Synthesis of triblock copolymer of polycaprolactone and polylysine

Chen, Zhao

2020

Chen, Z. (2020). Synthesis of triblock copolymer of polycaprolactone and polylysine.
Master's thesis, Nanyang Technological University, Singapore

<https://hdl.handle.net/10356/145589>

<https://doi.org/10.32657/10356/145589>

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**SYNTHESIS OF TRIBLOCK COPOLYMER OF
POLYCAPROLACTONE AND POLYLYSINE**

CHEN ZHAO

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2020

**SYNTHESIS OF TRIBLOCK COPOLYMER OF
POLYCAPROLACTONE AND POLYLYSINE**

CHEN ZHAO

**A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering**

2020

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Acknowledgements

First, I would like to express my heartfelt gratitude towards my main research supervisor, Professor Chan Bee Eng, Mary, for her patient guidance and details-oriented instructions through my whole research journey in NTU as a Master student. Without her careful supervision, this endeavor would not have been possible. Besides, I would also like to express my appreciation to my TAC members, Professor Xu Rong and Associate Professor Chew Sing Yian for their co-supervision.

Second, I am truly thankful to Dr. Yogesh, Dr. Raju, Dr. Wei Guangmin and Dr. Kim Chanjin for their proper mentoring and hands-on experiments teaching in the lab, which allow me to learn and master fundamental experimental skills to synthesize and characterize targeted compounds safely and with accuracy. When I face difficulties during the experiment or data analysis, they are always willing to help me out and teach me wholeheartedly.

In addition, I am extremely thankful to senior PhD students, Li Jianghua, Zhang Kaixi, Si Zhangyong, Hou Zheng, Wu Yang, Wang Liping, Zhong Wenbin and Zhang Penghui. They offer their selfless help and experience sharing which encourage and support me to get over hardship during the research. Also, I really enjoy the discussion with them, which helps me solve the puzzles in the experiments and find the direction towards the next step. Besides, I would like to thank Ruan Lin and Sheethal for helping me do the MTT assay and guiding me to perform experiments carefully in the biosafety cabinet.

I also want to express my thankfulness to SCBE technician staff, Dr. Wang Xiujuan, Dr. Yu Shucong, Zhu Wenting, Gan Chew Mei and Tan Yong Zen for their assistance and management of major facilities, such as NMR, DLS and TEM. They secure the equipment ready to use and function in good condition.

This work and Mr Chen Zhao were supported by Singapore Ministry of Education Tier 3 grant (MOE2013-T3-1-002) and Nanyang Technological University scholarship.

Last but not the least, I would like to thank my parents for supporting me to embark my postgraduate study in NTU and making me be positive and believe in myself to get through the difficult times during the studies.

Abbreviations

ATRP	Atom Transfer Radical Polymerization
AMP	Antimicrobial Peptide
Boc	Butoxycarbonyl
Cbz	Benzyloxycarbonyl
DMF	N,N-Dimethylformamide
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DIAD	Diisopropyl Azodicarboxylate
EPS	Extracellular Polymeric Matrix
GPC	Gel Permeation Chromatography
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
MDR	Multidrug Resistance
MHB	Mueller-Hinton Broth
NCA	N-Carboxyanhydride
NHP	N-Hydroxyphthalimide
OD	Optical Density
PPh ₃	Triphenylphosphine
PCL	Polycaprolactone
ROP	Ring-Opening Polymerization
RAFT	Reversible Addition-Fragmentation Chain Transfer
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic Acid
TEA	Triethylamine

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Summary

Polypeptide copolymers are a promising candidate that plays an important role in biomedical realms, such as drug delivery, gene transfer, biomedical devices, and consumer products. For combating a multidrug resistant bacterium, a peptide-based cationic block copolymer attracts widespread attention mainly because of the ease and low cost of production as well as the diversity in design and functionality. As an ideal biomimetic alternative for antimicrobial peptides (AMPs), polypeptide copolymers are mostly synthesized through the ring-opening polymerization (ROP) of amino-acid N-carboxyanhydride (NCAs), and their properties can be tuned by controlling the block sequence, composition, secondary structure, and overall molecular weight and polydispersity. During this study, we synthesized a series of ABA type triblock copolymers ($pK_nC_{40}K_n$), namely, poly(lysine-*b*-caprolactone-*b*-lysine). The target products were successfully made, as supported by the characterization of 1H -NMR, ^{13}C -NMR and Dimethylformamide Gel Permeation Chromatography (DMF GPC). The formation of quasi-micrometer particles is created by the self-assembly of the amphiphilic $pK_nC_{40}K_n$ via a nanoprecipitation method and varying the length of lysine units. Different morphologies such as micelle, vesicle, rod, and worm-like are observed by TEM. By using DLS analysis and zeta-potential measurements, the hydrodynamic radius of these quasi-micrometer particles ranges from 182.4 nm to 1025 nm, and the surface charge stays highly positive above 40 mV. The bioassay indicates that the $pK_nC_{40}K_n$ has a moderate biocide efficacy against *E. coli* and MRSA and a superb biocompatibility at 62.5 ppm. Intriguingly, after the encapsulation of thymol, the quasi-micrometer particles perform a noticeable synergistic effect against Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) with a 16-times improvement in biocidal efficacy.

CHAPTER 1: Introduction

1.1 Background

Effective antibacterial therapy is increasingly needed for combating the rise in multidrug resistant pathogens, which might result in a severe explosion of infections and pose a threat to the public health system. Since penicillin, as the first antibiotic, was discovered by Alexander Fleming in 1928 and driven by an increasing need of medical applications in the upcoming century, scientists have found thousands of antibiotics, and some of them are still in administration today. Antibiotics have led to the near eradication of many life-threatening infectious diseases [1]. However, their effectiveness and easy access have also resulted in their overuse, which brings concerns about the resurgence of deadly infections. According to the World Health Organization (WHO), persistent infections are estimated to cause 10 million deaths annually if we cannot suppress the current trend by 2050 [2]. This situation calls for instant actions towards discovering novel antibiotics and the corresponding treatment strategies. Although antibacterial research stays an active field, from the industrial perspective, the investment in the development of new antibiotics and treatment strategies is far from enough. The main reason is so called the “auto-obsolence” of antibacterials [1]. Considering the targets of antibacterials are acute infections and diseases, if the antibacterial is effective, the disease will be cured in a short timeframe. Thus, the patients will not continue treatment which results in the short lifecycle of drugs. Hence, the low profitability of antibacterial drugs demotivates large pharmaceutical companies (e.g., GSK, Pfizer, Novartis, etc.) who are more inclined to invest into the development of drugs that cure chronic diseases, such as cancers or hypertension [1]. All in all, the potential infections caused by multidrug resistant pathogens is definitely a great challenge for the 21st century healthcare system. In addition, the expected advances in antibacterial research will most likely come from the academic field and small- or medium-size biotechnology enterprises.

In the academic field, antimicrobial cationic polymers have received universal notice among researchers and play a significant role in developing antibacterial therapies because of the low possibility of inducing serious microbial drug resistance. There are two essential features of antimicrobial cationic polymers: one is the cationic groups, and the other is hydrophobicity. In general, antimicrobial cationic polymers can be attracted onto a negatively charged microbial membrane, and subsequently, the hydrophobic parts may insert into the membrane and cause disruption. For improving the overall performance of antimicrobial cationic polymers, there are mainly two considerations: one is increasing the antimicrobial activity over a wide spectrum of bacteria, and the other is decreasing the hemolytic effect towards mammalian cells.

1.2 Problem Statement and Motivation

First, peptide-based amphiphilic block copolymers are regarded as a promising choice to combat multidrug-resistant microbes, mimicking natural antimicrobial peptides. The key question of improving the overall performance is balancing the killing efficacy and cytotoxicity. One advantage of block copolymers is that their property can be adjusted by controlling the corresponding block length.

Second, due to their amphiphilic nature, amphiphilic block copolymers can self-assemble into different interesting architectures, such as micelles, rods, lamellae or vesicles. Further, these architectures can serve as a platform which can deliver loaded antibacterial agents to improve antibacterial efficacy. Essential oils, as natural plant-derivative antibacterial agents, are attracting increasing attention because of their universal applications in the food industry, cosmetics, pharmaceuticals and the consumer-care industry. The encapsulation of essential oils is an important topic for improving its bioavailability, and it is interesting to investigate how best the performance will be improved by encapsulating essential oils inside the cationic nanoparticles (proved to be quasi-micrometer particles by DLS and TEM).

1.3 Objectives and Scope

In this study, one objective is to achieve the optimal overall performance by tuning the length of the cationic block.

The other objective is to investigate the synergistic effect of cationic antibacterial nanoparticles plus an essential oil, thymol.

1.4 Dissertation Overview

Chapter 1 provides the background of this research project as well as motivation and research objectives.

Chapter 2 reviews the literature about MDR and current strategies to tackle the MDR problem.

Chapter 3 describes the methodologies for conducting the corresponding experiments.

Chapter 4 elaborates upon the obtained results and analyzes the principles behind them.

Chapter 5 summarizes the whole study and presents the general conclusion and suggestions for future work.

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CHAPTER 2: Literature Review

2.1 Introduction to Multidrug Resistance (MDR)

Inappropriate treatments and the lack of effective supervision of antibiotic use is bringing global public health dangerously close to a “postantibiotic” era. According to a recent report published by the Interagency Coordination Group on Antimicrobial Resistance (IACG), the spread of antimicrobial resistance poses a great threat to the agricultural industry, food-supply chains and medical service systems [1]. Without taking valid and timely actions to inhibit the development of MDR, drug-resistant infections will inevitably cause up to 10 million deaths worldwide each year and global economic damage on par with that of the 2008 financial crisis by 2050 [2-4]. The rise in MDR is a result of the adaptation of surviving bacteria under a cytotoxic level of biocides; the bacterial community then acquires resistance by evolving a range of self-protective mechanisms after horizontal gene transfer [5].

