Development of pH-triggered, Self-assembling Peptide Amphiphiles as Tumor Targeting Imaging Vehicles.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Arijit Ghosh, BSc., MSc., MS
Graduate Program in Chemistry.

The Ohio State University

2014

Dissertation Committee:
Dr. Joshua E. Goldberger, Advisor
Professor. Heather Allen
Professor. Dennis Bong
Professor. John Davidson
Abstract

The early detection of cancer is recognized by the American Cancer Society as the most effective way to improve survival outcomes, but can only be accomplished by developing diagnostic agents that can target smaller, earlier stage tumors. For example, a state-of-the-art cancer-specific imaging technique is $^{18}$F-Fluorodeoxyglucose Positron Emission Tomography (FDG-PET), which can locate tumors in vivo with a spatial resolution of ~1 cm. Magnetic Resonance Imaging (MRI) has far greater tissue contrast resolution than PET and spatial resolution of <1 mm, but lacks an imaging agent that can target a cancer hallmark, glycolytic metabolism. Developing stimuli-responsive imaging pharmaceuticals that localize passively in vivo via one of cancer’s generic hallmarks rather than specific biomarkers can prove effective in developing an MRI agent that can specifically image cancer.

One attractive hallmark target is the acidic extracellular microenvironment of tumor tissue (pHe 6.6−7.4) that arises due to the enhanced rate of glycolysis in cancer cells. Creating a material that is nano-sized in blood, but upon reaching the acidic extracellular tumor environment, transforms into a larger, more slowly diffusing object could serve as a novel mechanism for achieving high accumulation of imaging agents at the tumor site compared to the bloodstream.

Work in this dissertation includes the design, synthesis and characterization of self-assembling peptide amphiphile (PA) MRI contrast agents that reversibly transform
from spherical micelles into nanofibers (microns in length) when entering the acidic tumor vasculature. The PA molecules consist of four segments: a hydrophobic alkyl tail, a β-sheet-forming peptide sequence, a charged amino acid sequence and a DO3A-Gd MRI moiety, where DO3A=1,4,7-tris (carboxymethylaza) cyclododecane-10-azaacetyl amide. The PAs were synthesized via the solid phase technique, purified by High Performance Liquid Chromatography and characterized by mass spectrometry, analytical HPLC and peptide content analyses. Circular dichroism spectroscopy, critical aggregation concentration measurements and transmission electron microscopy were used to characterize the transitions and create concentration-pH phase diagrams for selected PAs. Finally, fluorescence anisotropy (FA) was used to probe self assembly in blood serum.

After extensive assessment of structure-property relationships in a series (>40) of rationally designed PAs, we developed the chemical insight for how this transitional pH can be systematically tuned with alkyl chain length, β-sheet sequence, and number of charged residues. A ratio of one hydrophobic to three charged amino acids was necessary to enable this transition in the desired pH range. Finally, we successfully created a vehicle that transitions in blood serum at pH 7.0 using FA with 1.5% of the PA labeled with a Ru(bipy)$_3^{2+}$ fluorophore. Surprisingly, albumin does not bind to these anionic PAs as it does to cationic and hydrophobic surfactants, but instead promotes nanofiber formation due to a molecular crowding effect. We also established that 150 mM NaCl, 2.2 mM CaCl$_2$, and 1.8 mM of 20 kDa PEG replicates the ionic strength and crowding of pure serum. MRI relaxivity values of water protons in presence of the PA were found to
be higher than that for a commercially available Magnevist control, providing a secondary mechanism for enhanced tumor detection.
Dedication

This document is dedicated to my parents and friends.
Acknowledgments

I would like to take this opportunity to thank the variety of people who have contributed significantly to my research and made it a success. First of all, I would like to thank my advisor, Dr. Josh Goldberger for welcoming me to join his group in my 3rd year of graduate studies, whose expert guidance has led to the realization of this dissertation. I would then like to thank Prof. Michael Tweedle whose periodic comments have immensely helped me improve on my work. Next, I would like to acknowledge the constant motivation and support from Dr. Shankaran Kothandaraman, Dr. Natarajan Raju, Dr. Krishan Kumar and Dr. Li Gong for being available whenever I needed help with certain laboratory procedures.

I would like to extend my heartfelt gratitude to the entire Goldberger group, past and current members notably Mr. Mark Haverick, Mr. Keith Stump, Mr. Eric Dobson, Mr. Michael Nicholl, Mr. Aaron Manos, Miss Ashley Wallace and Mr. Christian Buettner for their support in numerous important aspects related to the project and the dissertation. They were always available to help me whenever there were impediments in the way. I would also like to thank Mr. Richard Montione (CMIF, OSU), Dr. Xiangyu Yang (Department of Radiology, OSU) and Dr. Tanya Young (NMR facility manager, OSU) for their assistance with my TEM, MRI and NMR measurements respectively, along with the Biophysical Interaction Characterization and the Analytical Spectroscopy Facility in the department of Chemistry, OSU. It would be a shame if I didn’t cordially
thank all my friends and well-wishers in Columbus particularly Shiladitya Sen, Spencer Porter, Chitrak Gupta, Swagata Dey, Maxx Arguilla, Minette Ocampo to name a few, for their love and encouragement over the past six years. They made life bearable during times of frustration and disappointments. Last but not the least, thanks to my loving parents and family members back in India for always being my source of inspiration.

Finally, I would like to acknowledge the OSU Research Foundation and the Pelotonia Cancer Foundation for funding this research.
Vita

March 2003 .............................................. The Frank Anthony Public School, India
2006 ........................................................ B.Sc. Chemistry, Jadavpur University, India
2008 ........................................................ M.Sc. Chemistry, Jadavpur University, India
2010 ........................................................ M.S., Department of Chemistry, The Ohio State University
2010-present .......................................... Graduate Teaching Assistant, Department of Chemistry, The Ohio State University
2010-present .......................................... Graduate Research Assistant, Department of Chemistry, The Ohio State University

Publications


• Dobson, E. Ghosh A. and Goldberger, J. Programming pH triggered self assembly via isomerization of peptide sequence. In prep

Fields of Study

Major Field: Chemistry
## Table of Contents

Abstract ........................................................................................................................................... ii

Dedication ......................................................................................................................................... v

Acknowledgments ............................................................................................................................. vi

Vita.................................................................................................................................................... viii

List of Tables ...................................................................................................................................... xiv

List of Figures ..................................................................................................................................... xv

List of Abbreviations ......................................................................................................................... xxiii

Chapter 1: Introduction..................................................................................................................... 1

1.1 Motivation – The cancer problem .............................................................................................. 1

1.2 Nanotechnology in cancer therapy – Targeting strategies ..................................................... 2

1.3 Hallmarks of cancer ................................................................................................................... 6

1.4 Research objective ..................................................................................................................... 6

1.5 Molecular self-assembly .............................................................................................................. 7

1.6 Magnetic resonance Imaging (MRI) ......................................................................................... 9

1.7 Dissertation highlights ............................................................................................................. 10

Chapter 2: Experimental methods, materials and synthesis schemes ......................................... 120

2.1 Synthesis of PAs ....................................................................................................................... 20
2.2 Synthesis and Purification of tri-tert butyl ester of DO3A................................. .22
2.3 Conjugation of PAs with tri-tert-butyl ester form of DO3A........................... 24
2.4 Chelation of PA-DO3A with Gd\(^{3+}\)................................................................. 24
2.5 Conjugation of PA\(_2\) and Mouse Serum Albumin with Ru(bipy)\(^{2+}\) NHS dye ..... 25
2.6 HPLC Purification of PAs... ................................................................. 26
2.7 ESI-TOF Mass spectrometry ................................................................. 26
2.8 Analytical HPLC................................................................. 27
2.9 Peptide content analyses...................................................................... 27
2.10 Circular Dichroism (CD) Spectroscopy................................................... 28
2.11 Critical Aggregation Concentration (CAC) determination using the pyrene
   1:3 method.............................................................................................. 29
2.12 Transmission Electron Microscopy (TEM)............................................... 30
2.13 pH titrations of PAs................................................................................ 31
2.14 MRI relaxivity....................................................................................... 32
2.15 Zeta-potential Measurements............................................................... 33
2.16 Dynamic Light Scattering (DLS)........................................................... 33
2.17 NMR.................................................................................................... 33
2.18 UV-Vis spectrophotometry..................................................................... 34
2.19 Fluorescence and Fluorescence Anisotropy (FA).................................... 34
Chapter 3: Results and Discussion: Peptide design, synthesis and characterization - Fine
tuning the pH -trigger of self assembly...................................................... 43
5.4 FA in pure and dilute blood serum................................................123
5.5 FA reversibility, kinetics and stability of morphology transition............126
5.6 FA detection limit............................................................................126
5.7 Effect of salts and serum albumin on self assembly.............................127
5.8 ‘The Macromolecular Crowding Effect’ – Simulation with PEG..............129
5.9 PA structure re-optimization – Transition in pure serum.........................130.

Chapter 6: Conclusions and Future work.................................................164

6.1 Conclusions.......................................................................................164
6.2 Future directions...............................................................................166

References..............................................................................................168

Appendix: Rights and Permissions..........................................................186
List of Tables

Table 1. β-sheet forming properties of amino acids........................................57
Table 2. Peptide content analyses of PAs 3.1-3.5.............................................70
Table 3. CD transition pH and pKa values of PAs 3.1-3.4.................................81
Table 4. Peptide content analyses of PAs 4.1-4.4...........................................106
List of Figures

Figure 1.1. Schematic representations of active and passive tumor targeting with nanodrugs .............................................................. 12
Figure 1.2. The architecturally defective tumor vasculature ......................... 13
Figure 1.3. Nanoparticle biodistribution and clearance .................................. 14
Figure 1.4. Glycolysis in tumor cells leading to acidic extracellular pH .......... 15
Figure 1.5. Schematic representation of proposed research .......................... 16
Figure 1.6. Nanostructures formed via molecular self-assembly ..................... 17
Figure 1.7. Plot showing change in MRI intensity as a function of contrast agent concentration ................................................................. 18
Figure 1.8. The structure of the DO3A derivative of the macrocyclic metal chelator DOTA ((1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)) ................................. 19
Figure 2.1. Synthesis Scheme of PAs using the solid phase method and Fmoc chemistry ................................................................. 37
Figure 2.2. Synthesis Scheme of the tri-tert-butyl ester DO3A derivative .......... 38
Figure 2.3. a) PA-DO3A conjugation and b) Gd\(^{3+}\) chelation Scheme .............. 39
Figure 2.4. Schematic representation of the Circular Dichroism set up ............ 40
Figure 2.5. Schematic representation of Critical Aggregation Concentration measurements using the pyrene 1:3 method ........................................... 41
Figure 2.6. Schematic representation of the Fluorescence Anisotropy set up...........42
Figure 3.1. PA design strategy and generic structure........................................58
Figure 3.2. Structures of synthesized PAs..........................................................59
Figure 3.3. Analytical HPLC traces of PAs 3.1-3.6.................................................61
Figure 3.4. ESI-Mass spectra of PAs 3.1-3.6.........................................................64
Figure 3.5. pH-dependent CD spectrum of 10 µM PA 3.1......................................71
Figure 3.6. pH reversibility of morphology transition in 10 µM PA 3.1......................72
Figure 3.7. TEM images of 10 µM PA 3.1 at pH a) 6.0 and b) 8.0.........................73
Figure 3.8. CAC measurement of PA 3.1 at pH 6.6..............................................74
Figure 3.9. pH dependent CD spectra of PA 3.1 at concentrations of a) 15 µM and b) 30µM..........................................................75
Figure 3.10. pH dependent CAC measurements of PA 3.1.................................76
Figure 3.11. TEM images of 0.5 mM PA 3.1, measured at pH a) 6.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 3.1 as determined via CD (red squares) and CAC (blue diamonds) measurements. The white area corresponds to a self assembled region where the morphology is uncertain due to lack of suitable experimental techniques. All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl2..........................................................77
Figure 3.12. pH dependent CD spectra of 10 µM a) PA 3.2 b) PA 3.3 c) PA 3.4......78
Figure 3.13. pH reversibility of morphology transition in 10 µM PA 3.3....................79
Figure 3.14. pH titration curves of 10 µM a) PA 3.1 b) PA 3.2 c) PA 3.3 and d) PA 3.4..........................................................80
Figure 3.15. pH dependent CD spectrum of 10 µM PA 3.5.................................82

Figure 3.16. TEM images of 0.5 mM PA 3.5, measured at pH a) 4.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 3.5 as determined via CD (red squares) and CAC (blue diamonds) measurements. All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl₂.................................................................83

Figure 3.17. pH dependent CD spectra of PA 3.5 at concentrations of a) 20 µM and b) 0.5 mM.................................................................................................................................84

Figure 3.18. pH dependent CAC measurements of PA 3.5........................................85

Figure 3.19. DLS measurements for 2 mM PA 3.5 in salt solution at pH 8.0 and 6.0.....86

Figure 3.20. pH dependent CD spectra of PA 3.6 at concentrations of a) 20 µM and b) 0.5 mM.................................................................87

Figure 3.21. pH dependent relaxivity of water protons in presence of various concentrations of PA 3.5.................................................................88

Figure 3.22. a) Chemical structure of Arsenazo III dye b) pH dependent Absorbance spectra of 25 µM Arsenazo III only and Arsenazo-Gd complex control c) pH dependent Absorbance spectra of 25 µM Arsenazo III and 50 µM PA 3.5. The blue spectrum represents the difference between the traces at pH 4.73 and 5.81.................................89

Figure 3.23. Concentration dependent CD transition pH values (blue diamonds) of PA 3.5 in Hank’s balanced salt buffer, superimposed on the phase diagram of PA 3.5 in 150 mM NaCl and 2.2 mM CaCl₂.................................................................90

Figure 3.24. pH dependent CD spectra of 50 µM PA 3.5 in Hank’s balanced salt solution at 37 deg C (body temperature).................................................................91
Figure 4.1. Structures of synthesized PAs.........................................................101
Figure 4.2. Analytical HPLC traces of PAs 4.1-4.4...............................................102
Figure 4.3. ESI-Mass spectra of PAs 4.1-4.4..........................................................104
Figure 4.4. pH dependent CD spectra of a) 10 µM PA 4.1 b) 10 µM PA 4.4..............107
Figure 4.5. pH dependent CD spectra of a) 10 µM PA 4.2 b) 10 µM PA 4.3...........108
Figure 4.6. pH reversibility of morphology transition in 5 µM PA 4.4..........................109
Figure 4.7. pH dependent CAC measurements of PA 4.1......................................110
Figure 4.8. pH dependent CAC measurements of PA 4.4......................................111
Figure 4.9. TEM images of 100 µM PA 4.1, measured at pH a) 5.5 and b) 8.3 c)
Concentration-pH self assembly phase diagram of PA 4.1 as determined via CD (red
squares) and CAC (blue triangles) measurements. The concentration and pH at which
TEM were obtained are labeled in c) (red circles). All measurements were conducted in
150 mM NaCl and 2.2 mM CaCl₂.................................................................112
Figure 4.10. TEM images of 100 µM PA 4.4, measured at pH a) 6.6 and b) 9.9 c)
Concentration-pH self assembly phase diagram of PA 4.4 as determined via CD (red
squares) and CAC (blue triangles) measurements. The concentration and pH at which
TEM were obtained are labeled in c) (red circles). All measurements were conducted in
150 mM NaCl and 2.2 mM CaCl₂.................................................................113
Figure 4.11. TEM images of 100 µM PA 4.2, measured at pH a) 6.0 and b) 10.0. c)
Concentration-pH self assembly phase diagram of PA 4.2 as determined via CAC
measurements. The concentration and pH at which TEM were obtained are labeled in c)
(red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl$_2$.

Figure 4.12. TEM images of 100 µM PA 4.3, measured at pH a) 6.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 4.3 as determined via CAC measurements. The concentration and pH at which TEM were obtained are labeled in c) (red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl$_2$.

Figure 4.13. Concentration-pH self assembly phase diagram of PA 4.1 (blue) overlaid on the same for PA 3.1 (fade pink).

Figure 4.14. Concentration-pH self assembly phase diagram of PA 4.4 (blue) overlaid on the same for PA 3.5 (fade pink).

Figure 4.15. Schematic representation of the effect of Ile’s position on PA self assembly, a) Ile next to the palmitic acid core b) Ile in the second position.

Figure 5.1. PA3.1-Rubipy – Palmitoyl – IAAAEEEK(Rubipy)-NH$_2$.

