B.

2.4.6 UV-Visible Spectroscopy (UV-vis)
A Hewlett-Packard 8453 spectrophotometer was employed to record spectra using standard 1-cm path length quartz cuvettes. Peptide concentrations in solution were determined by tryptophan absorbance at 290 nm, using an extinction coefficient of 4900 M⁻¹ cm⁻¹. Tryptophan absorbance measurements at 290 nm, as opposed to 280 nm, were made to minimize the absorbance from conjugated polymers. Titration of helix bundle-polymer conjugates with heme was studied by UV-vis spectroscopy. Spectra were recorded after addition of each aliquot of heme (1-2 µL, of 200-500 µM heme in DMSO). A full description of heme titration experiments was reported previously.¹⁴

2.4.7 Dynamic Light Scattering (DLS)
DLS size measurements were made on a Malvern Zetasizer Nano-ZS with a 633-nm laser and a scattering angle of 170° to determine the hydrodynamic radius of samples in solution. Samples were passed through 0.22 µm filters prior to analysis.
2.4.8 Circular Dichroism (CD)
Characterization of peptide secondary structure was conducted by circular dichroism (CD) on a Jasco J810 spectropolarimeter equipped with a Jasco PTC-424S Peltier temperature controller. 1 mm path length quartz cuvettes were used for peptide concentrations \( \geq 10 \) µM, and 1 cm cuvettes were used for concentrations <10 µM. CD spectra were collected from 260 to 190 at 0.5 nm intervals, a rate of 50 nm/min, a response time of 4 s, and a bandwidth of 1 nm. Three spectra were recorded for each sample and averaged. Ellipticity was reported as the mean residue ellipticity ([θ], deg cm\(^2\) dmol\(^{-1}\)) and calculated as described previously.\(^{27}\) Temperature dependant changes of secondary structure were investigated by recording CD spectra as the temperature increased from 5 to 95 °C at a rate of 1 °C/min, with a 1 min equilibration time at each temperature before the measurement was taken.
Chapter 3

Effect of Alkyl Length of Peptide-Polymer Amphiphile on Cargo Encapsulation Stability and Pharmacokinetics of 3-Helix Micelles

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There is a great need to develop particles in size range of 10-30 nm, with tunable stability to optimize their use as nanocarriers. Our group recently reported design of 15 nm 3-helix micelles with the shell headgroup based on protein tertiary structure that leads to their excellent stability. The focus of the present study is to quantify the effect of core composition on the stability of 3-helix micelles and to decouple the contribution of micellar core from shell on their stability, using amphiphiles with different alkyl tails, namely C16 and C18. Both amphiphiles form well-defined micelles, ~17 nm in size, and show good stability, which can be attributed to the headgroup design. C18-micelles exhibit slightly higher kinetic stability in presence of serum proteins at 37°C, where the rate constant of subunit exchange is 0.20 hr\(^{-1}\) for C18-micelles vs. 0.22 hr\(^{-1}\) for C16-micelles. The diffusion constant for drug release from C18-micelles is approximately half of that for C16-micelles. The differences between the two micelles are significantly more pronounced in terms of their in vivo stability and extent of tumor accumulation. C18-micelles exhibit significantly longer blood circulation time of 29.5 hrs, whereas C16-micelles have a circulation time of 16.1 hrs. The extent of tumor accumulation at 48 hours after injection is ~ 43% higher for C18-micelles. The present studies underscore the importance of core composition on the biological behavior of 3-helix micelles. The quantification of the effect of this key design parameter on the stability of 3-helix micelles provides important guidelines for carrier selection and use in complex environment.

3.1 Introduction
Micellar nanoparticles based on amphiphilic self assembly have been studied extensively for use as nanocarriers for imaging and drug delivery.\(^1\)\(^-\)\(^{13}\) Control over stability of micellar particles is critical to optimize their pharmacokinetics, biodistribution and clearance mechanism.\(^14\)\(^-\)\(^{17}\) Amphiphilic block copolymers (BCP) constitute one of the most relevant families of materials that have been employed as micellar nanocarriers.\(^2\)\(^-\)\(^4\), \(^18\)\(^-\)\(^{20}\) Fundamental understanding of the effect of different molecular design parameters on micelle stability is critical to generate micelles with requisite stability and blood circulation time. Tailoring the chemical composition and physical state of core is commonly employed to tune BCP micelle stability, which has been an effective approach for a range of micellar systems with different core forming blocks such as polyesters, polypeptides and polyethers.\(^18\), \(^21\), \(^22\) Increasing the length and degree of hydrophobicity of core forming block leads to greater intermolecular interactions and higher micelle stability.\(^19\), \(^25\)\(^25\) Micelles with a frozen/solid-like core composed of crystalline or high glass transition temperature (\(T_g\)) polymeric blocks exhibit greater stability.\(^26\)\(^-\)\(^{28}\) These studies have been instrumental to identify design parameters that can be used to tune micelle stability, and stable polymeric micelles with size of 50-150 nm have been reported.\(^10\), \(^29\), \(^30\) Recent studies have established advantages of smaller nanocarriers in size range of 10-30 nm, in terms of favorable transport properties in blood circulation, facile extravasation from vasculature to tumor tissues, and homogeneous distribution in tumor.\(^17\), \(^31\), \(^32\) Hence, there is a great need to generate 10-30 nm micelles, where stability can be controlled and tuned without changing the size and structure of micelles. Chemical cross-linking between micellar head groups has been established as an effective route for stabilization of individual polymeric micelles with sizes of 20-40 nm.\(^5\), \(^11\), \(^33\) Amphiphiles
with short hydrophobic alkyl tails have been widely explored for the design of nanocarriers, 10-20 nm, in size.\textsuperscript{8, 34, 35} Increasing the length of hydrophobic alkyl tails in PEG-lipid micelles has been shown to decrease \textit{in vitro} subunit exchange and leads to higher stability.\textsuperscript{36} However, DSPE-PEG micelles exhibit limited \textit{in vitro} stability in biological environment similar to conditions after intravenous injection and short blood circulation time of 2 hours.\textsuperscript{8, 37, 38} It is critical to establish the effect of relevant design parameters on stability of 10-30 nm micellar nanocarriers to optimize their biological behavior.

We recently reported the design of 15 nm micelles based on self assembly of an amphiphile with headgroup being PEG conjugated coiled-coil 3-helix bundle.\textsuperscript{17} Our previous work has established the importance of headgroup design in 3-helix micelle shell on its excellent stability.\textsuperscript{39} The protein tertiary structure in the micellar headgroup allows to position entropic repulsions between the polymer chains that slows the subunit exchange and stabilizes 3-helix micelles. The hydrophobic tails provide an additional handle to tune the stability of 3-helix micelles. However, the stability of 3-helix micelles as function of tail hydrophobicity has not been quantified. Furthermore, in addition to understanding the effect of alkyl tail on the \textit{in vitro} stability of 3-helix micelles, it is equally, if not more, critical to evaluate the effect of relevant design parameters on \textit{in vivo} stability of micelles to establish if the results from \textit{in vitro} analytical characterization can be used as a guidance for \textit{in vivo} studies and predict micelle stability in biological environment. These quantitative studies provide critical guidelines to design and formulate 3-helix micelles with tunable stability for a range of applications for nanocarriers.

We present a systematic investigation of the effect of hydrophobicity of core forming alkyl chains on the size, core packing, drug release kinetics, and \textit{in vitro} and \textit{in vivo} stability, of 3-helix micelles. Our initial design consists of an amphiphile comprised of a coiled-coil peptide (1coi) conjugated to stearic acid (C18) at N-terminus and PEG-2000Da (P2K) at the middle of peptide backbone, which is called “1coi-dC18-P2K”. Conjugation of PEG-750Da (P750) chain at C-terminus results in “1coi-dC18-P2K-P750”, and micelles based on this amphiphile were used for \textit{in vivo} evaluation.\textsuperscript{17} Detailed physical characterization of kinetic stability of 3-helix micelles was performed using micelles assembled from “1coi-dC16-P2K”, without P750 chains required for stealth layer on micelle surface.\textsuperscript{39} In the present study, we investigate the exclusive effect of alkyl chain on assembly and stability of micelles from two amphiphiles, which we denote as “C16-micelle” and “C18-micelle”. Both amphiphiles have identical headgroup based on peptide-PEG conjugate 1coi-P2K-P750, where the peptide forms a 3-helix coiled-coil bundle, and the hydrophobic alkyl tails are C16 and C18, respectively (Figure 3.1). The phase transition temperature for alkyl chain packing in micelle core is higher for C18-micelles ($T_m \sim 24^\circ C$), relative to C16-micelles ($T_m \sim 15^\circ C$). Förster Resonance Energy Transfer (FRET) with dye encapsulated micelles show that C18-micelles exhibit slightly higher \textit{in vitro} stability with slower rate of subunit exchange and half-life of 3.46 hours, in presence of serum albumin, relative to C16-micelle (half-life 3.15 hours). Using DOX as a model cargo, release rates of DOX from micelles were consistent with their \textit{in vitro}...
stability based on FRET. The C18-micelles exhibit lower extent of initial and overall DOX release with smaller Higuchi rate constant of 0.024 hr$^{-1/2}$, compared to C16-micelles with rate constant of 0.034 hr$^{-1/2}$. However, the difference in blood circulation time of the two micelles is more pronounced than the difference in terms of in vitro half-life of micelles as shown by FRET studies. In vivo pharmacokinetic studies showed that circulation half-life ($t_{1/2,b}$) is 16.1 hrs for C16, and 29.5 hrs for C18-micelles. These systematic studies show that the not so large differences between the two micelles in terms of in vitro stability are amplified in complex biological environment and are reflected in differences in terms of in vivo stability and circulation time. From the perspective of fundamental understanding of 3-helix micelle stability, these results explain the importance of enthalpic contributions from alkyl chains in micelle core towards micelle stability. These results also provide a useful guideline to tailor the blood circulation time of 3-helix micelles with the choice of alkyl chain in micelle core. Different circulation times would be desirable for different applications. Faster accumulation at target along with rapid blood clearance is advantageous for imaging with high contrast, while longer circulation times would be preferable for targeting blood vessels and other components in vasculature and would facilitate passive targeting of tumor. The quantitative evaluations in this study provide a useful guideline to tailor the in vivo stability and the blood circulation time of 3-helix micelles for different biological applications.

Figure 3.1: Schematics of 3-helix micelle depicting the choice of two alkyl tails with different chain length and degree of hydrophobicity.
3.2 Results and Discussion

3.2.1 Structure of C16 and C18-micelles
Both amphiphiles form well defined, ~15 nm micelles with uniform size distribution as confirmed by DLS and SEC (Appendix A.2.1). Circular dichroism (CD) measurements show that there are no adverse effects of conjugation of C16 and C18 chains on the secondary and tertiary structure of 1coi, with peptide helicity ~ 82% in both amphiphiles, maintained at the level similar to peptide alone (A.2.2). Temperature dependent measurements (A.2.2) show that the peptide structure is stabilized after conjugation of hydrophobic alkyl tails, as observed previously.\textsuperscript{40, 41} This set of study shows that the overall effect of conjugation of C16 and C18 alkyl chains on the hydrodynamic size of micelles, and structure and thermal stability of peptide in micellar shell is similar, and any differences in the stability and pharmacokinetics between C16 and C18-micelles would potentially be due to the conformation of alkyl chains and interaction between chains in the micelle core.

Detailed quantification of the size of 3-helix micelles based on alkyl chains with different structure and hydrophobicity was investigated by solution small angle X-ray scattering (SAXS) measurements. Figure 3.2 shows the SAXS measurements for C16 and C18-micelles.

![SAXS Measurements](image)

Figure 3.2: SAXS of C16- and C18-micelles dissolved in phosphate buffer (25mM, pH 7.4), both at concentration of 5 mg/ml. Fitting of the data (solid lines) in q range of 0.03-0.1 Å\textsuperscript{-1} to a core-shell spherical form factor yields a core radii of ~2.8 nm for C16-micelles, and ~ 3 nm for C18-micelles. Shell thickness is calculated to be ~5.7 nm for both micelles.
The fit of data to a core-shell spherical model indicates that radius of the core is ~ 2.8 nm and thickness of shell is ~ 5.7 nm for C16-micelles, giving overall diameter ~ 17 nm. For C18-micelles, the core radius is ~ 3.0 nm with shell thickness ~ 5.8 nm, which corresponds to diameter of ~ 17.6 nm. The size of micelles with P750 on the surface is larger than that of lcoi-dC16-P2K micelles (14.8 nm) as reported previously and can be attributed to the shell thickness of P750 layer. These results confirm that the size of 3-helix micelles with C16 and C18 chains are not significantly different from each other, which allows us to investigate the effect of alkyl chain hydrophobicity, exclusively, on stability and biological performance of 3-helix micelles. Any differences in terms of in vitro stability, pharmacokinetics, and biodistribution observed between the two micelles can be safely attributed to the effect of core chain hydrophobicity, without any contribution from the size differences between the micelles. Hence, 3-helix micelles provide a good model system to understand the effect of a relevant design parameter (alkyl chain hydrophobicity in the present study), exclusively on the biological stability of smaller particles, < 20 nm in size, intended for nanocarrier applications.

