

Theranostic Antibacterial Agents Based on Biodegradable Polymer Nanoarchitectures

A thesis

submitted in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

by

Ruma Ghosh

Reg. No. 20163457



Department of Chemistry

Indian Institute of Science Education and Research

Pune - 411008

January, 2023

Dedicated to

My Family



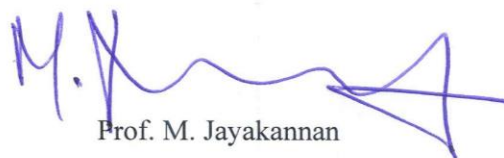
भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान पुणे
INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE
(An Autonomous Institution of Ministry of Human Resource Development, Govt. of India)
Dr. Homi Bhabha Road, Pune - 411 008.

Prof. Manickam Jayakannan
Department of Chemistry

CERTIFICATE

Certified that the work done in the thesis entitled “*Theranostic Antibacterial Agents Based on Biodegradable Polymer Nanoarchitectures*” submitted by **Ms. Ruma Ghosh** was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: 16th January 2023
Pune, Maharashtra, India

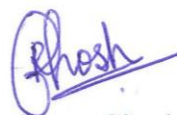

Prof. M. Jayakannan
(Thesis Supervisor)

16/01/2023

Declaration

I declare that this written submission represents my ideas in my own words, and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.

Date: 16th January 2023



Ruma Ghosh
20163457

Acknowledgements

This has been one of the most exciting journeys of my life so far. I learned a lot and grew as a person in these six years. I am thankful to spend formative years of my life in the beautiful IISER campus and interact with a lot of good people. This place will always be a part of me.

Foremost, I would like to extend my sincere respect towards my thesis supervisor Prof. M. Jayakannan for his constant support, guidance, and encouragement throughout the course of my PhD. His out of the box approach always taught us to look at a problem from different perspectives and never to give in to any situation. He has always been very approachable and kind to us. I am grateful to have him as my supervisor.

Another important member of my research career is my Research Advisory Committee, Dr. Harinath, and Dr. J. Nithyanandhan. They had always encouraged me and supported me to grow intellectually. They were always open to discussions and I cannot thank them enough for giving me their precious time. I would also like to thank Dr. S. K. Asha for allowing me access to her lab facilities and always encouraging us with a smile on her face. I would also like to thank Dr. Amrita Hazra mam for extending me facility to do antibacterial research. Thankyou mam, it would not have been possible without you.

I would like to thank Dr. K. N. Ganesh, Former Director, and Prof. Jayant B. Udgaonkar, Present Director for extending state-of-the-art amenities provided by IISER, Pune. I thank IISER, Pune for supporting me financially for the first six years and DST project for providing us funding for research facilities.

I would like to take this opportunity to express my heartfelt gratitude to IISER administration, Chemistry Technical staff, Mayuresh sir, Mahesh sir, Sandeep sir, Ganesh sir, Yathish sir, Megha ma'am, Hemlata ma'am, Santhosh sir and Vijay sir at IISER Pune imaging facility, Saurav sir at BD Biosciences FACS facility, Suraj Sir from National Centre for Gene Function in Health and Disease (NFGFHD).

I thank my all my colleagues, including lab members, past and present, for providing a good working environment. My seniors Dr. Sonashree, Dr. Bhagyashree, Dr. Nilesh, Dr. Mehak, Dr. Dheeraj for helping me through my PhD and making me a better researcher,

especially Mehak for being such a good friend and always being there for me. Present members Rahul, Mishika, Khuddus, Utreshwar, Shahid, Parshuram for maintaining a good lab environment and making this journey enjoyable. My 2016 batchmates, Rashmi, Neetu, Subhajit, Padmini, Anand, Puneeth, Abdul for all the fun filled adventures and sharing this journey along with me. I also thank Rupali for teaching me basic protocols of working in microbiology lab.

Lastly, I would like to thank my family without whom nothing would have been possible. Thank you for having faith in me and encouraging me. My father, who has never stopped me from taking my own life decisions. My mom, who has pleasantly supported me always and she is the reason of our smiles even at hard times and my sister, she has supported me blindly and always encouraged me to do better in life. They are the pillars of my life. My complete family including all my uncles and aunts and my cousins. Thank you all for being so nice people and providing a nurturing environment for us kids in our childhood. I wish your blessings always stay with me and guide me through the rest of my journey.

Ruma Ghosh

Synopsis

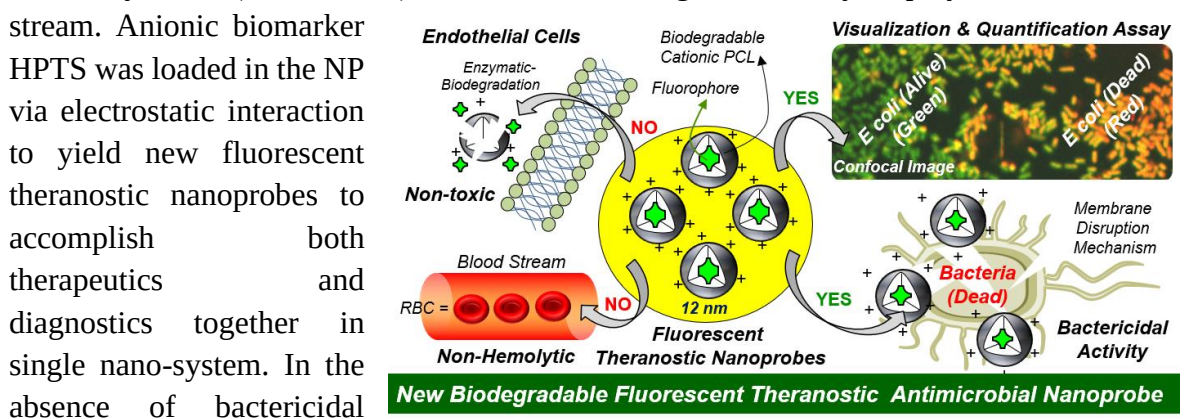
Infections caused by the bacteria and microbes are continued to be one of the most challenging problems that requires continuous attention of the scientific community in handling the healthcare of the society. Currently, beta-lactam antibiotics (like Penicillin) are used clinical drugs for the treatment of bacterial infections; however, unprecedented mutations in microbes quickly develop high resistance against majority of these antibiotic drugs. Antimicrobial are seen as a nature's selection for the treating bacterial infectious; however, tedious multi-step synthesis for large scale production, hemolytic side effects and in vivo toxicity pose huge challenge for AMPs long-term feasibility in therapeutics. Last one-decade, synthetic cationic polymers are emerging as a new alternative artificial antimicrobial agent to treat bacterial infections. The next generation antimicrobial polymers are essential to be *smart multitasking probes* having capability to exhibit both therapeutics and diagnostics together (theranostics) in single system so that maximum impact could be accomplished in single dose of drug administration. Further, the probe or antimicrobial polymers come in direct contact with the healthy tissues (or cells), proteins and red blood cells (RBCs) while testing the activity which introduce unwanted toxicity issues, and therefore, the incorporation of biodegradation property is essential component in theranostic probe design for safer biomedical applications. To address this important problem, this thesis work is emphasized in designing new "*biodegradable theranostic antibacterial nanoprobes*" based on cationic polycaprolactone (PCL) platform. The thesis has been divided into four chapters:

- **Chapter 1** provides brief account on the antimicrobial antibiotics, antimicrobial peptides, and synthetic macromolecules. The role of the polymer structure and their function on antimicrobial action has been reviewed in detail and efforts have been put to bring the impact and need for the next generation biodegradable-based antimicrobial agents for both therapeutics and diagnostic purpose.
- **Chapter 2** describes the development of new class of cationic polycaprolactone, study their structure-property relationship, test their biological activity, and further employ them as *theranostic nanoprobe* for direct visualization and qualitative determination of bactericidal activity by confocal *fluorescent* assay which could distinguish live and dead bacteria in real case scenario.
- **Chapter 3** is empathized to develop confocal image-based FRET-gate probe technology. Aggregation induced emission (AIE) active chromophore was chemically conjugated on the cationic biodegradable polycaprolactone (PCL) to yield blue-luminescent "*lighting up antimicrobial FRET donor*" on the bacteria membrane surface. The membrane slit-through action of the TPE donor polymer "**Gate-Opened**" and allowed the localization of CV at the peptidoglycan layer that brought together the donor-acceptor chromophores at the nano-confinement for membrane-reinforced FRET action.
- **Chapter 4** is dedicated to expand the scope of the PCL polymer design to make linear and star block copolymers having identical chemical composition but differ in their polymer topology. The topology of polymers was found to play significant role

on their biomaterials action; and these results revealed that the star-shaped polymer seems to be outperforming its linear counterpart in terms of antimicrobial activity, hemolysis and cytotoxicity in mammalian cell lines. Azithromycin- a ribosomal inhibiting antibody was encapsulated in the star-polymer to study the combination effect of antimicrobial action by the polymer membrane disruption mechanism and enzyme-inhibiting pathway by the antibody together in a single nanoplatform.

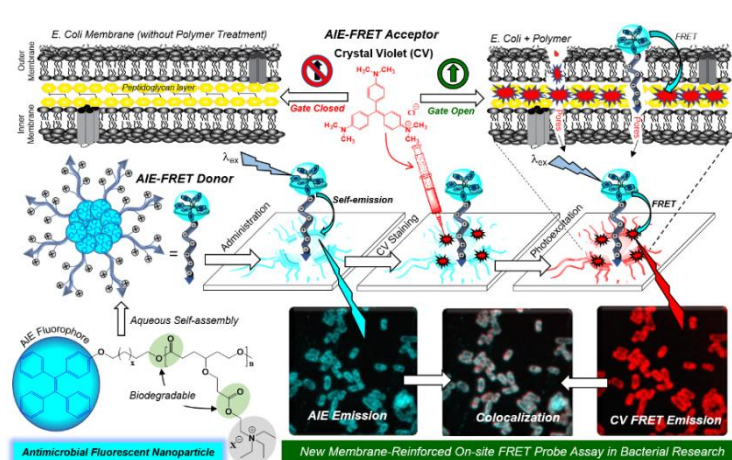
The last chapter provide the overall summary of the thesis with future perspectives. The detail account on chapters 2 to 4 is described below in detail.

In chapter 2, new cationic-charged polycaprolactone (PCL) was tailor-made and self-assembled into well-defined tiny 5.0 ± 0.1 nm aqueous nanoparticle (NP) with Zeta potential of +45 mV to maximize the impact on the bacterial membrane disruption. Electron microscopic studies were evident for membrane disruption mechanism with respect to their high selectivity for antibacterial activity at 10.0 ng/mL concentration was accomplished in gram-negative bacteria *E. coli*. Hemolysis assay revealed that RBCs were extremely stable in presence of polymer with % hemolysis values as negligible as 0.64 ± 0.003 with a selectivity index (HC_{50}/MIC_{50}) $>10^{11}$ times showing non-toxicity of polymer in the blood stream.



activity, the theranostic probe function exclusively as bio-marker to exhibit strong green-fluorescent signals in live *E. coli*. Once it became active, the theranostic probe induce membrane disruption on *E. coli*. which enabled the co-staining of nuclei by red-fluorescent propidium iodide. As a result, live and dead bacteria could be visualized as green and orange signals (merging of red+green), respectively, during the course of antibacterial activity by the theranostic probe. This has enabled the development of new image-based fluorescence assay to directly visualize and quantitatively estimate the real-time antibacterial activity. Time-dependent bactericidal activity was coupled with selective photo-excitation in confocal microscope to demonstrate the proof-of-concept of the working principle of theranostic probe in *E. coli* with very good reproducibility.

In chapter 3, A smart aggregation induced emission (AIE) theranostic polymer was built to act as real-time *lighting up probe* while breaching the bacterial membrane and also tightly regulates the outer-membrane gate-opening event for the permeabilization of peptidoglycan binding AIE-active biomarker crystal violet (CV) in the construction of the first-ever membrane-reinforced FRET probe for real-time visualization and quantification in bacterial research. For this purpose, AIE-activated cationic fluorescent amphiphilic polycaprolactone homo- and block copolymers were designed and developed by ring opening polymerization methodology. These polymers were readily self-assembled through selective segregation of hydrophobic TPE-chromophores at the core and hydrophilic cationic chains at the periphery to yield 20.5 ± 6.5 nm in size, strong blue-fluorescent nanoparticles in water. Electrostatic binding of these cationic-TPE nanoparticles on the *E. coli* membrane exhibited remarkable antimicrobial activity along with “lighting up” phenomena by

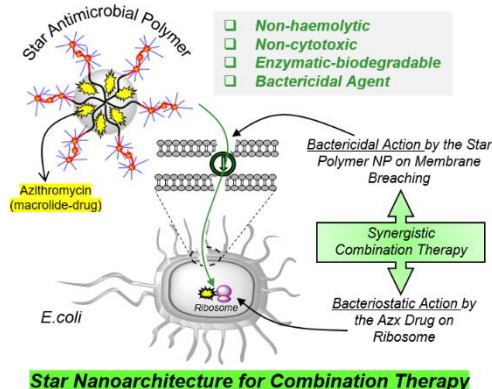


exhibiting significant fluorescence signal enhancement upon binding to the bacterial membrane and enabled the real-time visualization and quantification of membrane disruption by confocal microscopic image readout. Membrane-binding fluorescent signal enhancement in the TPE polymer substantiated its role as

excellent energy donor and its ability to in-situ gate-open for CV staining (as acceptor) brought the TPE-CV chromophores at appropriate nano-confinement in the peptidoglycan layer and facilitated the efficient photoexcitation energy transfer for the occurrence of FRET. This FRET probe is principally non-functional in solution (or in media) and exclusively active only on bacterial membrane, and thus, it opened new avenue for the development of real-time confocal image-based assay by direct visualization and quantification of bactericidal activity. Detail photophysical studies were done to establish the functioning of the FRET probe and selective photoexcitation in confocal microscope imaging protocols were optimized to demonstrate the proof-of-concept successfully in gram negative bacteria *E. coli*. Time dependent confocal microscopic assay revealed that the FRET probe was efficient in membrane disruption and nearly 90 % killing was accomplished in less than 4 h.

In chapter 4, the role of polymer topology control is investigated in detail by carefully designed 1D rod-shape (linear) and 3D star-shaped diblock (non-linear) core-shell cationic polymers of similar molecular weights, repeating units, size, morphology, and hydrophilic/hydrophobic content. These two different polymeric topologies were explored to understand the effect of chain architecture on infiltrating the biological membranes. Even though, minimum inhibitory action (MIC_{98}) for both the architectures were similar, star architecture was found to be less toxic to WT-MEF cell lines (mammalian cells) and RBCs.

Moreover, effective loading of a hydrophilic bioprobe (HPTS) facilitated direct imaging of *E.coli* cells while encapsulation of hydrophobic clinical broad-spectrum bacteriostatic macrolide antibiotic (azithromycin) maximized the antibacterial action impact via synergistic combination therapy. The development of such polytherapy treatment builds the foundation for substantial enhancement of drug efficacy along with reduced drug resistance by reducing their systemic dose amount.



The tailor-made biodegradable polymer structures have been studied extensively on gram negative bacteria *E.coli* and the study could not be expanded to other microbes like gram positive (*S. aureus*) due to the non-availability of facilities to handle the infectious bacterial strains. Nevertheless, the in-depth knowledge generated in this thesis work towards the electrostatic adherence of positively charged polymer NP on the negatively charged bacterial membrane would open up new avenue for futuristic engineering of smart macromolecular systems in biomaterial research. The PCL linear and star-shaped antimicrobial polymers and the bacterial-membrane reinforced FRET probe development in this thesis work, in principle could be expanded to many other biodegradable systems to study for both fundamental understanding as well as making therapeutic formulation in improving the treatment of infectious diseases in long-term.

TABLE OF CONTENTS

Chapter 1: Introduction

1.1 Antimicrobial Research in Health Care	2
1.2 Small molecule based Clinical Antibacterial Therapeutics	6
1.3 Antimicrobial Peptides (AMPs)	9
1.4 Polymer based antimicrobial agents	11
1.5 Biodegradable antibacterial polymers	21
1.6 Aim of the thesis	35

Chapter 2: Development of Polycaprolactone based Biodegradable Antimicrobial Polymers

2.1 Introduction	40
2.2 Experimental Methods	43
2.3 Result and Discussion	57
2.4 Conclusion	89

Chapter 3: Theranostic FRET gate to Visualize and Quantify Bacterial Membrane Breaching

3.1 Introduction	92
3.2 Experimental Methods	96
3.3 Result and Discussion	108
3.4 Conclusion	140

Chapter 4: Tweaking Polymer Topology for Combination Therapy in Bacterial Research

4.1 Introduction	144
4.2 Experimental Methods	149
4.3 Result and Discussion	156
4.4 Conclusion	181

5. Overall Conclusion and Future Direction

6. List of Publication

7. References

8. Animal Usage Approvals

9. Appendix

Introduction

1.1 Antimicrobial Research in Health Care

The study of interactions between prokaryotic and eukaryotic organisms is fundamental to understand their coexistence, ecology, and productivity. These interactions could be symbiotic (a mutually beneficial relationship), parasitic (one organism feeding on another organism) or commensal (one organism gains benefit while another is neither benefitted nor harmed) are extremely important for their metabolic and regulatory processes.¹ Herein, symbolic examples of various micro and macro-organisms are shown organized based on their relative sizes as shown in Figure 1.1.

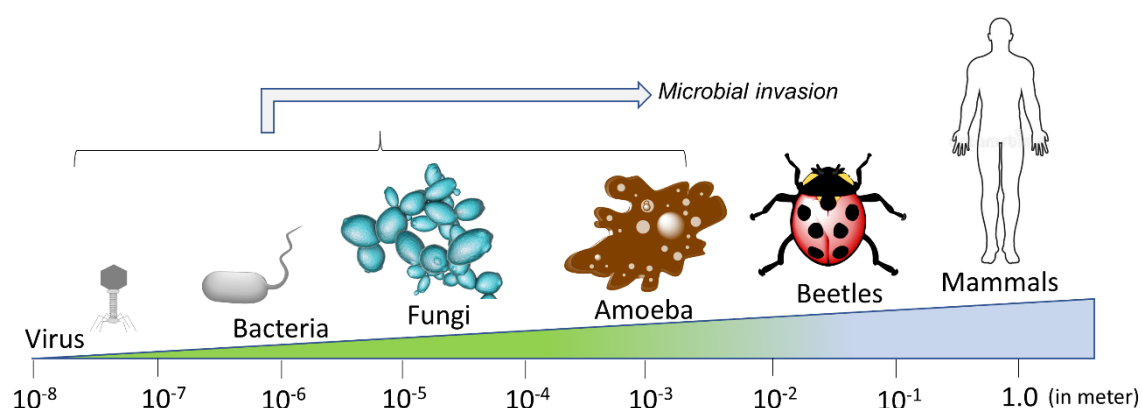


Figure 1.1: Schematic representation of various organisms based on their sizes.

The growing inflation of microbial invasion in various aspects of environment has primarily infiltrated global health care at large.² According to a recent report in science news, resistant bacterial infections are leading cause of death around the world wherein around 1.27 million deaths were estimated in 2019 due to antimicrobial resistance which was more than HIV or malaria.³⁻⁵ Antimicrobial resistance (AMR) to frontline antibiotics is a gruesome concern as it would pose a risk to infections which were earlier routinely abolished by drugs but will not be effective in the present.⁶⁻⁹ *Escherichia coli* (*E.coli*), *Klebsiella pneumoniae* and *Staphylococcus aureus* are among the top three bacteria responsible for fatal drug-resistant infections. A photograph of *Klebsiella pneumoniae*, one of the most infectious bacterial resistant culture grown in a culture dish is shown in Figure 1.2. These pathogens along with other hospital-acquired infections (HAIs) causes non-treatable infections to patients with weakened immune systems causing diseases such as pneumonia, tuberculosis, diarrhea and infections of the blood, wounds, and the urinary tract.¹⁰⁻¹² For almost every antibiotic, there has been a steady decrease in drug susceptibility with time. If the present trend continues,

world would slowly move towards “post-antibiotic” era where AMR would claim lives as much as 10 million by 2050 (as shown in Figure 1.3).¹³



Figure 1.2: A photograph of culture dish showing *Klebsiella pneumoniae* as one of the bacteria responsible for fatal drug-resistant infections worldwide in 2019. (Adapted from <https://www.sciencenews.org/article/antimicrobial-resistance-cause-death-antibiotic-bacteria>)

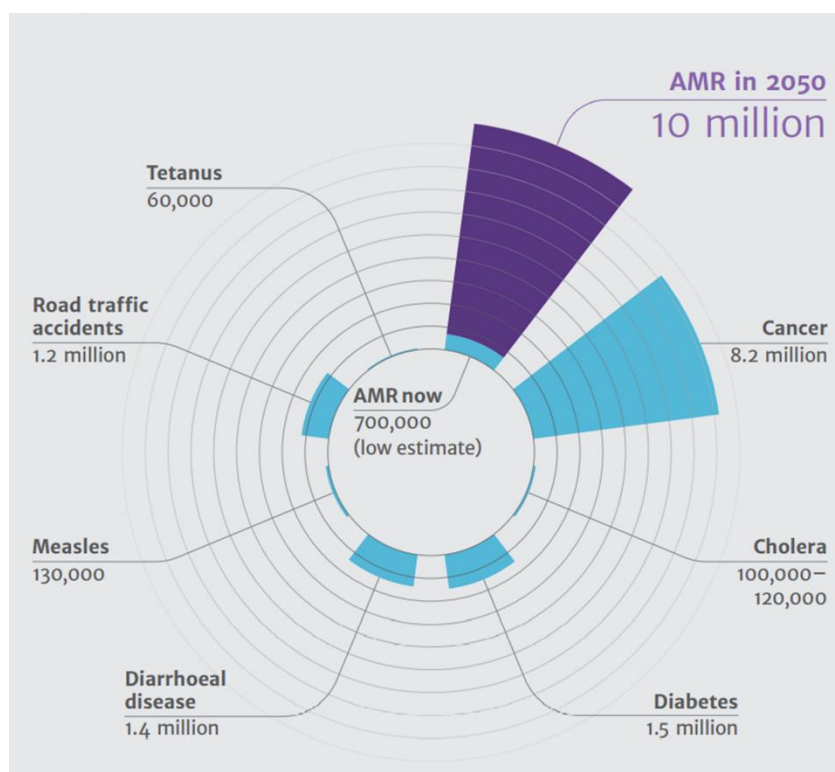


Figure 1.3: Deaths attributable to AMR every year, comparable to major causes of deaths (adapted from *The Review on Antimicrobial Resistance, 2014* Chaired by Jim O’Neill, Wellcome Trust)

This emphasized that funding and promoting antimicrobial research for the development of new antibacterial drugs along with novel approaches in understanding drug resistant mechanisms of bacterial infection, enhancing host defense system, combination therapy of drugs are primary steps required to be taken towards this problem. Different antimicrobial agents in the market can be broadly classified into three different generations based on their developmental sequence as shown in Figure 1.4. The first generation comprises of clinical antibiotics which are extremely efficient antimicrobial agents. But due to their singular mode of action, it is easy for microbes to develop resistance against them. This is mainly due to the fast-doubling time of micro-organisms. For example; in case of *E. coli*, the doubling time is around twenty minutes under optimum conditions, which means in an hour there would be 3 generations (n). So, in six hours, the number of generations would be $6 \times 3 = 18$ generations and the number of cells would be 2.5×10^5 cells, according to the formula $F_i = F \times n$ wherein, F_i = Total number of cells, F = initial number of cells and n = number of generations. As the time progresses, there is a fast exponential growth in cells which increases the chances of mutations and inclusion of resistant genes in the affected populations which leads to reduced drug efficacy.

The next generation comprises of natural antimicrobial peptides (AMPs) which are also extremely efficient and since their mode of action is generally membrane disintegration, it is difficult to develop resistance against them. They are extremely effective but due to their toxicity to RBCs, mammalian cell lines, unwanted interaction to blood protein, immunogenicity, nephrotoxicity, and cardiotoxicity their activity is compromised.¹⁴ To compensate the same, synthetic antimicrobial agents came into existence which comprises third generation antibacterial agents. They are generally composed of cationic amphiphilic macromolecules which are efficient antimicrobial agents as wells as carriers to encapsulate antimicrobial drugs (generation first) in their nano-cavity to show dual action for synergistic combination therapy. The opportunity to tune their architecture in order to bring in additional properties is an added advantage towards these antimicrobial agents. They can be broadly divided into two categories biodegradable polymers which are prone to undergo microbial degradation and non-biodegradable polymers which are made up of non-degrading materials. A few examples of synthetic antimicrobial agents are shown in Figure 1.4. This thesis provides a detail explanation for the antimicrobial agents.

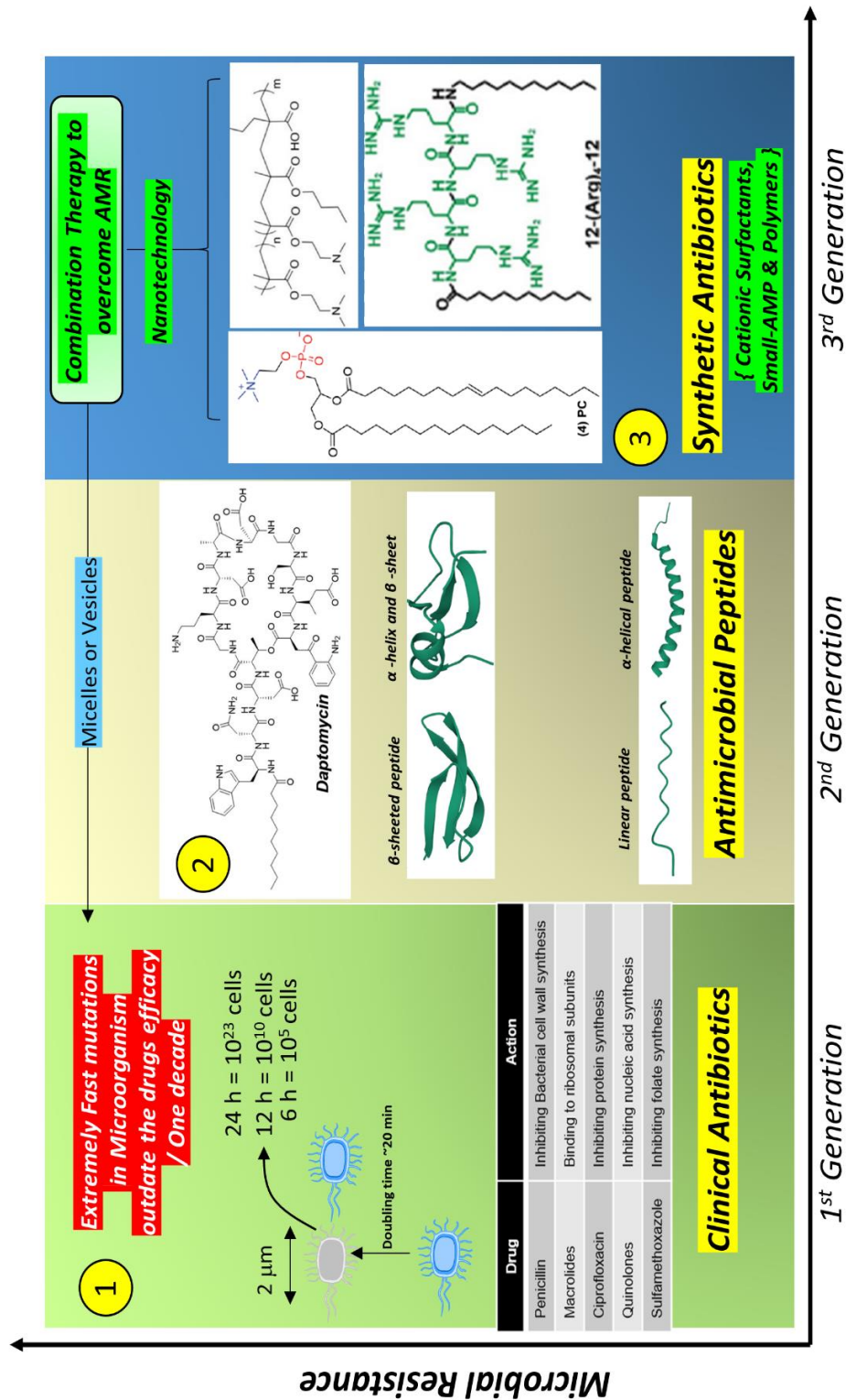


Figure 1.4. Plot showing various antimicrobial agents categorized into different generations based on their advancement and complexity (1) Small molecule based clinical antibiotics comprises generation 1 (2) Antimicrobial peptides (AMPs) comprised generation 2 (3) Polymer based macromolecules comprises generation 3 along with their combination therapy with drugs.

1.2. Small molecule based Clinical Antibacterial Therapeutics

The first-generation antibacterial agents basically comprise of small molecule based clinical antibiotics categorized as penicillin, macrolides, ciprofloxacin, quinolones etc. They are extremely efficient classes of antibiotics with a specific mode of action which involves inhibiting one or the other cellular machinery for bacterial inhibition.^{15,16} But unfortunately, due to extremely fast mutations in micro-organisms, their efficacy gets compromised within a decade which gives rise to antimicrobial resistance.¹⁷⁻²⁰ The antibacterial agents are survival vectors against microbial invasion and they can be divided based on their target site. For example, quinolones inhibit the DNA synthesis by targeting catalytic activity of topoisomerase IV and DNA gyrase.²¹ Rifamycin are responsible for inhibiting RNA synthesis²², Sulfonamides interfere with folic acid synthesis due to their structural similarity to para-amino benzoic acid (PABA).²³ Penicillin and Cephalosporins target bacterial cell wall synthesis machinery by inhibiting the action of penicillin binding proteins (PBPs)²⁴, Polymyxins disrupt the cell membrane integrity so on and so forth.²⁵ (see Figure 1.5a).

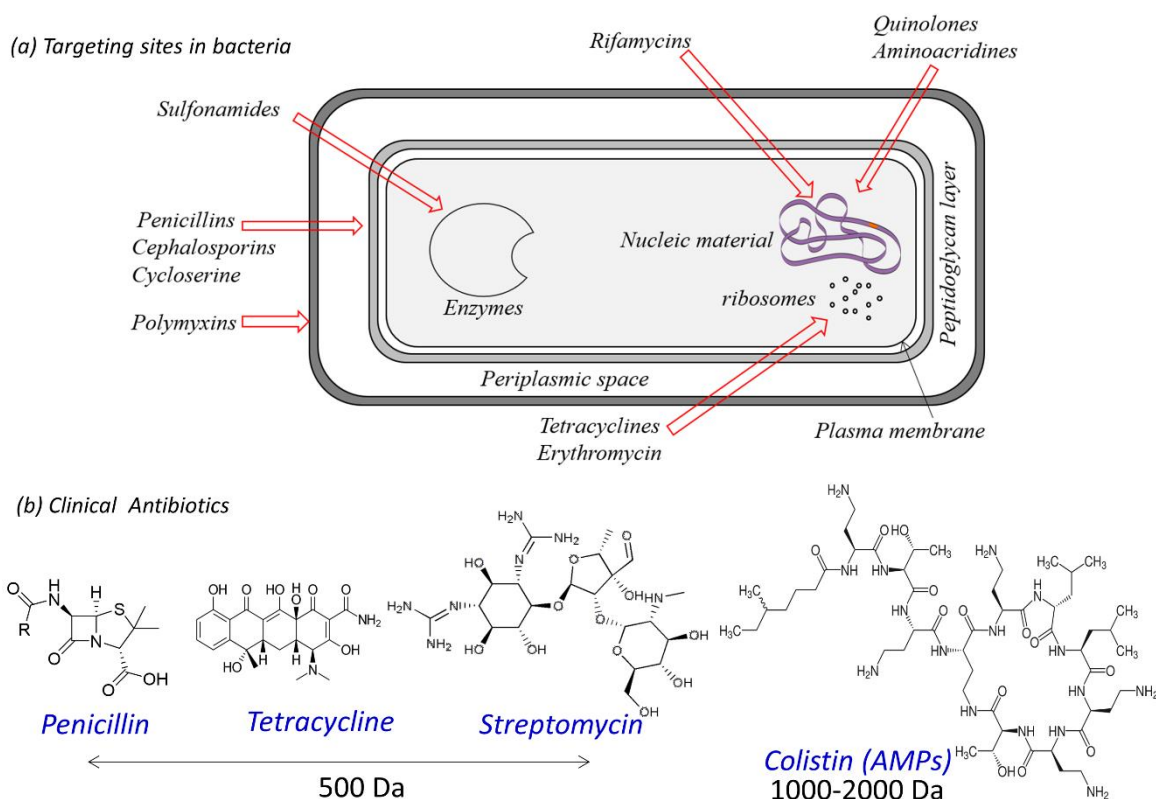


Figure 1.5. (a) Diagrammatic representation of bacterial cell along with different antibiotics and their specific target sites. (b) Structural representation of few clinical antibiotics differentiated based on their molecular weights.

Based on molecular weights, antibiotics can be characterized into two categories, compounds in the size range of 500 Da (including Penicillin, Streptomycin, Tetracycline) and secondly compounds with higher molecular weight which includes cationic oligopeptides i.e., antimicrobial peptides (AMPs) which are in the size range of 1000-2000 Da as shown in Figure 1.5b. Some of the recent breakthrough advancements in understanding the mechanism of action of few antibiotics is described. Beta-lactam Antibiotics are one of the most-widely used therapeutic class of medicine. They comprise a group of antibiotics which include beta-lactam ring in their structure i.e., Penicillin, cephalosporin, and other beta-lactams. They are known to disrupt peptidoglycan (PG) biogenesis by targeting penicillin binding protein (PBPs) which are responsible for cell wall synthesis. Their primary target involves covalently binding and modifying transpeptidase (TP) active site of PBPs which induces imbalance in the activities of PG synthases and PG hydrolases enzymes and ultimately leads to cell lysis.²⁶ It has been recently developed that beyond simply blocking the active site of transpeptidase, these beta lactams cause cell wall degradation by inducing depletion of cellular PG precursors.

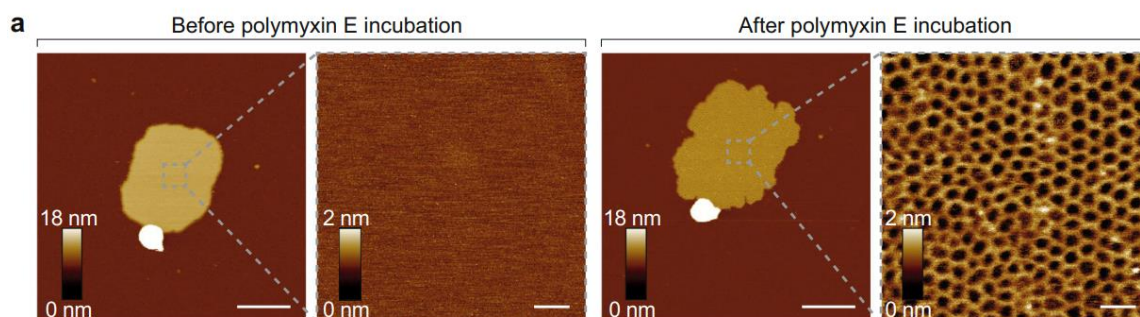


Figure 1.6: Polymyxin forms two-dimensional crystalline structures in bacterial outer membranes that correlate with antimicrobial activity. Scale bars, 200 nm (overview) and 20 nm (zoom-in). (adapted from *Nat. Commun.* **2022**, 13, 6195)

One interesting example includes petrification of bacterial membrane by polymyxins. Polymyxins (Polymyxin E, also known as colistin and polymyxin B) are a class of antibiotics which are last-resorts against multi-drug resistant infections. They are known to interact with lipopolysaccharide (LPS) through cationic side chains and hydrophobic interactions which would alter the outer membrane stability due to replacement of divalent cationic (Mg^{2+} and Ca^{2+}) from its surface.²⁷ This would destabilize and permeabilize the outer membrane followed by its progression into LPS molecules at inner membrane and ultimately lead to cell death.

It has been recently discovered that polymyxin crystallizes the cell membrane of bacteria (see Figure 1.6).²⁷ It forms hexagonal crystals with LPS and divalent cations, which converts the flexible bilayer membrane to thin brittle sheets which would make them vulnerable and easy to break. In order to elucidate resistance mechanisms in bacteria, fluorophore conjugated antibiotics are extremely helpful. Through this class of antibiotics, drug target interactions can be tracked at microscopic level (at bacterial surface) and macroscopic level (within organelles, whole cells as well as entire animals). The four major applications of fluorescent antibiotics involve resistance studies, toxicity studies, determination of mode of action via target visualization and diagnosis. One example of each of these applications are shown in Figure 1.7.²⁸ In order to access drug accumulation in wild type and mutant *Escherichia coli*, fluorescent trimethoprim (TMP) conjugates were utilized. Trimethoprim was covalently conjugated to three comparative fluorophores, 7-(dimethylamino)-coumarin-4-acetic acid (DMACA), 7-nitrobenzofurazan (NBD), and dansyl (DNS, dimethylaminosulfonyl).²⁹ They were treated with wild type *E. coli* and efflux pump deficient *E. coli* mutant strain.

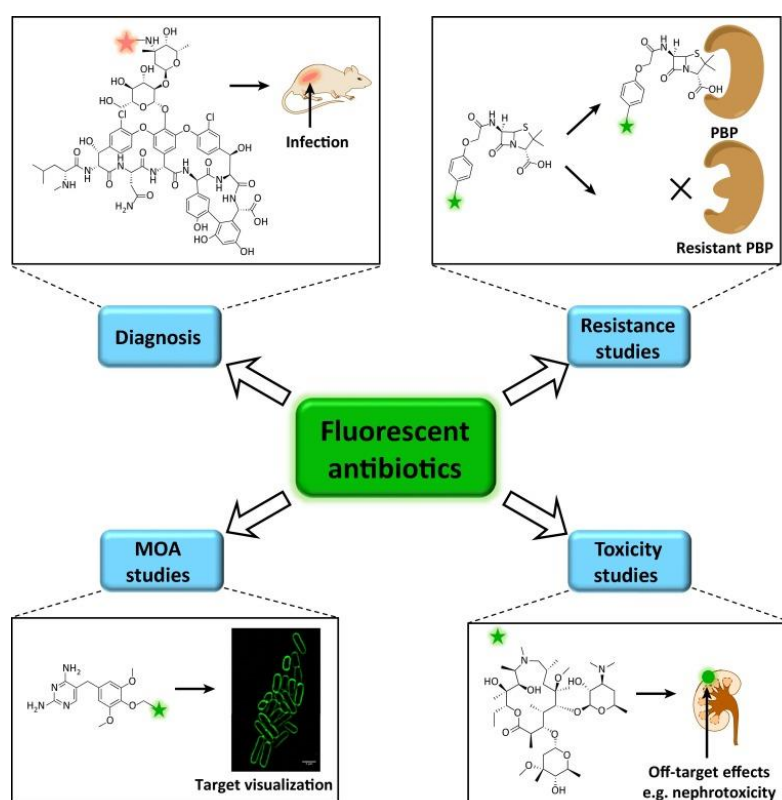


Figure 1.7: Visual representation of various applications of fluorescent antibiotics, including: study of bacterial resistance, examining toxicity, determination of mode of action (MOA) and diagnostic imaging of bacterial infection. (adapted from Trends Biotechnol. **2018**, 36, 523-536)

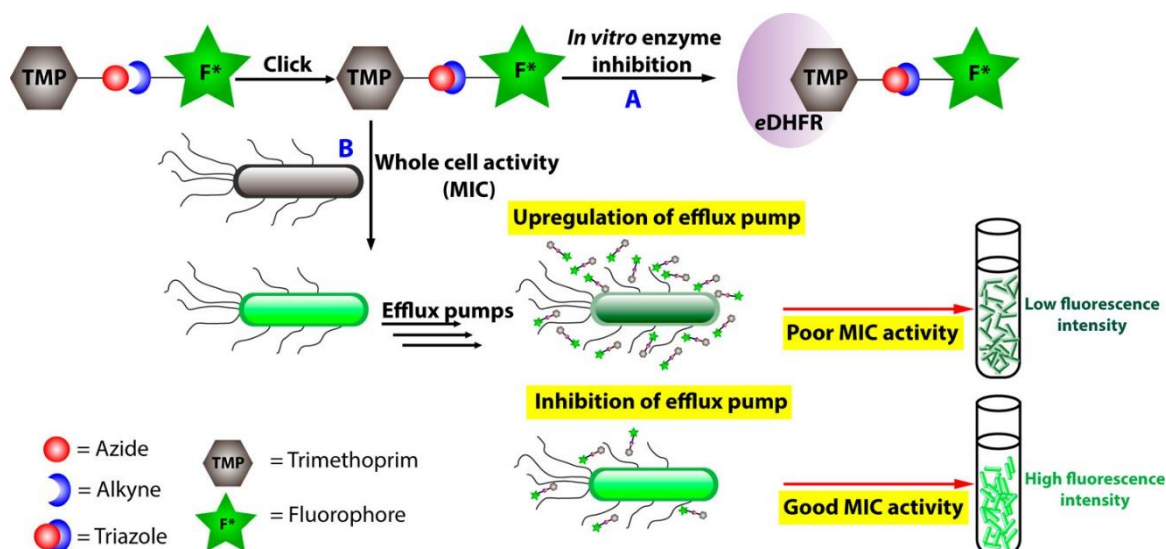


Figure 1.8: Use of fluorescently labeled antibiotics to assess efflux in Gram-negative bacteria without cell lysis: (A) *in vitro* enzyme inhibition (IC₅₀), enzyme binding and inhibition by fluorescent antibiotic; (B) whole cell activity (MIC), fluorescent probe loses MIC activity due to efflux pumps. Fluorescence intensity can quantify the efflux effect. (adapted from ACS Infect. Dis. **2016**, 2, 688–701)

It was observed that fluorophore conjugated TMP probe showed *in vitro* inhibition of dihydrofolate reductase (eDHFR) which confirmed the retention of drug's activity upon fluorophore conjugation (as shown in Figure 1.8).²⁹ Incubation of TMP with two different strains of *E. coli* at MIC concentration, revealed efflux pump dependent change in fluorescence intensity of fluorophore. In case of *W.T. E. coli* wherein efflux pumps were upregulated, less fluorescence intensity was observed due to poor accumulation of drug. For efflux pump deficient *E. coli* mutant, high fluorescence intensity was observed due to retention of drug. Another example includes, fluorescently labelled macrolide to visualize cellular and tissue distribution of azithromycin.³⁰

1.3. Antimicrobial Peptides (AMPs)

AMPs are another class of naturally occurring antibiotics which are also a part of our innate immune system. They have a broad activity to kill fungi, bacteria, viruses and even cancer cells.³¹⁻³⁴ Lysozyme is the first reported human antimicrobial protein which was identified by Alexander Fleming in 1922.³¹ Cationic AMPs consist of 10 to 50 amino acids with cationic and hydrophobic residues facing opposite sides of three-dimensional structure. These are water soluble oligopeptides due to positive charge (generally +2mV to +9mV). They represent three different basic secondary structures: a) alpha helix, b) beta sheet and c) extended AMPs. Alpha-helical AMPs include magainin, LL37 etc.^{35,36} which does not

exhibit secondary structure in aqueous environment but in non-polar environment, like bacterial membrane. Beta-sheet structure in defensins and bactenecins are stabilized by disulfide bonds. Extended AMPs does not contain a particular secondary structure but rich in amino acids like histidine, arginine, glycine, or tryptophan, for example indolicidin.

The antimicrobial action is due to the presence of cationic and hydrophobic residues. The positive charge ensures accumulation on the polyanionic surface of bacterial membrane due to the presence of acidic units such as lipopolysaccharides and teichoic acids in case of gram-negative and gram-positive cells respectively.³¹ Once they interact with the anionic surface of the cytoplasmic membrane, the hydrophobic part of the antimicrobial peptide starts disrupting the membrane through one of the known models for membrane disruption as shown in Figure 1.9.

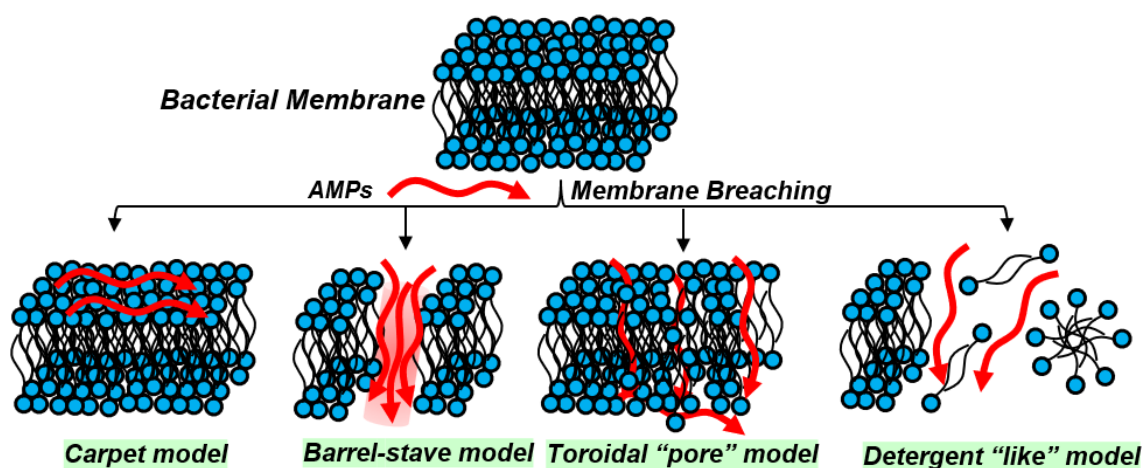


Figure 1.9: Schematic Representation of different models by which antimicrobial peptides (AMPs) disrupt bacterial membrane

In case of carpet model, there is a distribution of individual peptide chains on the surface of bacterial membrane in horizontal position which results in swamping of membrane charge by a “carpet” of peptides at the surface while for barrel-stave model, individual peptide chains arrange vertically across the bilayer membrane which give rise to formation of barrel-like assembly as shown in Figure 1.9. Toroidal “pore” formation leads to membrane cleavage via formation of pores by individual chains while formation of detergent-like dissolution of patches of membrane is predicted in detergent “like” model.³² Few examples of AMPs include nisin, gramicidin, defensins, thionin, indolicidin etc. The widespread development of resistance against AMPs by bacterial colonies is unlikely as compared to small molecule-based antibiotics like penicillin. AMPs attacks low-affinity

targets (bacterial membrane) as compared to high-affinity targets of conventional antibiotics.³⁷ Therefore, it is difficult and more challenging for bacteria to develop a single resistance mechanism and defend themselves by changing the composition of membrane and reorganize its lipids. Moreover, according to recently published reports, AMPs are not only categorized as potent antibiotics but also efficient modulators of inflammatory responses to bacterial toxins.^{38,39} It is important to control pro inflammatory responses because excessive production of proinflammatory responses can lead to harmful consequences like sepsis. For example, in human body, AMPs like LL-37 and bovine lactoferricin are reported to reduce secretion of TNF-alpha and IL-6 during recognition of foreign endotoxins like lipopolysaccharides (LPS). Moreover, LL-37 also limits the secretion of cytokines like TNF-alpha, IL-1beta, IL-6 and IL-8 from primary monocytes during an unwanted invasion of lipopolysaccharide (LPS) and lipoteichoic acid (LTA).

Although many AMPs are at various clinical trials such as polymyxin, colistin, daptomycin, gramicidin etc. their clinical success has been limited due to various factors like in vivo toxicity, difficulty in industrial scalability and unwanted protease degradation. With an exception of polymyxin, AMPs are more prone to induce nephrotoxicity and neurotoxicity during their intravenous administration.⁴⁰ Due to tedious synthesis, it is difficult to increase the scalability of AMPs in industrial scale.^{40,37} One of the most common disadvantages of natural peptides is their lability to proteases. Enzymes like chymotrypsin can lead to unwanted degradation which would reflect on their pharmacokinetics.³⁷

1.4 Polymer based Antimicrobial agents

This is a class of artificial antimicrobial agent which self-assemble into nanoparticles in the size range of 10-200 nm. They are positively charged polymeric nanoparticles with hydrophobic component responsible for membrane disruption action. They are extremely stable in aqueous environment due to positive charge (as shown in Figure 1.10). Moreover, due to larger hydrophobic cavity, they can easily accommodate drugs and dyes in their nanocavity and can be used for combination therapy or as theranostic agents respectively (as shown in Figure 1.4).⁴¹⁻⁴⁵ In these kinds of macromolecular architectures, we have the flexibility to tune their structure and thus could make them more biocompatible and less hemolytic. These polymeric biomaterials can be used as antimicrobial hydrogel⁴⁶⁻⁴⁹, antimicrobial coatings⁵⁰⁻⁵⁴, antimicrobial adjuvants⁵⁵⁻⁵⁹, immunomodulating and antibiofilm agents^{42, 60-63} for preventing microbial infections. For the treatment of topical infection,

hydrogels are best scaffold as they have high water retention ability and biocompatibility⁴⁸. Polymeric adjuvants are widely investigated along with antibiotics to increase the permeability of gram-negative bacteria as well as inhibiting the efflux pumps. Moreover, they are also recognized as immunomodulators as they control the release of pro-inflammatory cytokines such as TNF- α and IL-6. The over-expression of such cytokines leads to hyperinflammation and can lead to sepsis. The classification of these antimicrobial agents based on their molecular weights are shown in Figure 1.10.

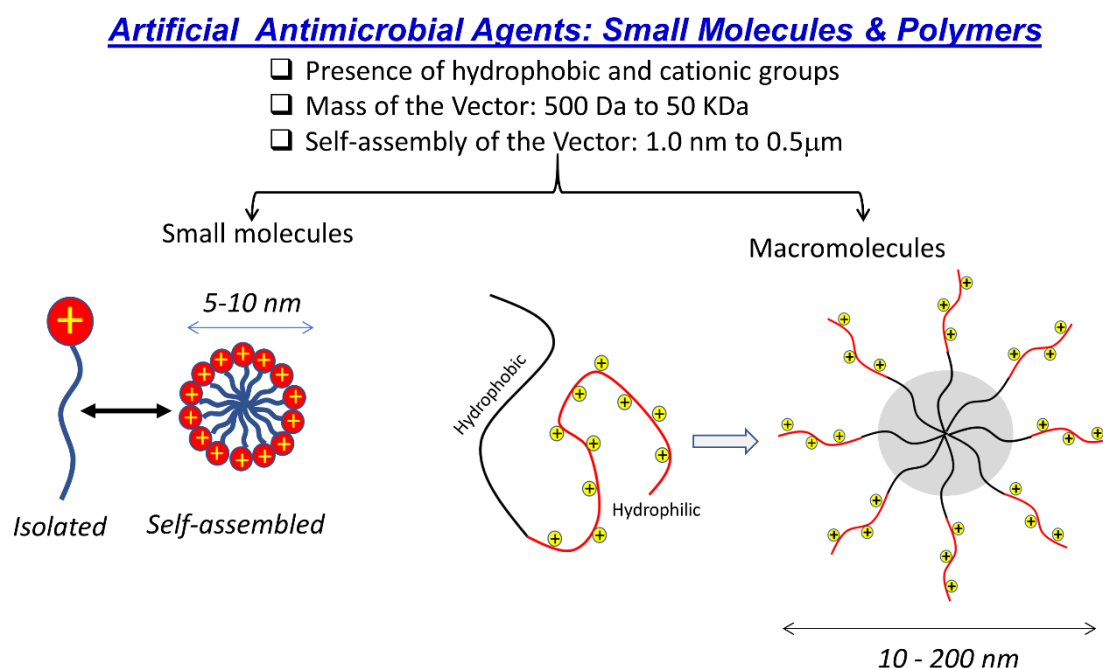


Figure 1.10: Diagrammatic representation of two broadly classified types of artificial antimicrobial agents: (a) Small amphiphilic molecules and (b) Large amphiphilic macromolecules

Small Amphiphilic molecules are a class of synthetic antimicrobial agents which are mimics of AMPs but since they are man-made, they are found to be less toxic and omits the shortcomings of natural AMPs. They can show antimicrobial activity in free as well as in self-assembled state as shown in Figure 1.10. In 2014, Yinfeng et al. developed a series of truncated derivatives containing 24 residues of amino acids known as GI24 of antimicrobial peptide PMAP-36.⁶⁴ These derivatives were compared to a clinically relevant AMP, melittin and it was observed that they were more antibacterial than melittin (MIC in the range of 1 to 4 μ M) with extremely low hemolytic activity. The membrane permeabilizing action of GI24 was determined by SEM and TEM techniques and the micrographs show that GI24 shows bacterial disruption through membrane disrupting action (see Figure 1.11). Upon comparing

GI24 with PMAP-36 and melittin, it was observed that GI24 treated cells showed more disrupted cells with visible pores on the surface.

