2-24-2015

Design, Synthesis and Applications of Polymer Biomaterials

Frankie Costanza
University of South Florida, frankie@mail.usf.edu

Follow this and additional works at: https://scholarcommons.usf.edu/etd
Part of the Polymer Chemistry Commons

Scholar Commons Citation

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Design, Synthesis and Applications of Polymer Biomaterials

by

Frankie Costanza

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Chemistry College of Arts and Sciences University of South Florida

Major Professor: Dr. Jianfeng Cai, Ph.D.
Kirpal Bisht, Ph.D.
Edward Turos, Ph.D.
Chuanhai Cao, Ph.D.

Date of Approval:
February 24th, 2015

Keywords: Antimicrobial polymers, drug delivery, amphiphilic, micelle

Copyright © 2015, Frankie Costanza
DEDICATION

To Michael and Victoria Costanza
ACKNOWLEDGMENTS

I would like to express my gratification to my parents Michael and Victoria Costanza who have always been my beacon of light through my hardest of times over the past many years. This Doctorate would not be possible without their love and support for me during this time of my life. I would also like to express my gratification to my mentor, Dr. Jianfeng Cai, for allowing me to work in his laboratory as well as providing me with guidance, support and understanding as I have endured numerous personal issues over the years. Without his compassion, I would have not accomplished this milestone in my life. It is through him that I can see what a truly hard working mentor and researcher really is. He has accepted all members of his group into his home, and into his family, thereby making everyone feel as though they belong here. For these reasons I can say with great respect, that it has been an honor to work and learn in your laboratory. I would also like to thank Dr. Kirpal Bisht, Dr. Edward Turos, and Dr. Chuanhai Cao for their support and insights over the years as well as for the use of their laboratory instruments. Next, I would like to thank the entire Cai group as my support system for the times when research became the most frustrating. Without their professional help, I would not have been able to conduct many experiments as efficiently. In particular I would like to thank Shruti Padhee for the antibacterial assays and Yan Wang for the in vivo mouse studies. Lastly, I would like to thank Zoya Khan for being the foundation of my social support system over the past 3 years. All of the mentioned above people have always stood by me and helped me achieve this
degree with their support and patience, and again, I would like to thank each and every one of you.

F.C.
# TABLE OF CONTENTS

**LIST OF TABLES** ................................................................. iii

**LIST OF FIGURES** ............................................................... iv

**ABSTRACT** .............................................................................. vi

**CHAPTER 1: INTRODUCTION** .................................................. 1
   1.1 Antibacterial Polymers. ...................................................... 1
   1.2 Drug Delivery Polymers. ................................................... 5
   1.3 Outline of Dissertation. ................................................... 6
   1.4 References. ................................................................. 7

**CHAPTER 2: INVESTIGATION OF ANTIMICROBIAL PEG-POLY(AMINO ACID)s** ....... 10
   2.1 Introduction. ............................................................... 10
   2.2 Materials and Methods. ................................................ 12
   2.3 Results and Discussion. ................................................ 15
   2.4 Conclusions. ............................................................. 23
   2.5 References. ............................................................... 23

**CHAPTER 3: PEG-POLY (AMINO ACID)s – ENCAPSULATED TANSHINONE IIA AS POTENTIAL THERAPEUTICS FOR THE TREATMENT OF HEPATOMA** ................. 28
   3.1 Introduction. ............................................................... 28
   3.2 Materials and Methods. ................................................ 31
   3.3 Results and Discussion. ................................................ 36
   3.4 Conclusions. ............................................................. 47
   3.5 References. ............................................................... 48

**CHAPTER 4: PEG-POLY (AMINO ACID)s/MICRORNA COMPLEX NANOPARTICLES EFFECTIVELY ARREST THE GROWTH AND METASTASIS OF HEPATOMA** ............ 52
   4.1 Introduction. ............................................................... 52
   4.2 Materials and Methods. ................................................ 55
   4.3 Results and Discussion. ................................................ 59
   4.4 Conclusions. ............................................................. 68
   4.5 References. ............................................................... 69

**CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS** ......................... 73
APPENDIX A: SUPPORTING INFORMATION FOR INVESTIGATION OF PEG-POLY(AMINO ACID)s.

A1. Synthesis of PEG-poly(amino acid)s. ......................................................... 76
A2. The Morphology of PEG-poly(amino acid)s in water. ................................. 79
A3. Antimicrobial activity. ............................................................................. 80
A4. Hemolytic activity. ................................................................................... 81
A5. Fluorescence microscopy. .......................................................................... 81
A6. SEM of bacteria after treatment with polymer P5. ................................. 82
A7. References. .............................................................................................. 83
LIST OF TABLES

Table 2.1: The antimicrobial and hemolytic activities of PEG-poly(amino acid)s. .............. 17

Table A1: Molecular weights of the polymers. ......................................................... 79
# LIST OF FIGURES

- Figure 1.1: Difference between bacterial and mammalian cell membranes. .................. 3
- Figure 1.2: Graphical representation of our drug delivery system used for miRNA-139. ...... 6
- Figure 2.1: Synthesis of PEG-poly( amino acid)s. .................................................. 16
- Figure 2.2: The nanomorphology of the PEG-poly( amino acid)s. ............................... 17
- Figure 2.3: Fluorescence micrographs of *B. subtilis* treated with polymer. ................. 21
- Figure 2.4: SEM pictures of bacteria treated with polymer. ...................................... 22
- Figure 3.1: The structure of Tanshinone IIA. ............................................................ 30
- Figure 3.2: Synthesis and NMR of the PEG-poly ( amino acid)s. .................................. 38
- Figure 3.3: Nanomorphology of poly ( amino acid)s. ................................................. 39
- Figure 3.4: In vitro cytotoxicity of nanoparticles in MHCC97-H hepatoma cells. ........... 40
- Figure 3.5: Biodistribution of TanshinoneIIA NPs. .................................................... 42
- Figure 3.6: Tumor inhibitory effect of TSIIA nanoparticles in vivo. .............................. 43
- Figure 3.7: (A) Kaplan-Meier overall survival analysis and (B) Life extended rate of the MHCC97-H hepatoma-bearing mice treated with TSIIA nanoparticles. ................. 44
- Figure 3.8: Representative histopathological sections of the tumors from tumor-bearing mice. 45
- Figure 3.9: Image of hepatoma-bearing mice. ......................................................... 47
- Figure 4.1: Synthesis and NMR of PEG-poly( amino acid)s. ...................................... 61
- Figure 4.2: SEM pictures of prepared nanoparticles. .................................................. 62
- Figure 4.3: The tumor growth curves. ................................................................. 63
- Figure 4.4: Survival time of hepatoma-bearing mice in different groups. ....................... 64
- Figure 4.5: Image of orthotopic hepatoma-bearing mice. ........................................... 66
Figure 4.6: The immunohistochemical staining of CD31 antigen..........................67
Figure 4.7: Wound closure healing assay in miR-139 nanoparticles......................68
Figure A1a: Synthesis of N-Carboxyanhydrides...........................................76
Figure A1b: Synthesis of PEG-OTs.................................................................76
Figure A1c: Synthesis of PEG-N₃......................................................................77
Figure A1d: Synthesis of PEG-NH₂.................................................................77
Figure A1e: Synthesis of Polymer P3...............................................................78
Figure A2: SEM image showing the morphology of PEG-poly(amine acid)s...........79
Figure A3: NMR of Polymer P2........................................................................84
Figure A4: NMR of Polymer P3........................................................................85
Figure A5: NMR of Polymer P4........................................................................86
Figure A6: NMR of Polymer P5........................................................................87
Figure A7: NMR of Polymer P6........................................................................88
Figure A8: NMR of Polymer P7........................................................................89
Figure A9: NMR of Polymer P8........................................................................90
Figure A10: NMR of Polymer P9......................................................................91
Figure A11: NMR of Polymer P10.................................................................92
ABSTRACT

The emergence of antibiotic resistant bacteria has prompted the research into novel kinds of antibacterial small molecules and polymers. Nature has solved this issue with the use of cationic antimicrobial peptides, which act as nonspecific antibiotics against invading species. Herein, we have tried to mimic this general mechanism in a biocompatible and biodegradable polymer micelle based on the polymerization of naturally occurring amino acids lysine and phenylalanine linked to a PEG tether. This amphiphilic structure allows for the spontaneous collapse into stable nanoparticles in solution, which contains a hydrophilic outer layer and a hydrophobic core. Our polymers have shown activity against clinically relevant strains including Methicillin Resistant S. epidermidis, B. subtilis, K. pneumoniae, and P. aeruginosa.

To further the application of our biopolymers, we have used them as drug delivery vehicles as well. First, we have used an anionic analogue based on glutamic acid to encapsulate a super hydrophobic drug Tanshinone IIA, and use it against a hepatoma bearing mouse model. Second, we have used a cationic analogue to form a complex with miRNA-139 and use it against a hepatoma bearing mouse model as well. In both cases, our PEG poly(amino acids)s have shown promising efficacy in drastically reducing the tumor size compared to the control only. Taken together, our results show that our nanoparticles have the potential to be versatile biomaterials as antibacterials as well as drug delivery vehicles in vivo.
CHAPTER 1: INTRODUCTION

1.1 Antibacterial Polymers

Clinics today are facing an increasing problem of infections associated with drug resistance to conventional antibiotics\(^1\). One of the most common infections which account for more than half of all reported skin infections, and causes an estimated 20,000 deaths annually is from methicillin-resistant *Staphylococcus aureus* and a similar strain from methicillin-resistant *Staphylococcus epidermidis*\(^2\). This problem becomes even more pronounced during surgery because bacteria can easily colonize the surface of living and non-living substrate including the raw patient’s tissue, as well as surgical devices, synthetic implants, orthopedics, and catheters, etc\(^3\). However, this problem is not limited to the clinic and also applies to drinking water, food packaging and agriculture\(^4-6\). This brings about an inherent need to develop new antibacterials that are broad spectrum by design, but more importantly, are intrinsically difficult for the bacteria to develop resistance.

In designing a new broad spectrum anti-bacterial material, there is one general structural feature that has been employed by many different researchers to regain activity against gram positive and gram negative bacteria. Generally speaking, this involves having a proper balance of cationic charge, which draws the material close to the negatively charged bacterial membrane, and hydrophobic residues, which interact with the phospholipid bilayer\(^3,7,8\). This theology is based on the natural defense mechanisms of many living organisms which synthesize cationic antimicrobial peptides (CAPs) when under attack. While each peptide is different and unique to
the particular organism, they all generally consist of about 12-50 residues, in which approximately 50% of them are hydrophobic. While the mechanism of these CAPs is still under some debate, it is generally believed that it does not interact with any receptor on the cell surface. Instead, CAPs (as well as broad spectrum antimicrobial polymers [AMPs]) target the natural differences between bacterial and mammalian cells. When a bacterial cell is approached by an AMP or CAP, hydrophobic interactions prevail and may sink the AMP or CAP into the bacterial membrane, and thus places a torsional strain on the membrane bound phospholipids, which can further lead to membrane disruption and subsequent cell lysis.

Eukaryotic mammalian cells (including human erythrocytes) contain up to 25% cholesterol in their lipid bilayers and are predominantly composed of zwitterionic (overall neutral) phospholipids such as phosphatidylcholine, phosphatidyethanolamine, and sphingomyelin. Furthermore, most of the lipids in mammalian cells that do have a net negative charge are actually located on the inner leaflet of the cell instead of the outer leaflet (Figure 1.1). Bacteria on the other hand, have a structurally different outer membrane. First, they lack cholesterol in their membranes, which is thought to act as a stabilization factor for mammalian cells. This alone can make bacterial membranes easier to disrupt compared to mammalian ones. Second, the outer leaflet of the bacterial cell are generally composed of negatively charged phospholipids such as phosphatidylglycine, cardiolipin and phosphatidylserine. More specifically in gram positive cells, some negative charge is thought to arise from lipoteichoic acids (which links the outer thick peptidoglycan layer to the inner cell membrane) and its associated negatively charged phosphate groups linked via phosphodiester bonds. In contrast, gram-negative cells have only a thin peptidoglycan layer, but contain a second outer cell membrane on top of this with the outer leaflet being composed mostly of lipopolysaccharides
that are highly anionic. The outer membrane of gram negative cells do not contain teichoic acids, but they do contain phosphate groups in a similar environment as the phosphates in teichoic acids. Discounting surface layer antigens, it can be said that the outer membranes of gram positive and gram negative bacteria carry an overall net negative charge, which is greater than that of mammalian cells which are relatively neutral. Therefore, a more electrostatically favorable interaction exists between CAPs and AMPs with bacteria as compared to mammalian cells.