3.2.2 Packing of Alkyl Chains in C16 and C18-micelles
DSC was performed to characterize the packing of alkyl chains in micelle core. DSC thermograms of both micelles show an endothermic phase transition, consistent with a change in alkyl chain packing in the core of micelles, with temperature (Figure 3.3).

![DSC thermograms for C16 and C18-micelles](image)

Figure 3.3: DSC thermograms for C16 and C18-micelles dissolved in phosphate buffer (25mM, pH 7.4), both at concentration of 2 mg/ml. The core phase transition temperature C16-micelle core is 15°C, and that for C18-micelle core is 24°C.
For C16-micelles, the transition temperature is 15°C, and the enthalpy associated with the phase transition is ~ 0.408 cal/g. For C18-micelles, the transition temperature is 24°C, and the enthalpy associated with transition is ~ 0.674 cal/g. The higher transition temperature in C18-micelles is consistent with higher melting point of C18 ($T_m \sim 70°C$) compared to C16 ($T_m \sim 63°C$). The transition enthalpies for both chains in micelles core are significantly smaller compared to the enthalpy of melting of C16 and C18 chains in bulk solid state. This decrease is due to amphiphilic self assembly of micelles, which results in packing of alkyl tails in a curved geometry in micelle core, with smaller phase transition enthalpies. However, the larger enthalpy of transition associated with C18 chains relative to C16 chains in 3-helix micelle core is consistent with higher hydrophobicity of C18 chains and greater extent of molecular interaction between C18 chains. These results are in agreement with previous studies, where increase in the chain length of hydrophobic tails leads to an increase in the melting temperature of alkyl chains in their hydrophobic core.  

Previous studies with DSPE-PEG have shown that stability of micelles is sensitive to temperature, with slower subunit exchange and higher micellar stability at temperatures below the transition temperature of alkyl chain in micelle core. The effect of the two alkyl tails on stability of 3-helix micelles at physiological temperature (37°C) was studied to determine their suitability for use both in vitro and in vivo. This is above the transition temperatures for the chain packing in both C16 and C18-micelles, and hence the alkyl chains in micelle core are expected to be in disordered/molten state. We hypothesize that potentially stronger interactions between alkyl chains in the core of C18-micelles as indicated by DSC, would impact micelle stability.

### 3.2.3 In Vitro Stability of C16 and C18-micelles

Quantification of in vitro half-life of 3-helix micelles based on different alkyl chains was performed by monitoring the changes in FRET from C16 and C18-micelles co-encapsulated with donor-acceptor pair (DiO-DiI) dyes. As shown in Figure 3.4a and 3.4b, the emission spectra show a major peak at 565 nm and a minor peak at 505 nm. The major peak is due to the excitation of the acceptor dye due to transfer of energy from the donor and indicates FRET from both of the micelles. The increase in emission intensity at 505 nm as function of time indicates subunit exchange and release of FRET dyes from the micelle into solution. Both C16 and C18-micelles show slight increase in the peak intensity at 505 nm. However the rate of change is comparatively higher for C16-micelles, suggesting faster kinetics of subunit exchange. The FRET ratio of $I_{505}/(I_{505} + I_{565})$ is used as an estimate for efficiency of energy transfer and the relative stability of micelles. Figure 3.4c shows the normalized FRET ratio for the two micelles as function of time. FRET ratio for C16- micelles decreases from 0.977 at 10 minutes to 0.967 at 1 hour, and decreases form 0.985 to 0.984 for the same time period for C18-micelles. After 12 hours, FRET ratio for C16 and C18-micelles are 0.892 and 0.945 respectively. The change of FRET ratio with time, fitted to an exponential decay, gives the decay rate constants (k) for C16 and C18-micelles as 0.22 hr$^{-1}$ and 0.20 hr$^{-1}$ respectively. Figure 3.4c also plots the rate of decrease in FRET efficiency of DSPE-PEG.
micelles. Much faster decay of FRET ratio, with $k = 1.24 \text{ hr}^{-1}$, indicates significantly faster exchange kinetics of DSPE-PEG micelles. These results establish that both C16 and C18-micelles exhibit significantly higher stability than DSPE-PEG micelles. The unique headgroup design based on a protein tertiary structural motif imparts the 3-helix

![Emission spectra](image)

Figure 3.4: Emission spectra of (a) C16-micelles and (b) C18-micelles encapsulated with donor/acceptor pair of FRET dyes. Micelles are dissolved in phosphate buffer (25mM, pH 7.4) at concentration of 0.2 mg/ml. The changes in fluorescence intensity are monitored for 12 hours in presence of serum albumin (50 mg/ml) at 37°C. The excitation wavelength was 450 nm and the emission spectra are recorded from 475 to 650 nm. (c) Plot of normalized FRET ratio as function of time for different micelles. The solid lines are the exponential fits to the decay observed.

However, it is critical to decouple the contribution of micellar core to 3-helix micelle stability. The higher value of $k$ for C16-micelles relative to C18-micelles indicates faster rate of subunit exchange and lower stability for C16-micelles at 37°C in presence of serum albumin (50 mg/ml). The half-life ($t_{1/2}$) corresponding to the first order decay constants are 3.15 and 3.46 hours for C16 and C18-micelles, respectively. The quantification of decay rate and half-life for 3-
helix micelles with different core composition indicates that the micelles with C18 chains exhibit slightly higher in vitro stability. The alkyl chains in the core of C16-micelle (with $T_m \approx 15^\circ C$) and C18-micelle (with $T_m \approx 24^\circ C$) would be in fluid state at 37°C. We hypothesize that the activation barrier for subunit exchange is inversely related to the difference in temperature of experiment and transition temperature for alkyl chain packing, as given by $(37^\circ C - T_m)$. The values of this temperature difference are 22°C and 13°C for C16 and C18-micelles respectively. The smaller temperature difference $(37^\circ C - T_m)$ for C18-micelles and greater hydrophobic interactions between the C18 chains lead to slower subunit kinetics and result in their higher stability relative to C16-micelles.

### 3.2.4 Cargo Release from C16 and C18-micelles

The effect of alkyl tail on cargo release kinetics from 3-helix micelles was quantified using DOX as a model drug. The loading for DOX was 7.3 ± 0.5 wt% in C16-micelles, and 7.8 ± 0.4 wt% in C18-micelles. The size, uniformity of size distribution and aqueous solubility of 3-helix micelles were maintained after incorporation of DOX for both amphiphiles.

Quantification of drug release by dialysis bag technique showed sustained release from both micelles (Figure 3.5a). For C16-micelles, ~8% of DOX is released within 1 hour, followed by ~22% release after 24 hours, whereas, 1% DOX is released from C18-micelles within initial first hour, followed by ~10% release at the end of 24 hours. The release of DOX from both micelles is comparable to other block copolymer formulations that exhibit DOX release in the range of 20-40% after 24 hours. The slower release

Figure 3.5: (a) Release profile of DOX from C16 and C18-micelles. Drug loaded micelle solutions (3mg/ml, 2ml) were placed in dialysis bags with MWCO 3500 Da, and release was monitored by quantification of drug in micelles at different times as determined by
HPLC. (b) Fits of DOX release to Higuchi model for diffusive release. Solid lines show thefits of the data to Higuchi equation, given by \( f = kt^{0.5} \), where \( f \) is fractional drug release, \( t \) is time, and \( k \) is Higuchi constant. The values of \( k \) are: 0.024hr\(^{-1/2}\) for C16-micelles; and 0.034hr\(^{-1/2}\) for C18-micelles.

Release of DOX from C18-micelles is consistent with their higher stability and lower subunit exchange. In absence of degradation and swelling of micelles, DOX release from both micelle formulations could be fitted to Higuchi model based on diffusive release with reasonable accuracy (Figure 3.5b). The Higuchi rate constants for DOX release from C16 and C18-micelles obtained from linear fits are 0.034 and 0.024 hr\(^{-1/2}\) which correspond to diffusion constants of 1.57x10\(^{-20}\) and 7.85x10\(^{-21}\) cm\(^2\)/s respectively. Diffusion constants in range of 10\(^{-15}\) - 10\(^{-18}\) cm\(^2\)/s have been reported for the release of variety of cargo molecules from block copolymer micelles.\(^{26, 46}\) The diffusion constants for DOX release from 3-helix micelles are significantly smaller, which suggests small drug leakage from 3-helix micelles. However, diffusion constant of DOX from C18-micelles is an order of magnitude lower than that from C16-micelles. Core hydrophobicity dictates micelle stability and micelle-drug interactions, both of which affect drug release. The results from this study confirm that increased core hydrophobicity results in slower rate of DOX release from 3-helix micelles. These observations agree well with the effect of core hydrophobicity on the stability of 3-helix micelles.

3.2.5 In Vivo Stability and Biodistribution of C16 and C18-micelles

The in vivo stability of C16-micelles in mice was assessed by PET in the present study and compared to that of C18-micelles reported previously.\(^{17}\) \(^{64}\)Cu labeled micelles were intravenously administered to mice bearing NDL tumors. PET image shown in Figure 3.6a indicates significant radioactivity from C16-micelles at 24 hours after injection. The activity from C16-micelles at 24 and 48 hours is reduced significantly compared to C18-micelles. These observations are consistent with the quantification of radioactivity from the two micelles. The blood radioactivity from \(^{64}\)Cu labeled C16-micelles at 24 and 48 hours after injection was 12.03 ± 0.32 % ID/cc and 5.81 ± 0.23 % ID/cc. The corresponding blood activity for C18-micelles was 25.74 ± 1.77 % ID/cc and 15.17 ± 1.58 % ID/cc. These results suggest higher in vivo stability of C18-micelles, with extended blood circulation. Figure 3.6b shows the blood radioactivity from C16-micelles as a function of time, compared against activity from C18-micelles as reported earlier.\(^{17}\) The rate of decrease of activity is higher for C16-micelles, which indicates lower stability in blood circulation relative to C18-micelles. The pharmacokinetics of C16-micelles was fitted to a biphasic model, which provides an estimate of \( \beta \)-phase blood circulation half-life (\( t_{1/2, \beta} \)) as 16.1 hrs. This value is significantly smaller compared to circulation half-life of 29.5 hrs for C18-micelles as reported previously.\(^{17}\) The difference in blood circulation time between the two micelles is significantly larger compared to differences between the two in terms of their in vitro stability and drug release kinetics. This amplification of the difference in terms of in vivo stability of 3-helix micelles underscores the critical role of core hydrophobicity on their biological performance.
Figure 3.6: Evaluation of *in vivo* micelle stability by positron emission tomography (PET). (a) Coronal view of PET images of $^{64}$Cu labeled C16 and C18-micelles after intravenous administration in mouse. The images were acquired immediately after and at 0, 3, 6, 24 and 48 hours after injection. (b) Blood radioactivity (%ID/cc) of $^{64}$Cu labeled C16 and C18-micelles. Curves were fitted as two-phase exponential decay. For C16-micelles, fit is given by $Y = 36.61e^{-0.043t} + 24.73e^{-0.74t}$, with $t_{1/2,\alpha} = 16.12$ hr; and for C18-micelles, fit is given by $Y = 45.32e^{-0.0235t} + 16.42e^{-1.27t}$, with $t_{1/2,\beta} = 29.52$ hr.
The radioactivity observed for $^{64}$Cu labeled C16-micelles in different clearance organs were $3.1 \pm 0.3\%$ ID/g in the spleen, $4.3 \pm 0.5\%$ ID/g in the liver, and $3.3 \pm 0.1\%$ ID/g in the kidney, not significantly different from C18-micelles as reported previously (Figure 3.7). Based on these results, it is apparent that accumulation of 3-helix micelles in reticuloendothelial system is minimal, which is advantageous for their long circulation in blood. We hypothesize that 3-helix micelles based on self assembly would eventually disintegrate into their biocompatible smaller subunits, $< 10kDa$ in molecular weight, that are potentially cleared through kidneys.

![Figure 3.7: Comparison of radioactivity (%ID/g) of C16 and C18-micelles in major clearance organs indicates insignificant differences between the two micelles in terms of their clearance profile.](image)

The greatest differences in terms of biodistribution of C16 and C18-micelles are reflected in their tumor accumulation. Transverse PET images (Figure 3.8a), show that both micelles accumulate in tumor, 24 hours after administration. The extent of tumor accumulation is higher for C18-micelles, 24 and 48 hours after administration. In order to describe these differences quantitatively, Figure 3.8b plots the accumulation of two micelles in tumor tissue as function of time. Tumor accumulation is at its maximum at 24 hours after injection for both micelles. At 48 hours, the accumulation levels for C16 and C18-micelles are $3.7 \pm 0.5\%$ ID/g and $5.7 \pm 0.6\%$ ID/g respectively. The significantly higher activity from C18-micelles is consistent with their longer blood circulation time that leads to higher extent of tumor accumulation mediated by EPR effect. These results are consistent with earlier studies that have shown increased tumor uptake for nanocarriers with longer blood circulation time that leads to more efficient passive targeting of the tumor tissue.47, 48 These results underscore the significance of the core
hydrophobicity of 3-helix micelles on their long-term tumor accumulation, which is critical to maximize the exposure of therapeutic carries based on 3-helix micelles to the disease site.