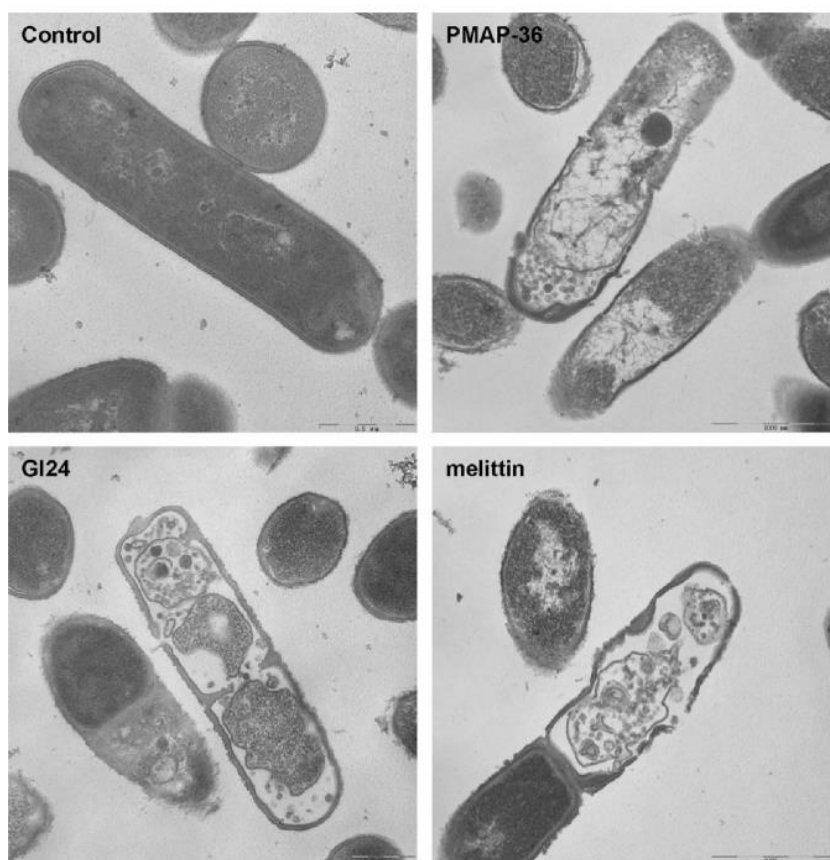
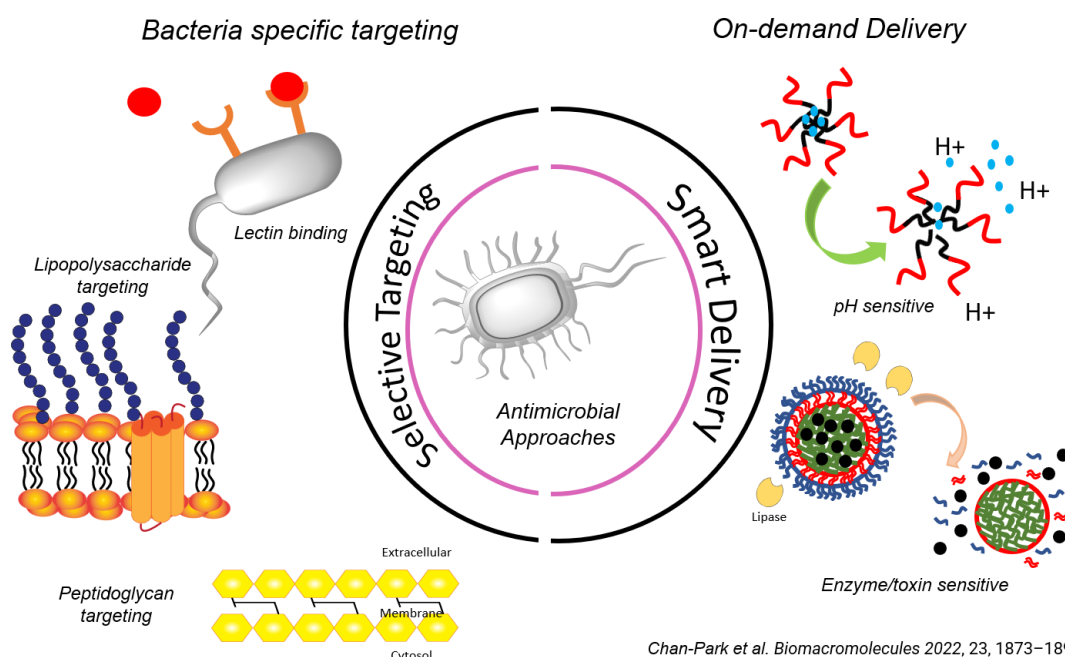


Figure 1.11: Transmission electron microscope micrographs of *E.coli* cells treated with various peptides and control (no treatment) (adapter from PLoS One 2014, 9 , e86364)

Lucia et al. developed an antibacterial WMR peptide which was a modified sequence of myxinidin.⁶⁵ In order to induce self-assembly, WMR was also attached to a peptide sequence (AAAAAAA) with a hydrophobic tail. Induction of self-assembly is extremely important to reduce cellular toxicity and hemolytic activity. These designed polymers self-assembled onto fibers and eradicated the formation of biofilms of *Pseudomonas aeruginosa* and *Candida albicans*. The importance of various self-assembled structures were further determined by Nathalia et al. wherein it was observed that micellar self-assembled nanoparticles are more effective with lower MIC values than rod-like structures.⁶⁶ They further concluded that parameters such as charge and hydrophobicity plays an important role in correlating such structure-activity relationships. Synthetic amphipathic sulfono- γ -AApeptides were developed by Jianfeng Cai and coworkers to mimic magainin 2 and exhibit bactericidal activity.⁶⁷ These smart antimicrobial agents are multifunctional in nature due to

various bacteria specific targeting sites and on-demand delivery options due to tunability in stimuli responsiveness. Bacteria specific targeting can be divided into three major targeting sites which includes (i) lectin binding site, (ii) lipopolysaccharide binding site and (iii) peptidoglycan targeting site as shown in Figure 1.12.



Chan-Park et al. *Biomacromolecules* 2022, 23, 1873–1891

Figure 1.12: Smart antimicrobial designs by bacterial-specific targeting and on-demand delivery approaches. (Adapted from *Biomacromolecules* 2022, 23, 1873–1891)

Lectin binding: Lectins are proteins which are responsible for adhesion of bacteria to host cells. These are carbohydrate binding proteins and due to their abundance in bacterial envelope, they are promising targeting sites. For example, FimH are proteins which are present on the tip of *E. coli* fimbriae and they are widely explored as targeting sites for mannose and glucose.⁶⁸ In case of *P. aeruginosa*, lectins such as LecA and LecB show high binding affinity for galactose and fucose. So, it can be summarized that glycan functionalized molecules can be used for lectins on bacteria.⁶⁹

Lipopolysaccharide (LPS) binding: LPS are major component of gram-negative bacteria's outer membrane. Its three major components are O-antigen, the core region and lipid A. The negatively charged phosphate head group in the inner core region is responsible for anionic charge on the membrane. LPS is also a highly toxic endotoxin which triggers immunogenic and inflammatory response in humans. It has been understood that cholic acid

derivatives bind strongly to LPS.⁷⁰ Pegylated antimicrobial peptide KYE28 is known to conjugate with LPS.⁷¹

Peptidoglycan targeting molecules: β -lactam containing antibiotics are known to target transpeptidase activity of peptidoglycan binding proteins (PBPs) thus interfering with peptidoglycan stability.⁷² Moreover, peptidoglycan look alike substrates are also an efficient strategy to inhibit the bacterial growth. Recently, peptidoglycan oligomers (PGOs) are utilized for bacteria specific metabolic labelling.⁷³⁻⁷⁵ These oligomers are incorporated into the cell wall and easily polymerized through trans glycosylation. Bacteria-triggered activation of nano-formulations can be broadly classified into two different responsiveness which includes pH sensitivity and enzyme responsiveness.

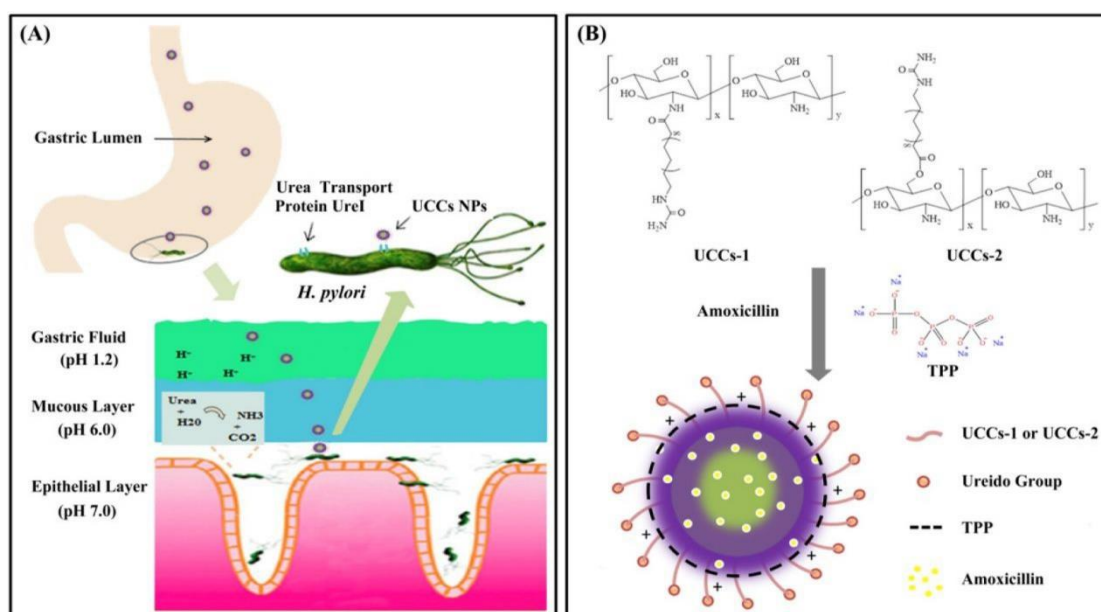


Figure 1.13: (A) *H. pylori* colonization and the strategy for eradicating *H. pylori* using the pH-sensitive ureido-conjugated chitosan/TPP targeted nanoparticles. (B) Representation of the prepared amoxicillin-loaded ureido-conjugated chitosan/TPP nanoparticles. (adapted from *Biomacromolecules* 2022, 23, 5, 1873–1891)

pH sensitivity: pH can be used as a stimulus for infected site targeted delivery as bacterial growth causes change in physiological pH. So, any pH responsive nanomaterial which would show swelling and deswelling with respect to change in pH can be used as pH responsive delivery system. For example, *Helicobacter pylori* is an infective bacterial strain which causes bacterial infection in stomach. It could sustain in the harsh acidic condition of stomach by increasing the local pH inside the mucosal layer by hydrolyzing urea to ammonia and bicarbonate.⁴⁴ So, it is extremely difficult to eradicate this infection as its colonies are

hidden beneath the mucus layer where it is in replicative phase which is completely inaccessible by antibiotics, while in the gastric acidic environment it exists in non-replicating coccoid state which is not susceptible by antibiotics (see Figure 1.13a). This suggests that in order to eradicate this infection pH dependent polymer/polypeptide is required to be tested. One of such examples include, poly(γ -glutamic acid)- graft-arginine (γ -PGA-g-Arg) zwitterionic polypeptide⁷⁶, which could self-assemble and load an antibiotic at pH below 3 through electrostatic complexation. But at neutral pH, due to deprotonation of free amines, self-assemblies are disrupted which leads to release of loaded cargo. This has helped to release cargo at neutral infection site and eradicate the infection as shown in Figure 1.13b.

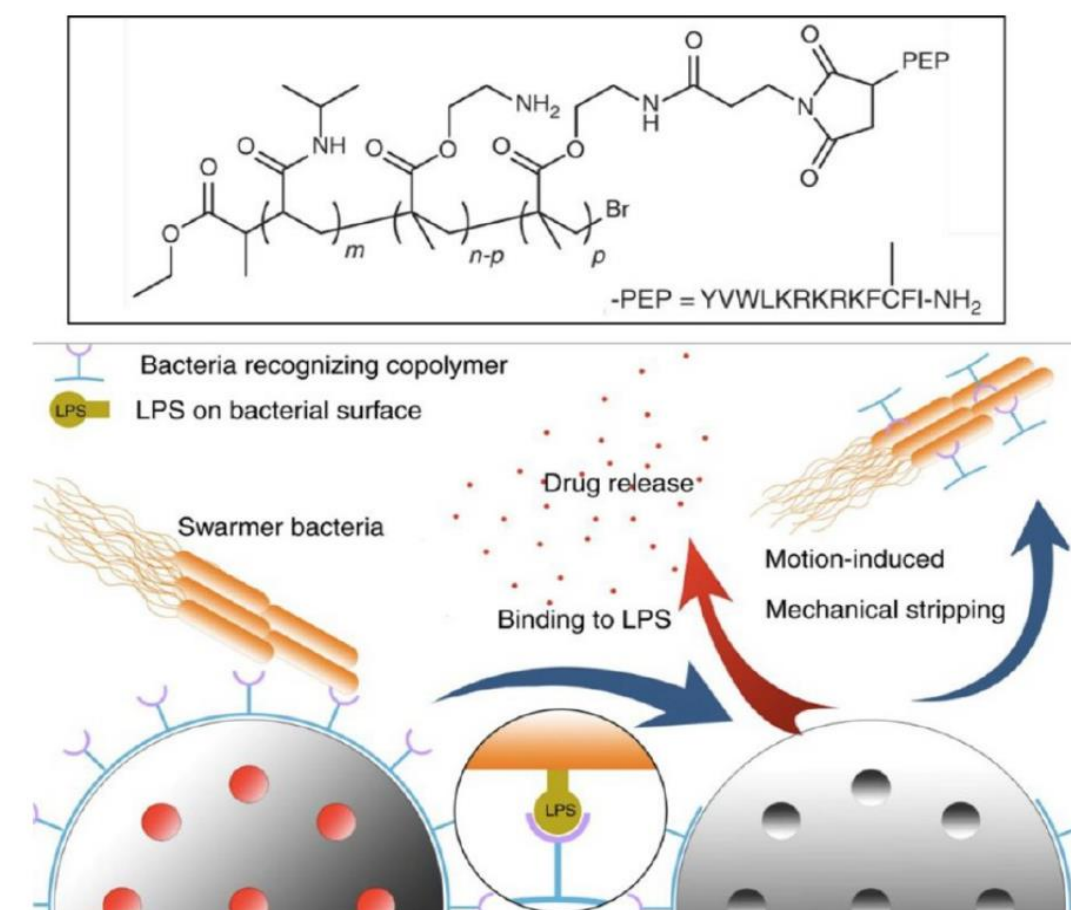


Figure 1.14: Lipopolysaccharide-affinity copolymer senses the rapid motility of swarmer bacteria to trigger antimicrobial drug release via a mechanism of motion-induced mechanical stripping (MIMS). (adapted from *Nat. Commun.* **2018**, 9, 4277)

Enzyme/toxin responsiveness: Drug release can also be controlled by release of bacterial toxins and enzymes in the affinity of a bacterial infection. Various virulence factors

produced by bacterial infections include LPS, exotoxins, lipases, gelatinase, hyaluronidase, metalloproteinase aureolysin, serine glutamyl endopeptidase, and cysteine proteinase staphopain A etc.⁷⁷⁻⁸³ One such example involves detection of LPS on the surface of gram-negative bacteria by polymer which would lead to release of antibiotics from the nano-assemblies as shown in Figure 1.14.⁷⁷ A copolymer pNIPAAm-co-pAEMA was coated on the surface of tobramycin-loaded mesoporous silica nanoparticles. Upon interacting with a moving gram-negative bacterium, the polymer would interact to LPS and ultimately “peel off” the polymer from silica surface which would lead to release of drug.

Polymers as drug adjuvants: Polymers are not only responsible as sole antibacterial agents but they can also work along with antibiotics as adjuvants to enhance their effectivity. Polymeric adjuvants can be broadly classified based on their two objectives: (a) Membrane permeabilizers and (b) Efflux pump inhibitors as shown in Figure 1.15.

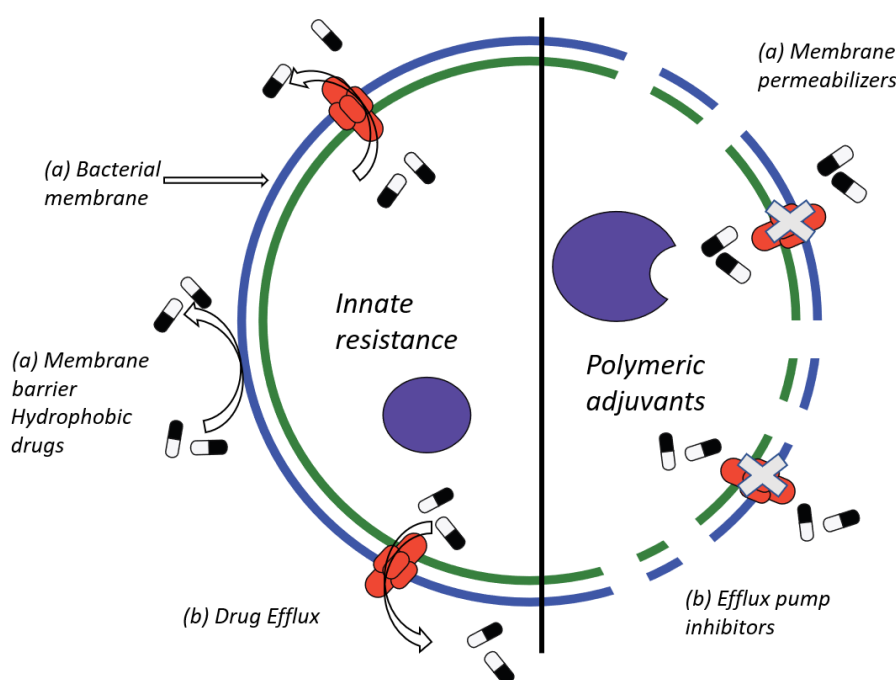


Figure 1.15: (a) Bacterial membrane-related intrinsic resistance mechanisms (left) and combination therapy with outer membrane permeabilizers and efflux pump inhibitors (right). (Adapted from Chem. Sci. **2022**, 13, 345–364)

Bacterial cells, especially gram-negative bacteria have intrinsic resistance to many small molecule-based antibiotics due to the presence of outer-membrane. Mostly small and hydrophilic molecules gain the access to cross the outer-membrane membrane through porin proteins present across the membrane. And even though, a hydrophilic drug crosses the

cytoplasmic membrane, its accumulation is highly restricted by presence of efflux pumps which pumps out foreign substances once they encounter the same. Efflux pumps are one of the major contributors of multi-drug resistant phenotypes.^{84,85} So, membrane permeabilizers and efflux pump inhibitors are extremely important to tackle multi-drug resistance.

(b) Chemical structures of antibiotic adjuvants

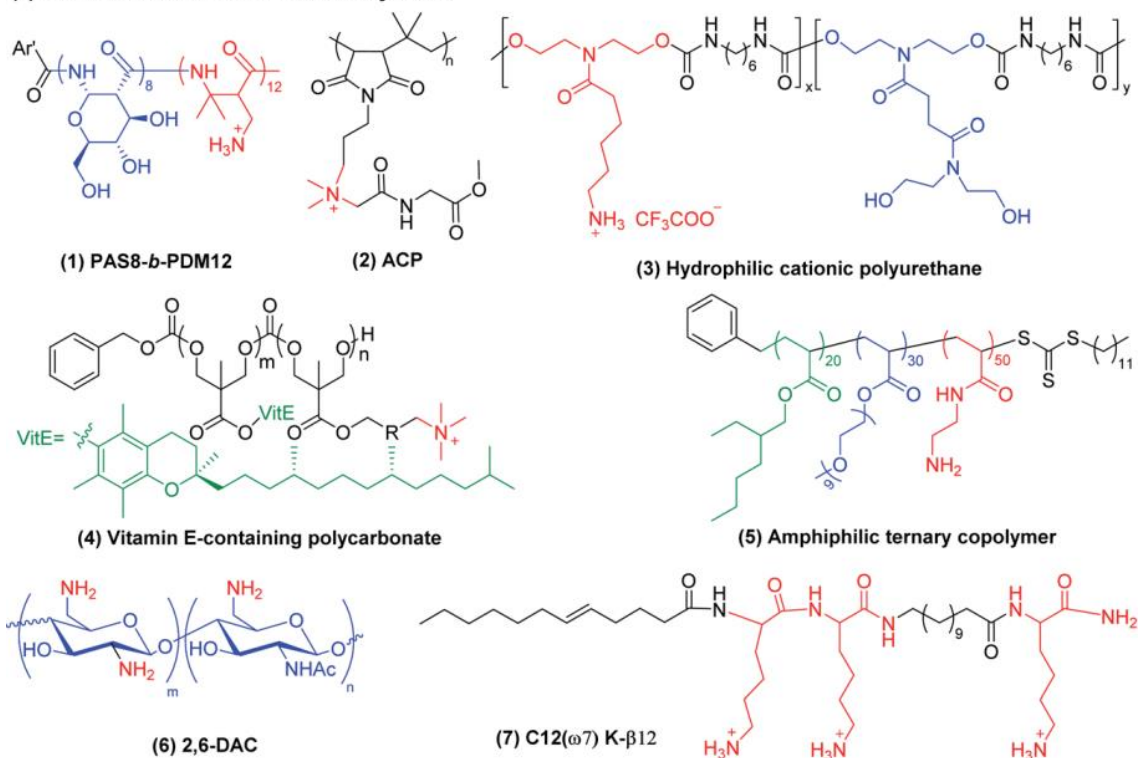


Figure 1.16: Chemical structures of some of the outer-membrane permeabilizers and efflux pump inhibitors. (Adapted from doi: Chem. Sci. 2022,13, 345-364)

Polymeric adjuvants also work as outer-membrane permeabilizers. Divalent cations form ionic bridges with anionic phosphate groups in lipid A. Cationic polymers replace the divalent cations like Ca^{2+} and Mg^{2+} from the outer-membrane and thus compromise the membrane integrity. Few of such examples are shown in Figure 1.16. A glycosylated block-co-beta-peptide polymer⁸⁶ (shown in Figure 1.16(1)) interacted with bacterial membrane with electrostatic and hydrogen bonding. A glycine conjugated polymer (as shown in Figure 1.16 (2)) showed more antibacterial activity as compared to rifampicin.⁸⁷ Not only the replacement of divalent cations, cationic polymers disrupt the bilayer membrane by producing pores in the membrane surface which would allow higher efflux of antibiotics into the bacterial cells. A synergistic killing has been demonstrated between amphiphilic ternary copolymer (structure shown in Figure 1.16 (5)) and doxycycline and colistin against *P.*

aeruginosa and *E. coli*.⁸⁸ Chan Park et al. demonstrated chitosan derivatives such as 2,6-diamino chitosan (see structure in Figure 1.16 (6)) can be utilized to enhance the activity of wide array of antibiotics such as amikacin, tobramycin, novobiocin and tazobactam by synergistically working towards lung infection models.⁸⁹ Moreover, they are also responsible for efflux pump inhibition. Efflux pumps are transmembrane proteins which are present across the bilayer membrane. They are responsible for “pumping out” antibacterial agents and toxins out of the bacterial cytoplasm. They are one of the most important antibacterial resistant phenotypes responsible for multi-drug resistance. β -lactams, macrolides, tetracyclines, quinolones, trimethoprim, sulfonamides are some of the most important antibiotics which are susceptible to efflux pump effects.⁹⁰⁻⁹⁵ Cationic polymers target bacterial cytoplasmic membrane due to which transmembrane proton motive force (PMF) gets disrupted. Most of the efflux pumps are powered by transmembrane proton motive force and due to disruption of cytoplasmic membrane, efflux pumps are inactivated. Due to this efflux pump inhibiting action of polymers, the accumulation of antibiotics in the bacterial cytoplasm is enhanced. One such example of cationic lipo- polypeptide is shown in Figure 1.16 (7).⁹⁶ This C12 chain containing polypeptide was able to potentiate tetracycline and erythromycin antibiotics by disrupting efflux pumps. The glycosylated co-beta-peptide polymer shown in Figure 1.16 (1)⁸⁶ was able to enhance the uptake of novobiocin through affecting efflux pumps in gram-negative bacteria.

Polymers as antibiofilm agents: Biofilm is a community of micro-organisms which are embedded in extracellular polymer matrix (EPS) which is a self-produced polymeric substance that provides them resistance to unfavorable environmental conditions.^{97, 98} It has been depicted by UN National Institutes of Health that more than 80% of hospital-acquired infections are bio-film related.⁹⁹ The maturation of a biofilm can be divided into two consecutive steps” Firstly, the bacteria attach itself to a favorable substrate and secondly there is a 3-D growth of matrix as shown in Figure 1.17. As compared to a planktonic bacterium (freely floating), biofilm bacteria are 10-1000 times more resistant to antibiotics.¹⁰⁰ In order to eradicate the initial bacteria attachment, hydrophilic non-fouling surfaces are extensively used while for eradication of mature biofilms, cationic polymers with lipophilic entities play significant role in antibiofilm action.

(a) Biofilm Formation

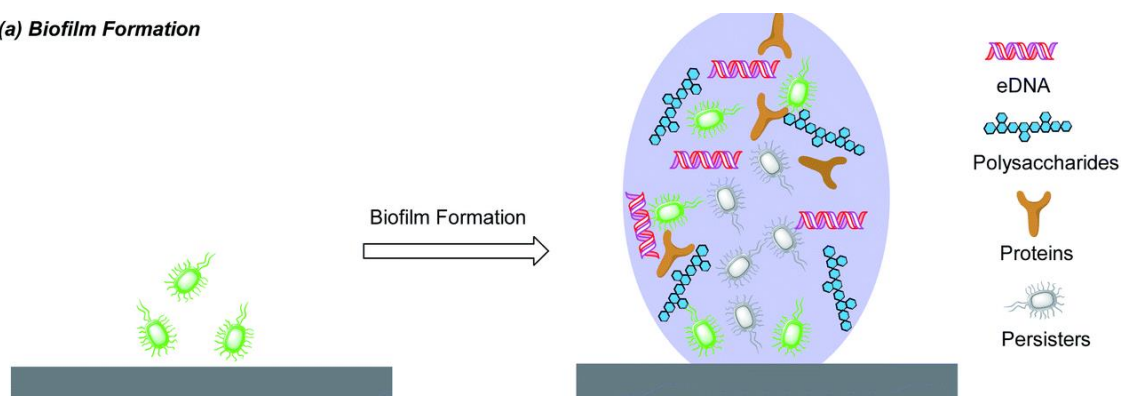


Figure 1.17: Diagrammatic representation of biofilm formation.(adapted from *Chem. Sci.* **2022**,13, 345-364)

Lipophilic entities help in enhanced penetration into the biofilms while cationic moieties are responsible for interacting with anionic bacterial membrane.¹⁰¹ Some of the structures of antibiofilm polymers are shown in Figure 1.18. Quaternary ammonium substituted chitosan polymers plays an important role as antibiofilm polymer as shown in Figure 1.18(1).¹⁰² Herein, the authors found that upon replacing the lipophilic unit with hydrophobic groups like stearyl, lead to 4-fold reduction in minimum biofilm eradication concentration (MBEC). It was also observed by Kuroda et al. that dispersal and eradication of biofilm mass depends on both cationic and hydrophobic components. To understand the same, they compared homopolymer (PE₀) to that of amphiphilic copolymer (PE₃₁), structures are shown in Figure 1.18 (2) and (3).¹⁰³ It was observed that homopolymer could only disperse the biofilm while copolymer was able to disperse and eradicate the biofilm formation. Other amphiphilic polymers include single chain polymeric nanoparticles (SCPNs), polysaccharide based amphiphilic polymer, polycarbonate based quaternized polymer etc.¹⁰⁴⁻¹⁰⁶

(b) Chemical structures of antibiofilm polymers

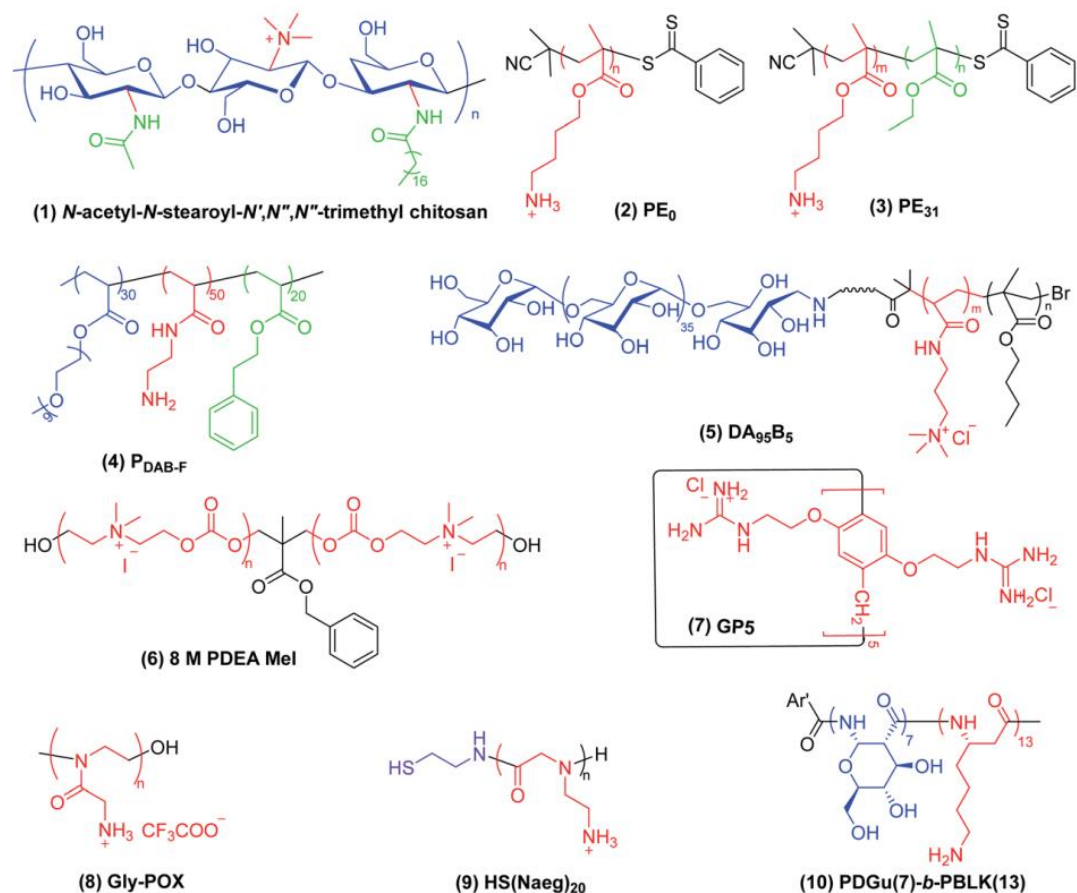


Figure 1.18: Chemical structures of antibiofilm polymers. (Adapted from Chem. Sci. 2022,13, 345-364)

1.5 Biodegradable antibacterial polymers

The biodegradable antibacterial polymers can be broadly classified into four different categories which includes polypeptides, aliphatic polycarbonates, polysaccharides, poly(l-lactic acid) (PLLA), poly-lactide-co-glycolide (PLGA) and polycaprolactones.¹⁰⁷⁻¹⁰⁹ The phenomenon of biodegradation could be either due to microbial digestion or enzymatic action during in vivo applications. In the case of bacterial degradation, microbes excrete some extracellular enzymes to disintegrate the polymer outside the cells. Small oligomers and monomeric units are generated which are comparatively consists of smaller molar mass units are transported across the semi-permeable membrane of bacterial cells. Inside the cell, these molecules are further metabolized into CO₂ and N₂ based on the metabolic pathways.^{110,111} In enzymatic degradation, various lysosomal enzymes like esterase, trypsin etc. in the mammalian cells degrade the polymers during their in vivo applications.^{112, 113}

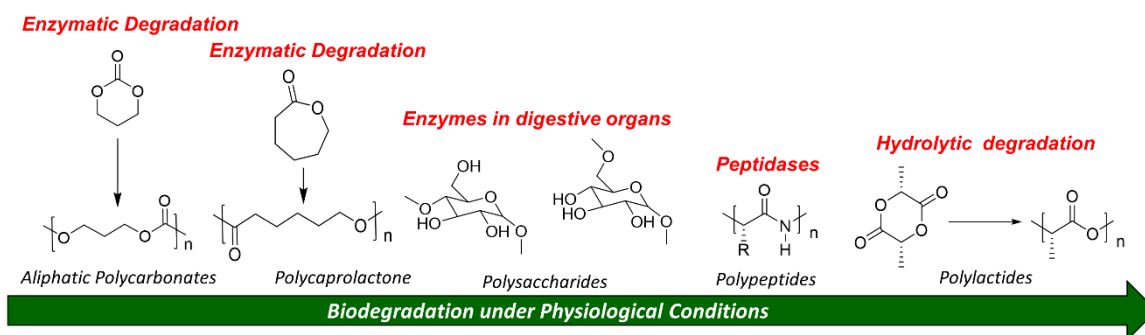


Figure 1.19: Diagrammatic Representation of various biodegradable polymers and their environmental stability

Different biodegradable polymers can be classified based on their environmental stability and rate of biodegradation as shown in Figure 1.19. Polypeptides are a class of synthetic antimicrobial agents which are developed by conjugating amino acids by peptide linkages.¹¹⁴ The cationic polypeptides used for antimicrobial activity are poly-L-lysine, polyarginine, polyhistidines, poly-ornithinines etc. Polymyxin B and polymyxin E (colistin) are clinically relevant circular oligopeptide based antimicrobial agents (see Figure 1.20).¹¹⁵ They are an extremely efficient broad-spectrum class of antimicrobial polymers. One major drawback of polypeptides is *in vivo* toxicity like nephrotoxicity, neurotoxicity, high immunogenicity, short life-span in blood etc.¹¹⁶ For example, in case of poly-L-lysine, ϵ -Poly-L-lysine is edible, biocompatible, and non-toxic as compared to α -Poly-L-lysine based on the pKa of the free ammonium ion of the growing chain.^{117,118} ϵ -Poly-L-lysine is a naturally occurring polypeptide derived from *Streptomyces albulus* and it has been approved by the U.S. Food and Drug Administration (FDA).¹¹⁹ Its mode of action involves absorption on the surface of membrane of microbes and resulting in damage to the cells. Copolymers of ϵ -Poly-L-lysine (EPL) and polycaprolactone (PCL) has been developed to investigate the interaction of polymers with bacteria.¹¹⁸ Upon cell membrane disruption, there has been an increase in generation of reactive oxygen species and reduction in alkaline phosphatase activity.

Copolymers based on polypeptides and polycaprolactone were also explored to develop antibacterial polymers with low cytotoxicity and efficient antimicrobial activity. They were synthesized via ring opening polymerization followed by coupling reaction (see Figure 1.20).¹²⁰ A series of novel antibacterial polymers (PCL₁₆-b-K_n) were developed based on the natural antimicrobial peptides (AMPs). The synthesis involves development of cbz protected Lysine NCA to develop poly(Z-Lys)_n and then conjugating diisocyanate at one end of the growing chain to develop Poly(Z-Lys)_n-HDI. This polymer was then coupled with

PCL-NH₂ followed by deprotection of cbz unit which lead to development of PCL-b-K_n. By varying the units of lysine units, authors could draw a conclusion to show the effect of balance between hydrophobic and cationic units in antibacterial activity.

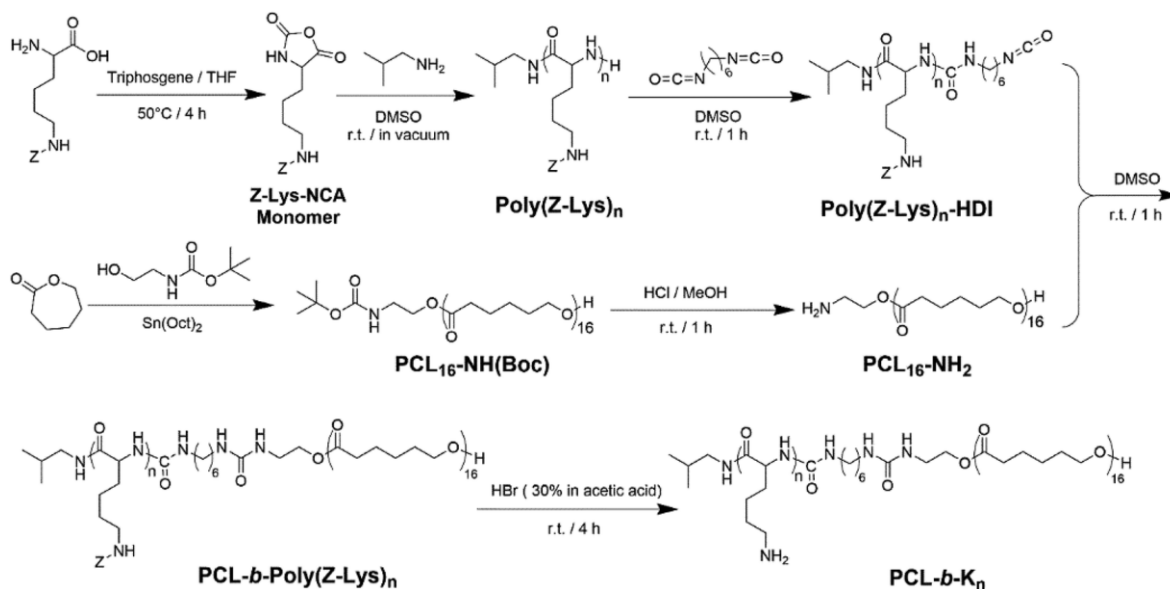


Figure 1.20: Synthetic strategy for the development of series of PCL₁₆-b-K_n polymers. (adapted from Polym. Chem. 2019, 10, 945)

Targeting groups like folic acid were also attached to polypeptide-PCL copolymers in order to enhance their cancer targeted drug delivery as the treatments to cure cancer make the tissue or organ also very susceptible to bacterial infection due to reduced immunity. The design strategy for such polymers is shown in Figure 1.21 and 1.22.¹²¹ Multi-layer films of poly(arginine) and hyaluronic acid are built layer by layer in order to strongly inhibit bacterial growth.¹²² These PAR30/HA films inhibits bacterial growth by contact-killing mechanism due to presence of mobile polyarginine (PAR30) chains. pH-dependent antimicrobial activity has also been demonstrated for arginine rich polypeptides.¹²³ Its binding and antimicrobial activity was enhanced at acidic pH of 5.5. This was due to the basicity of the imidazolium units in histidine moieties.

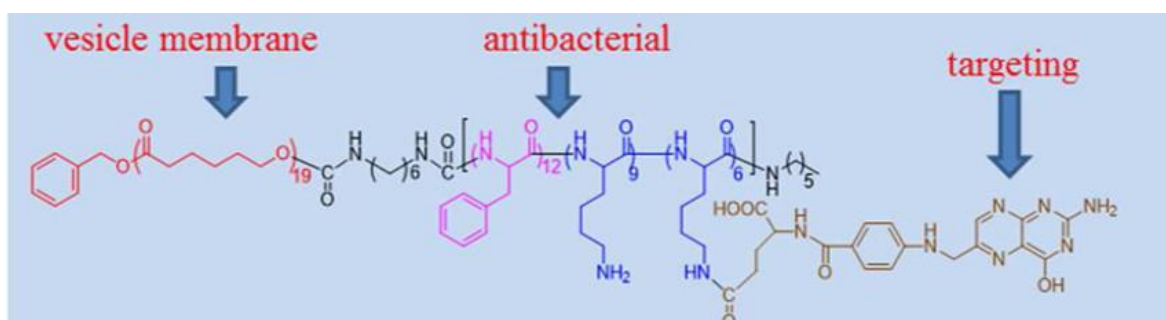


Figure 1.21: Design and synthetic strategy for PCL and polypeptide based antimicrobial polymers, their self-assembly and membrane disrupting action.(Adapted from *Bioconjugate Chem.* **2015**, 26, 4, 725–734)

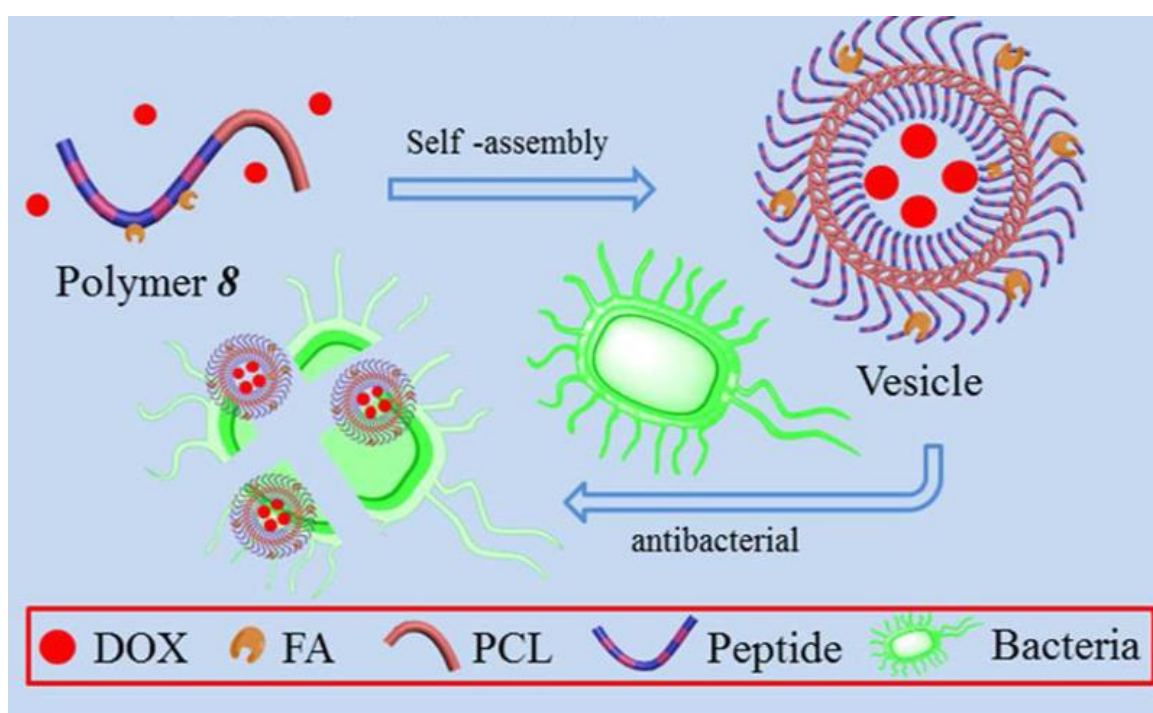


Figure 1.22: Design and synthetic strategy for PCL and polypeptide based antimicrobial polymers, their self-assembly and membrane disrupting action.(Adapted from *Bioconjugate Chem.* **2015**, 26, 4, 725–734)

Aliphatic polycarbonates are another class of mechanically tunable biodegradable polymers which are non-toxic and biocompatible in nature. They are prone to undergo hydrolytic degradation but with appropriate functionalization, its hydrolytic stability can be enhanced.¹²⁴ Aliphatic polycarbonates are also prone to undergo enzymatic degradation but the rate of degradation is different in *in vivo* and *in vivo* environment. The rate of biodegradation for polycarbonates is slower than polyesters. Note: Aromatic polycarbonates does not undergo microbial degradation. The slow enzymatic degradation rate is responsible for its biocompatible nature. Aliphatic polycarbonates such as poly(ethylene carbonate)

(PEC), poly(1,3-trimethylene carbonate) (PTC), poly(butylene carbonate) (PBC), and poly(hexamethylene carbonate) (PHC) are generally synthesized by ring opening polymerization of their respective cyclic monomers.¹²⁵ The synthesis and micellar formation of the first biodegradable polycarbonate based antimicrobial polymer is shown in Figure 1.23. Cationic triblock polycarbonate was developed by sequential ring-opening polymerization of chlorine substituted trimethylene carbonate monomer (MTC-CH₂)₃-Cl followed by nascent trimethylene carbonate monomer (MTC). The quaternization of chlorine units led to the development of cationic polycarbonate based triblock copolymer which readily formed micelles in aqueous environment and showed antibacterial activity through electrostatic interaction and steric hindrance.¹²⁶

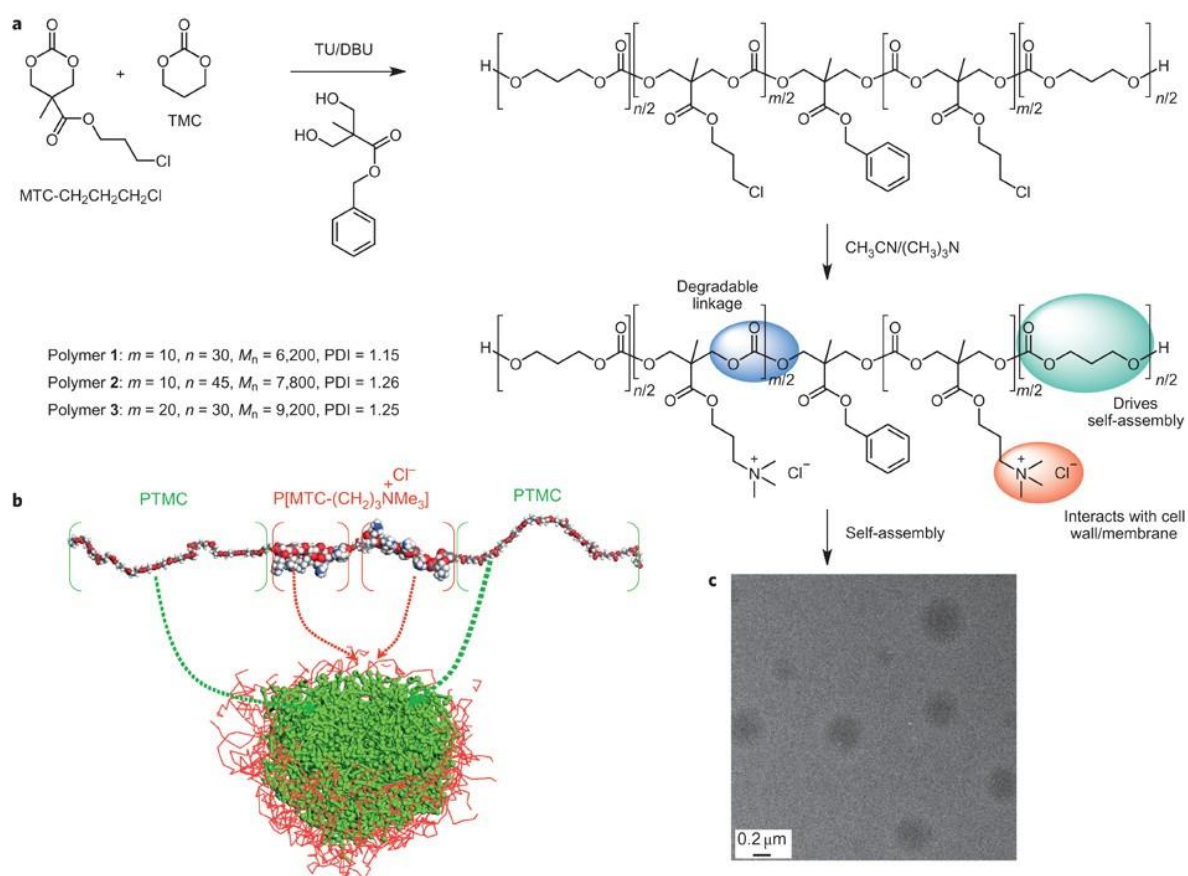


Figure 1.23: Synthetic scheme for the development of polycarbonate based triblock copolymer and their self-assembly. (adapted from *Nat. Chem.* **2011**, 3, 409-414).

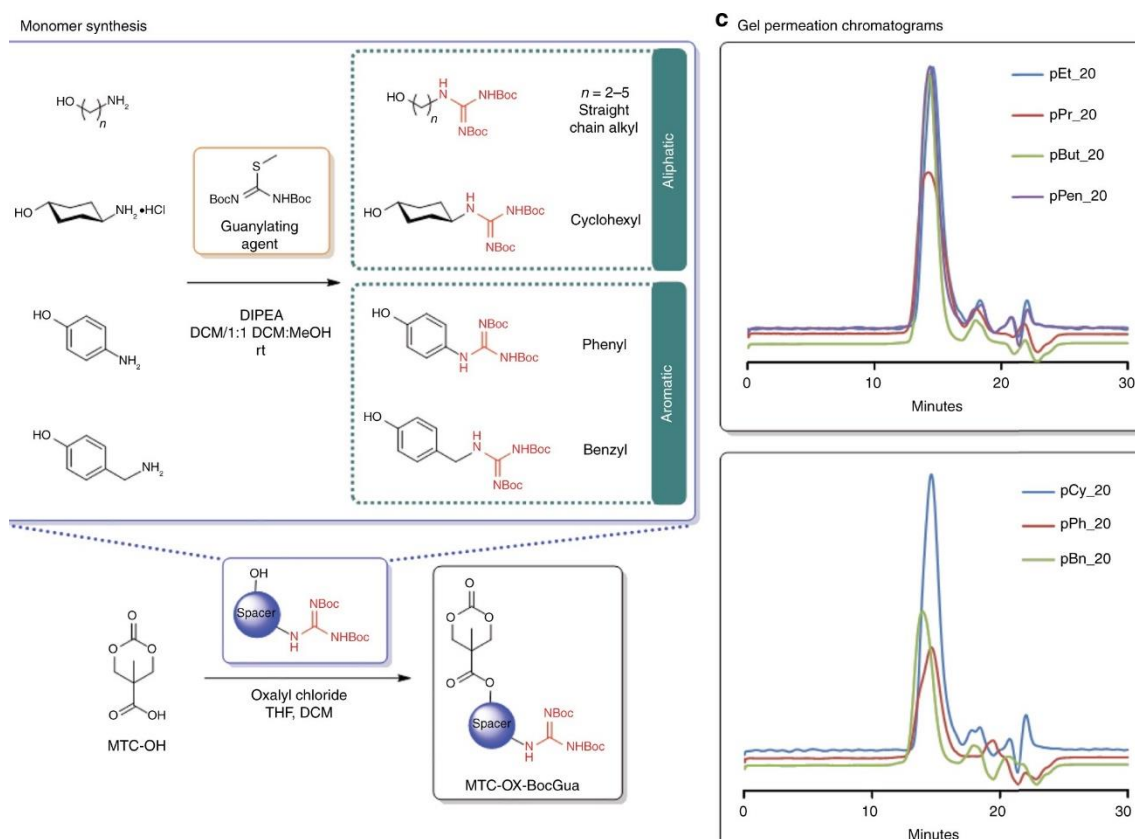


Figure 1.24. Design strategy for development of various trimethylene carbonate functionalized monomers conjugated through guanylation agent. (adapted from *Nat. Commun.* **2018**, 9, 917)

The mechanism of membrane perturbation was visualized through TEM imaging. The morphological changes in MRSA and *Enterococcus faecalis* were visualized before and after the polymeric treatment and the membrane was observed to be completely perturbed due to polymer action and empty spaces at cytosol suggested leakage of cytoplasmic content. Moreover, various guanidinium-functionalized polycarbonates are used as antibacterial agents and found effective against multi-drug resistant (MDR) bacteria (see Figure 1.24 and 1.25).¹²⁷ These guanidinium containing antimicrobial polymers has appropriate spacer units for studying the influence of different spacers in antibacterial activity. Unlike antibiotics, these guanidinium functionalized polymers were found to be unaffected by induction of drug resistance and they also showed high in vivo treatment efficacy against multi-drug resistant bacterial infection like *A. baumannii*-, *E. coli*-, *K. pneumoniae*-, methicillin-resistant *S. aureus* and *P. aeruginosa*.

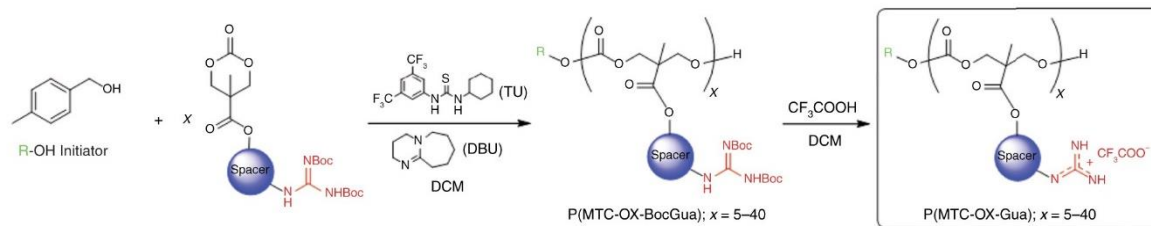


Figure 1.25. Synthetic scheme of guanidinium-functionalized polycarbonates ($P(\text{MTC-OX-Gua})$) by metal-free ROP followed by deprotection of BOC units. (adapted from *Nat. Commun.* **2018**, 9, 917)

Poly-L-lactides are a class of biodegradable polymers which are converted into short segments of lactic acid once degraded by enzymes. Moreover, these polymers are also bioassimilable in nature which means that the byproducts produced (lactic acids and glycolic acids) are eliminated via Krebs cycle as water and carbon dioxide.¹²⁸ Polyester Poly lactide-co-glycolide (PLGA) is a copolymer of poly-lactic acid (PLA) and poly-glycolic acid (PGA). PLA could be highly crystalline (PLLA) or completely amorphous in nature (PDLA) due to the orientation of methyl groups in growing polymer chain. PGA is extremely crystalline as it does not contain any side chain methyl groups. The degradation rate of PLGA is also dependent on molar composition of PLA and PGA. Higher contain of PLA units, results in increased hydrophobicity and reduced degradation in water.¹²⁸ Electrospun fibers of Poly(lactic-co-glycolide) and poly(dioxanone) (PDO) are used to load ciprofloxacin in the meshed fibers and showed controlled antibiotic release in the presence of *S. aureus* and *E. coli*.¹²⁹ Determination of zone of inhibition for various polymeric samples were carried out for day 1, day 4 and day10. It can be clearly observed that for all PLGA based polymers, sustained drug release has been obtained even after 10 days of incubation.

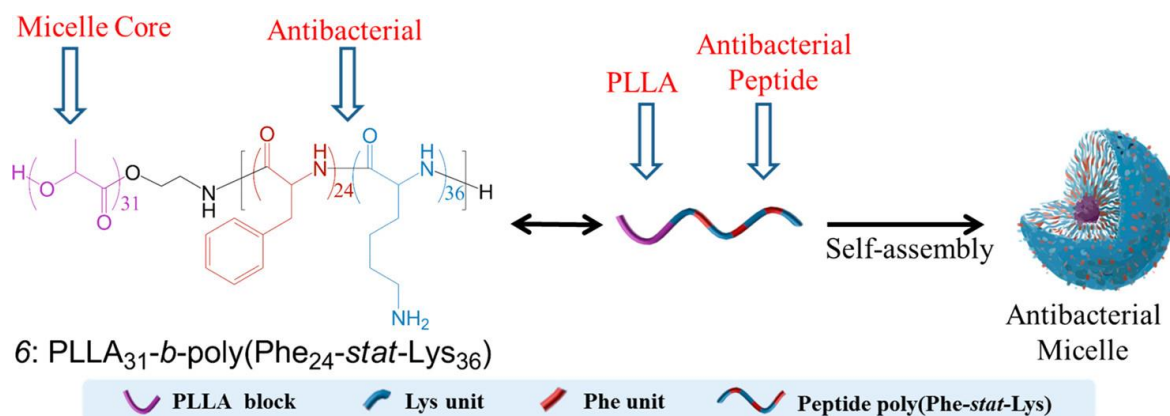


Figure 1.26. Self-assembly of PLLA and Polypeptide based copolymers. (adapted from *Biomacromolecules* **2016**, 17, 3922–3930)

Poly(L-lactide)-block-poly(phenylalanine-stat-lysine) were designed to show antibacterial activity against gram-negative and gram-positive bacteria.¹³⁰ In aqueous environment, PLLA forms the core of the self-assembled nanoparticles while polypeptide forms the coronas as shown in Figure 1.26. The antibacterial mechanism involves electrostatically interact of polymeric micelles with negatively charged bacterial membrane followed by bacterial membrane rupture. Poly(lactide-co-glycolide) (PLGA) microspheres loaded with totarol antibiotic has been used as slow antibiotic releasing scaffold.¹³¹ Post-polymerization functionalization of PLA surfaces through photochemical thiol-yne chemistry led to the development of hydrophobic and hydrophilic surface modified PLA polymeric surfaces. The schematic strategy to develop fluorophore modified PLA surfaces is shown in Figure 1.27.¹³² These polymers exhibited very efficient antibacterial activity against four bacterial strains: *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*. These polymers also exhibited biofilm eradicating property after 72 h of incubation. PLGA based polymers appeared to be idea polymers for loading antibacterial drug like rifampicin and moxifloxacin.