Antibiotics act on bacterial essential activities via different modes of action, such as suppressing the construction of the cell wall; disrupting the cell membrane, inhibiting the synthesis of proteins, DNA or RNA; and interfering with folate synthesis (Figure 2.1). However, bacteria reproduce exponentially by binary fission, and the overuse of antibiotics increases the possibility of developing defensive mechanisms through directed evolution. The overuse of antibiotics — especially taking antibiotics even when they are not the appropriate treatment — promotes antibiotic resistance. For example, an antibiotic is a proper treatment for strep throat, which is caused by the bacterium *Streptococcus pyogenes*. However, it is not the right treatment for most sore throats, which are caused by viruses. The specific mutation of genes endows bacteria with the ability to survive. The protective modes include the modification or overexpression of drug targets, the reduction of the intracellular drug concentration by decreased uptake or enhanced efflux, or the production of enzymes that inactivate the

antibiotics [5, 6]. Worse still, the in vivo or in vitro formation of biofilm, a slimy extracellular matrix made of the extracellular polymeric matrix (EPS) inhabited by a consortium of microorganisms, causes the contamination of surfaces of medical devices and resilience to disinfection [7]. Bacteria can be simply and empirically classified into two categories, gram-negative and gram-positive (Figure 2.2). The former has a cell wall with a thinner layer of peptidoglycan in-between the outer membrane and inner membrane. The latter has a cell wall composed of a dense layer of peptidoglycan at the outermost site [8]. Among all the kinds of bacteria, ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) is a group of six notorious bacterial pathogens that resist a broad spectrum of commonly used antibiotics, including the drugs of last resort (e.g., penicillin, vancomycin, methicillin, and carbapenems), and are the leading reason of nosocomial infections all over the world [9].

Figure 2.1 Antibiotic target sites and mechanisms of resistance [6]

Figure 2.2 A schematic diagram of Gram-positive and Gram-negative bacterial cell envelopes [8]

2.2 Current Strategies to Combat MDR

Due to the easy access and loose regulation of antibiotic administration, the abuse of antibiotics has promoted MDR in recent decades, and researchers have found many alternative and innovative approaches to curb the emergence of antibiotic resistance, ranging from new drug discovery to antibacterial polymeric nanosystems.

2.2.1 Discovery of New Small Molecule Antibiotics

The increasing disparity between the clinical need for new antibiotics and the development of new antibiotics calls for interdisciplinary collaboration. Traditionally, drug discovery relies heavily on creating a successive generation of antibiotic classes by the chemical modification of known antibiotic scaffolds (Figure 2.3) that are natural or synthetic [10]. It is true that enriching the pool of lead compounds in drug design by mining underexplored scaffolds is one possibility. Environmental microbes have been a great source of natural antimicrobials. Approximately 16,500 antibiotics have been isolated from environmental microbes as molecular scaffolds, most of which spring from Actinomycetales [11]. In addition to directly searching for natural antibiotics,

antibiotic adjuvants offer an orthogonal strategy that is complimentary to new antibiotic discoveries. These chemicals are not antibiotic-active but directly block resistance or improve the host response to infection [12].

Figure 2.3 Synthetic tailoring is widely used to create successive generations of antibiotic classes [10]

For example, antimicrobial peptides (AMPs), also known as host defense peptides, are a class of natural antibiotics created by multicellular organisms to defend the host against microbial invasion. Many potent peptides are cationic AMPs, although there are some anionic AMPs [13]. Cationic AMPs have been universally studied in the past decade because of their ability to kill a broad spectrum of bacteria. Cationic AMPs consist of a sequence of L-amino acids with a size ranging from 12-50 nm, a net positive charge of +1~10 at neutral pH due to excess lysine or arginine residues and a sequence that includes approximately 30-60% hydrophobic amino acids. Regarding the

secondary structure, AMPs are normally composed of four conformations: α -helix, β -sheet, and cyclic and random coils. Although the precise model of the killing process is not fully understood, it is believed that most cationic AMPs attach to the lipoteichoic acid of Gram-positive bacteria or to the lipopolysaccharide (LPS) of Gram-negative bacteria via electrostatic attraction and hydrophobic interaction, permeabilize the outer layer and subsequently disrupt the cytoplasmic membrane and affect internal targets [14]. Cationic AMPs are good templates for deriving analogs, and therefore, researchers have generated derivatives by building a substitution library of target AMPs in order to confirm which substitutions can improve activity [14].

However, these derivatives and analogs still cannot meet the increasing clinical demand since the progress of discovering and developing them is limited. In the past decades, it is noteworthy that computer-aided investigation has played an increasingly pivotal role in assisting with new antibiotic design and discovery. With the help of recent breakthroughs in computer science, virtual and high-throughput screening and algorithm-driven methods are becoming new tools to accelerate antibiotic discovery. By resorting to the power of computer science, the autonomous generation of antibiotic candidates with expected properties would be a windfall for medical chemists [15-17].

Except for manually searching for the untapped source of natural AMPs and their variants, building algorithm models as predictors based on a known database (e.g., the Collection of Antimicrobial Peptides) would be a faster method to design novel antimicrobials for testing and screening. The in-silico generation of more cost-effective sequences that are broad-spectrum and highly active without the associated cytotoxicity is widely investigated to overcome the shortcomings of synthesizing AMPs, which are costly, liable to proteolytic degradation and toxic to host cells [17]. Additionally, computational simulation (e.g., molecular dynamics) is helpful for the further understanding of the mode of action of AMPs in contact with the bacterial membrane. For instance, the universally accepted interaction model is membrane disruption and

translocation, as depicted in Figure 2.4 [17].

Figure 2.4 Possible mechanism and model of membrane disruption and/or translocation by antimicrobial peptides [17].

Apart from computer science, synthetic biology is another realm where scientists can create new biocidal agents based on natural systems, such as making chimeric chemical entities by shuffling biological assembly modules in secondary metabolic pathways, re-engineering and leveraging phage-derived products to achieve programmable therapeutics [11]. Environmental microbes utilize secondary metabolic pathways to assemble building blocks into small molecule antibiotics with a fine structure. Through these biosynthesis pathways, a variety of small molecules with the desired properties are produced. These biochemical cascades have inherent modularity, which makes them an ideal platform for synthetic biology. By manipulating the related enzymatic complexes, such as polyketide synthases and other tailoring enzymes, the process of biochemical pathways is modulated in order to develop new chemicals as designed.

Further on the gene level, advances in DNA synthesis and assembly technologies have offered the capabilities to tune the biosynthetic pathways by manipulation of genetic expression. Additionally, protein engineering has provided opportunities to modify the proteins, such as polypeptides, based on structure-activity relationships and direct their evolution with peptide sequence control. Therefore, together with genetic engineering, synthetic biology will become a powerful toolbox to produce new molecules through engineering the biosynthetic process.

2.2.2 Antivirulence Therapy

Compared to classical antibiotic therapy, antivirulence therapy has a few advantages [18], considering that the survival of microbes does not rely on virulence factors, and therefore, an antivirulence strategy is less likely to trigger the fast growth of resistance. In addition, this therapy provides an increased number of pharmacological targets (Figure 2.5). Finally, this therapy makes it possible to generate antimicrobial agents that exhibit new antimicrobial mechanisms. To target microbial virulence, natural compounds isolated from plants are the main reservoir for antivirulence therapy. Among all the classes of plant metabolites, phenolic compounds and derivatives (mainly flavonoids) are a major category (Figure 2.6) that cause cytoplasmic membrane damage and/or reduced membrane fluidity and that restrain the synthesis of nucleic acid and the cell wall and inhibit energy metabolism. For virulence factors, the ideal targets would be quorum sensing and biofilm formation, since their inhibition is believed to cause important disruptions to bacterial fitness [18].

Figure 2.5 Bacterial virulence factors as targets for antivirulence agents [18].

Figure 2.6 (A) Number of active compounds classified into chemical classes, and (B) the number of active compounds classified into phenolics and derivatives

2.2.3 Antibacterial Polymeric Nanosystems

The formation of polymeric nanosystems has recently been investigated as a method to develop new and effective antimicrobial agents to inhibit the rise in drug-resistant infections. Motivated by the great progress in controlled polymerization techniques, researchers have produced a variety of nanomaterials with tunable biological properties. The development of antibacterial polymeric nanoparticles yields many advantages over their linear counterparts because their shapes and sizes are tunable in order to attain different nanostructures with distinct properties that can also be applied to targeted therapy (e.g., drug delivery or gene delivery). For example, the presentation of a cluster of functional groups on the surface of nanoparticles enables stronger cell recognition and binding tendencies. Moreover, the construction of different hierarchical nanostructures [19], such as micelles, vesicles or star-like constructs, enables the efficient encapsulation of cargos that wait to be released at targeted sites in an adjusted pharmacokinetic profile. In addition, the creation of inorganic-polymer hybrid nanoparticles offers novel pathways for synergistic therapy and/or diagnosis [20].

The current antibacterial polymeric nanosystems can be generally categorized into 2 classes: those containing microbicidal agents and the intrinsic antibacterial nanosystems. The widely investigated microbicidal agents include traditional antibiotics, antimicrobial peptides [21], essential oils [22], inorganic nanoparticles (e.g., silver, titanium oxide, zinc oxide and Fe_3O_4) and other types of nanomaterials known as ‘nanoantibiotics’ [23]. These ‘nanoantibiotics’ exert their killing efficacy via various mechanisms, as summarized in Figure 2.7. For the intrinsic antibacterial polymer nanosystems, there are biocidal moieties in the structure, such as quaternary ammonium groups, phosphonium groups, polycations or those that generate reactive oxygen species upon light irradiation [24].

Figure 2.7 Various mechanisms of ‘nanoantibiotics’

In antibacterial polymeric nanosystems, amphiphilic block copolymers have attracted substantial attention in academia mainly because of their great potential to make functional nanomaterials and promising applications of drug delivery and release [25, 26]. By varying the composition, length, and architecture (Figure 2.8) of block copolymers, their morphology is tunable after self-assembly in the solution (Figure 2.9). The synthesis of block copolymers can be conducted via the sequential addition of monomers via controlled polymerization, such as by ATRP, RAFT, NCA ROP and many others [26].