Figure 1.1: Difference between bacterial and mammalian cell membranes.
In applying this theology to designing a functional biomaterial, we have developed amphiphilic nanoparticles based on naturally occurring amino acids linked to a mono methyl poly ethylene glycol (mPEG) tether. PEG is an FDA approved water soluble polymer for in vivo use due to its excellent biocompatibility and enzyme degradability\textsuperscript{13}. The use of PEG here is highly advantageous in that it is highly hydrophilic and helps the ensuing polymer collapse into a micelle as it has a hydrophobic core. Equally as important, PEG has non-adhesive antifouling properties\textsuperscript{14}, which prevent protein and cell adhesion to the nanoparticle. This prevents the aggregation of debris on the outside of the particle which would hinder its activity due to the particle being blocked from interacting with bacterial membranes. This most accepted reason for this property is due to a layer of tightly bound water molecules that surrounds the PEG surface, which effectively prevents protein adhesion\textsuperscript{15}.

To ensure the polymer is fully biocompatible, the bulk of the material is made from poly amino acids. Briefly, positively charged, negatively charged, and hydrophobic natural L-amino acids (lysine, glutamic acid, and phenylalanine respectively) were cyclized via triphosgene into N-carboxyanhydride (NCA) monomers and purified by recrystallization. Then, mPEG-NH\textsubscript{2} was used as the initiator for the polymerization and various equivalents of the amino acid monomers were added to develop a library of polymers. To lessen the hydrophobicity of the core, we have used a hybrid core that is composed of a random set of charged residues to hydrophobic residues. In theory, by having the charged segments (which have favorable electrostatic interactions to approach the bacterial membrane) next to the hydrophobic moieties, this allows the hydrophobic phenylalanine rings to move closer to the bacterial membrane and increase the membrane depolarization leading to cell lysis. Taken together, our compounds show broad spectrum activity
against both gram positive and gram negative strains and form nanoparticles in the sub 200nm range.

1.2 Drug Delivery Polymers

Due to our polymers exhibiting excellent water solubility as well has having a defined secondary nanoparticle structure with a hydrophobic core; we anticipated that they would also make suitable drug delivery vehicles for hydrophobic drugs. As a proof of concept, we have used a super hydrophobic drug, Tanshinone IIA, to be delivered by our PEG poly amino(acid)s in a live mouse model. Tanshinone IIA is a small molecule isolated from *salvia miltiorrhiza* and exhibits broad spectrum anti-tumor activity16-18. While its direct mechanism of action is unknown, it is believed to have multiple intra- and extra- cellular targets19. For a molecule such as Tanshinone which is highly insoluble in water and thus, unable to be administered in vivo, a drug delivery vehicle must be used. We have used a glutamic acid analogue of our polymers for this purpose. Its negative charge prevents it from aggregation with many human serum proteins, many of which are also negatively charged, and our hybrid core is hydrophobic enough to ensure that Tanshinone IIA is encapsulated and able to be administered in vivo. We have used a hybrid core instead of a purely hydrophobic core because it is hydrophobic enough to drive the collapse of the micelle and encapsulate the drug, but also contains less electrostatic interactions with the drug which will aid in its release to occur more rapidly. Our results show that compared to Tanshinone IIA alone, our encapsulated Tanshinone IIA has reduced the hepatoma tumor volume by approximately 70%, which allows it to be a suitable carrier for this drug.

To further the clinical application of our polymers, we have also used a lysine analogue of our nanoparticle to encapsulate and deliver a small interfering RNA, known as miRNA-139, to be administered in a live mouse model as well. MicroRNAs are small non-coding RNA
molecules that regulate post-transcriptional gene expression. Since they can bind to any specific mRNA sequence, they have potential for the regulation of virtually any gene, and have already been implicated in many biological functions such as cell proliferation, metastasis, differentiation and apoptosis\textsuperscript{20,21}. For our delivery system of miRNA-139, we have used a lysine analogue of our polymers due to its cationic nature, which has electrostatically favorable interactions with the anionic charged miRNA. Again, we have used a hybrid core so as to limit hydrophobic interactions, as well as facilitate the release of the miRNA due to the less tightly packed hydrophobic core. Compared to miRNA-139 alone, our encapsulated miRNA-139 has shown the ability to stunt the growth of a hepatoma tumor by approximately 75\% in a live mouse model (Figure 1.2). Taken together, these results show that our PEG poly amino(acid)s show great versatility in delivering novel therapeutic agents, as well as inhibiting the proliferation of a broad spectrum of bacterial strains.

Figure 1.2. Graphical representation of our drug delivery system used for miRNA-139 in vivo.
1.3 Outline of the dissertation

In this dissertation, we discuss the design, synthesis, and applications of mPEG poly amino (acid)s as well as testing of the compound in a live mouse model.

In chapter 2, we outline the synthetic design behind our positively charged polymer library for use as broad spectrum antibacterial agents against both Gram positive and Gram negative strains. The in vitro testing is also described.

In chapter 3, we describe the drug delivery application of a negatively charged polymer for the delivery of a super hydrophobic drug, Tanshinone IIA. Its in vivo application is discussed and data for its tumor suppression in a live mouse model is presented.

In chapter 4, we describe the drug delivery application of a positively charged polymer for the delivery of miRNA-139. The rational of the design, techniques used, and data for the in vivo tumor suppression of our delivery system is presented.

In chapter 5, we summarize the major findings of this dissertation and then conclude with the future directions of this research.

1.4 References


CHAPTER 2: INVESTIGATION OF ANTIMICROBIAL PEG-POLY(AMINO ACID)S

Note to reader

This chapter has been previously published and has been reproduced by permission of The Royal Society of Chemistry. Original citation:


2.1 Introduction

The extensive use of antibiotics in recent decades for medical and agricultural purposes has elicited the widely known issue of bacterial resistance. As such, the development of new antimicrobial agents with novel mechanisms has been a constant area of research. This includes the need for new antibacterial materials to prevent contamination in food packaging, storage, medical supplies, and drinking water, in addition to drug discovery. One approach to circumvent resistance could be the development of antimicrobial peptides (AMPs). Cationic AMPs exist in most organisms and are the first line of natural defense against infections. Although they possess different secondary structures or sometimes even random structures in solution, they are believed to adopt an amphipathic conformation when interacting with negatively charged bacterial membranes, and subsequently disrupt the function of bacterial membranes. As this biophysical interaction lacks defined bacterial targets, this mechanism of action is not expected to develop resistance as easily as traditional antibiotics. Based on such
recognition, many classes of AMP mimetics have been developed. These small peptidomimetics are capable of mimicking the function and mechanism of AMPs, and are shown to have potent and broad spectrum antimicrobial activity. However, their step-by-step preparation is tedious, and they are generally not economical to produce in substantial quantities, limiting the potential of widespread use as affordable biomaterials in the future.

Recently, there have been some advances in polymer antimicrobial agents such as polymers based on methacrylates, norborenes, amidoamines, and others. These polymers contain both hydrophobic components and cationic components, which are critical for the disruption of bacterial membranes. Many of them have also been shown to kill both Gram-positive and Gram-negative bacteria, including clinically relevant pathogens. In addition, they are generally prepared through a one-pot polymerization, allowing the generation of products in large amounts. As such, there is promising potential for these synthetic materials to be used as antimicrobial agents to treat bacterial infections. However, the development of biodegradable antimicrobial nanoparticles is very rare. Poly(amo-no acid)s, synthesized by ring-opening polymerization, show attractive features for further exploration. They have been widely used for drug delivery and other biomedical applications as they are completely biodegradable. However, their antimicrobial development is rare. In this study, we designed, synthesized and studied the antimicrobial activity of biodegradable polymer nanoparticles - PEGylated amphiphilic poly (amino acids). The PEG is introduced to the polymers because PEG (polyethylene glycol) is essentially non-toxic and biocompatible. Meanwhile, PEGylation promotes the formation of a corona which comprises the outer shell of the nanoparticles. Additionally, PEG is widely available, inexpensive, highly water soluble and easily modifiable by size and end group functionality. It has also been used in the development of peptide
nanoparticles with a hydrophilic corona which would enhance the stability of the micelle and increase circulation time.\textsuperscript{31-33} Another attractive aspect of using PEG in a block type antimicrobial nanoparticle is that it has non-adhesive antifouling properties which can prevent protein and cell adhesion.\textsuperscript{29} This is a key feature of the material as any cellular debris that can potentially adhere to the outer surface would likely inhibit its activity. Another report shows that PEG does not reduce activity to bacteria, while also may potentially decrease toxicity to human cells.\textsuperscript{19} As such, we also expected that introduction of a PEG segment to poly(amino acid)s will promote the formation of nanoparticles, which may facilitate bacterial membrane disruption, and therefore possess improved antimicrobial activity.

\textbf{2.2 Materials and Methods}

(All polymers were prepared by a similar method). 0.4 g of PEG-NH\textsubscript{2} was dissolved in 10 ml of anhydrous dioxane and purged with N\textsubscript{2}. Separately 0.49 g (20 eq) of Z-lysine NCA was dissolved in 5 ml anhydrous dioxane, passed through a 2 micron filter to remove any insoluble decomposed NCA, thoroughly purged with N\textsubscript{2} and then added via syringe. The reaction was then allowed to proceed for 3 days under an active N\textsubscript{2} atmosphere. Then 10 equivalents of Z-lysine NCA and 15 equivalents of phenylalanine NCA were dissolved in 5 ml anhydrous dioxane, passed through a 2 micron filter, purged, and added via syringe. Polymerization was allowed to continue for another 3 days. The clear solution was then precipitated into ether and the product was collected via filtration. The polymer was then dissolved in 10 ml of TFA and to this solution, 10 equiv. of HBr in AcOH (33\% v/v conc.) was added and stirred for 4 hours. The product was then precipitated into ether and collected via filtration. Finally, the compounds were
dissolved in DMSO (dimethyl sulfoxide) and purified via dialysis (MWCO 3,500) for 3 days. The water was changed daily and the final product was achieved after freeze drying.

The bacterial strains used for testing the efficacy of polymers were collected from the American Type Culture Collection (ATCC) and were multi-drug resistant *S. epidermidis* (RP62A), *B. subtilis* (BR151), *K. pneumoniae* (ATCC 13383) and multi-drug resistant *P. aeruginosa* (ATCC 27853). The antimicrobial activities of the polymers developed were determined in sterile 96-well plates by the 2-fold serial-dilution method. Bacterial cells were grown overnight at 37 °C in 5 mL medium after which a bacterial suspension of approximately $10^6$ CFU/mL in Luria broth or trypticase soy was prepared ensuring that the bacterial cells were in the mid-logarithmic phase. Aliquots of 50 µL bacterial suspension were added to 50 µL of medium containing different concentrations of polymers for a total volume of 100µL in each well. The 96-well plates were incubated at 37 °C for about 20 h. The Biotek microplate reader was used to measure the optical density (OD) at a wavelength of 600 nm after about 20 h. The experiments were carried out as three independent biological replicates, each in duplicate. The lowest concentration at which complete inhibition of bacterial growth is observed is defined as the minimum inhibitory concentration (MIC).