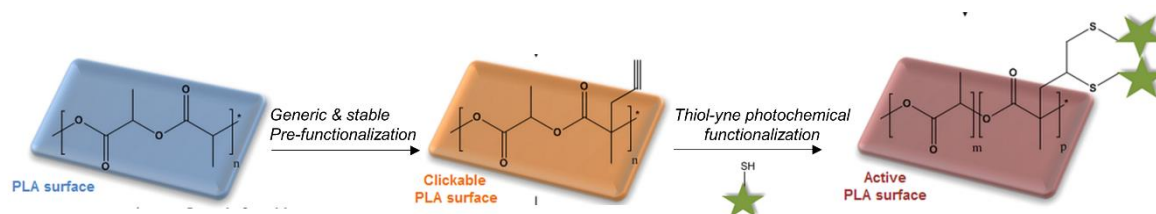


Figure 1.27: Schematic strategy for development of photochemically modified PLA surfaces by thiol-yne chemistry. (Adapted from *Biomacromolecules* **2014**, 15, 4351–4362)

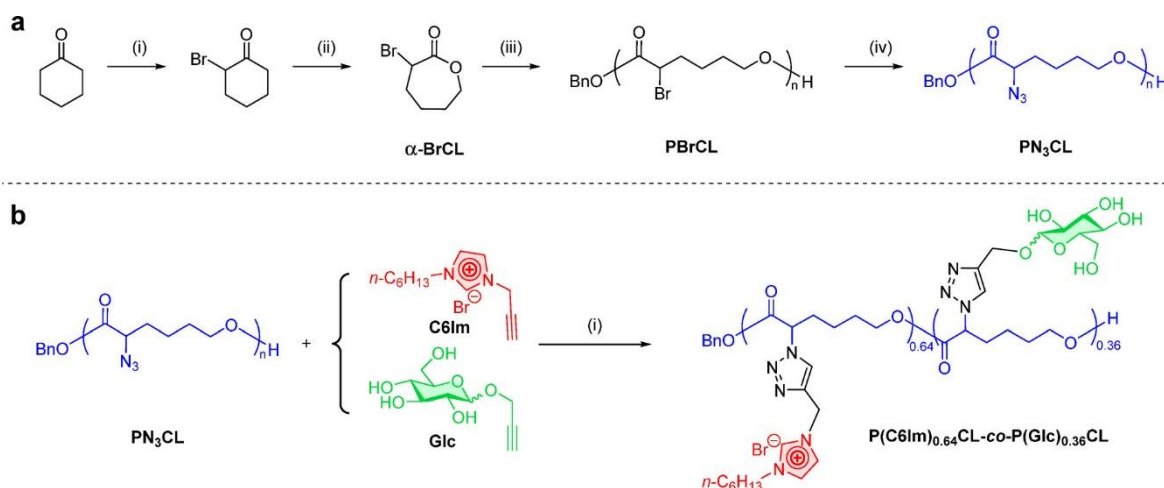


Figure 1.28. Design and synthesis of Poly(ϵ -caprolactone) based antimicrobial polymers. (i) NBS, NH₄OAc, Et₂O, rt, 30 min; (ii) *m*-CPBA, DCM, rt, 24 h; (iii) BnOH, Sn(Oct)₂, 80 °C, 48 h; (iv) NaN₃, DMF, rt, overnight. (b) Synthesis of P(C6Im)_{0.64}-co-P(Glc)_{0.36}CL in a one-pot reaction: (i) CuI, Et₃N, DMF, 40 °C, overnight (Glc = glucose). (Adapted from *Biomacromolecules* **2019**, 20, 949–958)

Polycaprolactones (PCL) are one of the most widely utilized biodegradable polyester for drug-delivery applications. It is Food and Drug Administration (FDA) approved polymer which is widely explored as drug-delivery agents, suture, and adhesive barrier.¹³³ Initially, it has only been used as a hydrophobic block along with antimicrobial polymers for encapsulating drug or providing required amphiphilicity for self-assembled structures.^{134–136} In 2016, first report on cationic group functionalized polycaprolactone was published in *Biomacromolecules*.¹³⁷ Herein, α -bromo substituted caprolactone monomer was developed which underwent ring opening polymerization to develop Br containing polymers (PBrCL) as shown in Figure 1.29. Polymer was reacted with NaN₃ to develop azide functionalized polycaprolactones (PN₃CL). These polymers were grafted with alkyl imidazolium units and different sugar units to maintain hydrophilic/hydrophobic content and mimic properties of cationic antimicrobial peptides. No considerable difference in antibacterial activity was observed in terms of minimum inhibitory concentration (MIC) with different sugar moieties like glucose, mannose, galactose, glucosamine etc. but only mannose and glucose containing polymers were less hemolytic activity and better compatibility to NIH 3T3 fibroblasts. Silver containing nanoparticles were developed by PCL-AMPs based diblock copolymer wherein critical micellar concentration (CMC) dependent bacterial inhibition was observed.¹³⁴ For gram negative bacterial cells, minimum bactericidal concentration (MBC) was observed to be higher than CMC while for gram-positive bacterial cells, MBC was obtained to be lower than CMC (as shown in Figure 1.29). This accounted for the role of different morphologies

in solution towards antibacterial action. Polycaprolactone-based molded mesh has been used to deliver antibacterial agents to infected wounds of mice, excellent mechanical properties, low molecular weight, and low cost.¹³⁸⁻¹⁴⁰ Electrospun molds based on PCL and imaging contrast agents (CA) are reported for non-invasive monitoring of biodegradation of synthetic implants. Computed tomography imaging showed a decrease in iodine concentration which accounted for the scaffold degradation as shown in Figure 1.30.¹⁴¹

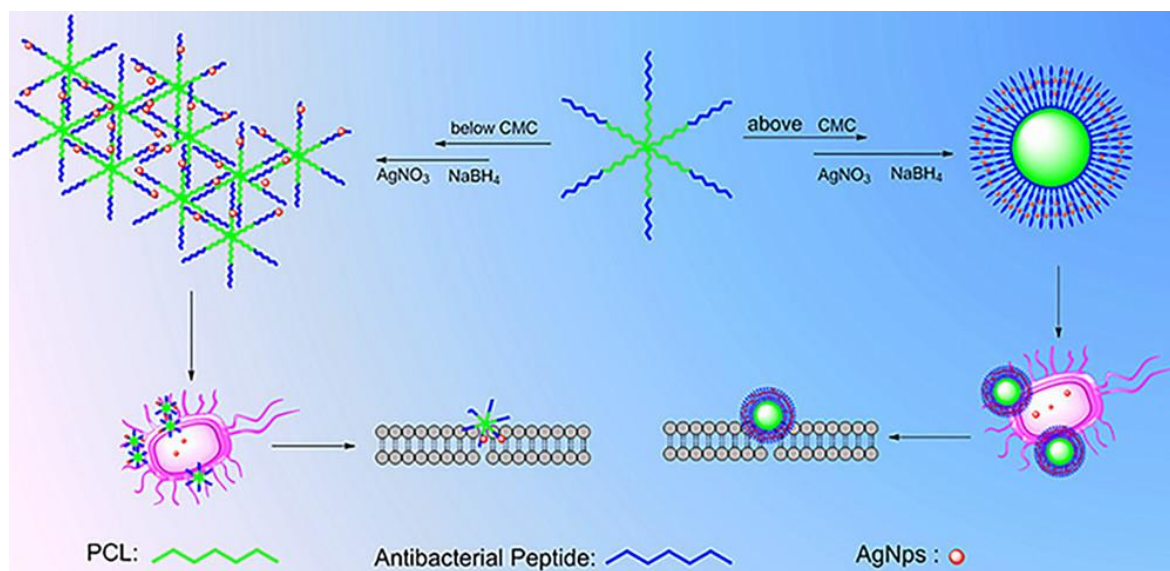


Figure 1.29. Proposed antibacterial mechanisms of the AgNPs@PCL-b-AMPs (below CMC) and nanocomposite AgNPs@PCL-b-AMPs with assembled structure (above CMC) (adapted from *Bioconjugate Chem.* **2020**, 31, 51–63)



Figure 1.30. The polycaprolactone based elastomeric biomaterial PCL2000-U4U and the contrast agent I-U4U interact via supramolecular hydrogen bonding interactions between the bisurea (U4U)

units (blue boxes). Simply mixing both components in the desired ratio in solution followed by electrospinning produces small caliber vascular grafts (photo). The in-situ TE scaffolds are implanted as aortic interposition grafts in rats and are monitored in time using CT, visualizing their degradation noninvasively. (Adapted from *Macromol. Biosci.* **2020**, 20, 2000024)

From our group carboxylic functionalized PCL based biodegradable polymers were introduced by Surnar et al. in 2013 wherein a stimuli responsive polymer was developed for delivery of cargo under gastrointestinal tract (GI).¹⁴² These vesicular nano-assemblies were able to load hydrophobic and hydrophilic drugs in their nano-cavity. This was a first attempt in developing carboxylic acid-based PCL nano-assemblies for oral drug delivery. Subsequently, this design strategy was further employed for cisplatin stitching in these core-crosslinked nanoparticles.¹⁴³

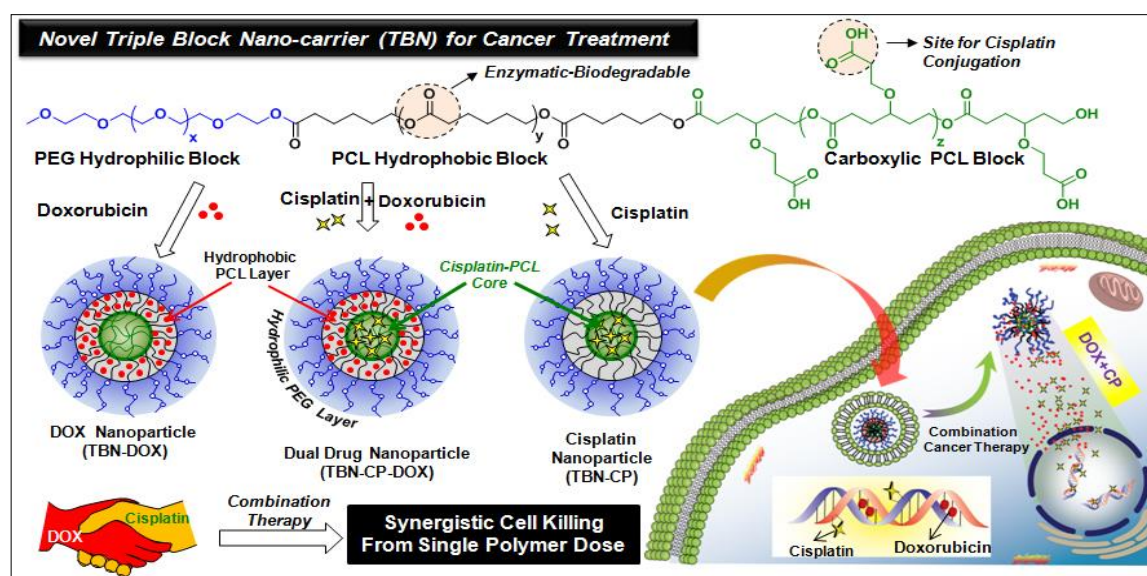


Figure 1.31: Design and development of Triple block nanocarrier (TBN) for combination therapy of cisplatin and DOX. (Adapted from *Biomacromolecules* **2016**, 17, 4075–4085)

These core-shell nanoparticle design was able to successfully deliver cisplatin only upon the action of lysosomal enzymes which disrupted the nanoparticle. This strategy was extremely helpful for reducing detoxification of cis-platin by cytoplasmic thiols. Moreover, triple block nanocarriers were also developed for combination therapy for two antagonistic drugs wherein cisplatin was conjugated at the core while DOX was loaded in the hydrophobic compartment as shown in Figure 1.31.¹⁴⁴ This triple block nano-carrier was comprised of three different polymeric blocks, PEG hydrophilic block for its affinity towards aqueous environment, PCL hydrophobic block for encapsulating hydrophobic anticancer drug DOX and lastly carboxylic acid functionalized PCL block to conjugate cisplatin. They

showed synergistic action in the intracellular compartment of breast cancer cell lines towards eradicating cancerous cells.

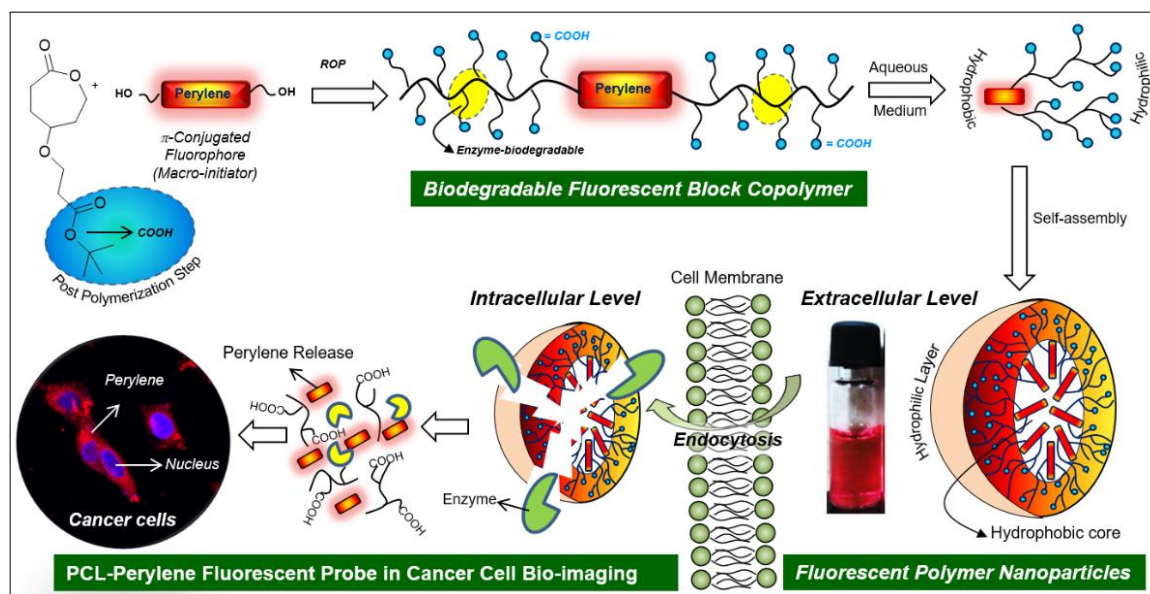


Figure 1.32. Design strategy for the development of perylene bisimide based PCL block copolymer and its bio-imaging application. (Adapted from *Biomacromolecules* **2016**, 17, 1004–1016)

Kulkarni et al. developed hydroxyl functionalized perylene bisimide (PBI) initiator was used for the ring opening polymerization of PCL block copolymer.¹⁴⁵ These fluorescent polymers were utilized for bio-imaging of cancerous cells as shown in Figure 1.32. The design strategy of introducing a π -conjugated fluorophore in the polymeric network was to build a fluorescent polymer which would cleave in the intracellular compartment by lysosomal enzymes and it would ultimately lead to release of free perylene molecules which could be used for fluorescently labelling the cancerous cells. Perylene shows aggregation caused quenching (ACQ) property in aqueous environment which limits its fluorescent property but carboxylic acid functionalized PCL backbone in the polymeric nano-assemblies reduced its ACQ property in aqueous environment. These stable fluorophore conjugated nanoparticles yielded as enzyme-responsive nanoparticles for bio-imaging.¹⁴⁶ Malhotra et al. developed new classes of block and random copolymer consisting of carboxylic functionalized caprolactone polymer and non-functionalized polycaprolactone polymer.¹⁴⁷ These two polymeric topologies were studied to understand the role of block and random copolymer in enzymatic degradation, drug loading and drug release capability in breast and cervical cancer cell lines (see Figure 1.33). Both block and random polymers were extremely efficient nanocarriers for intracellular delivery of anticancer drug DOX to nucleus.

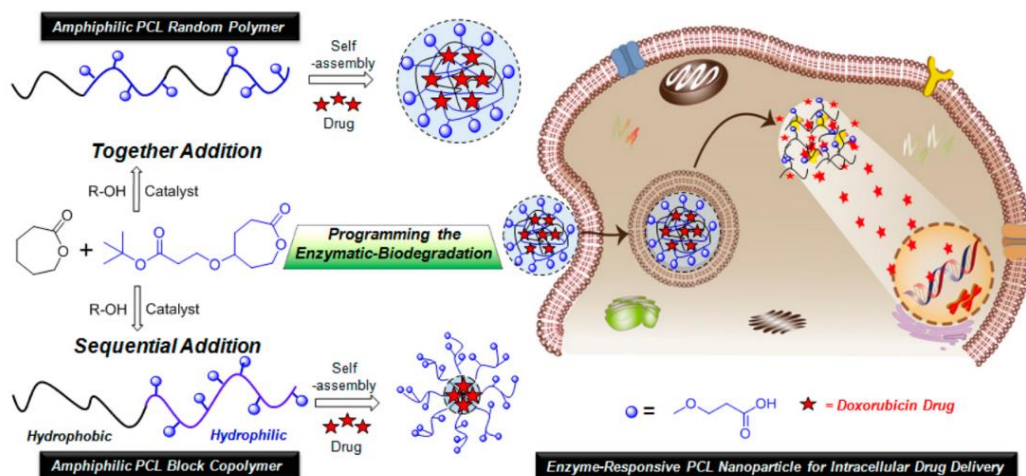


Figure 1.33. Topology driven enzymatic biodegradation of amphiphilic polymers (block and random copolymers). (Adapted from *Macromolecules* **2016**, 49, 8098–8112)

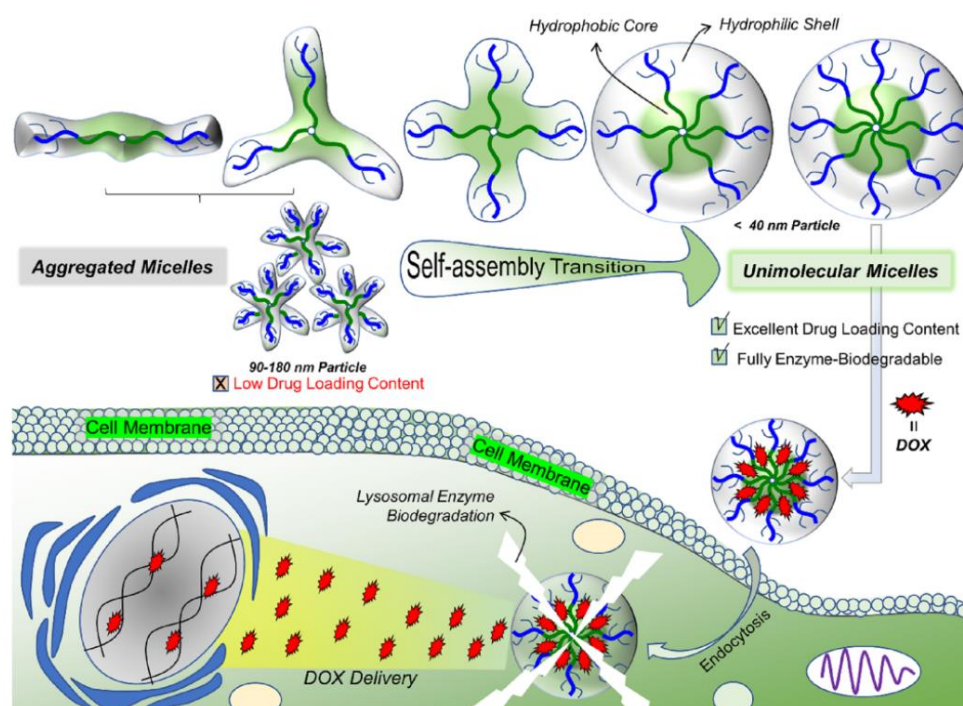


Figure 1.34: Development of fully PCL-based enzymatically biodegradable amphiphilic star block copolymers to study the impact of the arm number toward unimolecular micelle formation and anticancer drug delivery. (Adapted from <https://doi.org/10.1021/acsbiomaterials.2c01201>)

Recently, from our group Pranav et. al. published an article on structurally engineered unimolecular star block copolymers as anticancer drug delivery systems as shown in Figure 1.34.¹⁴⁸ These PCL based star block copolymers were developed with various arm numbers from three, four, six and eight to understand the influence of degree of branching on unimolecular nature of star polymers. The anticancer drug loading content in these unimolecular micelles were found to be 3-4 times higher than aggregated micellar assembly. They were found to be extremely efficient drug loading carriers which can be used for next generation drug delivery agent. Dr. Mehak et. al studied design and development of star-shaped macromolecular architectures and studied their unimolecular micelle formation. They were characterized by DLS, SAXS, FE-SEM, AFM and HR-TEM. These unimolecular micelles were also explored in our group for *in vivo* delivery of anti-cancer drugs and breaching blood brain barrier wherein studies are conducted to understand the biocompatibility and pharmacokinetics of the drug loaded polymeric scaffold. It was observed that as compared to free drugs, these systems were extremely efficient to cross blood brain barrier which is responsible for efficient drug delivery. The effect of surface charge was also studied to understand the cellular internalization mechanisms for different charge species. They were further explored to study the encapsulation of Nile red, IR-780, HPTS, Cardiogreen to visualize the uptake of these nanoparticles by various endothelial and neuronal cell lines. In summary, polycaprolactone based biodegradable polymers were mostly focused as a scaffold to deliver anticancer or anti-inflammatory drugs for cancer treatment. In order to broaden the horizon, in this thesis work, cationic polycaprolactone based systems are not only explored as antibiotic delivery agents but also as potent antibacterial agents. Different structural architectures were explored to understand the role of molecular architecture on antimicrobial activity and mechanism of bacterial membrane breaching.

1.6 Aim of the thesis

The aim of this thesis basically encompasses the design and development of biodegradable cationic antimicrobial polymers for effective bacterial inhibition. These polymers are designed considering three important aspects of antimicrobial polymers; Firstly, they should be non-toxic to mammalian cells (as shown in Figure 1.35(1)). Their non-toxicity in intracellular compartment of mammalian cells can be correlated to their enzymatic biodegradation. These polymers should be cleavable at lysosomal enzymatic condition to ensure that they do not accumulate in the cytosol. This property would be helpful during *in vivo* applications as well in long-term. The second factor included non-toxicity to RBCs (see Figure 1.35 (2)). This is also an important property of antimicrobial polymers during their intravenous delivery to an infected organ or tissue environment. The hemolytic

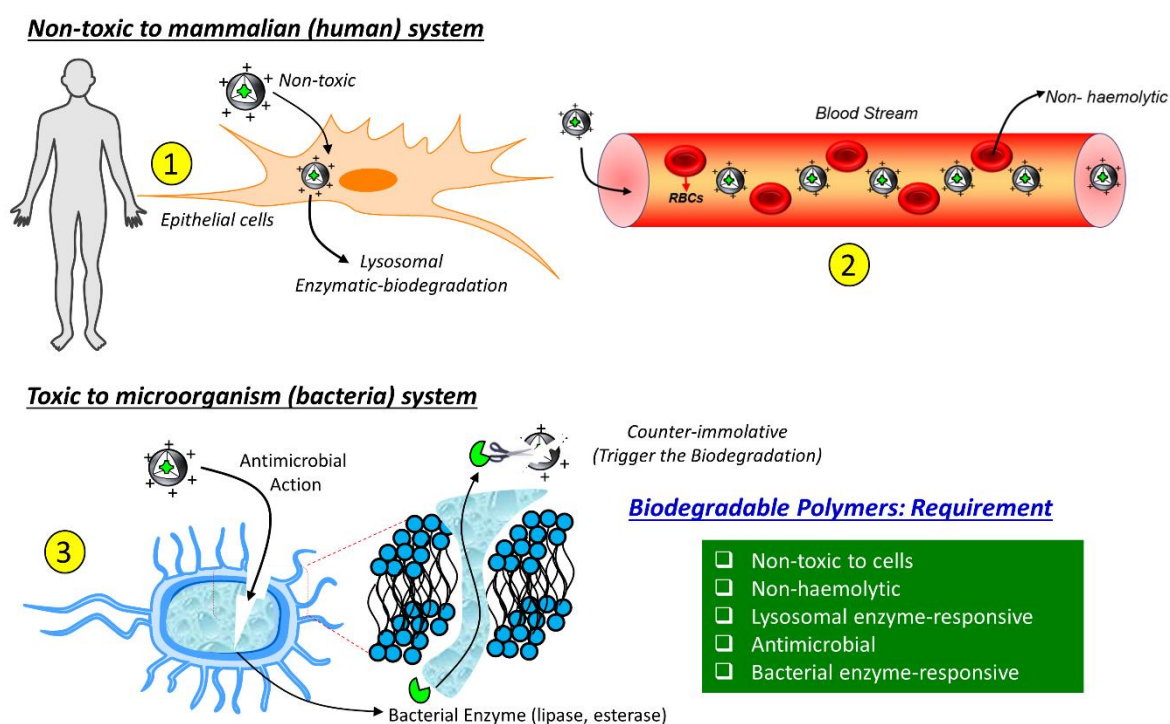


Figure 1.35: Diagrammatic Representation enlisting the basic features of antimicrobial polymers.

activity should be negligible at MIC concentration and the selectivity index also should be higher towards bacterial cells rather than RBCs. The third factor involves polymeric biodegradation by bacterial enzymes (Figure 1.35(3)). During an antimicrobial action of polymer at a bacterial infection, the exotoxins released by bacteria like lipases or intracellular enzymes like esterases should be able to degrade polymeric backbone to ensure non-toxicity in the environment. This kind of a phenomenon is counter-immolative to

polymer as the process has been triggered by the polymer by cleaving the bacterial membrane would initiate greater efflux of enzymes from the bacteria. In this work, functionalized polycaprolactone based polymers are employed to develop antibacterial polymers and they have been tested against *E. coli* as a model bacterium.

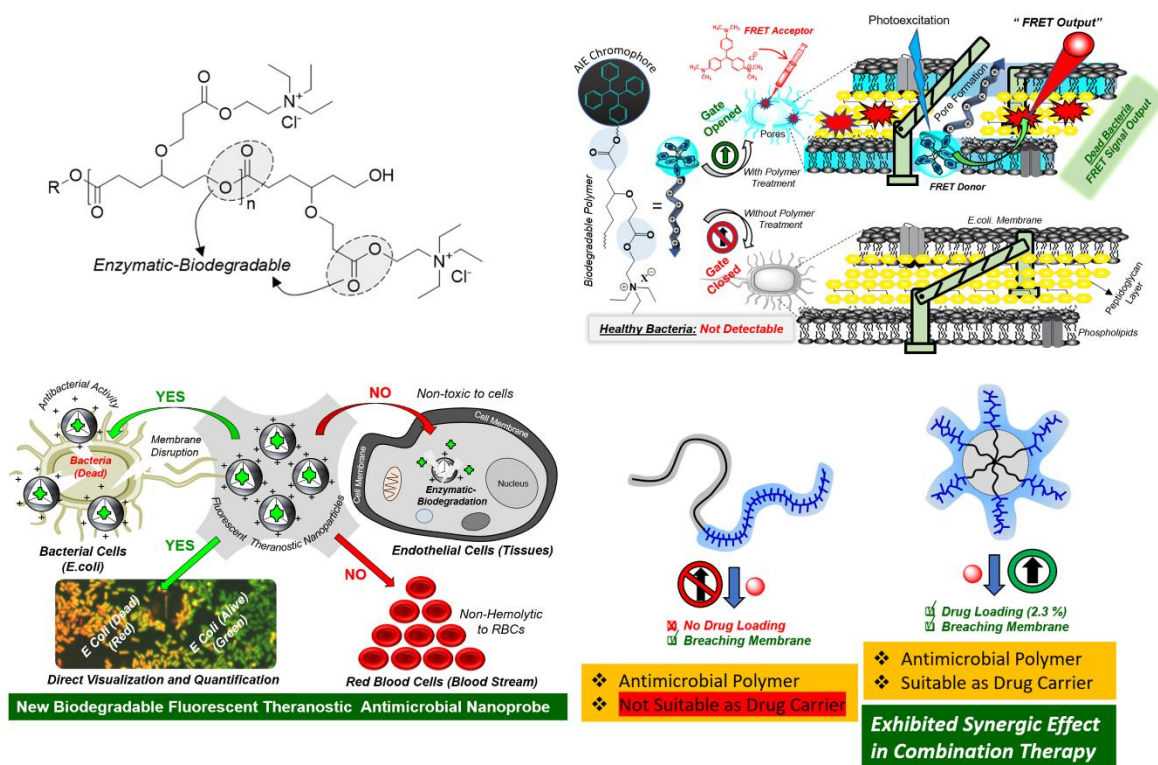


Figure 1.36: Schematic representation of consolidate thesis work

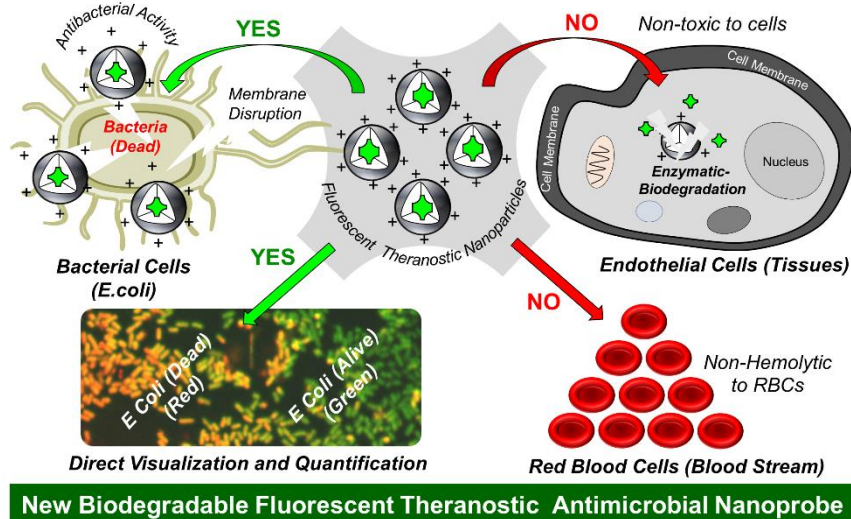
This thesis work is aimed to design and develop a new collection of PCL based antimicrobial polymers which was non-toxic to mammalian cell lines and erythrocytes. We employed the polymers to understand some of the prevailing questions of antimicrobial research revolving around the mechanism of membrane breaching mechanism by polymers and we tried our best to understand these core concepts. My entire thesis work can be broadly classified into three subsequent chapters and the outline is shown in Figure 1.36. The chapter 2 basically comprises of design and development of quaternary amine functionalized cationic antimicrobial homopolymers of various chain lengths. These polymers were found to be extremely selective to bacterial cells (*E. coli*) as compared to RBCs and mammalian cell lines. Encapsulation of an anionic fluorophore along with a membrane-impermeable dye (propidium iodide) facilitated its direct visualization and quantification of dead and alive bacterial cells at a given point of time by confocal microscopy imaging. In chapter 3, TPE

conjugated homopolymer was taken forward to understand the membrane breaching mechanism of polymer on the surface of bacterial by utilizing a FRET probe along with crystal violet (CV). An array of TPE conjugated polymers were prepared containing cationic, anionic, and neutral charges and their membrane binding and membrane disruption action was scrutinized. Through this FRET probe, we could easily distinguish between antimicrobial and non-antimicrobial polymers. Further in chapter 4, efforts were taken to study the topology dependent antimicrobial activity of linear and star shaped block copolymers wherein both the polymers had exact molecular weight, size, and zeta potential. Although the MIC value of both the polymers were equivalent, only star polymer was able to load azithromycin (macrolide antibiotic) in its nanocavity and could show synergistic antimicrobial action by combination therapy.

Lastly, the summary of entire work has been provided with future perspectives.

Chapter 2

Development of Polycaprolactone based Biodegradable Antibacterial Polymers



Abstract

This chapter reports one of the first examples of *biodegradable fluorescent theranostic nanoprobe* design strategy for simultaneous visualization and quantitative determination of antibacterial activity for the treatment of bacterial infections. Cationic-charged polycaprolactone (PCL) was tailor-made through ring opening polymerization methodology and it was self-assembled into well-defined tiny 5.0 ± 0.1 nm aqueous nanoparticle (NP) having Zeta potential of +45 mV. Excellent bactericidal activity at 10^{-2} $\mu\text{g/mL}$ concentration was accomplished in gram-negative bacterium *Escherichia coli* while maintaining their non-hemolytic nature in mice red blood cells (RBC) and non-toxic trend in wild type mouse embryonic fibroblast cells with selectivity index of $> 10^4$. Electron microscopic studies are evident of the *E. coli* membrane disruption mechanism by the cationic NP with respect to their high selectivity for antibacterial activity. Anionic biomarker 8-hydroxy-pyrene-1,3,6-trisulfonic acid (HPTS) was loaded in the NP via electrostatic interaction to yield new fluorescent theranostic nanoprobe to accomplish both therapeutics and diagnostics together in single nano-system. The theranostic NP was readily degradable by bacteria-secreted lipase enzyme as well as by lysosomal esterase enzymes at the intracellular compartments in < 12 h and support their suitability for biomedical application. In the absence of bactericidal activity, the theranostic nanoprobe functions exclusively as bio-marker to exhibit strong green-fluorescent signals in live *E. coli*. Once it became active, the theranostic probe induce membrane disruption on *E. coli* which enabled the co-staining of nuclei by red fluorescent propidium iodide. As a result, live and dead bacteria could be visualized via green and orange signals (merging of red + green), respectively during the course of antibacterial activity by the theranostic probe. This has enabled the development of new image-based fluorescence assay to directly visualize and quantitatively estimate the real-time antibacterial activity. Time-dependent bactericidal activity was coupled with selective photo-excitation in confocal microscope to demonstrate the proof-of-concept of the working principle of theranostic probe in *E. coli* with very good reproducibility. The new theranostic nanoprobe creates platform for simultaneous probing and treating the bacterial infections in a single nano-design which is very useful for long-term impact in healthcare applications.

2.1 Introduction

Ever-increasing demand to treat the infectious diseases caused by bacteria, virus and other microorganisms in the clinical contamination, wound healing, and respiratory chronic obstructive pulmonary diseases has become major concerns in healthcare and society.^{149,12} Multi-drug resistance and steadfast mutations of microorganisms¹⁵⁰ further obscure the treatment of the infectious diseases with existing drugs or antibiotics.¹⁵¹ Development of new diagnostic tools and methods are also equally important to accomplish timely required therapeutic efficacies (as shown in Figure 2.1).¹⁵²

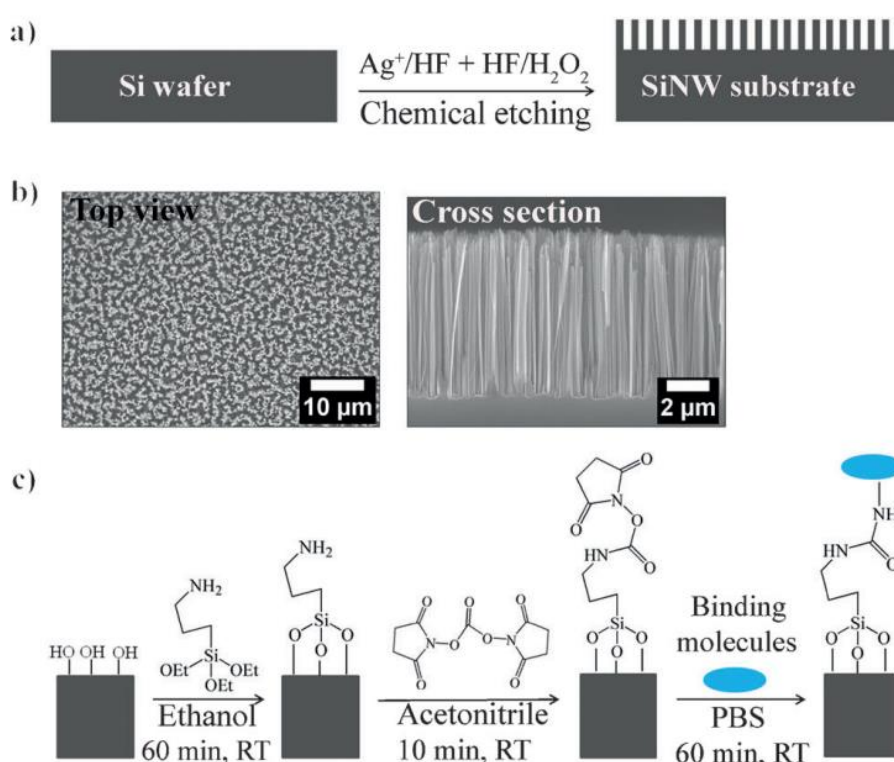
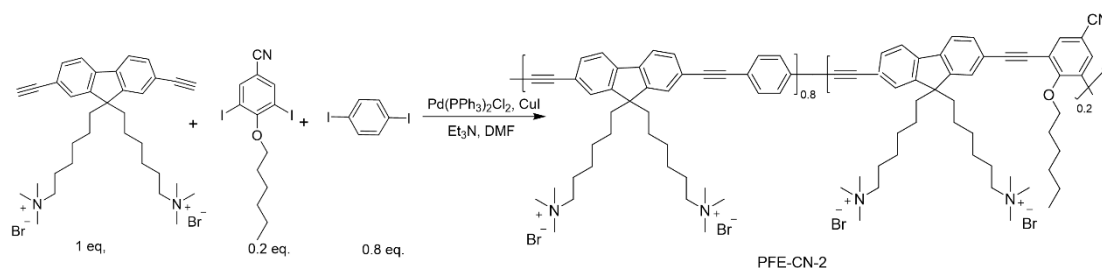


Figure 2.1. a) A schematic of the 3D SiNW substrate fabrication, which is based on silver-assisted chemical etching. b) SEM images of a typical SiNW substrate. c) Surface chemical modification of the silicon substrates (including SiNW and flat silicon) with bacteria-binding molecules. (Adapted from *Angew. Chem. Int. Ed.* **2014**, 53, 5837-584).

The next generation antibacterial candidates are essential to be *smart multitasking probes* having capability to exhibit both therapeutics and diagnostics together (theranostics) in single system so that maximum impact could be accomplished in single dose of drug administration.^{153,154} Among the diverse diagnostic tools; fluorescent techniques provide excellent opportunity because it requires very low probe concentrations and it could be also

employed under non-invasive protocols under physiological condition. Visible light to NIR active fluorescent chromophores were encapsulated or tagged in antimicrobial polymers and antimicrobial peptides in the construction of fluorescent theranostic probes. Gold/Silver nanoparticles based photoacoustic¹⁵⁵ and fluorescent imaging,^{156,157} NIR fluorophore tagged silica-polyelectrolyte nanoparticles¹⁵⁸ and ROS-responsive photodynamic ablation,¹⁵⁹ cyanine dye conjugated-siderophore,¹⁶⁰ Ru-fluorescent complexes,^{161,162} Gelatin nanoparticles,¹⁶³ DNA nanoscaffolds,¹⁶⁴ AIE chromophores¹⁶⁵ tagged fluorescent star-polyacrylates,¹⁶⁶⁻¹⁶⁸ poly(ionic liquid)s¹⁶⁹ and luminescent π -conjugated polymers based cationic polyelectrolytes¹⁷⁰⁻¹⁷² are other important systems explored for antimicrobial activity and bio-imaging in bacterial theranostics. One such example of cationic conjugated polymer is shown in Scheme 2.1.



Scheme 2.1. Synthesis of PFE-CN-2. (Adapted from *Macromolecules* **2018**, 51, 7239–7247)

The theranostic probes have made significant advancement in the treatment of infectious diseases; however, it is important to note that these probes were either build on non-biodegradable C-C or C-N polymer backbones (or metal nanoparticles).¹⁷³⁻¹⁷⁵ As a result, the biodegradation aspect of these theranostic probes is not understood which is a very crucial factor for their long-term impact in antibacterial research. Further the probe molecules (or polymers) come in direct contact with the healthy tissues (or cells), proteins and red blood cells (RBCs) while testing their antimicrobial activity,¹⁰⁸ and therefore, the incorporation of biodegradation property is essential component in theranostic probe design for safer biomedical applications. Aliphatic biodegradable polycarbonate nanostructures were successfully developed to address the mitigating drug resistance, selectively lysis microbial membranes, balance the charge and hydrophobicity, etc.,^{176, 177} Few efforts have been taken in the past to incorporate biodegradable aliphatic polyester segments such as polycaprolactone (PCL) in the antimicrobial blends,¹⁷⁸ imidazolium bearing PCL,¹³⁷ or block copolymers based on polymethacrylates,¹⁷⁹ polypeptides,¹⁸⁰⁻¹⁸² polysaccharides,¹⁸³

phosphonium¹⁸⁴ or phosphoesters,¹⁸⁵ etc. However, neither the biodegradation aspects (except phosphoesters¹⁸⁵) nor the theranostic probe concept was explored in these examples. Therefore, up to our knowledge, there is no effort that has been taken to combine two important concepts- biodegradation and theranostics together in a single polymer (or another nano-system) for studying the antimicrobial activity in the literature. Here, this task has been accomplished by cleverly designing new “*biodegradable theranostic antibacterial nanoprobes*” in enzymatic-biodegradable cationic polycaprolactone (PCL) platform and the new *theranostic fluorescent nanoprobe* was successfully employed for direct visualization and qualitative determination of bactericidal activity. This new concept is shown in Figure 2.2.

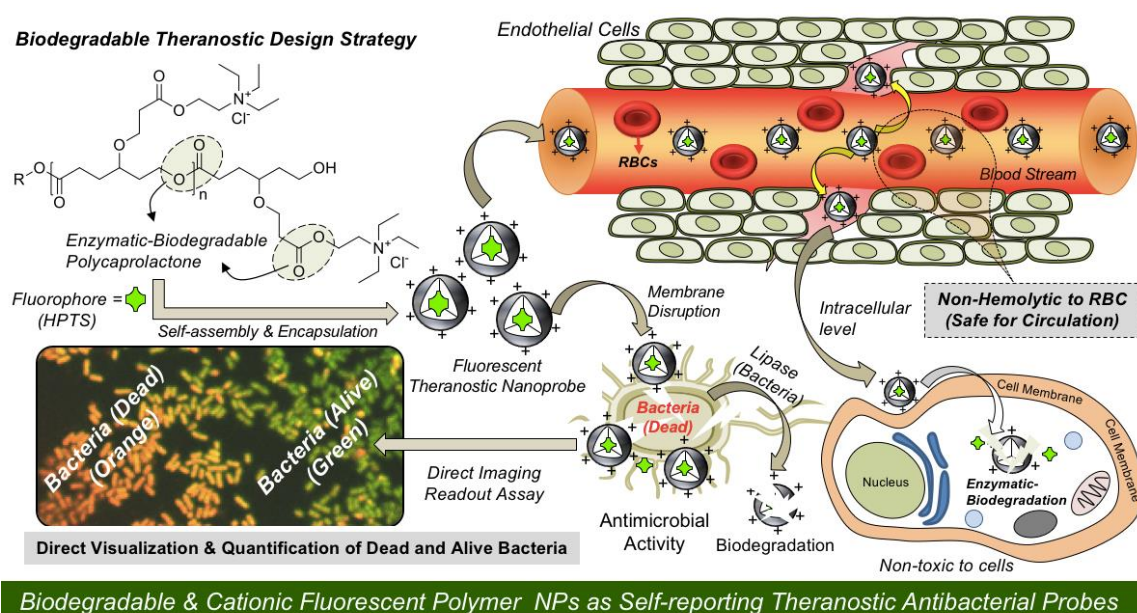


Figure 2.2. Structural engineering of new cationic polycaprolactone based biodegradable fluorescent theranostic nano-probes for direct visualization and estimation of antibacterial activity.

The cationic polymer fluorescent theranostic nanoprobe was built based on aliphatic polyester backbone (as shown in Figure 2.2); thus, it is readily susceptible to undergo two types of enzymatic-biodegradation: (a) by bacteria-secreted lipase enzyme bio-degradation of the polymer backbone¹⁸⁶⁻¹⁸⁸ during their encounter with microorganism for antibacterial activity, and (b) by lysosomal enzymes at the intracellular compartments of endothelial cells in the event of their unwanted encounter with mammalian cells in healthy tissues. For this purpose, substituted PCL polymer design was chosen since it is readily accessible for enzymatic-biodegradation based on our one-decade experience in the development of

biodegradable polymer nano-scaffolds for drug delivery in cancer research.^{142,144-147, 189} Briefly, substituted-caprolactone monomer was tailor-made through multi-step organic synthesis and it was subjected to ring opening polymerization (ROP) to make desired chain length cationic amphiphilic PCL. The cationic PCL self-assembled into tiny nanoparticles in water and it was found to be highly suitable to impact antibacterial activity in gram-negative bacterium *Escherichia coli*. The cationic polymers were found to be non-toxic to mammalian cells and they barely interacted with the RBCs and are largely non-hemolytic in blood. The loading of anionic fluorescent marker HPTS (8-Hydroxy pyrene-1,3,6-trisulfonic acid) yielded green-fluorescent theranostic polymer probe to differentiate the live or dead bacteria by green or orange-color (co-stained with PI) fluorescence, respectively. Bio-imaging protocols in confocal microscope analysis enabled the quantitative estimation of the degree of the bacterial killing by direct visualization. Selective photo excitation experiments in confocal microscope were coupled with time-dependent incubation assay to establish the proof-of-concept with very good accuracy. This new concept and the custom designed theranostic probe have very good potential for long-term implication in diagnostics and therapeutics in treating the infectious diseases which is yet to be reported in the literature.

2.2 Experimental Methods

2.2.1 Materials: 1,4-Cyclohexane diol, *tert*-butylacrylate, potassium *t*-butoxide, molecular sieves (4Å), pyridinium chlorochromate (PCC), *m*-chloroperbenzoic acid, 2-chloro ethanol, triethylene glycol monomethyl ether (TEG), tin(II) 2-ethylhexanoate (Sn(Oct)₂), pyrene, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS), lipase immobilized in Sol-Gel-AK from *Pseudomonas cepacia* were purchased from Sigma Aldrich and used without further purification. Triethyl amine was obtained from Thomas Baker and it was vacuum distilled and stored under NaOH pellets before use. TEG was dried in the vacuum oven prior to use. Tetrahydrofuran (THF), methanol, petroleum ether, ethyl acetate, trifluoroacetic acid (TFA) solvents were distilled and purified prior to use. Gram-negative bacterium *E. coli* (ATCC 25922) was purchased from National Collection of Industrial Microorganisms (NCIM), India. LuriaBertani Broth, Miller and Luria Bertani Agar, Miller were obtained from HIMEDIA. Wild-type Mouse Embryonic Fibroblast cells (WT-MEF) were maintained in DMEM (Gibco) containing 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin–streptomycin at 37 °C under a 5 % CO₂ humidified atmosphere. Cells were trypsinised using 0.05 % trypsin (Thermofischer) and seeded in 96 well flat-bottomed plastic plates

(Eppendorf) for all assays. Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, DAPI, Propidium Iodide (PI), paraformaldehyde, glycerol, glutaraldehyde was obtained from Sigma Aldrich chemicals. Mice Red blood Cells were obtained from C-57 B1/6 Mice from IISER Animal House facility. Gram negative bacterium *E. coli* (ATCC 25922) was purchased from National Collection of Industrial Microorganisms (NCIM), India. Mice Red blood Cells were obtained from C-57 B1/6 Mice from IISER Animal House facility.

2.2.2 Methods: Bruker 400 MHz spectrophotometer was used for recording NMR samples in CDCl₃ solvent using TMS as the standard. Gel Permeation Chromatography (GPC) data acquisition was carried out by a Viscotek VE 1122 pump, Viscotek VE 3580 RI, 3210 UV-Vis and Light scattering detectors. Samples for GPC were prepared in THF and executed from method files obtained after calibration using polystyrene standards. The mass determination for all the compounds was carried out using high-resolution mass spectrometry-electrospray ionization-quantitative time-of-flight liquid chromatography-mass spectrometry (HRMS-ESI-Q-TOF LC-MS) and Applied Bio system 4800 PLUS matrix-assisted laser desorption/ionization (MALDI) TOF/TOF Analyzer. The Perkin-Elmer thermal analyzer STA 6000 model was used to determine the polymer's thermal stability at 10 °C/min heating rate under nitrogen atmosphere. Thermal properties of polymers were analyzed using a TA Q20 differential scanning calorimeter (DSC), wherein the polymers were first heated to melt in order to remove any pre-thermal history and then subsequent heating-cooling cycles were recorded at 10 °C/min under inert conditions to generate the respective thermograms. Water Contact angle (WCA) was measured at room temperature (25 °C) and image was recorded within a minute to reduce the evaporation effects. GBX Model (DIGIDROP instrument) was used for the experiment. Perkin-Elmer Lambda 45 UV-Visible Spectrophotometer was used for the absorption studies. Emission spectra for determining the Critical Micelle Concentration (CMC) were recorded using a SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer. A 450W Xe lamp serves as the source of excitation at room temperature. Pyrene was used as the fluorophore, which has excitation maxima at 337 nm. The emission spectrum gives five distinct vibronic levels, amongst which the intensity of levels I₁ and I₃ are known to be extremely sensitive of the hydrophobic environment. As a result, pyrene when encapsulated within a micelle will give different I₁ and I₃ values. The ratio of I₁/I₃ v/s log C is plotted in order to determine the critical aggregate concentration (CAC). Dynamic light scattering (DLS) and Zeta Potential

measurement was performed using Nano ZS-90 setup (Malvern instrument). DLS utilizes a 633 nm laser as the light source, and the detector collects the scattered light at 90° angle. This gives information about the correlation function [$g^2(t)$], from which the diffusion coefficient (D) is calculated using cumulant method and further the diameter of the particle is determined using the Stock-Einstein equation. The experiment was performed thrice with independent amphiphilic solutions to yield reproducible data. FE SEM analysis requires the sample to be drop casted on silicon wafer followed by gold-coating to make it conducting and the experiment was done on a Zeiss Ultra Plus scanning electron microscope. Transmission electron microscopic (TEM) images were acquired by drop casting the samples onto Formvar-coated copper grid via a Jeol JEM2200FS 200 KeV systems instrument. Time-resolved fluorescence lifetime measurements (TCSPC) are recorded using a SPEX Fluorolog HORIBA JOBINVYON fluorescence spectrophotometer. A LED source of 340 nm is used as excitation source. Confocal microscopic analysis was done using Leica SP8 microscope. Optical density (OD_{600 nm}) of the bacterial samples was measured in EnSpire Multimode Plate Reader under the Perkin Elmer-IISER Pune, Centre of Excellence facility. A Xenon flash lamp was used as the excitation source. The instrument is designed with filter monochromator facility. For Hemolysis experiment, the project has been approved by Institutional Animal Ethics Committee (IAEC), case number of approved license is IISER/IAEC/2018-02/04. **Note:** Animal usage for this project is approved by Institute Animal Ethics Committee (IAEC) at IISER Pune and the approval number is IISER/IAEC/2018- 02/04.

2.2.3 Synthesis of tert-butyl 3-((-4-hydroxycyclohexyl)oxy)propanoate (Compound-1):

1,4 Cyclohexane diol (35.00 g, 302.00 mmol) was dissolved in dry THF (500.00 mL) under nitrogen. A catalytic amount of potassium t-butoxide (1.00 g, 9.00 mmol) was added to the solution and it was refluxed for 30 minutes. It was cooled and then t-butyl acrylate (30.00 g, 241.00 mmol) in dry THF (50.00 mL) was added dropwise at 25 °C using a dropping funnel under nitrogen and the reaction mixture was refluxed for 36 h. THF was evaporated using rotavapor and the residue was precipitated into cold DCM. The residue was filtered and the filtrate was concentrated to yield viscous yellow liquid as product. The product was further purified by passing through silica column using ethyl acetate and hexane (1:5 v/v) as eluent. Yield = 33.00 g (40%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 3.65 (m, 3H, O-CH₂- and O-CH), 3.26–3.36 (m, 1H, CH-OH), 2.44 (t, 2H, -CH₂CO-), 1.97–1.82 (m, 4H, -CH₂-),

1.63–1.30 (m, 4H, $-\text{CH}_2-$), 1.43 (s, 9H, $-\text{CH}_3$). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 171.33, 80.54, 74.31, 69.57, 68.43, 64.07, 63.66, 36.85, 32.64, 30.46, 29.32, 28.15 and 27.59. FT-IR (cm^{-1}): 3618, 2961, 1715, 1413, 1361, 1252, 1157, 1106, 957, 895, 847, 756, 686, 603 and 516. HR-MS (ESI⁺): m/z $[\text{M} + \text{H}^+]$ for $\text{C}_{13}\text{H}_{24}\text{O}_4$ Calculated= 245.1675 and Found = 245.0711.

2.2.4 Synthesis of t-Butyl-3-((4-oxocyclohexyl)oxy)-propanoate (Compound-2): The above compound-1 (30.00 g, 123.00 mmol) was dissolved in dry DCM (400.00 ml) under inert atmosphere and stirred at 25°C. Molecular sieves (4Å) were added along with PCC (53.00 g, 246.00 mmol) and the reaction mixture was stirred for 6 h. The reaction mixture was filtered and washed with DCM and further purified by passing over silica column using ethyl acetate and hexane (1:10 v/v) as eluent to obtain a colorless liquid product (**2**). Yield = 28.00 g (98%). ^1H NMR (400 MHz, CDCl_3) δ ppm: 3.71 (m, 3H, $\text{O}-\text{CH}_2-$ and $\text{O}-\text{CH}-$), 2.56 (m, 2H, $-(\text{CO})\text{CH}_2-$), 2.48 (t, 2H, $-\text{CH}_2-$), 2.23 (m, 2H, $-\text{CH}_2-$), 2.06 (m, 2H, $-\text{CH}_2-$), 1.87 (m, 2H, $-\text{CH}_2-$), 1.42 (s, 9H, $-\text{CH}_3$). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 170.82, 130.94, 128.24, 80.40, 72.60, 68.50, 63.87, 57.32, 37.04, 36.85, 36.41, 30.25 and 27.89. FT-IR (cm^{-1}): 3618, 2961, 1718, 1413, 1361, 1252, 1157, 1106, 957, 895, 847, 756, 686, 603 and 516. HRMS (ESI⁺): m/z $[\text{M} + \text{H}^+]$ for $\text{C}_{13}\text{H}_{22}\text{O}_4$ Calculated= 243.1518 and Found = 243.0547.