Figure 2.8 Representative architectures of block copolymers

Figure 2.9 Various self-assembled structures formed by amphiphilic block copolymers

Of the block copolymers, polypeptide-based copolymers are an important class with an inherent antibacterial efficacy that can also be used as a platform for controlled release. More significantly, the biocompatibility can be easily improved by post modification when compared to that of natural antimicrobial peptides, which are highly cytotoxic. For making synthetic antibacterial polypeptides with a defined structure at a large scale and low cost, the pioneering work of Deming et al. developed the technique for the controlled polymerization of NCA. Today, NCA ROP is the most common technique for synthesizing polypeptide copolymers.

In 2015, Wang et al. [27] successfully synthesized a novel antibacterial peptide-based copolymer, which is biocompatible and biodegradable, and forms vesicles that act as an ‘armed’ drug carrier in aqueous solution (Figures 2.10 and 2.11). The statistical combination of lysine and phenylalanine affords the vesicle coronas that are active against bacteria. The vesicle membrane is made from PCL, which improves the biocompatibility and can be biodegradable after drug release. In addition, the folic acid-conjugated design might enable the vesicles to gain access to the cancer cells to

achieve directed delivery. This copolymer achieved an MIC of 16 ppm against both gram-negative *E. coli* and gram-positive *S. aureus*.

Figure 2.10 The illustration of antibacterial vesicles formed by PCL₁₉-b-poly[F₁₂-stat-K₉-stat-(K-FA)₆]

Figure 2.11 TEM images of PCL₁₉-b-poly[F₁₂-stat-K₉-stat-(K-FA)₆] vesicles stained by phosphotungstic acid

In 2016, the Du group [28] reported the preparation of PLLA₃₁-b-poly(Phe₂₄-stat-Lys₃₆), which forms a micelle that penetrates the bacterial cell membrane, as revealed by TEM studies, and achieves an MIC of 40 ppm against both *E. coli* and *S. aureus*.

Figure 2.12 The illustration of antibacterial micelle formation by PLLA₃₁-b-poly(Phe₂₄-stat-Lys₃₆)

In 2017, the Zhou group [29] developed a series of amphiphilic diblock copolypeptides (K₃₀-b-F₁₅, K₃₀-b-F₃₀, and K₃₀-b-F₄₅) via NCA ROP and achieved MICs of 8 ppm and 2 ppm against *E. coli* and *S. aureus*, respectively. Then, using the same method, these authors made a series of triblock copolymers K₁₀-b-F_n-b-K₁₀ and investigated the relationship between the length of the middle block and the antibacterial efficacy, cytotoxicity, and self-assembly behavior. The poly(K₁₀-b-F₂₀-b-K₁₀) achieved the lowest MIC of 4 ppm [30]. These examples of recent work studying polypeptide-based copolymers, their morphology, and the corresponding MIC values against *E.coli* are collected in Table 2.1.

Table 2.1 A series of polypeptide copolymers reported in the literature. (F: Phenylalanine; K: Lysine; All MICs are measured against *E. coli* ATCC35218)

Copolymers	Morphology	MIC (ppm)
PCL ₁₉ -b-Poly[F ₁₂ -stat-K ₉ -stat-(K-FA) ₆]	Vesicle	16
PCL ₁₉ -b- Poly(F ₁₂ -stat-K ₁₅)	Vesicle	6.85~9.21
Poly(F ₁₂ -stat-K ₁₅)	N/A	32
PCL ₄₇ -b- Poly(F ₃₈ -stat-K ₂₀)	Vesicle	64
PLLA ₃₁ -b-Poly(F ₂₄ -stat-K ₃₆)	Sphere-like micelle	300
Poly(F ₃₀ -b-K ₁₅)	Rod-like micelle	16
Poly(F ₃₀ -b-K ₃₀)	Vesicle	8
Poly(F ₃₀ -b-K ₄₅)	Vesicle	8

Poly(K ₁₀ - <i>b</i> -F ₁₀ - <i>b</i> -K ₁₀)	Vesicle	8
Poly(K ₁₀ - <i>b</i> -F ₂₀ - <i>b</i> -K ₁₀)	Vesicle	4
Poly(K ₁₀ - <i>b</i> -F ₃₀ - <i>b</i> -K ₁₀)	Vesicle	16

2.3 Recent advances in the fabrication of polypeptides via Ring-opening polymerization (ROP) method

Proteins are very important functional biomacromolecules that play essential roles in conducting various cellular and life activities. Protein, as highly complex substances, possesses a variety of features at different structural levels from composition to higher ordered structures (e.g. quaternary structure). Design protein-mimic biomaterials have been the goal of polymer or biochemistry scientists to achieve the desired function. For combating drug-resistant pathogens, antimicrobial peptides (AMPs), also known as the host-defense peptides, have great potential as untapped resources to investigate. AMPs have unparalleled strong points compared to traditional antibiotics: they have broad-spectrum activities [31, 32], the rapid outset of killing, less probability of developing drug resistance [33], and fewer side effects [34,35]. However, AMPs are normally toxic to mammalian cells and the cost of separating them from nature is quite high. Hence, to circumvent the obstacles, researchers started to design AMP-mimic synthetic polymer, such as polypeptides.

Polypeptides are an important class of biomaterials that can be applied to various applications, such as gene/drug delivery system (e.g. self-assemblies of polypeptides), tissue engineering scaffold, wound care, 3D cell culture, and antimicrobial agents [36]. In past decades, synthetic polypeptides, the analogs of the natural protein, are attracting more and more attention from researchers mainly because of their biodegradability, biocompatibility, and versatility of chemical design. The common methods of making polypeptides include solid-phase peptide synthesis (SPPS) [37], microbial synthesis [38,39], and N-carboxyanhydrides (NCA) chemistry [40]. SPPS and microbial

synthesis of polypeptides can achieve monodisperse materials with sequence control. As for microbial synthesis, it can only use natural amino acids as raw materials and as for the SPPS, it can typically make short polypeptides (shorter than 50 residues) with low yields and tedious operation [41-43]. Synthesis of polypeptides via NCA chemistry has been the mainstream due to that it enables making polypeptides at a much lower cost and at large scale(kilograms) and in good yield [44, 45]. Additionally, NCA chemistry opens vast possibilities of producing protein function mimic polypeptides whose side chains are modified either through the functional monomer route or the post-polymerization modification route. Also, D-enantiomer NCAs and unnatural amino acid NCAs can be polymerized [45]. It is worthy to be noticed that the level of control in NCA polymerization is already comparable to that in other synthetic polymerization reactions where complex polymer architectures have been prepared. Although the limitations of NCA polymerization restrain control over molecular weight and curb emergence of the well-defined block copolymer, the recent improvements of ROP of NCA chemistry have been a breakthrough [46]. Now NCA chemistry can already offer the facial, more convenient way, and less stringent environment to produce high molecular weight polypeptide-based block copolymer with monodisperse distribution at large scale. And many excellent articles have reviewed the development of synthetic polypeptides in various aspects [41,43,45,47-57].

In this section, we will concentrate on the recent development of fabrication of polypeptides.

2.3.1 Fabrication of polypeptides

2.3.1.1 Synthesis of α -Amino Acid N-Carboxyanhydrides

Normally, the NCAs are produced by the phosgenation of side-chain protected amino acid. In the polymerization stage, the five-member ring can be opened in conjunction with the release of one molecule of CO₂, and then primary amine can be exposed [58]. The exposure of primary amine manifests as active species to open the ring of the next

NCA monomer. This step is repeatable until all the monomers are consumed and finally polypeptides are produced. The first synthesis of α -amino acid NCAs dates back to 1906. After Hermann Leuchs firstly distillate, he coincidentally discovered the α -amino acid NCAs. After that, preparation of polypeptides became one of the hot topics and α -amino acid NCAs became quite attractive types of building blocks. The “Leuchs method” for preparing α -amino acid NCAs requires high temperature and crucial halogenation step. Alternatively, the second synthetic method that is universally utilized nowadays to make NCAs is the “Fuchs-Farthing method”. The proposed mechanism of NCAs synthesis by this method is displayed in Figure 1 (A). Subsequent efforts to improve the synthesis condition are the use of diphosgene or triphosgene which are liquid or solid and hence make them easier and safer to handle. In the whole synthesis process, the key factor is the production of by-products hydrochloride which can cause ring-cleavage and the forming of undesirable by-products. Hence, the choice of solvent is important. The common solvents used for the synthesis of NCAs are THF, dioxane, toluene, or ethyl acetate. To remove the unwanted HCl, washing the NCA solution with aqueous sodium bicarbonate at 0 °C can be one way to go. Besides, other purification methods are also proposed such as using HCl scavengers like pinene and limonene which can prohibit the formation of by-products [59]. Subsequently, a more recent improvement was reported by Deming [45,60]. They utilized flash column chromatography which was effective to remove impurities especially for those that cannot be recrystallized.

Figure 1. The proposed mechanism of synthesis of NCA and side reactions. Reproduced with the permission from ref.56.

However, NCA still suffers from a tedious purification process, stringent environmental settings, and high expertise of synthetic skills. Recently, N-thiocarboxyanhydrides (NTA) are found to be more moisture-resistant and heat-resistant than NCA and hence open new toolbox to design polypeptides more conveniently with facile synthesis set-up. (Figure 2) Zhang et al. [61] showed that well-defined polypeptides with controlled polymer dispersity and molecular weight can be attained from interfacial ROP of NTA monomers in the open air. Consequently, the polymerization condition is so mild that we don't need to worry too much about moisture and heat. Due to NTA monomer's moisture stability, researchers can conduct ROP in air and open vessels [62, 63]. Besides, polypeptides with pendant hydroxyl groups can be obtained directly without protection because the NTA monomers exhibit wonderful resilience towards hydroxyl group [64]. A detailed review of characteristics of NTA compared to NCA was done by Ling Jun et al [65]. (Table 1).

Figure 2. Synthetic route towards NTAs[61]. Copyright 2017. Reproduced with permission from ref.58.