Figure 5.2. Analytical HPLC trace of PA3.1-Rubipy.

Figure 5.3. ESI-Mass spectrum of PA3.1- Rubipy (Mol. Wt. 1834 g/mol). The doubly and triply charged peaks are due to Ru$^{2+}$ and H$^+$.

Figure 5.4. a) Fluorescence spectra of PA3.5-Tb in salts and pure serum at different excitation wavelengths b) Fluorescence spectra of PA3.5-Tb in salts with and without pyrene c) Fluorescence spectra of PA3.5-Tb in serum with and without pyrene.

Figure 5.5. Concentration-pH self assembly phase diagram of the PA mix (blue) overlaid on the same for PA 3.5 (fade pink).
Figure 5.6. pH dependent CAC measurements of the PA mix.........................137
Figure 5.7. pH dependent CD spectra of a) 10 µM PA mix b) 50 µM PA mix........138
Figure 5.8. pH reversibility of morphology transition in 100 µM PA mix.............139
Figure 5.9. a) pH dependent FA of 100 µM PA mix in salts. The inset shows the fluorescence emission from the PA3.1-Rubipy in the mixture b) pH dependent CD spectra of the same solution. The color for each point in a) and trace in b) both the figures correspond to similar pH values.................................................................140
Figure 5.10. a) Fluorescence emission from 100 µM PA mix in serum and serum auto-fluorescence background b) pH dependent fluorescence of 100 µM PA mix in 1.5% (v/v) serum.................................................................................................141
Figure 5.11. pH dependent FA of 100 µM PA mix in pure mouse serum..............142
Figure 5.12. a) $^{13}$C-NMR spectra of 3 mM PA3.5-Lu at pH values of 7/85 and 5.71 in 150 mM NaCl and 2.2 mM CaCl$_2$ b) $^{13}$C-NMR spectra of 3 mM PA3.5-Lu at pH 7.79 in serum and serum background.................................................................143
Figure 5.13. pH dependent FA of 100 µM PA mix in various concentrations of dilute serum. Also shown are control measurements with 1.5% PA3.1-Rubipy only in 1.5% (v/v) serum and MSA-dye conjugate.................................................................144
Figure 5.14. a) pH and time dependent FA of 1.5% PA3.1-Rubipy only in 1.5% (v/v) serum b) pH and time dependent FA of MSA-dye conjugate.........................145
Figure 5.15. a)TEM image of 100 µM PA mix in 1.5% (v/v) serum at pH 6.85 b) TEM image of 100 µM PA mix in 1.5% (v/v) serum at pH 9.21 c) TEM image of 1.5% (v/v) serum control at pH 5.0.................................................................146
Figure 5.16. pH reversibility of morphology transition of 100 µM PA mix in 1.5% (v/v) serum

Figure 5.17. a) pH and time dependent FA showing the kinetics of morphology transition in 100 µM PA mix b) Time dependent FA of 100 µM PA mix at pH 8.55 c) Time dependent FA of 100 µM PA mix at pH 6.43

Figure 5.18. a) Fluorescence emission from 10 and 30 µM PA mix in 1.5% (v/v) serum b) Fluorescence emission from 10 and 30 µM PA mix in pure serum c) pH dependent FA of 10 and 30 µM PA mix in 1.5% (v/v) serum d) pH dependent FA of 30 µM PA mix in pure serum

Figure 5.19. pH dependent FA of 100 µM PA mix in salts, different serum concentrations and the corresponding mouse serum albumin (MSA) concentrations

Figure 5.20. CD transition points of 100 µM PA mix in 3.0 and 4.0 mM CaCl₂, 150 mM NaCl overlaid on the phase diagram of the same in 2.2 mM CaCl₂ and 150 mM NaCl

Figure 5.21. pH dependent FA of 100 µM PA mix in salts, 1.5% (v/v) serum, 7.8 µM MSA and 26 µM 20 kDa PEG

Figure 5.22. pH dependent FA of 100 µM PA mix in salts and 1.8 mM 20 kDa PEG

Figure 5.23. a) PA 5.1 – Palmitoyl-MAAAEEEKEK(DO3A:Gd)-NH₂ b) PA5.1-Rubipy-Palmitoyl-MAAAEEEKEK(Rubipy)-NH₂

Figure 5.24. Analytical HPLC traces of PAs

Figure 5.25. ESI-Mass spectra of PAs
Figure 5.26. pH dependent FA of various concentrations of PA mix 2 in pure serum...158

Figure 5.27. a) pH reversibility of morphology transition of 100 µM PA mix 2 in pure serum b) pH and time dependent FA of 100 µM PA mix 2 showing stability of the nanoparticles in pure serum.........................................................159

Figure 5.28. CD transition points of various concentrations of PA mix 2 in pure serum (red) and 1.8 mM 20 kDa PEG, 150 mM NaCl, 2.2 mM CaCl₂ (blue) overlaid on the phase diagram of pure PA 5.1 in 1.8 mM 20 kDa PEG, 150 mM NaCl, 2.2 mM CaCl₂ (fade orange).................................................................160

Figure 5.29. pH dependent CD spectra of a) 10 µM PA 5.1 b) 500 µM PA 5.1........161

Figure 5.30. pH dependent CAC measurements of PA 5.1...............................162

Figure 5.31. pH dependent CD spectra of a) 20 µM PA mix 2 b) 100 µM PA mix 2...163
List of Abbreviations

ATP- Adenosine triphosphate
CD- Circular dichroism
CAC-Critical aggregation concentration
CCC- Critical coagulation concentration
CTA- The citric acid cycle
DCM- Dichloromethane
DIPEA- N,N-Diisopropylethylamine
DLS- Dynamic light scattering
DMF- N,N-Dimethylformamide
DO3A- 1,4,7,10-tetraazaacyclododecane-1,4,7-triacetic acid tri t-butyl ester
EPR- Enhanced permeability and retention
ESI-MS- Electrospray ionization mass spectrometry
FA- Fluorescence anisotropy
FDA- Food and drug administration
FMOC- Fluorenylmethyloxycarbonyl chloride
HBTU- O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluroniumhexafluorophosphate
HATU- O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluroniumhexafluorophosphate
HOAt- 1-Hydroxy-7-azabenzotriazole
**HOBt**- 1-Hydroxybenzotriazole hydrate

**HPLC**- High performance liquid chromatography

**MeCN**- Acetonitrile

**MRI**- Magnetic Resonance Imaging

**MSA**- Mouse serum albumin

**Mtt**- Methyl trityl

**NADH**- Nicotinamide adenine dinucleotide

**NHS**- N-hydroxy succinamide

**NMR**- Nuclear magnetic resonance

**PA**- Peptide amphiphile

**PEG**- Polyethylene glycol

**TEM**- Transmission electron microscopy

**TFA**- Trifluoracetic acid

**TIS**- Triisopropylsilane

**TLC**- Thin layer chromatography

**TOF**- Time of flight

**VEGF**- Vascular endothelial growth factor
Chapter 1

Introduction

1.1. Motivation – The cancer problem:

Cancer is the second most common cause of death in the US exceeded only by heart
diseases, responsible for nearly 1 in every 4 deaths.\(^1\) In 2013, about 580,350 people were
estimated to die of some form of cancer, almost 1600 people a day.\(^2\) According to
published reports by the American Cancer Society, early detection via appropriate
screening methods is paramount to decreasing cancer mortality.\(^3\) For instance, it is known
that the 98.6% 5-year survival rate for patients with localized breast cancer is reduced to
23.3% once the cancer has metastasized.\(^4\) Some early screening efforts such as
colonoscopies for colon cancer, pap-smears for cervical cancer, and mammograms for
breast cancer, have been shown to result in curative treatments. Despite all these
advances, current diagnostic tests for many cancer types yield numerous false positives
requiring subsequent imaging and biopsies for verification. For example, screening for
prostate cancer requires either monitoring for elevated levels of a prostate-specific
antigen in the blood, or detecting an enlarged prostate \textit{via} digital rectal examination.
Both tests have a 70% false positive rate since infection or non-cancerous inflammation
of the prostate can exhibit the same symptoms.\(^5\) Recently, data analyzed from a series of
colorectal, lung and ovarian cancer screening trials showed that after 14 tests, the cumulative risk of having at least 1 false-positive is 60.4% for men, and 48.8% for women.\(^6\) Hence, there is a pressing long term need to develop targeted imaging pharmaceuticals and methods that would be able to easily and non-invasively detect tumors of all types with high sensitivity.

1.2. Nanotechnology in cancer therapy – targeting strategies:

A crucial step in increasing early stage cancer detection is designing imaging molecules capable of selectively targeting tumors. Nanoparticles (1-1000 nm) are known to achieve this site-selective targeting\(^7\)-\(^10\) via two main strategies – *Active targeting*, in which a nonspecific vehicle is imparted affinity towards its target via binding of this vehicle with another molecule (usually referred to as a targeting moiety or vector molecule) capable of specific recognition and binding to the target site.\(^11\) The underlying rationale of this strategy is that the avid and specific interaction between the nanocarriers and the target cells would selectively increase the rate and extent of drug delivery and imaging agents to the target tumor cells.\(^11\)-\(^12\) **Figure 1.1**\(^11\) illustrates the concept of active targeting. Among commonly used targeting moieties are antibodies and their fragments, lectins, other proteins, lipoproteins, hormones, charged molecules, mono-, oligo- and polysaccharides, and some low-molecular-weight ligands.\(^13\)-\(^16\) For example, tumor targeting using folate-modified liposomes has shown increased cytotoxicity because
folate receptors are frequently over-expressed on tumor cells. Another commonly used approach is to conjugate the anti-HER2 antibody to nanocarriers that binds to the HER2 protein over-expressed on certain tumor types. There is, however, a major limitation to this strategy. Although this approach often leads to initial improvements in a patient’s condition, the gains are many times followed by the aggressive reappearance of tumor growth and eventual death of the patient. This mysterious and devastating phenomenon was recently elucidated by a study finding genetic deviations not only between cancerous cells and healthy cells, but also among cells within a single tumor due to constant mutations. Tumor heterogeneity is the downfall of personalized medicine because, while the targeted cancerous cells may be killed off, any cancer cells present without the targeted surface markers return (and in greater numbers).

The second strategy, Passive targeting is based on the architecturally defective vasculature of tumors, also shown in Figure 1.1. An actively growing tumor needs to form new blood vessels in order to receive nutrients and sustain its growth. New vessel formation in tumors is initiated by an imbalance of proangiogenic and antiangiogenic factors. In normal tissues, the balance between these factors maintains the normal structure of the vascular networks in order to ensure optimal function. In tumors, however, the proangiogenic molecules (e.g. VEGF, basic fibroblast growth factors) are usually over-expressed in addition to vascular permeability factors such as bradykinin, nitric oxide etc., which causes formation of chaotic and irregular blood vessels. Unlike normal vessels, they lack an orderly branching hierarchy into smaller vessels and
are heterogeneous in their spatial distribution, dilated and tortuous, leaving avascular spaces of various sizes. The vessel wall structure is abnormal with wide interendothelial junctions and fenestrae with pore diameters as large as 10-100 nm (Figure 1.2). The targeting is achieved as the nanocarriers remain in the bloodstream where the vessel structure is normal, but would extravasate through the leaky vasculature at the tumor site selectively. Also, the impaired lymphatic drainage in tumors contributes to retention of the nanoparticles. This phenomenon, known as the Enhanced Permeation and Retention (EPR) effect, also entails that the nanoparticles stay in circulation long enough to ultimately accumulate at the tumor site in optimal concentrations. It is a typical practice to coat the particles with the hydrophilic Polyethylene glycol (PEG) as “stealth” layers in order to escape opsonization and consequently removal by the body’s immune system. For instance, the FDA approved formulation Doxil (or Caelyx), a PEGylated liposome that encapsulates the anticancer drug doxorubicin is known to exhibit enhanced circulation time, good drug retention and is six times more effective than free doxorubicin. Caron et al. showed that nanoparticles of low negative zeta-potential (surface charge), high solubility, and of between 20 and 200 nm diameter tend to evade immune system recognition and organ clearance leading to longer circulation lifetimes, being the ideal for EPR effect as illustrated in Figure 1.3. With regards to shape, cylindrical polymeric fibers have been shown to have a 10 times longer circulation time in the bloodstream than their spherical counterparts. However, it was recently shown that, when targeting cancer, the high interstitial fluid pressure of tumor tissue results in
diffusion becoming the most important mass transport process.\textsuperscript{21} Therefore, vehicles of small diameter (ideally 10-30 nm) permeate tumor tissue most easily.\textsuperscript{34} Unfortunately, therapeutic attempts at exploiting this effect have been ineffective to a certain extent because these small vehicles leave the tissue as easily as they enter, leading to decreased accumulation. It is known that EPR can improve the delivery of systemically administered vehicles to the tumor microenvironment by only 20-30\% over the bloodstream, which on its own is not optimal to yield a high tumor to background signal.\textsuperscript{35}

Hence, it is extremely crucial to design vehicles rationally so as to overcome the aforementioned challenges in tumor targeting in order to achieve significant accumulation of therapeutic and diagnostic agents at the tumor site. The ideal imaging agent would circulate in blood as a small vehicle (~10 nm) to maximize tumor penetration rate, and transition into a larger, more slowly diffusing vehicle in the tumor’s interstitial microenvironment in response to a cancer hallmark to maximize tumor retention. Non-hallmark trapping mechanisms will inevitably suffer from tumor heterogeneity and evolutionary loss of receptor expression.
1.3. Hallmarks of cancer:

Cancer tissue is known to exhibit six “hallmarks” irrespective of type and location - the ability to resist cell death, induce angiogenesis, enable replicative immortality, activate invasion and metastasis, evade growth suppressors, and sustain proliferative signaling.\textsuperscript{36} The third hallmark, constant replication of tumor cells, causes an increased demand for energy, specifically in the form of Adenosine Triphosphate (ATP). Most tumor cells use glycolysis as the main form of energy production with the net products of pyruvic acid, ATP, NADH, and \( \text{H}^+ \) (Figure 1.4).\textsuperscript{37} Pyruvic acid is then shuttled through the Citric Acid (TCA) cycle, where it is converted into cellular energy.\textsuperscript{37} This constant glycolytic cycle causes an increased production of \( \text{H}^+ \), which is further released into the extracellular environment of tumor tissue and cells. The constant flux of protons into the extracellular matrix causes it to be slightly more acidic than normal tissue and blood. The extracellular pH (pHe) of “normal” blood and tissue is 7.4 and is typically maintained constant throughout the body. However, tumor cells exhibit an abnormally acidic extracellular environment with the pHe ranging from 6.6-7.4.\textsuperscript{37-38}

1.4. Research objective:

In this work, we have adopted a hybrid (active/passive targeting) approach, by designing a dynamic system that is capable of sensing and transforming its molecular structure from spherical micelles (10-20 nm) to larger, more slowly diffusing nanofibers
(10-20 nm, 200-1000 nm in length) upon encountering the acidic pH of the tumor environment. **Figure 1.5** illustrates this idea. The small diameter micelle will maximize tumor penetration, and the much larger, much more slowly diffusing nanofiber formed in the tumor’s interstitial microenvironment will maximize retention. For a cylindrical micelle, the translational (DT) diffusion constant scales with cylindrical length (L) as $\text{DT} \sim L^{-1}$.\(^{39-40}\) In serum, a 12 nm spherical micelle has a diffusion constant of $2 \times 10^{-12}$ m\(^2\)s\(^{-1}\), whereas a 1 μm long nanofiber with the same diameter has a DT of $1 \times 10^{-13}$ m\(^2\)s\(^{-1}\).\(^{41}\) This 20 fold decrease in translational diffusion is expected to significantly improve tumor retention. Trapping vehicles with a replenishing hallmark mechanism will not suffer from tumor heterogeneity, and will allow targeting of most cancer phenotypes.