**Figure 3.8:** (a) Transverse view of sliced PET images of $^{64}$Cu labeled C16 and C18-micelles in mouse, indicate significant accumulation of micelles in tumor, 48 hours after intravenous administration. Arrow shown indicates the tumor location. (b) Tumor accumulation of micelles as function of time shows that C18-micelles have significantly higher extent of tumor localization, 48 hours after intravenous administration.

### 3.3 Conclusion

In summary, we present the systematic investigation of the effect of core hydrophobicity on size, core packing, *in vitro* stability, cargo release kinetics, and pharmacokinetics of 3-helix micelles. Micelles with more hydrophobic core composed of C18 alkyl tails exhibit higher stability with slower rate of subunit exchange in presence of serum proteins. C18-micelles have slower and extended cargo release with smaller rate of drug diffusion from
the micelles, relative to C16 micelles. The difference between the two micelles in terms of their in vivo circulation stability and extent of tumor accumulation are more pronounced compared to the differences in the in vitro stability and cargo release kinetics. These results underscore the critical role played by core composition on the biological behavior of 3-helix micelles. This study delineates the exclusive effect of core composition on the stability of 3-helix micelles and shows that small increase in length of core forming alkyl chains (C16 to C18) leads to significant differences between the their in vivo circulation stability. This may be attributed to the complex biological environment in circulation including dynamic blood flow conditions and presence of a large number of proteins that act as traps for amphiphilic assemblies, which has a critical impact on the distinction between pharmacokinetics of injected carriers. The results from this study underscore the significance of choice of alkyl tails on the stability of 3-helix micelles in complex biological environments. They also serve as important guidelines to design nanocarriers based on 3-helix micelles with tunable in vivo circulation times intended for different biological applications.

3.4 Experimental

3.4.1 Synthesis of Peptide-Polymer Conjugate

The design of the two amphiphilic conjugates is based on a 3-helix bundle peptide designed de novo, 1coi (EVEALEKKVAALECKVQALEKKVEALEHGWA). Aliphatic tails of palmitic acid (C16) and stearic acid (C18) were conjugated to N-terminus of peptide to investigate the effect of hydrophobicity of alkyl tails on assembly and stability of C16 and C18-micelles. The details about materials and the synthesis of amphiphilic peptide-polymer conjugate have been described previously. The important steps in the synthesis of conjugate are the solid phase conjugation of alkyl tails to peptide N-terminus, and PEG chain with molecular weight of 750 Da (P750) to peptide C-terminus. Peptides were then cleaved off the resin by standard procedures. Cysteine at position 14 facilitates the site specific coupling of maleimide functionalized PEG of molecular weight 2000 Da (P2K) to the middle of the peptide sequence in aqueous buffer. The resulting conjugate was purified by reverse-phase high pressure liquid chromatography (RP-HPLC) with water/isopropanol gradient and subsequently lyophilized. The conjugates are referred to as 1coi-dC16-P2K-P750 and 1coi-dC18-P2K-P750.

3.4.2 Biophysical Characterization of C16 and C18-micelles

C16 and C18 micelles were prepared by direct dissolution of the lyophilized powder in phosphate buffer (25 mM, pH 7.4). The solutions were annealed at 70°C for an hour to allow equilibration of micelle assembly. The annealed solutions were cooled down to room temperature before starting the experiments. Dynamic Light Scattering (DLS) size measurements were made on a Malvern Zetasizer Nano-ZS with a 633 nm laser and a scattering angle of 17°. Size exclusion chromatography (SEC) was carried out on a BioSep-SEC-S 4000 column (Phenomenex), with flow rate was 1 ml/min and 25 mM phosphate buffer, pH 7.4, was used as the elution solvent. Small angle X-ray Scattering (SAXS) was carried out at beamline 7.3.3 at the Advanced Light Source, Lawrence Berkeley National Laboratory. Details on the sample preparation and data analysis for
SAXS measurements can be found in our earlier work. Circular dichroism (CD) measurements were made on a Jasco J810 spectropolarimeter from 260 to 190 nm at 0.2 nm intervals, a rate of a 100 nm/min, a response time of 4 s, and a bandwidth of 1 nm. Differential Scanning Calorimetry (DSC) was performed on a VP-MicroCal (GE), as temperature was increased from 5 °C to 60 °C at a rate of 1 °C/min. DSC thermograms were obtained after concentration normalization and baseline correction with Origin software provided by MicroCal. The enthalpy change associated with alkyl chain phase transition was calculated by using area under the curve function in Igor Pro 6. A concentration of 2 mg/ml was used for both CD and DSC measurements. 

**In vitro** stability of micelles in serum albumin was studied by Förster Resonance Energy Transfer (FRET) from micelle encapsulated donor/acceptor dyes, pair as described previously. A lipophilic FRET pair, 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO, donor) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, acceptor), were used to measure the energy transfer upon mixing. The emission spectra were recorded in the range of 475 to 650 nm for 12 h with excitation wavelength at 450 nm. The efficiency of energy transfer between the dyes is characterized by FRET ratio, defined as I_{565}/(I_{565} + I_{505}), where I is fluorescence intensity at respective wavelength.

### 3.4.3 Loading and Release of DOX from Micelles

The loading of DOX in 3-helix micelles was performed by thin film hydration method as described in earlier work. The release profiles of DOX from micelles were studied using dialysis bag technique at 37°C. The solution of DOX-loaded micelles (3mg/ml, 2 ml) was placed in a dialysis bag (Spectrum Labs, MWCO 3500 Da). The dialysis bag was immersed in 500 ml of PBS solution (25 mM, pH 7.4) in a glass beaker, which was stirred at 600 rpm. 10 µl of solution was drawn from dialysis bag at desired time intervals and the drug concentration was analyzed using RP-HPLC. DOX release profiles were fitted to Higuchi model for diffusive release, described by $Q = 2C_o(Dt/\pi)^{1/2}$, where Q is the amount of drug released per unit area, $C_o$ is the initial drug concentration in micelle, t is the time in seconds, and D is the apparent diffusion constant. Higuchi equation can be rearranged to obtain fractional drug release, f, as $f = kt^{1/2}$, with Higuchi rate constant, $k = (36D/\pi R^2)^{1/2}$, where R is radius of micelle.

### 3.4.4 PET Imaging and Biodistribution

All animal studies were conducted under a protocol approved by the University of California, Davis, Animal Care and Use Committee. $^{64}$CuCl$_2$ was purchased from Washington University (St Louis, MO). The details of synthesis of radiolabeled micelles and PET imaging scans are described in previous work. In brief, the pharmacokinetics and in vivo biodistribution of C16 and C18-micelles were studied by positron emission tomography (PET) after intravenous administration of micellar solution in female FVB mice ($n = 6$ for C16-micelles, $371 \pm 107$ mCi and $0.68 \pm 0.15$ mg per mouse) and $n = 4$ for C18-micelles micelles, $407 \pm 9$ mCi and $0.69 \pm 0.008$ mg/mouse) bearing NDL tumors bilaterally within the mammary fat pads.
## Chapter 4

**Biological Evaluation of 3-Helix Micelles as Nanocarriers for Doxorubicin**

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Optimization of size, stability, cargo retention, and carrier degradation to control accumulation and drug release from nanocarriers in tumor tissue and to ensure proper clearance to avoid side effects is required to facilitate the translation of nanoplatforms as clinically relevant anticancer therapeutics. We have designed 15 nm micelles based on self-assembly of polymer conjugates of coiled-coil 3-helix bundles that demonstrate excellent in vivo kinetic stability. Designing stable drug nanocarriers, 10-30 nm in size, would have significant impact on their transport in circulation, tumor penetration and therapeutic efficacy. In the present study, biological properties of 3-helix micelles loaded with doxorubicin (DOX) were characterized to validate their potential as a nanocarrier platform. DOX-loaded micelles could be formulated as an aqueous suspension with ~8 wt% drug content without disrupting the core-shell structure of micelles. The encapsulated DOX interacted with alkyl chain core that further stabilizes the micelle and micelles can be disassembled by proteolytic degradation of peptide shell. DOX-loaded micelles exhibited high stability in terms of size and drug retention in concentrated protein environments similar to conditions after intravenous injections. DOX-loaded micelles were cytotoxic to PPC-1 and 4T1 cancer cells confirming the release of drug from micelles. In vivo studies on intravenous administration of these stable 15 nm particles clearly demonstrated improved tumor half-life and reduced toxicity to healthy tissues from DOX-loaded micelles compared to free DOX. Drug loaded 3-helix micelles with size < 20 nm, display extended stability, little drug leakage, substantial cytotoxicity towards cancer cells, favorable biodistribution and greatly reduced toxicity towards healthy tissues, and thus meet many of the critical requirements for safe and effective nanocarriers for cancer therapeutics.

4.1 Introduction

Many anticancer drugs have limited solubility and require delivery carriers and/or complex formulations to improve their pharmacokinetics, biodistribution, toxicity profile and efficacy.\textsuperscript{1-12} For nanocarrier design, size is the most critical property since it determines nanocarrier transport in the circulation, extravasation from vasculature into the tumor tissue, subsequent diffusion into the tumor and clearance pathway.\textsuperscript{13-16} Liposomes and protein-drug conjugates, such as Doxil and Abraxane, are two of the nanocarrier platforms currently in clinical use as anticancer therapeutics.\textsuperscript{17, 18} However, Doxil, with a diameter of ~100-130 nm, can only extravasate into tumor via enhanced permeability and retention (EPR) effect, but fails to penetrate deep into tumor tissues with consequently modest therapeutic benefits.\textsuperscript{19, 20} Smaller nanoparticles, 10-30 nm in size, have been shown to rapidly diffuse throughout the tumor matrix\textsuperscript{13-15} and exhibit greater extent of cellular accumulation and faster internalization in cancer cells.\textsuperscript{21-23}

Several formulations based on micelles of PEG-polyesters and PEG-polypeptides, typically in the range of 30-100 nm, are at different stages of clinical trials for cancer therapy.\textsuperscript{24, 25} Recent studies have shown deeper tumor penetration, greater intratumoral drug concentration and enhanced anticancer efficacy with 30 nm oxaliplatin loaded micelles in human pancreatic adenocarcinoma xenografts.\textsuperscript{15} Small nanocarriers, 10-30 nm in size, based on dendrimers\textsuperscript{26} and peptide micelles\textsuperscript{27} with chemically conjugated...
anticancer drugs have shown enhanced therapeutic effect after a single intravenous administration in C-26 colon carcinoma-bearing mice. These findings suggest that drug loaded nanocarriers in the range of 10-30 nm, with high stability, and better tumor accumulation offer the best possibility for optimal therapeutic efficacy. However, maintenance of carrier stability after intravenous administration has presented a significant challenge. We have developed 15 nm spherical micelles, called 3-helix micelles, based on amphiphilic peptide-PEG conjugates where the head-group self-associates into a 3-helix bundle. These micelles alone demonstrated good in vivo kinetic stability, and favorable biodistribution in terms of minimal accumulation in liver and spleen. 3-helix micelles with their small size and high biological stability appear to be a unique platform for the design of drug loaded nanocarriers and investigation of the effect of drug incorporation on their structural and biological properties is critical to establish their utility as a nanocarrier platform.

The present study is focused on the evaluation of 3-helix micelles as viable nanocarriers to meet critical requirements in terms of cargo loading, biological stability, tumor accumulation, triggered drug release and reduced off-target toxicity. We used doxorubicin (DOX) as the model anticancer drug, to undertake a systematic in vitro and in vivo validation of 3-helix micelles as a viable drug carrier. DOX-loaded micelles exhibited resistance to protein adsorption and high drug retention with little release in concentrated protein environments mimicking the conditions in the blood after intravenous injection. Micelles could be disassembled by protease mediated degradation of peptide based micellar shell, which is critical for drug release and clearance to minimize side effects. DOX-loaded micelles were cytotoxic to a range of cancer cells confirming the release of drug from micelles. Intravenous administration of DOX-loaded micelles indicated selective tumor localization 72 h after injection, and resulted in reduced toxicity to healthy tissues. These studies established that drug half-life and toxicity of DOX-loaded micelles are favorably altered relative to free drug. The present study clearly indicates the potential of 3-helix micelles as a viable nanocarrier platform that meets many critical requirements for drug formulation.

4.2 Results and Discussion

4.2.1 DOX Loading in 3-Helix Micelles
DOX is a highly potent anticancer drug and its efficacy in a variety of in vivo tumor models as well as in humans is well documented. However, the use of DOX is severely limited by its poor aqueous solubility, stability and high cardiotoxicity. There have been a number of studies focused on the design of different nanocarrier based DOX formulations to evaluate their potential as safe and effective anticancer therapeutics. Hence, DOX is a good model drug to explore the potential of 3-helix micelles as a nanocarrier platform.