Table 1. A comparison of NCA and NTA

<p>Amino Acid NCA</p>	<p>NTA (thiol-analogues of NCAs) <u>Gained growing attention recently</u></p>
Advantages	
<ul style="list-style-type: none"> • High reactivity and productivity • Widely used and studied 	<ul style="list-style-type: none"> • More stable against moisture and heat • Less side reactions and easy to store
Disadvantages	
<ul style="list-style-type: none"> • Moisture and heat-sensitive • Anhydrous environment is required during synthesis • Poor shelf life because of the impurities 	<ul style="list-style-type: none"> • kinetically less favorable toward nucleophilic

Nevertheless, NCA has been conventionally synthesized using phosgene and its derivatives. Phosgene is highly toxic and needs to be carefully handled. More recently, the Takeshi group reported their study about the environment-friendly, well-defined synthesis of NCAs based on phosgene-free transformation. Both artificial and naturally occurring amino acids were successfully converted to relevant urethane derivatives by utilizing diphenyl carbonate which is less toxic than phosgene (Figure 3) [66].

Figure 3. Polypeptide synthesis according to the utilization of urethane derivatives of amino acids. Copyright 2019 Society of Chemical Industry

2.3.1.2 Polymerization of α -Amino Acid N-Carboxyanhydrides

The α -amino acid NCA's chemical structure is depicted in Figure 4. There are four reactive sites (two electrophilic sites plus two nucleophilic sites) on NCA's ring.

Therefore, the ring can be opened via several concurrent mechanisms which make it difficult to prepare well-defined polypeptides with controllable chain-length and low dispersity.

Figure 4. Structure and reactive centers in NCA.

There are two main mechanisms accepted for the polymerization of NCA, which are “amine mechanism” (AM) and “activated monomer mechanism” (AMM). The amine mechanism is a nucleophilic ring-opening chain growth process where the polymer could grow linearly with monomer conversion (Scheme 1). The NCA polymerization usually is realized through AMM (Scheme 2) when aprotic bases are utilized. The strong base can deprotonate the NCAs, which become nucleophilic. And under such conditions, the NCA monomer can react with another NCA monomer. Hence, based on the AMM, initiators that polymerize NCAs are usually tertiary amines such as triethylamine.

Scheme 1. Proposed mechanism for NCA polymerization initiated by nucleophilic amines

Scheme 2. Proposed mechanism for NCA polymerization initiated by activated monomers

The major challenge for both the AM and AMM for NCA polymerization is the so-called “carbamate mechanism”. To overcome such weakness, further research employed different methods to promote controllability or even optimization of environmental conditions.

Several pioneering works reported by Deming group[60, 67-69] overcome the negative points of the traditional NCA initiator systems. They successfully substituted the amines which are routinely utilized in the initiation of NCA ROP. For example, Deming et al. [60] synthesize copolypeptides with predictable molecular characteristics and low dispersity by utilizing the bipyNi(COD) as initiator which curbs side reactions of chain-transfer and termination. Later, they reported the use of a cobalt-based initiator that brought about similar outcome. Although this transition-metal complexes strategy had uncommonly merits, one major drawback was the introduction of metal ions which are toxic and needed to be easily eliminated from the systems. The author claimed that using precipitation or dialysis after the polymerization would be enough to eliminate the metal ions.

Schlaad's group reported the second strategy [70]. They argued that forming hydrochloric salts by controlling the protonation of the amine initiator could be one way to reduce secondary reactions. More recently, Lu et al. [71] discovered that polymerization of NCAs mediated by HMDS can contain controlled and living. In their study, they provided a metal-free pathway to make block copolypeptides. Similarly, Zhao et al. [72] reported the living NCA ROP by utilizing aminoalcohols as initiators based on hydrogen-bonding organocatalysis. An array of precisely defined linear polypeptides with different molecular weights and the low dispersity (1.02-1.05) were successfully synthesized.

Hadjichristidis and co-workers [73] supposed that impurities found in the traditional NCA polymerization gave rise to the uncontrollable characteristics. They proposed the strategy that optimizing environmental conditions by using high vacuum technique (HVT) to generate and keep the essential requirements for living polymerization. HVT can ensure that reaction system is free of impurities during the whole synthesis process. This technique was later confirmed by Messman et al. as well [74].

Lower down the reaction temperature can control the polymerization of NCA. Vayaboury et al. [75] investigated the polymerization of N ϵ -TFA-L-lysine NCA in DMF by nonaqueous capillary electrophoresis (NACE). Either at room temperature or higher temperature, the researcher attained such polypeptides and distinctly identified side reactions (Table 2). Nevertheless, there were almost no dead polymer chains when the reaction temperature was under 0 °C.

Table 2. Characterization of poly(N ϵ -TFA-L-lysine) from NACE experiments.

Reproduced with permission from ref. 72.

M/I	Temperature (°C)	Living Polymers (°C) ^a	Dead Polymers (Peaks B and C) (%) ^a
50	50	20	80
50	Room	22	78
50	0	99	1

^a From NACE experiments.

Habraken et al. [59, 76] recently studied the polymerization of various NCAs (Figure 5) at 0 °C. They conducted MALDI-TOF and further confirmed that side reactions vanished at 0 °C. In all investigated cases, all results confirmed that it is a straightforward and practicable method to attain various well-defined homo- or copolypeptides via NCA polymerization at 0 °C.

Figure 5. List of NCAs applied in the polymerization at 0 °C. Reproduced with permission from ref. 56. Copyright 2010, Royal Society of Chemistry

Another attractive method of constructing polypeptides was demonstrated by Heise et al.[56, 77]. They used cyclohexylamine as initiator which was created in-situ by the UV. The author used MALDI-TOF and real-time FTIR analysis to attain evidence for the suggested photoinitiation mechanism and the attachment of the initiator to the polypeptide chain. It was believed that NCA photopolymerization brings about new opportunities to design polypeptides both in solution and on surfaces.

More recently, Liu's group [78] showed that LiHMDS initiates an exceptionally fast NCA polymerization procedure. This process can be done in an open vessel and completed within minutes. Polypeptides with variable chain length and narrow molecular weight distribution (1.08-1.28) were easily obtained in this manner. This method allows for swift polypeptide library synthesis for high-throughput screening.

In 2018, Zhang et al. [79] reported a metal-free, versatile, and eco-friendly imidazolium

hydrogen carbonate (IHC)-mediated fast NCA polymerization. (Figure 6) The use of an air-stable organocatalyst, IHC $[\text{NHC(H)(HCO}_3\text{)}]$, allows for high conversion of monomers within a few minutes under mild conditions. To further expand this methodology, Zhang et al. synthesize topologically nanoengineered antimicrobial polypeptides (TNAMPs) (Figure 7) and compare the effect of architectural differences on their antimicrobial efficacy [80]. It is noteworthy to be noticed that s-TNAMPs showed the strongest biocidal effect by the contrast of l-TNAMPs, h-TNAMPs, and c-TNAMPs at similar K to BE ratios. Before this work, Qiao et al. demonstrated that star-shaped architecture exhibited markedly higher antibacterial efficacy than their linear analogues [81], although Coelho et al. claimed that the polymer architecture had a minor effect on antibacterial activity [82].

Figure 6. Synthesis of linear or cyclic polypeptides by using $[\text{NHC(H)(HCO}_3\text{)}]$.

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Figure 7. Synthesis of tailored TNAMPs. Reproduced with permission from ref.77. Copyright 2020 Royal Society of Chemistry.

2.3.2 Conclusions and Perspectives

The past decade has witnessed the noticeable advances of NCA chemistry and the synthesis of functional polypeptides and hybrid materials for various applications. The ROP of NCA has been a growing important tool for making synthetic polypeptides which mimic the desired function and properties of natural proteins. In this review, we have shown how researchers synthesize and optimize the design of the polypeptide materials to achieve desired functions and biomedical performance.

Since the first time of synthesis and polymerizations of NCAs reported by Hermann Leuchs in 1906, chemists have made a lot of effort to optimize the NCA chemistry to achieve controllable living polymerization with high molecule weight, monodisperse and at large scale [83]. The goal of achieving controlled living polymerization was firstly finished by Deming to curb chain transfer and termination side reactions [84]. Afterward, a few new living initiation systems were developed as followed. These living initiation systems contain primary amine hydrochloride, high-vacuum technique (HVT) [73], low temperature technique [75], organosilicon initiators [85-87]. Also, different research groups are trying to overcome NCA's inherent sensitivity to moisture and low stability upon storage. From a synthetic perspective, the recent advances in ROP of NCA enable the making of polypeptides much easier with faster speed, less stringent requirements of synthesis set-up. Typical examples include thiol-analogues of

NCA monomers (NTAs) [65], LiHMDS initiating system [78], and IHC-mediated systems [79]. However, laborious procedures for the synthesis, purification, and handling of moisture sensitive NCA monomers, as well as the strict demands for controllability are still the main hurdles and barriers for broader application of NCA ROP. Therefore, the future of NCA chemistry will still focus on further improve the adaptability of NCA ROP into wider applications for larger-scale production. For example, the improving stability of NCA monomers, phosgene-free synthesis, creating new NCA monomers, and simplifying the NCA chemistry. As for NTA, it gets over several drawbacks of NCA but brings about new problems such as low reactivity, difficulty to get high molecular weight products and developing controlled NTA polymerization in polar solvents.

Polypeptide-based block copolymer with different architectures has been an interesting topic and has drawn increasing attention in academia due to unique properties, assembly behavior, and biomedical applications [88-90]. Generally, the polypeptides-based block copolymer could be classified into three categories based on their architecture, namely linear polypeptides, grafted and highly branched polypeptides, and star-shaped polypeptides. From the viewpoint of structure-properties relationship, we can see the different design of polypeptide-based block copolymer's composition and architecture will lead to different desired functionalities and biomedical performance at different level [91-94]. Many studies are still focusing on the correlation between gene delivery performance (transfection efficiency and biocompatibility) and self-assemblies or architectures. However, the research about the relationship between polypeptide-based block copolymer's self-assembly (micelles, vesicles, etc.) or architectures and antimicrobial performance is still less. Therefore, to curb the rising antibiotic-resistance problem, it is meaningful to expand future endeavors along that direction. Also, polymers with complex architecture are often difficult to characterize and analyze. The development of new analytical methods for the characterization/purification of complex macromolecular architectures is important in the future.