Freshly drawn human red blood cells (hRBC’s) were used for the assay. The blood sample was washed with PBS buffer several times and centrifuged at 700g for 10 min until a clear supernatant was observed. The hRBC’s were re-suspended in 1X PBS to get a 5% v/v suspension which was used to perform the assay. 50 µL of different polymers solutions were added to sterile 96-well plates. Then 50 µL of 5% v/v hRBC solution was added to make up a total volume of 100 µL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1 X PBS and 0.2% Triton-X-100 respectively. The 96 well plate was incubated at
37 °C for 1h and centrifuged at 3500 rpm for 10 min. The supernatant (30 µL) was then diluted with 100 µL of 1XPBS and hemoglobin was detected by measuring the optical density at 360nm by Biotek microtiter plate reader (Type: Synergy HT).

\[
\text{% hemolysis} = \left( \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}}}{\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}}} \right) \times 100
\]

A double staining method with DAPI (4’,6-diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (propidium iodide, Sigma) as fluorophores was used to visualize and differentiate the viable from the dead B. subtilis cells. DAPI being a double stranded DNA binding dye, stains all bacterial cells irrespective of their viability. Ethidium derivatives such as propidium iodide (PI) is capable of passing through only damaged cell membranes and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. The bacterial cells were first stained with PI and then with DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride). The bacterial cells were grown until they reached mid-logarithmic phase and then ~2x10^3 cells were incubated with 100 µg/mL polymer P5 for 4 h. Then the cells were pelleted by centrifugation at 3000 g for 15 min in an Eppendorf microcentrifuge. The supernatant was decanted and the cells were washed with 1X PBS several times and then incubated with PI (5 µg/mL) in the dark for 15 min at 0 °C. The excessive PI was removed by washing the cells several times with 1X PBS several times. Lastly, the cells were incubated with DAPI (10 µg/mL in water) for 15 min in the dark at 0°C. Finally, the excessive DAPI solution was removed by washing it with 1X PBS. The controls were performed following the exact same procedure for bacteria without P5. The bacteria were then examined by using the Zeiss Axio Imager Z1 optical microscope with an oil-immersion objective (100X). 7,8
MRSE and *B. subtilis* were grown to an exponential phase and approximately about 2 x 10^6 cells were incubated with the polymer for about 20 h. The cells were then harvested by centrifugation (3000 g) for 15 min. After pelleting the cells, they were washed three times with DI water. The cells were then fixed with 2.5% (w/v) glutaraldehyde in nanopure water for about 30 min, followed by extensive wash with DI water to get rid of any excess glutaraldehyde. Graded ethanol series (30%, 50%, 70%, 95% and 100%, 5 min each) were then used to dehydrate the cells. Following the dehydration of cells, hexamethyldisilazane was added for about 2 minutes. Then, about 2 μL of sample was added to a silicon wafer followed by Au/Pd coating, and the samples were observed at 25KV with a HITACHI S-800 scanning electron microscope.

**2.3 Results and Discussions**

To test our hypothesis, we synthesized a series of PEGylated poly(amino acid)s containing both hydrophobic groups and cationic groups (Figure 2.1) via ring-opening polymerization of N-carboxyanhydrides (NCAs). NCAs can easily be synthesized and attained in large quantities from natural amino acids at low cost, which makes them attractive in designing new materials. The synthetic procedure was adapted from a previously published protocol. Briefly, Phe-NCA and Z-Lys-NCA (Figure 2.1) were prepared through the treatment of amino acids phenylalanine or Z-Lysine with triphosgene, respectively. Next, CH3O-PEG-NH2 was used as the initiator to react with NCAs to generate poly(amino acid)s via the ring-opening polymerization. In the synthesis of two polymers (*P2* and *P3*), to ensure the formation of cationic shell of nanoparticles, Z-Lysine-NCA was first added into the flask containing the initiator and reacted for three days at the room temperature. Subsequently, Z-Lysine-NCA and Phe-NCA were then added in one batch to form a hybrid segment, which forms the core structure of
nanoparticles in solution. Such a random core structure was expected to facilitate the interaction with the bacterial membranes through both charge and hydrophobic forces. The random core was also designed to limit the packing density of the phenylalanine rings, which allows for a more overall amphipathic material. As such, a series of polymers containing varying numbers of hydrophobic and hydrophilic groups were prepared (Table 2.1). To test if the charge is critical to the polymers’ antimicrobial activity, one negatively charged polymer was synthesized using both Glu-NCA and Phe-NCA.

The structures of these polymers was characterized by the $^1$H NMR (Appendix A3-A11), and their nanomorphology was evaluated by scanning electron microscopy. As expected, most of these poly(amino acid)s form nanoparticles in water with sizes ranging from 50 to 200 nm (Figure A2). The zoomed-in pictures (Figure 2.2b and 2.2c) clearly show that these nanoparticles

Figure 2.1 Synthesis of PEG-poly(amino acid)s.

The structures of these polymers was characterized by the $^1$H NMR (Appendix A3-A11), and their nanomorphology was evaluated by scanning electron microscopy. As expected, most of these poly(amino acid)s form nanoparticles in water with sizes ranging from 50 to 200 nm (Figure A2). The zoomed-in pictures (Figure 2.2b and 2.2c) clearly show that these nanoparticles
contain a hydrophobic core and a cationic shell including a PEG corona. As expected, polymer P9 and P10 do not form nanoparticles due to the lack of the hydrophobic components. These polymers were tested against a range of Gram-positive and Gram-negative bacteria. Their antimicrobial activity is shown in Table 2.1.

**Figure 2.2** The nanomorphology of the PEG-poly(amino acid)s P5. a-c, SEM pictures. a is a zoomed-out view, while both b and c are the zoomed-in views. d is the schematic illustration of PEG-poly(amino acid)s core-shell structure.
Table 2.1. The antimicrobial and hemolytic activities of PEG-poly(amino acid)s. The microbial organisms used are *K. pneumoniae* (ATCC 13383), and *P. aeruginosa* (ATCC 27853), methicillin-resistant *S. epidermidis* (RP62A), *B. subtilis* (BR151). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits growth after 24 h. HC_{50} is the concentration causing 50% hemolysis. The antimicrobial peptide Magainin II was included as a control.

<table>
<thead>
<tr>
<th>#</th>
<th>MR SE (µM)</th>
<th>B. Subtilis (µM)</th>
<th>K. Pneumoniae (µM)</th>
<th>P. Aeruginosa (µM)</th>
<th>mPE G (mw)</th>
<th>Lysine Content b [c Phenylalanine Content d]</th>
<th>% hydrophobic content</th>
<th>hemolytic activity HC_{50} (µM)</th>
<th>MW (Theoretical)</th>
<th>Average size of nano particles (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>5000</td>
<td>Glu 20 (10 15)</td>
<td>33</td>
<td>-</td>
<td>10880</td>
<td>70-80</td>
</tr>
<tr>
<td>P2</td>
<td>55</td>
<td>2.8-5.5</td>
<td>27.5-55.3</td>
<td>&gt;100</td>
<td>5000</td>
<td>10 (10 10)</td>
<td>33</td>
<td>9</td>
<td>9030</td>
<td>80-90</td>
</tr>
<tr>
<td>P3</td>
<td>9.2-22.6</td>
<td>4.5-9</td>
<td>45.3</td>
<td>&gt;100</td>
<td>5000</td>
<td>20 (10 15)</td>
<td>33</td>
<td>25</td>
<td>11045</td>
<td>40-50</td>
</tr>
<tr>
<td>P4</td>
<td>6.5-13</td>
<td>1.6-3.2</td>
<td>65</td>
<td>&gt;100</td>
<td>5000</td>
<td>(10 10)</td>
<td>50</td>
<td>10</td>
<td>7750</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>200-250</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>5.3-10.5</td>
<td>5.3-10.5</td>
<td>26.3-52.6</td>
<td>21-52.6</td>
<td>2000</td>
<td>(10 10)</td>
<td>50</td>
<td>6</td>
<td>4750</td>
<td>80</td>
</tr>
<tr>
<td>P6</td>
<td>9.1-18.2</td>
<td>45.5-91.6</td>
<td>45.5-91.6</td>
<td>45.5-91.6</td>
<td>2000</td>
<td>(10 15)</td>
<td>60</td>
<td>5</td>
<td>5485</td>
<td>80-100</td>
</tr>
<tr>
<td>P7</td>
<td>6.7-13.3</td>
<td>3.4-6.7</td>
<td>&gt;100</td>
<td>33.3-83.6</td>
<td>2000</td>
<td>(20 20)</td>
<td>50</td>
<td>3</td>
<td>7500</td>
<td>80-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>200-250</td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>1.4-11.4</td>
<td>5.7-83.6</td>
<td>&gt;100</td>
<td>28-256</td>
<td>2000</td>
<td>(30 20)</td>
<td>40</td>
<td>3</td>
<td>8780</td>
<td>100-110</td>
</tr>
<tr>
<td>P9</td>
<td>5.5-11</td>
<td>5.5-20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>&gt;50</td>
<td>4560</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>6.5-12.8</td>
<td>6.5-12.8</td>
<td>63.6-128</td>
<td>&gt;100</td>
<td>2000</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>&gt;50</td>
<td>3920</td>
</tr>
</tbody>
</table>

Magainin II: 30 16 40 40 2478
The negatively charged PEG-poly(amino acid)s P1, which contains glutamate residues, did not show any antimicrobial activity under the tested conditions. This is consistent with the hypothesis that negatively charged bacterial membranes interact weakly with molecules bearing negative charge due to electrostatic repulsion. The outer membrane of bacteria has a net positive charge and thus the lack of activity of P1 is expected. The impact of PEGylation on the hemolytic activity can be illustrated by P4, which contains a longer PEG corona than P5, but has less hemolytic activity. It suggests that PEGylation does decrease the hemolytic activity of polymers compared to the non-PEGylated analogues, although the impact is not significant. However, a long PEG corona may prevent the interaction of the polymer with the bacterial membrane and thus less reactive against bacterial membranes. This is evidenced by the fact that P4 is not as broad-spectrum active as P5. It must be noted that hemolytic activity is more tightly related to the hydrophobic content of the polymers. Polymers P9 and P10 do not contain any hydrophobic content, and they do not show discernible hemolytic activity even up to 50 µM. Also, they are not active against Gram-negative bacterial membranes, indicating that the existence of hydrophobic segments, and thus the formation of nanoparticles are important for the disruption of membranes of Gram-negative bacteria. However, they are highly selective against Gram-positive bacteria, including clinically-related multi-drug resistant S. epidermidis, suggesting their potential application as novel antimicrobial biomaterials. This is consistent with other research and is likely due to the inability of the material to penetrate the sturdy outer membrane of Gram negative bacteria. Increasing hydrophobic contents lead to the most potent antimicrobial polymers P5 and P6, which are active against all the tested bacteria. In fact, both P5 and P6 have comparable antimicrobial activity to the antimicrobial peptide magainin II against the tested strains. Our results also suggest that the selectivity between antimicrobial
activity and hemolytic activity can be tuned. P2 and P3 have similar hydrophobic content and antimicrobial activity; however, P3 is less hemolytic than P2. It is possible that the cationic shell (outer layer) of P3 is thicker than P2, which prevents the hydrophobic contents from non-specifically interacting with the membrane due to increased hindrance. These effects are not evident in the other synthesized polymers, in which the hydrophobic Phe-NCA and cationic Lys-NCA were added simultaneously to produce hybridized core structures, and as such, their selectivity is low. Therefore, we speculate the addition of a cationic shell (b portion), such as a poly-lysine segment, into polymer P5 could allow for a more selective polymer to be obtained with higher antimicrobial activity but less hemolytic activity.

We also examined the impact of particle size and molecular weight of poly(amino acid)s on the antimicrobial activity. Although molecular weights of poly(amino acid)s do not have an obvious impact on the antimicrobial activity and hemolytic activity, it seems that the size of nanoparticles does affect these activities in some way. The smaller sized particles tend to have lower toxicity to blood cells. For instance, P3 has the smallest particle size and it has the least hemolytic activity. A similar trend is observed for other polymers, except for P5. We hypothesize that the larger the particle size, the less specific interaction it will have with red blood cells, thereby allowing the compound to be more hemolytic.

The ability of the polymers to damage bacterial membranes was first evaluated by fluorescence microscopy using a double staining approach. In this assay, B. subtilis was co-stained by DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide). DAPI can pass through the intact bacteria membranes irrelevant of bacterial viability; however, PI can only stain bacteria by intercalating nucleic acids in their nucleus after the membranes have been disrupted. Figure 2.3 shows B. subtilis with intact cell membranes do not show fluorescence
after the incubation with PI. However, after 2 h treatment of *B. subtilis* with the most potent polymer P5, a strong red fluorescence was observed inside the bacterium, indicating PI has permeated into the cell’s nucleus. This demonstrates that the membranes of *B. subtilis* have been disrupted.

**Figure 2.3.** Fluorescence micrographs of *B. subtilis* treated with 100 µg/ml polymer P5 for 2 h. a1, control, no treatment, DAPI stained; a2, control, no treatment, PI stained; a3, control, no treatment, the merged view. b1, P5 treatment, DAPI stained; b2, P5 treatment, PI stained; b3, P5 treatment, the merged view.