### 1.5. Molecular Self-assembly:

In order to develop nanomaterials capable of stimuli-induced shape transformation, the power of molecular self-assembly was judiciously exploited. Molecular self assembly is the spontaneous aggregation of amphiphilic surfactant type molecules into well-defined supramolecular structures mediated by weak, non-covalent interactions, notably hydrogen bonding, electrostatic interactions, hydrophobic and van der Waals interactions.\(^{42-45}\) Although these bonds are relatively weak in isolation, their cumulative effect is significant and governs the structural conformation of all
biomacromolecules found in nature. The relative balance in these interactions and consequently the morphology of self-assembled structures can be precisely controlled via changes in monomer structure and external factors like ionic strength, pH, temperature etc. Peptide Amphiphiles (PAs) are a class of bio-compatible, self assembling molecules that typically comprise of a peptide sequence modified with a fatty acid tail. These molecules self assemble because of hydrophobic interactions that stabilize secondary-structure motifs on their surface as a result of peptide tethering and crowding. Bowerman *et al.* studied amyloid structures via aggregation of monomers through noncovalent weak interactions that form β-sheet like conformations. They determined that the hydrophobic and aromatic amino acids found in amyloid structures, favor β-sheet formation through the synthesis and characterization of a library of peptides containing these amino acids. Additionally, *Stupp et al.* have developed amphiphilic peptide nanomaterials to mimic biological processes and enhance tissue regeneration (Figure 1.6).

In addition to the hydrophobic interactions of the tail, peptide head-group interactions are important for controlling stability and shape in the aggregated state. Solution ionic strength, pH and even mixing PAs of complimentary charge have been found to trigger self-assembly via screening of charges between neighboring PAs. Through rational design of sequence and structure, it is possible to fine-tune the environmental dependence of these PA self-assembled structures.
1.6. Magnetic Resonance Imaging (MRI):

MRI is a noninvasive diagnostic tool with < 1 mm spatial resolution based on the relaxation of water protons in response to an externally administered magnetic field.\(^6^3\) MRI was chosen over FDG-PET, another commonly used diagnostic imaging technique due to the latter’s poor tissue contrast and spatial resolution (~ 1 cm) and hence, inability to detect sub-1 cm early stage lesions.\(^6^4^-6^5\)

The addition of a contrast agent in MRI, such as the paramagnetic ion Gd\(^{3+}\), increases the \(T_1\) based relaxivity \((r_1)\) of water, therefore increasing signal intensity (Tumor to background ratio, TBR > 2) in the concentration range 10-1000 \(\mu\)M, as shown in Figure 1.7.\(^6^6^-6^8\) However, free (i.e. unchelated) Gd\(^{3+}\) in the blood is highly toxic.\(^6^9\) Clinically available contrast agents are chelated to macrocyclic chelators neutralizing any toxic effects.\(^7^0^-7^2\) The commonly used macrocyclic chelator, DOTA (1,4,7,10-tetraaza cyclododecane tetraacetic acid) is extremely effective at binding Gd\(^{3+}\), with a binding constant of 1.0x10\(^{24}\).\(^7^3^-7^4\) Conjugation of a DOTA:Gd chelate to a molecule targeted for cancer is an approach used to increase detection of tumors that has not generally succeeded because biochemical tumor targets (e.g. cell surface proteins) are only available to nM concentrations, while Gd\(^{3+}\) in \(\mu\)M concentrations (as mentioned before) is required to enhance TBR in an MRI scan.\(^7^5\) The approach taken in this research could localize Gd\(^{3+}\) in the \(\mu\)M range.

This work utilizes a DOTA:Gd derivative, known as DO3A (DO3A=1,4,7-tris (carboxymethylaza) cyclododecane-10-azaacetyl amide, Figure 1.8) conjugated to PA
molecules to create dynamic pH-triggered, self-assembling contrast agents that should be capable of tumor detection with higher signal and improved sensitivity.

1.7. Dissertation Highlights:

In Chapter 2, all experimental methods, materials and syntheses schemes used in this research are discussed in detail. Chapter 3 presents our PA design strategy and characterization of pH-triggered self assembly morphology transitions in a rationally developed PA molecule using CD, CAC and TEM measurements in 150 mM NaCl, 2.2 mM CaCl$_2$ that simulates the ionic strength of blood. This transitional pH was further fine-tuned via systematic changes in PA structure. Addition of the imaging tag was found to shift the transition to acidic values due to an increase in the PA headgroup size. Finally, MRI relaxivity measurements were conducted and compared with that of a commercially available contrast agent as a standard control. Chapter 4 explores the effect of changing the peptide sequence in the hydrophobic region on the pH trigger of self assembly. Moving the hydrophobic amino acid away from the palmitic acid tail was found to shift the transition to more basic pH values, indicating an increase in the hydrophobicity of the PA molecule. In Chapter 5, our PA self assembly behavior is probed in mouse blood serum with FA measurements, using a Ru(bipy)$_3^{2+}$ dye tagged PA spiked into our original PA molecule in small concentrations. Contrary to the typical notion of serum albumin binding to amphiphiles and disrupting self assembly, pure serum
was found to facilitate nanofiber formation even at basic pH values and no transition was observed in the desired pH range. This was attributed to the crowding effect of albumin which tends to stabilize the more compact, self-assembled form of amphiphilic molecules via simulating serum’s crowded environment with 20 kDa PEG molecules in addition to the usual salts. Finally, the PA structure was re-modified to successfully create a vehicle that transition in pure serum and a concentration–pH phase diagram was constructed in our simulated serum conditions. Chapter 6 presents the main conclusions of this dissertation and highlights the future work (currently in progress) for the project.
Figure 1.1. Schematic representations of active and passive tumor targeting with nanodrugs.\textsuperscript{11} (Reproduced with permission from reference 11).
**Figure 1.2.** The architecturally defective tumor vasculature (right, N = normal, T = tumor).²⁴ (Reproduced with permission from reference 24).
Figure 1.3. Nanoparticle biodistribution and clearance.\textsuperscript{30} (Adapted from reference 30).
Figure 1.4. Glycolysis in tumor cells leading to acidic extracellular pH.$^{37}$ (Reproduced with permission from reference 37).
Figure 1.5. Schematic representation of proposed research.
Figure 1.6. Nanostructures formed via molecular self-assembly.57 (Reproduced with permission from reference 57).
Figure 1.7. Plot showing change in MRI intensity as a function of contrast agent concentration.\textsuperscript{65} (Adapted from reference 65).
Figure 1.8. The structure of the DO3A derivative of the macrocyclic metal chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).\textsuperscript{70} (Adapted from reference 70).
Chapter 2

Experimental Methods, Materials and Synthesis Schemes

2.1. Synthesis of PAs:

All amino acids used in this research were purchased from AnaSpec Inc. PAs were synthesized on a 0.5 mmol scale in a linear fashion from the C-terminus to N-terminus direction using Fmoc chemistry. For all PAs containing lysine, a Sieber resin (AAPPTEC) was used for synthesis. All other peptides were made on a Rink Amide Resin purchased from AAPPTEC. The following procedure was used:

The resin was swollen in a shaker vessel with dichloromethane (DCM) for 30 minutes, the DCM was removed and dimethyl formamide (DMF) was added to the vessel and further shaken for 30 minutes. After the liquid was removed, 10% N,N-Diisopropylethylamine (DIPEA) in DMF was added to the vessel and mixed for 10 minutes. For deprotection, 20% piperidine in DMF was used to remove the Fmoc protecting group of the amine moiety on the resin. A Kaiser test protocol confirmed removal of the Fmoc protecting group. The test corroborates the presence of free primary amines via the formation of a ninhydrin complex that renders the solution blue.76
Coupling of the acid to the amine end of resin was done through activation using O-Benzotriazole N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU) or 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). The coupling solution contained 4.0 Eq. of amino acid, 3.96 Eq. of HBTU/HATU, 4 Eq. of N-Hydroxybenzotriazole (HOBt) or 1-Hydroxy-7-azabenzotriazole (HOAt), and 8 Eq. of DIPEA with respect to peptide allowing at least 3 hours of coupling per amino acid. The surfactant Triton X-100 was added to the coupling solution and to the latter amino acids to aid in coupling efficiency. The Kaiser test is used again, this time to confirm coupling and the absence of free amine groups (solution remains yellow). Resin cleavage of the peptide was done by addition of the following cocktails: a solution of 1% Trifluoroacetic acid (TFA), 1% Triisopropyl silane (TIS), 2% Anisole and 94% DCM was used for the PAs that would be conjugated to DO3A and a solution of 95% TFA, 1% TIS, 2% Anisole, 2% water was used for PAs with no imaging tags at all and the PA to be conjugated to the Ru-(biPy)$_3$ NHS ester dye (PA$_2$). The TFA was removed under vacuo and the PA was precipitated using two 20 mL portions of either cold water (PAs for DO3A conjugation) or cold diethyl ether (PAs with no imaging tags, PA$_2$). The crude peptide was filtered and washed with cold water/cold diethyl ether. **Figure 2.1** illustrates the scheme of solid phase peptide synthesis used in this research.
2.2. Synthesis and Purification of tri-tert butyl ester of DO3A:

All reagents were purchased from Sigma Aldrich and used without further purification unless specified. 30 g of cyclen (1,4,7,10-tetraazacyclododecane) was combined with powdered, dry 42.9 g of sodium acetate in 400 mL of N,N-dimethylacetamide in a round bottom flask and stirred for 30 minutes with an overhead glass rod stirrer. The round bottom flask containing the slurry was placed in an ice bath until it reached 0°C. 77.1 mL of tert-butyl bromoacetate was dissolved in 150 mL of N,N-dimethylacetamide and added drop-wise to the slurry at 0°C over a period of 25 minutes. The slurry equilibrated back to room temperature and was stirred for 5 days. A separate solution was made by dissolving 30 g of potassium bromide (KBr) in 2 L of deionized water (Millipore) followed by stirring and heating to a temperature of 50°C. After the KBr solution reached 50°C, it was added to the slurry forming a yellow colored solution. The pH was adjusted to 9.0 by the addition of powdered sodium bicarbonate and checked via litmus paper. Precipitation of the desired product, acetic acid tert-butyl ester hydrobromide (4,7-bis-tert-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl), settled for 4 hours without stirring, followed by vacuum filtering and drying, yielding a white powder. 10.0 g of acetic acid tert-butyl ester hydrobromide was dissolved in 50 mL of acetonitrile (MeCN) and combined with 5.1077 g (2.2 eq.) of finely powdered, dry potassium carbonate and stirred for 30 minutes. Benzyl bromo acetate 2.927 mL (1.1 eq.) was added drop-wise to the solution and stirred overnight at 50°C-60°C. The mixture was cooled to room temperature and vacuum filtered, with the desired product in the
filtrate. The solid was washed twice with MeCN. The combined MeCN was removed by evaporation under vacuum, yielding the tri-tert-butyl ester form of DO3A, a viscous yellow gel. NMR and ESI-MS confirmed the presence of the desired product. The crude product was purified using flash-column chromatography using 50.0 g of silica gel for every 1.0 g of tri-tert-butyl ester form of DO3A using DCM as the mobile phase. The product was eluted from the column using a gradient elution, starting with 2% MeOH in DCM to 6% of MeOH in DCM. The elution of the desired product was followed by TLC, using 10% MeOH in DCM as the mobile phase. Pure fractions were combined and the solvents evaporated under vacuum. The residue was then dissolved in approximately 50 mL of MeOH in deionized water (Millipore) at a ratio of 9:1. Palladium on carbon catalyst was added to the solution in 20% by weight with respect to tri-tert-butyl ester form of DO3A. The sample was hydrogenated under 50-psi hydrogen pressure overnight followed by filtration of the solid catalyst. The filtrate containing DO3A was evaporated under vacuum to remove the methanol then 100 mL of deionized water was added to the solution. Diethyl ether (50 mL) was added 3 times to the solution in a separatory funnel to extract the non-hydrogenated product. Solvent was removed by evaporation and the solution was freeze-dried to remove remaining deionized water, yielding a yellowish powder. NMR and ESI-MS were used to confirm the presence of DO3A and check purity. The synthesis scheme is shown in Figure 2.2
2.3. Conjugation of PAs with tri-tert-butyl ester form of DO3A:

A certain mass of the PA was dissolved in pyridine followed by addition of 2 eqv. of tri-tert-butyl ester form of DO3A, 2 eqv. of HATU and 4.4 eqv. of DIPEA. The solution was left to stir overnight. ESI-mass spectrometry was used to monitor conjugation. The product was then precipitated with cold water, filtered and dried under vacuum. The dried solid was then dissolved and stirred in 20 ml of 95% TFA, 1% TIS, 2% water and 2% anisole for about 20-24 hours to remove the tert-butyl groups on the glutamic acids in PA and the carboxylic acids in DO3A (Figure 2.3a). The excess TFA was removed under vacuum. The final de-protected product (PA-DO3A) was precipitated with cold ether, filtered and dried under vacuum before HPLC purification.

2.4. Chelation of PA-DO3A with Gd$^{3+}$:

10-15 mg of previously prepared and purified PA-DO3A was dissolved in 1.0-2.0 mL of water and combined with 2 eq. of GdCl$_3$ in 0.01 M HCl. The reaction was set to stir in an oil bath at 60°C for 30 min. The pH of the solution was gradually adjusted from approximately pH 2.0 to ~5.0 using small amounts of 0.050 M NaOH. The resultant solution was stirred for 24 hours at 60°C (Figure 2.3b). A small sample was removed and analyzed by ESI-mass spectrometry to determine the extent of reaction completion. The pH of the solution was then raised over a period of an hour using ammonium hydroxide to precipitate excess Gd$^{3+}$ as Gd(OH)$_3$, then filtered using a 0.2 μm syringe
filter. The solution was dialyzed against de-ionized water to remove NaCl and any remaining free Gd\(^{3+}\). The buffer water for dialysis was changed 4-6 times over a period of 24 hours. The PA-DO3A:Gd solution was freeze-dried to recover a white fluffy powder, with an yield of ~65-70%.

2.5. Conjugation of PA\(_2\) and Mouse Serum Albumin with Ru(bipy)\(_3\)^{2+} NHS dye:

Bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-rutheniumm N-succinimidyl ester-bis(hexafluorophosphate) was purchased from Sigma Aldrich and used without further purification. 5 mg of the dye was dissolved in 500 µL of dimethyl sulfoxide (DMSO) to make a stock solution. 10-15 mg of PA\(_2\) was dissolved in 1 mL of 0.2 M NaHCO\(_3\) (aq.) solution and stirred for 2-3 hours, followed by slow addition of 100 µL of the dye stock solution. The reaction solution was left to stir overnight at room temperature. Typically, 25-33% conjugation efficiency was achieved. ESI-mass spectrometry and analytical HPLC were used to track the coupling. Excess free dye was removed by dialysis (500-1000 Da cut-off Millipore tubing) against de-ionized water. The PA-RubiPy was then separated from unreacted PA\(_2\) via analytical HPLC using a linear gradient of 10% to 100% MeCN containing 0.1% NH\(_4\)OH (v/v) over 30 minutes. The same procedure was followed for conjugation of the dye to mouse serum albumin (MSA -Sigma Aldrich) except no analytical HPLC purification was done.
2.6. HPLC Purification of PAs:

The crude PAs were dissolved in 0.1% NH₄OH v/v (aq) at approximately 10-20 mg/mL by vigorously shaking and sonicating until the solution turned clear. To aid in dissolution, an additional drop of concentrated NH₄OH was added to the solution. The PA solution was filtered first using a 0.45 μm syringe filter (Whatman), followed by a 0.2 μm syringe filter. The sample was purified on a Shimadzu preparative HPLC (dual pump system controlled by LC-MS solution software) with an Agilent PLRP-S polymer column (Model No. PL1212-3100 150 mm x 25 mm) under basic conditions. The product was eluted with a linear gradient of 10% to 100% MeCN containing 0.1% NH₄OH (v/v) over 60 minutes. The purity of the collected fractions was verified using an electro-spray ionization (ESI) time-of-flight (TOF) mass spectrometer (Bruker) and a Shimadzu Analytical HPLC system.

2.7. ESI-TOF Mass spectrometry:

A small amount (~1-2 mg) of the crude/pure PA or 5-7 drops of the purified fractions (from HPLC) was dissolved in MeOH and serially diluted two more times for optimal signal-to-noise ratio (~10⁴-10⁵ intensity units). 1-2 drops of concentrated ammonia were added to aid in dissolution if required. About 100-200 μL of the most diluted sample was then injected into a Bruker Dalotonics MicroTOF LC-MS with electrospray ionization and TOF mass analyzer using a 1 mL syringe followed by
collection of mass spectra. Either positive or negative mode of collection was used depending on the PA sample.