The highest DOX loading that could be obtained in 3-helix micelles was ~ 8 wt%. DOX-loaded 3-helix micelles with different drug loadings were prepared to investigate the effect of drug content on micelle structure. Emission spectra of free DOX and DOX-
loaded micelles with different drug loadings are shown in Figure 4.1. Fluorescence of DOX in micelles is significantly reduced compared to fluorescence of free drug at the same concentration. This fluorescence attenuation is attributed to fluorescence self-quenching of drug molecules in close proximity with each other and indicates encapsulation of drug in micelles.\textsuperscript{38,43}

![Emission spectra of free DOX and DOX-loaded micelles with different drug loadings](image)

Figure 4.1: Emission spectra of free DOX and DOX-loaded micelles with different drug loadings in phosphate buffer (25mM, pH 7.4) at 40 µg/ml DOX concentration. The excitation wavelength is 480 nm and the emission spectrum is collected from 510-640 nm. The fluorescence from DOX-loaded micelles is significantly lower compared to free DOX, due to fluorescence self-quenching of DOX, that indicates incorporation of DOX in 3-helix micelles.

SEC (Figure 4.2) indicates that the elution profile of DOX-loaded micelles with different drug loadings is similar to blank micelles. DLS showed that size of DOX-loaded micelles with highest drug loading of 8 wt% is \(\sim\)15 nm, and is not significantly different from blank micelles (Appendix A.3.1). Thus, drug content in 3-helix micelles can be systematically varied without adversely affecting the size and aqueous solubility of micelles.

The drug loading capacity of micelles is determined primarily by the compatibility of the drug with micellar core.\textsuperscript{44,45} Non-covalent interactions such as hydrophobic, electrostatic and hydrogen bonding dictate the miscibility of the drug with micelle components, and
Figure 4.2: SEC chromatograms of blank and DOX loaded 3-helix micelles (1 mg/ml, injection volume 30 µl) with different loadings in phosphate buffer (25 mM, pH 7.4). Elution is monitored at 220 nm. Blank micelles and DOX-loaded micelles show relative homogeneous distribution with minimal aggregation.

are important factors governing the loading capacity of a micellar system.\textsuperscript{3, 46, 47} However, geometrical factors especially, micellar core size and molecular volume of drug, are critical in determining the drug capacity of micelles. DOX loadings in the range of 12-15 wt% have been reported for block copolymer micelles, 30-40 nm in size.\textsuperscript{38–40,43} The cores of these micelles are larger in size compared to the alkyl chain based core of 3-helix micelles. The maximum DOX loading that could be achieved in 3-helix micelles was ~ 8 wt%, which is comparable to nanocarriers that have demonstrated high \textit{in vivo} efficacy.\textsuperscript{26, 27} We believe that 3-helix micelles in the unique size range of 10-20 nm, with
high drug content, would have significant implications for biological performance of nanocarriers in terms of biodistribution and efficacy.

4.2.2 Structural Characterization of DOX-loaded 3-Helix Micelles
Micelles with 8 wt% DOX were studied in greater detail in terms of their size, structure, biological stability and drug release kinetics. Negatively stained TEM (Figure 4.3a), provides further evidence that assembly of 3-helix micelles is maintained in presence of DOX. CD (A.3.2) shows α-helical conformation and coiled-coil tertiary structure of the peptide is maintained after incorporation of DOX. Figure 4.3b shows DSC thermograms for DOX-loaded micelles and unloaded micelles to probe the alkyl chain packing in the micelle core.

![Figure 4.3: (a). Negatively stained TEM of DOX-loaded micelles with 8wt% loading in phosphate buffer (25mM, pH 7.4) showing presence of spherical micelles. (b). DSC thermograms for unloaded and DOX-loaded micelles with 8wt% loading in phosphate buffer (25mM, pH 7.4), both at concentration of 2 mg/ml.](image)

For unloaded micelles, the endothermic peak with a transition temperature ~24°C, corresponds to the melting of alkyl chain packing in the micelle core. The transition temperature for the DOX-loaded micelles is increased to ~ 33°C, which indicates interaction of DOX with stearic acid in the micelle core. The enthalpy change associated with transition in unloaded micelles is 0.674 cal/g, and that for DOX-loaded micelles is 0.474 cal/g. These results indicate the formation of a distinct thermodynamic phase in the core of DOX-loaded micelles, different from the core of empty micelles, possibly formed by the co-assembly of drug with alkyl chains. Based on these studies, the peptide structure in the shell of DOX-loaded 3-helix micelles is maintained and drug is encapsulated within the micellar core.
4.2.3 Stability and Drug Retention of DOX-loaded Micelles

Post intravenous administration, injected nanocarriers are introduced in a highly concentrated protein environment. Resistance to nonspecific protein adsorption and retention of therapeutic agent are critical requirements for stable nanocarriers to achieve extended blood circulation with minimum cargo leakage. The stability of DOX-loaded 3-helix micelles was investigated in presence of serum albumin under physiological conditions. Figure 4.4 shows the SEC chromatogram of DOX-loaded micelles incubated in serum albumin for 20 h at 37°C. The chromatograms of serum albumin and fresh DOX-loaded micelles indicate distinct elution profiles for the two components. The overlapping signals monitored at 220 nm and 480 nm for DOX-loaded micelles confirm the encapsulation of DOX in 3-helix micelles. After incubation of DOX-loaded micelles with serum albumin for 20 h, the chromatogram shows minimal differences from the chromatograms of the individual components. This confirms that the size and the uniformity of size distribution of DOX-loaded micelles are not significantly altered in protein rich biological environment. The encapsulation stability of drug within the micelle was investigated in presence of albumin, by monitoring fluorescence of DOX-loaded micelles over time.

Figure 4.4: SEC chromatograms for serum albumin (SA), DOX-loaded micelles and DOX-loaded micelles incubated in SA (10 mg/ml) at 37°C for 20 hours. Elution profile at 220 nm monitors the absorbance of albumin and peptide, and elution at 480 nm monitors the absorbance of DOX.
Figure 4.5 shows a very gradual change in the fluorescence of DOX-loaded micelles in presence of serum albumin for 20 h. The emission spectrum of an equivalent amount of DOX-loaded micelles dissolved in methanol is shown as a reference for fluorescence intensity from the free drug as methanol disrupts the structure of micelles. The inset reveals a slow rate of recovery for DOX fluorescence, only 12% after 20 hours, which suggests stable encapsulation of DOX in micelles with slow release in presence of serum albumin. Quantification by dialysis bag release confirmed slow and extended release, with ~ 11% DOX released from 3-helix micelles after 20 hours. The results from these studies indicate high resistance of DOX-loaded 3-helix micelles towards nonspecific protein adsorption and extended drug retention in highly concentrated protein environments.

![Fluorescence Spectrum](image)

Figure 4.5: Drug release from DOX-loaded micelles (DOX concentration, 20 µg/ml) with 8 wt% loading in presence of serum albumin (50 mg/ml, 37°C) for 20 hours. The excitation wavelength is 480 nm and emission spectra are collected from 510-650 nm for 20 hours.

4.2.4 Cytotoxicity and Proteolytic Degradation of 3-Helix Micelles

In addition to cargo stability during transport, an equally important requirement for effective anticancer nanocarriers is to control drug release at the disease site after tumor localization and/or cellular internalization. For stable nanocarriers, the modulated cargo release is critical to ensure antitumor effect. MTT assay showed that DOX-loaded micelles exhibited concentration and time dependent in vitro cytotoxicity towards PPC-1
and 4T1 cancer cells (Figure 4.6). DOX-loaded micelles effectively suppressed the growth of these cancer cells by 36 h and the rate and extent of cytotoxicity was comparable to that of free DOX. On the basis of stability studies, there is negligible drug release from micelles in cell culture medium and thus, the high toxicity of DOX-loaded micelles is due to release of DOX after cellular internalization.

![Figure 4.6: In vitro cytotoxicity of DOX-loaded micelles for PPC-1 and 4T-1 cancer cells measured by MTT. Both cells were treated with PBS control, free DOX at 5 µg/ml and 1 µg/ml, DOX-loaded micelles at drug concentration of 5 µg/ml and 1 µg/ml and blank micelles with peptide concentration matched to 5 µg/ml DOX-loaded micelles. Viability was evaluated, 36 and 72 hours after treatments. DOX-loaded micelles inhibited the growth of both cells effectively, similar to free drug. Statistical comparisons were performed by ANOVA followed by Tukey's multiple comparisons test. ** p < 0.0001 vs. PBS control and blank micelles at the same time point; * p < 0.05 vs. PBS control and blank micelles at the same time point.](image)

We hypothesize that the 3-helix peptide should be cleavable by proteolytic degradation. 3-helix micelles were incubated with proteinase K, a broad spectrum protease that cleaves peptide bonds adjacent to carboxyl group of aliphatic and aromatic amino acids. The sequence of 1coi peptide shows multiple sites of proteolytic cleavage by proteinase K. MALDI spectrum of a micelle solution shows a peak at ~ 7200 Da, the mass of the amphiphilic building block, dC18-1coi(P2K)-P750 (Figure 4.7). MALDI spectrum of micelles incubated with proteinase K indicates the disappearance of amphiphile peak and the appearance of peaks associated with PEG chains attached to small peptide fragments, which suggests degradation of peptide. In order to confirm micelle disassembly under proteolytic conditions, fluorescence of dye labeled micelles was monitored in presence of proteinase K. For this study, 3-helix micelles were labeled with fluorescein that self quenches when placed close to other fluorescein molecules. Figure 4.8 shows that the emission intensity of fluorescein labeled micelles is significantly lower compared to free
fluorescein in solution at the same concentration due to self quenching of dye fluorescence on the micelle surface. After addition of proteinase K, fluorescence intensity starts to increase potentially due to degradation of peptide in the micellar shell, which leads to fluorescence recovery of the quenched dye. These results confirm the proteolytic degradation of peptide in micellar shell and subsequent disassembly of 3-helix micelles. 3-helix micelles undergo proteolytic disassembly after cellular internalization, resulting in DOX release that leads to cytotoxicity as demonstrated by MTT assay. The disassembly of 3-helix micelles can be triggered by proteases for drug release and clearance to minimize side effects. This unique feature opens new opportunities to design micelles where drug release can be triggered in response to tumor-specific proteases.

Figure 4.7: MALDI-TOF spectra of the micelle solution before and after incubation with proteinase K, verifying the degradation of the peptide after addition of protease and presence of the PEG2K and PEG750 left after.
Figure 4.8: Emission spectra of fluorescein labeled micelles before and after addition of proteinase K. Self quenching of dye on micelle surface leads to lower fluorescence compared to free dye in solution. After addition of proteinase K (150 µg/ml, phosphate buffer, pH 7.4), fluorescence increases rapidly. The excitation wavelength is 480 nm and emission spectra are collected from 490-650 nm. Inset plots the rate of fluorescence recovery after proteinase K addition and shows very fast increase in fluorescence and indicates micellar disassembly in protease environment.

4.2.5 Intravenous Delivery of DOX-loaded Micelles
3-helix micelles with their small size and extended stability present a unique nanocarrier platform for systemic delivery. Intravenous administration of radiolabeled empty 3-helix micelles without drug in NDL tumor bearing mice confirmed significant tumor accumulation (5.7 ± 0.9 % ID/g), comparable to 100 nm, long circulating liposomes (4.3% ID/g).\textsuperscript{32} Time dependent PET imaging after tumor localization indicated that 3-helix micelles were highly mobile in tumor tissue, whereas liposomes did not show any detectable movement as function of time, which could be attributed to the significantly smaller size of 3-helix micelles compared to liposomes. The dynamics of nanocarriers in size range of 10-20 nm in tumor would have significant implications on tissue penetration and therapeutic efficacy, which requires further investigation. In the present study, DOX-loaded 3-helix micelles were intravenously administered to NDL tumor bearing FVB mice to investigate the biodistribution of drug loaded micelles. Figure 4.9 shows the fluorescence images from different organs, 24 and 72 h after injection of DOX-loaded micelles. Fluorescence in tumor confirms accumulation of DOX-loaded micelles due to EPR effect. Tumor fluorescence increases by 2.1 fold (p value = 0.0028) from 24 to 72 h, whereas, very low fluorescence is detected for other organs during this time period.
Fluorescence in different organs at these time points after free DOX injections cannot be detected as clearance time of DOX from blood circulation is on the order of tens of minutes. The selective fluorescence signal in tumor over extended time period indicates that distribution of DOX in tumor is favorably modified by encapsulation in 3-helix micelles, leading to longer tissue half-life. Thus, incorporation of DOX in 3-helix micelles increases the tumor exposure relative to free drug. Equally important, fluorescence in non-tumor sites including heart, at 24 and 72 h after injection was significantly lower. The fluorescent intensity in heart 24 hours after administration DOX-loaded 3-helix micelles was lower compared to intensity after Doxil administration as quantified previously. These results suggest reduced accumulation of DOX-loaded 3-helix micelles in heart, which is critical to minimize cardiomyopathy associated with DOX.