2.4 Common methods to determine minimal inhibitory concentration (MIC) of antimicrobial substances and MTT assay

Agar dilution and broth dilution are the most used techniques to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics and other substances that kill or suppress the growth of bacteria [95]. As for agar dilution, the suspension where defined numbers of bacterium are spotted onto agar plates which contained various concentrations of antibiotics. Afterwards, the appearance of colonies indicates growth of the microbes. As for broth dilution, it uses liquid growth medium which includes geometrically increasing concentrations of the antimicrobial agents. The final volume of the test defines whether the method is called macro-dilution or microdilution. After incubation, the presence of turbidity reveals growth of microbes [95].

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is used for assessing cell viability. Mitochondrial succinate dehydrogenase is responsible for catalyzing enzymatic reduction of MTT to MTT-formazan. MTT assay is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity of a cell although reducing agents and enzymes located in other organelles, such as the endoplasmic reticulum are also involved.

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CHAPTER 3: Experimental Methodologies

3.1 Summary of Chemicals and Equipment for Characterization

Chemicals: Butanediol(Sigma Aldrich), ϵ -caprolactone(Sigma Aldrich), Sn(Oct)₂(Sigma Aldrich), THF(Sigma Aldrich), hexane(Sigma Aldrich), CH₂Cl₂, DIAD(Sigma Aldrich), NHP(Sigma Aldrich), PPh₃(Sigma Aldrich), hydrazine, methanol(Sigma Aldrich), TMSCl(Sigma Aldrich), TEA(Sigma Aldrich), N- ϵ -Boc-protected lysine(Sigma Aldrich), diphosgene(Sigma Aldrich), NaOH(Sigma Aldrich), TFA(Sigma Aldrich).

Equipment: Bruker Advance II NMR spectrometer (300 MHz), Agilent PolarGel-M column (Serial No. 0006206222-37, MW range: 1000~500,000), Malvern Nano-Zetasizer with cuvette (DTS1070, Disposable Folded Capillary Cell), JEOL JEM 2100PLUS, Tecan600 (for MIC measurements), Graphpad (for MTT measurements).

3.2 The Synthesis of Poly (Lys-CL-Lys)

All the synthesis procedures were conducted according to ref [1]. The synthesis of hydroxy-terminated PCL was conducted based on the literature [2]. Lysine(Boc)-NCA was synthesized according to ref [3]. A typical synthesis route is depicted as in the following Figure 3.1.

Figure 3.1 Synthetic route of the Poly (Lys-CL-Lys) copolymer

3.2.1 Synthesis of Amino-ended Polycaprolactone (NH₂O-PCL-ONH₂)

Butanediol (257.11 mg, 2.853 mmol) and ϵ -caprolactone (13.862 mL, 114.12 mmol) were introduced to a round bottom flask. The flask was flushed with argon and sealed tightly with a septum. The viscous reaction mixture was introduced to a preheated 110 °C oil bath to remove the moisture. Afterwards Sn(Oct)₂ (0.02 mL, 0.0618 mmol) was added to the mixture using a 1 mL syringe, and the reaction was carried out in an argon atmosphere. After 8 hours, the reaction was cooled down to room temperature to terminate the reaction. Removed the septum, then added 5 mL THF into the solution and kept stirring overnight. The solution was precipitated in excess hexane to solidify the product and repeated 3 times. The solid was dried under vacuum at 50 °C for at least 2 hours to obtain HO-PCL-OH. The obtained HO-PCL-OH (4.9164 g, 1.056 mmol) was dissolved in a CH₂Cl₂ (50 mL) solution with NHP (0.5538 g, 4.224 mmol) and PPh₃ (0.9135 g, 4.224 mmol). Afterwards, DIAD (0.83 mL, 4.224 mmol, ρ = 1.03 g/mL) was added into the mixture, which was cooled with an ice–water bath. Then, kept stirring for 18 hours, and subsequently, the mixture was concentrated by evaporating

some volume of DCM ($< 30\text{ }^{\circ}\text{C}$). Cold methanol was used for precipitating the compound. The solid was washed 2 more times to obtain NHP-O-PCL-O-NHP (4946 g/mol) and removed excess reagents. Hydrazine (0.022 mL, 0.682 mmol) was added dropwise to the CH_2Cl_2 solution of NHP-O-PCL-O-NHP. Afterwards, the mixture was stirred for another 30 min. After some time, the white solid precipitated out in the solution and was removed by filtration. The filtrate was dropped into cold methanol to purify the polymer. Decanted the liquid, and the solid was completely dried in a lyophilizer to obtain the oxyamino-ended polymer (10.654 g, 69.2%) as a macroinitiator. The product was characterized by ^1H -NMR and DMF GPC.

3.2.2 Ring-Opening Polymerization of Lysine N-carboxyanhydride (Lys-NCA)

Lys(Boc)-NCA was prepared according to the procedure [3], as illustrated in Figure 3.2. First, 4.9 g (19.9 mmol) of N- ϵ -Boc-protected lysine was carefully weighed in a three-neck rounded bottom flask. Then, it was completely dried under vacuum for 1 hour. Then, suspended monomer in anhydrous THF (150 mL) and flushed the suspension by dry argon. Afterwards, 5 mL (39.8 mmol) of absolute TMSCl and 5.5 mL (39.8 mmol) of absolute TEA were injected gradually via a 10 mL syringe. Kept stirring for 2 hours and added 2.4 mL (19.9 mmol) of diphosgene by injection. Refluxed the suspension gently for another 2 hours. Led the dry argon flow through the suspension. Two gas washing bottles were prepared and filled with an aqueous NaOH solution in order to absorb the gas of phosgene and HCl. Afterwards, it was filtered to remove TEA salts and unreacted lysine, followed by reduced-pressure evaporation. Subsequently, the product was dissolved completely in absolute THF. The solution was precipitated with anhydrous hexane and stored in the fridge for another 1 hour. After filtration, the solid was washed with hexane and recrystallized with THF/hexane. The Lys(Boc)-NCA monomer was stored in a $-80\text{ }^{\circ}\text{C}$ fridge.

Figure 3.2 Synthetic scheme of Lys(Boc)-NCA

$\text{NH}_2\text{O-PCL-ONH}_2$ was dissolved in anhydrous THF in a Schlenk tube and degassed it by argon under vigorous stirring. Lys(Boc)-NCA was weighed in a glass vial and dissolved in anhydrous THF under an argon atmosphere. The monomer solution was injected into the Schlenk tube and the reaction progress was monitored by FTIR. After 24 hours, the solution was condensed via rotary evaporation, then dropped into excess diethyl ether to precipitate and washed 3 times. The obtained solid, PLys(Boc)-*b*-PCL-*b*-PLys(Boc), was dried under vacuum before deprotection. The product was characterized by $^1\text{H-NMR}$ and DMF GPC.

To remove the protecting group, weighed a certain amount of PLys(Boc)-*b*-PCL-*b*-PLys(Boc) in the flask. Added the excess TFA under the argon atmosphere to completely dissolve the compound in an ice-water bath. After completely being dissolved, the ice-water bath was removed. Then, the solution was brought to room temperature. After 24 hours, the solution was precipitated in excess diethyl ether and centrifuged to collect the solid. After being dried over the vacuum, the final product PLys-*b*-PCL-*b*-PLys (pKCK) was attained. For removing unwanted low-molecular-weight impurities, the product was dialyzed against water by using a regenerated cellulose membrane (MWCO = 3500) followed by lyophilization. The product was characterized by $^1\text{H-NMR}$ and DMF GPC.

3.3 NMR Characterization

NMR is a powerful technique for obtaining information about local atomic structures. Like an electron, neutrons and protons all have the intrinsic quantum property of spin. The spin of the nucleus could be considered as a magnetic moment vector, resulting in the nucleus behaving like a tiny magnet with north and south poles. The spin of the nuclei can experience a transition between the two energy states by the absorption of photons. The absorbed or released photons give the magnetic resonance signals.

All NMR characterizations were conducted via a Bruker Advance II NMR spectrometer (300 MHz) at room temperature. For sample preparation, all products were completely dissolved in the chosen deuterated solvent by vortex and heating when necessary.

3.4 Gel Permeation Chromatography (GPC) Study

GPC is a common technique for measuring the molecular weight and its distribution for a polymer. The column is inert and has pores of different sizes. The inertness is the important feature of the column, and it means that there is no interaction between the column and analytes. Larger analytes will cost less time in the pores and are eluted faster. The smaller analytes will enter the pores more easily and therefore spend more time in these pores, increasing their retention time.

The molecular weight and polydispersity of block copolymers were measured by an Agilent PolarGel-M column (Serial No. 0006206222-37, MW range: 1,000~500,000) with HPLC-grade DMF and 1 mg/mL LiBr as the eluent at a flow rate of 1 mL/min at the temperature of 25 °C. All samples were prepared at a concentration of approximately 1 mg/mL and filtered against a 0.45 μm Nylon filter. The data was calculated relative to polystyrene standards.

3.5 Self-Assembly of Nanoparticles by Nanoprecipitation

DMSO was chosen to completely dissolve pKCK to obtain a clear solution in a glass vial. Then, 1 mL deionized water was slowly added via a syringe pump at a speed of 2 $\mu\text{L}/\text{min}$ under mild stirring until reaching 50% water concentration. Then, the solution was dialyzed against DI water for 3 days to get rid of DMSO by utilizing the regenerated cellulose dialysis tube (MWCO = 3500). During this period, the deionized water was renewed every 3 hours. The nanoparticles were characterized via DLS and TEM.

3.6 Dynamic Light Scattering (DLS)

DLS is a common and convenient analytical technique that is used to determine the size distribution profile of particles or polymers in solution. All DLS measurements were conducted via a Malvern Nano-Zetasizer at 20 °C to obtain information about the hydrodynamic diameters of the nanoparticles. All samples were prepared at a concentration below 1 mg/mL and filtered against a 0.45 μm PES filter into the chosen cuvette (DTS1070, Disposable Folded Capillary Cell).