2.8. Analytical HPLC:

~1-2 mg of the previously purified PAs was dissolved in ~1-3 ml de-ionized water. To aid in dissolution, an additional drop of concentrated NH₄OH was added to the solution. Fractions from HPLC purification were used directly without further treatment. The PA solution was filtered using a 4 mm Millipore 0.2 μm syringe filter followed by injection of 20 µL into a Shimadzu analytical HPLC (dual pump system controlled by LC-MS solution software) with a Phenomenex Gemini 3μ C18 column (Model No 00G-4439-E0) under basic conditions. The product was eluted with a linear gradient of 10% to 100% MeCN containing 0.1% NH₄OH (v/v) over 35 minutes. Fractions greater than 95% purity were combined; the MeCN was removed under vacuum before freeze-drying.

2.9. Peptide Content Analyses:

Peptide content analysis was performed on lyophilized samples by the UC Davis Molecular Structure Facility to verify the amino acid stoichiometry and determine the residual salt concentration. The relative residue stoichiometry was within ± 5% of the
expected values for all amino acids. All further CD, and CAC measurements were scaled by these factors to determine the true concentration.

2.10. Circular Dichroism (CD) Spectroscopy:

Circular Dichroism is defined as the difference in absorption of left- \( A_{\text{left}} \) and right-circularly \( A_{\text{right}} \) polarized light that occurs in molecules containing one or more chiral centers, as given by equation 2.1\(^{77}\):

\[
\text{CD} = A_{\text{left}} - A_{\text{right}} \tag{2.1}
\]

After passing through a chiral molecule, the unequal amplitude vectors of the left- and right-circularly polarized light (due to differential absorption) add to form a vector that traces an ellipse and the resultant light thus gets elliptically polarized. The ‘ellipicity’ of this light is proportional to the absorption difference and is plotted as a function of wavelength in a typical CD spectrum.\(^{77}\) CD spectroscopy is an excellent tool for analyzing secondary structures of macromolecules, particularly proteins and peptides.\(^{78}\) When the chiral polypeptide backbone in proteins/peptides is aligned to form distinct secondary structures (\( \alpha \)-helix, \( \beta \)-sheet, random coil etc.), its optical transitions are shifted or split into multiple transitions due to ‘exciton’ interactions. This leads to characteristic CD fingerprints for each of these structural elements.\(^{78-79}\) Figure 2.4
illustrates a schematic representation of the CD set up.

In this research, CD measurements were done on a Jasco-815 Circular Dichroism spectrometer using 0.5-1 cm path length quartz cuvettes. 10-500 μM PA solutions (or PA mixtures with 1.5% PA-Rubipy) were prepared in 150 mM NaCl and 2.2 mM CaCl₂ by dilution from a concentrated PA stock (0.5-1 mM, pH 9). For simulated serum experiments, the solution also contained 1.8 mM 20 kDa Polyethylene Glycol (PEG). Double de-ionized Milli-Q water was used for preparing all solutions. The solutions were then heated at 80°C for 30 minutes in a water bath and gradually cooled to room temperature. An Accumet XL15 pH meter (Fisher Scientific) coupled with an Orion Ross Ultra semi-micro electrode (8103BNUWP, Thermo Scientific) was used to adjust the pH of the solution to the desired value followed by collection of the CD spectra. Each trace shown was averaged over 3 accumulations and was baseline subtracted using aqueous solutions containing salts only. All spectra are cut-off below 200 nm due to absorption by NaCl and CaCl₂.

2.11. Critical Aggregation Concentration (CAC) determination using the pyrene 1:3 method:

Critical Micelle Concentration (CMC) is defined as the lowest surfactant concentration at which micelles begin to form. Here, the more generic term ‘aggregation’ has been used in place of ‘micelle’ since our PA monomers have been
found to self assemble into either spherical micelles or nanofibers depending on structure and pH of solution.

For the CAC measurements, a series of solutions of the PA (pure or mixed with 1.5% PA-Rubipy) with concentrations ranging from 100-300 nM to 500-700 M were prepared using serial dilutions in 150 mM NaCl and 2.2 mM CaCl₂ (containing 1.8 mM PEG in the simulated serum experiments). The PA stocks were heated and cooled at room temperature for equilibration prior to dilution. The final concentration of pyrene in each solution was fixed to be 6 M. This was followed by pH adjustment of the solutions using careful additions of HCl or NaOH. 100 L of each solution was transferred to a 96-well plate and the fluorescence emission of pyrene was monitored using a hybrid reader fluorimeter (BioTek Synergy H4) at room temperature. The excitation wavelength was set at 335 nm. The ratio of the intensities of emissions at 376 nm and 392 nm were then plotted as a function of the PA concentration (log scale). The CAC was determined from an abrupt change in the slope of the plot using the least-squares fitting technique. Figure 2.5 shows a schematic representation of the CAC measurements using the pyrene 1:3 method.⁸¹


TEM images were obtained using solutions of either 10-800 M PA in 150 mM NaCl or 2.2 mM CaCl₂ or 100 M PA mixture in 1.5% (v/v) serum solution. The PAs in
serum, however, were not heated to avoid destroying/denaturing serum proteins. This was followed by pH adjustment using either HCl or NaOH. 5 μL of this solution was pipetted onto a Carbon Formvar grid (Electron Microscopy Sciences) and allowed to sit for 2 minutes before being wicked dry using filter paper. The samples were then negatively stained using 1 wt% uranyl acetate and imaged under a FEI Tecnai G2 Bio TWIN TEM system, operating at 100 kV. All TEM experiments were performed in duplicate.

2.13. pH titrations of PAs:

The titration measurements were conducted on 10 μM PA solutions prepared in 150 mM NaCl and 2.2 mM CaCl₂ using milli-Q water. The solution was heated at 80°C for 30 minutes followed by slow cooling at room temperature for equilibration. The pH of the solution was then adjusted to 4 using HCl. Finally, an Accumet XL15 pH meter (Fisher Scientific) coupled with an Orion Ross Ultra semi-micro electrode (8103BNUWP, Thermo Scientific) was used to track changes in pH of the solution as NaOH solution was added in small increments. pKa values were obtained from the second inflection points of the first derivative plots of the titration data. The first transition corresponds to neutralization of excess HCl. The calculated pKas reflect the average pKa for all four glutamic acids.
2.14. MRI relaxivity:

An MR relaxometry phantom was built by fixing 5 mm NMR sample tubes containing PA samples at 10 M, 150 M, 300 M, and 500 M concentrations and pH values 4 and 10 in a 600 ml beaker filled with deionized water. The samples also contained 150 mM NaCl and 2.2 mM CaCl\(_2\). The phantom was scanned on a 1.5 Tesla Signa Excite MRI scanner using an 8-channel phased-array head coil (GE Healthcare, Milwaukee, WI, USA). \(r_1\) relaxometry data were acquired using an Inversion Recovery Fast Spin-Echo (IR-FSE) sequence with the following parameters: inversion time \(T_1 = 50/100/200/400/600/800/1200/1600/3200\) ms; repetition time \(TR = 8000\) ms; echo time \(TE = 11\) ms; echo train length = 10; 90° flip angle; 100 × 100 mm\(^2\) field-of-view (FOV); 0.5 mm in-plane resolution; 2 mm slice thickness; single coronal slice placed at the center of the phantom. Sample longitudinal relaxation rates (\(r_1\)) were calculated by fitting the MR signal intensities observed at different TIs (\(S(TI)\)) to a three parameter model:

\[
S(TI) = |S_0 \times (1 - C \times exp(-TI \times r_1))| \tag{2.2}
\]

Where \(S_0\) is the equilibrium signal, \(C\) is a constant accounting for imperfect inversion of magnetization. The \(r_1\) relaxivity was calculated as the slope in the linear relationship between sample concentrations and their \(r_1\) relaxation rates.
2.15. Zeta-potential Measurements:

Zeta-potential measurements were conducted using a Malvern Zetasizer Nano ZS on 100 µM PA 3.5 in 150 mM NaCl and 2.2 mM CaCl₂ at pH values of 6.6 and 7.4. The solutions were equilibrated via heating at 80°C followed by slow cooling to room temperature prior to the measurements. For each solution, the potential value was averaged over three measurements.

2.16. Dynamic Light Scattering (DLS):

For DLS measurements, a 2 mM solution of PA 3.5 was prepared in 150 mM NaCl and 2.2 mM CaCl₂ at pH values of 6.0 and 8.0. The solutions were then filtered through 0.45 µm filters, sonicated for 15 minutes and re-filtered through 0.22 µm filters to eliminate any dust particles that might interfere with light scattering. This was followed by collection of DLS data using a Malvern Zetasizer Nano ZS equipment. Each measurement was averaged over three scans.

2.17. NMR:

$^{13}$C NMR was conducted on a Bruker DRX 500 NMR spectrometer using a 500 MHz magnet. 1 mM solutions of a PA variant containing Lu$^{3+}$ and palmitic acid with a $^{13}$C label were prepared in 150 mM NaCl, 2.2 mM CaCl₂ and 10% D₂O (v/v) in water
and serum. The aqueous solutions were heated and cooled as usual. The solutions were then pH adjusted using 0.1-1 M HCl and NaOH followed by collection of $^{13}$C NMR spectra. Background salt solution and serum spectra were collected at similar pH values and each spectrum was averaged over 10,000 scans.

2.18. UV-Vis Spectrophotometry:

Absorbance measurements were done on a Varian Cary 100 UV-visible spectrophotometer using 1 cm path length cuvettes to ascertain the lower limit of pH at which Gd$^{3+}$ starts to leach out of the PA. 50 µM solutions of PA 3.5 were prepared in water containing 25 µM Arsenazo III dye at a pH of ~7.0. The pH of the solutions were then adjusted using 0.1-1 M HCl followed by collection of absorbance spectra in the wavelength range 450-700 nm using the medium scan rate. All spectra are background subtracted using water blank. Controls containing the dye only at various pH values and dye-Gd complex were also measured.

2.19. Fluorescence and Fluorescence Anisotropy (FA):

FA measures the extent of decorrelation of the polarized emission from a fluorescent dye with respect to the polarization of the excitation light, which linearly depends on the tumbling rate of the dye containing rotating unit in solution and
consequently its size/volume and molecular weight, as given by equations 2.3 and 2.4 below:\textsuperscript{83-84}

\[ FA = \frac{FAo}{1 + \frac{\tau}{\phi}} \quad (2.3) \]

and \[ \phi = \frac{\eta V}{RT} \quad (2.4) \]

where, \( FAo \) is the dye’s intrinsic anisotropy, \( \tau \) is the fluorescence lifetime, \( \phi \) and \( V \) are the rotational correlation time and molecular volume of the dye containing rotating species, \( \eta \) is the solvent viscosity, \( R \) is the gas constant and \( T \) is the temperature.

Single molecules or \(~10 \text{ nm} \) spherical micelles are expected to exhibit high tumbling rates in solution and consequently, a higher extent of polarization scrambling of the fluorescent light relative to bulky, slow-diffusing micron-sized cylindrical nanofibers. This is reflected in an abrupt increase in FA values when the larger morphology is formed.

Fluorescence and FA measurements were done using 100 \( \mu \text{M} \) PA mixture in either 150 mM NaCl and 2.2 mM CaCl\(_2\) solution or various serum (MP Biomedicals) concentrations (0.75-100\% v/v) diluted in the same salt solution. PA samples in serum or MSA were not heated and cooled. All samples were then pH adjusted using HCl/NaOH solutions, transferred to a 96-well plate followed by collection of fluorescence emission.
from PA-RubiPy first parallel \((I_{\text{par}})\) and then perpendicular \((I_{\text{perp}})\) to the excitation polarization using a hybrid reader fluorimeter (BioTek Synergy H4) at room temperature. The excitation wavelength used was 458 nm and the emission was monitored using a 640/20 nm filter. The same method was followed for control experiments involving serum background samples, dye conjugated MSA, only 1.5% PA-RubiPy in serum and 100 µM PA mixture in MSA and salt solution. FA was calculated using the following equation (2.5):

\[
FA = \frac{(I_{\text{par}} - I_{\text{perp}})}{(I_{\text{par}} + (2 \times I_{\text{perp}}))}
\]  

(2.5)

A schematic representation of the FA set up is given in Figure 2.6.
Figure 2.1. Synthesis Scheme of PAs using the solid phase method and Fmoc chemistry.
Figure 2.2. Synthesis Scheme of the tri-tert-butyl ester DO3A derivative.
Figure 2.3. a) PA-DO3A conjugation and b) Gd$^{3+}$ chelation Scheme
Figure 2.4. Schematic representation of the Circular Dichroism set up.
Figure 2.5. Schematic representation of Critical Aggregation Concentration measurements using the pyrene 1:3 method.
Figure 2.6. Schematic representation of the Fluorescence Anisotropy set up.
Chapter 3

Results and Discussion: PA design and characterization – Fine tuning the pH trigger of Self Assembly

3.1. PA design and characterization:

Designing nanomaterials that can spontaneously change shape and size in response to specific physiological stimuli has the potential to exploit the differential diffusion kinetics to amplify the accumulation of these agents.\textsuperscript{85-90} In one example, Chien \textit{et al.} have demonstrated that peptide-polymeric amphiphiles can be triggered to undergo a self-assembly transition \textit{in vivo} from spherical micelles to aggregated assemblies using endogenous matrix metalloproteinases, leading to a higher concentration of material at the tumor site relative to the rest of the body.\textsuperscript{91} For cancer, one particularly attractive stimulus is the slightly acidic extracellular microenvironment of tumor tissue (pH 6.6–7.4) that arises due to the enhanced rate of glycolysis.\textsuperscript{36-38} There are numerous examples of materials that incorporate acid-cleavable linkages that degrade under the lysosomal (pH 5.0–5.5) or the slightly acidic tumor environment to release cargo.\textsuperscript{92-95} However, there are far fewer examples of materials that reversibly transform to larger,
more slowly diffusing morphologies in response to the extracellular cancer pH. Creating a material that, upon reaching the acidic extracellular tumor environment, transforms into a bulky, more slowly diffusing object could serve as a novel mechanism for achieving a higher relative concentration of imaging, drug delivery, or radiotherapeutic agent at the tumor site compared to the bloodstream. Although a multitude of self-assembling materials have pH-dependent assembly behavior, there are very few biologically compatible systems designed for *in vivo* use, with assembly behavior that can be reversibly triggered at neutral pH values (6.6−7.4) in an ionic environment that resembles serum. Both the concentration and the valency of the ionic environment play key roles in mediating the self-assembly of charged systems. Thus, developing systems that function under the stringent set of conditions for *in vivo* use requires considerable insight and optimization.

To develop materials capable of reversible pH-triggered morphological changes, we sought to design amphiphilic molecules that would exist as either single molecules or spherical micelles under normal physiological conditions (pH 7.4) and would self-assemble into nanofibers upon encountering the acidic environment (pH 6.6−7.4) of the tumor vasculature. PAs are an attractive class of molecules in this regard since they are biocompatible, can spontaneously self-assemble into a variety of morphologies, and have intermolecular forces that can be precisely tuned with the peptide sequence using a wide choice of charged, hydrophilic and hydrophobic amino acids.50-51
We hypothesized that our PA molecules should comprise of a hydrophobic alkyl tail, a β-sheet-forming peptide sequence, and a charged amino acid (anionic) sequence to exhibit amphiphilic character. Traditional colloid theory states that a repulsive “double layer” potential, \( \psi_0 \) exists at the surfaces of charged particles in liquids and decays exponentially with distance \( x \) as shown in eq. 3.1 below:\(^{98}\)

\[
\psi_x = \psi_0 e^{-\kappa x}
\]  

(3.1)

where the characteristic decay length of the potential is known as the Debye length, \( 1/\kappa \). The magnitude of \( 1/\kappa \) scales with the inverse square of the counter-ion concentration, as well as a prefactor that decreases with increasing ion valence. Decreasing the repulsive interaction of the charged region either via electrostatic screening or by lowering the degree of side-chain ionization with pH is expected to induce assembly into nanofibers via reduction in the head-group size.\(^{93-94}\) Hence, it may be possible to design a peptide sequence with a spherical micelle to nanofiber transition, which can be triggered by the protonation state of the charged residues under constant physiological salt concentrations. For instance, it was shown that upon addition of 5 mM Ca\(^{2+}\), a specific PA (palmitoyl-VVAAEEEEEIKVAV) underwent a spherical-to-nanofiber transition at pH 7.4.\(^{58}\) Consequently, by balancing the attractive and repulsive forces of the hydrophobic and charged regions respectively by amino acids in the hydrogen bond forming region, fine control of the self-assembly may be possible. The
hydrophobic region is typically composed of amino acids known to form hydrogen bonds in the form of β-sheets that bridges the carbonyl and amine groups of neighboring molecules in the peptide backbone. Berg et. al. examined the Zinc-finger peptide through a series of mutations at a solvent exposed area capable of forming a β-sheet secondary structure. They were able to rank each amino acid’s propensity for β-sheet formation by calculating the ΔΔG from a titration study using metal ions, Cobalt (II) and Zinc (II). The generated data (ΔΔG) corresponded well with the statistical propensity for each amino acid residue from crystallographic data of amino acids that form β-sheets in 64 different proteins. The ΔΔG and the probabilities of the amino acids to form β-sheets have been tabulated in Table 1 and were used as a guide to develop the dynamic systems in this study.