Figure 4.9: Biodistribution of DOX-loaded micelles after intravenous administration. Fluorescence images of tumor, 24 and 72 hours after injection indicate the presence of DOX fluorescence in tumor for extended period of time. Fluorescence in other organs at these time points is significantly lower, which indicates low accumulation in these organs. The different organs are labeled as 1: heart, 2: liver, 3: spleen, 4: kidney, 5: skin, 6: tumor.

Figure 4.10 depicts the images of mouse skin 25 days after administration of DOX-loaded micelles and Doxil. The absence of skin lesions is clearly evident for the mouse treated with DOX-loaded micelles, which indicates that skin toxicity from DOX is significantly reduced after its formulation in 3-helix micelles. On the other hand, accumulation of Doxil in skin increases over time as evident by skin damage. This study focused on evaluation of accumulation of DOX-loaded 3-helix micelles shows that off-target exposure to heart and skin from 3-helix micelles is significantly reduced compared to Doxil, which is encouraging for the safety profile of DOX formulated in 3-helix micelles.
4.3 Conclusion

In summary, we demonstrate that 3-helix micelles based on amphiphilic peptide-PEG conjugates can incorporate 8 wt% DOX and can be formulated in aqueous buffer in a reproducible manner. The self-assembly of amphiphiles into 15 nm core-shell spherical micelles is maintained after DOX incorporation. DOX-loaded micelles exhibit high stability in terms of size and DOX retention in protein-rich environment similar to conditions after intravenous injections. The disassembly of 3-helix micelles can be triggered by proteolytic degradation of peptide, which allows controlling drug release and clearance to minimize side effects. DOX-loaded micelles exhibit significant toxicity towards a range of cancer cells. *In vivo* studies of intravenous administration clearly establish that DOX-loaded micelles improve tissue half-life and reduce off-target toxicity compared to free drug. These findings are encouraging and suggest the potential of 3-helix micelles as a viable nanocarrier platform that meets a number of critical requirements for nanomedicine.

4.4 Experimental

4.4.1 Synthesis of Peptide-Polymer Conjugate

The design of amphiphile is based on a 3-helix bundle peptide designed *de novo*, 1coi (EVEALEKKVALECKVQALEKKVEALEHGW)

The peptide was synthesized on a Prelude solid-phase synthesizer (Protein Technologies) using standard 9-fluorenethylmethyl carbamate (Fmoc) chemistry. The details about materials and the synthesis of amphiphilic peptide-polymer conjugate have been described previously. The important steps in the synthesis of conjugate are the solid phase conjugation of...
stearic acid tails to peptide N-terminus, and PEG chain with molecular weight of 750 Da (P750) to peptide C-terminus. Peptides were then cleaved off the resin by standard procedures. Cysteine at position 14 facilitates the site specific coupling of maleimide functionalized PEG of molecular weight 2000 Da (P2K) to the middle of the peptide sequence in aqueous buffer. The resulting conjugate was purified by reverse-phase high pressure liquid chromatography with water/isopropanol gradient and subsequently lyophilized. The conjugate is referred as dC18-1coi(P2K)-P750.

4.4.2 Loading of DOX in 3-Helix Micelles
DOX was loaded into micelles by thin film hydration method. dC18-1coi(P2K)-P750 and DOX were dissolved in methanol in a glass vial and the solvent was evaporated in vacuum oven for 3 h. The dried film containing the conjugate and drug was rehydrated with 25 mM phosphate buffer, pH 7.4, and the solution was stirred for 16 h to allow assembly into drug loaded 3-helix micelles. Free drug was removed by centrifugation followed by spin ultrafiltration (Amicon centrifugal filters, MW cutoff: 3000 Da). The concentrate was washed with water and lyophilized to obtain DOX-loaded micelles as a dry red powder. DOX-loaded micelles were dissolved in methanol and loading was determined by absorbance of DOX at 480 nm. 3-helix micelles with DOX loadings of 1, 2, 4, and 8 wt% were prepared. For all experiments, lyophilized powder of DOX-loaded micelles was dissolved in 25 mM phosphate buffer, pH 7.4.

4.4.3 Structural Characterization of DOX-loaded 3-Helix Micelles
Size exclusion chromatography (SEC) was carried out on a BioSep-SEC-S 4000 column (Phenomenex). The flow rate was 1 ml/min with 25 mM phosphate buffer, pH 7.4, as the elution solvent. The elution profile was monitored with a UV-Vis detector at wavelengths of 220 nm and 480 nm. Dynamic Light Scattering (DLS) size measurements on drug loaded micelles were made on a Malvern Zetasizer Nano-ZS with a 633 nm laser and a scattering angle of 17°. FEI Tecnai 12 transmission electron microscope (TEM) at 120 kV was used to study the morphology of DOX-loaded micelles. DOX-loaded micelles solution (5 µl, 0.2 mg/ml) was dropped onto a discharged holey carbon coated grid (Ted Pella 01824), followed by phosphotungstic acid (5 µl, 2 wt %, pH 3.3) solution. Circular dichroism (CD) measurements were made on a Jasco J810 spectropolarimeter from 260 to 190 nm at 0.2 nm intervals, a rate of a 100 nm/min, a response time of 4 s, and a bandwidth of 1 nm. Differential Scanning Calorimetry (DSC) was performed on a VP-MicroCal (GE). Both blank and DOX-loaded micelles (600 µl, 2 mg/ml) were analyzed, as temperature was increased from 5 °C to 60 °C at a rate of 1 °C/min. DSC thermograms were obtained after concentration normalization and baseline correction with Origin software provided by MicroCal.

4.4.4 Stability and DOX Release in Serum Albumin
DOX-loaded micelles (10 mg/ml) and serum albumin (10 mg/ml) were mixed and incubated at 37 °C for 20 hours and the mixture was analyzed by SEC for any changes in size distribution. The release of DOX from 3-helix micelles was monitored by change in fluorescence of DOX-loaded micelles in presence of serum albumin (50 mg/ml). Fluorescence measurements were performed on LS-55 fluorometer (Perkin Elmer).
Micelle solution in quartz cuvettes was excited at 480 nm and the emission spectra were recorded from 510-650 nm for 20 hours. Serum albumin is added at the start with final concentration of 50 mg/ml. Fluorescence recovery is defined as \( \frac{[I(t) - I(0)]}{I(\text{MeOH}) - I(0)} \times 100 \), where \( I(t) \) is intensity at time \( t \), \( I(0) \) is intensity at \( t = 0 \) before introduction of albumin, and \( I(\text{MeOH}) \) is intensity in methanol.

4.4.5 Proteolytic Disassembly of 3-Helix Micelles
Fluorescence recovery of fluorescein labeled 3-helix micelles in presence of proteinase K was monitored to study proteolytic disassembly of micelles. 10 µl of concentrated proteinase K (final concentration: 150 µg/ml) was added to fluorescently quenched solution of dye labeled micelles (15 µM peptide, 30 mol% fluorescein). The increase in fluorescence after addition of protease was monitored for 3 hours. Fluorescein was excited at 480 nm and emission spectra were recorded from 490-640 nm. The degradation of peptide in the micellar shell was analyzed by MALDI-TOF spectroscopy (Applied BioSystems).

4.4.6 In Vitro Cytotoxicity
Cytotoxicity of free DOX, blank micelles and DOX-loaded micelles was investigated by MTT assay. PPC-1 human prostate cancer cells and 4T1 murine breast cancer cells were cultured in DMEM high glucose medium containing 10% FBS and 1% antibiotic and IMDM medium containing 10% FBS and 1% antibiotic, respectively. 24 h before experiments, cells were seeded at 2000 cells/well in 96-well tissue culture plates. Treatments were added in 100 µl medium/well in triplicate: 1) 25% PBS (v/v) in medium, 2) free DOX (1 µg/ml and 5 µg/ml), 3) DOX-loaded micelles (1 µg/ml and 5 µg/ml encapsulated DOX) and 4) blank micelles (concentration matched to the peptide content of the DOX-loaded micelles at DOX concentration of 5 µg/ml). After 36 to 72 h continuous incubation of cells with each treatment at 37°C in 5% CO₂, cytotoxicity was quantified via MTT assay. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/ml) was added to each well, and cells were incubated for 2 h at 37°C in 5% CO₂. After removal of medium, formazan crystals were dissolved in DMSO (100 µl/well) and absorbance was measured at wavelength 570 nm using a Tecan (San Jose, CA) Infinite® M1000 microplate reader. Statistical comparisons were performed by ANOVA followed by Turkey's multiple comparisons test.

4.4.7 Intravenous Administration of DOX-loaded Micelles
All animal experiments for intravenous administration were conducted under a protocol approved by the University of California, Davis, Institutional Animal Care and Use Committee (Davis, CA). NDL tumors were transplanted into the inguinal mammary fat fads of five to six week old FVB mice. 52 Approximately after 3 weeks, when the size range of the NDL tumors was 5-10 mm, animals were anesthetized with isoflurane and received an intravenous (IV) injection of DOX-loaded micelles. 150 µl of DOX-loaded micelles at a concentration of 12 mg/ml was injected. Mice were euthanized with an IV injection of Euthasol (Western Medical Supply, CA) at 24 h and 72 h after administration. Blood was drawn and 50 ml of saline was perfused into the left ventricle. Organs of interest, such as heart, liver, spleen, kidneys, skin, and tumors, were harvested.
and scanned on a Maestro 2 imaging system (PerkinElmer, Massachusetts). Images were acquired at emission wavelength of 550 nm. For skin toxicity assay, DOX-loaded micelles (6mg DOX/kg) were administered to mice intravenously two times per week. Images were taken at three weeks after treatment started. Skin toxicity from DOX-loaded micelles was compared with the treatment from Doxil as previously reported.39
Chapter 5
Tuning Cargo Loading and Encapsulation Stability in 3-Helix Micelles

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3-helix micelles exhibit high in vivo kinetic stability and favorable biodistribution and are attractive as a nanocarrier platform. Small core size combined with headgroup design based on protein tertiary structures present unique challenges for drug formulation with high loading in 3-helix micelles. Using cargo molecules over a broad range of hydrophobicity, loadings in the range of 1.5-8 wt% could be achieved in 3-helix micelles, with no obvious dependence of loading content on drug hydrophobicity or molecular size. Molecular planarity was identified as the most critical parameter that dictates interaction of drugs with micelle core and the resulting loading content. Drugs with more planar structure have higher loading in 3-helix micelles. Polymeric lipid DSPE-PEG and amphiphilic block copolymers were mixed with 3-helix peptide conjugate to tune chain packing in micelle core and facilitate incorporation of drugs with non-planar structure with high loadings. Mixed 3-helix micelles could achieve higher loadings of 8 wt% for rapamycin (RAP), compared to 3 wt% loading in pure 3-helix micelles. RAP loaded mixed micelles exhibit slower and extended drug release with smaller diffusion constants. Mixed 3-helix micelles based on different polymeric amphiphiles provide a convenient approach to tune micelle drug interactions to achieve higher drug loading and slow release. The loadings of 8 wt% for multiple drugs in different formulations of 3-helix micelle are fairly close to the theoretical limit of loading dictated by the small core volume. These studies offer fundamental insights into molecular parameters that can be tuned to increase drug loading content in 3-helix micelles and are important to establish their utility as a versatile and stable drug formulation platform, 15-20 nm in size.

5.1 Introduction

Over 40% of emerging small molecule drugs require formulation development to improve their pharmacokinetics, biodistribution, toxicity profile and efficacy. Liposomes and block copolymer (BCP) micelles have been widely used to formulate hydrophobic drugs for intravenous administration. One of the most successful examples of liposomal drug formulation is that of DOXIL, ~100 nm in size, with drug loading of 11 wt%, which is in clinical use for the treatment of ovarian and breast cancer. BCP micelles, 40-150 nm in size, have been investigated extensively as nanocarriers with drug content over a broad range of 2-25 wt%. Smaller nanocarriers with 10-30 nm size have advantages of facile extravasation from vasculature, deep tumor penetration and more homogeneous intratumor distribution. However, small micelles tend to have extremely low kinetic stability and poor pharmacokinetics which restricts their utility for drug formulation. Newly developed 3-helix micelles based on amphiphilic peptide-polymer conjugates demonstrated excellent in vivo kinetic stability, selective and deep tumor distribution and minimal accumulation in liver and spleen. Intravenous administration of doxorubicin (DOX) loaded 3-helix micelles demonstrated longer half-life in tumor tissue and reduced toxicity towards healthy tissues relative to free drug.