The ability of the polymer P5 to compromise the integrity of bacterial membranes was further assessed by SEM (Figure 2.4). After treatment of B. subtilis with P5, the membranes of *B. subtilis* are completely disrupted (Figure 2.4b). The same disruption is also observed after the
treatment of MRSE with P5. Figure 2.4d shows MRSE after incubation with P5 in which the membranes of MRSE were damaged, leading to the aggregation of debris due to loss of membrane zeta potential. 7-9 It is important to note that these polymers do not have any defined targets and are interacting non-specifically, which would allow for some MRSE to survive once they become shielded from nearby debris, as shown in figure 2.4d. Figure 2.4e clearly shows that one core-shell nanoparticle from P5 is approaching the surface of a MRSE cell, while the other nanoparticle is already merging into the membrane of the bacteria. These results support that the polymer P5 kills bacteria via membrane disruption. Meanwhile, it is also possible that multiple polymer nanoparticles interact with the membrane of the same bacteria cell simultaneously to cause bacterial cell lysis.

![Figure 2.4 SEM pictures. a, B. subtilis, no treatment; b, B. subtilis, treatment with 100 µg/mL polymer P5. c, MRSE, no treatment; d, MRSE, treatment with 100 µg/mL polymer P5, a zoomed-out review; e, MRSE, treatment with 100 µg/mL polymer P5, a zoomed-in view.](image-url)
2.4 Conclusions

In summary, we have successfully synthesized a series of PEGylated poly (amino acids) via ring-opening polymerization of NCAs. The reaction is run in one pot, and large quantities can be produced easily. Our results show that these biodegradable nanoparticles have broad-spectrum activity against both Gram-positive and Gram-negative bacteria. PEGylation can not only promote the formation of nanoparticles, but also potentially decrease hemolytic activity to a certain extent, even though both hemolytic activity and broad-spectrum activity are primarily determined by the hydrophobic content. The selectivity of the polymers can be tuned by varying both the ratio and numbers of hydrophobic and cationic groups. Both fluorescence microscopy and SEM suggest that these PEGylated poly(amino acid)s kill bacteria by disrupting the integrity of their membranes. Surprisingly, the polymers containing only PEG and lysine segments, show remarkable selectivity against Gram-positive bacteria, and do not display any hemolytic activity under our experimental conditions. It may suggest that these polymers can be not only used to develop antimicrobial biomaterials to prevent contamination in food packaging, storage and medical supplies, but also used as potential therapeutics to treat gram positive bacterial infections. Furthermore, our core-shell PEG-poly(amino acid)s nanoparticles are also capable of encapsulating drug molecules, making it possible to load small molecular antibiotics and to kill bacteria more effectively and synergistically via membrane disruption as well as targeted inhibition of bacterial functions. Such dual-functional antimicrobial nanoparticles are currently being developed in our lab.
2.5 References


CHAPTER 3: PEG-POLY (AMINO ACID)s – ENCAPSULATED TANSHINONE IIA AS POTENTIAL THERAPEUTICS FOR THE TREATMENT OF HEPATOMA

Note to reader

This chapter has been previously published and has been reproduced by permission of The Royal Society of Chemistry. Original citation:


3.1 Introduction

There have been many developments of drug delivery systems using amino acid polymeric scaffolds including poly(sodium N-acryloyl-L-valinate-co-alkylacrylamide)\(^1\) as carriers of nonsteroidal anti inflammatories, poly (L-lysine)-block-poly(L-phenylalanine)\(^2\), poly-L-amino acid (homopolymer)\(^3\) as carriers for DNA delivery or doxorubicin as a model encapsulated drug, and poly(L-lactide-\(\beta\)-\(\gamma\)-benzyl glutamate)\(^4\), among many others. However, there are limitations to some of these such as inadequate water solubility and lack of a defined amphiphilic water soluble structure in aqueous medium. This reduces circulation time as the material is rapidly cleared by the kidneys during circulation. For those without a proper peptide backbone, the materials are not biodegradable, which is very undesirable\(^1\).

There have been other kinds of drug delivery systems unrelated to peptides. One intriguing example involves the emulsification of oleic acid as a surfactant in chloroform with
polystyrene allyl alcohol followed by a second emulsification with poly vinyl alcohol. A DNA plasmid (hydrophilic model) or doxorubicin (hydrophobic model) nanocapsule is achieved after evaporation of the organic solvent\(^5\). While this approach is certainly unique, its polymer backbone is not ideal for a biological system. Other interesting delivery systems utilize derivatives of chitin (one of the most abundant polysaccharides) to form nanoparticles from its derivative chitosan, and have shown a model encapsulation of doxorubicin as well\(^6,7\). This approach is valid as chitosan is non-toxic, biocompatible and very abundant, but there are issues with water solubility at physiological pH levels.

Another class of drug delivery systems that have been gaining traction over the past decade are those of liposomes, which are essentially made of phospholipid spheres in solution. They have been used for aptamer delivery and labeled with FTIC\(^8\), and have also been used as a delivery of the model drug doxorubicin,\(^9\) among many others\(^10\). However, a major limitation of liposomes is their lack of stability as they are made up of small molecules and therefore, their critical micelle concentration is higher than that of polymer based nanoparticles. Upon dilution in a physiological system, they have a greater capacity to fall apart and the controlled release of the drug is lost. One way of circumventing this issue is by attaching on PEG to prolong their circulation time and in vivo stability upon delivering doxorubicin\(^11\).

While many groups have reported a novel kind of drug delivery system based on various approaches already mentioned, often times only a model drug is used for encapsulation and for proof of principle. Here, we have used our delivery system to encapsulate Tanshinone IIA and test it in a live mouse model. Tanshinone IIA (Figure 3.1) is a lipo-soluble small molecule isolated from *salvia miltiorrhiza*, and displays broad-spectrum anti-tumor activity.\(^12-17\) It is believed to have multiple intra- and extra-cellular targets\(^18\) and therefore is less probable to
develop drug-resistance in tumor cells. Some known targets include inhibition of human esterases and carboxylesterases\(^ {19}\), inhibition of hypoxia inducible factor-1\(^ {20}\), dilation of coronary blood vessels via activating potassium channels\(^ {21}\), and induced maspin expression\(^ {22}\) among many more undiscovered targets. However, Tanshinone IIA (TSIIA) has some intrinsic drawbacks such as poor water solubility and a short half-life.\(^ {23}\) This has prompted the ongoing research to develop new delivery systems to increase the solubility of TSIIA and to improve its anti-tumor efficacy.\(^ {17,18,23}\) In order to advance the application and efficacy of TSIIA as a novel anti-cancer therapeutic, the exploration of new polymer delivery systems are needed. As such, herein we report the design and synthesis of amphiphilic PEG-poly(amino acid)s (PPAAs), which were used as a novel nanocarrier to encapsulate and deliver TSIIA. The method of delivery is that of passive diffusion out of the polymer and into circulation as the drug is noncovalently attached. Many other drug delivery systems utilize polymer drug conjugates in which the drug is covalently attached to the nanoparticle. Unless there is a clear method of release, this should be avoided as it does decrease the activity of the drug by making it harder to reach its target\(^ {24}\). However, this also does limit some potential toxic effects the drug may have as well.

![Figure 3.1 The structure of Tanshinone IIA.](image)

30
Compared to other polymer delivery systems, poly (amino acid)s have many attractive features. The synthesis of poly (amino acid)s is very straightforward, which can be achieved through the ring-opening polymerization of N-carboxyanhydrides (NCAs). NCAs can be obtained in one step upon treatment of side chain protected natural amino acids with phosgene or triphosgene followed by crystallization as a primary purification method. Since the subsequent polymerization is living, the monomers can be added batch by batch, which therefore allows for easy synthesis of poly (amino acid)s with multiple blocks. As there are 20 natural amino acids, the potential is limitless to obtain poly (amino acid)s that contain a diverse array of functional groups with tunable charges. Additionally, they are biocompatible and completely biodegradable, and have low toxicities, which further augment their application for drug delivery. Furthermore, poly (amino acid)s can be easily modified with other functional groups. For example, PEG can be attached to the terminus of poly (amino acid)s, which helps to prevent self-aggregation and prolongs its half-life, etc. Therefore, we expect that PEG-poly(amo acid)s will be an excellent carrier for the delivery of TSIIA and will improve its anti-tumor efficacy.

3.2 Materials and Methods

Amino acids were purchased from Purebulk and Chemimpex. Other chemicals were purchased either from Fisher Scientific or Sigma-Aldrich, and used directly without further purification. Tanshinone IIA (98.4\%) was purchased from Xian Guanyu Pharmaceutical Co., Ltd, China. MHCC97-H hepatoma cells, and BALB/c male athymic nude mice from Shanghai Cancer Institute; and CCK-8 Cell Proliferation Assay Kit was purchased from Cayman Chemical. D-Luciferin potassium salt was purchased from Synchem-Pharma Co., Ltd.
BALB/c male athymic nude mice (5 weeks old, body weight 14.8 ± 0.9 g) were purchased from the Department of Experimental Animals of Shanghai Fudan University. All animals were housed at 22°C on a 12-h light/dark cycle. All experiments were performed according to the recommendations of the local animal protection legislation for conducting animal studies approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine (SYXK 2005-0008).

CH₂O-PEG5000-NH₂ (0.4g, 0.08 mmol) was dissolved in 10 ml of anhydrous dioxane and purged with N₂. Separately 20 equiv. (0.42g, 1.6 mmol) of Glu (OBz)-NCA was dissolved in 5 ml anhydrous dioxane, passed through a 2 μM filter to remove any decomposed NCA, thoroughly purged with N₂ and then added via syringe. The reaction was then allowed to proceed for 3 days under an active N₂ atmosphere. Then, 10 equiv. (0.21g, 0.8 mmol) of Glu (OBz)-NCA and 15 equiv. of Phe-NCA (0.229g, 1.2 mmol) were dissolved in 5 ml anhydrous dioxane, passed through a 2 μM filter, purged, and added via syringe. Polymerization was allowed to continue for another 3 days. The clear solution was then precipitated into ether and the product was collected via filtration.

The polymer was dissolved in 10 ml of TFA and to this solution, 10 equiv. of HBr in AcOH (33% v/v conc.) was added and stirred for 4 hours. The product was then precipitated into ether and collected via filtration.¹¹,¹²

The polymer was dissolved in minimal DMSO and added to dialysis tubing (MWCO = 3,500) followed by immersion in water. Dialysis was carried out for 3 days, replacing the water daily. Any precipitate was then filtered out, and the clear filtrates were lyophilized to afford the final products.
5 mg TSIIA and 45 mg PEG-poly (amino acid)s were dissolved in 700 μL DMSO, followed by drop-wise addition of 30 mL 30% tert-butanol/water. The resulting solution was stirred for 3h at room temperature, and then lyophilized to produce a solid puffy cake. The solid was re-dissolved in 30% tert-butanol/water, stirred for 3h and lyophilized. The resulting solid was again dissolved in pure water, stirred for 3h, and the lyophilization provided the desired TSIIA-loading poly (amino acid)s nanoparticles.

The cytotoxicity of nanoparticles against MHCC97-H hepatoma cells was determined using CCK-8 cell viability assay. Briefly, MHCC97-H hepatoma cells (1×10^4/well) were seeded in 96 well and incubated overnight. The cells were then incubated for 24 h and 48h at 37°C/5% CO₂ using different concentrations (5, 10, 20μg/mL) of TSIIA, (50, 100, 200μg/mL) PEG-poly (amino acid)s nanoparticles, and (50, 100, 200μg/mL; containing 5, 10, 20μg/mL TSIIA) PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles. 10 µl cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc, Japan) was added to each well and the plates were incubated for an additional 1h at 37°C/5%. Finally, the cell viability was measured as the absorbance at 450 nm (A450) using a microplate reader (Bio-Rad, Model 680, Hercules, CA, USA). All experiments were performed in triplicate.