Herein, we have developed a specific PA design strategy for tuning the pH at which the self-assembly transition into nanofibers occur by tenths of a pH unit, in simulated serum salt solutions (150 mM NaCl, 2.2 mM CaCl2) at 10 M PA concentration. The ionic strength of the solution and valence of ions are of crucial importance because of the sharp dependence of self assembly on them. According to the Hardy Schulze’s rule, the critical coagulation concentration (CCC) of surfactants varies as the inverse of the ion charge to the sixth power. Hence, divalent ions are expected to have a significantly greater impact on the pH trigger of self assembly in our PAs than their monovalent counterparts. As one of our eventual goals is to develop Gd3+-based MRI contrast agents, 10 M is the minimum diagnostic concentration of these agents in
The PAs in this study contain a palmitic acid tail, an XAAA hydrophobic β-sheet region, where X is an amino acid with a non-polar side chain, and four glutamic acid (-EEEE-) residues. For two of the molecules, a DO3A-Gd imaging moiety was attached via the side-chain ε-NH₂ group of a lysine at the C-terminal end, as shown in Figure 3.1. For our purpose, a ratio of one strongly hydrophobic amino acid (tyrosine (Y), valine (V), phenylalanine (F), or isoleucine (I)) to four glutamic acids was essential to enable this transition in the desired pH range of 6.0–6.6.

Figure 3.2 enlists the PA molecules synthesized for this study. Figures 3.3 and 3.4 show the analytical HPLC traces and mass spectra respectively of the PA molecules used in this study. The mg of total PA / mg of solid, as obtained from peptide content analyses for selected PAs are listed in Table 2.

3.2. pH triggered self assembly behavior:

Our target PA concentration (10 μM) is below the detectable limit of conventional techniques to determine the morphology such as cryo-TEM and small-angle X-ray scattering. Consequently, CD spectroscopy was initially used to characterize the morphology of these PAs at various pH values. PA 3.1 was the first molecule synthesized that underwent a self-assembly transition in our desired pH range of 6.6–7.4 at 10 μM PA concentration in 150 mM NaCl and 2.2 mM CaCl₂, as illustrated in Figure 3.5. The secondary structure exhibited a superimposable random coil morphology at pH > 6.82. At
more acidic pH, the PAs start self-assembling into a structure with β-sheet character (formation of inter-strand H-bonds), which is indicative of a nanofiber morphology. The transition pH from random coil to β-sheet occurred at pH 6.6. We defined the transition pH to be the value at which the ellipticity at 205 nm rises to zero, followed by the appearance of a minimum at 218–220 nm.

### 3.3. pH reversibility of self-assembly:

The transition between random coil and β-sheet structure was rapid and reversible. At pH 7.7, HCl was added until the pH was 6.1, and the resulting β-sheet CD spectrum was collected within 3 min. An appropriate amount of NaOH was then added to reverse the pH back to 7.7, and random coil behavior was observed again. This process was repeated three times, and the CD spectra were found to be superimposable with respect to pH ([Figure 3.6](#)) indicating that this self-assembly transition occurs under thermodynamic equilibrium and requires 3 min or less to achieve the expected morphology.

Conventional TEM imaging was used to determine the morphology of 10 M PA 3.1 at pH 6.0 and 8.0, as shown in [Figure 3.7](#). The TEM grids were prepared within 3 min of pH adjustment. At pH 6.0, both individual and bundled fibers were present but dilute, and the isolated fibers had an average length of 590 ± 200 nm and an average diameter of 9.1 ± 1.5 nm. This fiber diameter corresponds to roughly twice the molecular
length from MM+ molecular simulations, corresponding approximately to the expected diameter of cylindrical fibers consisting of hydrophobically collapsed $\beta$-sheets. At pH 8.0, no fibers were present, confirming that the $\beta$-sheet character corresponds to the existence of fibers.

3.4. PA phase diagram:

When the CD spectra show a random coil morphology, the PA molecules could either be self-assembled into spherical micelles or exist as isolated molecules in solution. Because it is difficult to distinguish between staining artifacts and sample with TEM imaging at such a low concentration of sample, to determine the morphology under basic pH values, the CAC was measured for PA 3.1 at pH 6.6 using the pyrene 1:3 method (Figure 3.8). The CAC was found to be 6.0 $\mu$M, which is slightly below the 10 $\mu$M concentration at which the CD spectrum was obtained. These two values are in relative agreement considering the arbitrary nature of defining the transition pH from the CD spectrum. Thus, the random coil behavior corresponds to isolated molecules in solution, as opposed to spherical micellar morphology. To determine the overall influence of concentration and pH on the nature of this self-assembly transition, CD spectra were collected at 10−30 $M$ concentrations (Figure 3.9) and CAC measurements were performed at pH 4.0–10.0 (Figure 3.10). The transition points determined from both techniques were plotted to generate a concentration–pH self-assembly phase diagram.
PA 3.1 exhibited a strong dependence on both concentration and pH in the self-assembly transition. This concentration dependence was further confirmed via conventional TEM imaging. At pH 6.0 and 10.0, both isolated and bundled nanofibers were observed in samples prepared at 0.5 mM concentration (Figures 3.11a,b). At pH 6.0 and 10.0, the isolated nanofibers had average diameters of 9.4 ± 1.1 and 9.5 ± 1.2 nm, respectively.

3.5. Tailoring pH triggered self assembly via PA structure tuning:

By varying the β-sheet propensity of the amino acids in the β-sheet-forming region, the transition pH can be systematically tuned. In PA 3.2–PA 3.4, the isoleucine of PA 3.1 was substituted with the hydrophobic amino acids phenylalanine, valine, and tyrosine. pH-dependent CD spectra of PA 3.2–PA 3.4 at 10 μM also show a β-sheet-to-random coil transition at pH 6.0–6.6 (Figure 3.12). Similar to PA 3.1, this transition was observed to be reversible (Figure 3.13). Previous studies have shown that the propensity for β-sheet formation of these amino acids follows the trend I > F > V > Y. In PA 3.1–PA 3.4, the transition pH shifts to lower values with decreasing β-sheet propensity of the substituted hydrophobic amino acid (Table 3)\(^{102}\). The average pKa values for the glutamic acid residues in each molecule were determined from pH titration curves (Figure 3.14) to be in the range 4.6–4.9 (Table 3), with no specific correlation with the hydrophobicity of the PA. Therefore, the difference in self-assembly pH is not due to
changes in the pKa of the glutamic acid side chains. Rather, the transition is determined by the balance between the relative attractive forces of the β-sheet-forming and hydrophobic regions, and the repulsive forces of the deprotonated glutamic acids in the PA. With a stronger β-sheet-forming segment, the transition shifts to more basic pH. For PA 3.1 and PA 3.4, the transition at 10 μM occurred when 98.8% and 91%, respectively, of the glutamic acids were deprotonated.

3.6. Addition of DO3A:Gd imaging tag:

We then incorporated an MRI imaging moiety on the C-terminus of the PA. An additional lysine, conjugated to a 1,4,7-tris(carboxymethylaza)cyclododecane-10-azaacetylamide (DO3A) tag was linked to the C-terminus of PA 3.1 and PA 3.3 to produce PA 3.5 and PA 3.6. The molecule-to-nanofiber transition was still observed at 10 μM PA; however, the transition pH of PA 3.5 was shifted to 5.7 (Figure 3.15). Since this imaging moiety does not add excess charge, this shift toward more acidic pH is likely due to the greater steric hindrance and additional hydrophilicity of DO3A restricting the formation of the self-assembled state. The concentration–pH self-assembly phase diagram was mapped out for PA 3.5 (Figure 3.16c) using concentration dependent CD (Figure 3.17) and pH dependent CAC measurements (Figure 3.18). Under basic conditions and above the CAC, a random coil secondary structure was observed in the CD spectra, which are indicative of self-assembly into a spherical micelle phase. The
transition from nanofibers to spherical micelles was confirmed via TEM imaging at 0.5 mM PA 3.5 at pH 4.0 and 10.0, respectively (Figure 3.16a,b). The nanofibers and spherical micelles had diameters of 11.9 ± 1.6 and 10.0 ± 1.2 nm, respectively. In contrast to the nanofiber-to-molecule transition, the pH for the nanofiber-to-micelle transition showed relatively little concentration dependence. The nanofiber- to-micelle transitions at 0.5 mM and 20 μM PA occurred at pH 6.0 and 5.7, respectively. The steric bulk of the DO3A moiety increases the headgroup size of PA 3.5 relative to PA 3.1, thus inducing the spherical self-assembly morphology. Also, the zeta potentials for the spheres and fibers were found to be -11.6 mV and -8.4 mV respectively in 150 mM NaCl and 2.2 mM CaCl₂. In addition, DLS measurements (Figure 3.19) conducted on 2 mM PA 3.5 in our salt solution showed a distinct peak at d (size) = 10.8 nm at pH 8.0 indicating spherical micelles. At pH 6.0, however, no peaks were observed in the size range 1-500 nm proving that a larger non-spherical morphology is formed.

The shift in transition pH due to the change in β-sheet propensity still occurs when the DO3A:Gd moiety is present. For each concentration, PA 3.6 had a nanofiber-to-micelle transition that occurred 0.4 pH unit lower than for PA 3.5 (Figure 3.20). Because the same trend occurs in these PAs irrespective of the presence of a DO3A:Gd moiety, this strategy of altering the β-sheet propensity can be generally used to systematically shift the transition pH.
3.7. Relaxivity measurements:

The aim of using a contrast agent in MRI is to accelerate the relaxivity of water protons of the surrounding tissue and can be achieved by paramagnetic labels (DO3A:Gd in our case). The general theory of solvent nuclear relaxation in the presence of paramagnetic substances was developed by Bloembergen, Solomon and others. A Gd(III) complex induces an increase of both the longitudinal and transverse relaxation rates, $1/T_1$ and $1/T_2$, respectively, of the solvent nuclei. The observed solvent relaxation rate, $1/T_{i,\text{obs}}$, is the sum of the diamagnetic ($1/T_{i,d}$) and paramagnetic ($1/T_{i,p}$) relaxation rates.\textsuperscript{66}

The diamagnetic term, $1/T_{i,d}$, corresponds to the relaxation rate of the solvent (water) nuclei in the absence of a paramagnetic solute. The paramagnetic term, $1/T_{i,p}$, gives the relaxivity ($r_i$) enhancement caused by the paramagnetic substance which is linearly proportional to the concentration of the paramagnetic species, [Gd].\textsuperscript{66}

\begin{equation}
1/T_{i,\text{obs}} = 1/T_{i,d} + r_i[Gd], \quad i = 1,2
\end{equation}

Relaxivity values of water protons in the presence of PA 3.5 at 500 μM, at pH of 5.0 and 10.0, were found to be 8.3 and 6.6 mM\textsuperscript{−1}s\textsuperscript{−1}, respectively, using a 1.5 T magnet, as shown in Figure 3.21. These values were higher than those measured for a Magnevist control standard (4.5 mM\textsuperscript{−1}s\textsuperscript{−1}).\textsuperscript{102} This relaxivity increase from spherical micelles to nanofibers likely originates from the longer rotational correlation time when imaging
agents are coupled to large molecular weight objects, which has been well established for magnetic resonance agents coupled to polymers and PAs. The relaxivity of these systems is about 25–50% lower than that of other supramolecular assemblies with similar K(DO3A:Gd) linkages. This suggests that the DO3A:Gd motion is independently faster than that of the nanofiber due to the conformationally flexible E₄K tether, which can be further optimized. Regardless, the primary mechanism for tumor imaging relies on the increased local concentration of the more slowly diffusing nanofibers in the tumor environment compared to the bloodstream, but the improved relaxivity of fibers compared to spheres could serve as a secondary mechanism for enhanced tumor detection.¹⁰²

3.8. Gd³⁺ leaching – Arsenazo tests:

The stability of the DO3A:Gd chelate is known to be a function of solution pH. To corroborate that Gd³⁺ does not leach out of the complex and alter self-assembly behavior at low pHs, absorbance spectra of PA 3.5 were collected at various pH values in the presence of the dye 2,2′-(1,8-Dihydroxy-3,6-disulfonaphthylene-2,7-bisazo) bis-benzenearsonic acid 2,7-Bis(2-arsonophenylazo) chromotropic acid, commercially known as Arsenazo III¹⁰³ (Figure 3.22a). The dye itself exhibits an absorption peak at 550 nm¹⁰³, which remains constant in the pH range of 1-7 (Figure 3.22b). In the presence of divalent or lanthanide ions, it forms a 1:1 chelate complex, which is characterized by a
red shift in the absorbance to 580-590 nm along with the appearance of a 650 nm peak, also shown in Figure 3.22b. Figure 3.22c shows that the absorption of the dye in the presence of 50 µM PA 3.5 remains constant down to pH 5.39. As the pH is further lowered to a value of 5.08 and below, the absorption peak begins to red shift and the peak at 650 nm appears indicating leaching of Gd$^{3+}$ from the PA. Subtracting the spectrum at pH 5.81 from that at pH 4.73 gives the spectral profile of the metal ion-dye complex. Hence, the solution pH was kept above this lower limit of 5.0 for PAs containing the Gd-imaging tag.

3.9. Probing PA self assembly in a salt buffer and at body temperature:

One of our eventual goals is to develop a medium that represents blood serum conditions to test our PA self-assembly in. To evaluate the accuracy of our simulated salt solution of 150 mM NaCl and 2.2 mM CaCl$_2$ in simulating the ionic strength of serum, concentration dependent CD spectra were collected for PA 3.5 as a function of pH in Hank’s balanced salt solution, a commercially available buffer that simulates the salt concentrations in real blood. The transition points thus obtained were super-imposed on the phase diagram of PA 3.5 (Figure 3.23), and were found to shift to basic pH values by ~0.6-0.8 units at higher concentrations. This shift is desired since the pH values are closer to our physiologically relevant range of 6.6-7.4, proving that the ionic strength of our solution is not significantly different from the real value.
Self assembly is also known to depend on ambient temperature. All our measurements so far, have been conducted at room temperature (25 deg C). To probe the effect of body temperature on the pH trigger of our PA self assembly, pH dependent CD spectrum was obtained for the 50 µM PA 3.5 solution in Hank’s buffer at 37 deg C (Figure 3.24). The transition pH was found to shift ~0.8 units acidic (pH 6.27 at 25 deg C) which is expected because a higher temperature imparts greater kinetic energy to the PA molecules, thereby disrupting self assembly to a certain extent. Hence, a slightly acidic pH (relative to room temperature) is required to induce self-assembly of the PA molecules.
Table 1. β-sheet forming propensities of amino acids.99

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>G (kcal mol(^{-1}))</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine (I)</td>
<td>-0.56</td>
<td>1.57</td>
</tr>
<tr>
<td>Phenylalanine (F)</td>
<td>-0.55</td>
<td>1.23</td>
</tr>
<tr>
<td>Valine (V)</td>
<td>-0.53</td>
<td>1.64</td>
</tr>
<tr>
<td>Tyrosine (Y)</td>
<td>-0.5</td>
<td>1.31</td>
</tr>
<tr>
<td>Threonine (T)</td>
<td>-0.48</td>
<td>1.33</td>
</tr>
<tr>
<td>Alanine (A)</td>
<td>-0.35</td>
<td>0.79</td>
</tr>
<tr>
<td>Glutamic Acid (E)</td>
<td>-0.41</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Figure 3.1. PA design strategy and generic structure.
**Figure 3.2.** Structures of synthesized PAs.

**PA 3.1 - Palmitoyl-IAAAAEEE-NH₂**

**PA 3.2 - Palmitoyl-FAAAAAEE-NH₂**

**PA 3.3 - Palmitoyl-VAAAAEEE-NH₂**

**PA 3.4 - Palmitoyl-YAAAAEEE-NH₂**

Continued
Continued from Figure 3.2

**PA 3.5** - Palmitoyl-I\text{AAEEEEEK(DO3A:Gd)-NH}_2

**PA 3.6** - Palmitoyl-V\text{AAEEEEEK(DO3A:Gd)-NH}_2
Figure 3.3. Analytical HPLC traces of PAs.
Continued from Figure 3.3.