Drug molecules vary widely in terms of their chemical structure, physical properties, and molecular geometry and this structural diversity presents significant challenges for the design of a universal formulation system for different drugs. A critical criterion for drug formulation using nanocarriers is to achieve a high loading and understanding of
parameters that can be used to increase loading content is essential. Drugs loadings in the range of 4-15 wt% have been achieved in liposomes, 100-150 nm in size, depending on lipid composition and method of drug incorporation. Previous studies with BCP micelles have shown that chemical compatibility of drug with the micelle core and the physical space available in core are the predominant factors that dictate loading content. Drug loadings of 10-20 wt% have been obtained in 40-100 nm BCP micelles by tuning hydrophobic, ionic and hydrogen bonding interactions between drugs and core forming polymers. High loadings of 20-25 wt% have been achieved in 100-150 nm BCP micelles with larger cores that provide greater space for drug localization.

For smaller nanocarrier, small volume available for cargo loading is an inherent limitation. For 15 nm 3-helix micelle, where the core radius is comparable to shell thickness, the volume occupied by core is only 12.5%. As most hydrophobic drugs localize in the hydrophobic core, this poses a theoretical limit for cargo loading content. DOX loadings of 8 wt% could be achieved in 3-helix micelles without compromising micelle size and size distribution. It is critical to identify the molecular parameters that can be tuned to control drug loading content in 3-helix micelles, significantly smaller than conventional nanocarriers.

3-helix micelles are unique in terms of their subunit design, small size and exceptionally high stability, and all of these features would influence their drug loading capacity. This chapter presents a systematic study focused on encapsulation of a number of structurally diverse, clinically approved, difficult to formulate, drugs in 3-helix micelles. Drug loadings in the range of 1.5-8 wt% could be achieved, with no obvious dependence of loading content on drug hydrophobicity or molecular size. Quantification of molecular planarity using a simple model shows that drugs with greater planarity in their structure have more facile incorporation in the densely packed core of 3-helix micelles. These studies underscore the significance of three dimensional structures of drug molecules, over other physicochemical properties, for their incorporation in 3-helix micelles, where core size is comparable to molecular dimensions of the drug. Mixing 3-helix peptide conjugate with different amphiphiles such as DSPE-PEG and BCP allows tuning the composition and phase behavior in micelle core, which leads to enhanced drug loading for two structurally different drugs in mixed 3-helix micelles. We show that loading content for rapamycin (RAP) increases from 3 wt% in pure 3-helix micelles to 8 wt% after its formulation in mixed 3-helix micelles. RAP loaded mixed micelles exhibit slower and extended drug release with smaller diffusion constants. These studies provide critical insights into the molecular parameters that control the drug content and encapsulation stability of 15 nm 3-helix micelles, and are important to establish the utility of 3-helix micelles as a versatile nanocarrier platform for formulation of a range of clinically relevant drugs.

5.2 Results and Discussion

Six drugs with different hydrophobicity and molecular structure were investigated for their encapsulation in 3-helix micelles. Tamoxifen (TAM), paclitaxel (PAX), and doxorubicin (DOX) are highly potent anticancer drugs that are in clinical use
for treatment of a wide range of solid and metastatic tumors. Rapamycin (RAP) is widely used as an immunosuppressant and is emerging as an anticancer drug. Apomorphine (APO) is currently used in the treatment of Parkinson’s disease. Dexamethasone (DEX) is an approved drug for treatment of a number of inflammatory and autoimmune conditions. All of these drugs have low aqueous solubility in the range of 0.1-40 µg/ml, and would greatly benefit from nanocarrier formulations to increase their blood circulation time and minimize side effects associated with off-target accumulation. 17, 20, 23, 25, 41, 42 Table 5.1 lists the different drugs that were formulated in 3-helix micelles, and the loading obtained for each of the drugs.

Table 5.1 lists the different drugs that were formulated in 3-helix micelles, and the loading obtained for each of the drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Log P</th>
<th>Molecular weight (Da)</th>
<th>Solubility parameter, δₙ (J/cm³)¹/₂</th>
<th>Loading (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen (TAM)</td>
<td>5.93</td>
<td>371</td>
<td>19.11</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>Rapamycin (RAP)</td>
<td>4.85</td>
<td>914</td>
<td>20.29</td>
<td>3.3±0.2</td>
</tr>
<tr>
<td>Paclitaxel (PAX)</td>
<td>3.21</td>
<td>853</td>
<td>21.15</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Apomorphine (APO)</td>
<td>2.51</td>
<td>267</td>
<td>19.29</td>
<td>7.6±0.5</td>
</tr>
<tr>
<td>Dexamethasone (DEX)</td>
<td>1.93</td>
<td>392</td>
<td>20.37</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Doxorubicin (DOX)</td>
<td>1.91</td>
<td>543</td>
<td>20.06</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>17.90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: Physicochemical properties of the drugs that were investigated for their formulation in 3-helix micelles. Loadings obtained for each drug represent average from 5 different measurements.

Log P and molecular weight of these drugs vary over a significant range that allows investigation of the drug loading capacity of 3-helix micelles for cargoes with different molecular structure. Hansen solubility parameters based on non-polar interactions, δₙ, calculated for different drugs and micelle core component stearic acid, indicate that these drugs cover a broad range of compatibility with 3-helix micelle core. The primary mode of interaction between drugs and micelle would be hydrophobic interaction between the drugs and alkyl chains in micelle core, which would dictate the loading content in micelles. Previous studies have shown that loading content in micelles generally correlates with cargo hydrophobicity, with higher loading content for more hydrophobic drugs. 38, 43, 44
Drug loaded micelles could be reproducibly solubilized in aqueous buffer after the formulation process. Micelle size and size uniformity are maintained after incorporation of different drugs (Appendix A.4.1). The loading content of different drugs in 3-helix micelles varies from 1.5-8 wt% as shown in Table 5.1. For 3-helix micelles, there is no obvious dependence of loading content on drug hydrophobicity and cargo molecular weight (A.4.2). \textit{In vitro} release of drugs from 3-helix micelles shows drug and loading content dependant cargo retention in micelles. Drugs with lower loading in the range of 1.5-3 wt\%, exhibit significantly faster release, compared to drugs with higher loading of 7.5-8 wt\%, that show relatively slower and extended release from 3-helix micelles (A.4.3).

The loading capacity of 3-helix micelles has no obvious dependence on chemical structure and hydrophobicity of drug molecules. We believe that cargo geometry would play critical role in determining loading content in highly stable 3-helix micelles that are significantly smaller than conventional nanocarriers. The dimensions of drug molecules studied range from ~1-2.3 nm as assessed by Chem3D. For 3-helix micelles, the core radius is ~ 2.8 nm, comparable to molecular dimensions of drug molecules studied here. The built in MM2 simulation function in Chem3D that is designed to reproduce equilibrium covalent geometry of molecules was used to obtain energy minimized 3D structures for different drugs. A closer look at the 3D structure of different drugs indicates higher extent of planarity for molecules with higher loading content (Figure 5.1).

![Figure 5.1: Three dimensional structure of drugs assessed in Chem3D show higher planarity for drugs with higher loading in 3-helix micelles. Molecular size of drugs is comparable to core radius of 3-helix micelles.](image-url)
We speculate that more planar geometry of cargo molecules would facilitate their incorporation within alkyl chains in core of micelles, whereas it would be difficult to accommodate drugs with bulkier structure without severely affecting the alkyl chain packing (Figure 5.2). Considering high stability of 3-helix micelles and dense packing of alkyl chains in micelle core mediated by rigidity of shell due to protein tertiary structures, it would be energetically unfavorable for bulky drugs with isotropic geometry to get incorporated in 3-helix micelles.

![Drugs with planar structure could be incorporated in alkyl chain core of 3-helix micelles](image1)

![Drugs with less planar structure would not be incorporated in core of 3-helix micelles without significant chain disruption, which would be energetically unfavorable due to high stability of 3-helix micelle](image2)

Figure 5.2: Small space available in the core of 3-helix micelles, with dense alkyl chain packing could allow easier incorporation of drugs with more planar structure, relative to drugs with bulky structure.

In order to understand the quantitative dependence of loading content on drug geometry, cargo molecules were confined in a 3D box and planarity of molecules was quantified by defining an index based on the ratio of dimensions of the box. Figure 5.3 shows the geometric model that is used to define planarity index as the ratio of vertical and lateral dimensions of the box that surrounds the cargo molecule. Drugs with more planar structure have higher value of planarity index. Figure 5.3 shows that loading content for different drugs in 3-helix micelles increases with planarity index. This indicates that drug molecules with more planar and less isotropic structure have higher loading in 3-helix micelles. This simple geometric model confirms our hypothesis on the critical role of molecular geometry on cargo loading capacity of 3-helix micelles, and explains the
experimental results on loading content fairly well. Previous studies on polymeric micelles, larger than 30 nm, have established that drug loading efficiency of micelles is directly related to the drug hydrophobicity and chemical compatibility between the drug and core forming block.\textsuperscript{35, 39, 40} However, the results from this study indicate that geometric considerations in terms of 3D structure of drug molecule are critical in determining the drug-micelle interaction and the loading capacity of 3-helix micelles, 15 nm in size. These systematic studies underscore the significance of a structural feature of cargo molecule, which is not commonly investigated in detail for its effect on micellar loading capacity. These results provide useful guidelines to be considered for detailed simulation and theoretical studies on design of smaller micelles for drug formulation.

![Figure 5.3](image)

**Figure 5.3:** Quantification of planarity of drug molecules by defining an index based on the ratio of vertical and lateral dimensions of the cuboid that confines the drug molecule. Drugs with higher planarity in their structure have higher value of the index. Drug loading content in 3-helix micelles increases with the planarity index of molecules. Solid line is for visual aid.

The distinct effect of molecular structure of cargo on loading capacity in 3-helix micelles has its origin in the unique design features. The close packing of amphiphilic subunits based on protein tertiary structures results in small micelles with high radius of curvature and high local density of rigid subunits. High stability, combined with small size of 3-helix micelles severely restricts the accessibility of drug molecules for the small space available in micelle core. The phase behavior of micelle core also affects its interaction with different drugs. We hypothesize that thermal characteristics of micelle core can be tuned by mixing 3-helix peptide conjugate with polymer-lipid conjugates. Apart from changing core packing, rigidity in 3-helix micelle shell would be reduced by adding amphiphiles containing soft polymeric headgroups, which could modify the incorporation of drugs in mixed micelles. For this purpose, DSPE-PEG was mixed with peptide conjugate in equal weight proportions. The self assembly of two amphiphiles resulted in
well defined micelles with hydrodynamic diameter of ~ 12 nm with uniform size distribution (A.4.4). DSC thermogram of mixed micelles shows that transition temperature for core packing is significantly lower relative to 3-helix micelles (Figure 5.4).

![DSC thermograms for 3-helix and DSPE-PEG mixed 3-helix micelles](image)

Figure 5.4: DSC thermograms for 3-helix and DSPE-PEG mixed 3-helix micelles in phosphate buffer (25mM, pH 7.4), both at concentration of 2 mg/ml. The composition of mixed micelles is 1:1 wt/wt of 3-helix conjugate and DSPE-PEG. The distinct transition temperature for alkyl chains in core of the two micelles indicate difference in the phase behavior of the core forming chains in the two micelles.

The transition temperature for alkyl chain packing in 3-helix micelles is ~ 24°C. The broad peak for mixed micelle transition could be deconvoluted to two peaks corresponding to phase transition temperatures of 11°C and 16°C. This suggests that mixing 3-helix conjugate with polymeric lipid changes the phase behavior of core packing. These changes in alkyl chain conformation and packing in core of mixed micelles could potentially facilitate the interaction of molecularly diverse drugs with micelle core and increase the loading capacity of mixed 3-helix micelles.

In order to test this hypothesis, two drugs with different molecular structures, DOX and RAP, and distinct loading content in 3-helix micelles were selected to study their loading in mixed micelles. Encapsulation of DOX and RAP in mixed micelles results in significantly increased loading for both drugs relative to 3-helix micelles as shown in Table 5.2. DLS (A.4.5) confirms that self assembly of the amphiphiles into mixed micelles is maintained after incorporation of DOX and RAP at significantly high loading.
content without any adverse effect on uniformity of size distribution. Hence, we show that mixing 3-helix peptide conjugate with polymeric lipids can tailor the core packing of micelles and could be a viable strategy to increase cargo loading in 3-helix micelles.

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>DOX loading (wt%)</th>
<th>RAP loading (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-helix micelle</td>
<td>7.9±0.4</td>
<td>3.3±0.2</td>
</tr>
<tr>
<td>DSPE-PEG mixed 3-helix micelle</td>
<td>11.4±0.5</td>
<td>7.8±0.3</td>
</tr>
</tbody>
</table>

Table 5.2: Loading for two different drugs in DSPE-PEG mixed 3-helix micelle and 3-helix micelle. DSPE-PEG mixed 3-helix micelles have significantly higher loading relative to 3-helix micelles, for two structurally distinct drugs, DOX and RAP.

Increased loadings for DOX and RAP were achieved in DSPE-PEG mixed 3-helix micelles. Pure 3-helix micelles with 8 wt% DOX loading exhibit excellent encapsulation stability and meet several critical requirements as nanocarriers for DOX as discussed in chapter 4, and hence DOX formulation is not the focus of the present study. We focus on loading behavior and encapsulation stability of RAP in different 3-helix micelle based formulations. Figure 5.5 shows the release of RAP from different formulations at 37°C as monitored by dialysis technique.