2.2.5 Synthesis of 3-((4-oxocyclohexyl)oxy)propanoic acid (Compound-3): TFA (22.00 mL, 289.00mmol) was added to a solution of compound-2 (10.00 g, 41.00 mmol) in 25.00 mL DCM and the reaction mixture was stirred for 1 h at 25 °C. TFA and DCM were evaporated using rotavapor and the reaction mixture was given a few washes with DCM. The crude product was further purified by passing over silica column using ethyl acetate and petroleum ether (2:5 v/v) as eluent to obtain a light-yellow liquid product (**3**). Yield = 7.30g (98%). ^1H NMR (400 MHz, CDCl_3) δ ppm: 8.79 (s, 1H, $-\text{COOH}$), 3.77 (t, 2H, $-\text{O}-\text{CH}_2-$), 3.76 (m, 1H, $-\text{O}-\text{CH}-$), 2.65 (s, 2H, $-\text{CH}_2-$), 2.55 (m, 2H, $-\text{CH}_2-$), 2.25 (m, 2H, $-\text{CH}_2-$), 2.08 (m, 2H, $-\text{CH}_2-$), 1.90 (m, 2H, $-\text{O}-\text{CH}_2-$). ^{13}C NMR(100 MHz, CDCl_3) δ ppm: 212.26, 177.15, 73.00, 63.48, 37.04, 35.23, and 30.39. FT-IR (cm^{-1}): 3445, 2949, 1703, 1418, 1348, 1247, 1186, 1104, 1065, 956, 836, 735 and 587. HRMS (ESI⁺): m/z $[\text{M} + \text{H}^+]$ for $\text{C}_9\text{H}_{14}\text{O}_4$ Calculated = 187.0892 and Found = 187.0970.

2.2.6 Synthesis of 2-chloroethyl 3-((4-oxocyclohexyl)oxy)propanoate (Compound-4):

Compound **3** (6.0 g, 32.2 mmol) and 2-chloroethanol (16.0 g, 193.5 mmol) were refluxed at 60 °C for 6 h under nitrogen atmosphere. Unreacted 2-chloroethanol is removed using mixture of ethylacetate (50 mL) and distilled water (50 mL) and crude product is isolated in organic layer. The procedure is repeated thrice. Further purification was done by silica chromatography using ethyl acetate and hexane (1:20 v/v) as eluent to obtain a colorless liquid product. Yield = 7.0g (82 %). ¹H NMR (400 MHz, CDCl₃) δ ppm: 4.34 (t, 2H, O-CH₂), 3.79 (t, 2H, O-CH₂), 3.73 (m, 1H, -CH-), 3.68 (t, 2H, Cl-CH₂), 2.66 (t, 2H, CO-CH₂), 2.25 (m, 2H, -CH₂-), 2.08 (m, 2H, -CH₂-), 1.90 (m, 2H, -O-CH₂-). ¹³C NMR(100 MHz, CDCl₃) δ ppm: 211.50, 171.48, 73.17, 64.28, 63.74, 41.73, 37.19, 35.37 and 30.55. FT-IR (cm⁻¹): 3820, 3499, 2949, 2877, 1716, 1430, 1350, 1250, 1173, 1105, 1074, 1024, 960, 895, 821, 752, 666, 600, and 516. HRMS (ESI+): m/z [M + Na⁺] for C₁₁H₁₇ClO₄; Calculated = 271.0815 and Found = 271.0712.

2.2.7 Synthesis of 2-chloroethyl 3-((7-oxooxepan-4-yl)oxy)propanoate (Monomer-M1):

Compound **4** (5.0 g, 20.0mmol) was dissolved in 200 mL dry DCM and NaHCO₃ (4.2g, 50.0mmol) was added to the solution under nitrogen atmosphere at 25°C. m-CPBA (10.4 g, 60.0mmol) was added into the reaction mixture and then stirred at 25°C for 15 h. The reaction mixture was quenched with saturated aqueous solution of NaHCO₃ and Na₂S₂O₃ and the crude product was extracted in DCM and the organic layer was dried over anhyd. Na₂SO₄. DCM was evaporated completely and the crude product was purified by passing over silica column using ethyl acetate and hexane (2:5 v/v) as eluent to obtain a pale yellow coloured liquid product. Yield = 4.5g (84%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 4.41 (m, 1H, -CH-), 4.30 (t, 2H, O-CH₂), 4.00 (m, 1H, -CH-), 3.66 (m, 5H, -CH-), 2.90 (m, 1H, -CH-), 2.58 (t, 2H, CO-CH₂), 2.38 (m, 1H, -CH-), 1.99-1.76 (m, 4H, -CH-). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 178.30, 172.09, 71.66, 64.75, 60.39, 41.94, 36.13, 35.57, 30.13 and 28.78. FT-IR (cm⁻¹): 3057, 2881, 1731, 1433, 1355, 1265, 1182, 1120, 1069, 962, 896, 729 and 562. HRMS (ESI+): m/z [M + H⁺] for C₁₁H₁₇ClO₅; Calculated = 265.0765 and Found = 265.0850.

2.2.8 Synthesis of chlorine substituted Polycaprolactone (P10): Typical ring opening polymerisation procedure is described for polymer P-10. In a flame dried schlenk tube,

triethylene glycol (TEG) monomethyl ether (TEG, 31.0 mg, 0.2mmol as initiator) was taken in dry toluene (1.0 mL) and the solution was purged under nitrogen for 15 min. $\text{Sn}(\text{Oct})_2$ (38.0 mg, 0.1mmol, as catalyst) was added and the content was again stirred at 25 °C for 15 minutes under nitrogen purge. Monomer 3 (0.5 g, 2.0 mmol) was dissolved in 1.0 mL of dry toluene and added to the above mixture. The polymerization setup was immersed in oil bath at 110 °C and the reaction was continued for 48 h with constant stirring. At the end of the polymerization, the amount of toluene was reduced to 0.5 mL using rotavapour and viscous polymer solution was precipitated in 50 mL diethyl ether. The isolated polymer was re-dissolved in THF and precipitated again in diethyl ether, and purification was repeated at least twice to obtain high purity polymer. Yield: 450 mg (90 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.14 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.58 (t, 2H), 2.35 (t, 2H), 1.86-1.78 (m, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 173.91, 171.70, 76.10, 64.87, 61.67, 42.03, 35.59, 33.29, 30.10 and 29.28. FT-IR (cm^{-1}): 3391.56, 2951.26, 2685.41, 2496.17, 1728.88, 1643.34, 1453.24, 1356.90, 1256.62, 1174.13, 1091.27 and 659.48. A similar procedure was followed for other homopolymers with different number of repeating units varying = 25, 50, 80 and 100.

2.2.9 Synthesis of polymer P25: TEG (13.00 mg, 0.08 mmol) and $\text{Sn}(\text{Oct})_2$ (16.00 mg, 0.04 mmol) are taken in polymerization tube along with dry toluene (1.00 mL) and kept under nitrogen purging condition for 10 min. Chlorinated monomer 3 (0.50 g, 2.00 mmol) dissolved in 1.00 mL dry toluene is added to the tube in dry condition and the ROP and purification was carried out as described for polymer P10. Yield = 400.00 mg (80 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 3.70 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.59 (t, 2H), 2.37 (t, 2H), 1.86-1.78 (m, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 173.92, 171.68, 76.09, 64.60, 61.66, 42.03, 35.59, 33.29, 30.09 and 29.28. FT-IR (cm^{-1}): 3888, 3827, 2938, 1723, 1356, 1258, 1174, 1097, 1060, 966, 734, 652, 591 and 524.

2.2.10 Synthesis of polymer P50: TEG (6.50 mg, 0.04 mmol) and $\text{Sn}(\text{Oct})_2$ (8.00 mg, 0.02 mmol) are taken in schlenk tube along with dry toluene (1.00 mL) under nitrogen purge for 10 min. Monomer 3 (0.50 g, 2.00 mmol) dissolved in dry toluene (1.00 mL) is added to the above mixture and the ROP and purification was carried out as described for polymer P10. Yield = 350.00 mg (70 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.34 (t, 2H), 4.12 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.37 (s, 1H), 2.58 (t, 2H), 2.36 (t, 2H), 1.86-1.78 (m, 3H). ^{13}C -

NMR (100 MHz, CDCl_3) δ ppm: 75.55, 64.36, 61.54, 59.16, 58.71, 40.26, 37.59, 28.52 and 28.05. FT-IR (cm^{-1}): 3508, 2954, 1728, 1353, 1259, 1169, 1068, 969, 813, 733, 665, 596 and 517.

2.2.11 Synthesis of polymer P80: In a flame dried schlenk tube, TEG (4.00 mg, 0.03 mmol) along with dry toluene (1.00 mL) is taken. The system was nitrogen purged for 10 min. $\text{Sn}(\text{Oct})_2$ (5.00 mg, 0.015 mmol) is added to the same followed by addition of monomer 3 (0.50 g, 2.00 mmol). The ROP and purification was carried out as described for polymer P10. Yield = 350.00 mg (70 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.58 (t, 2H), 2.36 (t, 2H), 1.86-1.78 (m, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 128.69, 64.40, 64.23, 64.06, 63.51, 59.15, 37.98, 29.50, 28.52 and 28.05. FT-IR (cm^{-1}): 3824, 3452, 2951, 1730, 1354, 1256, 1173, 1098, 971, 808 and 665.

2.2.12 Synthesis of polymer P100: TEG (3.00 mg, 0.02 mmol) along with dry toluene (1.00 mL) is taken in a flame dried schlenk tube. The system was nitrogen purged for 10 min. $\text{Sn}(\text{Oct})_2$ (4.00 mg, 0.01 mmol) is added to the same followed by addition of monomer 3 (0.50 g, 2.00 mmol). The ROP and purification were carried out as described for polymer P10. Yield = 300.00 mg (60 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.58 (t, 2H), 2.36 (t, 2H), 1.85 -1.78 (m, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 73.01, 69.11, 64.22, 63.25, 49.54, 38.74 and 15.32. FT-IR (cm^{-1}): 3823, 2947, 1722, 1356, 1262, 1176, 1097, 1062, 964, 732, 657, 574 and 516.

2.2.13 Synthesis of triethyl amine functionalized PCL cationic polymer(CP-10):The synthetic protocol for triethyl amine quaternized polymer is described for chlorine substituted polymer **P-10**. **P-10** (200.0mg, 0.8 mmol) polymer was dissolved in 1.0 mL dry DMF in a melt tube under nitrogen atmosphere. The reaction mixture was given nitrogen purging for 15 min. Dry triethylamine (200 mg, 2.4 mmol) was added to the reaction mixture and then the reaction mixture was kept at 80 °C for 72 h in sealed condition. DMF was removed and polymer was re-dissolved in THF and precipitated in diethyl ether. For further purification, the product was dialyzed using dialysis tube (MWCO = 1000 Da) against water. Yield: 200 mg (80 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.34 (t, 2H), 4.14 (t, 2H), 3.69

(m, 6H), 3.46 (m, 1H), 3.37 (s, 1H), 3.11 (q, 2H), 2.58 (t, 2H), 2.35 (t, 2H), 1.86-1.78 (m, 3H), 1.41 (t, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 173.43, 171.00, 75.44, 67.89, 66.13, 64.27, 61.11, 53.88, 45.63, 41.54, 35.19, 32.61, 29.51, 28.63 and 25.50. FT-IR (cm^{-1}): 3891.02, 3823.94, 3733.97, 3232.53, 2951.69, 2728.95, 2325.56, 1729.56, 1455.61, 1351.06, 1267.48, 1179.03, 1093.54, 1020.78, 967.15, 833.04, 741.87, 608.32 and 518.09. A similar procedure was followed for other homopolymers as given below.

2.2.14 Synthesis of polymer CP25: P25 (200.00mg, 0.80 mmol) polymer was dissolved in 1.00 mL dry DMF along with dry triethyl amine (200.00 mg, 2.40 mmol) in a melt tube under nitrogen atmosphere. Menshutkin reaction and purification is performed as described for CP10. Yield = 190.00 mg (76 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.29 (t, 2H), 4.14 (t, 2H), 3.69 (m, 6H), 3.48 (m, 1H), 3.37 (s, 1H), 2.96 (q, 2H), 2.54 (t, 2H), 2.33 (t, 2H), 1.86-1.78 (m, 3H), 1.24 (t, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 173.97, 171.77, 75.85, 64.92, 61.01, 54.31, 46.06, 41.96, 35.61, 33.04, 30.01, 29.04, 28.39, 23.02, 14.50, 11.58 and 8.94. FT-IR (cm^{-1}): 3887, 3822, 3737, 3385, 2941, 2743, 2605, 2492, 1730, 1541, 1474, 1392, 1266, 1175, 1080, 1035, 851, 805, 738, 671, 567 and 516.

2.2.15 Synthesis of polymer CP50: P50 (200.00 mg, 0.80 mmol) polymer was dissolved in 1.00 mL dry DMF along with dry triethyl amine (200.00 mg, 2.40 mmol) in a melt tube under nitrogen atmosphere. Menshutkin reaction and purification is performed as described for CP10. Yield = 210.00 mg (84 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.29 (t, 2H), 4.14 (t, 2H), 3.69 (m, 6H), 3.48 (m, 1H), 3.37 (s, 1H), 2.96 (q, 2H), 2.54 (t, 2H), 2.33 (t, 2H), 1.86-1.78 (m, 3H), 1.24 (t, 3H). ^{13}C -NMR (100 MHz, CDCl_3) δ ppm: 173.97, 171.77, 75.85, 64.92, 61.01, 54.31, 46.06, 41.96, 35.61, 33.04, 30.01, 29.04, 28.39, 23.02, 14.50, 11.58 and 8.94. FT-IR (cm^{-1}): 3887, 3822, 3737, 3385, 2941, 2743, 2605, 2492, 1730, 1541, 1474, 1392, 1266, 1175, 1080, 1035, 851, 805, 738, 671, 567 and 516.

2.2.16 Synthesis of polymer CP80: P80 (200.00 mg, 0.80 mmol) polymer and triethyl amine (200.00 mg, 2.40 mmol) in dry DMF (1.00 mL) is taken and Menshutkin reaction is performed. Detailed procedure and purification were followed as described for CP10. Yield = 180.00 mg (72 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.58 (t, 2H), 2.36 (t, 2H), 1.86-1.78 (m, 3H). ^{13}C -NMR

(100 MHz, CDCl₃) δ ppm: 128.69, 99.82, 76.31, 64.23, 59.15, 37.98 and 28.52. FT-IR (cm⁻¹): 3824, 3452, 2951, 1730, 1354, 1256, 1173, 1098, 971, 808 and 665.

2.2.17 Synthesis of polymer CP100: P100 (200.00 mg, 0.80 mmol) polymer and triethyl amine (200.00 mg, 2.40 mmol) are taken in dry DMF (1.00 mL) and Menshutkin reaction is performed. Detailed procedure and purification is described for CP10. Yield = 175.00 mg (70 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 3.69 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.58 (t, 2H), 2.36 (t, 2H), 1.86-1.78 (m, 3H). ¹³C -NMR (100 MHz, CDCl₃) δ ppm: 143.66, 104.98, 103.13, 76.36, 69.11, 63.25 and 49.54. FT-IR (cm⁻¹): 3823, 2947, 1722, 1356, 1262, 1176, 1097, 1062, 964, 732, 657, 574 and 516.

2.2.18 Dye Encapsulation in the cationic polymer scaffolds: A detailed procedure of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS), an anionic dye loading is provided here. 5.0 mg of the polymer along with 0.5 mg of the dye were dissolved in 2.5 mL HPLC grade DMSO. To this mixture, Milli-Q water (12.5 mL) was added dropwise under constant stirring at 25 °C. The solution was kept to stir for 4 h at room temperature. The solution was transferred to the dialysis bag (MWCO = 1000 Da) and dialyzed against water for 48 h. The water was replenished with fresh milli-Q water frequently to ensure the complete removal of DMSO and unencapsulated dye. The drug loading efficiency (DLE) and drug loading content (DLC) were determined by UV-Visible spectroscopy using the following equations:

DLE (%) = {weight of drug incorporated in nanoparticle/weight of drug in feed} X 100%

DLC (%) = {weight of drug in incorporated in nanoparticle/weight of drug-loaded nanoparticles}X 100%

2.2.19 In vitro Enzymatic Biodegradation of HPTS encapsulated Cationic Polymeric Nanoparticle: (a) DLS is used to evaluate the rate of biodegradation of functionalized PCL based micelles. PCL being aliphatic polyester gets degraded in the presence of lipase enzyme. The degradation degree was evaluated by shift in size parameter of the micellar assemblies to micrometer range. This shift is corresponding to disruption in self-assembly due to chain degradation. 1 mg of lipase was incubated along with 10³ μ g/ml concentration of CP10+HPTS polymer in 2 mL milli-Q water at 37 °C. One control sample was also taken without the lipase enzyme. The change in diameter of nanoparticle was monitored with respect to incubation time. (b) Fluorescence Spectrophotometer is used to monitor the

increase in PL Intensity as dye is leached out of the self-assembled nanoparticle in the presence of esterase and lipase enzyme. 10^3 $\mu\text{g/mL}$ Polymer nanoparticle solution in PBS (for esterase) and milli-Q water (for lipase) is taken in a vial at pH 7.4 along with the concerned enzyme and steady state fluorescence spectra is recorded at HPTS emission intensity at 520 nm at various incubation time at 37 °C. Control vials in the absence of enzyme are also prepared and emission spectra of leached dye are recorded at 37 °C at pH 7.4.

Relative Release (%) = {PL Intensity for 100 % dye leaching/ PL Intensity for dye leaching at time 't'} X 100

2.2.20 Cell Viability Assay (MTT Assay): The biocompatibility of cationic polymers is determined by MTT assay on wild type mouse embryonic fibroblast (WT-MEF) cell lines.¹⁴⁷ In an atypical experiment, a fixed number of cells (10^3) were seeded in a 96-well plate (Eppendorf) in 100 μL DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin for a period of 16 h at 37 °C in a humidified 5% CO_2 -containing atmosphere. After successful adhesion of the cells at the bottom of the wells, the media was aspirated and freshly prepared solution of various polymer concentrations (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$) in media were added to each well and this was done in triplicates corresponding to each concentration along with the control wells (without polymer) and incubated for another 72 h. Post-incubation, the media was aspirated and 100 μL freshly prepared solution of MTT reagent (500 $\mu\text{g/mL}$) in sterile DMEM was added to each of the wells and incubated for another 4 h. The formation of purple formazan crystals was observed due to the reduction of MTT reagent by the mitochondrial dehydrogenase enzyme present in viable cells which were further dissolved by replacing the media with 100 μL fresh DMSO. Their absorbance was measured using the microplate reader at 570 nm (Varioskan Flash). The relative number of viable cells per plate was determined by correlating the O.D. observed for each polymeric concentration to the control wells absorbance considering it to be 100 percent.

2.2.21 Mice Blood Sample Collection and Hemolysis Assay: 0.8 mL blood was collected from orbital sinus of 7 weeks old, male C57B1/6 mice by retro-orbital blood collection technique. Required animal has been approved from animal ethics committee. The biocompatibility of the newly designed polymers in blood RBCs was determined by measuring their hemolytic activity. It was assessed by monitoring the amount of hemoglobin

released by mice erythrocytes. Freshly withdrawn blood was collected in K₃-EDTA collection tubes and centrifuged at 8000 rpm for 10 min at room temperature to separate blood plasma from the RBCs. It was washed thrice with PBS buffer and supernatant was carefully removed. The RBCs were finally diluted to the concentration of 5% (v/v) in PBS buffer. 100 μ L of the above RBC concentration was added in a 96-well plate (Eppendorf). 100 μ L of freshly prepared polymer concentrations (100 μ g/mL to 10⁻³ μ g/mL) in PBS were added to various wells of the 96-well plate. Negative (only PBS) and positive (DI water) controls were also incubated to determine the relative hemoglobin release. All concentrations were monitored in triplicates. The plate was then incubated for 1 h at 37 °C. The plate was centrifuged at 1400 rpm for 10 min. 100 μ L of the supernatant was transferred to a new 96-well plate and absorbance was measured at 576 nm. Percentage of viable erythrocytes was determined by using the formula:

$$\text{Hemolysis (\%)} = \frac{\text{OD}_{576\text{nm}} \text{ in polymer solution} - \text{OD}_{576\text{nm}} \text{ in PBS}}{\text{OD}_{576\text{nm}} \text{ in DI water} - \text{OD}_{576\text{nm}} \text{ in PBS}} \times 100$$

Selectivity Index = HC₅₀/MIC₅₀, where HC₅₀ and MIC₅₀ are corresponding to concentrations of cationic polymer required to cause 50 % hemolysis of RBCs by hemolysis assay and 50 % bacterial growth inhibition, respectively.

2.2.22 Critical Micellar Concentration (CMC): CMC is defined as the lowest concentration of the polymer required to form a stable self-assembled structure. It was determined by using pyrene as a fluoro-probe¹⁴⁷. Pyrene (0.12 μ g/mL, 0.6 μ M) solution was made in HPLC grade acetone and then acetone was evaporated at 60 °C, to get pyrene coated glass vials which were then incubated with different concentrations of polymeric system (500 μ g/mL to 1E-2 μ g/mL). The concentrations were sonicated for 4 h to allow pyrene to get inside the nano-assemblies. The solutions were incubated at room temperature overnight. Fluorescence reading of the solutions were taken along with nitrogen purging at excitation maxima 337 nm to remove any dissolved oxygen from the solution system. Inert gas purging reduces the chances of collisional deexcitation of fluorophore's excited state by molecular oxygen. Pyrene shows different behavior in different molecular environment which is reflected in the vibronic levels of the emission spectra. A steep change in the ratio of its first and third vibrational intensities (I₁/I₃) signifies the microenvironment around the probe.

2.2.23 Minimum Inhibitory Concentration (MIC): Minimum inhibitory Concentration is defined as the minimum concentration of the polymer required at which the observable growth of the bacteria is inhibited. It was determined by colony suspension assay. In the literature, two different methods have been reported for the determination of MIC, the colony suspension method and the broth microdilution method. These methods were employed for determination of MIC¹⁹⁰.

2.2.24 Colony Suspension Method: Polymer stock solutions were prepared in water. The stock solutions were serially diluted 10-fold by distilled water. *E. coli* cells were streaked from a glycerol stock maintained at -80 °C and plated onto a LB agar plate followed by an overnight incubation at 37 °C. The next day, 2-3 colonies were picked and the turbidity of the culture was maintained as 0.5 McFarland Standard which is equivalent to 0.08-0.1 OD at 600 nm.¹⁹⁰ The bacterial culture was then diluted to O.D_{600 nm} = 0.005 with LB broth corresponding to ~ 10⁶ Cfu/mL. 100 µl of this bacterial suspension was incubated along with 100 µl of polymeric stock solutions leading to final concentration of bacterial cells to be 5X10⁵ Cfu/mL. After 18 h incubation at 37 °C, the MIC₉₀ value is determined to be the lowest concentration at which killing was observed.

2.2.25 Growth (broth microdilution) Method: According to this method, the initial O.D₆₀₀ was maintained to be 0.7 O.D i.e., bacterial cells have just entered the stationary phase.¹⁹⁰ A single bacterial colony was incubated in 5 mL LB Broth for 12 h at 37 °C. The next day, the cells were pelleted down and resuspended in LB to an O.D₆₀₀ ~0.7. This stock solution of bacteria was incubated with different polymeric concentrations and incubated for 18 h at 37 °C. The minimum concentration that would lead to 90% retardation in bacterial growth was determined.

2.2.26 FE-SEM study for Antibacterial Mechanism: *E. coli* bacterial suspension (100 µL) was cultured to mid-logarithmic phase (10⁵ CFU/mL) and then incubated with CP10 polymer (100 µL, 2µg/mL) for 6 h. post-incubation, bacterial suspension was washed with sterile Phosphate buffer (X 3) and dehydrated with graded ethanol series. 2.5% formaldehyde solution was used to fix the morphology and subsequently dropcasted on the silicon wafer which was then dried in heating oven at 37 °C for 24 h. The dehydrated sample

was gold-coated for 2 min at 20 mA for imaging under high vacuum using a Zeiss Ultra Plus scanning electron microscope.

2.2.27 TEM study for Antibacterial Mechanism: Bacterial solution (0.1mL, 10^5 cells) was incubated with CP10 (0.1mL, 2 μ g/mL) polymer at its lethal concentration in an inoculation shaker at 37°C with continuous shaking. After 6 h solution was taken and centrifuged at 8000 rpm for 10 minutes, and supernatant was removed. Then the bacterial morphology fixation was done with Phosphate buffer (pH 7.0, 0.5mL) containing 2.5% glutaraldehyde and incubated overnight at 4°C. The samples were washed three times in PBS buffer (X2) and followed by dehydration with graded ethanol series. The sample (0.02mL) was spread on 400-mesh copper grids and dried. TEM images of the bacterial suspension were obtained using the Jeol JEM2200FS 200 KeV systems instrument with an acceleration voltage of 200 keV.

2.2.28 Determination of membrane action of cationic polymer using Zeta Potential:

10^3 μ g/mL solution of CP10 and CP10+HPTS polymer is prepared in PBS containing 45.7 mM NaCl, 0.9 mM KCl, 3.3 mM Na_2HPO_4 and 0.6 mM KH_2PO_4 at pH 7.4. *E. coli* ($\text{OD}_{600\text{nm}} = 0.8$) is taken in the above-mentioned polymeric solution. Time-dependent zeta potential is measured at 37 °C for 6 h. For the authenticity of the experiment, two controls are kept (a) *E. coli* bacterial suspension in PBS (b) CP10 polymer and time dependent zeta potential is determined.

2.2.29 Confocal microscopy experiments-Double Staining of Bacterial Cells: DAPI (4',6-diamidino-2-phenylindole dihydrochloride) is a cell permeable dye that stains dead and living bacteria¹⁷⁵ and PI (Propidium iodide) is a cell impermeable dye that stains dead bacteria with damaged membranes and these were used in the assay. Bacterial cell suspension (10^8 Cfu/mL) was incubated with CP10, CP50 and CP100 polymeric samples at 10 X MIC concentration. After 8 h incubation, 200 μ L of the cell suspension was taken, centrifuged and PBS (X 2) wash was given. PI (5 μ L, 56 μ g/mL in PBS) was added and left in dark for 15 min. Excess unbound dye was removed by giving PBS (X 2) wash. Then the bacterial suspension was washed with DAPI (5 μ L, 10 μ g/mL in water) and again incubated for 15 min in dark, followed by PBS (X 2) washes. The bacterial suspension was then

examined under oil-immersion objective (100 X) by using Leica SP8 optical microscope employing $\lambda 405$ nm (blue channel) and $\lambda 561$ nm (red channel) lasers.

2.2.30 Confocal Microscopy of HPTS loaded Polymer: 100 μ L CP10+HPTS polymeric nanoparticle (1×10^{-3} μ g/mL) and 100 μ L Free HPTS (8×10^{-3} μ g/mL) are incubated with *E. coli* ($OD_{600\text{ nm}} = 0.8$) in three biological replicates for 8 h at 37 °C. After that, the samples are washed with PBS (X 2). They are stained by PI (5 μ L, 56 μ g/mL in PBS) and kept at dark for 15 min. To remove excess dye, washed with PBS (X 2). The samples are examined under oil-immersion objective (100 X) by using Leica SP8 optical microscope employing $\lambda 405$ nm (blue channel) and $\lambda 561$ nm (red channel) lasers. In order to quantify the intensities their corrected total cell fluorescence (CTCF) was determined according to the following equation:

CTCF = Integrated Density- (Area of selected cell X Mean Fluorescence of background readings)

CTCF value is calculated by selecting fifteen regions of interest in similar looking bacterial cells and in the background and calculating their mean and standard deviation.

2.2.31 Time-dependent confocal Imaging of HPTS loaded CP10 polymer: For this experiment, HPTS loaded polymer (10 X MIC) was incubated with bacterial cell suspension ($0.1 OD_{600\text{ nm}}$, 10^8 cfu/mL) at 37 °C. At specified time intervals suspension is taken and washed with PBS (X 2) and stained with PI (5 μ L, 56 μ g/mL in PBS) and kept in dark for 15 min. The bacterial suspension was streaked in a glass slide using a loop and heat fixed. Two sequential emission ranges, 440-540 nm for HPTS (encapsulated in the polymeric system) and 567-707 nm for PI (externally stained) were fixed to collect individual emission coming from the two different fluorescent dyes and observed under oil-immersion objective (100 X) by using Leica SP8 optical microscope employing $\lambda 405$ nm (HPTS Excitation) and $\lambda 561$ nm (PI Excitation) lasers.

To calculate PI Intensity/ Total Intensity, three consecutive images were taken at a particular timepoint and integrated intensity for the complete image is calculated subtracting the background.

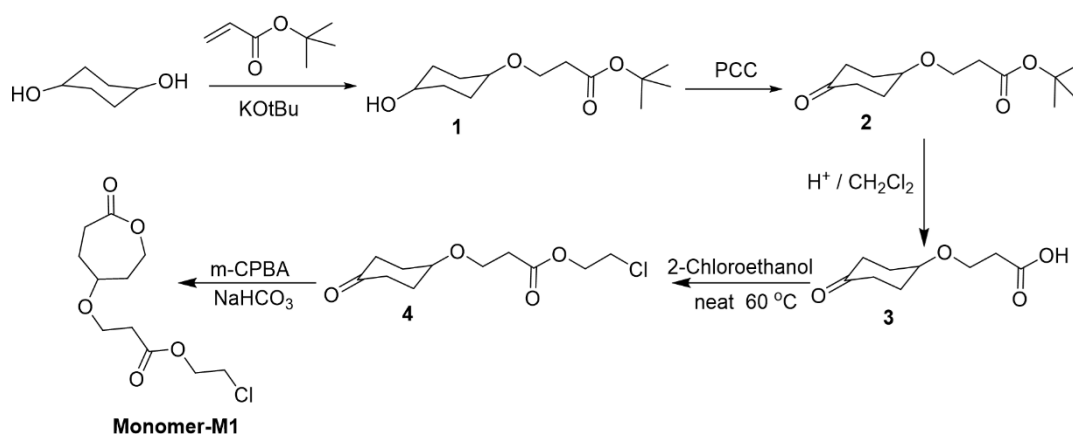
PI Intensity = Mean Intensities of three consecutive images at same time point obtained by excitation at $\lambda 561$ nm laser.

Total Intensity = Mean Intensity of three different images at same time point obtained at excitation source λ 405 nm.

2.2.32 Flow Cytometry: The cellular uptake of HPTS was determined through flow cytometry analysis of free HPTS and CP10+HPTS polymeric nanoparticles. 1×10^8 *E.coli* cells were incubated with free HPTS and CP10+HPTS nanoparticle for 4h at 37 °C. After the incubation, the cells were washed with PBS (X 2) and resuspended in 500 μ L of PBS. Flow Cytometry analyses was carried out using BD LSR Fortessa SORP cell analyzer. Excitation source of 405 nm was used with a band pass filter of 525 ± 25 nm.

2.3 Result and Discussion

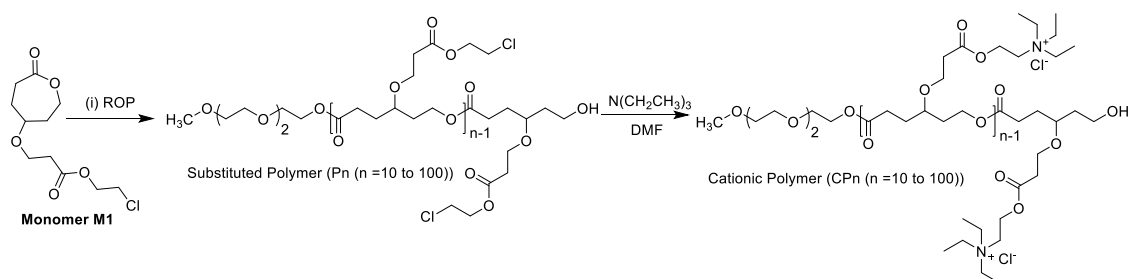
2.3.1 Synthesis of Amphiphilic Cationic Polycaprolactones



Scheme 2.2: Synthesis of chlorine substituted monomer. PCC is a short form of pyridinium chlorochromate $[C_5H_5NH]^+ [CrO_3Cl]^-$ and m-CPBA is m-chloroperbenzoic acid $[ClC_6H_4CO_3H]$.

The multi-step synthesis for chlorine substituted caprolactone monomer (Monomer M1) involves the utilization of commercially available 1,4-cyclohexanediol which undergoes Michael addition reaction to yield tert-butyl 3-((4-hydroxycyclohexyl)oxy)propanoate (Compound 1). The oxidation of aliphatic alcohol in compound 1 with PCC in dry DCM for 12 h yielded tert-butyl 3-((4-oxocyclohexyl)oxy)propanoate (compound 2) as shown in Scheme 2.2. The de-protection of compound 2 to develop carboxylic substituted cyclohexanone derivative 3-((4-oxocyclohexyl)oxy)propanoic acid (compound 3). It was subjected to reaction with 2-chloroethanol to yield 2-chloroethyl 3-((4-oxocyclohexyl)oxy)propanoate (compound 4)

which was ultimately oxidized by baeyer villiger oxidation using m-CPBA in presence of base yielded chlorine substituted ϵ -caprolactone monomer (Monomer M1).



Scheme 2.3. Synthesis of chlorine substituted polymer and triethyl amine functionalized cationic polymer. (i) TEG, $\text{Sn}(\text{OC}_8\text{H}_{17})_2$, Toluene, 110°C .

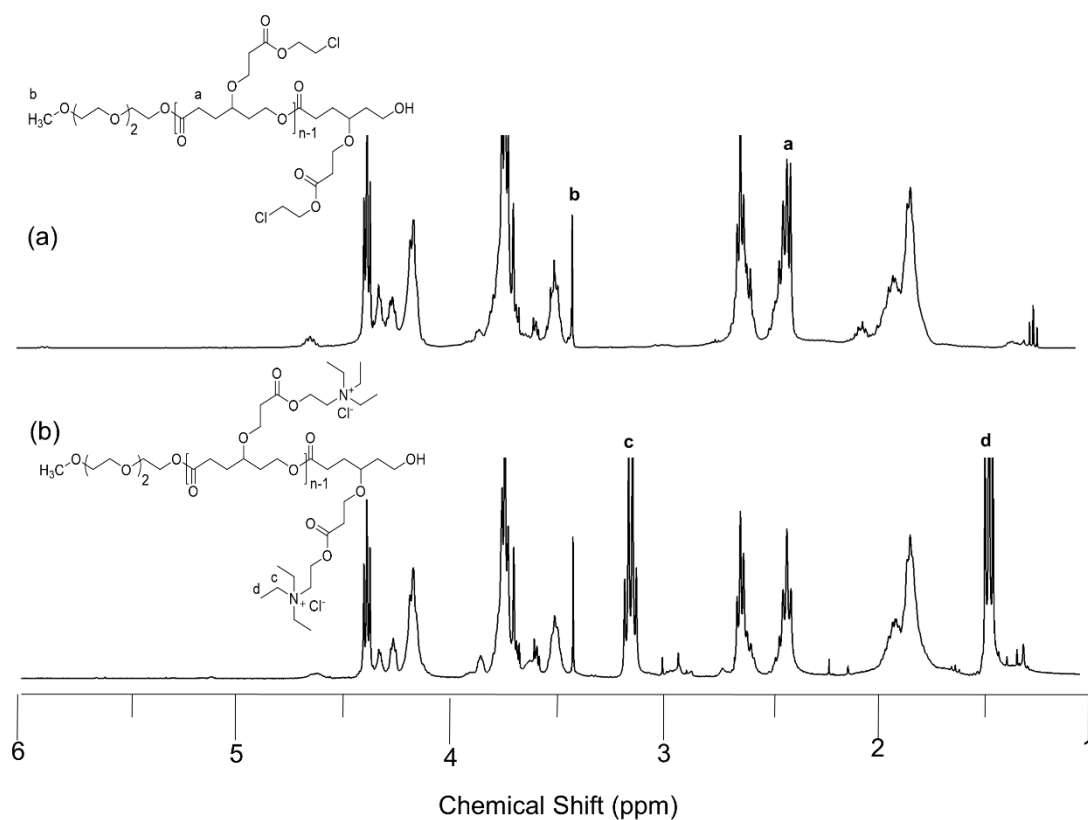


Figure 2.3. ^1H - NMR spectra of (a) chlorine substituted polymer (p10) and (b) triethylamine quaternized polymer (CP10) in CDCl_3

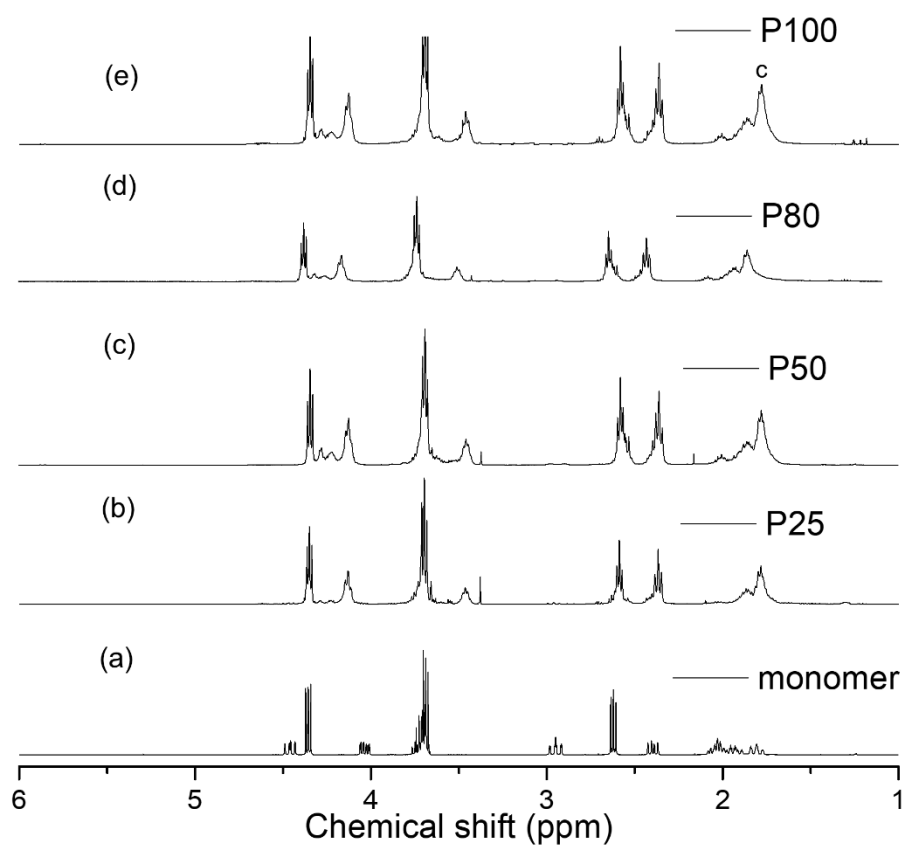


Figure 2.4. ^1H -NMR spectra of (a) monomer 3, (b) P25, (c) P50, (d) P80, (e) P100 in CDCl_3 .

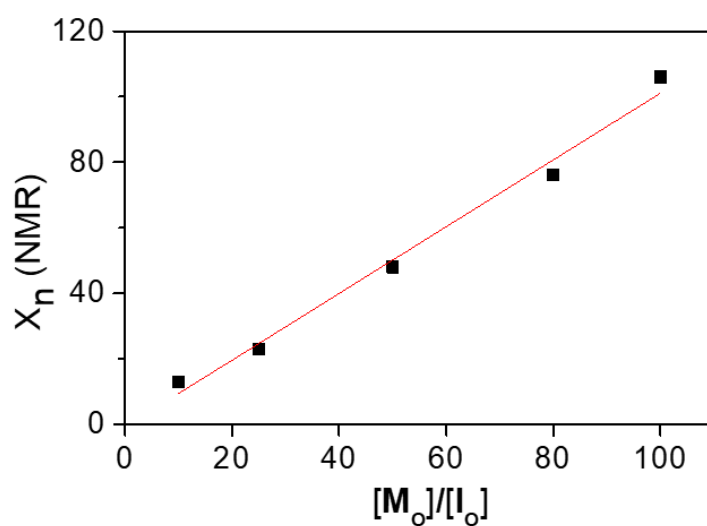


Figure 2.5. Plot of $[M_0] / [I_0]$ in the feed versus the degree of polymerization (n) determined from ^1H -NMR for various polymeric repeating units.

Ring opening polymerization (ROP) of monomer **M1** was carried out in dry toluene using triethyleneglycol monomethyl ether (TEG) and Tin (II) 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$) as initiator and catalyst, respectively (see Scheme 2.3). The ^1H -NMR spectra of P-10 is shown in Figure 2.3a. The degree of polymerization (n) in the ROP synthesized polymer was estimated by comparing $-\text{OCH}_3$ protons of the TEG initiator at 3.38 ppm (proton b) to that of polymer peak $-\text{COCH}_2$ at 2.35 ppm (proton a) in Figure 2.3a. The $[\text{M}_0]/[\text{I}_0]$ ratios were taken as 10, 25, 50, 80, 100 in the feed for varying the molecular weight of the PCL chains. ^1H -NMR spectra of monomer **M1** along with various chlorine-substituted PCL polymers are shown in Figure 2.4. The $-\text{COCH}_2\text{CH}_2$ protons in the ϵ -caprolactone ring appeared at 2.90 and 2.38 ppm being diastereotopic protons which upon polymerization appeared together at 2.35 ppm in the polymer spectrum (see Figure 2.4). The degree of polymerization was obtained as 10, 24, 52, 82, and 99 for the $[\text{M}_0]/[\text{I}_0]$ in the feed 10, 25, 50, 80 and 100, respectively. The plot of $[\text{M}_0] / [\text{I}_0]$ in the feed versus the degree of polymerization (n) exhibited a linear trend with slope of ~ 1.0 (see Figure 2.5) confirming very good control over the molecular weight of PCL polymers in the ROP process. The post polymerization functionalization of chlorine-substituted PCL into triethylamine quaternary ammonium salt was confirmed in Figure 2.3b. The chlorine substituted PCLs (referred as Pn) were reacted with triethylamine in DMF to produce quaternary ammonium salt of cationic PCL (referred as CPn), where n is the number of repeating units. The appearance of two new peaks at 3.11 ppm and 1.41 ppm in the polymer spectrum are assigned with respect to $-\text{NCH}_2\text{CH}_3$ (proton c) and $-\text{NCH}_2\text{CH}_3$ (proton d). Further, the comparison of the integration of peaks in polymer main chain (proton a) to that of a new peak (proton c) showed 1:1 ratio with respect to the expected chemical structure and confirmed the formation of the triethylamine quaternary ammonium salt of the PCL polymers. ^{13}C -NMR (see Figure 2.6) of the cationic polymer CP10 showed appearance of new peaks at 45.0 ppm and 8.7 ppm with respect to carbon atoms in $-\text{NCH}_2\text{CH}_3$ and $-\text{NCH}_2\text{CH}_3$ compared to its parent polymer P10. The ^1H -NMR spectra for other cationic polymers are shown in Figure 2.7.

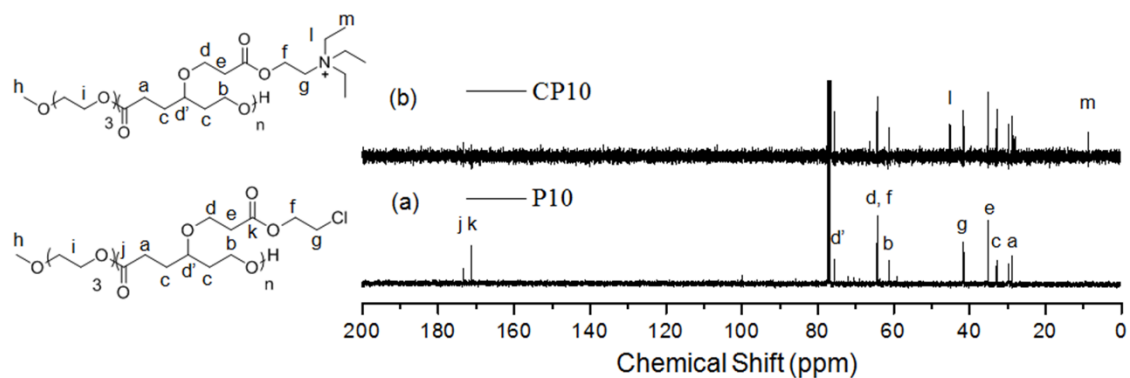


Figure 2.6. ^{13}C -NMR spectra of (a) P10 and (b) CP10 in CDCl_3 . The peaks are assigned by alphabets.

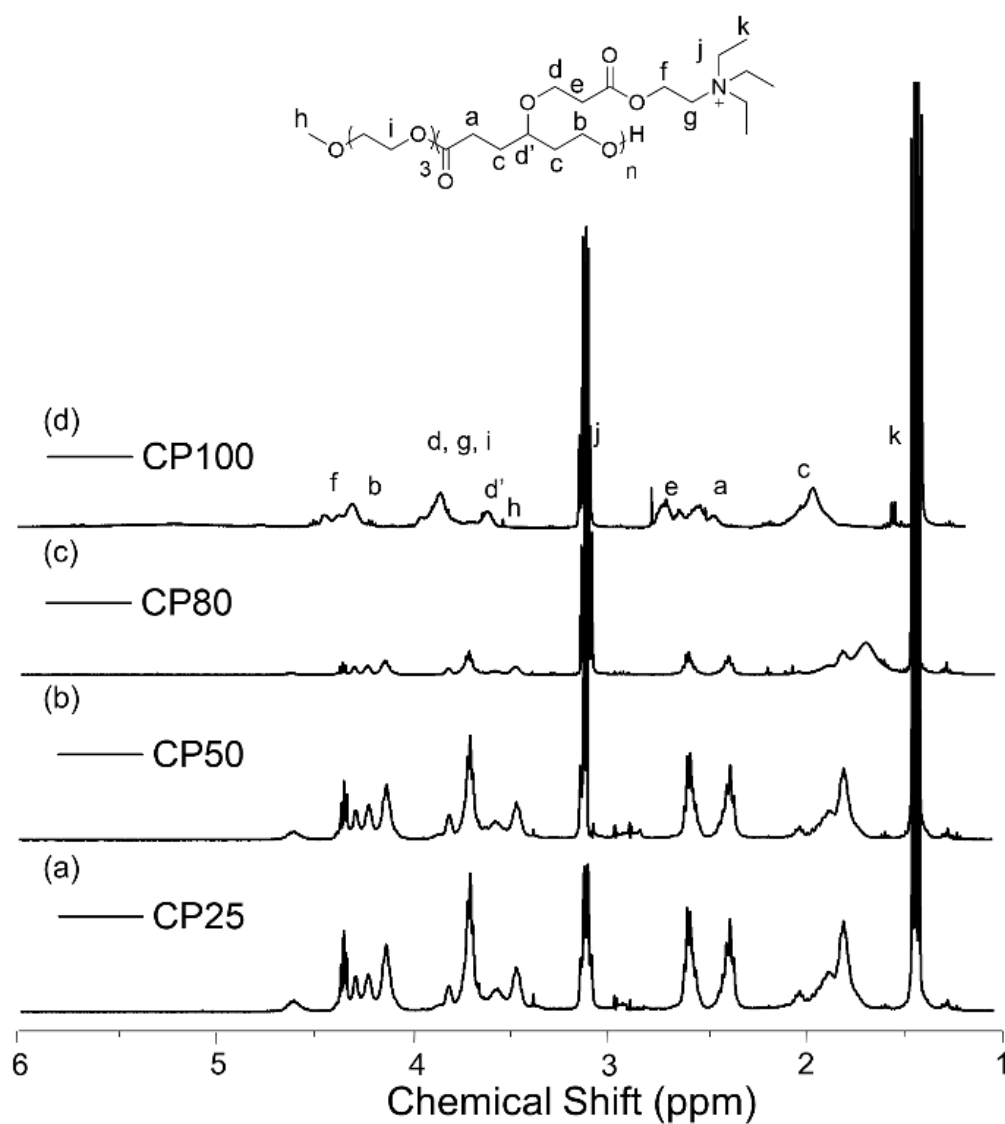


Figure 2.7. ^1H -NMR spectra of (a) CP25, (b) CP50, (c) CP80, (d) CP100 in CDCl_3 . The peaks are assigned by alphabets.

The molecular weights of the chlorinated PCL polymers were determined by gel permeation chromatography using polystyrene as the standard in THF solvent (see Figure 2.8). All the polymers showed monomodal distribution and the GPC molecular weights M_n , M_w , and polydispersity are shown in the table in Table 2.1. The M_n of the polymers determined by GPC showed large deviation from NMR which is attributed to the underestimation of the molecular weights by GPC (due to the limitation in usage of polystyrene standards for calibration). The PDI of the polymers were found to be ≤ 1.8 which is slightly higher for the substituted PCL polymers compared to PCL as observed in our earlier reports.⁴⁶

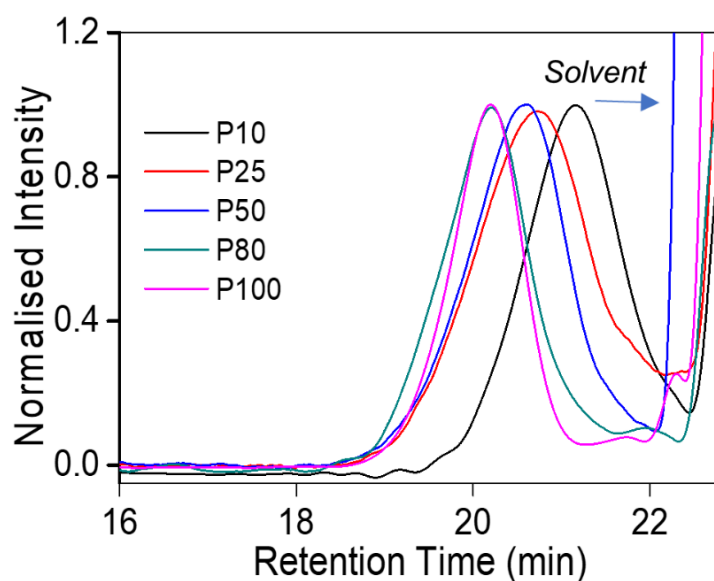


Figure 2.8: GPC plots of chlorinated PCL polymers P10, P25, P50, P80, P100 in THF.

Table 2.1. Table containing the degree of polymerization (n) estimated by ^1H -NMR, GPC molecular weights and the thermal properties of the polymers.

Table containing the molecular weights and properties of the polymers

Polymer	M_n (g/mol) ^a	M_n (g/mol) ^b	M_w (g/mol) ^b	(M_w/M_n)	T_D (°C) ^c	T_g (°C) ^d
P-10	3100	2800	5000	1.8	132	-27.55
P-25	6300	3600	5000	1.4	150	-37.72
P-50	12100	4300	7800	1.8	156	-37.74
P-80	20600	5700	10800	1.9	160	-29.92
P-100	25900	7200	13100	1.8	250	-27.33

^aMolecular weights are determined by ^1H NMR. ^bMolecular weights and PDI are determined by GPC using PS standard in THF at 25 °C. ^cDetermined by TGA at 10 °/min heating rate under N_2 . ^dDetermined by DSC at 10 °/min heating rate under N_2 .

MALDI-TOF MS spectrum of chlorine substituted polymer P-10 is given in the Figure 2.9. The spectra showed mass peaks corresponding upto 5 units having mass with respect to $P_n + 164 + 39$, where P represents the repeating unit mass and 164 is attributed to the end group mass. Unlike PCL chains, it was very difficult to detect high molecular weight of polymer chains in MALDI spectrum for substituted PCL. This may be partially associated with the slightly large polydispersity of the substituted PCL which hampered both desorption and TOF process during the sample analysis. Thermal stability of the newly designed chlorine substituted PCL polymers and cationic polymers were determined using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) under nitrogen at 10 °C/min (see Figure 2.10). The polymers were found to be stable up to 250 °C. DSC thermograms of the polymers showed only glass transition temperature (T_g) in the range of -37 to -27 °C with respect to their highly amorphous nature (see Table 2.1).

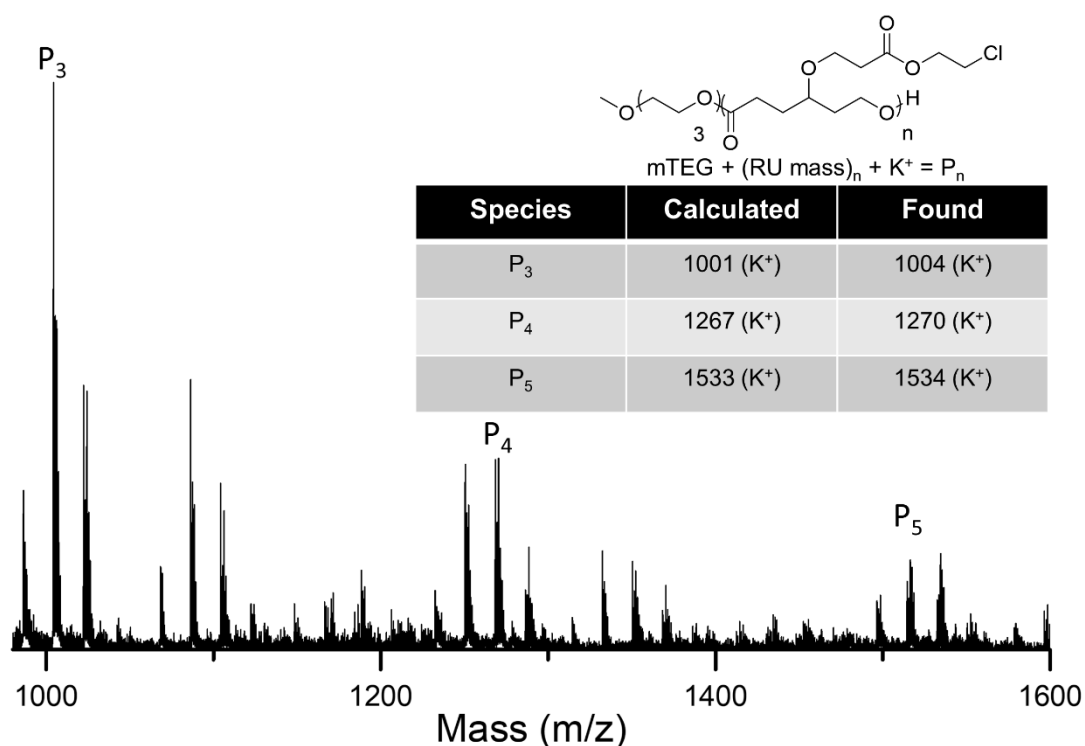


Figure 2.9. MALDI-TOF mass spectra of the P10 polymer in CHCA matrix with 2:1 composition of polymer and matrix

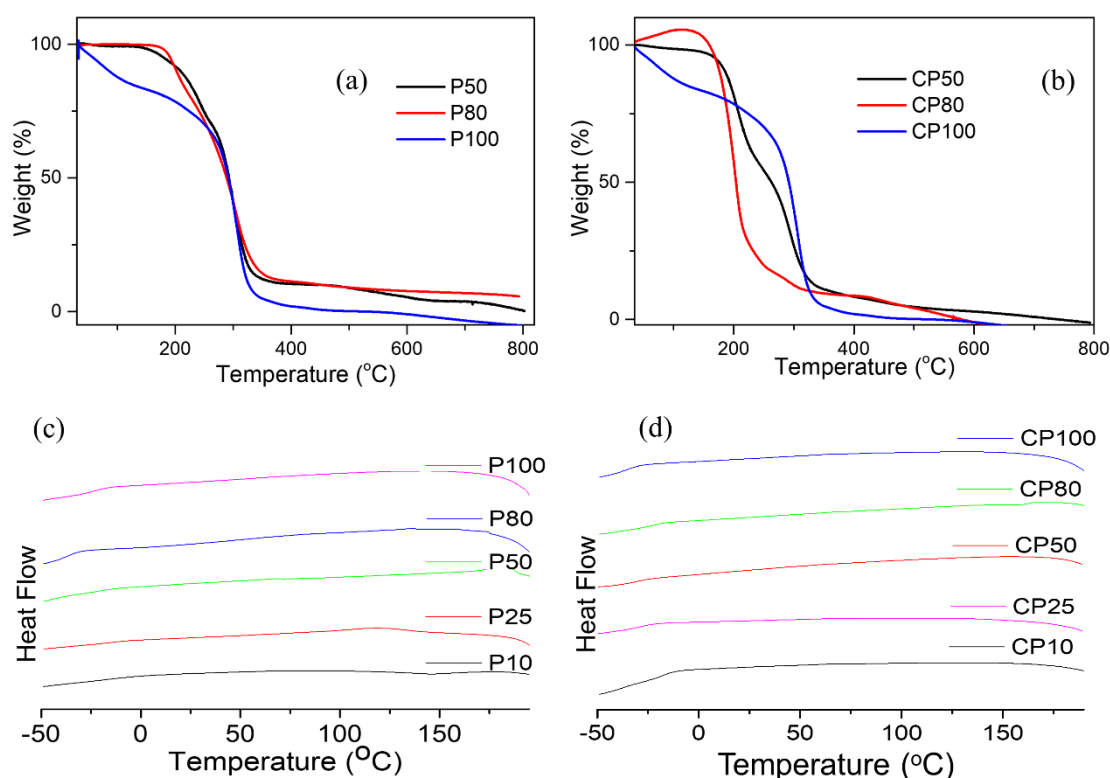


Figure 2.10. Thermogravimetric analysis of (a) P50, P80, P100 and (b) CP50, CP80, CP100 at 10 °/min heating rate under nitrogen. (c) DSC thermograms of P-n polymer at 10 °C/min in the cooling cycle under nitrogen. (d) DSC thermograms of CP-n polymer at 10 °C/min in the cooling cycle under nitrogen.