PA 3.3

PA 3.4

Continued
Continued from Figure 3.3.

PA 3.5

PA 3.6
PA 3.1 – Mol. Wt. 1098.29 g/mol

Figure 3.4. ESI-Mass spectra of PAs.
Continued from Figure 3.4.

**PA 3.2** – Mol. Wt. 1132.30 g/mol

Continued
Continued from Figure 3.4.

**PA 3.3** – Mol. Wt. 1084.26 g/mol
Continued from Figure 3.4.

**PA 3.4** – Mol. Wt. 1148.30 g/mol

Continued
Continued from Figure 3.4.

**PA 3.5** – Mol. Wt. 1767.09 g/mol

![Mass spectrum diagram]

- **M + Na**
- **M + 2Na**
- **M + H**
- **M + Na + K + 2H**

Continued
Continued from Figure 3.4.

PA 3.6 – Mol. Wt. 1753.06 g/mol
Table 2. Peptide content analyses of PAs 3.1-3.5

<table>
<thead>
<tr>
<th>PA</th>
<th>mg of PA/mg of solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>88%</td>
</tr>
<tr>
<td>3.2</td>
<td>73%</td>
</tr>
<tr>
<td>3.3</td>
<td>91%</td>
</tr>
<tr>
<td>3.4</td>
<td>61%</td>
</tr>
<tr>
<td>3.5</td>
<td>96%</td>
</tr>
</tbody>
</table>
Figure 3.5. pH-dependent CD spectrum of 10 µM PA 3.1.
Figure 3.6. pH reversibility of morphology transition in 10 µM PA 3.1.
Figure 3.7. TEM images of 10 µM PA 3.1 at pH a) 6.0 and b) 8.0.
**Figure 3.8.** CAC measurement of PA 3.1 at pH 6.6.
Figure 3.9. pH dependent CD spectra of PA 3.1 at concentrations of a) 15 µM and b) 30 µM.
Figure 3.10. pH dependent CAC measurements of PA 3.1.
Figure 3.11. TEM images of 0.5 mM PA 3.1, measured at pH a) 6.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 3.1 as determined via CD (red squares) and CAC (blue diamonds) measurements. The white area corresponds to a self assembled region where the morphology is uncertain due to lack of suitable experimental techniques. All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl₂.
Figure 3.12. pH dependent CD spectra of 10 µM a) PA 3.2 b) PA 3.3 c) PA 3.4.
Figure 3.13. pH reversibility of morphology transition in 10 µM PA 3.3.
**Figure 3.14.** pH titration curves of 10 µM a) PA 3.1 b) PA 3.2 c) PA 3.3 and d) PA 3.4.
Table 3. CD transition pH and pKa values of PAs 3.1-3.4

<table>
<thead>
<tr>
<th>PA</th>
<th>CD transition pH</th>
<th>Avg. pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>6.6</td>
<td>4.66 ± 0.10</td>
</tr>
<tr>
<td>3.2</td>
<td>6.6</td>
<td>4.94 ± 0.09</td>
</tr>
<tr>
<td>3.3</td>
<td>6.2</td>
<td>4.86 ± 0.11</td>
</tr>
<tr>
<td>3.4</td>
<td>6.0</td>
<td>4.70 ± 0.10</td>
</tr>
</tbody>
</table>
Figure 3.15. pH dependent CD spectrum of 10 µM PA 3.5.
Figure 3.16. TEM images of 0.5 mM PA 3.5, measured at pH a) 4.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 3.5 as determined via CD (red squares) and CAC (blue diamonds) measurements. All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl$_2$.$^{102}$
Figure 3.17. pH dependent CD spectra of PA 3.5 at concentrations of a) 20 µM and b) 0.5 mM.
Figure 3.18. pH dependent CAC measurements of PA 3.5.
Figure 3.19. DLS measurements of 2 mM PA 3.5 in salt solution at pH 8.0 and 6.0.
Figure 3.20. pH dependent CD spectra of PA 3.6 at concentrations of a) 20 µM and b) 0.5 mM.
Figure 3.21. pH dependent relaxivity of water protons in presence of various concentrations of PA 3.5.
Figure 3.22. a) Chemical structure of Arsenazo III dye\textsuperscript{103} b) pH dependent Absorbance spectra of 25 µM Arsenazo III only and Arsenazo-Gd complex control c) pH dependent Absorbance spectra of 25 µM Arsenazo III and 50 µM PA 3.5. The blue spectrum represents the difference between the traces at pH 4.73 and 5.81.
Figure 3.23. Concentration dependent CD transition pH values (blue diamonds) of PA 3.5 in Hank’s balanced salt buffer, superimposed on the phase diagram of PA 3.5 in 150 mM NaCl and 2.2 mM CaCl₂.
Figure 3.24. pH dependent CD spectra of 50 µM PA 3.5 in Hank’s balanced salt solution at 37 deg C (body temperature).
Chapter 4

Results and Discussion: PA structure-property relationships – Programming pH triggered self assembly via isomerization of peptide sequence

4.1. PA design and characterization:

Previous studies involving peptides and PAs have explored the relationship between sequence variation through the rearrangement of amino acids in the peptide’s primary structure and self-assembly morphology.\textsuperscript{105-108} For instance, Zhao et al. have shown that by rearranging the amino acid sequence in a short peptide pentamer, a variety of morphologies including nanofibrils, nanotubules, and a lack of regular structure can be induced depending on the programmed sequence.\textsuperscript{109} By creation of N-methylated glycine derivatives and alanine mutants, Paramonov et al. have shown that for an amphiphilic system (palmitoyl-GGGGGERGDS-COOH), the H-bonding in the four amino acids near the hydrophobic core is most essential for self-assembly of nanofibers.\textsuperscript{110} Coarse-grained molecular dynamics simulations suggest that for another amphiphilic system (palmitoyl-VVVAAAE), residues 2-5 tend to assume a β sheet secondary structure.
most readily and that hydrophobic residues collapse to some extent into the fatty acid aggregate core.\textsuperscript{111}

However, the effects of sequence isomerization have never been studied in a dynamically self-assembling system with an environmental trigger, for instance pH as in our case. Also, the effect of changing the position of an amino acid in the peptide primary structure on its self assembly behavior might not be as apparent as the effect of changing the identity of the amino acid. The purpose of this study was to establish a relationship between the position of the β sheet-forming residue isoleucine (Ile) in the primary peptide sequence and the pH of transition. To accomplish this, the previously studied PA 3.1 was isomerized to create palmitoyl-AIAAEEEE-NH\textsubscript{2} (PA 4.1), palmitoyl-AIAAEEEE-NH\textsubscript{2} (PA 4.2), palmitoyl-AAAIEEEE-NH\textsubscript{2} (PA 4.3) and palmitoyl-AIAAEEEEK(DO3A:Gd)-NH\textsubscript{2} (PA 4.4), as shown in Figure 4.1. Figures 4.2 and 4.3 show the analytical HPLC traces and ESI-mass spectra of the PAs. Table 4 lists the peptide content analysis results for the synthesized PAs. As usual, all measurements were performed in 150 mM NaCl and 2.2 mM CaCl\textsubscript{2}.

4.2. CD results:

pH dependent CD spectra were collected for PAs 4.1-4.4 to determine the prominent secondary structures of PAs at concentrations of 5 µM and greater. Previously, a β sheet CD signal has been correlated to a nanofiber morphology and a
random coil CD signal has been correlated to a spherical micelle or single molecule morphology. When using CD, the β sheet secondary structure is signaled by an ellipticity minimum at 218-222 nm and random coil, by an ellipticity maximum at 212 nm and a minimum at 196 nm. The transition from spherical micelle (random coil) to nanofiber (β sheet) was determined to take place just as the ellipticity at 205 nm first became positive. This transition was observed to be concentration dependent as well, with higher concentrations favoring a β sheet secondary structure even at high pH values. This concentration dependence was found to decrease with decrease in the PA’s hydrophobicity via incorporation of the bulky DO3A:Gd imaging moiety as a part of it’s hydrophilic head-group.

CD data for PA 4.1 at 5 µM revealed a transition from random coil to β-sheet between pH values of 7.05 and 7.12. At 10 µM this transition increased to pH values between 6.16 and 6.26, as shown in Figure 4.4a. At 12.5 µM the transition shifted higher still, to between pH 8.13 and 8.26. By concentrations of 15 and 30 µM, no transition could be found and the PA exhibited a β sheet signal exclusively independent of pH.

CD analysis of PA 4.4 at 5 µM revealed a transition between β sheet and random coil between pH values of 6.44 and 6.55. At 10 µM this transition point increased to between pH 7.09 and 7.15 (Figure 4.4b). By 25 µM, the transition was observed between pH 7.67 and 7.80. Between 50 µM and 150 µM, the transition seemed relatively constant, observed between pH 7.92 and 8.04 and between 7.94 and 8.05, respectively.
This decrease in the transitional pH values relative to PA 4.1 was expected due to the increase in the PA’s headgroup size.

CD spectra acquired for 10 µM PA 4.2 and PA 4.3, as showed in Figures 4.5a and b respectively, exhibited the β sheet morphology exclusively at all concentrations and irrespective of pH, indicating that moving the Ile to the third or fourth position in the PA primary structure significantly enhances its hydrophobicity and β sheet forming propensity.

The reversibility of the transition was also tested using CD. A sample of 5 µM PA 4.4 initially was brought to pH 8.55 and a CD spectrum was acquired. This solution was then acidified to pH 5.65 and another spectrum was recorded. The solution pH was again raised back to 8.55 and spectra were recorded every 15 minutes for 45 minutes (Figure 4.6). Although the spectra reflected a random coil signal, none of the spectra taken within the 45 minutes after returning to pH 8.55 were super-imposable with the original pH 8.55 spectrum.

4.2. CAC results:

CACs for PA 4.1 were determined to be 2.0 µM, 2.6 µM, 2.5 µM, 3.2 µM, and 13.4 µM for pH 5.0, 6.0, 7.0, 8.0, and 10.0 respectively. A clear relationship between pH and CAC is evident in these data and is illustrated in Figure 4.7, which shows the CAC determination for pH values 5.0 and 10.0 superimposed in a single chart. As expected,
the CAC value decreases with decrease in pH. Under basic conditions, the PA molecule exists in a highly ionized state, hence aggregation is inhibited by ionic repulsion. When the glutamic acid residues are more protonated (at acidic pH values) this ionic repulsion is lessened or eliminated, allowing aggregation at lower concentrations.

In determining the CACs for PA 4.4, a similar pH dependence was noted. The dependency is illustrated in Figure 4.8, which shows the CAC determination for pH values of 5.0 and 10.0 superimposed in a single chart. The CAC values for palmitoyl-AIAAEEEEK(DO3A:Gd)-NH$_2$ were determined to be 1.5 µM, 3.2 µM, 3.5 µM, 3.7 µM and 5.4 µM for pH values of 5.0, 6.0, 7.0, 8.0, and 10.0 respectively.

CACs for PAs 4.2 and 4.3 could only be determined under basic conditions due to their significant hydrophobicity. At Acidic pH values (< 7.0), these PAs were found to exist in the self assembled form at all concentrations down to the low nM range. For PA 4.2, the CAC values were 0.56 µM, 2.7 µM and 19 µM at pH 6.0, 7.4 and 10.0 respectively and for PA 4.3, they were 0.48 µM and 2.1 µM at pH 8.5 and 10.0 respectively.

4.4. TEM measurements and Isomerized PA Phase Diagrams:

Following the collection of CD and CAC data, the observed transition points were plotted with respect to pH and PA concentration to create phase diagrams for PA 4.1 (Figure 4.9c) and PA 4.4 (Figure 4.10c). TEM images confirmed the presence of
nanofibers in 100 µM PA 4.1 at pH 8.3 and pH 5.5, respectively (Figure 4.9a and b). TEM also confirmed the presence of long nanofibers (greater than 1 micron) in 150 µM PA 4.4 at pH 6.6 (Figure 4.10a), whereas at pH 9.9, a spherical micellar morphology was observed (Figure 4.10b) with an avg. diameter of ~9-10 nm. In each case that nanofibers were found, the diameter of an individual fiber was found to be ~10-11 nm.

The phase diagrams for PAs 4.2 (Figure 4.11c) and 4.3 (Figure 4.12c) exhibited the nanofiber morphology exclusively with minor low concentration regions where single molecules exist. TEM images (Figures 4.11a,b, 4.12a,b) show the fiber morphology at both acidic and basic pH values at PA concentrations of 100 µM or above.

4.5. Comparison with parent PAs:

By overlaying the phase diagrams of isomeric PAs 3.1 and 4.1 lacking the DO3A imaging moiety (Figure 4.13) and the phase diagrams of isomeric PAs 3.5 and 4.4 with the DO3A imaging moiety (Figure 4.14), the effects of sequence variation within the β sheet-forming region were analyzed. The creation of sequence variants in both cases resulted in lower CAC concentrations at nearly all pH values. Therefore, moving the Ile residue to the second position promotes single molecule aggregation.

By examining the CD data in each phase diagram, it is evident that by moving the Ile to the second β sheet forming position, aggregation from spherical micelle to nanofiber is promoted at higher pH. This is most evident in the DO3A:Gd-containing
molecules due to their lower concentration dependence of transition pH. For instance, at 10 µM, PA 4.4 transitions from sphere-to-nanofiber at pH 7.1. This transition pH is nearly 1.5 units higher than that for PA 3.5 at the same concentration. Notably, PA 4.4 shows much higher concentration dependence for its transition than PA 3.5. Between 10 and 500 µM, the pH of transition for PA 3.5 lies in the range 5.66-6.02 (less than 0.5 pH units). Contrastingly, the transition for PA 4.4 between 5 and 150 µM spans a range from 6.5 to 8.0 (1.5 pH units). This concentration dependence seems to decrease at high concentrations for PA 4.4, since between 50 and 150 µM the transition remains constant at pH 8.0.

Comparison of the CD data for non-DO3A:Gd-containing isomers is more difficult due to the high concentration dependence the sphere-to-nanofiber transition exhibits for both isomers. In general, PA 4.1 seems to favor the nanofiber morphology at higher pH values compared to PA 3.1. At 10 µM, PA 3.1 displays a sphere-to-nanofiber transition at pH 6.5, while its isomer shows a transition at pH 7.35 (a shift of nearly 0.9 pH unit). Additionally, PA 3.1 exhibits a solely nanofiber morphology above 20 µM concentration while its isomer is exclusively nanofibers at all concentrations above 15 µM.
4.6. Explanation of observed trends:

The synthesis and characterization of various constitutional isomers have revealed the major role that the position of β sheet-forming residues has on pH-dependent dynamic self-assembly in PAs. Shifting the hydrophobic Ile away from the palmitoyl tail by one residue in a DO3A-conjugated molecule, the transitional pH was found to increase by greater than 2 pH units and the concentration-dependence of this transition was found to increase below 25 µM. Regardless of whether DO3A was included in the molecular structure, isomerizing Ile to the second position resulted in an increased preference for the nanofiber morphology and an increased propensity for general aggregation at lower concentrations. With Ile in the third position, nanofibers formed regardless of pH and aggregation occurred at even lower concentrations. With Ile in the fourth position, flat nano-belts were observed at slightly acidic pH values and twisted nano-ribbons were observed at slightly basic pH values.

The drastic shift in transition pH between PA 3.1, PA 3.5, and their respective control systems is attributed to the fact that, when positioned next to the palmitoyl acid tail, the β sheet forming residues like Ile more readily associate with the hydrophobic tail, destabilizing the β sheet. As a hydrophobic residue, the positioning of Ile near the palmitoyl tail causes the residue to either participate in the hydrophobic collapse near the micelle’s core or to facilitate hydrogen bonding with neighboring PAs, assisting in the creation of β sheet secondary structure. Hydrophobic collapse precludes Ile from participating in hydrogen bonding, and vice versa. Additionally, this collapse interrupts
the formation of hydrogen bonds to a certain extent along the peptide backbone (Figure 4.15a). By creating distance between Ile and the hydrophobic core (via isomerization), hydrophobic collapse of the Ile becomes less favorable. Instead, the Ile hydrogen interacts with nearby residues of neighboring molecules (Figure 4.15b). This results in the PA more readily assuming a nanofiber morphology. This effect is even more pronounced in PAs 4.2 and 4.3 as distance from the hydrophobic core is increased. The extreme pH-dependence of CAC values for PA 4.3 is due to the proximity of the hydrophobic Ile to the acidic glutamic acid residues and the great distance between isoleucine and the hydrophobic core leading to formation of a secondary hydrophobic region away from the palmitic acid core.
**Figure 4.1.** Structures of synthesized PAs.