Figure 5.5: Release profile of RAP from 3-helix micelles and DSPE-PEG and BCP based mixed 3-helix micelles. Mixed micelles were prepared from 1:1 wt/wt mixture of peptide...
conjugate and the respectively added amphiphile. Drug loaded micelle solutions (3mg/ml, 2ml) were placed in dialysis bags with MWCO 3500 Da, and release was monitored by quantification of drug in micelles at different times as determined by HPLC.

The release of RAP from DSPE-PEG mixed 3-helix micelles is significantly slower compared to 3-helix micelles. 22% of RAP is released from DSPE-PEG mixed 3-helix micelles after 8 hours, whereas 53% of RAP is released from 3-helix micelles after 8 hours. Drug release from micelles is dictated primarily by micelle stability and micelle drug interactions. DSPE-PEG mixed 3-helix micelles exhibit lower stability compared to 3-helix micelles. In case of release being controlled by micelle stability, drug release from mixed micelle formulations should be faster relative to pure 3-helix micelles. FRET studies on RAP formulations of the two micelles confirm that RAP formulated DSPE-PEG mixed 3-helix micelles exhibit lower stability relative to RAP formulated pure micelles (A.4.6). However, RAP release from mixed micelles is slower compared to 3-helix micelles. These studies suggest that stability differences between RAP formulated pure and mixed micelles cannot explain the release of RAP from these formulations. Difference between release profiles are explained on the basis of the mechanism of drug release as discussed later in the following section.

We show that mixing peptide conjugate with DSPE-PEG provides a simple way to tune drug loading content in 3-helix micelles. In order to extend the families of amphiphiles that can be mixed with 3-helix peptide conjugate to tune loading, amphiphilic BCPs were used. Polyethyleneglycol-b-polycaprolactone (PEG(2K)-b-PCL(2.8K)) could be mixed with peptide conjugate up to equal weight proportions to generate BCP mixed 3-helix micelles without adversely affecting the homogeneity of size distribution of micelles (A.4.7). BCP mixed 3-helix micelles exhibit reasonably high stability, although the rate of subunit exchange is faster relative to 3-helix micelles (A.4.8). RAP loadings of 8.4±0.5 wt% could be achieved in BCP mixed 3-helix micelles. Release of RAP is significantly slower from these formulations, with 17% release after 8 hours (Figure 5.5). These set of studies show that two different amphiphiles could be mixed with 3-helix peptide conjugate to obtain mixed micelles with significantly higher RAP loading in the range of 7.8-8.4 wt%, and greater RAP encapsulation stability relative to pure 3-helix micelles.

RAP formulations in mixed 3-helix micelles were further characterized to understand the mechanism of drug release. In absence of degradation and swelling of micelles, RAP release from pure and mixed micelle formulations could be fitted to Higuchi model based on diffusive release with reasonable accuracy (Figure 5.6). Higuchi rate constant for RAP release from 3-helix micelle obtained from linear fit is 0.208 hr^{-1/2}, which corresponds to diffusion constant of 5.93x10^{-19} cm^2/s. Rate constants for DSPE-PEG and BCP mixed 3-helix micelles are 0.096, 0.072 hr^{-1/2}, with diffusion constants of 1.26x10^{-19} and 0.72x10^{-19} cm^2/s respectively for RAP release from these formulations. 3-helix micelles with lower RAP content have faster rate of diffusion, where as mixed micelle formulations with higher loading exhibit lower rate of drug diffusion. Based on geometric considerations regarding drug incorporation in micelle core as discussed earlier, we
expect RAP, with bulky molecular structure, to be loosely associated with 3-helix micelles, which in turn could explain faster release of RAP with shorter diffusion path length. Significantly smaller diffusion constants for RAP release from mixed micelles indicate better drug incorporation within mixed micelles compared to 3-helix micelles. We speculate that changes in the core packing and shell rigidity after mixing peptide conjugate with other amphiphiles changes the interaction between drug and micelles, and potentially the site of drug localization. Slower diffusive release of RAP from relatively less stable mixed micelles suggests that site of RAP localization within mixed micelles is different from that of 3-helix micelles. These changes in the site of drug localization in mixed micelles with modified chain packing in core lead to stronger retention of RAP within mixed micelles. Better retention RAP due to its different site of localization in mixed 3-helix micelles explains extended release and smaller rates of drug diffusion from mixed micelles relative to pure 3-helix micelles. Collectively, these studies demonstrate that formulation in mixed 3-helix micelles is a viable strategy to tune the intermolecular interactions between drugs and micelle and could be used to increase drug loading content and to prolong the release.

![Figure 5.6: Fits of RAP release to Higuchi model for diffusive release. Solid lines show the fits of the data to Higuchi equation, given by \( f = kt^{0.5} \), where \( f \) is fractional drug release, \( t \) is time, and \( k \) is Higuchi constant. The values of \( k \) obtained by fits are: 0.208 hr\(^{-1/2}\) for 3-helix micelles; 0.096 hr\(^{-1/2}\) for DSPE-PEG mixed 3-helix micelles; and 0.072 hr\(^{-1/2}\) for BCP mixed 3-helix micelles.](image-url)
5.3 Conclusion
In summary, we present a systematic study to identify the molecular parameters that dictate drug loading content in 3-helix micelles. The results on formulation of structurally diverse cargo in 3-helix micelles show that 3D structure of cargo molecule has a critical effect on their interaction with micelle core and drug incorporation in 3-helix micelles. Drugs with greater planarity have higher loading content in the densely packed core of 3-helix micelles. 3-helix peptide conjugates could be mixed with polymeric amphiphiles to tune the core packing that changes the interaction of bulkier drugs with micelle and increases their loading in mixed 3-helix micelles. Using RAP as a model drug that has a non-planar structure, we show that mixed 3-helix micelles based on two different amphiphiles, DSPE-PEG and PEG-PCL BCP allow effective drug formulation with significantly increased loading of 8 wt%. Release of RAP from mixed micelles is much slower compared to pure 3-helix micelles, with smaller rate of drug diffusion. Slow diffusive release of RAP from mixed micelles suggests small drug leakage and has important implications in terms of encapsulation stability during storage and blood circulation. These studies demonstrate that mixed 3-helix micelles based on two different amphiphiles present a promising platform to increase drug loading content in 3-helix micelles without comprising the advantages offered by their small size and high stability. Work described earlier in this dissertation showed that 3-helix micelles are sensitive to proteolytic degradation which results in their disassembly and enables drug release. Collectively these results suggest that 3-helix micelles are highly promising as a nanocarrier platform with tunable cargo loading and encapsulation stability, and triggered/controlled drug release at an intended target site mediated by micelle degradation.

5.4 Experimental

5.4.1 Synthesis of Peptide-Polymer Conjugate
The details about materials and the synthesis of amphiphilic peptide-polymer conjugate have been described previously. The important steps in the synthesis of conjugate are the solid phase conjugation of alkyl tails to peptide N-terminus, and PEG chain with molecular weight of 750 Da (P750) to peptide C-terminus. Peptides were then cleaved off the resin by standard procedures. Cysteine at position 14 facilitates the site specific coupling of maleimide functionalized PEG of molecular weight 2000 Da (P2K) to the middle of the peptide sequence in aqueous buffer. The resulting conjugate was purified by reverse-phase high pressure liquid chromatography (RP-HPLC) with water/isopropanol gradient and subsequently lyophilized.

5.4.2 Formation of Mixed Micelles and Biophysical Characterization
Mixed 3-helix micelles based on polymeric lipid DSPE-PEG, and amphiphilic BCP polyethylene glycol-b-polycaprolactone (PEG(2K)-b-PCL(2.8K)) were prepared by thin film hydration. Equal amounts of 3-helix peptide conjugate and the mixing amphiphile were dissolved in methanol and the solvent was evaporated in vacuum oven for 3 h. The dried film was rehydrated with 25 mM phosphate buffer, pH 7.4, and the solution was stirred for 16 h to allow assembly of mixed 3-helix micelles. The solution was
concentrated by spin ultrafiltration and the concentrate was lyophilized till further use. Dynamic Light Scattering (DLS) size measurements were made on a Malvern Zetasizer Nano-ZS with a 633 nm laser and a scattering angle of 17°. Size exclusion chromatography (SEC) was carried out on a BioSep-SEC-S 4000 column (Phenomenex), with flow rate was 1 ml/min and 25 mM phosphate buffer, pH 7.4, was used as the elution solvent. Differential Scanning Calorimetry (DSC) was performed on a VP-MicroCal (GE), as temperature was increased from 5 °C to 60 °C at a rate of 1 °C/min. DSC thermograms were obtained after concentration normalization and baseline correction with Origin software provided by MicroCal. In vitro stability of micelles in serum albumin was studied by Förster Resonance Energy Transfer (FRET) from micelle encapsulated donor/acceptor dyes, pair as described previously.  

5.4.3 Drug Loading and Release from Micelles

The drug loading in micelles was performed by thin film hydration method as described before. Drug release profiles from micelles were studied using dialysis bag technique at 37°C. The solution of drug loaded micelles (3mg/ml, 2 ml) was placed in a dialysis bag (Spectrum Labs, MWCO 3500 Da). The dialysis bag was immersed in 500 ml of PBS solution (25 mM, pH 7.4) in a glass beaker, which was stirred at 600 rpm. 10 µl of solution was drawn from dialysis bag at desired time intervals and the drug concentration was analyzed using RP-HPLC. Drug release profiles were fitted to Higuchi model for diffusive release, described by \( Q = 2C_o(Dt/\pi)^{1/2} \), where \( Q \) is the amount of drug released per unit area, \( C_o \) is the initial drug concentration in micelle, \( t \) is the time in seconds, and \( D \) is the apparent diffusion constant. Higuchi equation can be rearranged to obtain fractional drug release, \( f \), as \( f = kt^{1/2} \), with Higuchi rate constant, \( k = (36D/\pi R^2)^{1/2} \), where \( R \) is radius of micelle.

5.4.4 Physicochemical Properties and Structure of Drugs

Six different drugs covering a broad range in terms of molecular structure were chosen for formulation in 3-helix micelles. LogP values used as a quantitative measure of hydrophobicity were obtained from DrugBank. Hansen solubility parameter accounting for non-polar and VanderWaals interactions, \( \delta_d \) was calculated by group contribution method. Drug molecules are divided into smaller functional groups and the contributions from different groups were accounted together to evaluate \( \delta_d \). Molecular dimensions of drug molecules were obtained from Chem3D Pro12.0. Energy minimized 3D structure of molecule were obtained by MM2 simulation function. Molecules arranged according to fixed reference system were confined in the smallest box to define molecular dimensions of different cargo.
Chapter 6
Potential of 3-Helix Micelles for Brain Delivery

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6.4 Experimental Section ............................................. 75
6.1 Introduction
In US, over 10000 patients are diagnosed each year with Glioblastoma multiforme (GBM), about half are alive 1 year after diagnosis, and 25% after two years. The long-term prognosis for patients with GBM after conventional therapy is poor, and has remained relatively static over the last 30 years. Many novel strategies have been developed for the treatment of GBM, but few have progressed beyond early-phase clinical trials. Chemotherapeutic treatment for GBM has been largely ineffective because of limited access and poor penetration of drugs due to blood brain barrier (BBB). Drug containing nanoparticles have been shown to cross BBB by passive diffusion across the tight junctions in the barrier and receptor mediated cellular internalization pathways. However, there have been limited reports that have shown enhanced therapeutic effect from intravenously administered nanocarriers for the treatment of GBM.

Challenges associated with delivery across BBB have led to the development of alternate routes for drug delivery with more facile access to brain tissue. Convection-enhanced delivery (CED) of therapeutic agents into brain is a promising treatment strategy, allowing direct infusion of high concentrations of therapeutic drugs, while essentially eliminating systemic toxicity. CED is a pressurized infusion technique that allows even distribution of therapeutic agents at high concentrations over a larger area in brain tissue, than in the absence of CED, that is, by diffusion alone. Cerebrospinal fluid (CSF) delivery by intrathecal injection represents another way in which brain anatomy may be exploited to distribute therapeutics broadly in the central nervous system. Liposomes and adeno-associated virus (AAV) particles have been delivered by these two routes to rodent and non-human primates and have shown penetration within brain tumors in rat xenograft models and canine patients with spontaneous brain tumors.