2.3.2 Self-assembly of Cationic PCL

The newly designed cationic PCL structure has hydrophobic backbone and hydrophilic quaternary ammonium salt as pendant group in each repeating unit; thus, the structure possessed appropriate amphiphilicity to self-assemble in aqueous medium as shown in Figure 2.11a. It is also important to mention that the chlorine-substituted PCL polymer was completely insoluble in water due to the lack of the hydrophilic component in the polymer. For self-assembly studies, the cationic polymer (up to $5 \times 10^3 \mu\text{g/mL}$) was dissolved in water at 55 °C and subjected to sonication for 10 minutes at 25 °C to obtain clear solution.

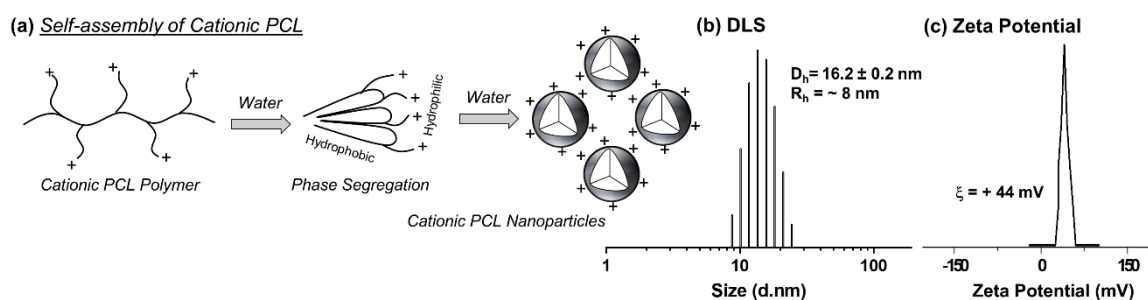


Figure 2.11. (a) Self-assembly of cationic PCL polymer in aqueous medium into spherical nanoparticles. (b) Dynamic light scattering histograms of CP10. (c) Zeta potential plot of CP10.

The size of the polymer nano-assemblies was determined by dynamic light scattering (DLS) and the histogram for CP10 is shown in Figure 2.11b. The cationic polymer CP10 showed the existence of particle sizes of 16.2 ± 0.2 nm with narrow distribution having polydispersity < 0.1 . Further, the sizes of the nano-aggregates were found to be almost similar irrespective of the molecular weight or chain length of the cationic polymers. DLS typically gives hydrodynamic diameter of the nano-aggregates (D_h) from which the hydrodynamic radius could be readily determined ($R_h = D_h/2$). The nanoparticles showed Zeta potential values as $+44.6$ mV (see Figure 2.11c) with respect to good cationic polymer nanoparticles. To visualize the size and shape of the cationic PCL nano-assemblies, the sample CP10 was subjected to electron microscope (FE-SEM and HR-TEM) analysis (see Figure 2.12a and 2.12b). FE-SEM showed the existence of large number of tiny spherical nanoparticles of average sizes of 8.8 ± 0.1 nm (as shown in the histogram).

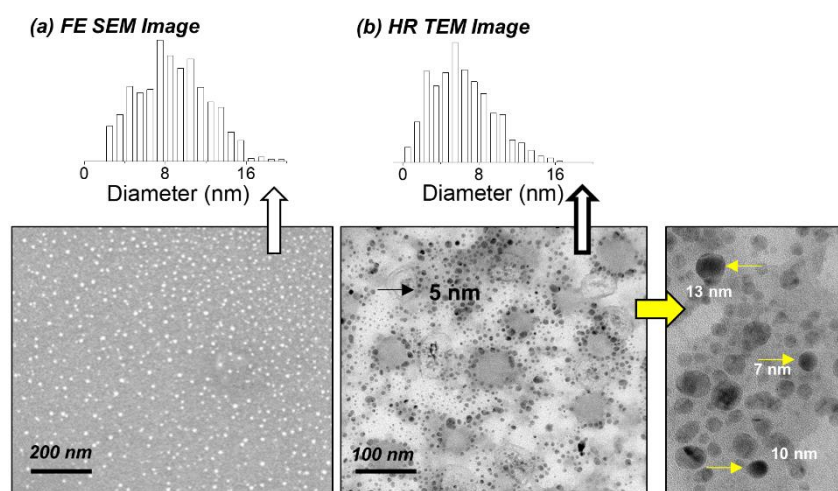


Figure 2.12. (a) FE-SEM image of CP10 polymer nanoparticles. (b) HR-TEM image of CP10 nanoparticles.

HR-TEM image showed the average size of the nanoparticles as 6.2 ± 0.1 nm and the existence of nanoparticles having sizes as small as 5 nm (see the expanded image in Figure 2.12b). The size of the nanoparticles observed in the solution aggregates (DLS data) and the HR-TEM images were comparable which suggested that the cationic nanoparticles indeed existed as stable nanoparticles in aqueous solution. Further, DLS size and zeta potential of the cationic PCL polymers did not show much change despite ten order magnitude difference in the molecular weights between the entire range of CP10 and CP100 (see Table 2.2). The chlorine substituted and its corresponding cationic polymers were subjected to water contact angles (WCA) measurements to study their amphiphilicity (see Figure 2.13). It was observed that for PCL based chlorinated polymers, the water contact angle measurement had come in the range of 85° to 93° while in case of quaternized polymers WCA fell in the range of 29° to 47° (see Table 2.2). This difference in the interaction of water droplet to polymeric samples in cationic and chlorine PCL samples could be accounted by the hydrophilic and hydrophobic nature of the polymers, respectively.

Table 2.2. Table contains the DLS size, Zeta potential, CMC (Critical Micellar Concentration) and WCA (Water Contact Angle) of the polymer nanoparticles. DLS and Zeta potential are recorded for $100 \mu\text{g/mL}$ concentration of polymer in water and electron microscope imaging are done for $100 \mu\text{g/mL}$ solutions.

Table containing Properties of cationic polymer

Polymer	DLS Size (nm) ^a	Zeta Potential (mV) ^b	CMC (g/mL) ^c	WCA ($^\circ$) ^d
CP10	16.2 ± 0.2	44.6	5×10^{-7}	47
CP25	14.7 ± 4.2	49.8	4×10^{-7}	38
CP50	23 ± 11	40.6	4×10^{-7}	39
CP80	9.75 ± 0.75	39.5	1×10^{-7}	29
CP100	17.1 ± 6.6	41.3	1×10^{-6}	31

^aSize determined by dynamic light scattering at 37°C using polystyrene cuvette. ^bSurface charge determined at 37°C using Zetasizer cuvettes with folded capillary and in-built electrodes. ^cCMC determined using pyrene ($0.6 \mu\text{M}$) as probe. ^dWater Contact Angle measured by placing a water droplet on thin layer of polymer film made on a cover slip

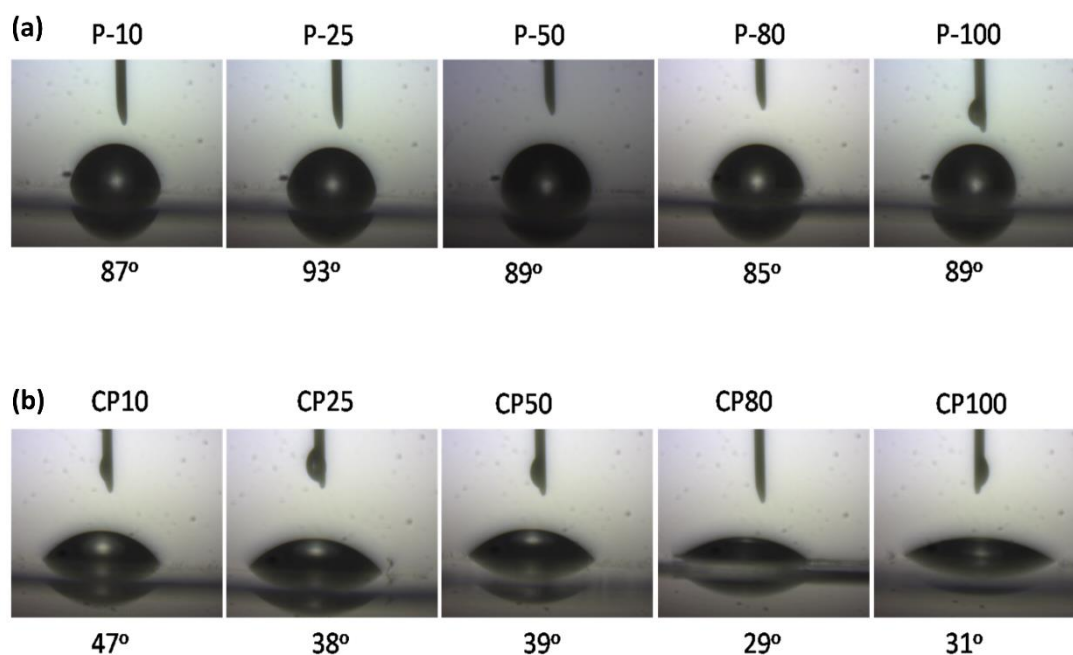


Figure 2.13: Water Contact Angle of (a) P10, P25, P50, P80 and P100 (b) CP10, CP25, CP50, CP80 and CP100 measured by placing a water droplet on thin layer of polymer film made on a cover slip.

The critical aggregation concentration (CAC) of the cationic PCL was determined using pyrene as probe. A 0.6 μM concentration of pyrene was incubated with various concentrations of the polymer ranging from 10^{-5} to 10^{-3} $\mu\text{g/mL}$ in water.¹⁴⁷ Emission spectra of pyrene was recorded by excitation at 337 nm. The ratio of I_1 and I_3 could be interpreted as the location of pyrene i.e., hydrophobic (inside polymeric micelle) or hydrophilic (unloaded in water) compartment. For that purpose, I_1/I_3 peak intensities of pyrene were plotted against the concentration of polymer solution (see Figure 2.14) and the break points in these plots gave the CAC values of the polymers that are summarized in Table 2.2. The CAC of the polymers was obtained in the range of 10^{-4} to 10^{-3} $\mu\text{g/mL}$ concentration range irrespective of the molecular weights of the cationic polymers. All the above studies are pointing out that the self-assembly of the cationic PCL polymers are predominately driven by its amphiphilic structure and the molecular weights (CP10 to CP100) seems to be relatively less influential.

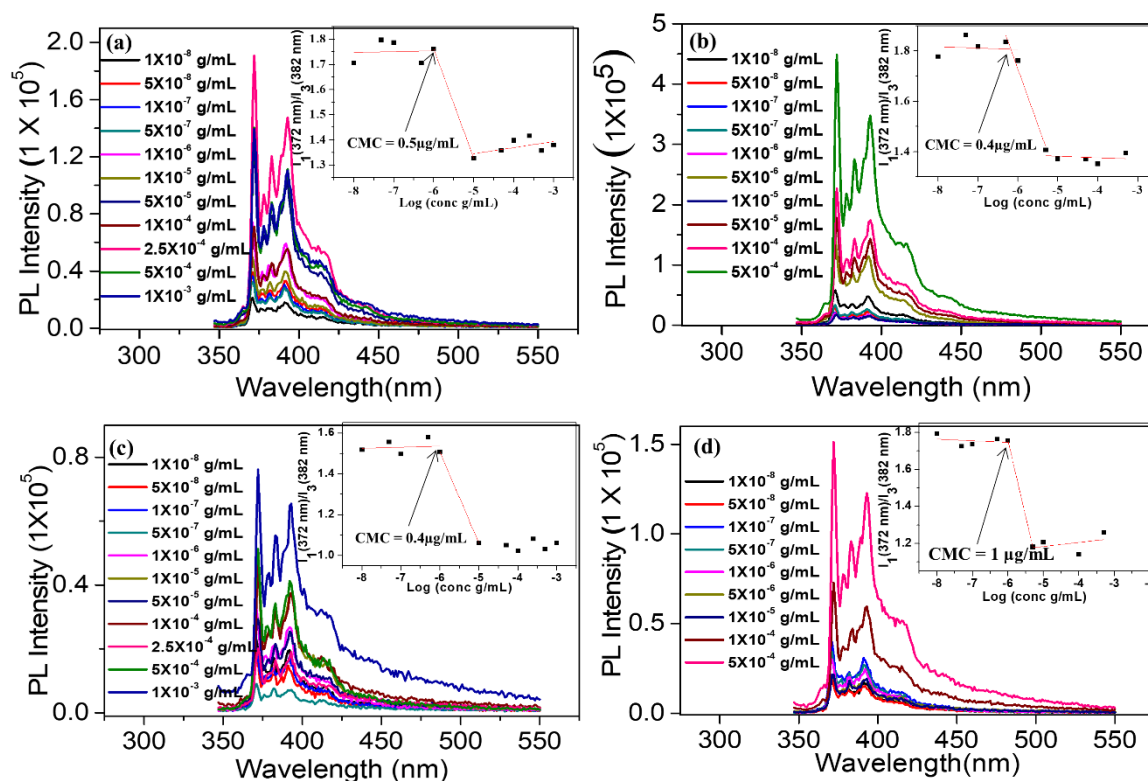
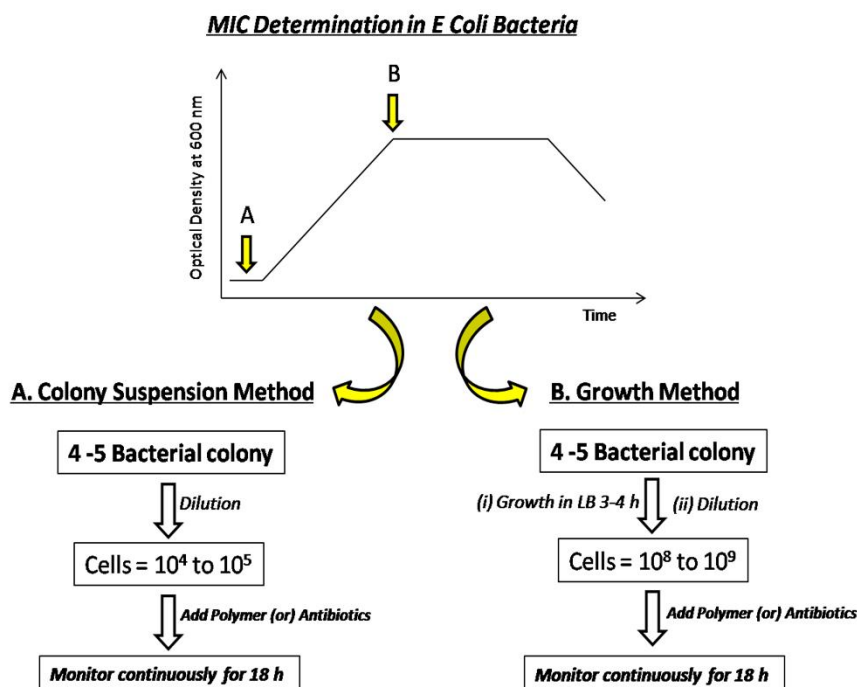


Figure 2.14: Emission spectra of pyrene at various concentrations to determine Critical Aggregation Concentration (CAC) of (a) CP10, (b) CP25, (c) CP50 and (d) CP100 using pyrene as a probe in water. Plot of I_1/I_3 peak versus logarithmic concentration of polymer is inset within respective emission plots.

2.3.3. Antibacterial Activity and Mechanism

The cationic PCL nanoparticles were tested for antibacterial activity using gram negative bacterium *E. coli* using two independent methods namely, the colony suspension method and the growth (or broth microdilution) method.¹⁹⁰ These two methods are schematically shown in Figure 2.15 and the experimental procedures were optimized against *E. coli* using the antibiotic ampicillin exhibiting MIC in the range of 16 $\mu\text{g/mL}$. In the colony suspension method, the cationic PCL was administrated at the initial stage of the log phase (10^5 CFU/ml) and the bacterial viability was continuously monitored by recording the turbidity at 600 nm for 18 hours. Antibacterial activity (OD at 600 nm) of polymer CP10 at various polymer concentrations ranging from 1×10^{-9} $\mu\text{g/mL}$ to 1×10^{-2} $\mu\text{g/mL}$ are shown in Figure 2.16a. The positive control (without any polymer) showed a continuous increase in the turbidity of the culture with an increase in the incubation time whereas the negative control (with ampicillin) showed more than 99 % inhibition in the *E. coli* growth.



Reference: Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **2008**, 3, 163.

Figure 2.15: Schematic representation showing a bacterial growth curve and the two methods (A) Colony Suspension method and (B) Growth method based on the initial bacterial density to determine the minimum inhibitory concentration (MIC) for *E. coli*.

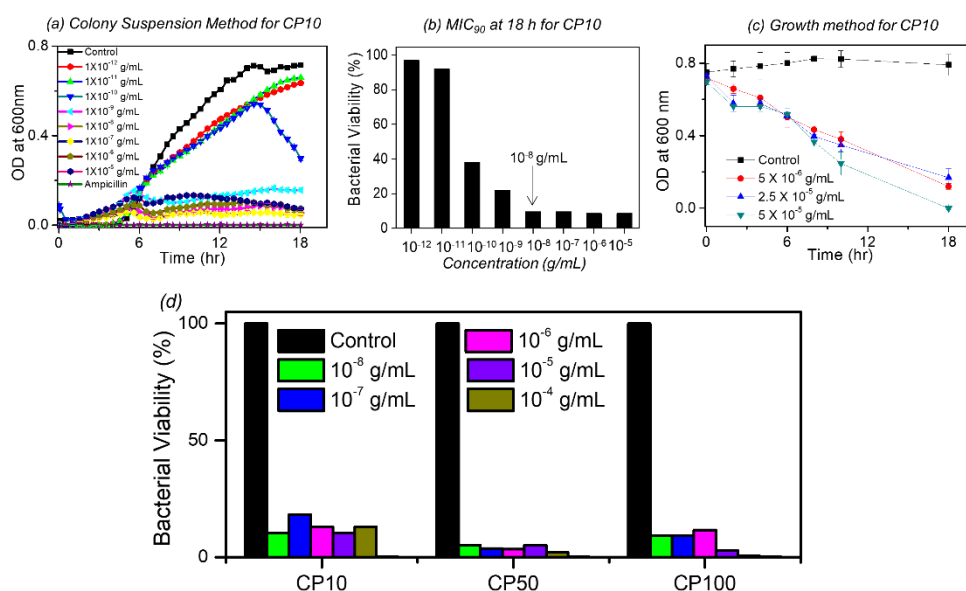


Figure 2.16. (a) Growth inhibition of *E. coli* using colony suspension method, showing the growth of the *E. coli* culture monitored at 600 nm over a period of 18 h without polymer (positive control), in the presence of Ampicillin (negative control), and at various concentration of CP10. (b) Plot of percentage bacterial viability versus the concentration of CP10 polymer. (c) Bactericidal activity of

the polymer against *E. coli* using broth microdilution method, the turbidity of the *E. coli* culture monitored at 600 nm over a period of 18 h without polymer (positive control) and at various concentration of CP10.

Interestingly, the CP10 polymer showed concentration dependent inhibition of the *E. coli* growth, with very low polymer concentration (10^{-9} to 10^{-7} $\mu\text{g/mL}$) not showing any growth inhibition, while with an increase in the polymer concentration ($>10^{-6}$ $\mu\text{g/mL}$), a significant reduction in the turbidity of the culture was observed corresponding to the suppression of the *E. coli* growth in the broth. To further stress the bacteriostatic property of the polymer, the rate of exponential growth (k) and the generation time (t_{gen}) of the bacterial culture was determined.^{191, 192} During exponential growth of a bacterial culture, the rate of growth is the number of generations of bacteria produced per hour and the generation time is the time taken by bacteria to double the growth. It was observed that for the control, growth rate (k) was 2.23 gen/h and generation time (t_{gen}) was 27 min/gen as reported earlier.¹⁹³ At very low polymer concentration (1×10^{-9} $\mu\text{g/mL}$), k and generation time (2.46 gen/h and 24 min/gen respectively) were almost identical to that of the positive control. Interestingly at higher polymer concentration (1×10^{-5} $\mu\text{g/mL}$), k and generation time were obtained as 0.61 gen/h and 97.68 min/gen, respectively. It clearly specifies that the growth rate of *E. coli* was highly reduced in the presence of cationic polymer CP10 and the time taken by the bacterium to double itself was increased by 70 min. To determine the MIC at 90 % inhibition of the *E. coli* growth (MIC_{90}), the bacterial viability $[(\text{OD}_{\text{polymer}}/\text{OD}_{\text{control}}) \times 100]$ was plotted for each polymer concentration as shown in Figure 2.16b. From the on-set of the break-point, the MIC_{90} for the CP10 polymer was estimated to be 10^{-5} $\mu\text{g/mL}$. The antibacterial activity of the cationic polymers was also tested by broth microdilution method. An overnight culture of *E. coli* grown in LB broth ($\text{OD}_{600 \text{ nm}} = 0.8$, 10^8 CFU/mL) at the late log phase was incubated with cationic PCL polymer for 18 h. The polymer concentration was chosen to be 10-100 folds higher than the MIC_{90} observed in the colony suspension method to compensate for the presence of large number of *E. coli* cells in the broth. A positive control with *E. coli* grown in the absence of polymer showed no significant changes in growth as shown in Figure 2.16c indicating that the cells were stable and viable in the stationary phase under the experimental conditions. However, upon exposure to the polymer, a significant reduction in the bacterial density was observed over time and the bacterial viability was $< 2\%$ post 18 hours of incubation. A comparative antibacterial activity of three different molecular weight cationic polymers (CP10, CP50 and CP100) in the colony suspension assay is shown in

Figure 2.16d. The MIC₉₀ value for all the three polymers falls in the range of 10^{-5} $\mu\text{g/mL}$. The closer observation of the data in Figure 2.16d revealed that the higher molecular weight polymer chains in CP50 and CP100 could accomplish a larger inhibition of the growth of *E. coli* with bacterial viability $< 2\%$ compared to that of the shorter chains (CP10, $\sim 10\%$ viability).

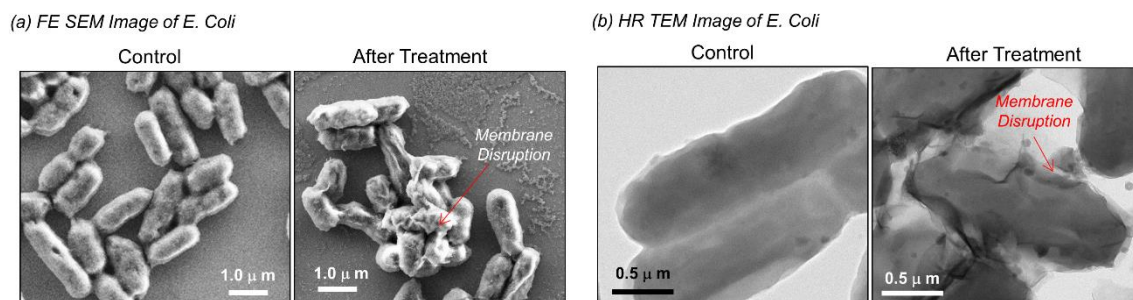


Figure 2.17. a) FE-SEM images of *E. coli* before and after treatment with cationic PCL polymer CP10. (b) HR-TEM images of *E. coli* before and after treatment with cationic PCL polymer CP10. For sample preparation, CP10 polymer ($2 \mu\text{g/mL}$) was incubated with *E. coli* bacteria for 6 h before dehydration with graded ethanol series and fixation with 2.5 % (w/v) formaldehyde solution.

To study the mechanism of action of polymeric nanoparticles on *E. coli*, the cells were subjected to morphological analysis by electron microscopy. *E. coli* (10^7 - 10^8 cells) were incubated with CP10 polymer for 6 h and their images were captured before and after exposure to the polymer. Field emission scanning electron microscope (FE-SEM) image of the control cells of *E. coli* showed its rod-like shape with intact membranes in Figure 2.17a. After the treatment with the polymer CP10, the cell membrane was observed to be ruptured followed by the interaction between the polymer and the *E. coli* cell membrane. In order to further understand the mechanism of action at a higher resolution, high resolution transmission electron microscope (HR-TEM) images for the *E. coli* before and after exposure to CP10 polymer were also captured as shown in Figure 2.17b. It was clearly observed that the action of polymer leads to leakage of the contents of bacterial cell with a completely compromised membrane. The electron microscope images are evidence of the *E. coli* membrane disruption by the cationic PCL polymers and its bactericidal action. Hence, it may be concluded that the newly designed cationic PCL polymer is very efficient in inhibiting growth of the gram-negative bacterium. *coli* through membrane disruptive pathways as typically expected for cationic polymer systems. The molecular weights of the polymers seem to have relatively less influential on the overall antibacterial activity. Interestingly, the MIC₉₀ data revealed that the PCL cationic polymers are capable of not just

inhibiting the bacterial growth, but also to kill the cells. Thus, these cationic polymers may be very useful with wide ranges of application in both material science and biomedical field.

2.3.4 Hemolysis, Cytotoxicity and Selectivity Index

Under physiological conditions, the cationic polymers encounter direct interaction with three important biological species as shown in Figure 1: (i) electrostatic interaction with anionic bacterial membranes, (ii) hemolysis of red blood cells (RBC) in bloodstream, and (iii) adhesion to healthy tissues and cells. Though a significant difference exists in the negative potential of the bacterial membranes, RBCs, and mammalian cell membranes; they have definitively high affinity towards the cationic polymers via electrostatic interactions. Therefore, it is essential to test the exclusive affinity of the newly designed cationic PCL polymers towards bacterial membranes compared to RBCs and healthy cells. To visualize the effect of cationic polymer on the bactericidal activity double-staining method was employed using DAPI and Propidium Iodide (PI) dyes.

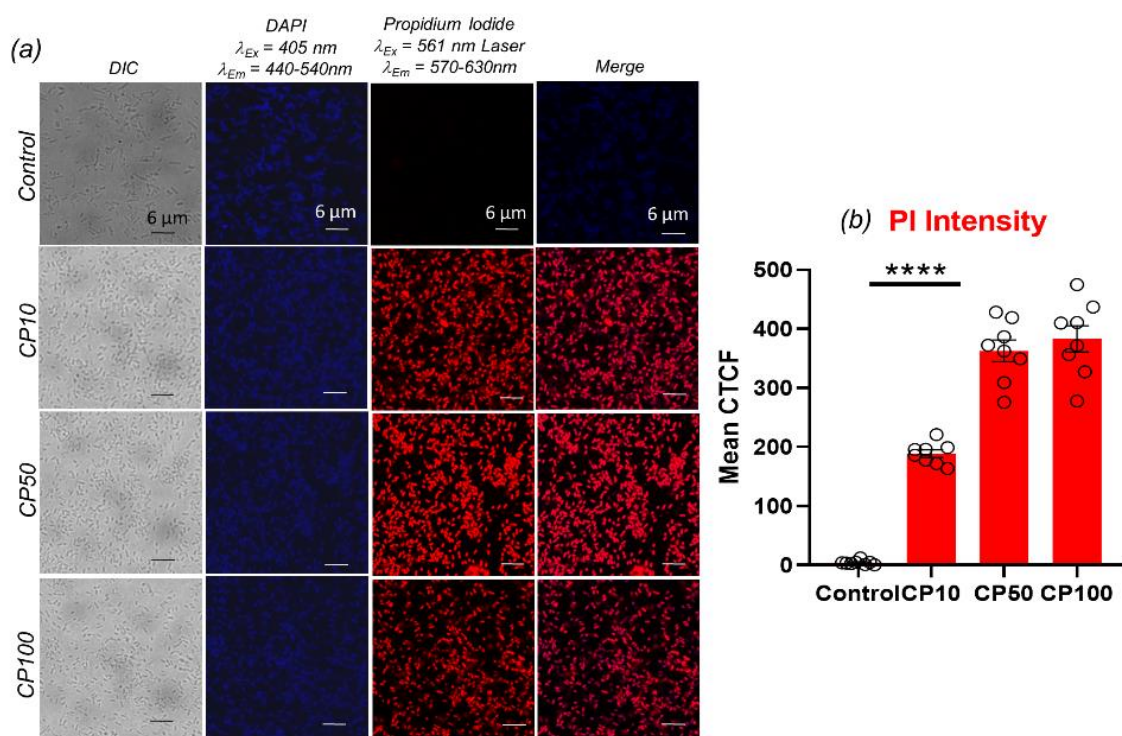


Figure 2.18. (a) Confocal microscope images in *E. coli* cells control (first column) and incubated with cationic PCL nanoparticle CP10, CP50, CP100 (100 μg/mL, (second column)). The cells were stained by DAPI (blue) and PI (red) [Incubation time = 8 h], (*E. coli*) = 10^8 CFU). (b) Plot of Mean CTCF calculation for PI Intensity of Control and CP10, CP50 and CP100 polymers. (**** $P < 0.0001$) Each point represents mean $\pm$ SEM ($n = 8$).

DAPI is known to stain all bacterial cells irrespective of their viability while PI being a cell-impermeable dye, only stains the nucleic acid of membrane compromised cells.¹⁷⁵ Bacterial cells (1×10^8) were incubated with CP10 at 10^2 $\mu\text{g/mL}$ polymer concentration for 8 h. In confocal microscope, the bacterial cells were excited with 405 nm and 561 nm lasers for DAPI ($\lambda_{\text{em}} = 440\text{-}540$ nm) and PI ($\lambda_{\text{em}} = 567\text{-}707$ nm), respectively and the images are shown in Figure 2.18a. In control experiment (without polymer), the cells stained by DAPI showed very good blue emission whereas there was no red emission from PI-staining confirming that the bacterial membrane is intact and *E. coli* are largely alive. The images for *E. coli* incubated with the cationic polymer CP10 showed the blue emission from DAPI channel as well as exhibited strong red emission from PI which corroborates to the *E. coli* membrane disruption followed by antibacterial activity. The merged images show the co-localization of both DAPI and PI. Further, the corrected total cell fluorescence (CTCF) intensities were calculated from the PI-stained red channel and are shown in Figure 2.18b. The bar diagram corresponding to control (without polymeric nanoparticle) shows extremely low PI intensity which confirms that all the bacterial cells were largely alive with intact cell membrane. In case of polymer incubated bacterial samples (CP10), an exuberant increase in PI intensity is observed which reiterates that the cationic polymer exhibits antibacterial activity against *E. coli* due to which PI can stain the nucleic acid of dead bacteria. A similar observation was noticed for the incubation of CP50 and CP100 polymers along with *E. coli* (see Figure 2.18b). This observation recapitulates the antibacterial activity of the cationic PCL and their membrane disruption mechanism as observed in electron microscopic analysis in Figures 2.17a and 2.17b.

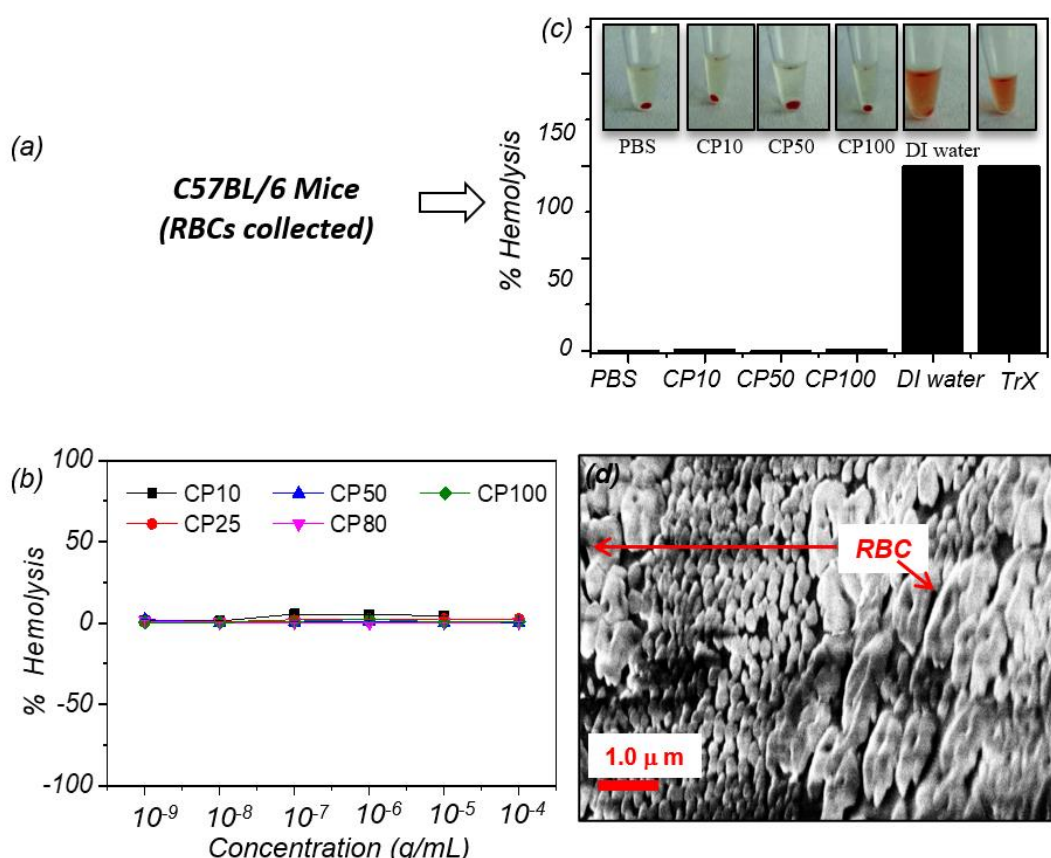


Figure 2.19. (a) Diagram showing C57BL/6 mice for hemolysis experiment. (b) Plot of % Hemolysis versus different concentration of polymer (CP10, CP25, CP50, CP80 and CP100) with C57BL/6 mice erythrocytes at 37 °C for 1 h. (c) Hemolysis assay for cationic polymer nanoparticles, PBS (negative control) and de-ionized water and 0.1 % Triton-X (positive control). (d) FE-SEM images of morphologically intact C57BL/6 mice erythrocytes after treatment with CP10 polymer at 37 °C for 1 h.

To test the interaction of cationic PCL with RBCs, hemolysis assay was performed in mice blood RBCs by absorption method following the earlier reported procedures.^{194, 195} The percentage hemolysis provides information about the relative disruption of erythrocytes on exposure to cationic polymer and it is determined by monitoring the hemoglobin release from mice red blood cells (RBCs). For this experiment, blood was procured from C57BL/6 mice as shown in Figure 2.19a. PBS buffer was taken as a negative control where no disruption to cells occurs while deionized water was used as positive control in which 100% RBC degradation occurs due to osmosis. The cationic PCL polymers with various number of positive charge units (CP10, CP50, CP100) were tested at various polymer concentrations ranging from 1×10^{-6} $\mu\text{g/mL}$ to 1×10^{-1} $\mu\text{g/mL}$ (see Figure 2.19b). The eppendorf containing RBCs in PBS (negative control) and DI water (positive control), and the polymers in PBS are shown in Figure 2.19c.

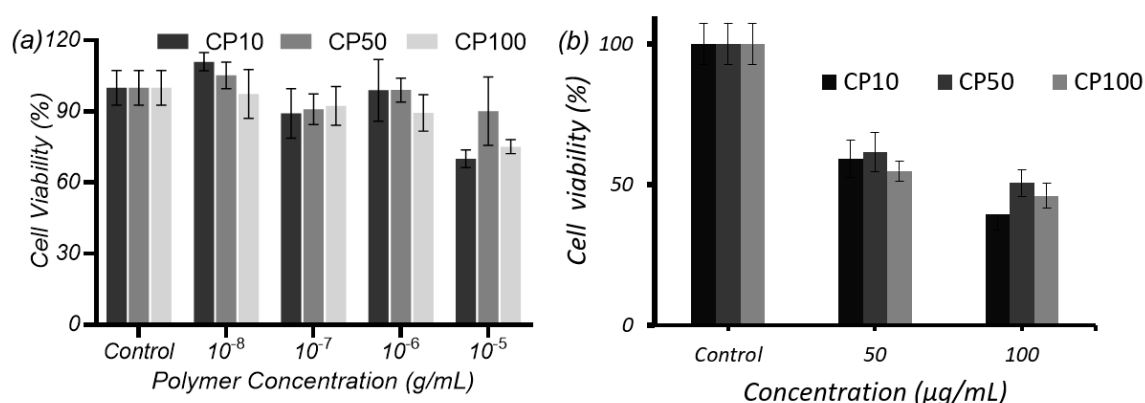


Figure 2.20. (a) Cytotoxicity data for cationic polymer nanoparticles CP10, CP50 and CP100 in WT-MEF cell lines. Comparative Cell Viability of CP10, CP50 and CP100 at higher concentrations (50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$) along with control (in the absence of polymer) in WT-MEF cell line at 37 °C for 72 h.

The DI water (positive control) is highly lytic to RBCs; thus, it caused 100% lysis and the eppendorf appeared to be red in color due to the leaching of hemoglobin. The RBCs were found to be very stable in PBS (negative control) and the polymers in PBS suggesting that there is no appreciable damage to the RBCs by the cationic polymers. The % hemolysis obtained for the three polymers CP10, CP50 and CP100 were 0.64 ± 0.003 , 0.038 ± 0.004 and 0.8 ± 0.007 respectively, which is almost negligible. Further to prove the stability of RBC morphology, FE-SEM image of RBCs were recorded after exposure to CP10 polymer and the image is shown in Figure 2.19d. The donut-shaped morphology of the RBCs was perfectly retained even after exposure to the cationic polymer indicating that the newly designed cationic PCL was non-toxic in blood stream. The cytotoxicity of cationic PCL polymers was investigated in wild-type mouse embryonic fibroblast (WT-MEF) cell line by *in vitro* MTT assay and the data is summarized in Figure 2.20a. Three PCL cationic polymers (CP10, CP50 and CP100) were scanned at various concentrations (1×10^{-2} to 1×10^1 $\mu\text{g/mL}$) in order to determine whether these polymers exhibit cytotoxicity towards healthy cell lines (for higher concentration, see Figure 2.20b). The percentage viability of the cells in the presence of polymers revealed that the polymers are non-toxic to cells up to a concentration of 1 $\mu\text{g/mL}$. However, at higher polymer concentration (10 $\mu\text{g/mL}$) the cells were viable only upto 75 %.

Table 2.3. Table contains MIC₅₀, HC₅₀ and selectivity index of all cationic polymers against *E. coli*.*MIC and Hemolysis Data of CPn Polymers*

Polymer	MIC ₅₀ ^a (g/mL)	HC ₅₀ ^b (g/mL)	Selectivity Index ^c
CP10	1x10 ⁻⁸	>10 ⁻⁴ (max. 10 ± 1.3%)	>10 ⁴
CP25	1x10 ⁻⁸	>10 ⁻⁴ (max. 1.1 ± 1.7%)	>10 ⁴
CP50	1x10 ⁻⁸	>10 ⁻⁴ (max. 2 ± 0.3%)	>10 ⁴
CP80	1x10 ⁻⁸	>10 ⁻⁴ (max. 1.1 ± 0.2%)	>10 ⁴
CP100	1x10 ⁻⁸	>10 ⁻⁴ (max. 2 ± 0.4%)	>10 ⁴

^a Determined in LB Broth against *E. coli*. ^b The average HC₅₀ values along with percentage errors for three independent experiments, respectively. ^c Calculated as the ratio of HC₅₀ and MIC₉₀.

To determine the relative selectivity of the polymers towards bacterial cells as compared to the RBCs, MIC₅₀ and HC₅₀ values of the various cationic polymers were determined and are shown in table in Table 2.3. Selectivity index for a particular polymer is determined by the factor of HC₅₀/MIC₅₀. It was observed that at the highest concentration upto which the polymers were scanned (10² µg/mL) for hemolysis activity was itself 10,000-fold higher than the concentration at which bactericidal activity was observed and at such high concentration as well only 10% hemolysis was observed (see Table 2.3). This shows that the polymers were highly selective (>10⁴ times) in their action and not toxic to blood stream while targeting the bacterial membrane disruption. This can be attributed to the higher anionic potential contributed by the phospholipid content in the bacterial membrane surface compared to mammalian RBCs.¹⁹⁶ This can be corroborated from the specific potential values of *E. coli*, RBCs¹⁹⁷ and normal cell lines¹⁹⁸ that are -20 mV, -15 mV and 0 mV, respectively. It could be observed that the anionic potential of bacterial membrane is larger in magnitude than the other two which contributes towards the high electrostatic interactions between cationic polymer and the bacterial membrane over the RBCs/normal cells and leads to excellent selectivity index as shown in the table in Table 2.3. It is important to note that the cationic polymers are active towards *E. coli* at very low concentration (10⁻² µg/mL) and the polymer chains are absolutely non-toxic to WT-MEF healthy cells at this low concentration range. Hence it may be mentioned that while administrating the cationic PCL polymer chains to accomplish the antibacterial activity, the polymers are very safe towards both RBCs and cells in the nearby healthy tissues.

2.3.5 Fluorescent Theranostic PCL Nanoparticles

To build fluorescent theranostic cationic PCL nanoprobe, the encapsulation capabilities of the cationic PCL based nanoparticles were tested for wide ranges of neutral and anionic fluorophores. Pyrene-1-sulfonic acid, 8-hydroxy-pyrene-1,3,6-trisulfonic acid (HPTS), Rhodamine B, Nile Red, Congo red and Methyl orange were attempted for encapsulation by dialysis method. Among them, only HPTS (anionic dye) was found to stabilize in the CP10 polymeric nanocavity (see Figure 2.21a). Under similar conditions, the high molecular weight analogues CP50 and CP100 did not load HPTS or other dyes. Both the molecular weight of the cationic polymers as well as the nature or charge of the dye played crucial role resulting in stable dye loading.

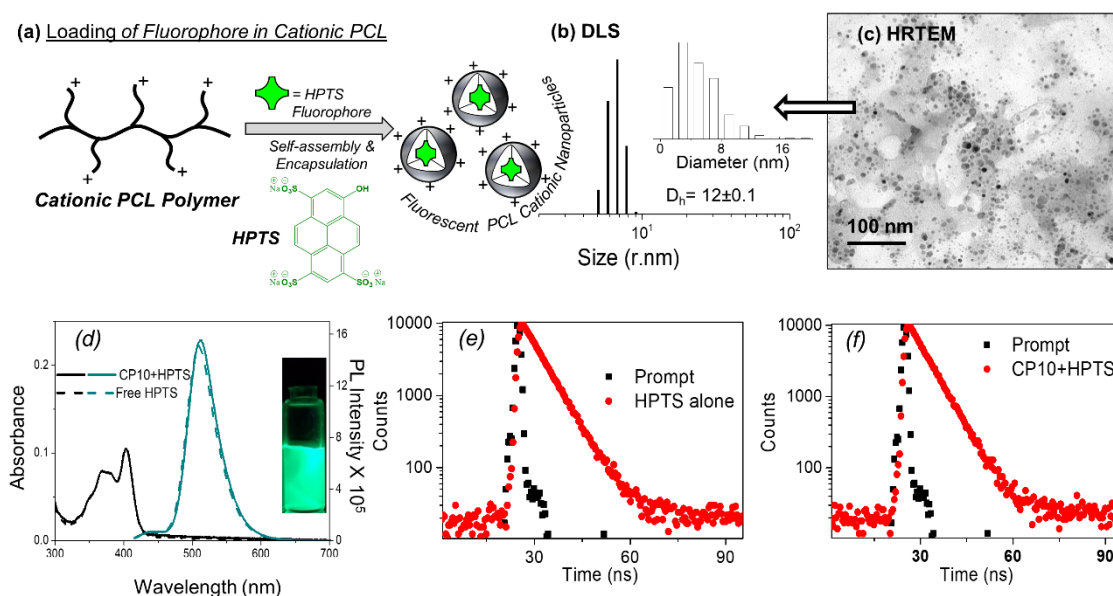


Figure 2.21. (a) Encapsulation of HPTS dye in the CP10 polymer. (b) DLS of CP10+HPTS. (c) HR-TEM image of CP10+HPTS. (d) Absorbance and emission spectra of CP10+HPTS and free HPTS in water ($\lambda_{exc} = 330$ nm). TCSPC decay profiles of (e) HPTS fluorophore and (f) CP10+HPTS along with prompt (LED Source: 340 nm).

The mechanism of loading an anionic dye (HPTS) in hydrophobic core of self-assembled CP10 polymer is driven by both electrostatic and hydrophobic interactions. Despite having identical planar aromatic ring, the pyrene-1-sulfonic acid (with one sulfonic acid group) was unable to load in the CP10 nanoparticles. This observation lays stress on the importance of both electrostatic and hydrophobic interactions to play a role in loading of HPTS in CP10. HPTS dye having a polyaromatic pyrene core along with three peripheral sulfonic acid groups has the appropriate balance required load in the cationic CP10 polymer

nanoparticle. The HPTS loaded CP10 polymer sample was referred to as CP10+HPTS and it is schematically shown in Figure 2.21a. The dye loading content (DLC) and dye loading efficiency (DLE) were determined as 0.8 % and 8.0 %, respectively, by absorbance spectroscopy. Though the DLC is relatively low; it is sufficient to be employed as a marker for biological studies. HPTS has been recently explored in the literature as fluorescent probe for cellular uptake in live lung cells and it was co-loaded with anticancer drugs in reverse micellar as FRET probe in cancer cell lines.¹⁹⁹⁻²⁰¹ In the present investigation, HPTS has been chosen for the first time for antibacterial research. The size of the CP10+HPTS was measured by DLS and its histogram showed the formation of small nanoparticle in the range of 12.0 ± 0.1 nm (see Figure 2.21b). The CP10+HPTS sample was subjected to HR-TEM imaging to measure the size and shape of the fluorescent cationic nanoparticles (see Figure 2.21c). The HPTS loaded nanoparticles exhibited the formation of spherical nanoparticles with average size of 5.0 ± 0.1 nm. The comparison of the HR-TEM images of CP10 and CP10+HPTS nanoparticles (see Figures 2.12b and 2.21c) revealed that the shape and size of the cationic PCL was retained even after the encapsulation. The size of CP10 nanoparticle determined by HR-TEM remains almost constant after HPTS loading (before loading 6.2 ± 0.1 and after loading 5 ± 0.1 nm). The difference of 1.0 nm is not very significant for self-assembled organic polymers; thus, it may be assumed that the size of the nanoparticles is retained almost identical. Since HPTS is a fluorescent dye, the absorbance and emission spectra of the CP10+HPTS were recorded and showed in Figure 2.21d. The nanoparticles (CP10+HPTS) and free HPTS showed similar absorbance maxima at 405 nm and emission maxima at 520 nm with respect to the HPTS probe. The fluorescent nanoparticle was also subjected to time resolved fluorescence decay study by TCSPC (Time correlated single photo counting) to study fluorescence life time (see Figure 2.21e and 2.21f). Fluorescence decay profile is known to be sensitive to the environmental effects and lifetime of the fluorophore could shed light on the local environment of the polymeric nanoparticles. Nano LED of 390 nm was used as the excitation source and signal was collected at 520 nm. Single exponential decay profile was fitted for both free HPTS and HPTS loaded polymeric sample showing an average fluorescence lifetime of 5.61 ns and 5.51 ns, respectively. The photophysical studies confirmed that anionic HPTS fluorophore did not alter its emission characteristics in the cationic polymer nano-cavity and is highly suitable for bio-imaging applications. Hence, the HPTS loaded fluorescent cationic polymer nanoparticles could be

employed as potential vectors for accomplishing both the bacterial imaging and antibacterial activity simultaneously in single polymer nano-platform.

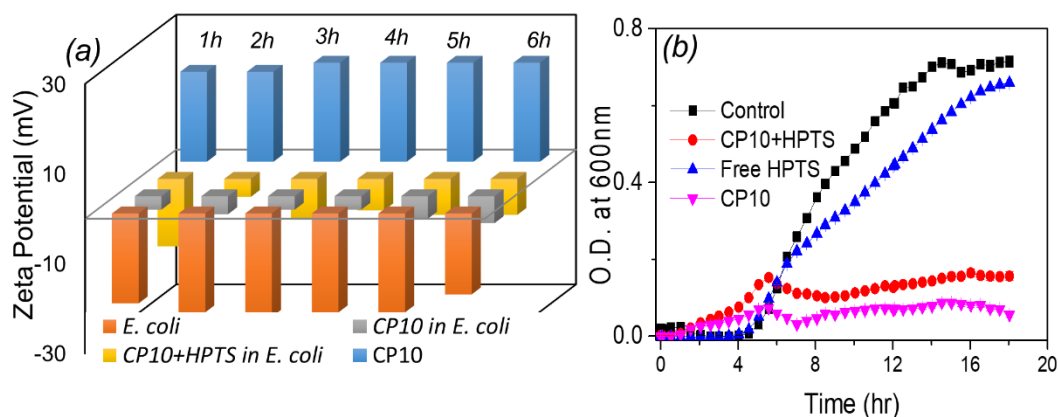


Figure 2.22. (a) Zeta potential of free *E. coli*, free CP10 polymer, *E. coli* incubated with CP10 polymer, and *E. coli* incubated with CP10+HPTS at various incubation time in PBS at 37 °C. The concentration of CP10 was maintained as 100 $\mu\text{g/mL}$ and the amount of *E. coli* was 10^5 CFU. (b) Antibacterial activity of CP10+HPTS, free HPTS, CP10 polymer by colony suspension method in *E. coli*.

A time-dependent Zeta potential for the action of polymer CP10 and CP10+HPTS on the *E. coli* in PBS at 37 °C was determined. In Figure 2.22a, *E. coli* exhibited -20.0 mV in PBS buffer and the negative potential was almost retained up to 6 h incubation. The negative potential is attributed to the phospholipid's composition on the *E. coli* membrane surface.²⁰² The polymer CP10 showed positive Zeta potential of +22 mV in PBS with respect to its cationic nature. A reduction in magnitude of positive charge (see Table 2.2) in zeta potential of CP10 polymer is observed due to change in solvent system from water to PBS buffer. Interestingly, the exposure of *E. coli* to the cationic polymer showed reduction in the negative charge potential of the bacterial membrane and the net charge potential showed move towards the neutral species. Under the identical conditions, the additions of CP10+HPTS into *E. coli* did not show much difference and the observation is like that of the CP10 polymer on *E. coli*. This trend in the Zeta potential revealed two important observations: (i) there is significant interactions between the negatively charged *E. coli* bacterial membrane and cationic polymer nanoparticles via electrostatic interactions, and (ii) the encapsulation of the anionic HPTS fluorophore in the cationic polymer did not affect the interaction of the polymer on *E. coli*. To check the antibacterial activity of the dye loaded nanoparticle (CP10+HPTS), colony suspension method was employed and bacterial viability was measured as a factor of turbidity for polymeric nanoparticle as well as free HPTS (see

Figure 2.22b). It was observed that free HPTS was non-toxic to bacterial cells while HPTS loaded nano-assemblies showed a decrease in viable bacteria (OD_{600nm} reduced to 0.2, 75 %) which was comparable to that of the CP10 polymer alone (OD_{600nm} reduced to 0.2, 75 %). This signifies that the encapsulation of HPTS anionic dye in cationic polymer CP10 does not hamper its antibacterial activity in *E. coli* (Figure 2.22b).

2.3.6 Enzymatic-Biodegradation of Cationic PCL

The cationic polycaprolactone polymer design carries aliphatic ester-linkages in the backbone as well as side chain that bear the cationic charge. Aliphatic esters are very well known to undergo enzymatic-biodegradation by the intracellular enzymes such as esterase enzymes in mammalian cells and bacterial lipase-enzyme.