The following study consisted of tissue distribution, targeted imaging and therapeutic evaluation was carried out when the tumor grew to approximately 0.1 cm³. Four to six week old Male BALB/c nude mice were subcutaneously injected with 2x10^6 human liver cancer cells MHCC97-H. After 7-10 days when implanted tumors reached 1-1.2 cm in diameter, the mice were sacrificed under sterilized condition, the xenografic tumors were dissected out, minced into 1mm³ pieces, and were then implanted into the right flanks of nude mice. When these tumors reached 1 cm in diameter, they were minced to 1-mm³ chunks to establish the model of the
orthotopic liver cancer. The procedure was first done by making a 1-cm incision along the lower edge of the left ribcage, which exposes the left lobe of liver. Then, a space was generated between the liver and its capsule using forceps, into which tumor chunks were inserted. Hemostatic measures were applied during surgery and no active bleeding was detected before closure.

To evaluate TSIIA biodistribution, the six HCC-bearing mice (three mice per group) were injected with TSIIA and TSIIA NPs at doses equivalent to 1 mg TSIIA per kg in 0.2 ml of PBS via the caudal vein. The heart, liver, spleen, lung, kidney, colon, and tumors were collected 24 h post-injection. Biodistribution was calculated as the percentage of injected dose (ID) per gram of tissue (%ID/g). Values are expressed as mean ± standard deviation (n = 3).

The forty-eight mice were classified into 4 groups on the basis of the solutions they were administered: control group (normal saline, 13.5 mL/kg), blank nanoparticles group (blank NPs, 10mg/kg), TSIIA solution group (TSIIA, 1 mg/kg), and PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles group (TSIIA NPs, 10mg/kg, containing TSIIA 1mg/kg), by injection through the vena caudalis at 1vic/2day. Fourteen days after the injection, six mice in each group were randomly evaluated for survival time. Life extended rate (L) was calculated as follows:

\[ L = \frac{ST_{test} - ST_{control}}{ST_{control}} \times 100\% \]

where \( ST_{control} \) and \( ST_{test} \) are the average survival time (days) for the mice administered with normal saline and with tested agents respectively. The toxicities of the NPs were evaluated by monitoring changes in body weight. The remaining six mice in each group were killed, and the tumors were removed for examination. The longest \((a)\) and the longest \((b)\) vertical dimensions of the tumor were measured. The size \((V)\) of the tumor was calculated using the following equation:
Representative formalin-fixed, paraffin-embedded tissue blocks from each tumor were analyzed by conventional hematoxylin-erosin (HE) staining. The degree of necrosis in each tumor was visually graded as follows: +, no necrosis present or slight necrosis in fragments; ++, mid-range necrosis, absence of nuclei from many cells with or without massive cytoplasmic damage; and ++++, severe necrosis, total loss of cytoplasm of the cancer cells.

The in vivo optical imaging in MHCC97-H hepatoma-bearing mice was evaluated using CRI Maestro TM In vivo Fluorescence Imaging System from Cambridge Research & Instrumentation (Massachusetts, USA). The twenty-four mice were classified into 4 groups: control group (normal saline, 13.5 mL/kg), blank nanoparticles group (blank NPs, 10mg/kg), TSIIA solution group (TSIIA, 1 mg/kg), and PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles group (TSIIA NPs, 10mg/kg, containing TSIIA 1mg/kg), by injection through the vena caudalis at 1vic/2day. Prior to in vivo imaging, the mice were anesthetized with phenobarbital sodium. D-luciferin solution (150 mg/kg) was injected i.p 5 min. before imaging. Exposure times of the bioluminescence imaging ranged from 10 to 30s. Fluorescence images were obtained at an excitation wavelength of 490 nm and emission wavelength of 535 nm. Exposure times ranged from 1 to 2 min. and the fluorescence images were captured using CRI Maestro TM In vivo Fluorescence Imaging System in vivo optical imaging.

3.3 Results and Discussion

To test our hypothesis, we synthesized a PPAA containing both hydrophilic and hydrophobic groups (Figure 2) via ring-opening polymerization of N-carboxyanhydrides (NCAs).
In an aqueous solution, the PEG-poly (amino acid)s are expected to form amphiphilic micelles containing a hydrophobic core and a hydrophilic corona. In our attempt, we prepared PPAAs containing glutamate and phenylalanine residues. In brief, Glu(OBz)-NCA and Phe-NCA were synthesized by reacting amino acids phenylalanine or H-Glu(OBz)-OH with triphosgene (Figure 3.2a). Then, CH$_3$O-PEG5000-NH$_2$ was used to initiate the ring-opening polymerization of the NCAs (Figure 3.2b) to produce the desired poly (amino acid)s. The core-shell nanostructures were achieved by first adding 20 equiv. Glu(OBz)-NCA and allowing the reaction to proceed for three days at room temperature. Subsequently, 10 equiv. Glu(OBz)-NCA and 15 equiv. Phe-NCA were added simultaneously in order to form an anionic-hydrophobic hybrid core in solution. This hybrid core design is different from other previously reported PEG-poly(amino acid)s di-block copolymers, in which the core contains only exclusively hydrophobic groups such as Phe. We anticipated the hybrid core to be advantageous as it is hydrophobic enough to encapsulate TSIIA, yet hydrophilic enough (due to the presence of anionic charges) to aid in its water solubility. This type of core can minimize the probability of aggregation, enhance the targeted delivery of TSIIA, and aid in its release as it is more weakly bounded to the core compared to previously reported literature. The sequence of the poly (amino acid)s (poly dispersity Index: 1.05 by gel permeation chromatography) was also confirmed by NMR (Figure 3.2c).

The PPAAs were then used to encapsulate TSIIA by adopting a previously reported protocol. Briefly, TSIIA and PEG-poly (amino acid)s were dissolved in DMSO, followed by drop-wise addition of 30% tert-butanol/water and shaken overnight. The solution was lyophilized and re-dissolved in 30% tert-butanol/water and again shaken overnight. This solution was again freeze dried and re-dissolved in water to be shaken overnight. Lastly, the solution was lyophilized to produce the final TSIIA-loaded nanoparticles.
Figure 3.2. a, synthesis of Phe-NCA and H-Glu(OBz)-NCA; b, synthesis of the PEG-poly (amino acid)s. c, $^1$H-NMR (800 MHz, DMSO-d$_6$) of the PEG-poly (amino acid)s. Amino acid residues shown in the bracket were added in one batch.

The nanomorphology of these NPs was confirmed by SEM. As expected, irrelevant of encapsulation of TSIIA, poly (amino acid)s all form nanoparticles in water with sizes ranging from 50 to 100 nm (Figure 3.3). Interestingly, poly (amino acid)s containing TSIIA show a smaller size
distribution, probably due to the strong hydrophobic interactions between TSIIA and Phe residues in the polymer, which leads to a tighter core in the nanoparticles.

**Figure 3.3** Nanomorphology of poly (amino acid)s. a, SEM image of poly (amino acid)s; b, SEM image of TSIIA-loading poly (amino acid)s. c, Size distribution of poly (amino acid)s obtained by dynamic light scattering.

Cytotoxicity results showed that PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles effectively increase the cytotoxicity for MHCC97-H hepatoma cells as compared with TSIIA alone (Figure 3.4). At 24 h post-incubation, the cell viability of MHCC97-H hepatoma cells was higher than that of 48 h post-incubation. The MHCC97-H hepatoma cells treated with increasing doses of PEG-poly(amino acid)s nanoparticles showed 94% to 98%
viability, respectively. This finding suggests that the blank PEG-poly(amino acid)s nanoparticles were non-toxic at each of the tested concentrations. Specifically, the cytotoxicity of PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles was significantly improved over time, revealing that the PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles can enhance the cytotoxicity of TSIIA for MHCC97-H hepatoma cells. This higher cytotoxicity is very beneficial for antitumor therapy in vivo.

Figure 3.4. In vitro cytotoxicity of nanoparticles in MHCC97-H hepatoma cells. (A) 24 h-treatment, and (B) 48 h-treatment. *p<0.05 when compared with TSIIA; **p<0.01 when compared with TSIIA.

Biodistribution of free TSIIA and PEG-poly (amino acid)s-encapsulated TSIIA nanoparticles in HCC-bearing mice in various tissues and organs is shown in Figure 3.5. After 24 h of injection, greater concentrations of free TSIIA were found in the heart, lung and kidney than TSIIA NPs. In tissues, TSIIA concentration 24h post-TSIIA NPs injection followed the order of tumor > liver > kidney > lung > spleen > colon > heart, whereas tumor accumulation of free
TSIIA was markedly lower. For TSIIA NPs, around 16.3% ID/g (injected dose / gram) was found in tumor at 24 h post-injection whereas for free TSIIA found was around 1.2%ID/g. This suggests that the TSIIA NPs does show mild selectivity for the tumor over other organ systems, but the TSIIA itself is being widely distributed. Overall, there were significant differences in the uptake of free TSIIA and TSIIA NPs by different tissues and organs. Therefore, with TSIIA loaded NPs, a greater amount of the drug reached the tumor site than would have if injected without a delivery system.

**Figure 3.5.** Biodistribution of TanshinoneIIA NPs. Data are shown as the percentage of injected dose per gram of organ weight (%ID/g). **P < 0.01 compared to the other groups. Data is presented as mean ± SD (n = 3).**
To assess the potential of poly (amino acid)s with encapsulated TSIIA as a novel anti-cancer agent, its in vivo therapeutic efficacy using a hepatoma-bearing mouse model was first evaluated, as TSIIA was previously reported to have significant activity against hepatoma.\textsuperscript{17,34-36} First, the ability of encapsulated TSIIA to prevent the growth of hepatic tumor was evaluated. After administration of the polymer nanoparticles, the tumor volume was measured at predetermined intervals so as to calculate the inhibition rate. As illustrated in Figure 3.6A, compared to the control group in which only saline was administered, poly (amino acid)s alone (denoted as blank NPs hereafter) did not stop tumor growth. Consistent with previous findings, TSIIA is an effective anti-tumor agent, which suppressed the growth of the hepatic tumor by 40%. However, as seen after 4 weeks of treatment, encapsulated TSIIA (denoted as TSIIA NPs) was a superior therapeutic agent, as it successfully arrested the growth of the hepatic tumor by 75%. Compared to TSIIA alone, the anti-tumor efficacy of TSIIA NPs inhibited another 50% tumor growth. The results demonstrate that the delivery of TSIIA NPs by PPAAs is an effective approach to treat hepatoma, suggesting the potential of further development of PPAAs for anti-cancer drug delivery.

With regard to toxic side effects, the body weight of the animals showed no significant difference between the control and treated groups (Figure 3.6B), but the difference of tumor size is conspicuous, suggesting that the PPAAs effectively enhanced antitumor efficiency with minimal toxicity. However, the slight decrease in body weight in the TSIIA only group shows that without the drug delivery platform, the drug itself is likely quite toxic.
Figure 3.6 Tumor inhibitory effect of TSIIA nanoparticles in vivo. (A) The tumor growth curves (mm$^3$), (B) body weights (g). Data is represented as the mean ± standard deviation (n = 6).

The effect on the survival time of mice with hepatoma was also determined. As shown in Figure 3.7A, all the mice eventually died of hepatoma. Among the four test groups, TSIIA NPs exhibited the most promising results to improve the overall survival rate, with the longest survival time of 37.50±1.87 days ($P<0.01$), compared with the saline control group that had the shortest survival time of 24.83±2.56 days. Interestingly, TSIIA alone did not substantially improve the survival time, which may be due to the insolubility and the short half-life. Blank NPs as well only showed a very marginal effect on the mouse survival. The results indicate that among the four groups, TSIIA NPs show the most potent anticancer activity, which further augments its potential as an anti-cancer nanomedicine (Figure 3.7B). Meanwhile, the results further support that PPAAs are excellent nanocarriers for therapeutic development. The TSIIA NPs have improved survival time by more than double compared to the TSIIA alone proving that a drug delivery vehicle is required for proper delivery and activity.
Figure 3.7. (A) Kaplan-Meier overall survival analysis and (B) Life extended rate of the MHCC97-H hepatoma-bearing mice treated with TSIIA nanoparticles. **p < 0.01 vs the other groups. Data is represented as the mean ± standard deviation (n = 6)

In addition, the degree of tumor putrescence in each group was also examined by electron microscope with conventional HE staining as illustrated in Figure 3.8. The necrosis area in the TSIIA NPs group was significantly greater than that of the other groups, which further demonstrated the powerful capability of preventing the growth of the tumor. The TanshinoneIIA group also displayed a certain extent of necrosis, which might be due to its high concentration in solution and its intrinsic anti-tumor efficacy and cytotoxic effects.
Figure 3.8. Representative histopathological sections of the tumors from tumor-bearing mice (HE Stained, magnification ×200). (A) minimal necrosis after treatment with normal saline; (B) minimal necrosis after treatment with blank nanoparticles; (C) mid-range necrosis after treatment with free Tanshinone IIA; (D) severe necrosis after treatment with TSIIA nanoparticles.