3-helix micelles, with 15 nm size and high stability, present a unique nanocarrier platform with potential applications in drug delivery to brain. This chapter presents a preliminary in vivo study to investigate the feasibility of 3-helix micelles as an agent for CED and CSF delivery. Local administration of 3-helix micelles to rodent brain by CED shows that 3-helix micelles distribute evenly through the entire striatum including the posterior lobe. Studies with doxorubicin (DOX) loaded 3-helix micelles show that micelle formulated drug has greater and more uniform distribution relative to free drug in striatum, 7 days after administration. In normal brain tissue, the toxicity from micellar drug is significantly lower compared to that from free drug. Quantification of drug concentration in brain tissue confirms slow leakage of DOX from 3-helix micelles in concentrated protein environment in brain. These studies indicate that tissue half-life and off-target toxicity from DOX are modified favorably after its formulation in 3-helix micelles. Intrathecal delivery to CSF in Rhesus monkey shows that 3-helix micelles can navigate the central nervous system through endogeneous flow of spinal fluid and nanocarriers. Collectively, these studies underscore the potential of 3-helix micelles to localize in different regions of brain by administration through different routes. These findings have significant implications for the development of 3-helix micelles as drug nanocarriers for GBM and other diseases of central nervous system.
6.2 Results and Discussion

6.2.1 Convection Enhanced Delivery (CED) of 3-Helix Micelles
Fluorescent 3-helix micelles labeled with Oregon Green (OG) were used to determine whether micelles would distribute effectively when infused into normal rat striatum. As shown in Figure 6.1, fluorescent 3-helix micelles distributed remarkably well when infused into rat striatum 90 min after infusion. In the left panel of the figure, broad distribution of labeled micelles into anterior striatum is evident, and this same robust distribution was evident throughout the entire brain striatum. H&E staining (shown in right) of the brain tissue, 7 days after infusion, shows minimum evidence of any brain damage or pathology. These results demonstrate the feasibility of direct infusion of 3-helix micelles into brain tissue in a safe and effective manner with broad distribution and minimum toxicity, which suggests potential of 3-helix micelles as a viable carrier for CED delivery.

Figure 6.1: Distribution of fluorescent 3-helix micelles administered by CED to rodent brain. Micelles show wide distribution throughout the entire striatum, 90 minutes after administration. H&E stain on tissue harvested a week after administration shows minimal toxicity from 3-helix micelles.

6.2.2 Distribution and Toxicity of DOX-loaded 3-Helix Micelles
The distribution and toxicity of DOX-loaded 3-helix micelles delivered by CED was investigated to explore their feasibility as a nanocarrier platform for localized delivery in normal rat brain. DOX-loaded micelles showed broader and more uniform distribution in the brain striatum compared to free DOX, seven days after infusion (Figure 6.2a). It is critical to minimize the intrinsic toxicity from DOX-loaded micelles to normal brain. H&E staining of the harvested brain tissue, a week after infusion, showed that DOX-loaded micelles displayed reduced toxicity, whereas free DOX resulted in significant tissue damage (Figure 6.2b). Distribution and toxicity profiles of DOX-loaded micelles indicate stable encapsulation of drug in 3-helix micelles with minimal release in
concentrated protein environment of normal brain tissue. These results clearly show that micelles can be locally delivered by CED, and tissue half-life of DOX can be extended with reduced toxicity towards healthy tissue after incorporation in 3-helix micelles. These findings would have significant implications on therapeutic efficacy of drug nanocarriers based on 3-helix micelles delivered by CED for brain cancer.

Figure 6.2: Brain tissue distribution and toxicity of DOX-loaded micelles and free DOX delivered to Sprague-Dawley rats by CED. (a) Fluorescence images of striatum 7 days after infusion indicate greater distribution of drug in 3-helix micelle formulation. (b) Optical microscopy of tissues after H&E staining, 7 days after infusion show significantly lower toxicity from DOX-loaded micelles compared to free DOX.
6.2.3 Quantification of Drug Half-Life in Brain
The frequency with which CED infusions can be performed into brain tumors is necessarily limited. There is a critical need to develop nanocarriers with long tissue-half-lives and extended drug release, that can be delivered by CED to brain tumors. In order to establish the utility of 3-helix micelles as a drug delivery vehicle with long tissue half-life, we quantified the concentration of drug as function of time in the brain tissue after administration of DOX-loaded 3-helix micelles. Figure 6.3 shows higher drug concentration in brain tissue, immediately after CED of DOX-loaded micelles compared to free DOX injection. This suggests that formulation of DOX in 3-helix micelles decreases the clearance of drug in the brain tissue. More, importantly, the rate of decrease of drug concentration in brain is much smaller for DOX-loaded micelles relative to free DOX. These results confirm minimal drug leakage from 3-helix micelles in normal brain tissue and suggest that tissue half-life for DOX is increased as a result of its formulation in 3-helix micelles. On the other hand, significantly lower drug concentration in tissue, 5 days after administration of free DOX, indicates low tissue half-life with rapid clearance. Collectively, these results suggest that formulation of DOX in 3-helix micelles allows overcoming a number of challenges associated with CED of free drug. Broader tissue distribution with increased drug half-life and reduced toxicity of DOX-loaded 3-helix micelles indicate that formulation of DOX in 3-helix micelles is an effective approach for CED delivery of a potent anticancer drug.

![Graph showing drug concentration in brain tissue](image)

Figure 6.3: Quantification of drug in brain tissue after administration of DOX-loaded micelles and free DOX delivered to Sprague-Dawley rats by CED. Tissue samples were harvested at the day of administration and after 5 days, and drug concentration was determined by liquid chromatography.

6.2.4 Cerebrospinal Fluid (CSF) Infusion of 3-Helix Micelles
3-helix micelles were delivered to non-human primates (NHP) by intrathecal administration to investigate their distribution in brain tissue through CSF. Previous studies have shown that intrathecal administration of 25 nm AAV particles in NHP
resulted in extensive transduction of neuronal and non-neuronal cells throughout the spinal cord, brainstem and neocortex. OG labeled 3-helix micelles were delivered to spinal cord of Rhesus monkey by intrathecal injection. Figure 6.4 shows that 3-helix micelles, shown in green, filled the perivascular space surrounding blood vessels 4 h after injection and distributed extensively through the paravascular pathways deep into the brain. In contrast to AAV particles, infusion of micelles generated prominent perivascular fluorescence not only in brainstem and cerebellum, but also in deeper regions in brain including ventral tegmental area and amygdala. These results show that 3-helix micelles present a promising nanocarrier platform for intrathecal delivery that can be transported to brain by CSF. We believe that that ultra-small size and high stability of 3-helix micelles has significant implications for their access to deeper regions in brain.

Figure 6.4: Cisternal injection of fluorescently labeled 3-helix micelles (shown in green) indicates rapid transport of these particles within different regions of the NHP brain. The micelles fill the perivascular space of blood vessels throughout the brain, particularly in the brainstem (BS), cerebellum (CB), ventral tegmental area (VTA), and amygdala (AM). The micelles are shown in green (white arrows) and presence of autofluorescent lipofuscin is shown in red

6.3 Conclusion
In summary, we demonstrate that 3-helix micelles can be delivered to brain by two different techniques. Micelles can be administered locally to brain tissue by CED in a safe and effective manner with broad distribution through the entire striatum. CED of
DOX loaded 3-helix micelles show that drug formulation in micelles results in increased tissue-life and reduced off-target toxicity compared to free drug. The administration of 3-helix micelles to spine shows that micelles can be transported through paravascular pathways deep into the brain. Small size of 3-helix micelles, combined with their high in vivo kinetic stability makes them highly attractive for delivery into the regions of the brain tissue, not accessible to commonly used nanocarriers. Anatomical targeting of 3-helix micelles by these two distinct routes of administration has important implications for their therapeutic delivery.

6.4 Experimental

6.4.1 Fluorescent and DOX-loaded Micelles for Brain Infusions

Fluorescent 3-helix micelles for CED and intrathecal infusions were prepared by mixing Oregon Green labeled 3-helix peptide-polymer conjugate (dC18-1CW(P2K)-OG) with micelle forming amphiphile (dC18-1CW(P2K)-P750). Both amphiphiles were synthesized according to previously described methods.21 10 mg dC18-1coi(P2K)-P750 and 1 mg of dC18-1CW(P2K)-OG were dissolved in 0.5 mL of methanol in a glass vial and the solvent was evaporated in vacuum oven for 3 h. The dried film was rehydrated with 2 mL of 25 mM phosphate buffer, pH 7.4, and the solution was stirred for 16 h to allow assembly of OG-conjugated fluorescent 3-helix micelles. The micelle solution was washed to remove any unincorporated OG and concentrated by spin filtration. The concentrate was washed with water and lyophilized to obtain OG labeled 3-helix micelles. Fluorescent micelle solution was prepared by directly dissolving the lyophilized fraction in phosphate buffer. DOX-loaded 3-helix micelles, with 8 wt% loading were prepared by dry film hydration method as described in section 4.4.2.

6.4.2 Convection-Enhanced Delivery (CED) of Micelles

All procedures were in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of California at San Francisco. 3-Helix micelles were administered to rat brain by CED to examine their distribution within the brain tissue. A total of 20 µL of free DOX and DOX-loaded micelles (DOX concentration: 0.2 mg/mL) were infused by CED at a rate of 0.5 µL/min in the striatum of normal Sprague-Dawley rats, as previously described.11 Briefly, after rats were anesthetized, a sagittal incision was made in the skin, and burr-holes were made in the skull by a drill. To minimize trauma and reflux, silica cannulae (O.D. 235 µm; I.D. 100 µm) were used for all infusions (Polymicro Technologies, Phoenix, AZ). The cannulae were attached directly to Nanofill-100 syringes placed in the pumps controlled by a Micro4 MicroSyringe Pump Controller (World Precision Instrument). The coordinates for the injection were taken from the rat brain atlas. A week after infusion, the rats were euthanized and brain tissue was sectioned and distribution of micelles was examined with optical microscope. The tissue sections were stained with hematoxylin and eosin (H&E) to look for pathology. For quantification of drug concentration as function of time, tissues harvested from animals were treated with methanol to extract the drug. DOX concentration in solution was determined using reverse phase liquid chromatography.
The concentration of drug was normalized by the mass of the brain tissue to describe the changes in tissue half-life for the two formulations.

6.4.3 Cerebrospinal Fluid (CSF) Infusion of 3-Helix Micelles
All procedures were in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of California at San Francisco. OG labeled 3-helix micelles were infused into spinal cord of Rhesus monkey to investigate their transport through CSF. Monkeys were sedated with ketamine/xylazine and placed in a prone position in a stereotactic frame with the head flexed. A 3 mL syringe was manually guided into spine until CSF was aspirated into the syringe. Once the correct position of the needle was verified, 2 mL of infusate (6mg/ml, OG labeled micelles) was infused at a rate of 0.5 μL/min. The duration of the infusion procedure was approximately 4 min with a 2 min waiting period prior to withdrawal of the needle. Animals were subjected to necropsy 4 h after micelle delivery and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA)/PBS. Brains were harvested, post-fixed in 4% PFA/PBS for 24-48 h and cryoprotected in 30% sucrose. All post-fixed brains were cut into 40 μm serial sections that were then processed for immunohistochemistry. Every second tissue section was collected and viewed with a Zeiss microscope with a filter appropriate for Cy-3 fluorescence.
Afterword

3-helix micelles hold great promise for drug formulation and the findings from the studies discussed here validate their potential as a safe and effective nanocarrier platform. The sensitivity of blood circulation time of 3-helix micelles to specific design parameters paves the way to explore the potential of generating 3-helix micelles with distinct circulation times that would be useful for different imaging and therapeutic applications. 3-helix micelles are sensitive to controlled disassembly mediated by proteolytic degradation of the micellar shell, which is critical for drug release and to ensure clearance. Incorporation of stimuli responsive features in 3-helix micelles can be expanded through the design of micelles that are sensitive to disease specific extracellular and intracellular triggers such as pH, reducing agents, and proteases. These studies would have critical implications for achieving spatial and temporal control over cargo release from 3-helix micelles for a particular therapeutics. 3-helix micelles present an attractive platform to design nanoparticles that can be internalized in cells by specific pathways, and can potentially be targeted to a cell organelle. Self-assembly based generation of 3-helix micelles offers a convenient and highly modular approach to control the composition and spatial distribution of cell targeting ligands on micelle surface, which would facilitate active targeting of therapeutic and diagnostic agents to diseased cells. 3-helix micelles, by virtue of their ultra small size and exceptional stability, offer tremendous promise for delivery of therapeutics to brain tissue. Studies aimed at detailed investigation of the potential of 3-helix micelles to navigate brain tissue after their administration by local and systemic routes are required. The findings from these studies would be instrumental in defining the scope of 3-helix as effective carriers for brain delivery.
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A.1.5

(a) UV−vis spectra of heme titrations into a ~ 6 μM solution of H10H24-PS recorded in a 1 cm path length quartz cuvette, upon addition of 0, 0.3, 0.7, 1.2, 1.7, 2.5, 3.3, 4.0, and 4.3 equiv of heme per 4-helix bundle. The peak at 413 nm, which corresponds to the absorbance of heme bound in the interior of the bundle. (b) The absorbance at 413 nm vs the [heme]/[4-helix bundle] ratio for H10H24 and H10H24-PS in phosphate/OG buffer solution. The ratio at which the slope changes in each data set indicates the number of hemes that each bundle binds.
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A.2 Supporting information for Chapter 3

A.2.1

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