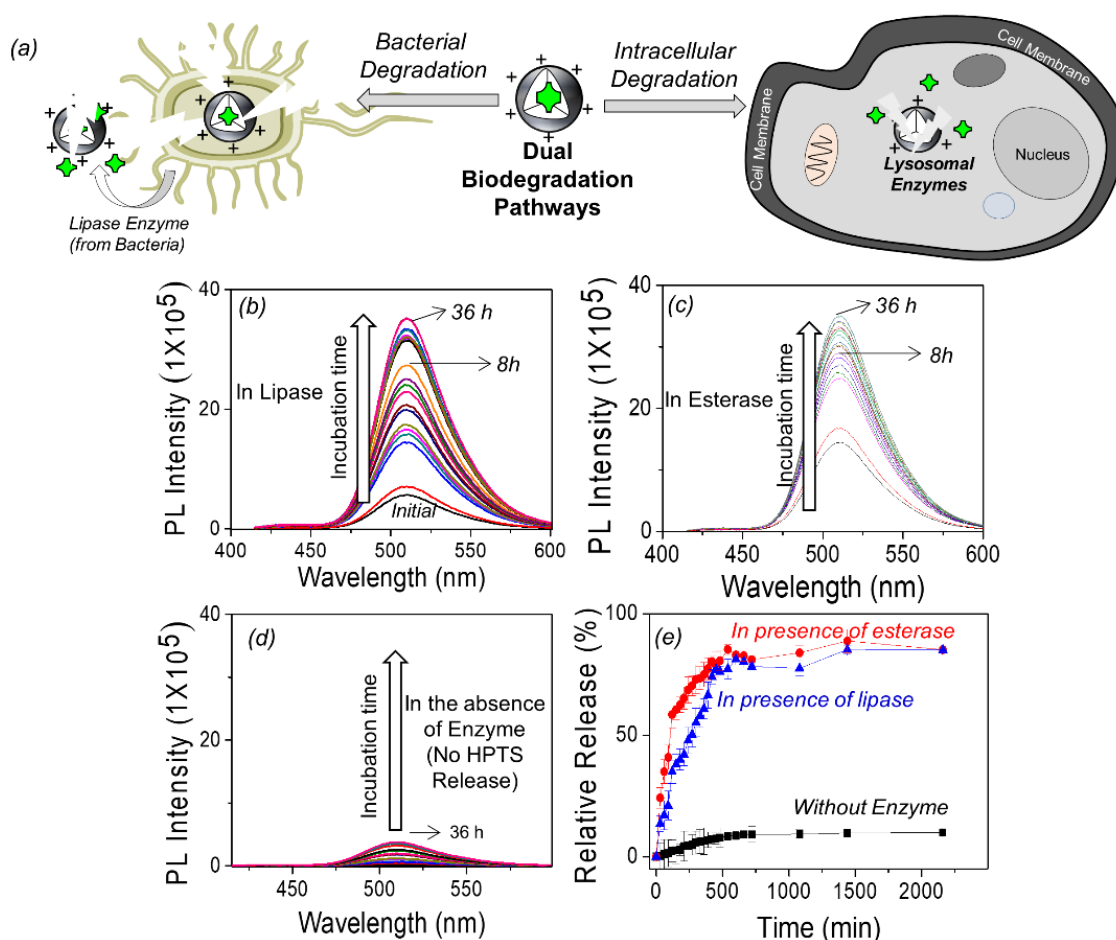


Figure 2.23. (a) Dual-biodegradation pathways for the cationic PCL nanoparticles by bacterial-enzymes and lysosomal-enzymes in healthy cells. (b) The fluorescence spectra of HPTS loaded polymer (CP10+HPTS) in presence of lipase in water at 37°C at various time intervals. (c) The fluorescence spectra of HPTS loaded polymer (CP10+HPTS) in presence of esterase in PBS at 37°C

at various time intervals. (d) Emission spectra of CP10+HPTS in the absence of enzyme (lipase or esterase) at various incubation time ($\lambda_{\text{exc}} = 330 \text{ nm}$). (e) Time dependent PL intensity plot of HPTS release from CP10+HPTS nano-assemblies in the absence or in the presence of enzyme at 37°C .

The biodegradation of the carboxylic ester chemical in lipids molecules are readily hydrolyzed by esterase and lipase enzymes depending upon the short or long aliphatic chains, respectively.²⁰³ Recent studies were also supported the presence of esterase and lipase enzymes in significant amounts in gram-negative bacteria *E. coli* for the catalytic activity of ester hydrolysis in lipids.²⁰⁴⁻²⁰⁶ Further, few reports recently reported the usage of lipase in the biodegradation of PCL chains.¹⁸⁴⁻¹⁸⁶ The PCL biodegradable polymers employed here for antibacterial activity have much higher mass than typical aliphatic units in lipids; thus, both esterase and lipase enzymes are selected for the enzymatic-biodegradation. In real-case scenario the custom designed cationic PCL is anticipated to come in direct contact with both healthy cells in tissues and bacteria as shown in Figure 2.2; thus, it is important to trace the biodegradability of the cationic PCL upon exposure to esterase and lipase enzyme action. Two different scenarios of degradation were accounted by these two enzymes as shown in Figure 2.23a. Thus, during the antibacterial action, the long chains of polymer are chopped off to smaller oligomeric units by the extracellular enzyme (like lipase) secreted by bacteria. So, the use of a microbial enzyme (i.e., lipase) to exhibit *in vitro* degradation of the polymeric system would lead to closer proximity to real scenario. Secondly, the unwanted cytotoxic effect introduced by the cationic polymers by crossing mammalian cell membranes through endocytosis process would be minimized by the intracellular biodegradation of the PCL chains by the lysosomal esterase enzymes.^{146, 147} Hence, dual biodegradation ability of the cationic PCL by the extracellular lipase (from bacteria) and intracellular esterase (from mammalian cells and bacterial cells) would provide ideal design of biodegradable polymers for accomplishment of antibacterial activity under physiological conditions.

Enzymatic-biodegradation analysis was performed by dialysis method using semi-permeable membrane of MWCO = 1000 Da by measuring the fluorescence intensity of the HPTS release from the nanoparticle. For this purpose, the CP10+HPTS nanoparticle was taken in the dialysis tube along with 10 U of *Pseudomonas* lipase or pig liver esterase enzymes and incubated in PBS buffer at 37°C for 36 h. During the incubation process, the enzymes will act on the polymer scaffold and degrade them gradually to release the HPTS from its cavity. The HPTS is a small molecule; thus, it could cross the semi-permeable membrane into the reservoir and the amount of HPTS accumulated in the reservoir was

monitored by recoding its fluorescence signal. Control experiment was also done without enzymes to study the leaching out of the HPTS from the nanoparticles under the similar incubation condition in PBS. The emission spectra of the HPTS recorded upon exposure of the CP10+HPTS nanoparticles to lipase and esterase enzymes at various incubation time is shown in Figure 2.23b and Figure 2.23c, respectively. It is very clear from the spectra that the more than 90 % of the HPTS released from the nanoparticles within 8 h of incubation. Emission spectra for the nanoparticles without enzymes are shown in Figure 2.23d.

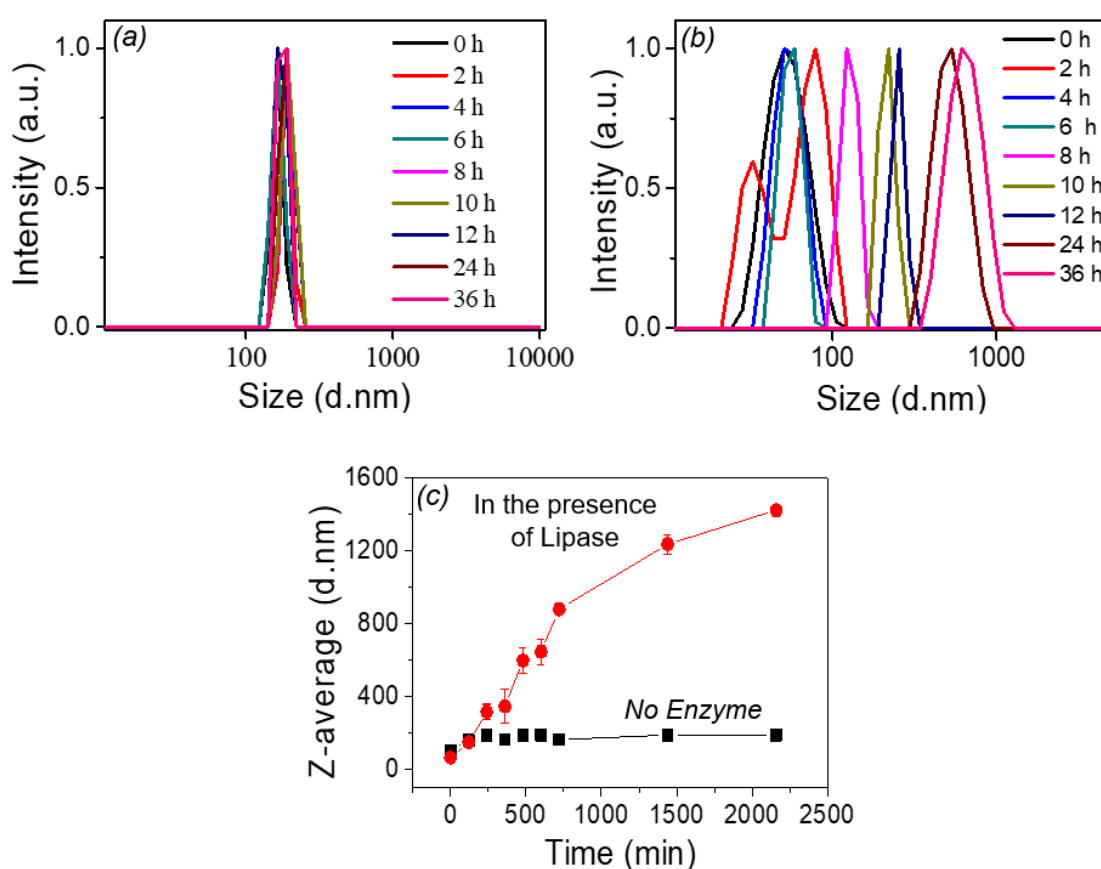


Figure 2.24. Determination of enzymatic biodegradation of CP10+HPTS nanoparticle by monitoring change in size using DLS instrument (Light source: 633 nm laser and collection of scattered light at 90° angle) (a) Control (in the absence of enzyme) (b) CP10+HPTS in the presence of lipase enzyme (c) Plot of Z-average (d.nm) with time in presence and absence of lipase enzyme.

It was observed that esterase enzyme showed similar enzymatic-biodegradation and HPTS release was like that of lipase whereas the HPTS release was negligible in the absence of enzyme. The relative fluorescence was determined by $(E_t/E_0) \times 100$ where E_t is the fluorescence intensity of HPTS in the reservoir at time “t” and E_0 is corresponding to initial

fluorescence of the HPTS in the reservoir and the data is shown in Figure 2.23e. It was observed that as the time progressed, the percentage release of HPTS from the polymeric nano-carrier increased in the presence of enzyme and 90 % of the loaded cargoes released in less than 8 h (see Figure 2.23e). On the other hand, in the absence of enzymes, only less than 8 % of HPTS released and this trend clearly supports the enzymatic biodegradation of the newly designed cationic PCL nano-assemblies. The enzymatic biodegradation of the polymer nanoparticle was also investigated by DLS in PBS buffer at 37 °C for 36 h and the data is shown in Figure 2.24. On incubating polymeric nanoparticle with enzyme would lead to enzymatic degradation of the polymer and the disassembly would create imbalance in the amphiphilicity of the polymer system. This would lead to the change in the size of the polymer self-assembly which could also be used as a measure to study the degradation kinetics. As it has been clearly shown in Figure 2.24b that as the incubation time increases the average size of the particle increases in presence of lipase enzyme. In the absence of the enzyme, there is no substantial change in the size of the polymer nanoparticles (see Figure 2.23a). The plots in Figure 2.24c showed that the size of the polymer nanoparticles increases significantly within 8 h of incubation upon exposure to the lipase. Self-assembly of polymeric nanoparticles is directed by the hydrophobic and hydrophilic components of the polymer chain. The enzymatic action of lipase disrupts this hydrophobic-hydrophilic balance resulting in the separation of the two components. The hydrophobic part undergoes phase separation from the aqueous medium and as a result, we observe the formation of large aggregates in solution which transcribes to the increase in size in the DLS measurement.²⁰⁷
²⁰⁸This trend is very well matching with the fluorescence-based biodegradation data in Figure 2.23e supporting the enzymatic-biodegradation of the cationic PCL polymer nanoparticles. Based on the above studies, it may be summarized that the HPTS loaded cationic PCL nanoparticle is very good biodegradable-cum-fluorescent probe for testing the antibacterial activity and bio-imaging in bacterial system. As it has been observed that the MIC values were unaffected by the polymeric chain lengths and was found to be in the same range (10^{-2} µg/mL) for all the polymers from CP10 to CP100. This leads to the conclusion that even when CP100 is enzymatically biodegraded into low molecular weights (for example 10-mer, equivalent to CP-10) still the antibacterial activity of the polymer was not compromised.

2.3.7 Theranostic Probe for Direct Visualization and Quantitative Determination

It is very important to note that DAPI stains both live and dead *E. coli* cells and PI stains only the dead cells. Thus, the confocal images shown in Figure 2.18 provide the indirect evidence for the antibacterial activity of the cationic PCL polymers; however, this method could not be used for the quantitative estimation of live and dead bacterial cells. This problem could be addressed by the custom designed HPTS loaded cationic polymer nanoparticles in the present investigation due to its three important features: (i) free HPTS can stain the *E. coli* membranes (green) without any disturbance, (ii) HPTS loaded CP10 polymer is capable of staining (green) both membrane intact *E. coli* as well membrane disrupted *E. coli*, (iii) and thus, the co-staining of PI (red) along with HPTS loaded CP10 nanoparticles (green) would enable the differentiation of alive *E. coli* (green) and dead *E. coli* (green + red) in the confocal microscope images of area of interest. To demonstrate this concept, *E. coli* were exposed to HPTS and HPTS loaded CP10 polymer and the images were recorded with PI staining as shown in Figure 2.25a. In the control (no treatment), no emission was observed in either HPTS channel or PI channel (after 8 h incubation) as can be seen from the first panel. The images showed the presence of viable bacteria under the experimental conditions. In the case of incubation with free HPTS, the bacterial cells showed green emission resulting from the adhesion of HPTS on the *E. coli* membrane; however, there was no appreciable red emission from the PI channel indicating that free HPTS is not an antibacterial agent for *E. coli* membrane disruption. This trend is very well matching with the MIC data in Figure 2.16. The merged images are clear of the co-localization of red and green colour together to exhibit the dead *E. coli* as orange in colour.

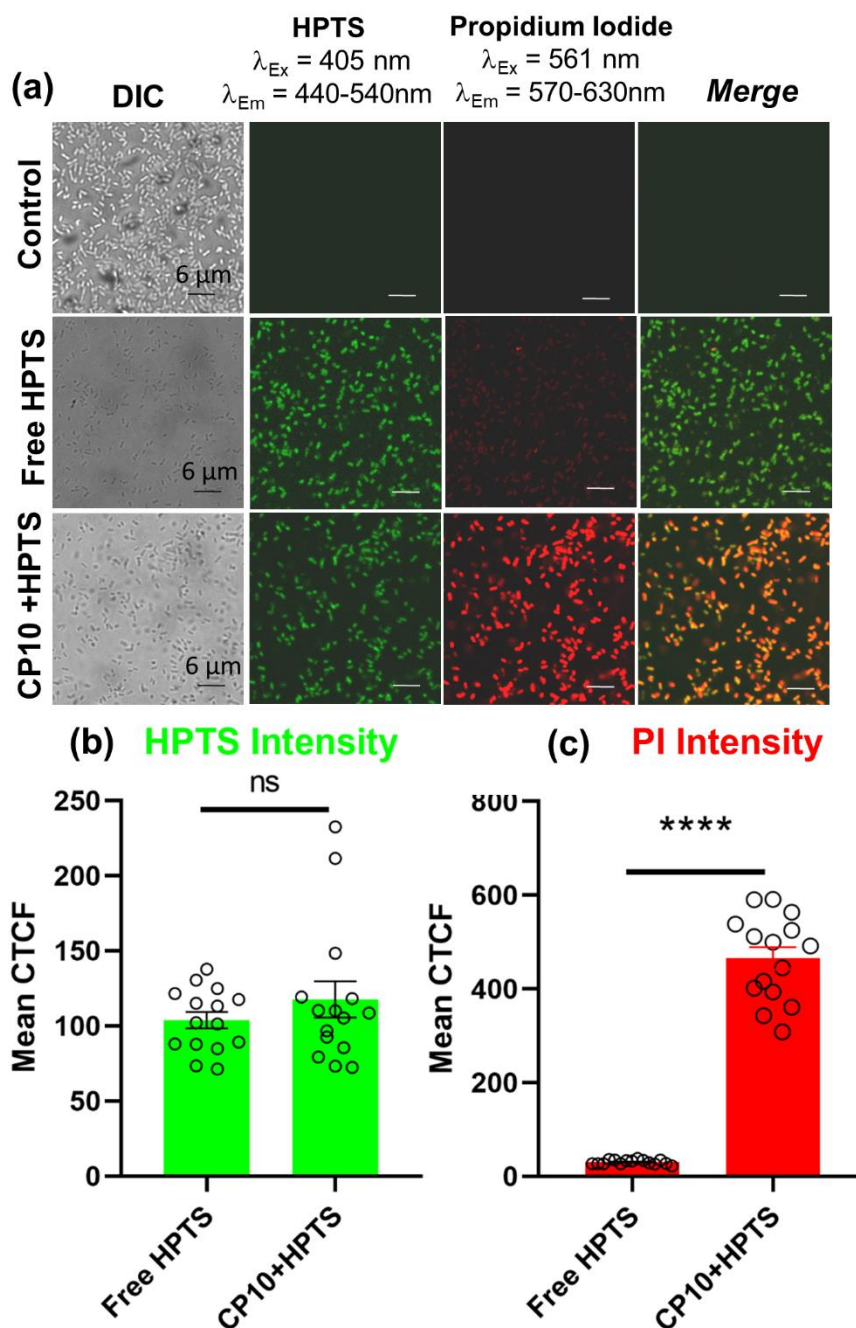


Figure 2.25. (a) Confocal microscope images in *E. coli* bacterium cells control (first panel), incubated with Free HPTS (second panel), CP10+HPTS (third panel). The cells were incubated for 8 h at 37 °C and stained by PI (red). (b) Calculation of Mean CTCF of HPTS and PI Intensity (c) for incubation of bacterial cells with Free HPTS and CP10+HPTS. (**** $P < 0.0001$) Each point represents mean $\pm$ SEM ($n = 15$).

The third panel corresponding to HPTS loaded CP10 polymer showed two important observations: (i) strong green emission with respect to the binding of HPTS on to the *E. coli* membrane, and (ii) the action of CP10 cationic polymer on the *E. coli* cell membrane

disruption which facilitates the entry of the membrane impermeable dye PI into the cell which in turn exhibited strong red emission from the PI staining. The merged images are clear of the co-localization of red and green colour together to exhibit the dead *E. coli* as orange in colour. The closer observation of the merged images further revealed that very few cells were only stained in green color with respect to the HPTS and no PI staining indicating that the polymer acted on majority of these *E. coli* bacteria accomplishing its antibacterial activity. This provides a unique opportunity to monitor the extent of the bacterial cell killing by the cationic PCL at the given point of incubation. The observations could be corroborated by determining the mean Corrected total cell fluorescence (CTCF) corresponding to the HPTS and PI intensity, respectively. As can be seen in Figure 2.25b, the HPTS intensity determined from the second and third panel corresponding to free HPTS and CP10+HPTS, respectively exhibited no significant difference in the mean CTCF values indicating that the adhesion of HPTS onto the *E. coli* membrane was similar in both cases. However, in the case of the PI intensity there was a drastic increase in the Mean CTCF values for CP10+HPTS compared to free HPTS (**** $P < 0.0001$), thus reinstating the fact that HPTS alone is not an antibacterial agent and the cationic polymer CP10 has an enhanced antibacterial effect caused by the membrane disruption (see Figure 2.25c). Thus, the cationic PCL polymers provide an ideal theranostic platform by exhibiting significant antibacterial activity as well as being able to validate the alive vs. dead bacteria.

The flow cytometry data was also found to be in compliance with the confocal microscopy images. *E. coli* (1×10^8 CFU/mL) was incubated with free HPTS and CP10+HPTS for 4 h to determine the relative uptake by bacterial cells as compared to control wherein no treatment was done. Herein, on maintaining the number of events at 10000 and voltage at zero for control (bacterial suspension in PBS without any fluorophore), CP10+HPTS polymeric nanoparticle and free HPTS exhibited 10-fold higher cellular internalization in *E. coli*.

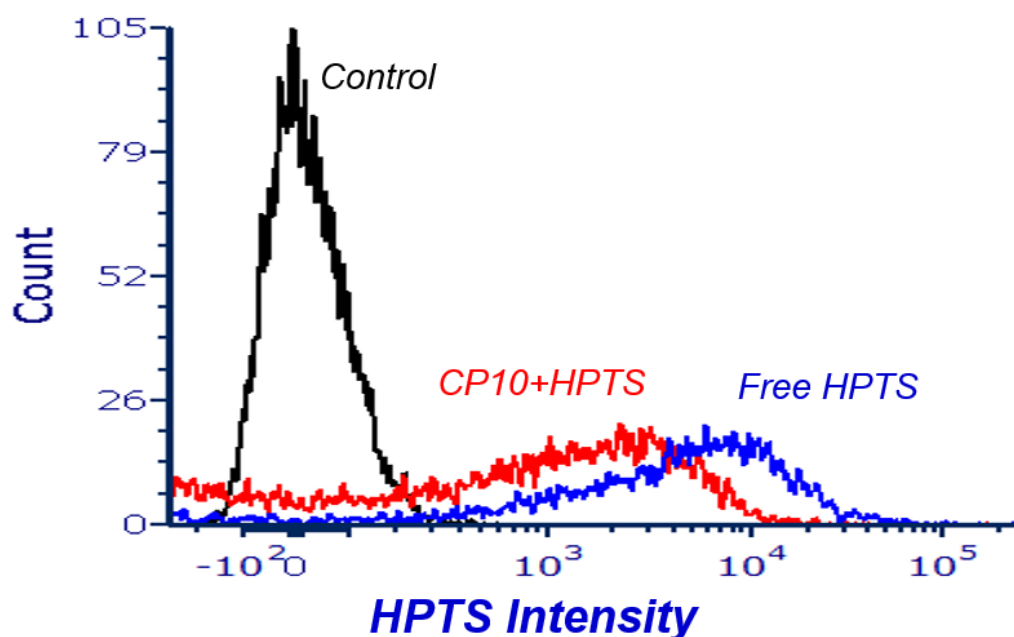


Figure 2.26. Flow cytometry plots of Control, CP10+HPTS and Free HPTS in *E. coli* after 4 h incubation (HPTS concentration = 8 $\mu\text{g/mL}$ and 10 000 cells are used).

Both the CP10+HPTS and Free HPTS loaded nanoparticles exhibited remarkable internalization in *E. coli* (see Figure 2.26) which confirms the internalization of cationic polymer CP10 and ultimately leading to membrane disruption and increase in bactericidal activity with time. Having established that the cationic polymers loaded with HPTS were in fact able to adhere to the bacterial membrane and cause membrane disruption resulting in bactericidal activity, the next obvious step was to study the kinetics of bacterial cell death in order to understand the progression of the antibacterial activity of the cationic polymers. In order to understand the kinetics of the action of HPTS loaded CP10 polymer, bacterial suspension was incubated with the same concentration of polymer (10 X MIC) at various time intervals (0.5 h, 2 h, 4 h, 6 h, 8 h) and then imaged under confocal microscopy (see Figure 2.27a).

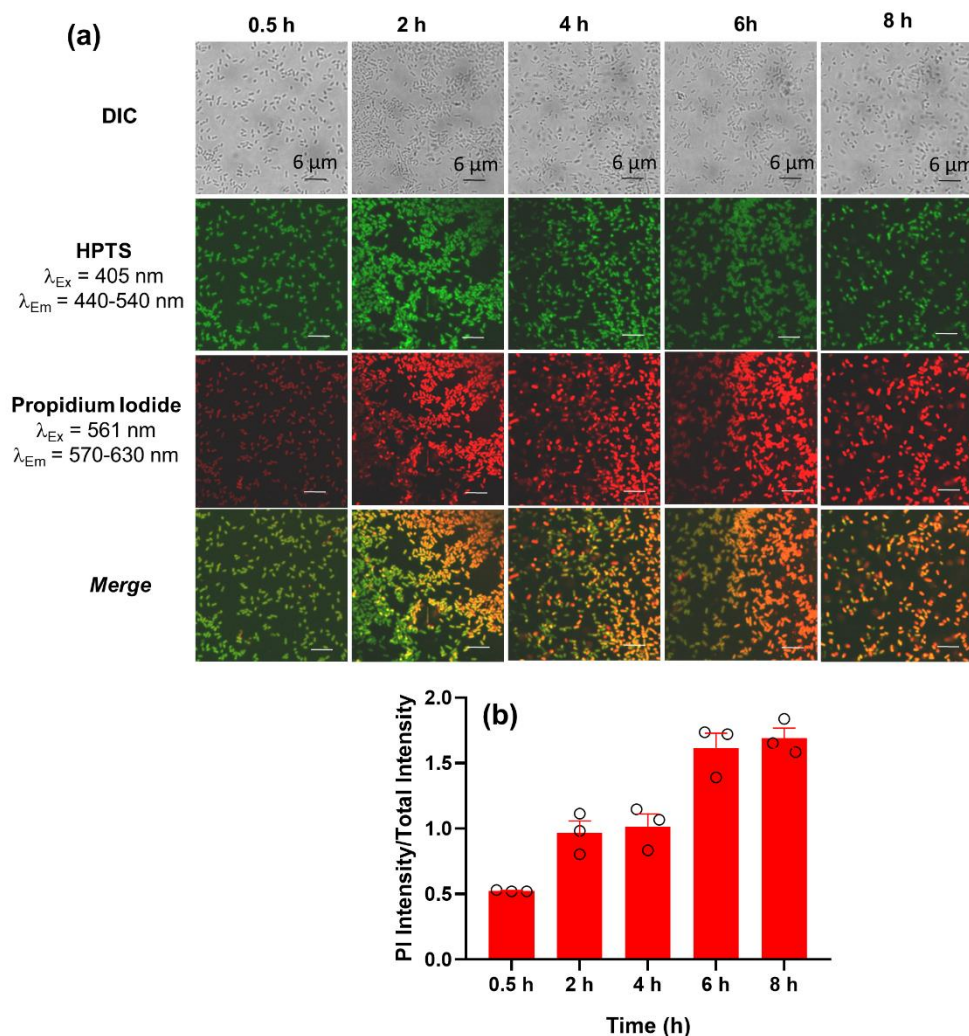


Figure 2.27. (a) Time dependent Confocal microscopic images showing bacterial population incubated with CP10+HPTS at 0.5 h (first panel), 2 h (second panel), 4 h (third panel), 6 h (fourth panel), 8 h (fifth panel) and simultaneous staining with PI. CFU/mL was maintained at 10^8 cells. (b) Plot of PI Intensity/Total Intensity with respect to various incubation time.

Bacterial cells stained by HPTS loaded polymeric nanoparticle were visualized through the blue channel while dead bacterial cells with destructed membrane were visualized through red channel corresponding to PI intensity. The merged images showing orange colour signifies the influence of cationic PCL polymer in the destruction of the bacterial membrane resulting in the staining by both HPTS polymer nanoparticle (green) and PI (red). As the time of incubation increases, the percentage of cells stained by PI also increases as is visible from the growing population of orange (green+red) colored bacterial cells in the merged image. It has also been illustrated graphically in Figure 2.27b where the percentage of PI-stained cells is calculated with respect to time. It is the ratio of the PI intensity compared to the total intensity from all the bacterial cells. The relative decrease in

the number of live bacterial cells could be easily observed which transcribes to a gradual increase in the PI-stained cell ratio with increasing the incubation time. Moreover, not only the number of cells but PI intensity signals from the dead cells also increased with time. The CTCF calculation clearly shows that the ratio of the intensity of red fluorescence (PI) to the total intensity increases gradually with time which proves the number of dead cells also increases with time and attains a plateau at 6 h. The present investigation is aimed to develop one of the first biodegradable theranostic probes based on the PCL and establish the proof-of-concept in antibacterial research which is indeed successfully demonstrated by direct visualization and quantification assay. It is important to mention that the theranostic concept and the newly designed cationic PCL polymer are not just restricted only to the HPTS or their validation in gram-negative bacteria *E.coli*. This polymer design could be further expanded to make block copolymer architectures with other polymeric systems and the resultant nano-assemblies could be employed as theranostic probe to study wide ranges of microbial systems including gram-negative, gram-positive, and multidrug resistance bacterial systems. The new generation PCL cationic polymers designed and developed here would be very useful for other biomedical application such as drug/gene delivery. Currently, the research work is being pursued in these directions to utilize the structural engineering aspects of PCL cationic polymers in biomedical research.

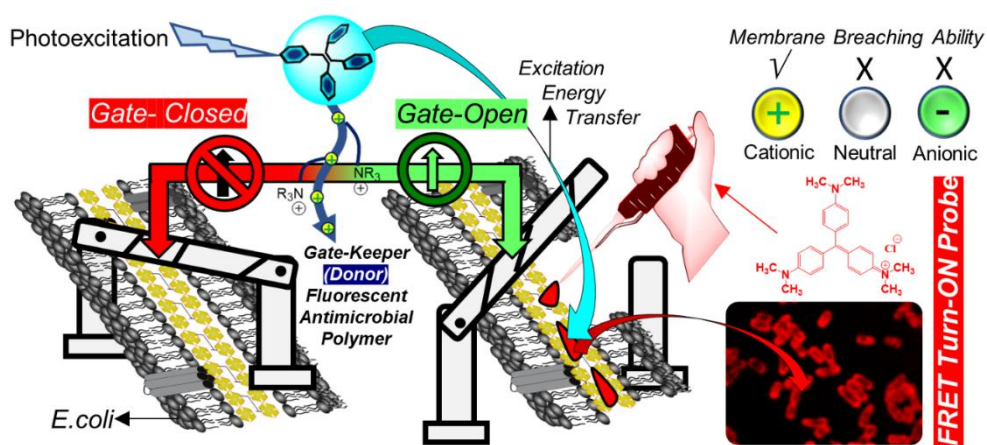
2.4. Conclusion

In conclusion, the present investigation has successfully demonstrated the simultaneous inhibition and quantitative diagnostic properties of new classes of fluorescent probe loaded biodegradable amphiphilic cationic polyester. The synthetic methodology of the polymer involves design and development of chlorine substituted ϵ -caprolactone monomer which was polymerized to produce chlorine substituted polycaprolactone polymers by solvent assisted ring opening polymerization methodology. These fully hydrophobic chlorine substituted polymers were then converted to cationic amphiphilic polymers by quaternary triethyl amine substitution. The aqueous self-assembly of these cationic polymers showed spherical nano-structures in the size range of 10 nm as corroborated by FE SEM and HR TEM images. Due to the presence of positive charge on the surface of nanoparticles, a positive zeta potential in the range of +40 to +50 mV is observed and it exhibited potent antibacterial activity against *E. coli* with $MIC_{90} = 10^{-2}$ $\mu\text{g/mL}$. Further, to lay stress in the bacteriostatic property of the polymer, generation time (t_{gen}) and growth rate (k) were determined at MIC_{90} concentration as 70 min with $k = 0.61$

gen/h. FE SEM and HR TEM images of polymer incubated *E. coli* bacterial cells demonstrated morphological changes like bacterial cell membrane disruptions which confirmed the membrane-lysis action of the polymer. Hemolysis assay revealed that RBCs were extremely stable in presence of polymer with % hemolysis values as negligible as 0.64 ± 0.003 with a selectivity index (HC_{50}/MIC_{50}) $>10^4$ times showing non-toxicity of polymer in the blood stream. *In vitro* MTT assay showed that polymers are non-toxic to healthy cells upto concentration of 10 $\mu\text{g/mL}$. The polymer was successfully able to permeabilize the bacterial membrane which enables PI to stain the nucleic acids of membrane compromised dead bacterial cells. To further enhance the understanding, direct visualization and quantification was enabled by encapsulating HPTS (anionic green fluorophore) as theranostic probe in the cationic polymeric nanoparticle. On incubating the *E. coli* cells with HPTS loaded cationic polymer nanoparticles, it is observed that bacterial cells are co-stained by both HPTS and PI demonstrating the antibacterial action of cationic polymer while free HPTS did not have antibacterial property since it did not fluoresce in the red region. It has been reinstated quantitatively by calculating the CTCF of HPTS intensity and PI Intensity for Free HPTS and CP10+HPTS samples which showed a clear distinction in the PI intensities of the samples. As the incubation time increases, number of PI-stained cells as well as the PI intensity signal from the dead cells increases which reaches the plateau region at 6 h. This shows that the combination of cationic polymer and encapsulated anionic dye (HPTS) can simultaneously be used to determine the antibacterial activity as well as visualize and qualitatively estimate dead bacterial cells. Presently, research work is focused on this direction of development of new functionalized cationic polycaprolactone polymers and utilizing its therapeutic potential in the field of biomedical science.

Chapter 3

Theranostic FRET Gate to Visualize and Quantify Bacterial Membrane Breaching



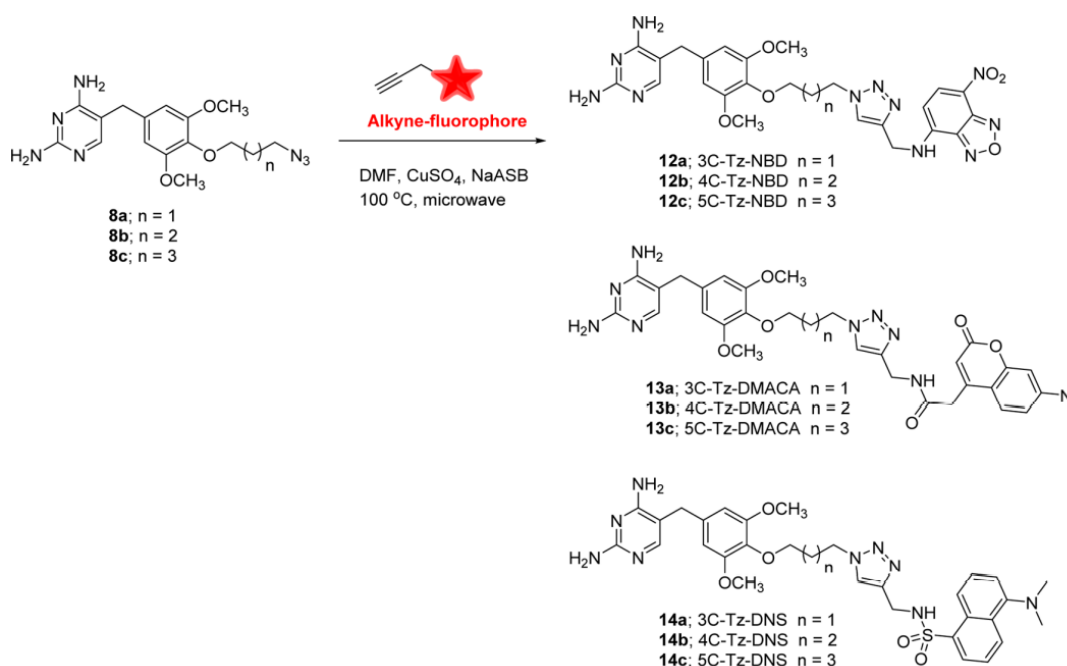
Bacterial Membrane Breaching By Antimicrobial FRET Probe

Abstract

Designing new antibacterial-cum-probe molecules to study real-time bacterial membrane breaching and concurrently develop inquisitorial image-based analytical tools is essential for the treatment of infectious diseases. An array of aggregation induced emission (AIE) polymer (donor) consisting of neutral, anionic, and cationic charges were designed and employed as antibacterial theranostic gate keepers for the permeabilization of peptidoglycan layer adherable crystal violet (CV, acceptor) in *E. coli* membrane. Electrostatic adhering of the cationic AIE-polymer and subsequent gate-opening aided fluorescent resonance energy transfer (FRET) probe activation on *E. coli* bacterial membrane. Energy transfer has been spectroscopically determined by TCSPC measurement wherein a decrease in lifetime of donor excited species in presence of acceptor suggested appropriate energy transfer between them. Selective photoexcitation energy transfer was coupled in confocal microscopy imaging to build a new visualization-based FRET assay for the quantification of bactericidal activity. Non-antibacterial AIE- polymers (neutral and anionic) did not breach the bacterial membrane resulting no FRET signal. Moreover, a decrease in number of *E. coli* colonies was further established by spread plate method upon polymeric incubation for 18 h. This technique also eradicated the possibility of photoexcitation energy transfer induced reactive oxygen species (ROS) generation by CV by incubating *E. coli* cells with only CV followed by irradiation with blue LED source. Tetraphenylethylene (TPE)-tagged polycaprolactone biodegradable platform was chosen to construct the membrane-reinforced FRET probes and their self-assembled tiny amphiphilic nanoparticles were employed as gate-keeper. Detail photophysical studies were done to establish the FRET probe mechanism and the proof-of-concept in gram negative bacteria *E. coli* membrane.

3.1 Introduction

Bacterial microorganism mutations have continuously challenged humanity to find smart diagnostic tools for early detection of infectious diseases and design efficient therapeutic drugs in the treatment and handling of public health care at large.²⁰⁹⁻²¹¹ Penicillin and other beta-lactam antibiotic drugs are used for treatment against bacterial infections; however, microbes are smart and quick in developing resistance against these clinical antibiotics and make them inefficient in the therapeutic regimens.²⁶ Currently, fluorescent antibiotics are used to study antibiotic function at the cellular level, rationalize the resistance mechanism, develop high throughput screening, etc, to discover new antibiotics for improving therapeutic efficacies.²⁸⁻²⁹ The development of Trimethoprim based fluorescent probes are shown in Scheme 3.1.



Scheme 3.1: Synthesis of Trimethoprim Probes Using Microwave-Assisted Click Chemistry. (adapted from *ACS Infect. Dis.* **2016**, 2, 688–701)

Antibacterial peptides (AMPs) are also employed as first level defence against the pathogenic microbe attack in bacterial infections.^{31, 40} Last one-decade, synthetic cationic polymers are emerging as an alternative antibacterial candidates for both gram-negative and gram-positive bacteria.^{41, 42, 48, 212} Cationic antibacterial polymers typically undergo non-specific carpet mechanism (similar to AMPs) to fragment the membranes to inhibit the cell

growth.^{62, 63, 213,214} Unlike in antibiotics; in-depth molecular level interaction between polymer analyte and bacterial membrane substrate was rarely understood. Several efforts have been taken to employ steady state fluorescence^{215, 216} and fluorescence resonance energy transfer (FRET) to study bacterial enzyme-responsiveness,^{217, 218} antibiotic screening,^{219,220} pathogen discrimination,²²¹ wash-free bacterial detection,²²² differentiation of bacteria over fungi,²²³ probe localization in living bacteria,^{224, 225} etc. Unlike in the area of antibiotics, in-depth molecular-level interaction between a polymer analyte and bacterial membrane substrate has been rarely understood. Furthermore, majority of π -conjugated chromophores²¹³ were found to undergo π -stack aggregation caused quenching (ACQ) in aqueous medium;²²⁶ therefore, it was rather difficult to differentiate the fluorescent signals in LB broth (or media, in free state) from bacterial membrane bound state.^{227, 218} An example of conjugated polymers-based development of supramolecular antibacterial/detection system is shown in Figure 3.1.²¹³

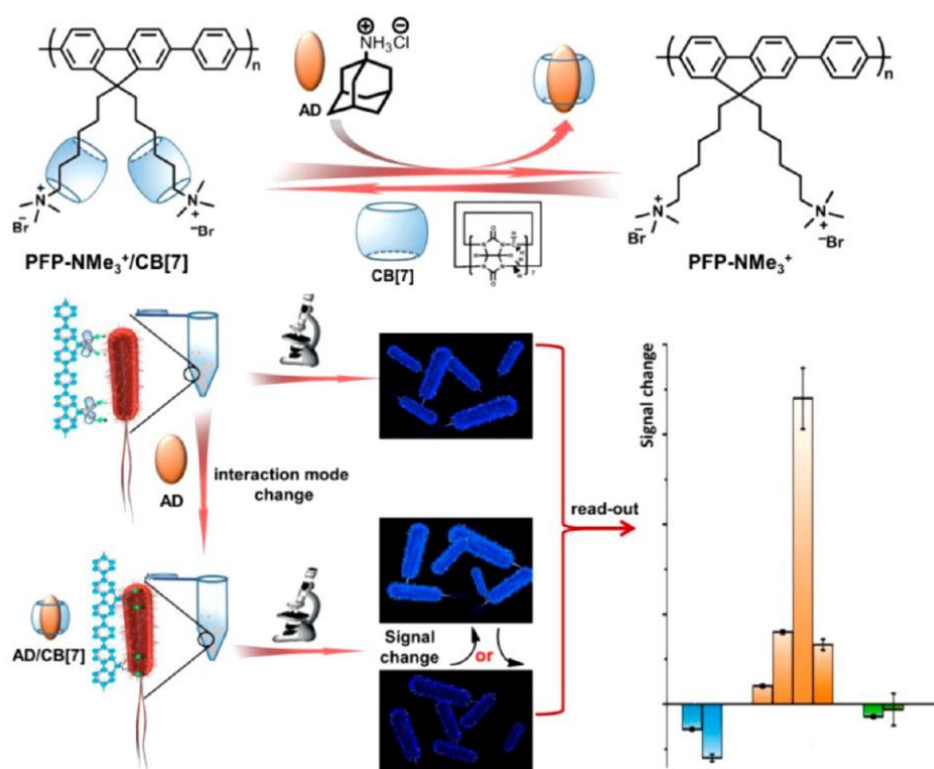


Figure 3.1: Supramolecular composite material formed by cationic conjugated polymer (PFP-NMe₃⁺) and cucurbit[7]uril (CB[7]) for controllable identification of microbes. (adapted from ACS Appl. Bio. Mater. 2021, 4, 370-386)

As an alternative, picking up aggregation induced emission (AIE) π -conjugated fluorophores²²⁹⁻²³¹ and building new AIE-assisted FRET probes is an elegant strategy to overcome the above limitations. AIE concept has been known in bacterial imaging;²³²⁻²³⁴ however, AIE-based FRET-probes are very rarely reported in bacterial research. This is partially associated with the scarcity of low band-gap AIE-acceptor chromophores ($E_g \leq 2.0$ eV, red-region) that could couple with the AIE donor chromophores having high HOMO-LUMO energy levels ($E_g \geq 3.0$ eV, blue-region).^{235, 236} Energy level diagram for FRET energy transfer in case of luminescent and non-luminescent acceptor is shown in Figure 3.2.

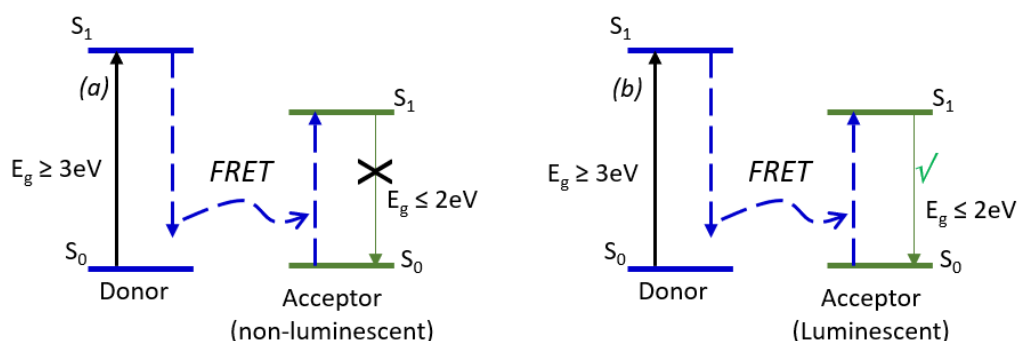


Figure 3.2: Energy diagram for FRET energy transfer for two cases (a) when the acceptor is non-luminescent and (b) when the acceptor is luminescent in nature.

Here, we report a new FRET pair between peptidoglycan-layer permeable biomarker crystal violet (CV, acceptor) along with tailor-made AIE-active TPE-antibacterial polymer (as a donor). CV has been chosen for this purpose since it has been reported as a fluorescent marker to study the traverse effect in *E. coli* peptidoglycan membrane²³⁷ and as a fluorescent discriminator for G-quadruplex complex, etc.²³⁸⁻²⁴⁰ CV-anchored magneto-fluorescent nanoparticles were also designed for detection of bacteria by μ -NMR and optical imaging as a point-of-care diagnostics.²⁴¹ Cadmium-free quantum dots and CV have been explored as FRET pair to generate photoactivated reactive oxygen species and solution-based spectroscopic methods (not image-based assay) was explored to study their antibacterial activity.^{242, 243} Until now, there is no effort has been made to explore image based FRET assay in bacterial research. In the present investigation, a novel strategy is introduced wherein on-site bacterial membrane-reinforced FRET gate was created by AIE-active polymer gate-keeper (donor) to permeabilize the CV biomarker (acceptor). The probe

became functional only upon the incidence of the antibacterial activity and breaching the bacterial membrane to stain the CV in the peptidoglycan layer. Upon antibacterial action, the membrane slit-through action of the TPE-polymer exclusively “**gate-opened**” to permeabilize CV in the peptidoglycan layer to **turn-On** the FRET signals. As a result, this strategy provides direct information for the polymer binding on the bacterial membrane via self-emission (probe-1) and cross-talking between polymer (donor) and membrane-bound CV (acceptor) by FRET (probe-2). In the absence of the antibacterial activity, the non-compromised bacterial membrane does not stain the CV and eventually the “**gate-closed**” which results no FRET signal. This new concept is shown in Figure 3.3.

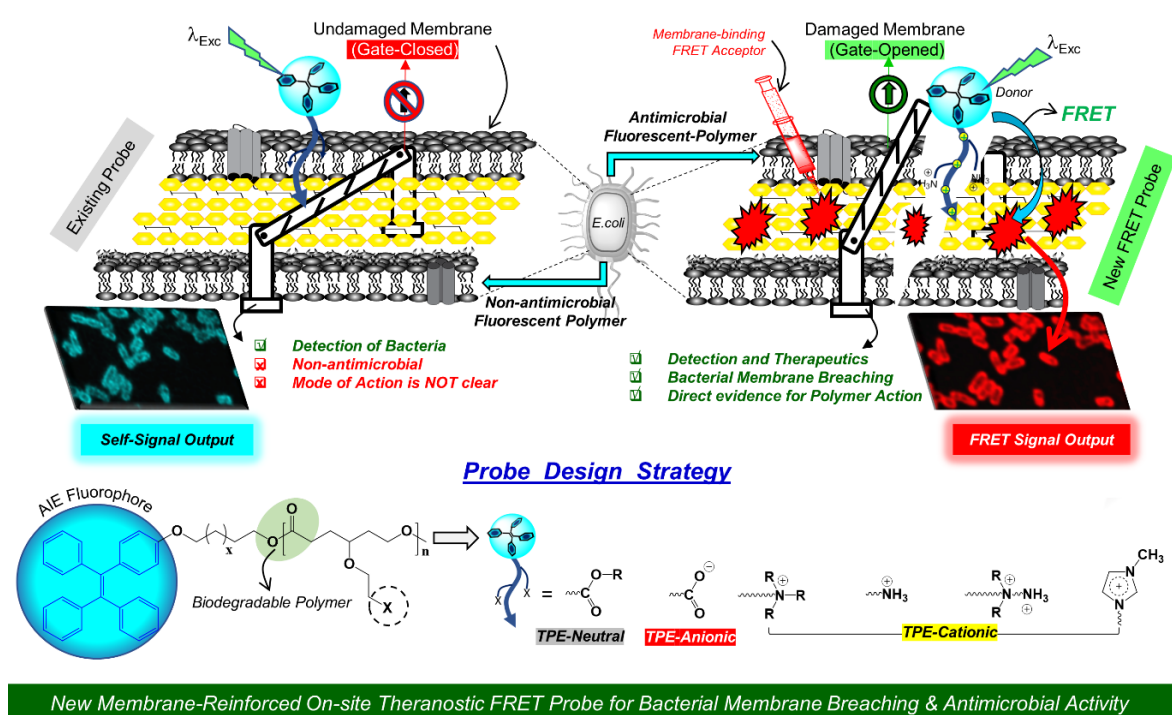


Figure 3.3. Development of membrane-reinforced on-site FRET probe-cum-antibacterial polymer to visualize and quantify the bactericidal activity in gram-negative bacteria *E. coli*.

To design new FRET probe, our expertise in biodegradable polycaprolactone (PCL) platform^{144-147, 188, 189, 244} and FRET probes²⁴⁵⁻²⁴⁷ in cancer research has become handy to build new “blue-luminescent” antibacterial PCL donor polymer. In our earlier attempt, PCL based cationic polymers were developed and their structure-property relationship was established on their antibacterial action on *E. coli*.²⁴⁴ In the present investigation, more than half-a-dozen TPE-conjugated PCL polymers having similar molecular weights but

possessing variable charges such as anionic, cationic, and neutral were designed by ring opening polymerization strategy. This enabled the detail investigation on the role of the polymer charges (cationic, anionic, and neutral) towards their ability to electrostatically bind on the surface of bacterial membranes for successful construction of FRET probes. These amphiphilic PCL polymers were self-assembled to yield blue-fluorescent nanoparticles in water. Electrostatic binding of these TPE nanoparticles on the *E. coli* membrane exhibited remarkable antibacterial activity depending upon the charge as well as functional groups in the pendant units. Detail steady state and time-resolved fluorescence decay photophysical studies were carried out to establish the FRET probe action. Selective photoexcitation experiments revealed diminished blue-emission from TPE and enhanced FRET-based red-luminescent signal from CV confirming efficient photoexcitation energy transfer from TPE to CV on the *E. coli* membrane. Time-dependent confocal microscope studies established that the newly developed FRET assay is an efficient new tool to visualize and quantify the real-time antibacterial activity on bacterial substrate.

3.2 Experimental Methods

Caprolactone (CL), 1, 12-dodecanediol, 4-toluene sulfonyl chloride (TsCl), 4- hydroxyl benzophenone, benzophenone, titanium tetrachloride (TiCl₄), zinc powder, were purchased from Sigma Aldrich and they were used without purification. For hemolysis experiment, C-57 B1/6 mice was collected from IISER Animal house facility. Gram's Crystal Violet obtained from SD Fine Chemicals. Paraformaldehyde, glutaraldehyde was obtained from Sigma Aldrich chemicals. Bruker 400 MHz spectrophotometer (in IISER) was used for recording all the monomer and polymeric NMR samples in CDCl₃ and D₂O solvents. For molecular weight and dispersity measurement, Gel Permeation Chromatography carried out by a Viscotek VE 3580 RI, and Light scattering detectors. THF was used as a mobile solvent for GPC analysis and calibrated using polystyrene standards. Mass analysis for small molecular weight compounds were determined using high-resolution mass spectrometry-electrospray ionization-quantitative time-of-flight liquid chromatography-mass spectrometry (HRMS-ESI-Q-TOF LC-MS) while for macromolecules, molecular weight distribution was determined through Applied Bio system 4800 PLUS matrix-assisted laser desorption/ionization (MALDI) TOF/TOF Analyzer. For time-dependent analysis of change

in optical density of *E. coli* colonies, Perkin-Elmer Lambda 45 UV-Visible Spectrophotometer was used. HORIBA JOBIN VYON SPEX Fluorolog fluorescence spectrophotometer was utilized for determination of Emission spectra. An excitation source of 450 W Xe lamp was used at room temperature. Dynamic light scattering (DLS) for determination of nanoparticle size and zeta potential utilized Nano ZS-90 setup (Malvern instruments). It measures the Brownian motion of particles which compares the motion to size of particles using stock Einstein equation. Three independent measurements were taken for reproducibility. FTIR has been recorded in Agilent ZnSe ATR system. FE-SEM was conducted using Zeiss Ultra Plus Field Emission Scanning Electron Microscope wherein sample was dropcasted on silicon wafer and gold-coating was done before analysis to make it conducting. For TEM (Transmission electron microscopic) images were obtained by drop casting the samples onto Formvar-coated copper grid (400 mesh) via JEM-2200FS Field Emission Electron Microscope. Confocal microscopic analysis was done using Leica SP8 microscope. The substituted caprolactone monomer 2-chloroethyl 3-((7-oxooxepan-4-yl)oxy) propanoate (monomer M1) was synthesized following reported procedure in Chapter 2. **Note:** Animal ethics committee approval number for this project is IISER-Pune/IAEC/2021-01/10.

3.2.1 Synthesis of 4-(1,2,2-triphenylvinyl) phenol (1): 4-Hydroxyl-phenylbenzophenone (3.2 g, 16.0 mmol), benzophenone (2.9 g, 16.0 mmol) and Zinc powder (2.6 g, 40.0 mmol) were taken in 250 mL two-necked flask, and dry tetrahydrofuran (THF) (80 mL) was added and the content was refluxed for 30 minutes under nitrogen atmosphere. The mixture was cooled to 0 °C, TiCl_4 (3.6 g, 19.2 mmol) was added, and the reaction mixture was refluxed for 12 h under nitrogen atmosphere. The reaction was cooled, dilute hydrochloric acid (1 N, 80 mL) was added, and the product was extracted into dichloromethane (DCM). The organic layer was dried over anhydrous Na_2SO_4 and crude product was purified by silica gel column using petroleum ether/ ethyl acetate (10:1 by volume) as eluent. Yield = 3.0 g (47 %). ^1H NMR (400 MHz, CDCl_3) δ ppm: 7.12 – 7.00 (m, 15H, -CH-), 6.91 – 6.89 (d, 2H, -CH-), 6.59 – 6.56 (d, 2H, -CH-), 4.66 (s, 1H, -OH-). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 154.46, 144.35, 140.88, 140.64, 136.83, 133.19, 131.82, 128.16, 128.06, 126.83, 126.71, 115.04. FT-IR (cm^{-1}): 3322.89, 2943.64, 2833.32, 1654.86, 1412.37, 1112.83, 1018.12 and 682.43. HR-MS (ESI+): m/z $[\text{M} + \text{H}^+]$ for $\text{C}_{26}\text{H}_{20}\text{O}$ Calculated= 348.43 and found = 348.15.

3.2.2 Synthesis of 12-hydroxydodecyl 4-methylbenzenesulfonate (2): In 250 mL round bottom flask, 1,12-dodecanediol (5.0 g, 25.0 mmol) and dry triethyl amine (2.5 g, 25.0 mmol) were dissolved in dry DCM (150 mL) under nitrogen atmosphere at 25 °C. 4-Toluene sulfonyl chloride (4.8 g, 25.0 mmol) dissolved in 50 mL Dry DCM was added to the reaction mixture dropwise in ice-cold condition. The reaction was continued for 10 h under stirring at 25 °C. DCM was evaporated and product was purified over silica column using ethyl acetate and hexane (1: 5 v/v) to obtain colorless liquid product. Yield = 3.1 g (35 %). ¹H NMR (400 MHz, CDCl₃) δ ppm: 7.79 - 7.77 (d, 2H, -CH-), 7.34 - 7.32 (d, 2H, -CH-), 4.02 - 3.99 (t, 2H, -O-CH₂-), 3.64 - 3.61 (t, 2H, HO-CH₂-), 2.44 (s, 3H, CH-CH₃), 1.65 - 1.52 (m, 4H, -CH₂-), 1.33-1.21 (m, 16H, -CH₂-). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 130.26, 128.36, 71.18, 63.59, 33.21, 30.04, 30.01, 29.94, 29.89, 29.85, 29.81, 29.37, 29.28, 26.17, 25.78, 22.10 and 0.45. FT-IR (cm⁻¹): 3317.03, 2941.07, 2832.56, 2047.32, 1650.58, 1449.57, 1113.03, 1019.02 and 673.88. HR-MS (ESI+): m/z [M + H⁺] for C₁₉H₃₂O₄S Calculated= 356.52 and found = 357.20.