3.7 Zeta-potential Measurement

All zeta-potential measurements were conducted via a Malvern Nano-Zetasizer at 20 °C to obtain information about the surface charge of the nanoparticles. All samples were prepared at a concentration below 1 mg/mL and filtered against a 0.45 μm PES filter into the chosen cuvette (DTS1070, Disposable Folded Capillary Cell).

3.8 Transmission Electron Microscopy (TEM)

TEM is a widely used technique to observe the sample surface structure or morphology. An electron beam is transmitted through the specimen to form images, which can be

magnified and focused on the screen.

The TEM was conducted by using a JEOL JEM 2100PLUS TEM machine. The sample preparation was conducted by the following procedure. First, the TEM tweezer was cleaned thoroughly by flushing with water, followed by rinsing with acetone, and then dried with tissue paper. Second, used the tweezer to clamp the copper grid and removed it. Dropped 7 μ L of sample onto the copper grid, and the excess solution was absorbed away from beneath the copper grid. After a repetition of 3 times, the copper grid was carefully placed on the paper in the glass dish. Finally, the sample was stored at room temperature overnight before being ready to use.

3.9 Encapsulation of Thymol

Essential oils (EOs) are aromatic compounds that are extracted from parts of plants (flowers, buds, seeds, leaves, bark, etc.) and are generally used as cosmetics additives, food flavoring, and preservative agents and pharmaceutical formulations [4]. Phenolic compounds comprise the largest class of essential oils, which widely exist in higher plants and play multiple roles in plant physiology [5]. Several species of terpene derivatives (e.g., thymol, carvacrol, carvone, and cinnamaldehyde) are depicted in Figure 3.2. Polyphenols have been studied over the course of the last 20 years for their potential application in treating chronic diseases, such as diabetes, cancers, and cardiovascular disease. Normally, polyphenols possess a wide spectrum of antibacterial efficacy and a variety of modes of actions to suppress several bacterial virulence factors [5]. Thymol, as one common component of essential oils, has been reported to have antibacterial activities against *E. coli* and *S. aureus*. It is believed that *S. aureus* appears to be more sensitive than *E. coli* is [6]. For the mechanisms of the antibacterial action of thymol, it has been reported that thymol can permeabilize and depolarize the cytoplasmic membrane to breach the membrane integrity, allowing the loss of intracellular ions (such as potassium ions) and decreasing the membrane potential [7].

Most of the essential oils are an oily liquid at room temperature and have a high vapor pressure, which will result in a quick decrease in efficacy. However, thymol is a colorless solid with a moderate water solubility. In this study, thymol was chosen due to its solubility and low vapor pressure, which makes achieving long-lasting effects easier.

Thymol

Carvacrol

Carvone

Cinnamaldehyde

Citral A

Citral B

p-Cymene

Eugenol

Limonene

Menthol

Figure 3.3 Chemical structures of eleven common essential oils.

The process of encapsulation in this study is described as follows, which is a modification of nanoprecipitation. First, dissolved a certain amount of thymol (1.28 g) in DMSO (250 mL) by pipette to make a clear solution at a concentration of 5.12 mg/mL. Then, added equal amount of pKCK(1.28 g) into the solution to achieve a total concentration of 10.24 mg/mL. The solution was sonicated for half an hour followed by a slow water addition to reach a 50% water concentration via a syringe pump at a speed of 2 μ L/min under mild stirring. Last, the solution was dialyzed against deionized water for 3 days in order to get rid of DMSO by utilizing a regenerated cellulose dialysis tube (MWCO = 3500). During this period, the deionized water was renewed every 3 hours.

3.10 Minimum Inhibitory Concentration (MIC) determination

The broth microdilution method was utilized to determine the MIC according to the CLSI standard protocol with a minor modification. A single bacterium colony was streaked out from the agar plate and inoculated aerobically at 37 °C overnight. The overnight culture was 1:100 diluted in fresh Mueller Hinton Broth (Difco®, Becton, Dickinson, and Company) and incubated for 4 hours to reach the exponential growth phase. The bacteria were further diluted by 1:1000 to obtain a bacteria suspension containing approx. 1×10^6 CFU/mL in fresh MHB. The polymer was dissolved in Deionized water (DI water) at a concentration of 10.24 mg/mL and subsequently diluted to 1.024 mg/mL in fresh MHB. A series of two-fold dilutions of the polymer was

prepared in a 96-well plate (Nunc, ThermoScientific), achieving a concentration gradient from 512 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$ in MHB. Next, 50 μL of bacteria suspension was added to each well of the 96 well plate to obtain a final volume of 100 μL per well. Afterwards, the plate was incubated at 37 °C for 18 hours, and the OD absorbance of each well was measured at a wavelength of 600 nm. The MIC value is defined as the lowest concentration at which more than 90% of bacterial growth was inhibited based on the OD600 nm readings.

3.11 MTT Assay

The MTT assay, also known as MTT colorimetry, is a method to detect cell survival and growth. The detection principle is that the succinate dehydrogenase in the living cell mitochondria can reduce the exogenous MTT to the water-insoluble blue-purple crystal formazan and deposit in the cell; however, the dead cell has no such function. An increasing number of cells results in an increase in purple coloring.

In this study, the metabolic activity of mouse 3T3 fibroblast cells after the treatment of a series of pKCKs was determined by the MTT assay. The 3T3 cells were cocultured in 96-well plates for 24 hours at 37 °C with pKCK polymers with a graded concentration series (62.5 ppm, 125 ppm, 250 ppm, and 500 ppm). Then, the culture medium that contains the block copolymer was discarded, and each well was washed with PBS buffer before the addition of the MTT solution. Afterwards, the plate was incubated for 4 hours at 37 °C, and subsequently, the medium was discarded followed by the addition of 100 μL of DMSO into each well. The relative cell viability was calculated according to the absorbance of each well at 570 nm. The statistical analysis is done by Software Graphpad

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CHAPTER 4: Results and Discussion

4.1 Synthesis and Characterization of pKCK

In this study, a series of triblock copolymers, $pK_nC_{40}K_n$ ($pK_7C_{40}K_7$, $pK_{10}C_{40}K_{10}$, $pK_{20}C_{40}K_{20}$), were synthesized in five steps, as shown in Scheme 4.1 (All reactions conditions and reagents are described in previous Chapter, please refer to Chapter III, 3.2). First, the HO-PCL-OH macroinitiator was synthesized via ROP initiated by butanediol and catalyzed by Tin(II) octanoate. Second, the hydrogen on the hydroxy end group was converted to NHP. Third, the hydrazination reaction was achieved by adding hydrazine to the solution to attain the amino-ended polycaprolactone, NH_2O -PCL- ONH_2 , macroinitiator. Fourth, PLys(Boc)-*b*-PCL-*b*-PLys(Boc) was synthesized via the NCA ROP of Lys(Boc)-NCA, which was used as prepared. A series of compounds with designed ratios was obtained by varying the ratio of the monomer to the macroinitiator. In the final step, the deprotection of Boc was achieved in excess anhydrous TFA, followed by precipitation in diethyl ether. The purified product was harvested as a white solid after dialysis against water, followed by freeze-drying. Its composition and molecular structure were confirmed by 1H -NMR, ^{13}C -NMR and DMF GPC.

Scheme 4.1 A typical synthesis scheme of $pK_{10}C_{40}K_{10}$

4.1.1 Synthesis and Characterization of the NH₂O-PCL-ONH₂ Macroinitiator

In this study, the block length of PCL was fixed as 40 repeating units by the ROP of ϵ -caprolactone with Sn(Oct)₂ as a catalyst. Through the Mitsunobu reaction, the conversion from hydroxy to oxyimino group was achieved.

As shown in Figure 4.1(A), peak a (2.23 ppm) is assigned to the -CH₂ beside the -C=O of PCL and set to be 1.00 as the internal reference. The spectrum also shows the peak e corresponding to the backbone of PCL at 4 ppm, which is assigned to the -CH₂ protons beside -OH. Peak f (3.57 ppm) is assigned to the protons of -CH₂ beside the oxygen atom on the butyl chain. The degree of polymerization (DP) of PCL_n can be calculated by A_f, and the results can be verified by A_c, A_{b+d} or A_e.

$$DP = \frac{A_a}{A_f} \times 2 = 40$$

As shown in Figure 4.1(B), Peak f (7.70~7.80 ppm) corresponds to the protons on the aromatic ring in NHP. The conversion rate from hydrogen to NHP can also be verified by calculating with respect to A_a and A_f.

$$\text{conversion rate} = \frac{\frac{A_a}{A_f}}{\frac{2 \times 20}{4}} = 100\%$$

Table 4.1 The integrals of different peaks and the degrees of polymerization

Spectrum	A _f	A _a	A _c	A _{b+d}	A _e
Fig. 4.1(A)	0.05	1.00	1.02	2.10	1.00
Fig. 4.1(B)	0.10	1.00	1.10	2.32	1.00
Fig. 4.1(C)	---	1.00	1.06	2.23	1.00

As shown in Figure 4.1(C), the complete disappearance of peak f, which is shown in Figure 4.1(B), indicates the full conversion from NHP to NH₂. In other words, the NHP is fully hydrazinated.

Figure 4.1 NMR spectra of (A) HO-PCL-OH, (B) NHP-O-PCL-O-NHP, and (C) NH₂O-PCL-ONH₂

4.1.2 Synthesis and Characterization of Lys(Boc)-NCA Monomers

The synthesis of Lys(Boc)-NCA was conducted via the diposgene method with the

aid of TMSCl and TEA. As shown in Figure 4.2, each proton can be assigned correspondingly. The peak assignment is summarized as the following [1]. $^1\text{H-NMR}$ (300 MHz, DMSO- d_6): δ [ppm] = 9.07 (1 H, $-\text{NH-CO-O-CO-}$), 6.90-6.70 (1 H, $-\text{NH-CO-O-C(CH}_3)_3$), 4.42 (1 H, $-\text{CO-CH-CH}_2-$), 2.90 (2 H, $-\text{CH}_2\text{-NH-CO-O-C(CH}_3)_3$), 1.79-1.56 (2 H, $-\text{CH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH-}$), and 1.45-1.12 (13 H, $-\text{CH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH-CO-O-C(CH}_3)_3$).