Subcutaneous xenografting has been a widely used model to evaluate the tumor growth in vivo. In our current report, we established an orthotopic murine liver cancer model by directly implanting human hepatic cancer cell line MHCC97-H-Luc into the mouse liver. This model has shown the ability to not only retain the morphology and biological characteristics of primary human liver cancer, but also to mimic its microenvironment with the cancer cells capable of metastasizing. To continuously observe and record in vivo tumor growth, we used a luciferase labeled human hepatic cancer cell line MHCC97-H-Luc for this model. Twenty days after implantation, when orthotopic tumors grew to an average size of 1 mm³, tumor-bearing animals were randomly allocated into 4 different treatment/injection groups: saline control, blank NPs, TSIIA, TSIIA NPs. Tumor size was determined on day 3, 6, 18, 24 and post-treatment. As shown
in Figure 3.9, on day 3, no significant difference of tumor size was observed among any experimental group. On day 14, we found significantly smaller tumors in the mice treated with TSIIA NPs compared to the controls (P<0.01). On day 24, tumors in the control groups grew to considerable size and had detectable metastasis, while tumors in TSIIA treatment group were smaller in spite of having some metastasis. In contrast, tumors were much smaller in the TSIIA-nanoparticle group (P<0.01), and more interestingly, exhibited the smallest metastatic sites. The results suggest that TSIIA NPs also inhibit tumor metastasis, as well as growth.

Figure 3.9. Image of hepatoma-bearing mice. The color bars (from blue to red) represent the change of fluorescence intensity from low to high. Data is represented as the mean ± standard deviation (n = 6)
3.4 Conclusion

In conclusion, we report the design and synthesis of PEG-poly (amino acid)s copolymer nanoparticles. The nanoparticles encapsulated Tanshinone IIA and enhanced its antitumor activity in vivo using a hepatoma-bearing mouse model. The increase in anti-tumor efficacy is not due to the polymer nanoparticles themselves; instead, it comes from Tanshinone IIA after encapsulated delivery. Our results suggest that PEG-poly (amino acid)s encapsulated Tanshinone IIA are a potential novel nanomedicine for the treatment of hepatoma, and meanwhile, PEG-poly (amino acid)s are an excellent class of nanocarriers for further development and delivery of hydrophobic anti-cancer therapeutics.

3.5 References


CHAPTER 4: PEG-POLY (AMINO ACID)S/MICRORNA COMPLEX

NANOPARTICLES EFFECTIVELY ARREST THE GROWTH AND METASTASIS OF HEPATOMA

Note to reader

This chapter has been submitted for publication and is currently in the peer review process.

4.1 Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate post-transcriptional gene expression.\(^1,2\) They function by specifically binding to their mRNA targets, inhibiting protein translation, or destroying target mRNAs.\(^3,4\) MiRNAs have been found to play key roles in various biological functions, including cell proliferation, metastasis, differentiation, and apoptosis.\(^5,6\) The aberrant expression of miRNA is associated with a variety of diseases including many types of cancers.\(^7,8\) Specifically, miRNAs can act as tumor suppressors or oncogenes, which makes them promising targets for anti-cancer therapeutic development.\(^9\) In contrast to small molecules, which generally act by hindering certain receptors, enzymes, ion channels and metabolic pathways, interfering RNA can regulate a specific gene transcript anywhere in the body.\(^10\) This allows enormous specification and diversification when targeting any kind of illness. Utilizing these natural processes of RNA interference, miRNAs show considerable promise as they are quickly becoming a next generation of therapeutics for diseases
such as cancer\textsuperscript{11}. Furthermore, since miRNAs are very small compared to a full size RNA, they can easily be synthesized which allow them to become economically feasible for industrial demands.

Despite the potential, there are significant challenges for the development of miRNA-based therapeutics. One problem is the delivery of miRNAs, as they are not cell permeable and are highly negatively charged.\textsuperscript{12-14} In addition, they also face obstacles such as short circulation time, aggregation with serum proteins, and rapid enzymatic degradation.\textsuperscript{15,16} This elicits a need to develop new kinds of nontoxic and biocompatible delivery systems that can further its clinical application. In theory, a suitable carrier should protect the miRNA from enzymatic degradation, so that it is transported it to the target of interest still intact. This should be followed by internalization and subsequent release inside the cell so that it can intersect the mRNA at the desired location.

As such, nanostructures such as polymeric micelles and liposomes have been widely used as nanocarriers for targeted delivery of miRNAs to specific cells or tissues.\textsuperscript{15-18} These non-viral transport mediums are less likely to provoke an immune response and are much less expensive to produce compared to their viral counterparts. In order for successful delivery, the miRNA must first form a stable complex with, and become shielded by the delivery system so as to prevent it from degradation in the body. Both of these conditions are met by using a PEG tether as a driving force for the collapsing of a stable nanoparticle as well as forming a protective shield before reaching the target. Next, the nanocarrier must be internalized via endocytosis, which is typically accomplished nonspecifically\textsuperscript{19}. Finally, the cargo or miRNA must be released from the nanocarrier before being degraded. Due to the less hydrophobic nature of our hybrid core, we
anticipate that our miRNA should be sufficiently released faster than endosomal enzymes can degrade the miRNA.

To date, the most widely used delivery systems for miRNA are cationic lipid based nanoparticles, many of which also use PEG as a shield and stabilizer\textsuperscript{20,21}. More specifically, liposomes have even shown the ability to directly target the hepatocytes for miRNA delivery\textsuperscript{10}. For this general purpose however, liposomes have many intrinsic drawbacks such as the premature release of the cargo, rapid clearance from the blood and uptake by macrophages\textsuperscript{22}. Other popular approaches are cationic polymeric based particles such as poly(2-(dimethylamino)ethyl methacrylate)\textsuperscript{23} which has been used to form a complex with DNA. Here, PEG was also used as a hydrator as well as to ameliorate the electrostatic interactions so as to allow decomplexation inside the cell more readily. The polymeric nature of the nanoparticle allows for greater stability compared to a lipid based liposome counterpart. However, it is important to note that both liposome and polymeric based nanoparticles are produced from self-assembly in solution, and as so, they are not exceptionally stable compared to a much more robust delivery system such as an inorganic gold nanoparticle\textsuperscript{24}. However, particles such as these are inherently more toxic and less biodegradable and so; researches must fine tune their delivery system to have a well-balanced profile of toxicity, stability, and biodegradability.

Among the different nanocarriers developed for drug delivery, poly (amino acid)s have many unique advantages. Poly (amino acid)s are polypeptides that can be synthesized conveniently using the ring-opening polymerization of N-carboxyanhydrides (NCAs).\textsuperscript{25} Since NCAs can be easily synthesized on a large scale from natural amino acids, poly (amino acid)s can be tuned straightforwardly with diverse functional groups and charges. Due to their intrinsic low toxicity and full biodegradability, they have been widely used for drug delivery.\textsuperscript{26,27,28}
Furthermore, the addition of a PEG segment to polymers has been shown to mitigate the problem of non-specific interactions between cationic nanoparticle/RNA complexes and negatively charged serum proteins due to its antifouling properties\textsuperscript{29}, prevent self-aggregation, and evade the immune system.\textsuperscript{15} However, to the best of our knowledge, the use of PEGylated poly (amino acid)s for the delivery of miRNAs is not yet reported. Herein, we report the development of PEG-poly (amino acid)s as a novel carrier for the delivery of miRNA-139. Low expression of miR-139 has been linked to a few cancers including hepatocellular carcinoma, colorectal cancer, leukemia, glioblastoma, etc.\textsuperscript{30-34} Up regulation of miRNA-139 has been shown to suppress tumor growth, migration and metastasis by targeting the 3’-untranslated region of some oncogenic mRNAs such as Rho-kinase 2,\textsuperscript{32} insulin-like growth factor receptor\textsuperscript{31} and MCl-1 mRNA.\textsuperscript{34} As such, systemic delivery of miRNA-139 is expected to lead to novel anti-cancer therapeutics. In this report, we show that the positively charged poly (amino acid)s with a PEG tail can form defined nanoparticles in aqueous solution. Such nanoparticles can be used to form a complex with the negatively charged miRNA-139. These PEG-poly (amino acid)s/miRNA-139 complex nanoparticles (denoted as miR-139 NPs hereafter for simplification) effectively suppress the growth of a hepatoma tumor in MHCC97-H hepatoma-bearing mice by significantly reducing 85% of the tumor size in four weeks, while subsequently also doubling their lifespan. In addition, these nanoparticles have also been shown to prevent hepatic tumor cell migration and metastasis. Our results suggest that PEG-poly (amino acid)s are promising non-viral gene vectors for the delivery of miRNAs for the treatment of cancers.
4.2 MATERIALS AND METHODS

Amino acids used for the synthesis of poly (amino acid)s were purchased from purebulk inc. The miRNA mimics and miRNA control were purchased from Biomics Biotechnologies (Nantong, China). MHCC97-H hepatoma cells, and BALB/c male athymic nude mice from Shanghai Cancer Institute; and CCK-8 Cell Proliferation Assay Kit was purchased from Cayman Chemical. D-Luciferin potassium salt was purchased from Synchem-Pharma Co., Ltd.

CH$_3$O-PEG2000-NH$_2$ was dissolved in 10 ml of anhydrous dioxane and purged with N$_2$. Separately 20 eq of Z-lys-NCA was dissolved in 5 ml anhydrous dioxane, passed through a 2 µM filter to remove any decomposed NCA, thoroughly purged with N$_2$ and then added via syringe. The reaction was then allowed to proceed for 3 days under an active N$_2$ atmosphere. Then 10 equiv. of Z-Lys-NCA and 15 equiv. of Phe-NCA were dissolved in 5 ml anhydrous dioxane, passed through a 2 µM filter, purged, and added via syringe. Polymerization was allowed to continue for another 3 days. The clear solution was then precipitated into ether and the product was collected via filtration.

The polymer was dissolved in 10 ml of TFA and to this solution, 10 equiv. of HBr in AcOH (33% v/v conc.) was added and stirred for 4 hours. Product was then precipitated into ether and collected via filtration.

The polymer was dissolved in minimal DMSO and added to dialysis tubing (MWCO = 3,500) followed by immersion in water. Dialysis was carried out for 3 days and the water was replaced daily. Any precipitate was then filtered out, and the clear filtrates were lyophilized to afford the final products.
To form the PEG-poly(amino acid)s/miR nanoparticle complex, 1 mg miRNA-139 and 15 mg PEG-poly (amino acid)s were dissolved in 200 μL DMSO, followed by drop-wise addition of 10 mL water. The resulting solution was stirred for 3h at room temperature, and the lyophilization provided the desired product.

The following targeted study consisting of tissue distribution, targeted imaging and therapeutic evaluation was carried out when the tumor grew to approximately 0.1 cm³. Male BALB/c nude mice, 4-6 weeks old, were subcutaneously injected with 2x10⁶ human liver cancer cells MHCC97-H. After 7-10 days, when implanted tumors reached 1-1.2 cm in diameter, the mice were sacrificed under sterilized condition, the xenographic tumors were dissected out, minced into 1mm³ pieces, which were then implanted into the right flanks of nude mice. When these tumors reached 1 cm in diameter, they were minced to 1-mm³ chunks for establishing the orthotopic liver cancer model. The procedure was first done by making a 1-cm incision along the lower edge of left ribcage, to expose the left lobe of liver. Then, a space was generated between the liver and its capsule using forceps, into which tumor chunks were inserted. Hemostatic measures were applied during surgery and no active bleeding was detected before closing abdominal incision. No fasting was applied to the animals.

The mice were classified into 4 groups on the basis of the solutions they were administered: control group(normal saline, 13.5 mL/kg), blank NPs(10mg/kg) group, miR-control NPs(10mg/kg) group, miR-139 NPs (10mg/kg) group, by injection through the vena caudalis at 1vic/2day. 7 days after the injection, six mice in each group were chosen randomly for evaluation of survival time. Survival rate (SR) was calculated as follows:
where \( ST_{control} \) and \( ST_{test} \) are the average survival time (days) for the mice administered with normal saline and with test agent respectively. The toxicities of the NPs were evaluated by monitoring changes in body weight.