3.2.3 Synthesis of 12-(4-(1,2,2-triphenylvinyl) phenoxy) dodecan-1-ol (3): In a 50 mL two-neck round bottom flask, **1** (2.0 g, 6.0 mmol) and K₂CO₃ (3.7 g, 27.0 mmol) were dissolved in 10 mL dry DMF and the mixture was stirred at 80 °C for 0.5 h. Compound **2** (3.5 g, 9.0 mmol) dissolved in dry DMF (10 mL) and it was slowly added to the reaction mixture under nitrogen atmosphere. After 12 h of stirring at 80 °C, DMF was removed through vacuum distillation. The crude product was extracted in DCM and purified by silica column using ethyl acetate and hexane (1:20 v/v) to obtain a yellow viscous liquid which was then precipitated in hexane to obtain a white solid (**3**). Yield = 2.2 g (97 %). ¹H NMR (400 MHz, CDCl₃) δ ppm: 7.15 - 7.01 (m, 15H, -CH-), 6.91 - 6.89 (d, 2H, -CH-), 6.59 - 6.56 (d, 2H, -CH-), 3.88 - 3.83 (t, 2H, -CH₂-), 3.65 - 3.62 (t, 2H, -CH₂-), 1.77 - 1.70 (m, 2H, -CH₂-), 1.60 - 1.53 (m, 2H, -CH₂-), 1.32 - 1.29 (m, 16H, -CH₂-). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 158.13, 144.47, 141.04, 140.40, 136.32, 132.94, 131.81, 127.70, 126.32, 113.69, 67.94, 63.22, 32.93, 29.69, and 25.87. FT-IR (cm⁻¹): 3316.92, 2939.56, 2830.46, 1658.64, 1448.38, 1113.19, 1020.17, 672.38. HR-MS (ESI+): m/z [M + H⁺] for C₃₈H₄₄O₂ Calculated= 532.75 and found = 531.46.

3.2.4 Synthesis of tert-butyl (2-hydroxyethyl) carbamate: Ethanol amine (10 g, 0.2 mol) was dissolved in water: THF (1:1) solvent composition. (BOC)₂O (42.5 g, 0.2 mol) was added to the solution in dropwise manner at 0 °C. The reaction mixture was stirred for 24 h at 25 °C. THF layer was evaporated and ethyl acetate was added to the reaction mixture. The product was isolated in water layer and pH was maintained at 6.5 followed by isolation of product in organic layer. Yield = 23 g (87.1 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 5.26 (bs, 1H, -NH-), 3.61 (t, 2H, -CH₂-), 3.20 (t, 2H, -CH₂-), 1.38 (s, 9H, -CH₃). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 156.87, 79.60, 62.12, 43.09, 28.42. FT-IR (cm⁻¹): 3341.35, 2974.91, 2942.04, 2863.81, 2820.24, 2776.57, 1690.33, 1516.29, 1456.60, 1390.47, 1364.89, 1270.17, 1249.51, 1167.57, 1100.30, 1073.51, 1040.23, 996.86, 887.56, 860.62, 838.38, 778.27 and 668.12.

3.2.5 Synthesis of tert-butyl (3-(dimethylamino) propyl) carbamate: 3-(N, N dimethyl amino) propyl amine (5.0 g, 48.9 mmol) was dissolved in 60 mL DCM. Solution of (BOC)₂O (11.2 g, 51.3 mmol) in 60 mL DCM was added dropwise at 0 °C and the reaction was continued at 25 °C for 12 h. Water was added to the reaction mixture and DCM layer was extracted thrice followed by brine wash. Product was extracted in DCM layer. Yield = 7.0 g (70 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 3.06 (t, 2H, -CH₂-), 2.34 (t, 2H, -CH₂-), 2.24 (s, 6H, -CH₃), 1.66 (m, 2H, -CH₂-), 1.44 (s, 9H, -CH₃). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 156.18, 78.91, 57.79, 45.51, 39.55, 28.50 and 27.61. FT-IR (cm⁻¹): 3351.30, 2975.04, 2941.97, 2863.61, 2819.77, 2777.13, 1690.27, 1517.86, 1456.74, 1390.43, 1364.88, 1270.14, 1249.55, 1167.78, 1100.28, 1073.49, 1040.23, 996.71, 888.07, 861.39, 838.31, 778.45 and 667.97.

3.2.6 Synthesis of tert-butyl 3-((-4-hydroxycyclohexyl) oxy) propanoate (Compound-A): A solution of 1,4 Cyclohexane diol (35.0 g, 302.0 mmol) in dry THF (500.0 mL) was prepared and catalytic amount of potassium t-butoxide (1.00 g, 9.00 mmol) was added to the same and refluxed for 30 minutes. A solution of t-butyl acrylate (30.0 g, 241.0 mmol) in dry THF (50.0 mL) was added dropwise at 25 °C under nitrogen and it was refluxed for 36 h. THF was evaporated and residue was precipitated into cold DCM. The product was purified column chromatography using ethyl acetate and hexane (1:5 v/v) as eluent. Yield = 28.0 g (42%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 3.74–3.63 (m, 3H, O–CH₂– and O–CH), 3.37

(m, 1H, CH-OH), 2.46 (t, 2H, -CH₂CO-), 1.82 (m, 4H, -CH₂-), 1.64 (m, 4H, -CH₂-), 1.43 (s, 9H, -CH₃).

3.2.7 Synthesis of t-Butyl-3-((4-oxocyclohexyl) oxy)-propanoate (Compound-B): A solution of compound-A (16.0 g, 65.5 mmol) was prepared in dry DCM (250.0 ml) and stirred at 25°C. Activated molecular sieves (4Å) and PCC (28.2 g, 13.1 mmol) was added and it was stirred for 6 h. The reaction mixture was filtered and washed with DCM and further purified by column chromatography using ethyl acetate and hexane (1:10 v/v) as eluent to obtain a colorless liquid product (2). Yield = 15.0 g (95%). ¹H NMR (400 MHz, CDCl₃) δ ppm: 3.72 (m, 3H, O-CH₂- and O-CH-), 2.55 (m, 2H, -(CO) CH₂-), 2.48 (t, 2H, -CH₂-), 2.22 (m, 2H, -CH₂-), 2.07 (m, 2H, -CH₂-), 1.87 (m, 2H, -CH₂-), 1.43 (s, 9H, -CH₃).

3.2.11 Synthesis of tert-butyl 3-((7-oxooxepan-4-yl) oxy) propanoate (M2): Compound B (8.0 g, 41.0 mmol) was weighed in a 250 mL round bottom flask and Dry DCM (50.0 mL) was added, followed by addition of NaHCO₃ (10.4 g, 123.0 mmol) and mCPBA (21.3 g, 123.0 mmol) to the reaction mixture. The reaction was continued at 25 °C for 12 h. Saturated solution of NaHCO₃ and Na₂S₂O₃ was prepared and product was extracted in DCM layer followed by column chromatography at 10 % Ethyl acetate/Pet Ether solution system. Yield = 7.8 g (92 %). ¹H NMR (400 MHz, CDCl₃) δ ppm: 4.45 (m, 1H, -CH-), 4.00 (m, 2H, -O-CH₂-), 3.66 (m, 1H, -CH-), 2.95 (m, 1H, -CH-), 2.46 (t, 2H, -CH₂-), 2.38 (m, 1H, -CH-), 2.01-1.79 (m, 4H, -CH₂-), 1.45 (s, 9H, -CH₃).

3.2.12 Synthesis of TPE-conjugated Cl substituted Polycaprolactone (TPE-ClPCL): In a flame dried Schlenk tube, compound 3 (214.0 mg, 0.4 mmol) was dissolved in 1.0 mL dry toluene and purged under nitrogen for 15 min at 25 °C. To this solution, Sn (Oct)₂ (81.0 mg, 0.2 mmol) was added maintaining the nitrogen atmosphere and reaction mixture is allowed to stir for another 15 min at 25 °C. Monomer 4 (1.0 g, 4.0 mmol, for M/I = 10) in 1.0 mL dry toluene was added to the polymerization tube. Polymerization was continued by constant stirring at 110 °C for 48 h under sealed conditions. The solvent was reduced to half and then 50 mL diethyl ether was used to precipitate the polymer. The polymer purification was repeated by dissolving THF and re-precipitated in diethyl ether. Yield = 0.8 g (67 %). ¹H

NMR (400 MHz, CDCl_3) δ ppm: 7.15 – 7.01 (m, 15H), 6.91 – 6.89 (d, 2H), 6.59 – 6.56 (d, 2H), 4.35 (t, 2H), 4.13 (t, 2H), 3.70 (m, 6H), 3.46 (m, 1H), 3.38 (s, 1H), 2.59 (t, 2H), 2.37 (t, 2H), 1.86-1.78 (m, 3H), 1.30-1.27 (m, 17H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 173.97, 171.36, 132.62, 127.70, 126.32, 113.66, 75.76, 64.27, 61.34, 41.71, 35.27, 32.20, 29.71 and 28.01. FT-IR (cm^{-1}): 3843.04, 3739.75, 3316.74, 2940.11, 2830.84, 1657.00, 1450.03, 1112.98, 1019.75 and 670.38. GPC molecular weights: M_n = 2000 g/mol, M_w = 3100 g/mol, and M_w/M_n = 1.5.

3.2.13 Synthesis of TPE-conjugated tert-butyl ester substituted Polycaprolactone (TPE-Neutral):

TPE-based initiator (3) (207.2 mg, 0.4 mmol) was taken in a schlenk tube and 1 mL dry toluene was added to the same and the solution was nitrogen purged for 15 min at 25 °C. $\text{Sn}(\text{Oct})_2$ (78.5 mg, 0.2 mmol) was added to the same under nitrogen condition followed by addition of monomer (1.0 g, 4.0 mmol, for M/I = 10) and polymerization was continued for 48 h at 110 °C under sealed condition. The precipitation was done in 50 mL diethyl ether. Yield = 1.0 g (82.8 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 7.12 – 6.99 (m, 15H), 6.92 (d, 2H), 6.63 (d, 2H), 4.13 (m, 2H), 4.04 (t, 2H), 3.86 (t, 2H), 3.66 (m, 2H), 3.44 (m, 1H), 2.43 (t, 2H), 2.37 (t, 2H), 1.89-1.75 (m, 4H), 1.45 (bs, 9H), 1.33-1.27 (m, 17H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 131.37, 127.70, 127.59, 77.25, 75.68, 64.42, 62.99, 41.60, 35.14, 32.76, 29.71, 28.69 and 28.05. FT-IR (cm^{-1}): 3391.18, 2944.50, 2603.28, 2496.23, 2311.96, 1730.71, 1642.74, 1508.84, 1455.77, 1395.38, 1359.94, 1241.83, 1169.35, 1103.43, 1065.29, 1036.55, 969.60, 910.96, 849.99, 807.49, 732.98, 702.12 and 668.04. GPC molecular weights: M_n = 3300 g/mol, M_w = 4800 g/mol, and M_w/M_n = 1.4.

3.2.14 Synthesis of TPE-conjugated carboxylic acid substituted PCL (TPE-Anionic):

Trifluoroacetic acid (TFA) was used for deprotection of tertiary butyl group protected polymer (TPE-Neutral). TPE-Neutral (200 mg) was dissolved in DCM (1 mL) in pear-shaped round bottom flask. TFA (2 mL) was added dropwise to the above reaction mixture in ice-cold condition and the reaction was continued for one hour in room temperature. TFA was evaporated by vacuum distillation and the resultant polymer was precipitated in 30 % diethyl ether/hexane. Yield = 150 mg (91.4 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 7.11 – 6.99 (m, 15H), 6.92 (d, 2H), 6.63 (d, 2H), 4.16 (m, 2H), 4.04 (t, 2H), 3.86 (t, 2H), 3.73 (m, 2H), 3.53 (m, 1H), 2.57 (t, 2H), 2.39 (m, 2H), 1.93-1.78 (m, 4H), 1.34-1.27 (m, 17H). ^{13}C

NMR (100 MHz, CDCl_3) δ ppm: 139.76, 131.41, 131.26, 127.56, 126.42, 113.57, 64.52, 29.72, 29.62, 25.94. FT-IR (cm^{-1}): 3445.99, 2953.39, 2924.48, 2854.44, 2377.96, 2344.78, 2311.47, 1730.92, 1606.74, 1550.20, 1507.79, 1456.42, 1417.94, 1377.13, 1362.22, 1244.85, 1177.10, 1103.83, 1062.25, 973.72, 828.94, 806.10, 775.24, 746.68, 720.95, 699.93 and 668.36.

3.2.15 Synthesis of TPE-conjugated Boc-protected ethanol amine substituted Polycaprolactone (TPE-NHBOC): TPE-Anionic (100 mg, 0.5 mmol) was dissolved in Dry DMF: DCM solvent mixture (4.5 mL: 1.2 mL). Once the polymer got completely solubilized, solution of tert-butyl (2-hydroxyethyl) carbamate (159.6 mg, 1.0 mmol) in 0.5 mL DMF was added to the same. Reaction was continued for 48 h at room temperature. After the completion of the reaction, reaction mixture was filtered and dialyzed for 48 h. The sample was lyophilized in order to determine the yield of the reaction. Yield = 120 mg (66.6 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 7.10 – 6.98 (m, 15H), 6.89 (d, 2H), 6.62 (d, 2H), 5.05 (s, 1H), 4.14 (m, 4H), 3.70 (m, 2H), 3.46-3.38 (m, 3H), 2.54 (t, 2H), 2.36 (t, 2H), 1.90-1.77 (m, 4H), 1.43 (s, 9H), 1.30-1.25 (m, 24H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 156.81, 49.22, 33.92, 28.40, 26.17, 25.61, 24.93. FT-IR (cm^{-1}): 3393.67, 2956.68, 2926.29, 2682.71, 2377.63, 2344.55, 2309.88, 1731.53, 1510.76, 1455.70, 1417.63, 1392.89, 1366.20, 1249.69, 1172.35, 1105.59, 973.31, 852.93, 775.95, 748.99, 700.00 and 668.20. GPC molecular weights: M_n = 1900 g/mol, M_w = 3100 g/mol, and M_w/M_n = 1.6.

3.2.16 Synthesis of TPE-conjugated amine substituted Polycaprolactone (TPE-Cat-NH₂): TPE-NHBOC (100 mg, 0.3 mmol) was dissolved in 1mL DCM and TFA (660.0 μL , 3.0 mmol) was added to the same dropwise in ice-cold condition. The reaction was continued for 2 h at 25 °C. TFA was removed azeotropically using DCM and polymer was precipitated in 40 mL diethyl ether. Yield = 50 mg (70.0 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 8.21 (bs, 24H), 6.98 (d, 2H), 6.77 (d, 2H), 4.37 (bs, 2H), 4.13 (m, 2H), 3.67 (m, 6H), 3.45 (m, 1H), 3.28 (bs, 2H), 2.55 (bs, 2H), 2.35 (bs, 2H), 1.86-1.78 (m, 4H), 1.30-1.27 (m, 17H). FT-IR (cm^{-1}): 3393.95, 3356.91, 3340.06, 2953.88, 2924.33, 2851.84, 2711.97, 2511.11, 2377.27, 2353.75, 2344.58, 2311.48, 1842.52, 1731.56, 1680.14, 1553.73, 1534.80, 1454.94, 1418.13, 1396.30, 1377.83, 1362.43, 1201.53, 1178.12, 1131.15, 1083.99, 970.07, 908.94, 832.37, 800.95, 768.81, 720.85, 668.49 and 657.24.

3.2.17 Synthesis of TPE-triethylamine substituted cationic polymer PCL (TPE-Cat-NEt₃): TPE-ClPCL polymer (265.0 mg, 1.0 mmol) in 1.0 mL dry DMF was taken in the polymerization tube under nitrogen atmosphere at 25 °C. Freshly dried triethylamine (416.0 µL, 3.0 mmol) was added to the reaction mixture and the quaternization was carried out by stirring at 80 °C for 72 h. DMF was removed through rotatory evaporator, re-dissolved in THF, and polymer was purified using diethyl ether. Yield: 196.0 mg (87 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 7.15 – 7.01 (m, 15H, -CH-), 6.91 – 6.89 (d, 2H, -CH-), 6.59 – 6.56 (d, 2H, -CH-), 4.34 (t, 2H), 4.14 (t, 2H), 3.70 (m, 4H), 3.46 (m, 1H), 3.10 (q, 6H), 2.54 (m, 2H), 2.36 (m, 2H), 1.86-1.78 (m, 4H), 1.40 (t, 9H), 1.26 (m, 16H). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 139.36, 127.34, 104.25, 63.57, 45.65, 34.75, 29.39 and 8.42. FT-IR (cm⁻¹): 3394.62, 2952.39, 2603.16, 2493.47, 1730.47, 1446.89, 1355.74, 1258.19, 1174.32, 1065.65 and 961.04.

3.2.18 Synthesis of TPE- cationic N-methyl imidazole substituted PCL (TPE-Cat-NMeImz): TPE-ClPCL (100.0 mg, 0.4 mmol) was dissolved in 1 mL dry DMF and N-methyl imidazole (155.0 µL, 1.9 mmol) was added through syringe under nitrogen atmosphere at 25 °C. The reaction was kept at 85 °C in sealed condition for 72 h. Polymer was precipitated in diethyl ether and vacuum dried. Yield: 100 mg (85 %). ¹H -NMR (400 MHz, D₂O) δ ppm: 8.66 (m, 1H), 7.44 (m, 2H), 4.54 – 4.21 (m, 4H), 3.93 (bs, 3H), 3.78 – 3.67 (m, 3H), 2.66 (m, 2H), 2.44 (m, 2H), 1.83 (m, 4H) and 1.21 (m, 20H). FT-IR (cm⁻¹): 3396.38, 3154.91, 2930.09, 2378.58, 2353.48, 2344.29, 2310.77, 1842.07, 1731.26, 1680.86, 1659.04, 1642.92, 1631.80, 1565.73, 1553.67, 1510.49, 1454.65, 1417.91, 1361.12, 1339.17, 1244.52, 1174.81, 1104.07, 1066.83, 970.96, 852.71, 751.92, 701.10 and 668.10.

3.2.19 Synthesis of TPE-cationic tert-butyl (3-(dimethylamino) propyl) carbamate substituted PCL (TPE-Cat-NR₂-NHBOC): TPE-ClPCL (200.0 mg, 0.8 mmol) polymer was dissolved in 1.00 mL dry DMF along with tert-butyl (3-(dimethylamino) propyl) carbamate (202.3 mg, 2.2 mmol) in a melt tube under nitrogen atmosphere. Menshutkin reaction and purification is performed as described for TPE-Cat-NEt₃. Yield = 250.0 mg (76.6 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 7.08 – 6.99 (m, 12H), 6.89 (d, 2H), 6.61 (d, 2H), 5.38 (s, 1H), 4.61 – 4.14 (m, 4H), 3.68 (m, 4H), 3.45 (s, 1H), 3.26 (m, 4H), 2.81 (s,

6H), 2.55 (m, 2H), 2.37 (m, 2H), 2.03 (m, 2H), 1.78 (m, 4H), 1.42 (s, 9H), 1.26 (m, 20H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 157.01, 132.91, 131.77, 127.99, 126.61, 113.91, 64.94, 55.87, 52.52, 43.53, 43.40, 30.00, 28.82 and 25.46. FT-IR (cm^{-1}): 3368.56, 2956.39, 2926.33, 2311.32, 1731.33, 1510.79, 1456.19, 1417.46, 1392.41, 1366.22, 1249.83, 1171.39, 1106.33, 971.52, 857.86, 776.44, 700.79 and 668.34.

3.2.20 Synthesis of TPE-cationic N^1 , N^1 -dimethylpropane-1,3-diaminesubstituted PCL (TPE-Cat-NR₂-NH₂): TPE-Cat-NR₂-NHBOC (100 mg, 0.2 mmol) polymer was dissolved in 1 mL DCM and TFA (159 μL , 2.0 mmol) was added to the same in ice-cold condition. The reaction was continued at 25 °C for 24 h. TFA was evaporated in rotatory evaporator and precipitated in diethyl ether. Yield = 60 mg (78.2 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 7.07 – 7.01 (m, 15H), 6.89 (d, 2H), 6.62 (d, 2H), 4.15 (m, 4H), 3.69 (m, 4H), 3.49 (m, 1H), 3.16 (m, 4H), 2.82 (s, 6H), 2.53 (m, 4H), 2.01 – 1.79 (m, 6H), 1.27 (m, 18H). FT-IR (cm^{-1}): 3480.09, 3417.74, 3393.34, , 2926.21, 2854.50, 2566.48, 2377.50, 1941.86, 1842.85, 1732.11, 1682.38, 1645.39, 1609.04, 1567.26, 1361.68, 1338.93, 1244.04, 1186.30, 1137.05, 1109.32, 1061.69, 969.30, 700.10 and 668.28.

3.2.21 Determination of Critical Aggregation Concentration (CAC): The AIE property of TPE-based cationic polymers facilitated the detection of CAC. The emission of the TPE fluorophore is regulated by restriction of intramolecular motions. At lower concentration, TPE conjugated cationic polymeric chains are dispersed freely in water showing faint fluorescence but as the concentration of the polymer reaches above CAC value, due to gradual phase segregation of hydrophilic and hydrophobic segments of the polymer, the AIE luminogen will aggregate in the hydrophobic core of the aggregated self-assembly. It leads to a pronounced AIE emission above CAC. Serial dilutions (10-fold) of polymeric samples were prepared (1×10^{-3} g/mL to 1×10^{-13} g/mL) in water and PL intensity was measured at $\lambda_{\text{exc}} = 330$ nm.

3.2.22 Solvent-dependent and composition-dependent AIE phenomenon: TPE based cationic amphiphilic polymers showed ultrafast solution state quenching in organic solvents where they were highly soluble while the amphiphilic nature of the polymers leads to formation of self-assembled nano-aggregates in aqueous medium. This restricted their

intramolecular motions and ultimately block their non-radiative decays which makes them highly emissive in aqueous medium. *TPE-Cat-NEt₃* polymeric solution (10^3 $\mu\text{g/mL}$) was prepared in three different solvent systems (DMSO, THF, and water) and their respective photoluminescence intensity was measured at $\lambda_{\text{exc}} = 330$ nm. The change in emission intensity on decreasing the solvation of TPE-based cationic polymers can be observed by increasing the water content in DMSO solutions. Various DMSO/Water solvent composition systems were prepared by increasing the fraction of water gradually while maintaining the total solvent volume as 3 mL. 10^3 $\mu\text{g/mL}$ concentration of *TPE-Cat-NEt₃* polymer is dissolved in each of the solvent systems and fluorescence was measured at $\lambda_{\text{exc}} = 330$ nm.

3.2.23 Particle size and Zeta Potential determination through DLS: In order to determine the average particle size and particle size distribution of the cationic nano-aggregates in water DLS measurement has been done. 100 $\mu\text{g/mL}$ concentration of cationic amphiphilic polymers were weighed and dissolved in 1 mL millipore water and hydrodynamic radius is determined along with polydispersity index. Similarly, zeta potential (surface charge) of the nano-aggregates were also determined using Zetasizer Nano ZS 90 setup (Malvern) to determine surface potential.

3.2.24 Photophysical Studies: The absorbance and fluorescence studies were conducted at optical density of TPE as 0.1. The excitation source used for TPE and CV were 330 nm and 620 nm respectively. For TCSPC lifetime measurement, nano-LED source of $\lambda_{\text{exc}} = 370$ nm for TPE excitation was used where the lifetime decay was obtained at $\lambda_{\text{em}} = 480$ nm. The decay profile was fitted by using EZ software.

3.2.25 Evaluation of Cellular Morphology using Transmission Electron Microscopy (TEM): TEM technique was implemented to observe any distortion in size or shape of the bacterial cells on account of polymer action. 1×10^5 bacterial cells were incubated with *TPE-Cat-NEt₃* polymer in a thermal shaker for 8 h at 37 °C. Samples were centrifuged at 8000 rpm for 10 min and redissolved in sterile PBS buffer. Bacterial samples were incubated with 2.5% glutaraldehyde overnight at 4 °C and ultimately dehydrated using graded ethanol series. 20 μL sample was dropcasted on 400-mesh copper grids and dried overnight at 25 °C

and then under heating oven at 37 °C. TEM images were obtained using JEM-2200FS Field Emission Electron Microscope with an acceleration voltage of 200 keV.

3.2.26 Bacterial Inhibition Assay via Colony Suspension Method: As reported earlier⁴⁹, in order to determine the antibacterial property of the cationic amphiphilic polymers, Minimum inhibitory concentration (MIC) is determined. It was the minimum concentration of the polymer required to inhibit the observable growth of bacteria. In colony suspension method, well-isolated *E. coli* colonies were picked up (usually 2 to 3) from a stock and bacterial inoculum was prepared in LB broth with turbidity of the suspension equivalent to 0.5 McFarland standard. It was then diluted to $O.D_{600\text{ nm}} = 0.005$ which corresponds to 1×10^6 CFU/mL. Serial dilutions of polymeric stock solutions (1×10^4 µg/mL to 1×10^3 µg/mL) were prepared. 100 µL of the polymeric solutions were incubated with 100 µL of bacterial suspension along with a positive and negative control containing only bacterial suspension and Ampicillin at its antibiotic concentration (16 µg/mL) respectively. Time-dependent scattering of the bacterial turbid solution was measured at $\lambda_{\text{exc}} = 600$ nm for 18 h using Perkin Elmer Enspire Multimode Plate Reader.

3.2.27 Crystal violet staining of *E. coli* (Gram-negative) bacterial cells: Enhanced outer-membrane permeability due to pore-forming ability of cationic polymer was corroborated with the retention of crystal violet dye in bacterial membrane. Bacterial cells (1×10^8 CFU/mL) were incubated with different polymers (10^3 µg/mL) along with a negative control (without polymer) for 8 h at 37 °C in a shaker incubator. Cells were pelleted and washed with phosphate buffer (pH 7.4, 1 mL). 10 µL of 10^4 g/mL CV solution in PBS buffer was added to the same and kept it in dark for 10 min. The resultant solution was given PBS washes (X 2) followed by addition of Gram's decolorizer (95 % ethyl alcohol) and kept for 15 seconds, and then washed with PBS again. The *E. coli* cells were fixed on a glass slide and examined under oil immersion objective (100x) by using the Leica SP8 optical microscope employing $\lambda_{561\text{ nm}}$ (red channel) laser.

3.2.28 FRET analysis through confocal imaging: The co-localization of two fluorochromes (TPE-based antibacterial polymer and Crystal violet) in a bacterial micro-environment sheds light on the alteration in bacterial membrane permeability by the

antibacterial polymer. It was achieved through FRET were observed upon donor excitation and detection of emission from acceptor fluorophore, and simultaneous reduction of donor fluorescence. *E. coli* (1×10^8 cells/mL) suspension was prepared in LB broth and incubated with TPE- polymers (10^3 $\mu\text{g/mL}$) for various time points at 37 °C. Cells were pelleted and washed thrice with phosphate buffer (pH 7.4, 1 mL). 10 μL of 10^4 mg/mL CV solution in PBS buffer was added to the same and kept it in dark for 10 min. The resultant solution was given PBS washes (X 2) followed by addition of Gram's decolorizer (95 % ethyl alcohol) and kept for 15 seconds, and then washed with PBS again. A control slide was also prepared which did not involve Gram's staining. The slides were observed under oil immersion objective (100x) by using the blue channel and red channel in Leica SP8 optical microscope

3.2.29 Hemolysis Assay: The bio-compatibility of polymeric samples was evaluated by in-vitro hemolysis assay by monitoring the hemoglobin release in the medium as an indicator for red blood cell lysis. Antibacterial polymers (*TPE-Cat-NH₂* and *TPE-Cat-NEt₃*) were incubated at their MIC concentration (100 $\mu\text{g/mL}$ in PBS) with 5 % v/v solution of RBCs in PBS. For non-antibacterial polymers (*TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NMeImz* and *TPE-Cat-NR₂-NH₂*) assessment was done at the highest concentration till which bacterial inhibition assay was demonstrated i. e. 500 $\mu\text{g/mL}$ in PBS. 1 % Triton X-100 solution and PBS were also taken as positive and negative controls respectively. 100 μL of polymeric sample was incubated with 100 μL of RBCs for 1 h in static incubator followed by centrifugation at 8000 rpm for 5 min. The observance of RBC pellet at the bottom of the eppendorf was confirmative of the non-toxicity of the sample. Absorbance was measured at $\lambda_{\text{abs}} = 570$ nm and % hemolysis was determined as mentioned in the reported procedure.²⁴⁴

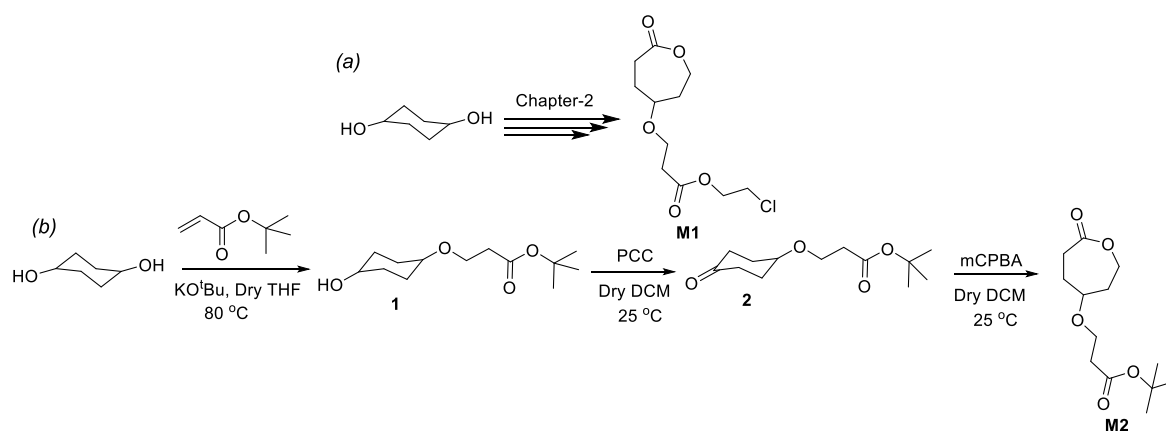
3.2.30 Colony Counting Experiment: A single colony was picked from an *E. coli*-streaked plate and 100 μL of *E. coli* (1×10^6 CFU/mL) was incubated with 100 μL of various polymeric samples followed by irradiation with a blue LED light source (Osram 58 W/865 LUMILUX T8), giving an incident light intensity of $\sim 499 \pm 19$ lux for 1 min. After irradiation, the samples were incubated for 18 h (Incubation is done for 18 h to replicate broth microdilution experiment). In order to count the number of viable colonies at the end

of 18 hours, diluted samples were spread evenly over agar surface and incubated for 18-20 h and colonies were counted.

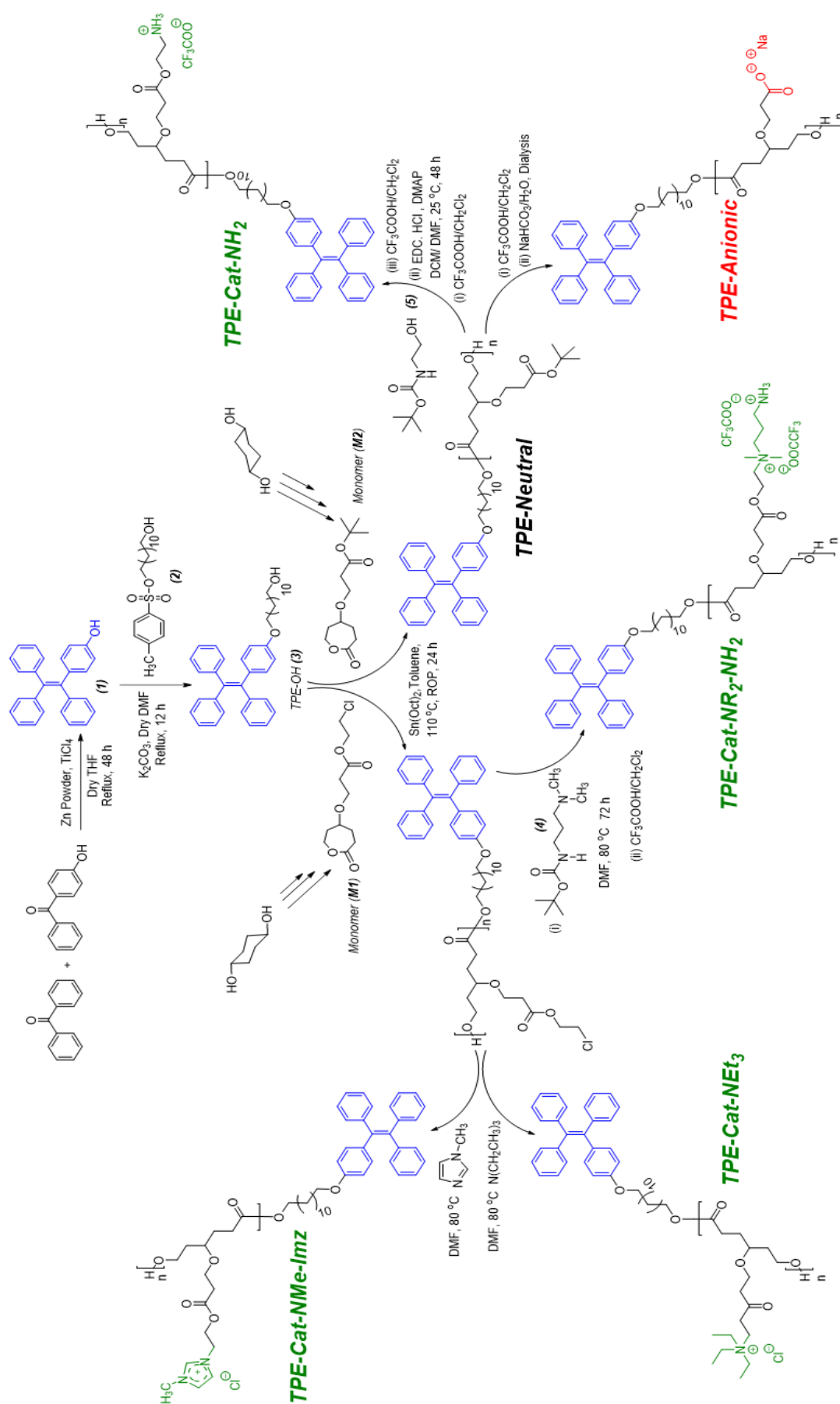
3.3 Result and Discussion

3.3.1 Synthesis of *tert*-butyl 3-((7-oxooxepan-4-yl)oxy)propanoate (M2)

1,4-Cyclohexane diol was subjected to multi-step reactions to yield γ -Cl substituted caprolactone monomer (M1, as described in chapter-2) and γ -tertiary butyl ester substituted caprolactone monomer (M2) as shown in Scheme 3.2. The synthesis of *tert*-butyl 3-((7-oxooxepan-4-yl)oxy) propanoate (monomer M2) involves three consecutive steps. Michael addition of 1,4-cyclohexanediol with *tert*-butyl acrylate in the presence of potassium *tert*-butoxide to yield *tert*-butyl 3-((4-hydroxycyclohexyl)oxy)propanoate (A) (as shown in Scheme 3.2). The mono substituted product (A) underwent PCC oxidation to yield *tert*-butyl 3-((4-oxocyclohexyl)oxy)propanoate (B) and subsequently Baeyer Villiger oxidation in the presence of mCPBA and NaHCO₃ to ultimately obtain monomer M2.



Scheme-3.2. Synthetic scheme of γ -Cl substituted caprolactone monomer (M1) and γ -tertiary butyl ester substituted caprolactone monomer (M2)



Scheme 3.3. Synthesis of TPE-conjugated Cationic, Anionic, and Neutral polycaprolactones

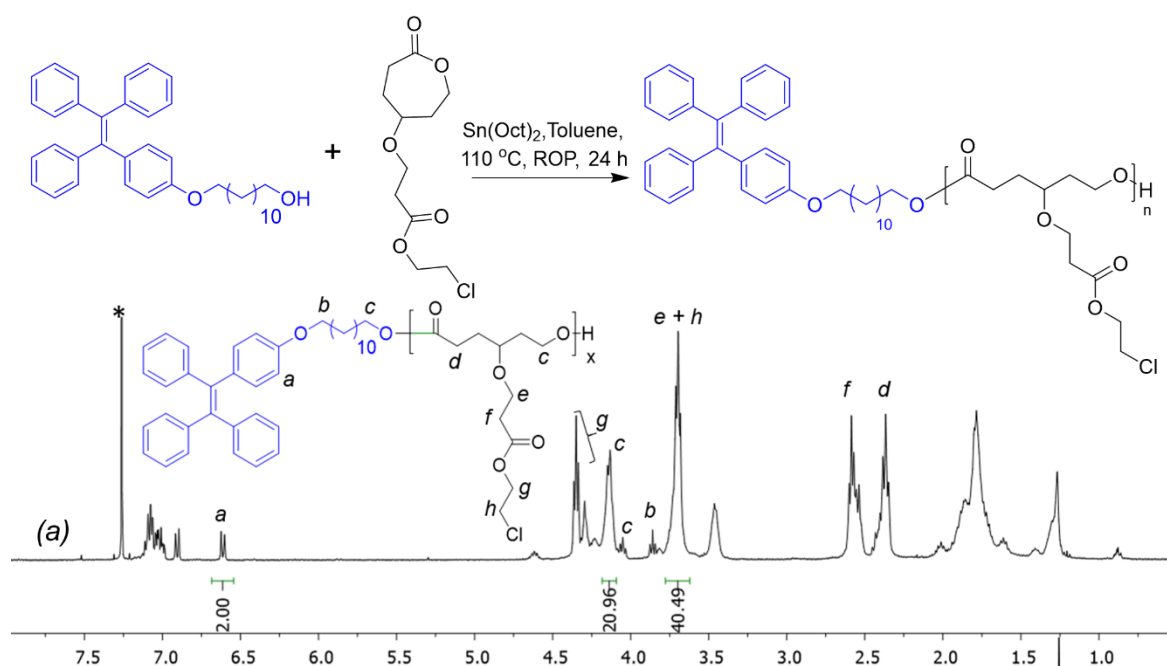


Figure 3.4. ^1H NMR spectrum of TPE-ClPCL in CDCl_3 . The expanded spectra from 0.5 ppm to 8 ppm is shown for clear visualization and the peaks are assigned by alphabets with respect to the types of protons shown in the chemical structure.

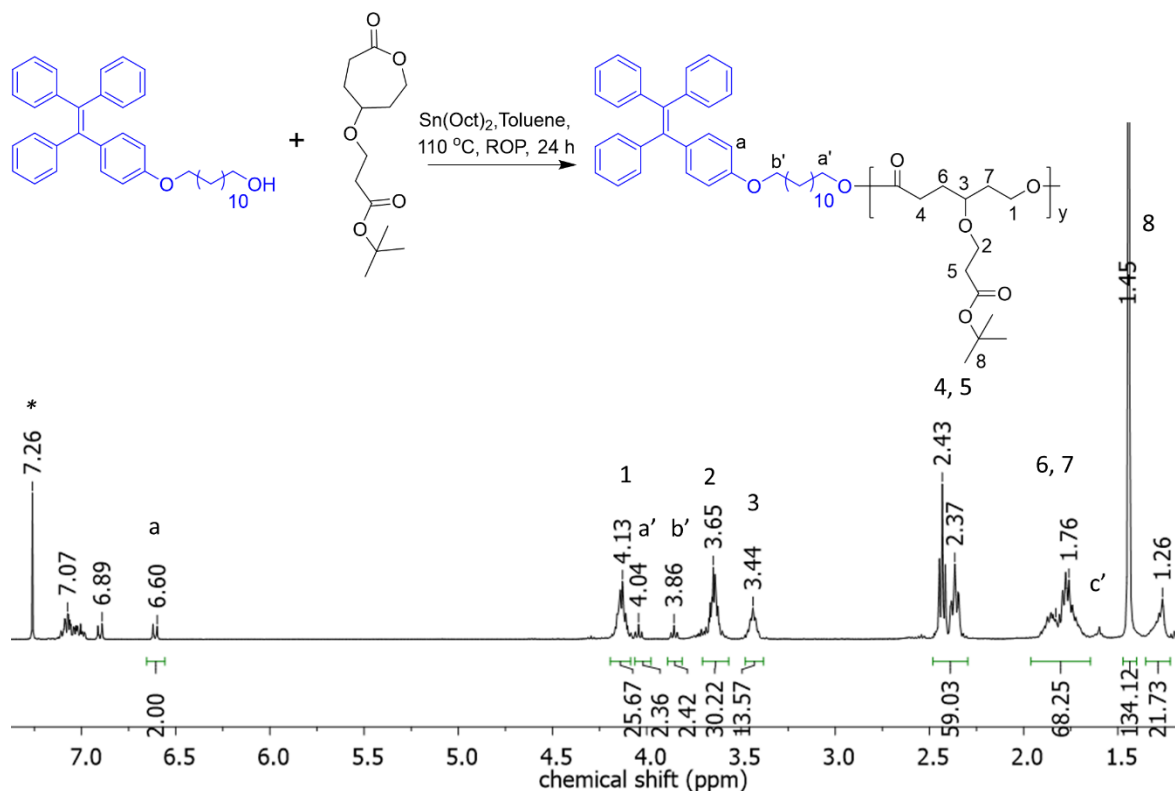


Figure 3.5. ^1H NMR spectrum of TPE-Neutral in CDCl_3 . The expanded spectra from 1 ppm to 8 ppm is shown for clear visualization and the peaks are assigned by numeric with respect to the types of protons shown in the chemical structure.

TPE-phenolic OH (1) was coupled with mono-tosylated alcohol (2) to make AIE-active hydroxyl functionalized tetraphenylethylene (3, TPE-OH) as shown in Scheme 3.3. TPE-OH was employed as an initiator for ring opening polymerization (ROP) of monomers M1 and M2 using Sn (Oct)₂ catalyst with M/I =10 to yield TPE-conjugated homopolymers *TPE-CIPCL* and *TPE-BPCL*, respectively. ¹H-NMR analysis enabled the determination of number of *CIPCL* and *BPCL* repeating units in the homopolymer as 10-11 units (Figure 3.4 and 3.5). In chapter-2,²⁴⁴ the PCL cationic polymers (non-luminescent system) showed saturation in their antibacterial activity beyond 10 repeating units; and thus, it is adopted in the present study. Deprotection of tert-butyl protecting group in *TPE-BPCL* polymer yielded carboxylic substituted PCL polymer (Figure 3.6), and the *TPE-BPCL* polymer is hereafter referred as *TPE-Neutral*. The conversion of -COOH units into its corresponding sodium salt -COO⁻Na⁺ resulted *TPE-Anionic* polymer. The carboxylic polymer was coupled with Boc-protected ethanol amine followed by deprotection to yield amine-salt substituted cationic polycaprolactone (referred as *TPE-Cat-NH₂*, exists as a -NH₃⁺CF₃COO⁻). These polymers were successfully characterized by ¹H-NMR spectroscopy (Figure 3.7 and 3.8). *TPE-CIPCL* polymer was derivatized into three different quaternized polymers using three different tertiary amines. N-methyl imidazole, triethyl amine and tert-butyl (3-(dimethylamino) propyl) carbamate to yield imidazolium ion substituted polycaprolactone (*TPE-Cat-NMe-Imz*), triethyl amine substituted polycaprolactone (*TPE-Cat-NEt₃*) and N, N-dimethyl substituted quaternized caprolactone polymer (*TPE-Cat-NR₂-NHBOC*), respectively, as shown in Scheme 3.3 (see NMR spectra in Figures 3.9-3.12). Size exclusion chromatograms of *TPE-CIPCL* and *TPE-BPCL* polymers showed monomodal distribution (see Figure 3.13) with PDI in the range of 1.4 to 1.5. The molecular weight of these fluorescent cationic polymers was found in the range of M_n ~ 5000 g/mol to 8000 g/mol.

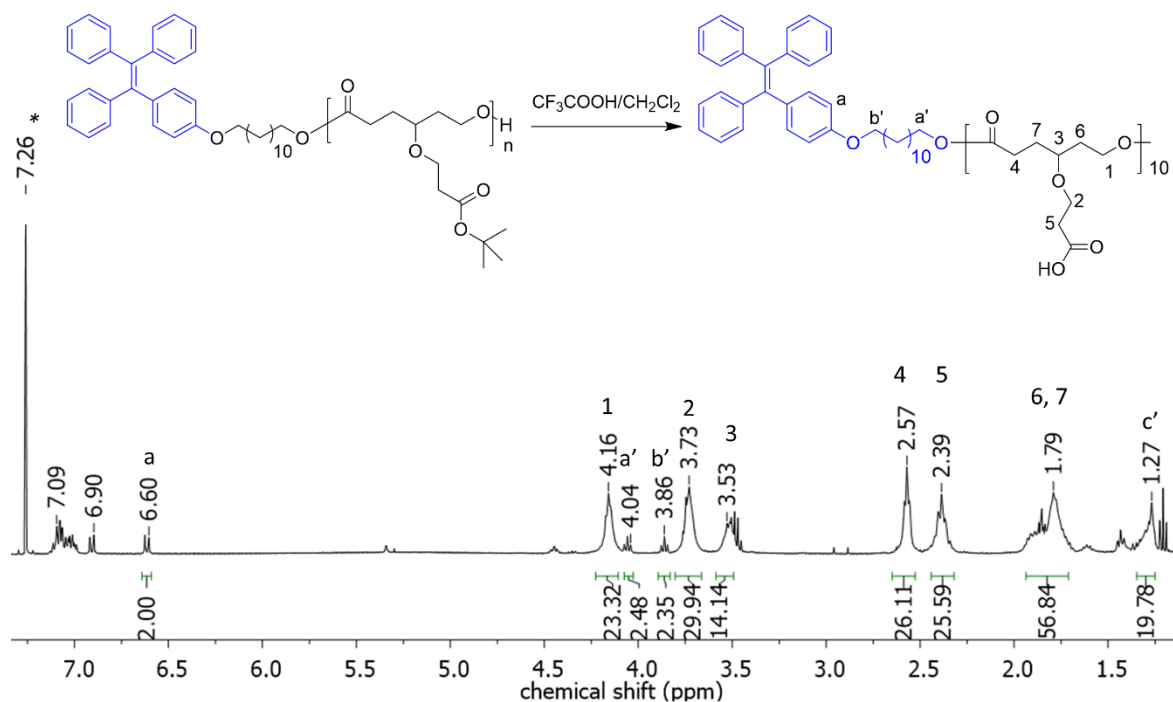


Figure 3.6. ^1H NMR spectrum of TPE-Anionic in CDCl_3 . The expanded spectra from 1 ppm to 7.5 ppm is shown for clear visualization and the peaks are assigned by numeric with respect to the types of protons shown in the chemical structure.

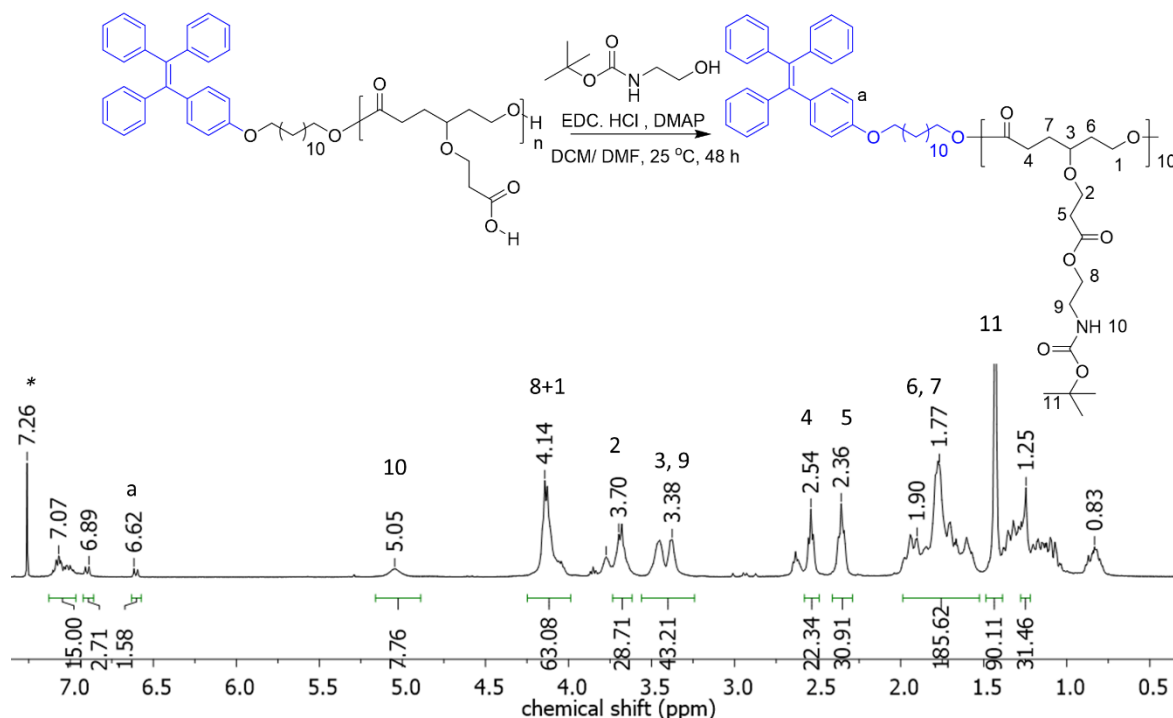


Figure 3.7. ^1H NMR spectrum of TPE-NHBOC in CDCl_3 . The expanded spectra from 1 ppm to 7.5 ppm is shown for clear visualization and the peaks are assigned by numeric with respect to the types of protons shown in the chemical structure.

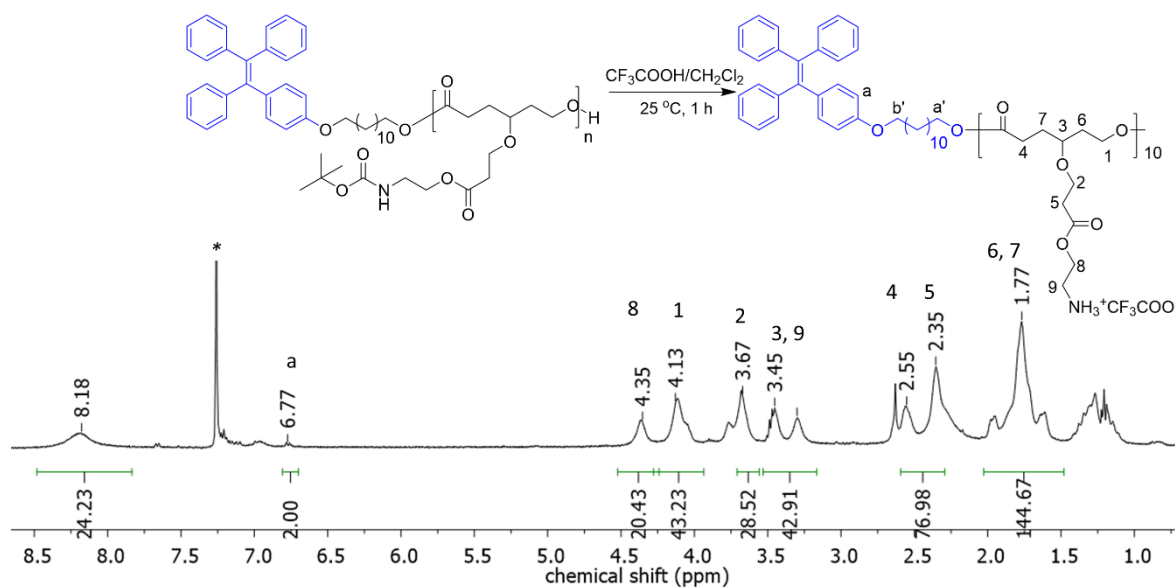


Figure 3.8. ¹H NMR spectrum of TPE-Cat-NH₂ in CDCl₃. The expanded spectra from 0.5 ppm to 8.5 ppm is shown for clear visualization and the peaks are assigned by numeric with respect to the types of protons shown in the chemical structure.

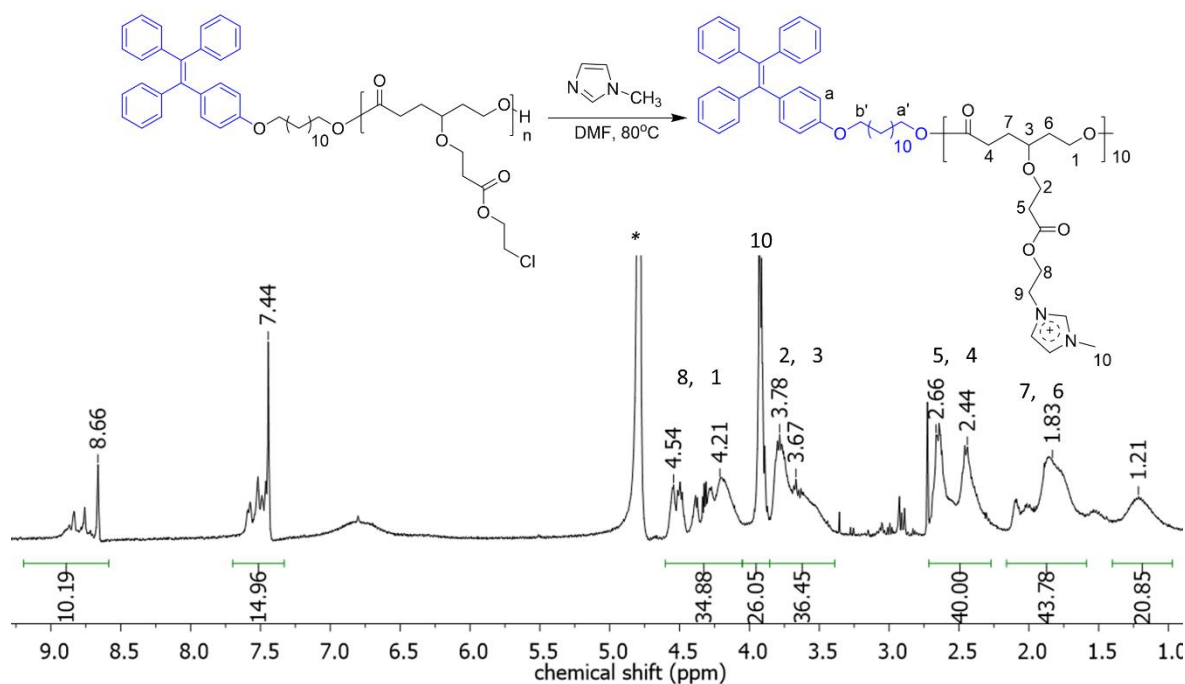


Figure 3.9. ¹H NMR spectrum of TPE-Cat-NMeImz in D₂O. The expanded spectra from 0.5 ppm to 9 ppm is shown for clear visualization and the peaks are assigned by alphabets with respect to the types of protons shown in the chemical structure.