Figure 4.2 NMR spectrum of Lys(Boc)-NCA

In this study, the reason why the Boc-protected lysine NCA was synthesized rather than the Cbz-protected lysine NCA is that there is a concern about the cleavage of the PCL backbone in the subsequent deprotection stage. If we use the Cbz-protected lysine NCA, after copolymerization, the Cbz protecting group will be removed in 33% v/v HBr/HAc. Under this condition, it is quite possible that the ester bond of PCL will be hydrolyzed if the quality of the reagent is not good enough or the experiment is not carefully executed [2-4].

For securing the intactness of the PCL chain without breaking, I investigated the degradation of commercial product PCL(14k) under excess TFA treatment, which is the same as the condition in the Boc protecting group removal.

As shown in NMR (Figure 4.3) and GPC data (Figure 4.4 and Table 4.2), by

comparison of the data obtained before and after the excess TFA treatment, there is no significant difference, which indicates the PCL stays intact without cleavage. In Figure 4.3, the characteristic peaks (a, b, c, d, and e) of PCL can be found. Figure 4.4 shows that the retention times of the product peaks are almost the same.

Figure 4.3 NMR spectra of PCL(14k) before and after the excess TFA treatment

Figure 4.4 DMF GPC traces of PCL(14k) before and after the excess TFA treatment

Table 4.2 Molecular weights and molecular weight distributions of PCL(14k) before and after the excess TFA treatment

GPC Trace	Retention Time (min)	Mw	Mn	PDI
Original	14.813	17887	10059	1.78
After TFA Treatment	14.905	16323	9818	1.66

4.1.3 Synthesis and NMR Characterization of a series of pKCK after Boc Group Deprotection

In this study, $\text{NH}_2\text{O-PCL}_{40}\text{-ONH}_2$ was used as a macroinitiator to initiate the ROP of Lys(Boc)-NCA. The triblock copolymers $\text{pK}_n\text{C}_{40}\text{K}_n$ ($\text{pK}_7\text{C}_{40}\text{K}_7$, $\text{pK}_{10}\text{C}_{40}\text{K}_{10}$, $\text{pK}_{20}\text{C}_{40}\text{K}_{20}$) were obtained by varying the feeding ratio of Lys(Boc)-NCA to macroinitiator. Typical NMR characterization results of $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$ are shown in Figure 4.5. Compared to Figure 4.5(A), in Figure 4.5(B) there is no peak found between 1.45-1.12 where the signals correspond to the Boc protecting group. The vanishing of peak k indicates the complete removal of the Boc group.

Figure 4.5 NMR spectra of (A) $\text{PLys(Boc)}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys(Boc)}_{10}$, (B) $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$ (^1H -NMR), and (C) $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$ (^{13}C -NMR)

In ^{13}C -NMR, as shown in Figure 4.6(C), two characteristic signals (peak a and peak h) are assigned to the carbonyl groups of PCL and PLys, respectively.

The corresponding integrals of the typical peaks are collected in Table 4.3. The degree of polymerization of lysine units is determined as the following equation according to the integrals of the peak a and peak i in Figure 4.6(B).

Table 4.3 The integrals of different peaks of PLys(Boc)₁₀-*b*-PCL₄₀-*b*-PLys(Boc)₁₀

Spectrum	A _a	A _i	A _e	A _{b,d,h}
Fig. 4.3(B)	2.00	1.09	2.09	6.39

$$\text{DP of Lysine} = \frac{2 \times 40 \times A_i}{2 \times A_a} = 21.80 \approx 22$$

The results for PLys₇-*b*-PCL₄₀-*b*-PLys₇ and PLys₂₀-*b*-PCL₄₀-*b*-PLys₂₀ are also obtained following the same process, and the ^1H -NMR spectra of them are shown in Figure 4.6 and Figure 4.7, respectively. The relating integrals of peaks are collected in Table 4.4 and Table 4.5, respectively. According to the integrals, the repeating units of lysine in these two block copolymers are calculated as 13 and 49, respectively.

Figure 4.6 ^1H -NMR spectrum of PLys₇-*b*-PCL₄₀-*b*-PLys₇

Table 4.4 The integrals of the different peaks of PLys(Boc)₇-*b*-PCL₄₀-*b*-PLys(Boc)₇

Spectrum	A _a	A _i	A _e	A _{b,d,h}
Fig. 4.3(A)	1.92	0.62	2.00	5.23

$$\text{DP of Lysine} = \frac{2 \times 40 \times A_i}{2 \times A_a} = 12.91 \approx 13$$

Figure 4.7 ¹H-NMR spectrum of PLys₂₀-*b*-PCL₄₀-*b*-PLys₂₀**Table 4.5** The integrals of the different peaks of PLys(Boc)₂₀-*b*-PCL₄₀-*b*-PLys(Boc)₂₀

Spectrum	A _a	A _i	A _e	A _{b,d,h}
Fig. 4.3(A)	2.00	2.46	2.12	8.77

$$\text{DP of Lysine} = \frac{2 \times 40 \times A_i}{2 \times A_a} = 49.2 \approx 49$$

4.1.4 GPC Characterization of a series of pKCK Block Copolymers

The molecular weights and molecular weight distributions were obtained by the DMF phase GPC measurement. Take PK₁₀-*b*-PCL₄₀-*b*-PK₁₀ (K: Lysine) as an example, the GPC curves of NH₂O-PCL₄₀-ONH₂, PLys(Boc)₁₀-*b*-PCL₄₀-*b*-PLys(Boc)₁₀ and PLys₁₀-*b*-PCL₄₀-*b*-PLys₁₀ are shown in Figure 4.8, and the related data are collected in Table 4.6.

From Figure 4.8, we can observe a clear peak shift towards the left side (lower retention time) from the single peak of the macroinitiator $\text{NH}_2\text{O-PCL}_{40}\text{-ONH}_2$ to the single peak of $\text{PLys(Boc)}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys(Boc)}_{10}$. This indicates the successful addition of Lys(Boc) repeating units to the macroinitiator with the increase in molecular weight. Then, after the deprotection of Boc groups, the peak shifts towards the right side (higher retention time) with the decrease in molecular weight. In view of the combination of NMR and DMF GPC results, $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$ is successfully obtained with a narrow distribution ($\text{PDI} = 1.19$).

Figure 4.8 DMF GPC curves of $\text{NH}_2\text{O-PCL}_{40}\text{-ONH}_2$, $\text{PLys(Boc)}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys(Boc)}_{10}$ and $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$

Table 4.6 The obtained GPC data of $\text{NH}_2\text{O-PCL}_{40}\text{-ONH}_2$, $\text{PLys(Boc)}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys(Boc)}_{10}$ and $\text{PLys}_{10}\text{-}b\text{-PCL}_{40}\text{-}b\text{-PLys}_{10}$

Curve	Retention Time (min)	Mw	Mn	PDI
Black	15.327	11794	8851	1.33
Red	14.700	20325	15658	1.29
Blue	15.088	13435	11259	1.19

The GPC curves of pKCK(7-40-7) , pKCK(10-40-10) , pKCK(20-40-20) are gathered in one plot, as shown in Figure 4.9, and the relating GPC data are collected in Table 4.7. Unfortunately, the molecular weight distributions of pKCK(7-40-7) and

pKCK(20-40-20) are substantially wider than that of pKCK(10-40-10), which might result from a slower chain propagation due to differences in the purity of the prepared Lys(Boc)-NCA. Additionally, the molecular weight of the pKCK block copolymers are higher than the results obtained by ^1H -NMR due to the calibration standards of GPC being polystyrene, which is different from our block copolymer. The retention times of pKCK(7-40-7), pKCK(10-40-10), pKCK(20-40-20) are 14.662, 15.088, and 15.233, respectively. Additionally, the corresponding number average molecular weights are 16870, 11259 and 9119, respectively.

Figure 4.9 DMF GPC curve of a series of pKCK block copolymers

Table 4.7 The obtained GPC data of a series of pKCK block copolymers and molecular weights calculated by ^1H -NMR

Curve	Retention Time (min)	Mw	Mn	PDI	Mn, _{NMR}
Black	15.233	13567	9119	1.49	6352
Red	15.088	13435	11259	1.19	7120
Blue	14.662	22606	16870	1.34	9680

4.2 Self-assembly of the pKCK block copolymers

Self-assembly is a phenomenon in which an ordered structure or pattern can form

without guidance from an outside source in a disordered system.

The formation of pKCK nanoparticles was achieved by the nanoprecipitation method or the so-called slow water addition method. It is known that the process of self-assembly is thermodynamic and driven by hydrophobic interactions among amphiphilic polymers. The mean size of the nanoparticles is determined by the DLS analysis, and the surface charge of nanoparticles is determined via the zeta potential measurement. To evaluate the morphology of nanoparticles, TEM images are taken.

The DLS measurement (Figure 4.10 and Table 4.8) shows that the hydrodynamic radius of pKCK(7-40-7), pKCK(10-40-10) and pKCK(20-40-20) are 705.2, 182.4 and 1025 nm, respectively. Accurately speaking, these particles are sub-micrometer particles or micrometer particles. Hence, the term “nanoparticles” will be fixed as “sub-micrometer particles” or “micrometer particles” or simply one term “quasi-micrometer particles” hereafter. All these particles possess a positive surface charge ranging from 43.3 to 55.9 mV. According to the theoretical hydrophilic fraction [5], pKCK(20-40-20) is the least hydrophobic amphiphilic copolymer that is expected to form a small micelle, however, it assembles into largest nanoparticle whose size by intensity is 1025 nm. This is contradicted by packing parameter theory [5, 6]. Most likely, the packing parameter theory is more applicable to AB type diblock amphiphilic copolymers, while in this case of an ABA triblock copolymer, the deviation from the predicted model is observed.