**PA 4.1** - Palmitoyl-AIAEEEEE-NH₂

**PA 4.2** - Palmitoyl-AAIAEEEE-NH₂

**PA 4.3** - Palmitoyl-AAAIEEEE-NH₂

**PA 4.4** - Palmitoyl-AIAEEEEEK(DO3A:Gd)-NH₂
Figure 4.2. Analytical HPLC traces of PAs.
Continued from Figure 4.2.

PA 4.3

![Graph for PA 4.3]

PA 4.4

![Graph for PA 4.4]
PA$s 4.4-4.3 -$ Mol. Wt. 1098.29 g/mol

Figure 4.3. ESI-Mass spectra of PAs.
Continued from Figure 4.3.

**PA 4.4** – Mol. Wt. 1767.09 g/mol
<table>
<thead>
<tr>
<th>PA</th>
<th>mg of PA/mg of solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>43%</td>
</tr>
<tr>
<td>4.2</td>
<td>48%</td>
</tr>
<tr>
<td>4.3</td>
<td>56%</td>
</tr>
<tr>
<td>4.4</td>
<td>48%</td>
</tr>
</tbody>
</table>

**Table 4.** Peptide Content analyses of PAs 4.1-4.4
Figure 4.4. pH dependent CD spectra of a) 10 µM PA 4.1 b) 10 µM PA 4.4.
Figure 4.5. pH dependent CD spectra of a) 10 µM PA 4.2 b) 10 µM PA 4.3.
Figure 4.6. pH reversibility of morphology transition in 5 µM PA 4.4.
Figure 4.7. pH dependent CAC measurements of PA 4.1.
Figure 4.8. pH dependent CAC measurements of PA 4.4.
Figure 4.9. TEM images of 100 µM PA 4.1, measured at pH a) 5.5 and b) 8.3 c) Concentration-pH self assembly phase diagram of PA 4.1 as determined via CD (red squares) and CAC (blue triangles) measurements. The concentration and pH at which TEM were obtained are labeled in c) (red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl$_2$. 
Figure 4.10. TEM images of 100 µM PA 4.4, measured at pH a) 6.6 and b) 9.9 c) Concentration-pH self assembly phase diagram of PA 4.4 as determined via CD (red squares) and CAC (blue triangles) measurements. The concentration and pH at which TEM were obtained are labeled in c) (red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl₂.
Figure 4.11. TEM images of 100 µM PA 4.2, measured at pH a) 6.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 4.2 as determined via CAC measurements. The concentration and pH at which TEM were obtained are labeled in c) (red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl₂.
Figure 4.12. TEM images of 100 μM PA 4.3, measured at pH a) 6.0 and b) 10.0. c) Concentration-pH self assembly phase diagram of PA 4.3 as determined via CAC measurements. The concentration and pH at which TEM were obtained are labeled in c) (red circles). All measurements were conducted in 150 mM NaCl and 2.2 mM CaCl₂.
Figure 4.13. Concentration-pH self assembly phase diagram of PA 4.1 (blue) overlaid on the same for PA 3.1 (fade pink).
Figure 4.14. Concentration-pH self assembly phase diagram of PA 4.4 (blue) overlaid on the same for PA 3.5 (fade pink).
Figure 4.15. Schematic representation of the effect of Ile’s position on PA self assembly,

a) Ile next to the palmitic acid core b) Ile in the second position.
Chapter 5

Results and Discussion: *In vitro* characterization – Probing peptide self assembly in blood serum

5.1. PA system design and characterization:

One of the great challenges in developing biocompatible, dynamic systems that can transform morphologies *in vivo*, is the difficulty in probing their self-assembly behavior in blood serum. Serum contains variable amounts of salt concentrations, as well as proteins such as albumin, that bind to amphiphilic molecules, enzymes and other macromolecules.\(^\text{112-115}\) Any spectroscopic technique for determining the self-assembly behavior of PAs in the presence of other proteins will require the addition of a chromophore to the molecular structure. However, any minor changes to the molecular structure of the PA can dramatically change the pH trigger of self-assembly. Indeed, we observed that the addition of just a single methyl group in the \(\beta\)-sheet sequence shifted the transition basic by 0.4 pH units.\(^\text{102}\) Thus, developing approaches for probing this transition in serum without significantly altering the self-assembly behavior is an essential prerequisite for understanding the influence of this dynamic trigger on *in vivo* biodistribution.
Here, we have developed a method to probe the pH-dependent self-assembly morphology of PAs in mouse blood serum without significantly changing the intrinsic self-assembly behavior of the material. We employed Fluorescence Anisotropy (FA) of a fluorescently-labeled PA for this purpose. FA measures the extent of decorrelation of the polarized emission from a fluorescent dye with respect to the polarization of the excitation light, which linearly depends on the tumbling rate of the dye containing rotating unit in solution and consequently its size/volume and molecular weight. Here, we chose Palmitoyl-IAAAEEEEK(DO3A:Gd)-NH₂ (PA3.5) as our model PA system, since it undergoes a concentration-independent transition from spherical micelles to nanofibers below a pH of 6.0. In the isolated state, this molecule has a molecular weight of ~1.8 kDa. A 10 nm spherical micelle formed from the molecule with an estimated aggregation number of 60-100 (~108-180 kDa) would therefore have a different FA than a micron-sized nanofiber, which has molecular weight orders of magnitude larger and scaled according to length. For example a 500 nm long fiber is expected to contain 3000-5000 monomers, with a molecular weight of 5-9 MDa. By extrapolation from the MW of previously published proteins, the spherical micelle is expected to have rotational correlation lifetimes of 50-100 ns, and the cylindrical nanofiber to be larger than ms. Therefore, our target dye needs to have a fluorescence lifetime comparable to these rotational correlation lifetimes. To distinguish between these two morphologies, bis(2,2’-bipyridine)-4’-methyl-4-carboxybipyridine ruthenium(II) was conjugated to the lysine ε-amine group of the PA Palmitoyl-IAAAEEEEK-NH₂ (PA3.1-Rubipy, Figure 5.1) via an
NHS ester linker. This dye has excitation and emission wavelengths of 458 and 630 nm respectively and a fluorescence lifetime of ~400-500 ns.\(^{118}\) Moreover, the dye's absorption wavelength is significantly shifted from that of serum proteins avoiding any excitation of the latter. The PA3.1-Rubipy was spiked into PA3.5 molecule in a small amount (1.5% of the total PA concentration) for FA measurements (PA mix). **Figures 5.2** and **5.3** show the analytical HPLC trace and ESI mass spectrum of PA3.1-Rubipy respectively.

Initial attempts in using ms lifetime luminescent Tb\(^{3+}/\)Eu\(^{3+}\) in place of Gd\(^{3+}\) in PA3.5 (PA3.5-Tb) as the fluorescent tag\(^{119-120}\) were unsuccessful due to masking of the fluorescence signal via serum auto-fluorescence\(^{121}\) (**Figure 5.4a**), even in the presence of pyrene which is known to act as a sensitizer enhancing lanthanide fluorescence intensity\(^{122}\) (**Figure 5.4 b,c**).

**5.2. PA mix phase diagram and reversibility:**

Self-assembly behavior is known to be extremely sensitive to minor changes in monomer structure and presence of impurities. To ensure that the addition of 1.5% PA3.1-Rubipy to PA3.5 does not significantly disrupt self-assembly behavior, a concentration-pH phase diagram was mapped out for this PA mixture in our 150 mM NaCl and 2.2 mM CaCl\(_2\) salt solution and compared with the phase diagram for pure PA3.5 (**Figure 5.5**).
CAC measurements via the pyrene 1:3 method were used to ascertain the single molecule to spherical micelle transitions at varying pH values ranging from 5.0-10.0 (two pH points shown in Figure 5.6 for clarity). CD spectroscopy was used to determine the pH points at which different concentrations (10-500 µM) of the PA molecules transitioned from a random coil to a β-sheet secondary structure (two concentrations shown in Figure 5.7). It has been previously shown that the random coil and β-sheet structures correspond to spherical micellar/isolated molecule and nanofiber morphologies respectively. The CD transition point was defined to be the pH value at which the ellipticity at 200 nm rose from a negative value (random coil) to zero accompanied by the appearance of a minimum at 218-220 nm. The addition of PA3.1-Rubipy was found to shift the CD transition points to more basic values by ~0.7 pH units. The CAC values, on the other hand were found to be 5x higher in the acidic region but comparable under basic conditions relative to pure PA3.5.

The transition was found to be rapid and reversible with respect to pH. This was tested by switching the pH of the PA mixture back and forth between 4.0-5.0 and 9.0-10.0 followed by collection of the CD spectra within 2-3 minutes (Figure 5.8) The CD curves showed almost superimposable secondary structures for the acidic and basic pH values, indicating the system to be under thermodynamic equilibrium. This reversibility was not observed with larger relative concentrations of PA3.1-Rubipy (≥3% of the total PA concentration).
5.3. CD-FA correlation:

pH dependent FA values of a 100 µM PA mix in the same isotonic salt solutions (Figure 5.9a) were then obtained. The inset in Figure 5.9a shows the fluorescence emission from the PA3.1-RubiPy in the mixture upon excitation with 458 nm light that was exploited for FA measurements. The FA values increased from 0.066 to 0.115 as the pH was carefully lowered from 8.35 to 5.31. The onset of the self-assembly transition was defined by the data point that first showed a higher value relative to the constant FA at basic pH values. The end of this transition was defined as the pH point below which the FA became constant again. The transition profile measured via FA correlated well with that obtained from the pH dependent CD spectra (Figure 5.9b). The FA transition onset at 6.98 is close to the most basic pH CD spectrum at 7.10 that started to deviate from a superimposable random coil morphology. Also, the FA transition midpoint at a pH of 6.68 was close to our previously defined CD transition spectrum at a pH of 6.62.

5.4. FA in pure and dilute blood serum:

The fluorescence of the PA mixture was detectable in pure mouse serum above the serum autofluorescence background. Figure 5.10a shows this fluorescence emission in blood serum along with the serum auto-fluorescence background. The fluorescence emission intensity increased with increasing amounts of PA3.1-Rubipy in serum and was
found to be superimposable under acidic and basic conditions (Figure 5.10b). The ~30 nm red shift in the PA3.1-Rubipy fluorescence emission in serum is commonly observed for Ru(bipy)$_3^{2+}$ in the presence of protein and lipid molecules due to changes in the dye's emission pathways. The FA in pure mouse blood serum was found to be pH independent with a constant value of ~0.20 (Figure 5.11). This constant high value results from the formation of nanofibers immediately after addition of the serum to the PA mixture even at basic pH values. The general increase in the absolute FA value in serum (~0.20) relative to salts (~0.11) was attributed to an increase in solution viscosity which increases rotational correlation times and consequently FA.$^{83}$

This immediate formation of nanofibers in pure serum was also confirmed via $^{13}$C-NMR spectroscopy using a $^{13}$C-labelled PA 3.5 (3 mM) containing the diamagnetic Lu$^{3+}$ in place of Gd$^{3+}$. The basis for using NMR to detect morphologies with distinctly different molecular weight is based on the idea that the NMR peak width is determined by the spin-spin relaxation ($T_2$) of the active nucleus, which in turn depends on the tumbling rate and consequently the size of the molecule.$^{124}$ A larger morphology, like the nanofibers have a slow tumbling rate and hence, a higher $T_2$ resulting in a significant broadening of the NMR peak. When measured in 150 mM NaCl and 2.2 mM CaCl$_2$, the NMR peak at 176 ppm ($^{13}$C=O from PA) and pH 7.85 was found to broaden out when the pH was reduced to 5.71 indicating formation of the bulky fibers, as shown in Figure 5.12a. However, in serum, a considerably broadened peak was observed even at a basic pH of 7.79, as shown in Figure 5.12b.
To elucidate the influence of serum on the self-assembly behavior, pH dependent FA values were collected for the 100 µM PA mixtures in solutions containing varying serum concentrations (0.75-4% serum v/v in 150 mM NaCl, 2.2 mM CaCl₂ solution) (Figure 5.13). The transition onset and endpoints shifted to more basic values upon addition of greater concentrations of serum, until no transition was observed in 4% serum. Similar to the values observed in the salt solution, the FA increased from ~0.065 to ~0.12 as the pH was lowered. At 4% serum, the FA was found to be constant at ~0.125. Also, FA values obtained for the corresponding serum background ranged from 0.003-0.005, indicating that there is no contribution from serum auto-fluorescence.

To confirm that this pH dependent jump in FA values in the serum samples were actually reflecting changes in self-assembly morphology and not just the PA3.1-Rubipy single molecules bound to the 70 kDa serum albumin proteins, a control FA experiment was conducted with a sample containing only 1.5 µM PA3.1-Rubipy isolated molecules condition in 1.5% serum (v/v). The FA values remained constant at ~0.06 over time at both the acidic and basic pH values (Figure 5.14a). A second control (MSA-dye) where the dye was directly conjugated to pure Mouse Serum Albumin (MSA) also showed a constant FA value of 0.04 as a function of pH and time (Figure 5.14b). The self-assembly morphologies were further confirmed via conventional TEM (Figures 5.15a, 5.15b) of the 100 µM PA mix in 1.5% serum at pH 6.85 (FA~0.11) and 9.21 (FA~0.06). At pH 6.85, nanofibers having diameters of ~12.3±1.9 nm were observed. At a pH value of 9.21, a distinctly different globular morphology having diameters of 10.7±1.4 nm was
observed, resembling spherical micelles that are typically only distinguishable in salt solutions at much larger PA concentrations (0.5-1 mM). A control sample containing 1.5% serum only at a pH 5 showed no fibers (Figure 5.15c). This imaging data proves that the higher FA values (~0.11) correspond to the nanofiber morphology.

5.5. Reversibility, stability and kinetics of morphology transition:

The transition was found to be reversible as a function of pH with a small hysteresis of 0.25 pH units (Figure 5.16). It was also found to be rapid, occurring within 3 minutes of pH adjustment as illustrated via the swift change in FA values as a function of time in Figure 5.17a. Stability of the nanostructures in 1.5% serum were tested for 100 μM PA mix via FA over time and shown in Figure 5.17b. At pH values of 8.55 and 6.43, the FA was constant at ~0.06 and ~0.14 respectively over a period of 200 minutes. The values were found to be ~0.05 and ~0.14 after a period of 2 days, indicating that the nanoparticles are significantly stable in solution.

5.6. FA detection limit:

The detection limit of FA measurements was tested with 30 and 10 μM of the PA mix in both dilute (1.5%) and pure serum. The fluorescence signals from these two
solutions in the dilute and pure serum are shown in Figures 5.18a and b respectively. In serum, the fluorescence intensity was observed above the serum auto-fluorescence background for the 30 µM solution only. FA data showed morphology transitions in the pH range 7.0-8.0 for the 30 and 10 uM solutions in 1.5% serum, with an expected basic shift observed for the 30 µM solution (Figure 5.18c). However, the FA was found to be constant around 0.20 for the 30 µM PA in serum indicating that the nanofiber morphology is formed exclusively even at such low concentrations (Figure 5.18d).

5.7. Effect of salts and serum albumin on PA self assembly:

The increased propensity for nanofiber formation in serum could be due to at least two probable factors: a higher ionic strength relative to the our salt solution, and/or the interaction with serum proteins. Serum albumin typically constitutes ~75-80% of all proteins in blood serum, having a concentration of 35-50 g/L.\textsuperscript{112} It is well known that serum albumin disrupts micelle formation via adsorption of isolated surfactant amphiphiles.\textsuperscript{125-126} If this occurred in our sample, we would expect a decrease in the concentration of PAs in solution, and consequently a shift in transition to lower pH values, as observed in our previous study. To determine the extent to which serum albumin impacts the transition shift, PA samples were prepared in 3.9 and 7.8 µM MSA, 150 mM NaCl and 2.2 mM CaCl\textsubscript{2}. These MSA concentrations correspond to the amounts
that would be present in 0.75 and 1.5% serum, respectively. pH dependent FA (Figure 5.19) measurements of these samples showed a transition onset to pH values that were 1.1-1.3 pH units more basic than without MSA, resulting in transitions that occur within 0.5 pH units compared to the 0.75 and 1.5% pure serum samples. This shows that the presence of serum albumin, surprisingly facilitates fiber formation.