The remaining mice in each group were killed, and the tumors were removed for examination. The longest \( (a) \) and the longest \( (b) \) vertical dimensions of the tumor were measured. The size \( (V) \) of the tumor was calculated using the following equation:

\[
V = \frac{ab^2}{2}
\]

The in vivo optical imaging of 4 groups in MHCC97-H hepatoma-bearing mice were evaluated using CRI Maestro TM In vivo Fluorescence Imaging System from Cambridge Research & Instrumentation (Massachusetts, USA).

Prior to in vivo imaging, the mice were anesthetized with Phenobarbital sodium. D-luciferin solution (150 mg/kg) was injected i.p 5 min before imaging. Exposure times of bioluminescence imaging ranged from 10 to 30 s. Fluorescence imaging was obtained with an excitation wavelength of 490 nm and emission wavelength of 535 nm. Exposure times ranged from 1 to 2 min. and the fluorescence images were captured using CRI Maestro TM In vivo Fluorescence Imaging System In vivo optical imaging.

Tissue sections of 5-μm in thickness were cut from formalin fixed and paraffin embedded tissue blocks. After deparaffinization and rehydration, endogenous peroxidases were quenched in 0.3% \( \text{H}_2\text{O}_2 \) diluted in methanol. Tissue sections were then incubated overnight at room temperature (RT) with mouse anti human CD31 antibody(1:400; Dako, Glostrup, Denmark).
Immunodetection was performed incubating the slide with a goat anti-mouse antibody and horseradish peroxidase (HRP)-streptavidin complex (both Dako) for 30 min at RT. Staining was visualized using 0.05% 3,3’-diaminobenzidine containing 0.0038% H2O2. Representative micrographs were taken under an Olympus BX-51TF microscope equipped with a DP23-3-5 camera. The CD31 positive microvessels in the tumor-bearing area were quantified by computerized image analysis. Four representative areas in tumor for CD31 staining were selected and photographed at 200 × magnification.

For cell motility assay, 8 × 105 cells were seeded onto 60-mm dishes and grown for 24 h. A linear wound was made by scraping a pipette tip across the confluent cell monolayer. Cells were rinsed with PBS and grown in 1640 supplemented with 10% FBS for additional 48 h. The cell motility in terms of wound closure was measured by photographing at three random fields at the time of wounding (time 0) and at 12, 24 and 48 h after wounding.

4.3 Results and Discussion

To test our hypothesis, we synthesized a PEGylated poly (amino acid)s containing both cationic and hydrophobic groups (Figure 4.1) via ring-opening polymerization of N-carboxyanhydrides (NCAs). The synthetic procedure was adapted from a previously published protocol. Briefly, Z-Lys-NCA and Phe-NCA were first synthesized through the treatment of amino acids phenylalanine or Z-Lysine with triphosgene, respectively (Figure 4.1a). Next, CH3O-PEG2000-NH2 was used as the initiator to react with both NCAs to produce poly (amino acid)s via the ring-opening polymerization (Figure 4.1b). In order to generate defined core-shell nanoparticles, 20 equiv. Z-Lysine-NCA was first added into the flask containing CH3O-PEG-NH2 and the reaction was allowed to proceed for three days at the room temperature. Subsequently, 10
equiv. Z-Lysine-NCA and 15 equiv. Phe-NCA were then added in one batch to form the cationic-hydrophobic hybrid core of nanoparticles in solution. We hypothesized that such a hybrid core would be superior to a pure hydrophobic core consisting of phenylalanines because the core is still positively charged, which increases the solubility as well as the electrostatic attraction with negatively charged miRNAs, but is still hydrophobic enough to cause the self-assembly into nanostructures. The NMR of the poly (amino acid)s is shown in Figure 1c.
Figure 4.1. **a**, synthesis of Phe-NCA and Z-Lys-NCA; **b**, synthesis of PEG-poly (amino acid)s. **c**, $^1$H-NMR (800 MHz, DMSO-d$_6$) of the PEG-poly (amino acid)s. Amino acid residues shown in the bracket were added in one batch, and the residues were randomly mixed.

The PEG-poly (amino acid)s/miRNA-139 complex nanoparticles (miRNA-139 NPs) were then prepared by adapting a previously reported protocol. $^{35,36}$ Briefly, 1 mg miRNA-139 and 15 mg PEG-poly (amino acid)s were dissolved in DMSO, followed by slow addition of water. The nanoparticles (NPs) were obtained after lyophilization. Meanwhile, miR-67 (miR-control), a miRNA that has a minimum sequence similarity to miRNAs in human and mouse, $^{37}$ was included as a negative control.

The nanomorphology of these NPs was confirmed by SEM. The PEG-poly (amino acid)s and their complexes with miRNAs form nanoparticles with sizes ranging from 50 to 200 nm (Figure 4.2). PEG-poly (amino acid)s nanoparticles themselves tend to aggregate together (Figure 4.2a); however, after forming complexes with miRNAs, the nanoparticles are more defined and
segregated (Figure 4.2b and 4.2c). It is possible that the presence of miRNAs close to surface of nanoparticles lead to electrostatic repulsion among nanoparticles.

**Figure 4.2.** SEM pictures of prepared nanoparticles. a, PEG-poly(amino acid)s (denoted as blank NPs thereafter); b, miR-139 NPs; c, miR-control NPs. Bar = 50 nm.

To evaluate the *in vivo* anti-tumor effect of miR-139 NPs, we tested these particles in a mouse xenograftic model of hepatoma cell line MHCC97-H. After four weeks, in comparison to the control groups (only saline, miR-139, blank NPs, or miR-control NPs), the miR-139 NPs treatment showed the most significant inhibition of the tumor growth, with treated tumors being 1/5 the average size of the ones in the control groups (*P* < 0.05) (Figure 4.3). Our result further indicated that the repressive effect on the tumor growth by miR-139 NPs was very specific, since blank NPs or scramble miR-control NPs did not exhibit any inhibitory effect on tumors. Although as anticipated, miR-139 alone did exhibit the ability to block the tumor growth, and the efficacy is much less than that of miR-139 NPs. These results clearly demonstrate that the novel miR-139 NPs are effective anti-tumor agents. It also indicates that PEG-poly(amino acid)s are very effective nanocarriers for the delivery and the *in vivo* efficacy of miRNAs.
Figure 4.3. The tumor growth curves (mm$^3$). Data is represented as the mean ± standard deviation (n = 6).

The effect on the survival time of mice with hepatoma was also determined (Figure 4.4). Although all the mice eventually died of hepatoma, administration of miR-139 NPs had greatly improved the life-span of mice bearing hepatoma. As seen in Figure 4.4, among the tested agents, the miR-139 NPs group showed that it had the most promising prospect to improve the survival condition, coupled with the longest survival time of 50.33 ± 3.08 days ($P<0.01$), compared with the saline control group with the shortest survival time of 25.50 ± 1.87 days. Neither NPs alone nor miR-control NPs had virtually any effect in the improvement of the survival time. Although as expected, miR-139 displayed certain antitumor efficacy, but it is far inferior to that of miR-139 NPs. Overall, the results demonstrate that the complexation of PEG-poly (amino acid)s with miRNAs greatly enhances the therapeutic efficacy of miRNAs.
Subcutaneous xenograft has been a widely used *in vivo* tumor growth model of cancer cells, but in many ways, has failed to retain and recapitulate characteristics of the primary tumors. In our current report, we established an orthotopic murine liver cancer model by directly implanting human hepatic cancer cell line, MHCC97-H, into a mouse liver. Not only does this model retains the morphology and biological characteristics of a human liver cancer, but it also mimics the microenvironment with which the cancer cells are capable of metastasizing in. To continuously observe and record in vivo tumor growth, we used a luciferase labeled MHCC97-H human hepatic cancer cell line and renamed it “MHCC97-H-Luc”. Twenty days after implantation, when orthotopic liver tumors grew to an average size of 1 mm$^3$, tumor-bearing animals were randomly allocated into 5 different treatment/injection groups similar to Figure 4.4. Tumor size was determined on day 7, 14, 21, and 28 post-treatment. As shown in Figure 4.5, on
day 7, no significant difference of tumor size was observed among mice, treated vs. controls. At day 14 and beyond, significantly smaller tumors were observed in miR-139-NPs treated mice compared to the controls (P<0.01). On day 28, tumors in saline, blank NPs and miR-control NPs group grew to such a considerable size that they nearly took over the whole livers, and showed detectable metastasis. Mice in miR-139 had smaller tumor sizes, however, the difference was marginal compared to other control groups. In contrast, tumors were much smaller in miR-139 NPs treatment group (P<0.01), and more interestingly, no metastatic sites were observed.

**Figure 4.5.** Image of orthotropic hepatoma-bearing mice. The color bars (from blue to red) represent the change of fluorescence intensity from low to high.

Since the loss of miR-139 was closely associated with aggressive pathologic features such as metastasis in primary human hepatoma cases, we hypothesized that miR-139 NPs could
impede the migratory and invasive capabilities of hepatoma cells. As such, we also evaluated the effect of miR-139 NPs on tumor angiogenesis. First, by immunohistochemistry staining, we showed that the expression of CD31, a blood vessel endothelial cell marker, was present in the control xenograft tumors, whereas its expression was weak or negative in the tumor treated with miR-139 NPs (Figure 4.6). Semi-quantitative analysis of the microvessel area revealed that the average MVD (microvessel density) value in miR-139 NPs treated tumor was significantly lower than that of the other control groups. (p< 0.01).

**Figure 4.6.** The immunohistochemical staining of CD31 antigen showed that there were micro-vessels around tumor cells (the brown parts). A, control group (saline, 13.5 mL/kg); B, blank NPs (10mg/kg) group; C, miR-control NPs (10mg/kg) group; D, miR-139 (0.5mg/kg)group; E, miR-139 NPs (10mg/kg)group. Quantitative analysis was performed by counting the number of tumor foci in 10 randomly selected high-power fields under microscope (×200).

Next, we assessed the anti-metastasis effect of miR-139 NPs on MHCC97-H cells in vitro by a wound healing assay. As shown in Figure 4.7, miR-139 NPs remarkably attenuated the migratory ability of MHCC97-H hepatoma cells, as indicated by the decrease in the numbers of
migrated cells, compared to the controls. These results are consistent with the previous findings in that miR-139 NPs played a significant role in hepatocarcinoma cell migration. \[32\]

**Figure 4.7.** Wound closure was delayed in miR-139 nanoparticles transfected cells in 12h, 24h and 36h time points in MHCC97-H cells

### 4.4 Conclusion

In conclusion, we designed and synthesized PEGylated poly (amino acid) nanoparticles, for the purpose of using them as nanocarriers to form a miR-139 NP complex. Although the use of nanoparticles for microRNA delivery is common, the employment of PEG-poly (amino acid)s for such a purpose is rare. We believed that PEG-poly (amino acid)s would be versatile carriers
because they are amendable to further modification on their functional groups. Meanwhile, the presence of a PEG tail is expected to minimize side reactions and to increase the bioavailability of microRNAs. Indeed, miR-139 complex nanoparticles effectively suppress tumor growth in a traditional xenograftic model, as well as in a newly established orthotopic liver cancer model of human hepatoma cell line MHCC97-H, and displays significant inhibitory impacts on cancer cell migration and metastasis. Our results suggest that PEG-poly (amino acid)s are potent non-viral gene vectors for the delivery of therapeutic miRNAs for the treatment of cancers.

4.5 References


CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, we have explored mPEG poly amino (acid)s as broad spectrum antibacterial polymers as well as versatile drug delivery polymers. In this chapter, we want to summarize our results and provide some perspective for future directions.

We have developed polymer micelles based on the ring opening polymerization of N-carboxy anhydrides by mPEG-NH₂. By using natural L-amino acids, our material is fully biodegradable and biocompatible which makes it very suitable to be used in vivo. While here we have only used three representative amino acids based on the cationic side chain of lysine, anionic side chain of glutamic acid, and hydrophobic side chain of phenylalanine, in theory, any natural amino acid can be used as long as the side chain is protected during the polymerization. With 20 natural amino acids, and hundreds more unnatural amino acids being used in research, this leads the way to develop new types of polymers with enormous diversity. Furthermore, instead of using PEG as an initiator, any other polymer containing a nucleophilic primary amine group can be used as an initiator to encourage even more diversity. Together, this allows one to attach a different tether, use different equivalents of monomers to control the polymer size, and use different amino acids which provide the polymer with different kinds of functionalization to be used in post polymerization modification.