Figure 4.10 The DLS profiles of (A) pKCK(7-40-7), (B) pKCK(10-40-10) and (C) pKCK(20-40-20)

Table 4.8 The characteristic properties of quasi-micrometer particles formed by the pKCK block copolymers

Block copolymer	Mean size by intensity (nm)	PDI	ζ -potential (mV)
pKCK(7-40-7)	705.2	0.406	55.9
pKCK(10-40-10)	182.4	0.158	43.3
pKCK(20-40-20)	1025	0.356	53.4

In aqueous solution, the pKCK(10-40-10) forms hard-sphere micelles and worm-like or rod-like quasi-micrometer particles, as shown in Figure 4.11. The theoretical hydrophilic fraction of pKCK(10-40-10) is determined as 36%, which is predicted to form a vesicle. However, under TEM observation, nanosized micelles were observed.

Figure 4.11 The morphology of pKCK(10-40-10) via TEM

For the other two block copolymers, pKCK(7-40-7) and pKCK(20-40-20), both formed large quasi-micrometer particles with distinct lamellar structures. The shaded area indicates the folded membrane of the quasi-micrometer particles (Figure 4.12).

Figure 4.12 The morphology of pKCK(7-40-7) and pKCK(20-40-20) via TEM

4.3 Antibacterial Properties of pKCK Block Copolymers with and without Thymol

The antibacterial properties of pKCK copolymer are measured via MIC testing. To investigate the synergistic effect, thymol is added. The copolymer and thymol both are dissolved in DMSO to obtain a clear solution followed by nanoprecipitation and dialysis, resulting in a better dispersion of thymol.

Table 4.10 summarizes the MIC results of the pKCK block copolymers and the chosen essential oil, thymol. For thymol and pKCK(7-40-7), the MIC values towards *E. coli* and MRSA are relatively high. The MIC of thymol is above 512 and the MIC of pKCK(7-40-7) is 256. Intriguingly, after the encapsulation of thymol in pKCK(7-40-7), the MIC towards MRSA decreases as much as 16-fold. The improvement in the MIC after the addition of thymol is also observed for pKCK(10-40-10) and pKCK(20-40-20). Conversely, the addition of thymol lead to worst MIC results against *E. coli*. In other words, the addition of thymol revealed the opposite trend against *E. coli* and MRSA after being treated with pKCK. The difference in the MIC results between *E.*

coli and MRSA is probably because MRSA is more susceptible to thymol than E. coli is [7] after the disturbance of the cationic block copolymer, pKCK.

Table 4.9 The MIC results of pKCK and/or thymol

No.	Compound	MIC ₉₀	
		E. coli 8739	MRSA BAA40
1	Thymol	> 512	> 512
2	pKCK(7-40-7)	256	256
3	pKCK(10-40-10)	64	64
4	pKCK(20-40-20)	64	64
5	pKCK(7-40-7) + Thymol	>512	16
6	pKCK(10-40-10) + Thymol	256	16
7	pKCK(20-40-20) + Thymol	256	16

4.4 Cytotoxicity of pKCK Block Copolymers

The cytotoxicity of the pKCK copolymers is measured by the MTT assay (Figure 4.13, Table 4.10). Generally, pKCK(10-40-10) is more toxic than the others probably due to its smaller size (182.4 nm) and its tendency to penetrate 3T3 cells' membranes. At a concentration of 62.5 ppm, pKCK(20-40-20) and pKCK(7-40-7) show a good biocompatibility since the relative cell viability is higher than 90%, while pKCK(10-40-10) displays a poor biocompatibility with a relative cell viability below 70%. Above the concentration of 125 ppm, all block copolymers exhibit a high toxicity towards the 3T3 cells because all cell viabilities are below 30%.

Figure 4.13 The MTT assays of a series of pKCK copolymers

Table 4.10 The MTT results of a series of pKCK copolymers

pKCK Conc. (ppm)	Relative Cell Viability(%)		
	pKCK (20-40-20)	pKCK (10-40-10)	pKCK (7-40-7)
62.5	89.946%±13.07%	66.416%±0.773%	106.55%±4.86%
125	21.029%±1.364%	25.614%±3.377%	21.887%±4.672%
250	27.231%±4.696	18.65%±1.398%	23.341%±1.751%
500	20.476%±1.843%	15.359%±3.482%	22.762%±1.15%

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CHAPTER 5: Conclusions and Future Work

5.1 General Summary

In this study, cationic peptide-based block copolymers (pKCK) with different lengths of the lysine block were successfully made via NCA ring-opening copolymerization. Additionally, their relative characteristics are measured via different characterization techniques such as NMR, GPC, TEM, DLS, Zeta potential measurement, MIC testing, and the MTT assay. The self-assembly structure of pKCK is clearly observed via TEM. The overall characteristics of pKCK are summarized in Table 5.1. Additionally, the encapsulation of thymol inside the quasi-micrometer particles was conducted via nanoprecipitation and the synergistic effect is investigated as well. The pKCK triblock copolymers are believed to disrupt the outer membrane of bacteria via electrostatic interactions.

Table 5.1 A summary of properties of pKCK copolymers

Copolymer Design	F ^a	MW ^b	Morphology	Size by intensity (nm)	ζ (mV)	MIC ₉₀		Relative Cell Viability at 62.5 ppm
						E. coli	MRSA	
						8739	BAA40	
pKCK (20-40-20)	53%	9680	Vesicle	1025	53.4	64	64	90%
pKCK (10-40-10)	36%	7120	Micelle	182.4	43.3	64	64	67.9%
pKCK (7-40-7)	28%	6352	Vesicle	705.2	55.9	256	256	107%

^aTheoretical hydrophilic fraction; ^bTheoretical molecular weight

5.2 Overall Conclusions

By tuning the length of the lysine block, one objective is to achieve the overall optimal performance. The total length of the lysine block varies from 14 to 20 to 40. With the increase in the lysine block, the best candidate of pKCK is pKCK(20-40-20), because it has the lowest MIC with a value of 64 and the highest relative cell viability, which is 90%. In this study, the increase in the lysine block does not lead to a significant increase in surface charge. For pKCK(10-40-10), it has the smallest hydrodynamic diameter, and its morphology is that of a micelle. Based on the cell viability test, pKCK(10-40-10) has the greatest cytotoxicity which results in only a 67.9% cell viability. The size of the quasi-micrometer particles also plays an important role in penetrating the bacterial outer membrane.

To investigate the synergistic effect of pKCK in combination with thymol, we find that MRSA is more susceptible to pKCK and thymol than *E. coli* is. This result is probably because gram-positive bacteria are surrounded by a thick peptidoglycan wall which repels small antimicrobial molecules, while Gram-negative bacteria have a rigid outer membrane (rich in lipopolysaccharides), which limits the diffusion of hydrophobic compounds through it. Additionally, with encapsulated thymol in amphiphilic quasi-micrometer particles, the MIC towards *E. coli* becomes higher, while the MIC towards MRSA becomes lower. The thymol and pKCK collectively exhibit a synergism towards MRSA.

5.3 Suggestions for Future Work

Table 5.1 summarizes the common essential oils that exhibit relatively low MIC values. Among them, citronellol was reported to have an MIC value of 5 against *E. coli*, and mangostin was reported to have an MIC value of 2 against *S. aureus*. Hence, it is possible to achieve the lower MIC after encapsulating citronellol or mangostin.

From the perspective of polymer design itself, the smaller size quasi-micrometer particles are, the higher cytotoxicity will be. As far as antibacterial efficacy is concerned, there is no evident trend between polymeric particles size and MIC against E.coli 8739 or MRSA BAA40. Hence, it is meaningful to investigate the relationship between particle size and killing efficacy. According to the literature, it is expected that the smaller size of polymeric particles is, the greater likelihood of improved biocidal efficacy will be. However, it is worthy to be noticed that the correlation between the particle size and sanitization capability is not linear relationship and is more like a step function. Therefore, achieving a better control of particle size and narrowing the size down to nano level will be a critical question. The size of self-assembled polymeric particles is affected by multi-factors (e.g. polymer itself, temperature, injection speed, stirring speed, solvent polarity, time and probably most important thing is manipulation technique). For making nanosize-controlled polymeric vehicles, the velocity of solvent removal and thus nuclei formation is the key instead of larger lumps or agglomerates. The strategy of reducing the size of polymeric particles is increasing solvent quality reduction and this can be done by lower down the polymer concentration [1]. By doing so, I will make a series of real polymeric nanoparticles (ranged from 1~100 nm) and study their impact on antimicrobial efficacy.

In the future, I plan to study the combination of pKCK quasi-micrometer particles or pKCK nanoparticles with different chosen essential oils to find the most promising combinations for combating the bacteria, E. coli and MRSA. To go further, guanidino groups can be further incorporated into the lysine chain by a simple replacement of the amino groups on PLL segments in order to mimic the transmembrane function of cell-penetrating peptides. After guanidination (Figure 5.1), pKCK will be converted to pKCK(AG1-AG3) with 3 different degrees of guanidination. Then, they will be used as new delivery tools to encapsulate chosen essential oils, and the synergistic effect will be studied as well.

Table 5.1 Common essential oils and their corresponding reported properties

No.	Essential Oil	Category	Appearance	Reported MIC (µg/ml)	
				E. coli	S. aureus
1	Epigallocatechin gallate (EGCG)	Esters	White solid	400 [2]	50~100 [2]
2	Terpinen-4-ol	Alcohols	Colorless liquid	600[3]	2500[3]
3	Citral	Aldehydes	Light yellow liquid	500[3]	300~600[3]
4	Perillaldehyde	Aldehydes	Yellow liquid	500[3]	N.A.
5	Linalool	Alcohols	Colorless liquid	600[3]	1250[3]
6	Terpineol	Alcohols	Colorless liquid	600[3]	2500[3]
7	Menthol	Alcohols	White solid	525[3]	600[3]
8	Carveol	Alcohols	Colorless liquid	200[3]	2000[4]
9	Citronellol	Alcohols	Colorless liquid	5[4]	375[4]
10	Mangostin	Xanthonoid	Yellow solid	N.A.	2[5]

Figure 5.1 Guanidination of pKCK

References

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