It is also well known that ionic strength of the solution can significantly change the self-assembly morphology. We have always characterized our PA molecules in 150 mM NaCl and 2.2 mM CaCl\(_2\) prior to serum. However, there is a significant variability in the concentrations of bound vs unbound Ca\(^{2+}\) ions in blood serum. To determine the influence of salt concentration on the transition, the CD transition points of the PA mixture in 150 mM NaCl solutions containing 3.0 and 4.0 mM CaCl\(_2\) (Figure 5.20) were measured. The CaCl\(_2\) concentration was varied since divalent cations more drastically affect the critical coagulation concentrations of amphiphiles. With 3.0 and 4.0 mM CaCl\(_2\), the transition onset was shifted to more basic pH values by 1-2 pH units. Had our observed shifts in the serum samples been due to the greater Ca\(^{2+}\) concentration, this would imply that pure serum would have to contain ~267 mM Ca\(^{2+}\). This incredibly high value is completely outside the known range of divalent salts in serum. This further suggests that the observed transition shifts are primarily due to interactions with serum albumin, with a minor effect from variability in ionic strength.
5.8. The ‘Macromolecular Crowding Effect’ – Simulation with PEG

The propensity of serum albumin to promote nanofiber formation can be attributed to the macromolecular crowding effect, according to which high concentrations of macromolecules like albumin in tend to preferably stabilize the more compact, self-assembled form of an amphiphilic molecule with significant hydrophobic character. This is primarily a steric effect and is commonly observed in crowded environments such as blood serum. To corroborate the crowding effect on self-assembly morphology transitions of our PA molecule, MSA was replaced by Polyethylene glycol (PEG), a hydrophilic polymer with molecular weight of 20 kDa, to simulate the crowded environment of serum. This was followed by collection of FAL data as a function of pH. A PEG concentration of 26 uM, normalized by the ratio of the molecular weights of MSA (67 kDa) and PEG., was used. As shown in Figure 5.21, the normalized PEG concentration of 26 uM shifted the transition to around where the transition in the presence of 7.8 uM MSA was observed proving that the increased propensity for nanofiber formation is due to the steric crowd of albumin. Also, using 1.8 mM PEG corresponding to MSA concentration in pure serum, nanofibers formed exclusively irrespective of pH (Figure 5.22), a result consistent with our previous findings in 100% serum.
5.9. PA structure re-optimization – transition in pure serum:

Since PA 3.5 was too hydrophobic to survive the crowding effect of albumin, the PA structure was re-modified by substituting the Ile by the weakly β-sheet forming amino acid methionine (PA 5.1, Figure 5.23a). The Rubipy analogue of the PA was synthesized and mixed with PA 5.1 (PA mix 2) for FA studies (PA5.1-Rubipy, Figure 5.23b). The analytical HPLC traces and mass spectra for these two PAs are shown in Figures 5.24 and 5.25 respectively. pH dependent FA measurements in pure serum finally showed morphology transitions in the pH range 7.4-7.1 for different concentrations of PA mix 2, as shown in Figure 5.26. Hence, methionine was found to possess the perfect hydrophobicity to enable this transition in the desired pH range in blood serum.

The transition was found to be rapid and reversible with a slight hysteresis of ~0.03 pH units as shown in Figure 5.27a, indicating that these structures exist under thermodynamic equilibrium. Also, they were found be considerably stable in serum as determined via time dependent FA measurements shown in Figure 5.27b. The FA values remained constant at ~0.24 and ~0.09 at pH values of 6.0 and 8.0 respectively over a period of 600 minutes.

To simulate the crowded environment of pure serum, an aqueous solution containing 1.8 mM PEG along with our usual 150 mM NaCl and 2.2 mM CaCl₂ was prepared. A concentration-pH phase diagram (Figure 5.28) was then constructed for the pure PA 5.1 in this simulated serum solution using transition points obtained via concentration dependent CD (Figure 5.29, CD at two concentrations shown) and pH
dependent CAC (Figure 5.30, CAC at two pH points shown for clarity) measurements. To evaluate the effectiveness of this simulated solution to mimic real serum, pH dependent FA values previously collected for various concentrations of PA mix 2 in pure serum (Figure 5.26) were super-imposed on the phase diagram. The transition points in serum were found to be shifted by ~0.5 unit to basic pH values due to the presence of 1.5% PA5.1-Rubipy which has been previously shown to cause this shift (Figure 5.5). Concentration dependent CD spectra (Figure 5.31, CD at two concentrations shown) were then collected as a function of pH for PA mix 2 and overlaid on the phase diagram to show the proximity of the transitions to those obtained in pure serum (within 0.08 pH unit).
Figure 5.1. PA3.1-Rubipy – Palmitoyl – IAAAEEEK(Rubipy)-NH$_2$. 

Ru(bipy)$^{2+}$ fluorophore

Exc. 450 nm, Em. 630 nm

Fluorescence lifetime ~ 400 ns
Figure 5.2. Analytical HPLC trace of PA3.1-Rubipy.
Figure 5.3. ESI-Mass spectrum of PA3.1-Rubipy (Mol. Wt. 1834 g/mol). The doubly and triply charged peaks are due to Ru$^{2+}$ and H$^+$. 

134
Figure 5.4. a) Fluorescence spectra of PA3.5-Tb in salts and pure serum at different excitation wavelengths b) Fluorescence spectra of PA3.5-Tb in salts with and without pyrene c) Fluorescence spectra of PA3.5-Tb in serum with and without pyrene.
Figure 5.5. Concentration-pH self assembly phase diagram of the PA mix (blue) overlaid on the same for PA 3.5 (fade pink).
Figure 5.6. pH dependent CAC measurements of the PA mix.
Figure 5.7. pH dependent CD spectra of a) 10 µM PA mix b) 50 µM PA mix.
Figure 5.8. pH reversibility of morphology transition in 100 μM PA mix.
**Figure 5.9.** a) pH dependent FA of 100 µM PA mix in salts. The inset shows the fluorescence emission from the PA3.1-Rubipy in the mixture b) pH dependent CD spectra of the same solution. The color for each point in a) and trace in b) both the figures correspond to similar pH values.
Figure 5.10. a) Fluorescence emission from 100 µM PA mix in serum and serum auto-fluorescence background  b) pH dependent fluorescence of 100 µM PA mix in 1.5% (v/v) serum.
Figure 5.11. pH dependent FA of 100 µM PA mix in pure mouse serum.
Figure 5.12. a) $^{13}$C-NMR spectra of 3 mM PA3.5-Lu at pH values of 7.85 and 5.71 in 150 mM NaCl and 2.2 mM CaCl$_2$ b) $^{13}$C-NMR spectra of 3 mM PA3.5-Lu at pH 7.79 in serum and serum background.
**Figure 5.13.** pH dependent FA of 100 µM PA mix in various concentrations of dilute serum. Also shown are control measurements with 1.5% PA3.1-Rubipy only in 1.5% (v/v) serum and MSA-dye conjugate.
Figure 5.14. a) pH and time dependent FA of 1.5% PA3.1-Rubipy only in 1.5% (v/v) serum b) pH and time dependent FA of MSA-dye conjugate.
Figure 5.15. a) TEM image of 100 µM PA mix in 1.5% (v/v) serum at pH 6.85 b) TEM image of 100 µM PA mix in 1.5% (v/v) serum at pH 9.21 c) TEM image of 1.5% (v/v) serum control at pH 5.0.
Figure 5.16. pH reversibility of morphology transition of 100μM PA mix in 1.5% (v/v) serum.
Figure 5.17. a) pH and time dependent FA showing the kinetics of morphology transition in 100 µM PA mix b) Time dependent FA of 100 µM PA mix at pH 8.55 c) Time dependent FA of 100 µM PA mix at pH 6.43.
Figure 5.18. a) Fluorescence emission from 10 and 30 µM PA mix in 1.5% (v/v) serum b) Fluorescence emission from 10 and 30 µM PA mix in pure serum c) pH dependent FA of 10 and 30 µM PA mix in 1.5% (v/v) serum d) pH dependent FA of 30 µM PA mix in pure serum.
Figure 5.19. pH dependent FA of 100 µM PA mix in salts, different serum concentrations and the corresponding mouse serum albumin (MSA) concentrations.
Figure 5.20. CD transition points of 100 μM PA mix in 3.0 and 4.0 mM CaCl$_2$, 150 mM NaCl overlaid on the phase diagram of the same in 2.2 mM CaCl$_2$ and 150 mM NaCl.
Figure 5.21. pH dependent FA of 100 µM PA mix in salts, 1.5% (v/v) serum, 7.8 µM MSA and 26 µM 20 kDa PEG.
Figure 5.22. pH dependent FA of 100 µM PA mix in salts and 1.8 mM 20 kDa PEG.
Figure 5.23. a) PA 5.1 – Palmitoyl-MAAAEEEEK(DO3A:Gd)-NH₂ b) PA5.1-Rubipy - Palmitoyl- MAAEEEEK(Rubipy)-NH₂.
Figure 5.24. Analytical HPLC traces of PAs.
PA 5.1 – Mol. Wt. 1784 g/mol

Figure 5.25. ESI-Mass spectra of PAs.
PA5.1 – Rubipy - Mol. Wt. 1857 g/mol

Continued from Figure 5.25.
Figure 5.26. pH dependent FA of various concentrations of PA mix 2 in pure serum.
Figure 5.27. a) pH reversibility of morphology transition of 100 μM PA mix 2 in pure serum b) pH and time dependent FA of 100 μM PA mix 2 showing stability of the nanoparticles in pure serum.
Figure 5.28. CD transition points of various concentrations of PA mix 2 in pure serum (red) and 1.8 mM 20 kDa PEG, 150 mM NaCl, 2.2 mM CaCl$_2$ (blue) overlaid on the phase diagram of pure PA 5.1 in 1.8 mM 20 kDa PEG, 150 mM NaCl, 2.2 mM CaCl$_2$ (fade orange).
Figure 5.29. pH dependent CD spectra of a) 10 µM PA 5.1 b) 500 µM PA 5.1.
Figure 5.30. pH dependent CAC measurements of PA 5.1.
Figure 5.31. pH dependent CD spectra of a) 20 μM PA mix 2 b) 100 μM PA mix 2.
Chapter 6

Conclusions and Future Work

6.1. Conclusions:

In summary, we have shown that through judicious design, it is possible to use the power of self assembly to create dynamic materials out of PAs that change shape and size in response to minute changes in pH, in solutions containing mono- and divalent cations (150 mM NaCl, 2.2 mM CaCl₂) like those found in blood serum. By balancing the attractive hydrophobic and hydrogen bonding forces and the repulsive electrostatic and steric forces, this transitional pH can be systematically shifted by tenths of pH units. This morphological change was found to be rapid and occurred reversibly under thermodynamic equilibrium, which is ideal for in vivo drug delivery and imaging applications. Also, it is expected that our PA nanoparticles will achieve a higher degree of selective tumor targeting over single molecule drugs, accumulating at higher concentrations (via transforming into fibers and getting trapped at the site) and leading to MRI signal and resolution improvement. In addition, the PA nanostructures increased the relaxivity of water protons greater than that observed with similar concentrations of a
commercially available FDA approved contrast agent Magnevist, providing a secondary mechanism for enhanced tumor detection.

The self-assembly transition pH was also found to be tunable via systematically changing the amino acid sequence of the hydrophobic region of the PA. Moving the Ile further away from the palmitic acid tail significantly increased the PA’s hydrophobic character facilitating the nanofiber morphology even at severely basic pH values, due to formation of a secondary hydrophobic region in the nanostructure. This approach provides an alternate handle to control self assembly morphology.

Finally, we have demonstrated an approach to successfully probe pH-dependent self-assembly transitions of PAs in pure blood serum using fluorescence anisotropy. This method allowed the accurate determination of the formation of distinct peptide nanostructures, in the presence of high concentrations of large molecular weight proteins, without significantly altering the intrinsic self-assembly profile. In contrast to the general notion of amphiphiles binding to serum albumin, the latter was found to promote self assembly and nanofibers formed at all pH values. This observation was attributed to the crowding effect of albumin and was confirmed via replacing it with a 20 kDa hydrophilic polymer, PEG only to exhibit the same effect as albumin. The PA structure was then successfully re-modified to enable this transition in the desired pH range in pure serum. We were also able to create a simulated serum solution containing our usual salts and the appropriate concentration of PEG to mimic the crowded environment of blood for future testing of PAs. Although in vivo tests will ultimately determine the performance of our
PAs in animal models, this technique promises to simplify the pathway from test tube to \textit{in vivo} experimentation.

\textbf{6.2. Future directions:}

The next crucial step that is currently in progress is to modify the PA structure with different analogues of PEG to increase circulation lifetimes of the PA micelles in the blood-stream. Phase diagrams of these PAs will be mapped out in the simulated serum solution to understand their concentration-pH self-assembly behavior and compared with FA measurements in pure serum.

Preliminary cyto-toxicity assays have shown that our PAs are considerably bio-compatible. \textit{In vivo} time dependent blood curves have demonstrated that the PA nanoparticles leave the blood stream and get internalized into organs within 20-24 hours of i.v. injections. Also, PA 3.5 was found to show a distinctly different bio-distribution profile than that of a spherical micelle control PA Palmitoyl-GGAAEEEEK(Do3A:Gd)-NH$_2$, indicating that a different morphology (fibers?) is formed, a result consistent with our \textit{in vitro} FA data. Future \textit{in vivo} work include obtaining extensive bio-distribution profiles and of PA 5.1 along with its PEGylated analogues followed by comparison with micelle-only and fiber-only PA controls, pH measurements of xenografted tumors in nude mice and ultimately time and concentration dependent MRI measurements of i.v. injected PAs in tumor bearing mice.
We strongly believe that this research will enable the development of MRI imaging probes that can target most cancer phenotypes. A diagnostic vehicle that accumulates high concentrations of probes in tumors by transitioning into a larger, more slowly diffusing entity in the acidic microenvironment has not yet been experimentally validated due to the challenge of creating a vehicle that undergoes such a transition in vivo.
References:


(34) Chauhan, V. P.; Stylianopoulos, T.; Martin, J. D.; Popovic, Z.; Chen, O.; Kamoun, W. S.; Bawendi, M. G.; Fukumura, D.; Jain, R. K. Normalization of tumour


Merbach, A. E.; Toth, E. The chemistry of contrast agents in medical magnetic resonance imaging; Wiley.


(87) Dong, H.; Hartgerink, J. D. Short homodimeric and heterodimeric coiled coils. *Biomacromolecules* 2006, 7, 691.


(105) Mehmet, S.; So, C. R.; Tammerler-Behar, C.; Hayamizu, Y.; Hayamizu Y. New polypeptide comprising specific amino acid sequence, used for making a structure such as for making patterned array e.g. nanowires and quantum dots on the inorganic solid surface e.g. graphite and graphene. Univ Washington; DIIDW:2013G68960


(119) Li, M.; Selvin, P. R. Luminescent polyaminocarboxylate chelates of terbium and europium - the effect of chelate structure. *Journal of the American Chemical Society* 1995, 117, 8132.

(120) Richardson, F. S. Terbium(iii) and europium(iii) ions as luminescent probes and stains for biomolecular systems. *Chemical Reviews* 1982, 82, 541.


Appendix: Rights and Permissions:

Chapter 1:

Figure 1.1: Reprinted with permission from Gullotti, E.; Yeo, Y. “Extracellularly Activated Nanocarriers: A New Paradigm of Tumor Targeted Drug Delivery”. Molecular Pharmaceutics 2009, 6, 1041. Copyright 2009 American Chemical Society.

- RightsLink permission provided below.
Figure 1.2: Reprinted with permission from Maeda, H. “Tumor-Selective Delivery of Macromolecular Drugs via the EPR Effect: Background and Future Prospects”. Bioconjugate Chemistry 2010, 21, 797. Copyright 2010 American Chemical Society.

- Rightslink permission provided below.
Figure 1.5. Reprinted with permission from Stubbs, M.; McSheehy, P. M. J.; Griffiths, J. R.; Bashford, C. L. Causes and consequences of tumour acidity and implications for treatment. *Molecular Medicine Today* 2000, 6, 15.

- Rightslink permission provided below.

- Rightslink permission provided below.

- Rightslink permission provided below.

---

Chapters 4-6: No previously published material.