Our first application with our polymer micelles utilizes the differences in electrostatics between bacterial membranes and eukaryotic membranes. Due to bacterial membranes having an overall more net negative charge on their outer membrane compared to eukaryotic membranes
which are overall more neutral, this has allowed us to develop cationic polymer micelles that selectively interact, and subsequently disrupt bacterial membranes more readily. The extent of hemolysis against erythrocytes can be tuned by lowering the amount of hydrophobic content in the polymers, but this also lessens its membrane disrupting ability. Also, our polymers allow for the further functionalization to increase the antibacterial activity due to its reactive amine groups that are still present. One could imagine attaching specific antibiotics or membrane targeting ligands to the polymer in an effort to synergistically kill the microbes with greater activity. As per our other two applications, antibacterial drugs could also be encapsulated by our polymers for a more systemic effect.

Next, we have shown the ability of a negatively charged polymer to encapsulate a super hydrophobic drug Tanshinone IIA and be delivered to a live liver cancer bearing mouse. Polymeric micelles such as ours are gaining traction in the field of drug delivery due to their small size, relatively narrow distribution of particles, ability to encapsulate hydrophobic drugs, and their ability to prolong the circulation time of the drug. There were no additional side effects noticed from our delivery method, and we observed a marked decrease in tumor size which exemplifies our delivery method as safe and effective. Furthermore, the nanoparticles formed are all less than 200 nanometers, which is thought of being the maximum size that a particle can be in order to cross the blood brain barrier should one try and use this delivery system to target brain chemistry. While unnecessary in this application, one could imagine loading a different kind of novel therapeutic into our polymer with the end goal of delivering the cargo through the blood brain barrier.

Lastly, we have used our system to show a proof of concept in delivering a negatively charged miRNA with our cationic based polymer. Again, no significant side effects were
observed and the tumor size was drastically reduced, which shows the versatility in our nanoparticles to deliver various kinds of cargo. However, the major limitation of our design is that it lacks targeting efficiency and the cargo is not being delivered directly at the site of interest. This drawback has spawned the latest research in our lab in which we are currently testing our nanoparticles with Tanshinone IIA, but this time with FTIC as a fluorescent labeling polymer, as well as with RGD as a targeting peptide. This is made possible by the many available functional groups that the polymer still possesses. At the time of this writing, the results of our site specific targeting and labeling have not yet arrived, and therefore are not present in this dissertation. However, we do expect that with targeting peptides such as RGD, our efficiency to deliver site specific cargo will increase.

Finally, we would like to conclude by stating that our research has further validated the use of mPEG poly amino (acid)s in being able to exhibit broad spectrum antibacterial activity in addition to acting as a versatile drug delivery nanoparticle. We anticipate that in research to come, this type of methodology will become more site specific as in our targeting approach, and that new types of functionalities will allow this class of materials to achieve its full potential in becoming a new generation of safe and effective polymer biomaterials.
APPENDIX A

Supporting info for: Investigation of antimicrobial PEG-poly(amine acid)s

A1. Synthesis of PEG-poly(amine acid)s

Figure A1a. Synthesis of N-carboxy anhydrides (NCAs):\(^1\)

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{O} & \\
\text{O} & \\
\text{R} & \\
\text{H} \\
\text{N} & \\
\text{O} & \\
\text{O} & \\
\text{O} & \\
\text{R} & \\
\end{align*}
\]

Typically, to a round bottom flask, 5 g of the amino acid was suspended in approximately 130 mL of anhydrous THF and heated to 60°C. Separately, 0.34 equivalents of triphosgene was dissolved in 20 mL of anhydrous THF and added dropwise to the solution under an active \( \text{N}_2 \) atmosphere (final concentration \(~0.15 \text{ M}\)). After the solution turned clear in \(~4\) h, any insoluble precipitates were filtered off. The solution was concentrated under vacuum and crystallized from THF/hexane. Three to four subsequent recrystallizations were performed until pure white compounds were obtained.
A1b. Synthesis of PEG-OTs:

Typically, to a solution of 10 g of mPEG (mw = 2000 or 5000) in 50 mL of DCM, 0.416 ml of TEA was added and stirred for 10 min. Then 3.8 g of p-toluensulfonyl chloride (10 eq.) dissolved in DCM was added and stirred for 20 h at room temperature. The solution was washed with citric acid to neutralize TEA and then dried over NaSO₄. The solvent was removed and product was precipitated into ether.

A1c. Synthesis of PEG-N₃:

5 g of mPEG-OTs was dissolved in 40 ml of DMF and 1.89 g (30 eq.) of NaN₃ was added. The reaction was heated to 90°C for 20 h. Then 200 ml of brine was added and extracted into DCM. The extracts were concentrated and then precipitated into ether.

A1d. Synthesis PEG-NH₂:

Excess water/brine was used to move all salts into the water layer along with DMF. The DCM extracts were dried over NaSO₄, concentrated under reduced pressure and precipitated into ether.
2 g of PEG-N₃ was dissolved in 50 ml of THF (minimal) and 200 mg of LAH (2 eq.) was added at 0 °C. The reaction was allowed to reach room temperature and was stirred overnight. Then 5 eq of water was added from a 4M NaOH solution and stirred until all the LiOH and Al(OH)₃ salts have precipitated out as a white solid. The salts were filtered off, and the solvent was removed to be re-dissolved in DCM. It was then washed with water/brine to remove any excess salts. DCM layers were collected, dried over NaSO₄, and precipitated into ether.
A1e. Synthesis of polymer P3

(The other polymers were prepared by a similar method). 0.4 g of PEG-NH$_2$ was dissolved in 10 ml of anhydrous dioxane and purged with N$_2$. Separately 0.49 g (20 eq) of Z-lysine NCA was dissolved in 5 ml anhydrous dioxane, passed through a 2 micron filter to remove any decomposed NCA, thoroughly purged with N$_2$ and then added via syringe. The reaction was then allowed to proceed for 3 days under an active N$_2$ atmosphere. Then 10 equivalents of Z-Lysine NCA and 15 equivalents of Phenylalanine NCA were dissolved in 5 ml anhydrous dioxane, passed through a 2 micron filter, purged, and added via syringe. Polymerization was allowed to continue for another 3 days. The clear solution was then precipitated into ether and the product was collected via filtration.

A1f. Removal of Z protecting groups:

Polymers were dissolved in 10 ml of TFA and to this solution, 10 equivalents of HBr in AcOH (33% v/v conc.) was added and stirred for 4 hours. Product was then precipitated into ether and collected via filtration.

A1g. Purification

The polymers were dissolved in minimal DMSO and added to dialysis tubing (MWCO = 3,500) followed by immersion in water. Dialysis was carried out for 3 days replacing the water daily. Any precipitate was then filtered out, and the clear filtrates were lyophilized to afford the final products.
Table A1. Molecular Weights of the polymers

<table>
<thead>
<tr>
<th>Polymer #</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW (calculated)</td>
<td>10880</td>
<td>9030</td>
<td>11045</td>
<td>7750</td>
<td>4750</td>
<td>5485</td>
<td>7500</td>
<td>8780</td>
<td>4560</td>
<td>3920</td>
</tr>
</tbody>
</table>

A2. The morphology of PEG-poly(amino acid)s in water.
Figure A2. SEM image showing the morphology of PEG-poly(amino acid)s

A3. Antimicrobial activity

The bacterial strains used for testing the efficacy of polymers were multi-drug resistant *S. epidermidis* (RP62A), *B. subtilis* (BR151), *K. pneumoniae* (ATCC 13383) and multi-drug resistant *P. aeruginosa* (ATCC 27853). The antimicrobial activities of the polymers developed were
determined in sterile 96-well plates by serial dilution method. Bacterial cells were grown overnight at 37 °C in 5 mL medium after which a bacterial suspension of approximately 10^6 CFU/mL in Luria broth or trypticase soy was prepared ensuring that the bacterial cells were in the mid-logarithmic phase. Aliquots of 50 µL bacterial suspension were added to 50 µL of medium containing different concentrations of polymers for a total volume of 100µL in each well. The 96-well plates were incubated at 37 °C for about 20 h. The Biotek microplate reader was used to measure the optical density (OD) at a wavelength of 600 nm after about 20 h. The experiments were carried out as three independent biological replicates, each in duplicate. The lowest concentration at which complete inhibition of bacterial growth is observed is defined as the minimum inhibitory concentration (MIC).

**A4. Hemolytic activity**

Freshly drawn human red blood cells (hRBC’s) were used for the assay. The blood sample was washed with PBS buffer several times and centrifuged at 700g for 10 min until a clear supernatant was observed. The hRBC’s were re-suspended in 1X PBS to get a 5% v/v suspension which was used to perform the assay. 50 µL of different polymers solutions were added to sterile 96-well plates. Then 50 µL of 5% v/v hRBC solution was added to make up a total volume of 100 µL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1 X PBS and 0.2% Triton-X-100 respectively. The 96 well plate was incubated at 37 °C for 1h and centrifuged at 3500 rpm for 10 min. The supernatant (30 µL) was then diluted with 100 µL of 1XPBS and hemoglobin was detected by measuring the optical density at 360nm by Biotek microtiter plate reader (Type: Synergy HT).

\[
\% \text{ hemolysis} = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}})}{(\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}})} \times 100
\]
A5. Fluorescence microscopy

A double staining method with DAPI (4’,6-diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (propidium iodide, Sigma) as fluorophores was used to visualize and differentiate the viable from the dead *B. subtilis* cells. DAPI being a double stranded DNA binding dye, stains all bacterial cells irrespective of their viability. Ethidium derivatives such as propidium iodide (PI) is capable of passing through only damaged cell membranes and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. The bacterial cells were first stained with PI and then with DAPI. The bacterial cells were grown until they reached mid-logarithmic phase and then ~2x10³ cells were incubated with 100 µg/mL polymer P5 for 4 h. Then the cells were pelleted by centrifugation at 3000 g for 15 min in an Eppendorf microcentrifuge. The supernatant was decanted and the cells were washed with 1X PBS several times and then incubated with PI (5 µg/mL) in the dark for 15 min at 0 °C. The excessive PI was removed by washing the cells several times with 1X PBS several times. Lastly, the cells were incubated with DAPI (10 µg/mL in water) for 15 min in the dark at 0°C. Then finally the excessive DAPI solution was removed by washing it with 1X PBS. The controls were performed following the exact same procedure for bacteria without P5. The bacteria were then examined by using the Zeiss Axio Imager Z1 optical microscope with an oil-immersion objective (100X).²,³

A6. SEM of bacteria after treatment with polymer P5.

MRSE and *B. subtilis* were grown to an exponential phase and approximately about 2 x 10⁶ cells were incubated with polymer P5 for about 20 h. The cells were then harvested by centrifugation (3000 g) for 15 min. After pelleting the cells, the cells were washed three times with
DI water. The cells were then fixed with 2.5% (w/v) glutaraldehyde in nanopure water for about 30 min, followed by extensive wash with DI water to get rid of any excess glutaraldehyde. Graded ethanol series (30%, 50%, 70%, 95% and 100%, 5 min each) was then used to dehydrate the cells. Following the dehydration of cells hexamethyldisilazane was added for about 2 min. Then about 2 µL of sample was added to a silicon wafer followed by Au/Pd coating, and then the samples were observed at 25KV with a HITACHI S-800 scanning electron microscope.

A7. References


Figure A3: NMR of Polymer P2

$^{1}$H-NMR (DMSO-$d_6$, 800 MHz)
Figure A4: NMR of Polymer P3

$^1$H-NMR (DMSO-d$_6$, 800 MHz)
Figure A5: NMR of Polymer P4

$^1$H-NMR (DMSO-$d_6$, 800 MHz)
Figure A6: NMR of Polymer P5
Figure A7: NMR of Polymer P7
Figure A8: NMR of Polymer P7
Figure A9: NMR of Polymer P8

$^1$H-NMR (DMSO-$d_6$, 800 MHz)
Figure A10: NMR of Polymer P9
Figure A11: NMR of Polymer P10

$^{1}$H-NMR (DMSO-d$_6$, 800 MHz)