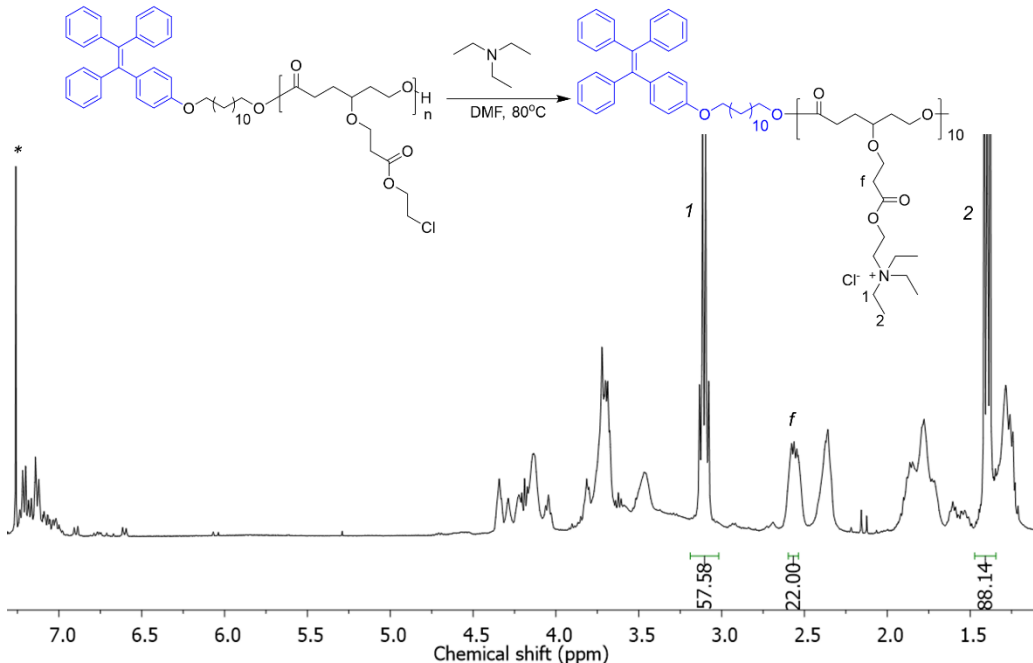


Figure 3.10. ^1H NMR spectrum of TPE-Cat-NEt₃ in CDCl₃. The expanded spectra from 0.5 ppm to 7.5 ppm is shown for clear visualization and the peaks are assigned by alphabets with respect to the types of protons shown in the chemical structure.

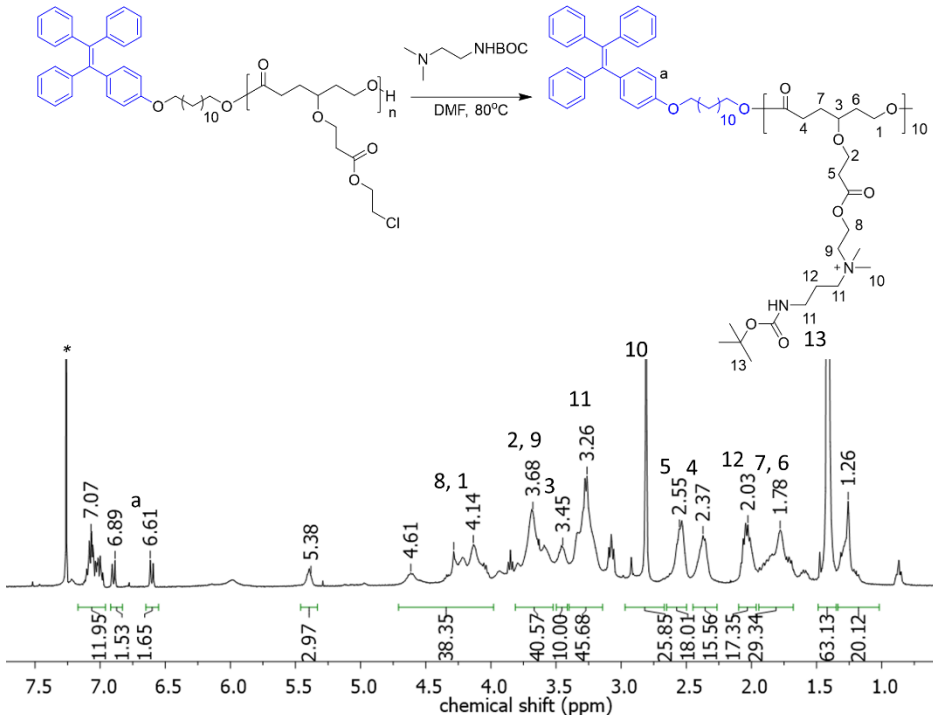


Figure 3.11. ^1H NMR spectrum of TPE-Cat-NR₂-NHBOC in CDCl₃. The expanded spectra from 0.5 ppm to 7.5 ppm is shown for clear visualization and the peaks are assigned by alphabets with respect to the types of protons shown in the chemical structure.

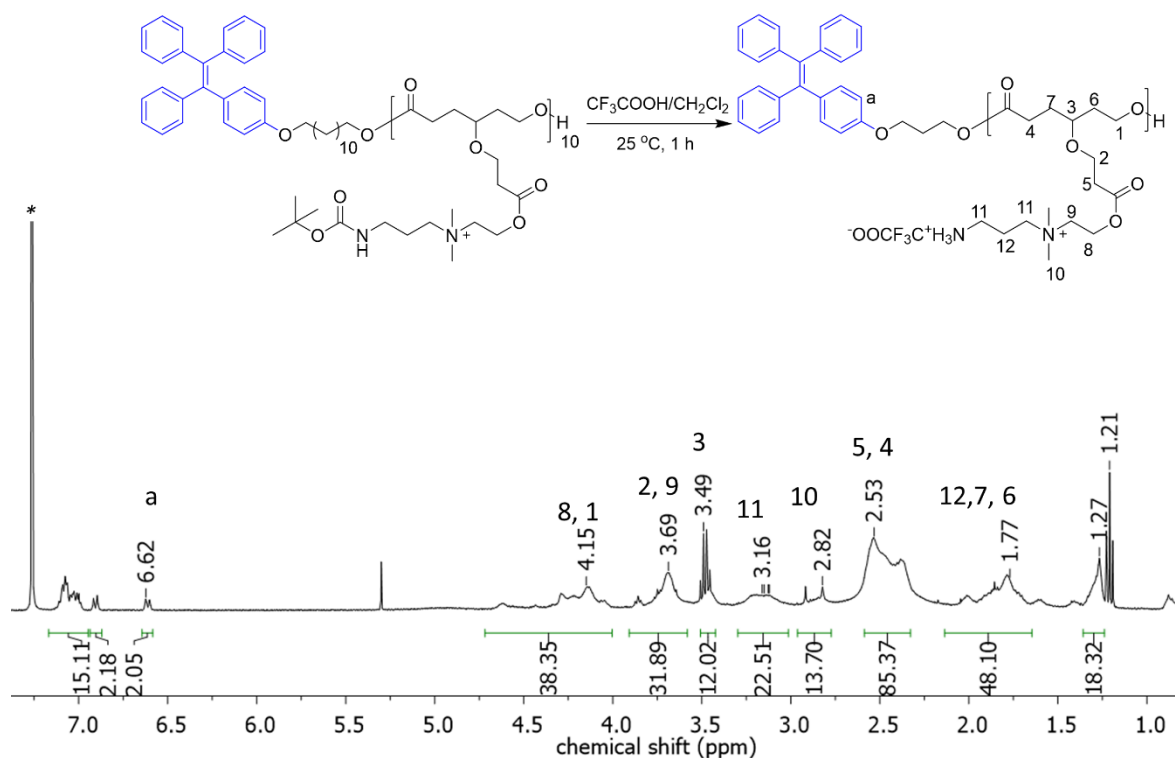
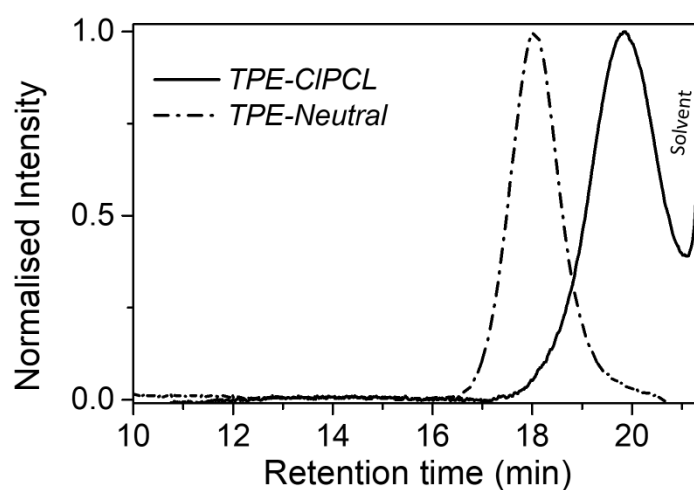


Figure 3.12. ¹H NMR spectrum of TPE-Cat-NR₂-NH₂ in CDCl₃. The expanded spectra from 0.5 ppm to 7.5 ppm is shown for clear visualization and the peaks are assigned by numeric with respect to the types of protons shown in the chemical structure.



Polymer	Mn g/mol	Mw g/mol	PDI
TPE-Neutral	3300	4800	1.47
TPE-CIPCL	2000	3100	1.55

Figure 3.13. GPC plots of (a) TPE-CIPCL₁₀ and TPE-Neutral along with tabulated data to show the number average molecular weight (Mn), weight average molecular weight (Mw) and PDI of polymers.

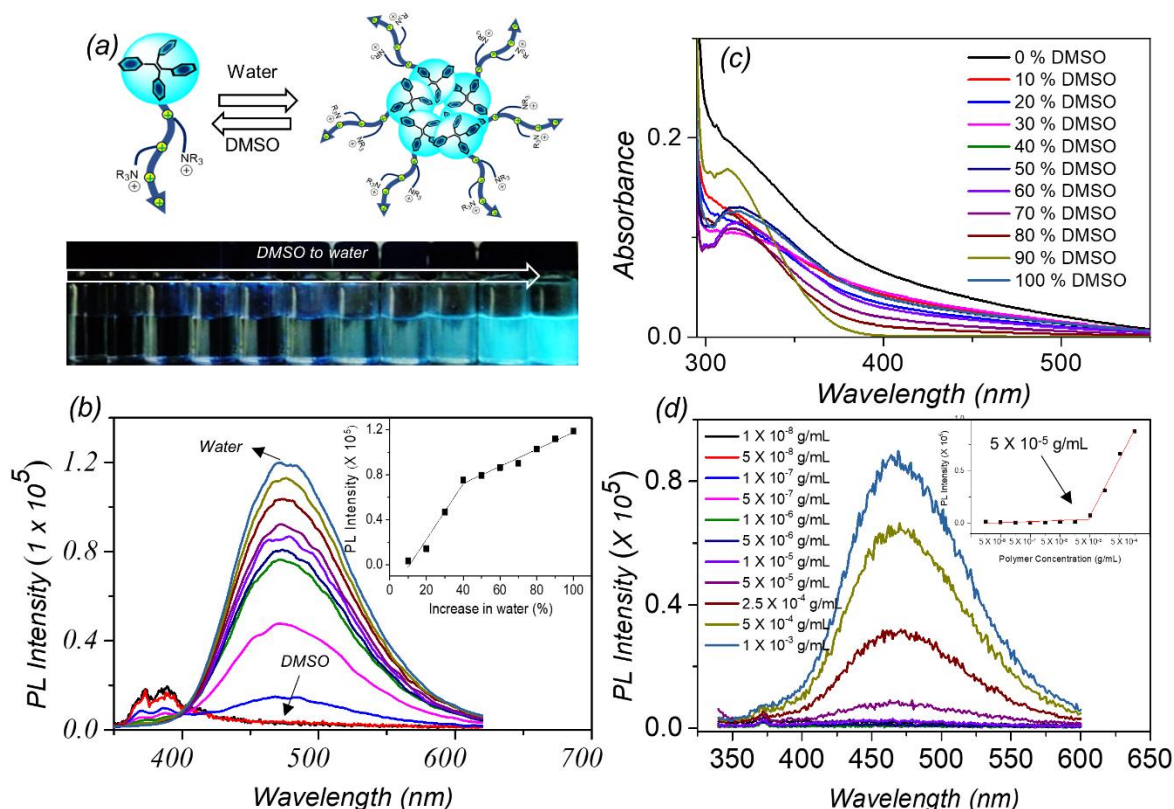


Figure 3.14. (a) Diagram showing self-assembly of TPE-polymers in DMSO and water (b) Plot showing PL Intensity at $\lambda_{em} = 480$ nm for various solvent compositions (inset) along with vials in photograph showing the gradual occurrence of AIE with increase in water-rich domain. (c) Plot showing absorbance profile for TPE-Cat-NEt₃ polymer at different DMSO + Water compositions. (d) Emission spectra of TPE-Cat-NEt₃ at $\lambda_{exc} = 330$ nm with increase in concentration of polymer. Plot showing concentration dependent change in PL Intensity $\lambda_{em} = 480$ nm (inset).

These newly designed cationic PCL polymers inherited amphiphilicity from its hydrophobic aliphatic polyester backbone and hydrophilic groups in the side chains in each repeating unit. The diagrammatic representation of one of the cationic polymers *TPE-Cat-NEt₃* is shown in Figure 3.14a, where the hydrophobicity arose from the bulky aromatic TPE unit and quaternized triethyl amine units were responsible for hydrophilicity. In aqueous medium, these amphiphilic polymers would adopt a phase segregation of TPE hydrophobic segment in the core and the cationic charge on the periphery to produce well defined micellar nanoparticle assemblies as shown in Figure 3.14a. As shown in the vials, TPE chromophores in the cationic polymer was completely dispersed in DMSO and its emission was switched-off. The increase in the water content in DMSO + water solvent mixture, the fluorescent characteristic of the TPE-cationic polymer was restored with strong blue-emission. In Figure

3.14b, upon increasing the water content in DMSO solution, a gradual rise in emission intensity was observed due to the increase in the AIE of the cationic nano-assemblies in aqueous medium with a constant absorbance maxima i.e., λ_{max} (Figure 3.14c) commenting on no change in molar absorptivity of TPE.

A gradual increment in TPE AIE fluorescence upon increasing the water content which might be due to the difference in self-assembled state at various DMSO/water compositions of *TPE-Cat-NEt₃* cationic polymer. This trend is attributed to the impact of the TPE chromophore hydrophobicity on the chain aggregation that restricts their intermolecular motion (RIR or RIV) even at lower water content that ultimately facilitates the TPE-AIE emission. Concentration dependent fluorescent spectra of *TPE-Cat-NEt₃* from 1×10^{-7} $\mu\text{g/mL}$ to 1×10^3 $\mu\text{g/mL}$ in water was recorded to determine the critical aggregation concentration (CAC, see Figure 3.14d). The emission intensity gradually increased at 480 nm with the increase in the concentration of *TPE-Cat-NEt₃* polymer. A clear break point at 50 $\mu\text{g/mL}$ polymer concentration was observed with respect to its CAC as shown in the inset plot.

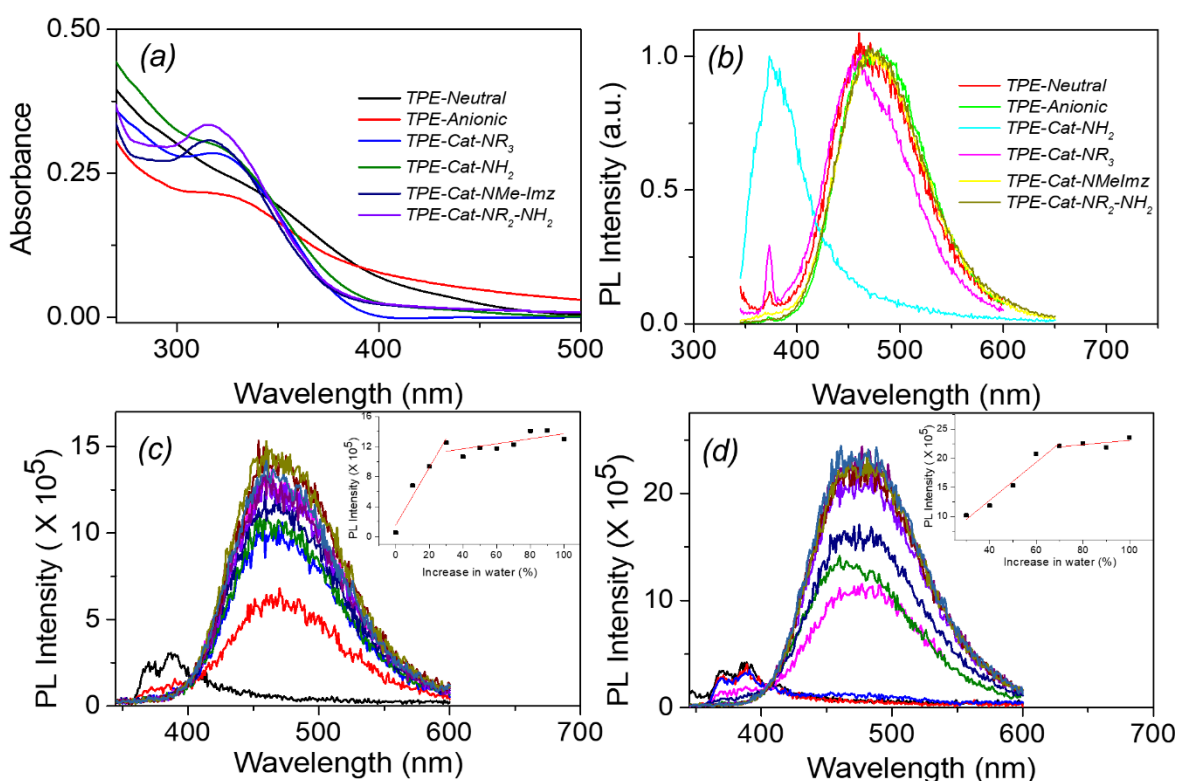


Figure 3.15. (a) Absorbance spectra for all the polymers are shown in water. (b) Plot showing normalized intensity profiles for all the TPE conjugated polymers. Plot showing PL Intensity at λ_{em}

= 480 nm for various solvent compositions for TPE-BPCL (neutral polymer, c) and TPE-CPCL (anionic polymer, d).

The absorbance and fluorescence spectra for all other TPE-polymers (*TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NH₂*, *TPE-Cat-NR₂-NH₂* and *TPE-Cat-NMe-Imz*) are shown in Figure 3.15a and 3.15b. To understand the role of the charges (cationic, or anionic) on the AIE property of the polymer, the solvent composition dependent AIE fluorescence of *TPE-Anionic* (anionic polymer) and *TPE-Neutral* (neutral polymer) were also studied and the details are shown Figure 3.15c and 3.15d. For *TPE-Neutral*, breakpoint at lower water content i.e., 30 % water in DMSO and for *TPE-Anionic*, breakpoint at higher water content i.e., 70 % of water in DMSO was observed. Hence, the polymer self-assembly in solvent mixtures (DMSO+water) was also influenced by the charge of the pendent units in the PCL chains. Nevertheless, all the TPE-conjugated polymers showed highest AIE emission in 100 % water confirming their suitability for biomaterial research.

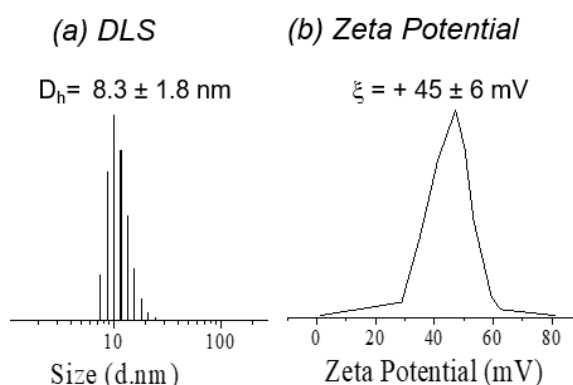


Figure 3.16. (a) Dynamic light scattering histogram and (b) Zeta potential plot for *TPE-Cat-NEt₃* polymer at 100 µg/mL concentration. (c) FE-SEM and (d) HR-TEM image of *TPE-Cat-NEt₃* polymer.

To determine the size of the polymeric nano-assemblies, *TPE-Cat-NEt₃* polymer was readily dispersed in water (100 µg/mL) by sonication, and subjected to dynamic light scattering (DLS) measurement. *TPE-Cat-NEt₃* sample showed tiny nano-assemblies in the size range of $8.3 \pm 1.8 \text{ nm}$ in diameter with $\text{PDI} < 0.1$ (see Figure 3.16a) and zeta potential of +45 mV (as shown in Figure 3.16b) which can be attributed to its positive surface charge. FE-SEM and HR-TEM images of the *TPE-Cat-NEt₃* showed the existence of spherical nanoparticles with an average size of $6.6 \pm 0.2 \text{ nm}$ and $6.5 \pm 0.3 \text{ nm}$, respectively (Figures 3.17a and 3.17b). For all the other cationic polymers (*TPE-Cat-NEt₃*, *TPE-Cat-NH₂*, *TPE-*

Cat-NR₂-NH₂ and *TPE-Cat-NMe-Imz*), neutral polymer (*TPE-Neutral*) and anionic polymer (*TPE-Anionic*), DLS histograms and Zeta potential values are shown in Figure 3.18. It can be clearly noted that, sizes of *TPE-Neutral* and *TPE-Anionic* nanoparticles are relatively larger (~ 150 nm) than cationic nanoparticles (~ 10 nm to 80 nm). This might be due to the insufficient amphiphilicity in polymeric structure of neutral and anionic (*TPE-Neutral* and *TPE-Anionic*) polymers. The values are tabulated for comparison in Table 3.1.

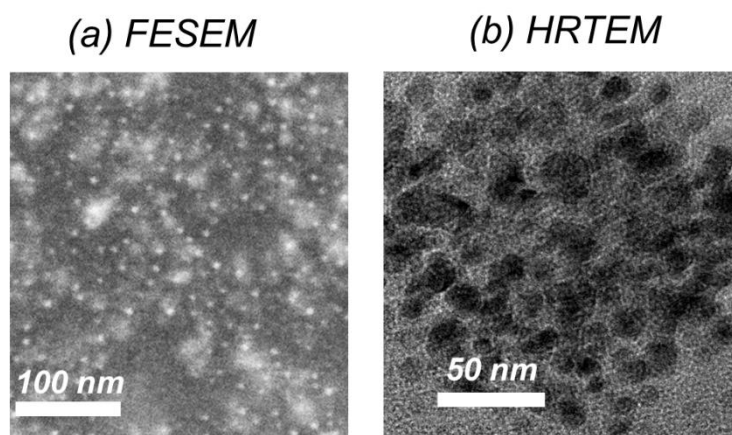


Figure 3.17. (a) FE-SEM and (b) HR-TEM image of *TPE-Cat-NEt₃* polymer.

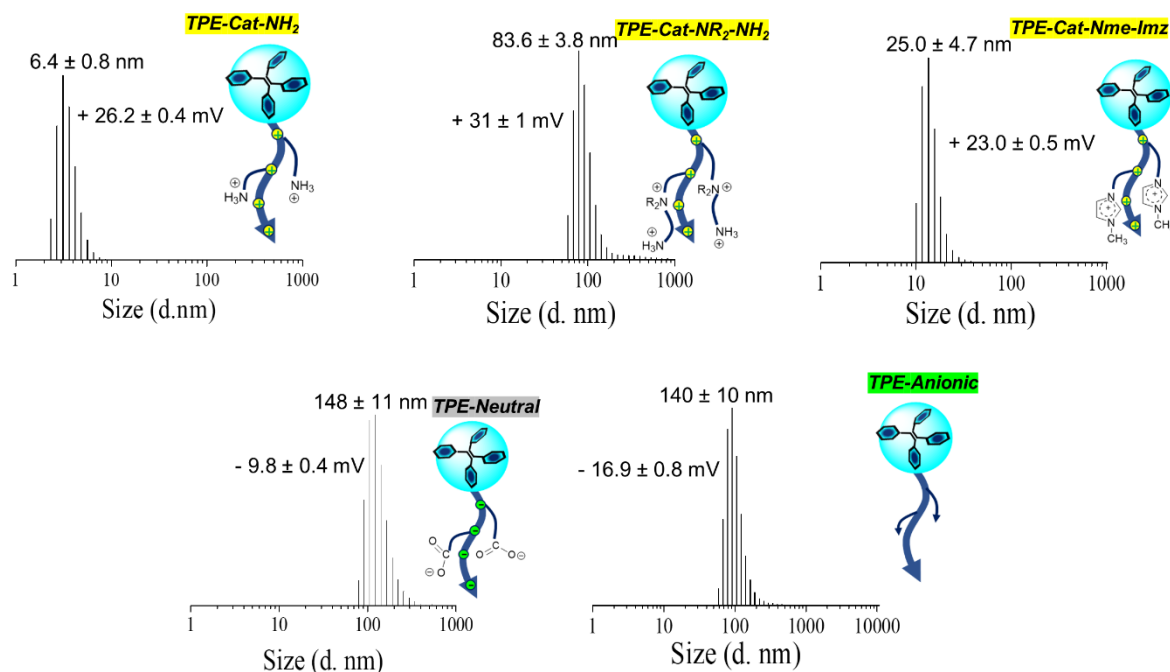


Figure 3.18: Plots of DLS Sizes along with Zeta potentials for all the polymers in water. Polymer concentration was maintained at 100 $\mu\text{g/mL}$.

Table 3.1. Table enlisting size and zeta potential, MIC_{90} , HC_{50} along with selectivity index the polymers (100 $\mu\text{g/mL}$ concentration)

Table containing values of size, zeta potential, MIC_{90} , HC_{50} and selectivity index

Polymer	DLS (d. nm) ^a	Zeta Potential (mV) ^a	MIC_{90} (mg/mL) ^b	HC_{50} (mg/mL) ^c	Selectivity Index ^d
TPE-Neutral	140.0 $\pm$ 10.0	-9.8 $\pm$ 0.4	NA	> 0.5	-
TPE-Anionic	148.0 $\pm$ 11.0	- 16.9 $\pm$ 0.8	NA	> 0.5	-
TPE-Cat-NEt ₃	8.3 $\pm$ 1.8	+45.0 $\pm$ 6.0	0.1	> 0.1	>10
TPE-Cat-NH ₂	6.4 $\pm$ 0.8	+26.2 $\pm$ 0.4	0.1	> 0.1	>1
TPE-Cat-NR ₂ -NH ₂	83.6 $\pm$ 3.8	+31.0 $\pm$ 1.0	NA	> 0.5	-
TPE-Cat-NMe-Imz	25.0 $\pm$ 4.7	+23.0 $\pm$ 0.5	NA	> 0.5	-

^aSize and zeta potential was determined by dynamic light scattering (DLS) instrument. ^bMinimum inhibitory concentration for 90% inhibition of *E. coli* growth was determined by colony suspension assay. ^cMinimum polymer concentration for 50 % inhibition of RBCs. ^d $\text{HC}_{50}/\text{MIC}_{50}$

The antibacterial activity of the polymers was determined by broth microdilution assay^{244, 247} by measuring the optical density (O.D. at 600 nm) of bacterial suspension upon action of polymer. Variable concentrations of the amphiphilic polymers (1 X 10 to 1 X 10³ $\mu\text{g/mL}$) along with control positive control (without polymer treatment) and negative control (ampicillin) were incubated with 10⁶ CFU/mL of bacterial suspension (0.005 O.D. at 600 nm) at 37 °C for 18 h. The decrease in turbidity of bacterial suspension over incubation time was direct readout of antibacterial action of polymers as shown in Figure 3.19. The MIC_{90} value was determined by comparing the optical density for control (when no polymer is added) at the end of 18 h to that of polymer incubated samples and MIC_{90} for TPE-Cat-NEt₃ polymer was observed to be 1x10² $\mu\text{g/mL}$ (Figure 3.19a). Similarly, for TPE-Cat-NH₂ (cationic polymer), the MIC_{90} value was observed to be 1x10² $\mu\text{g/mL}$ (Figure 3.19b). However, the other two cationic polymers TPE-Cat-NR₂-NH₂ and TPE-Cat-NMe-Imz did not show any considerable decrease in optical density at 600 nm for *E. coli* suspension (till maximum concentration, 5 X 10² $\mu\text{g/mL}$) (Figure 3.19c and 3.19d). This suggests that among the four units that were introduced as cationic charge in PCL polymer; both quaternary amine (⁺NEt₃) and primary amine (⁺NH₃) are responsible for ≥ 90 % inhibition of *E. coli* whereas imidazolium ion and the combination of primary amine and quaternary

amine ($^+NR_2-$ $^+NEt_3$) were found to be ineffective. Under the identical conditions, both *TPE-Neutral* (ester pendant unit) and *TPE-Anionic* polymers were also found to be non-antibacterial in nature (Figure 3.19e and 3.19f).

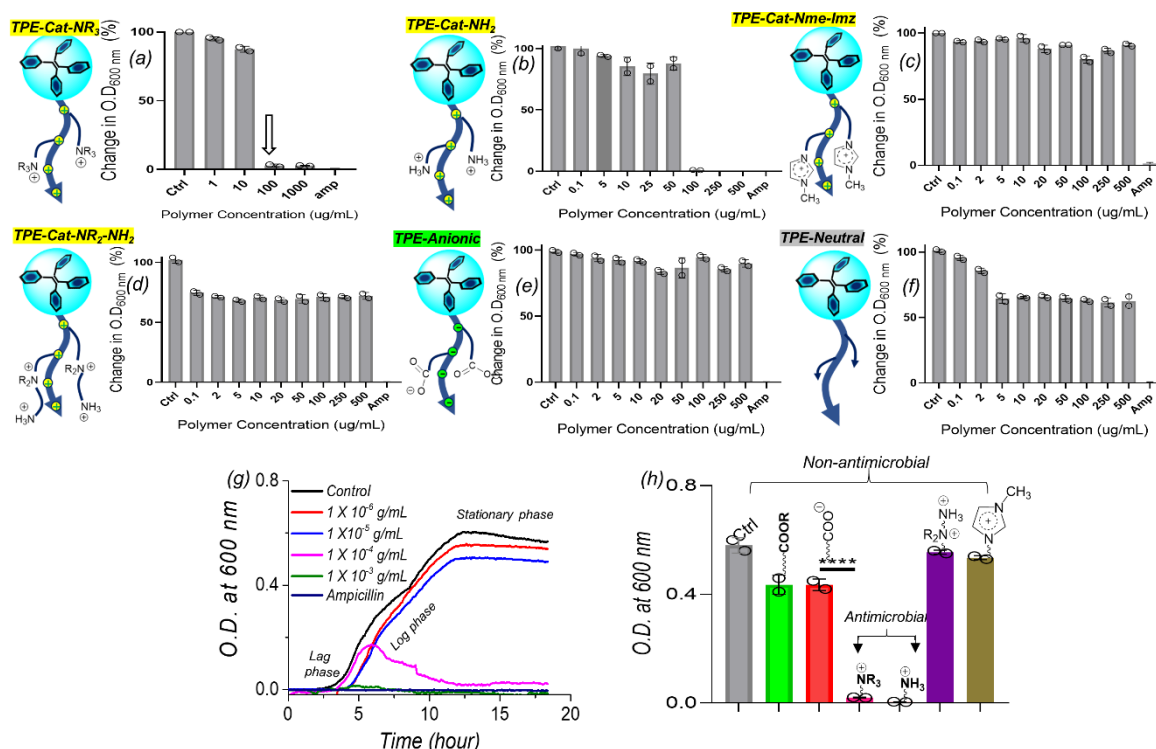


Figure 3.19. Determination of MIC_{90} for (a) *TPE-Cat- NEt_3* , (b) *TPE-Cat- NH_2* , (c) *TPE-Cat-NMe-Imz* and (d) *TPE-Cat- NR_2-NH_2* , (e) *TPE-Anionic* and (f) *TPE-Neutral*. (g) Broth microdilution assay showing the plot of optical density at 600 nm with time for *TPE-Cat- NEt_3* . (h) Comparative determination of MIC_{90} for various polymeric samples

The growth profile of *E. coli* cells without polymer treatment (control sample) comprises the following phases of bacterial growth curve which involves: (a) lag phase (during the initial 0 to 2 h) where there was no change in turbidity of the sample as the cells are metabolically active but did not start to divide, (b) log phase (for time interval ~ 2 h to 12 h) where exponential increase in *E. coli* density is observed, and (c) stationary phase (for time interval ~ 12 h to 18 h) marked by the plateau region where rate of cell division and cell death were roughly equal. In contrast, for *TPE-Cat- NEt_3* polymer incubated *E. coli* suspension, as the cationic polymer concentration was increased from 1×10^{-6} to 1×10^{-3} $\mu\text{g/mL}$, a gradual decrease in bacterial turbidity was observed as a measure of decrease in O.D. 600 nm of the bacterial suspension which marked the antibacterial action of the polymers (see Figure 3.19g). A comparative plot of various polymers with respect to their final optical

density value at the end of 18 h is shown in Figure 3.19h, where it is clearly visible that optical density of antibacterial polymer (*TPE-Cat-NEt₃*, *TPE-Cat-NH₂*) incubated *E. coli* samples was drastically less as compared to non-antibacterial polymers (*TPE-Cat-NR₂-NH₂*, *TPE-Cat-NMe-Imz*, *TPE-Neutral* and *TPE-Anionic*). This result suggested that the type of cationic units in the polymer backbone played a very significant role in determining the antibacterial activity.

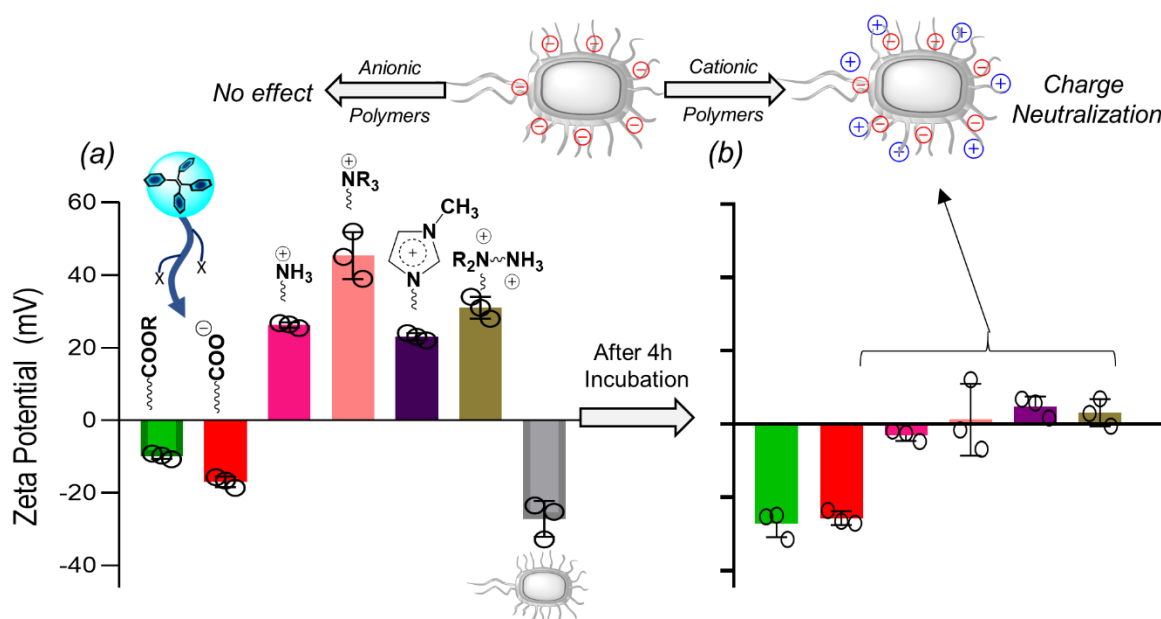


Figure 3.20. (a) Zeta potential values for all the polymers along with *E. coli*. (b) Change in zeta potential after 4 h incubation with *E. coli*.

To validate polymeric interaction with *E. coli* membrane, *E. coli* surface charge potential was monitored by Zeta potential. Zeta potential of nascent *E. coli* suspension (0.5 OD at 600 nm) was found out to be -25 mV due to presence of anionic units comprising of lipopolysaccharide and phospholipids on its surface.¹² Zeta potential of nascent polymers along with *E. coli* is shown in Figure 3.20a. After 4 h incubation of the polymeric samples with *E. coli*, the magnitude of zeta potential in Figure 3.20b for all the cationic polymers were reduced to ~-5 mV to +5 mV showing charge neutralization upon polymeric interaction with *E. coli* membrane. However, in the case of *TPE-Neutral* and *TPE-Anionic* polymers, the zeta potential of bacterial surface remained almost unchanged (see Figure 3.20b). This clearly suggests polymeric adherence onto the surface of *E. coli* can be determined through zeta potential measurement for cationic polymers due to charge neutralization but not in case

of neutral and anionic polymers as shown diagrammatically in Figure 3.20a. Moreover, irrespective of their antibacterial action, all cationic polymers were able to neutralize the *E. coli* surface charge potential by adhering on its surface. The comparison of the data in Table 3.1 and Figure 3.20b revealed even though all cationic polymers could adhere on the surface of *E. coli*; only *TPE-Cat-NH₂* and *TPE-Cat-NEt₃* breach the membrane due to their antibacterial activity.

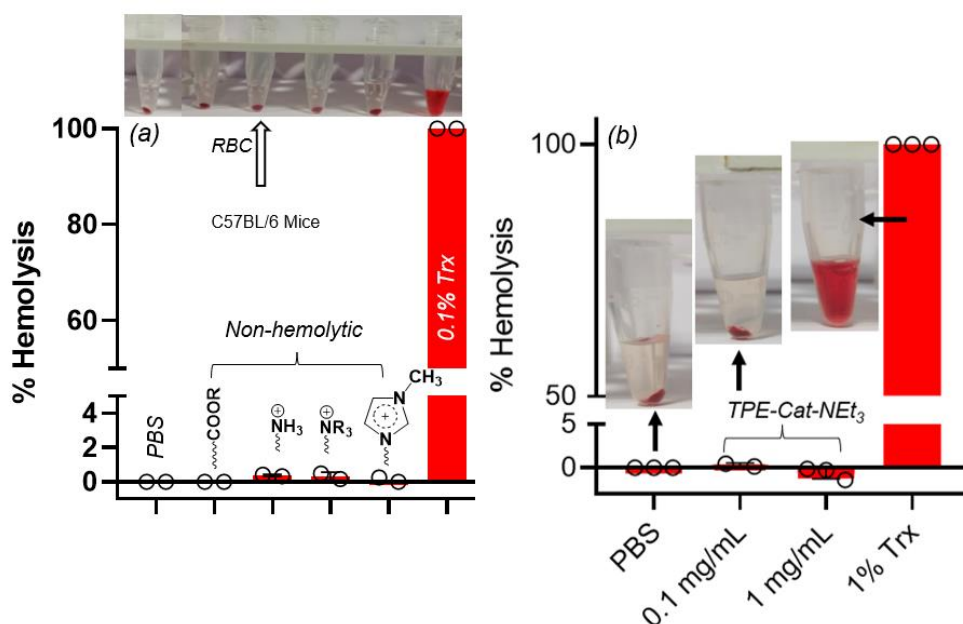


Figure 3.21. (a) Hemolysis activity determination for *TPE-Neutral*, *TPE-Cat-NEt₃*, *TPE-Cat-NH₂* and *TPE-Cat-NMe-Imz* along with PBS and 1% Triton-x. (b) Plot showing % Hemolysis value for *TPE-Cat-NEt₃* polymer at two different concentrations (100 $\mu\text{g/mL}$ and 10³ $\mu\text{g/mL}$) along with PBS (as negative control) and 1 % Triton-x (as positive control).

The hemocompatibility of TPE-polymers was ascertained by incubating them with RBCs of freshly withdrawn mice blood. After 1 hour of static incubation at 37 °C, the samples were taken and centrifuged at 8000 rpm for 5 min. The presence of an undisturbed RBC pellet at the bottom of the eppendorf was affirmative of polymer's hemocompatibility (see Figure 3.21). The percentage hemolysis was determined for four polymeric samples along with PBS as negative control, relative to 1 % Triton-x as positive control. The polymers were found to be completely non-hemolytic (see Figure 3.21a) with their HC₅₀ values to be higher than the concentration range at which hemocompatibility was checked (see Table 3.1). The selectivity index provides us a therapeutic window of a drug at which its efficiency is maximum without causing any unwanted toxicity. Upon analyzing the

selectivity index, it can be inferred that at MIC concentration, the quaternary amine and primary amine functionalized antibacterial polymers were found to be more selective towards *E. coli* than RBCs. The hemolysis activity was also checked at 10 times higher polymeric concentration ($10^3 \mu\text{g/mL}$) for *TPE-Cat-NEt₃* polymer (see Figure 3.21b). The polymer was non-hemolytic and the selectivity index could be improved by order of 10 times than the values reported in Table 3.1. In our earlier attempt⁴⁹, the PCL cationic polymer without TPE substitution showed selectivity index 1000 times better than the present system. It indicates that the TPE conjugation probably influenced on the hemolysis action of the PCL-based cationic polymer and the exact reason is not clear at present. In general, TPE-based antibacterial polymers are very new in the literature, and therefore, more insight into the structure-property relationship is further required to establish to rationalize their effect on hemolysis data. For the development of efficient cationic antibacterial polymer, balance between hydrophobic and hydrophilic content is extremely important. It has been known in the literature that lack of appropriate hydrophobicity leads to lower bactericidal activity (due to less membrane penetration) whereas selectivity is compromised at higher hydrophobic content due to non-specific polymer insertion into bilayer membrane of RBCs and mammalian cells.⁴² In the present case, the cationic polymers is designed with the minimum amount of sufficient cationic units (10-12 units per chains) that are required to maximize the impact on the bacterial membrane breaching based on existing structure-property relationship data.²⁴⁴ Further, the polymers were built on biodegradable and biocompatible PCL platform, and therefore, the antibacterial polymers were found to be more compatible to the RBCs and epithelial cells. Hence, the hemolysis data indicate that the newly designed polymers can be stated as safe to use.

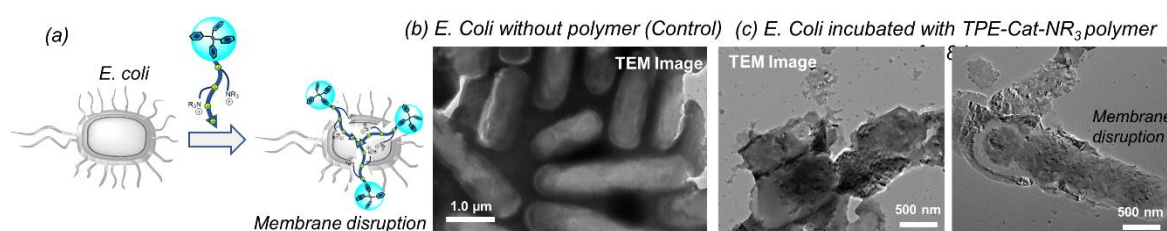


Figure 3.22. (a) Schematic representation of membrane disruption by cationic antibacterial polymer. TEM images of (b) *E. coli* and (c) TPE-Cat-NEt₃ incubated *E. coli*.

In order to understand the membrane disruption mechanism upon polymeric interaction, samples were viewed under electronic microscopy to observe membrane imperfections upon polymeric action as shown in Figure 3.22a. The morphological changes in *E. coli* cells were examined after its treatment with *TPE-Cat-NEt₃* homopolymer by HR-TEM imaging. The bacterial cells were incubated with *TPE-Cat-NEt₃* homopolymer for 8 h at 37 °C along with control sample (without polymer) and then examined by HR-TEM. It was observed that in case of control, the cell surface morphology appeared to be smooth and regular with clear edges in Figure 3.22b. On the contrary, as shown in Figure 3.22c, image of the bacterial membrane of cationic polymer treated sample showed completely compromised cellular membranes with apparent cellular debris nearby the membrane compromised *E. coli* cells. The appearance of blebs and wrinkles due to membrane disintegration ultimately led to release of cellular content and cell lysis. This observation suggested that the mechanism of polymeric killing involved surface association followed by membrane disruption which ultimately led to bacterial killing.

The AIE property of the cationic TPE-polymer on bacterial surface was studied using *E. coli* as a template by confocal microscope. The membrane rupturing capability of the TPE-polymer would facilitate the influx of propidium iodide (PI) to bind to the DNA at the intracellular level in membrane-compromised dead *E. coli*. PI is a membrane impermeable biomarker and it would undergo cellular internalization exclusively in the membrane compromised cells (not inner or outer membrane specific); thus, it is regularly employed as readout for non-fluorescent antimicrobial agents to infer the viability of the bacterial cells. Interestingly, the employment of fluorescent antimicrobial agents like in the present case along with PI, would enable the co-localization of signals in confocal microscope to differentiate site specific accumulation of probe molecules in the bacteria. By using selective photoexcitation of TPE chromophore at 405 nm laser and PI at 561 nm laser and the colocalization of confocal microscope images; one could directly visualize TPE on the bacterial membrane distinct from the PI-DNA at the intracellular compartment.

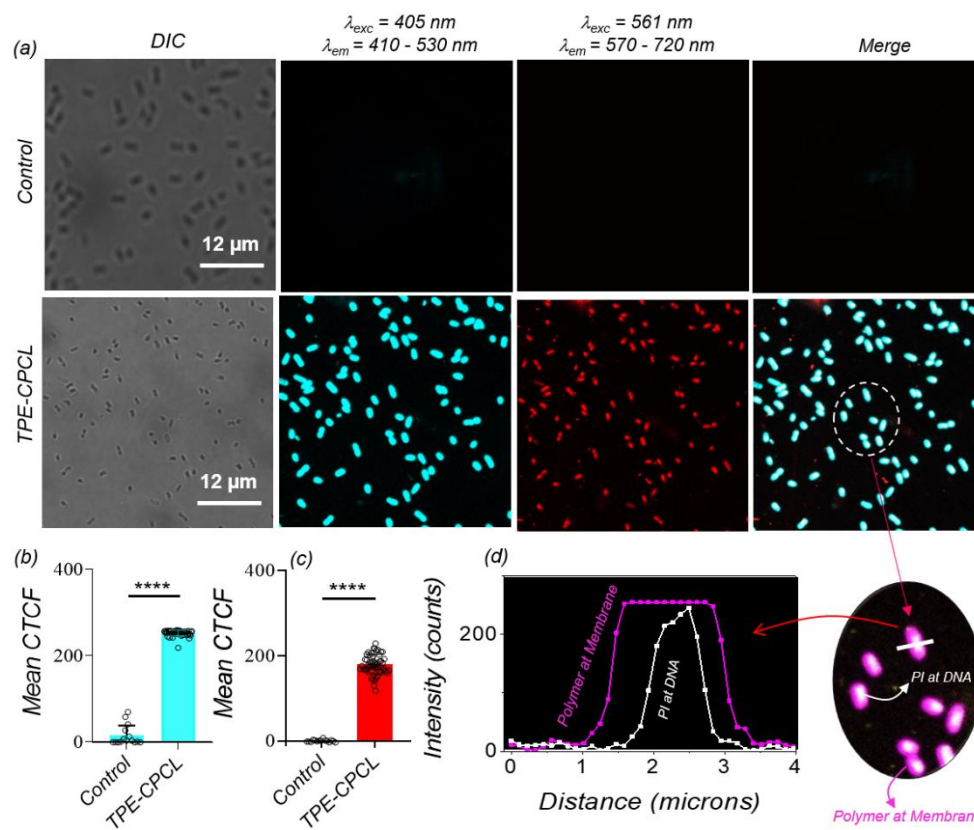


Figure 3.23. (a) Confocal image of *E. coli* bacterial cells without polymer (first panel) and incubated with TPE-CPCL polymer for 8 h (second panel). (b) Calculation of Mean CTCF value for TPE fluorescence and (c) propidium iodide intensity coming from bacterial cells for control (without polymer) and TPE-CPCL incubated bacterial cells. Each point represents mean $\pm$ SEM ($n = 15$), (**** $P < 0.0001$). (d) Energy profile plot showing change in fluorescence intensity with distance across a single bacterium along with a magnified CLSM merged image (magenta (pseudo color) for TPE-CPCL and green (pseudo color) for PI for better visualization).

E. coli (1×10^8 CFU/mL) was treated with TPE-Cat-NEt₃ homopolymer (1xMIC) and without polymer (control) for 8 h, and stained with PI to record the confocal images as shown in Figure 3.23. In the absence of cationic polymer, (control first panel), the bacterial cells were healthy and the membrane was not compromised which is evident by the absence of signals in the blue and red-channels (PI staining is not possible). Upon exposure to the TPE-Cat-NEt₃ incubation (second panel), the bacterial cells were observed to be brightly illuminated under confocal microscope in the blue-channel due to the binding of TPE chromophore on bacterial surface. The simultaneous membrane disruption by the cationic polymer enabled the PI entry and binding to the DNA which account for the strong emission

in the red-channel. The merged image of *TPE-Cat-NEt₃* incubated cells showed the colocalization of both the channels from *E. coli* cells. The corrected total cell fluorescence (CTCF) was calculated for the blue and red channels and showed in Figures 3.23b and 3.23c, respectively. Strong AIE-based emission was observed from the polymer incubated bacterial cells whereas no blue emission was observed in the control sample (Figure 3.23b). This confirmed that the polymeric interaction on the *E. coli* bacterial membrane did not alter the AIE phenomena of the probe and its blue-fluorescence emission retained. Further, the PI emission is minimal in the control while an exuberant increase after incubation with *TPE-Cat-NEt₃* (Figure 3.23c); this reinstates that PI could only enter into the cells followed by the membrane disruption by the cationic polymer. The precise location of both the fluorophores inside a single bacterium was determined by generating a horizontal fluorescence profile plot wherein a cursor line is drawn across an *E. coli* and fluorescence intensity for both the fluorophores (*TPE-Cat-NEt₃* polymer and PI biomarker) that fall along that line is plotted as shown in Figure 3.23d. *TPE-Cat-NEt₃* and PI emission were given magenta and green colors (instead of cyan and red), respectively, for distinct visualization of their colocalized part in a single bacterium. The intensity of the magnified image of bacterial cells showed distinct signals in the membrane from the cytoplasm. TPE emission from the cationic NP predominately visible in the bacterial envelope as evident for its membrane binding ability. The apparent white emission from the center of the bacteria due to colocalization of two complementary colors, magenta (from TPE) + green signals (from PI). This confirmed PI emission coming from inside the cell which is due to its interaction with bacterial DNA. Selective photoexcitation of TPE chromophore and observing the signals from PI (570-720 nm) was also carried out to find out the cross-talking between the two chromophores. There is no signal observed from PI (red channel) and it clearly rules out the interaction between TPE and PI. Therefore, the individual optical purity was retained for further colocalization analysis in the bacterial cells to confirm the polymer into the lipid hydrophobic layer of *E. coli* which causes membrane-disruption, and ultimately cell death.

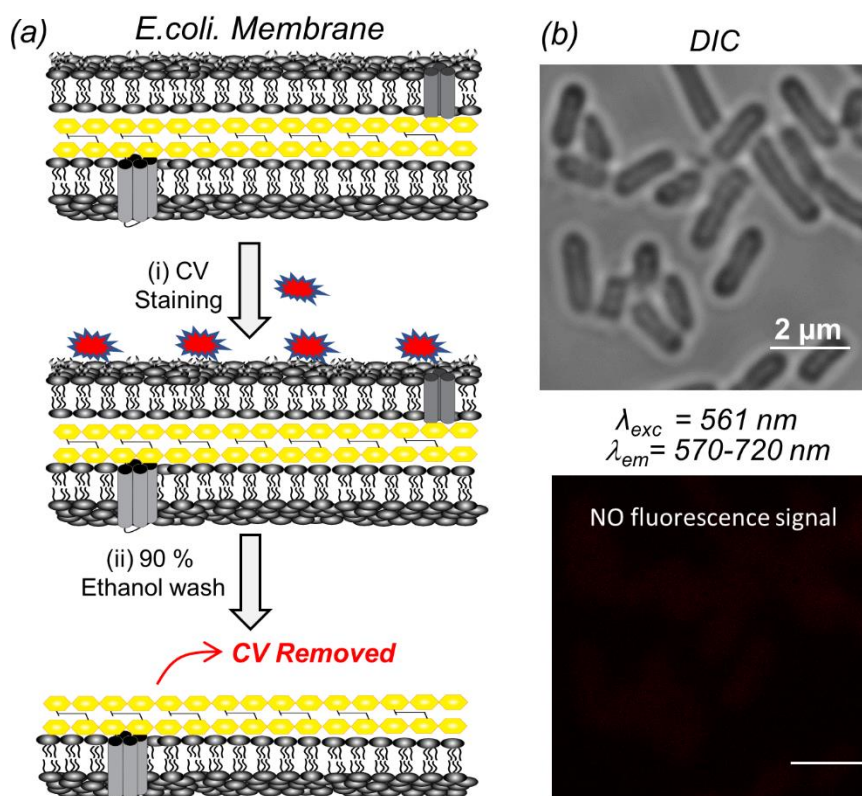


Figure 3.24. (a) Diagrammatic representation showing differential incapability of CV to stain gram negative cell lines through gram's staining. (b) Confocal microscopy images of *E. coli* bacterial cells (1×10^8 CFU/mL) stained with CV by Gram's stain procedure without polymer.

E. coli membrane has intact morphology and it does not allow CV to penetrate through the outer-membrane surface in gram-staining procedure.²⁴⁸ CV could be physically adsorbed on outer-membrane surface by electrostatic interactions; however, ethanol-wash protocol involved as part of the gram-staining procedure would remove CV completely from the outer membrane as shown in Figure 3.24a. Since CV does not stain *E. coli* in the gram-staining protocol, the CV cannot be visualized by fluorescence imaging in confocal microscopy. This point is validated in Figure 3.24 for *E. coli* samples in the native state and there is no fluorescence signal from CV. A non-fluorescent PCL-antibacterial polymer (CP-10, from Chapter-2), was chosen to disrupt the membrane so that the CV could be stained in the peptidoglycan layer as shown in Figure 3.25a. Once the CV is strongly bound on the peptidoglycan layer, it is not removable by ethanol wash in gram staining procedure, and therefore, one can image the CV signals in the confocal microscope. This point is validated

in the present investigation, by treating *E. coli* with non-fluorescent cationic PCL polymer (synthesized as per Chapter-2) for 8 h and the cells were stained with CV, washed with 90 % ethanol and fixed for confocal imaging. The cells were excited at 561 nm (CV excitation) and their signals were collected in the red-channels (570-720 nm). *E. coli* exhibited strong red-emission in Figure 3.25b confirming the outer membrane disruption by the polymer and subsequent permeabilization of CV in the peptidoglycan layer. This was further corroborated by observance of very minimal mean CTCF values in Figure 3.25c for red-channel in control (in the absence of polymer, Figure 3.24b) whilst a significant enhancement in CV signals with respect to their strong binding on membrane compromised cells (as shown in Figure 3.25c). This experiments clearly demonstrated CV binding exclusively on membrane compromised *E. coli* cells. This method is currently tested for *E. coli* which is gram-negative bacteria; however, a detail analysis is separately required to test the polymer action on gram positive bacteria membrane.

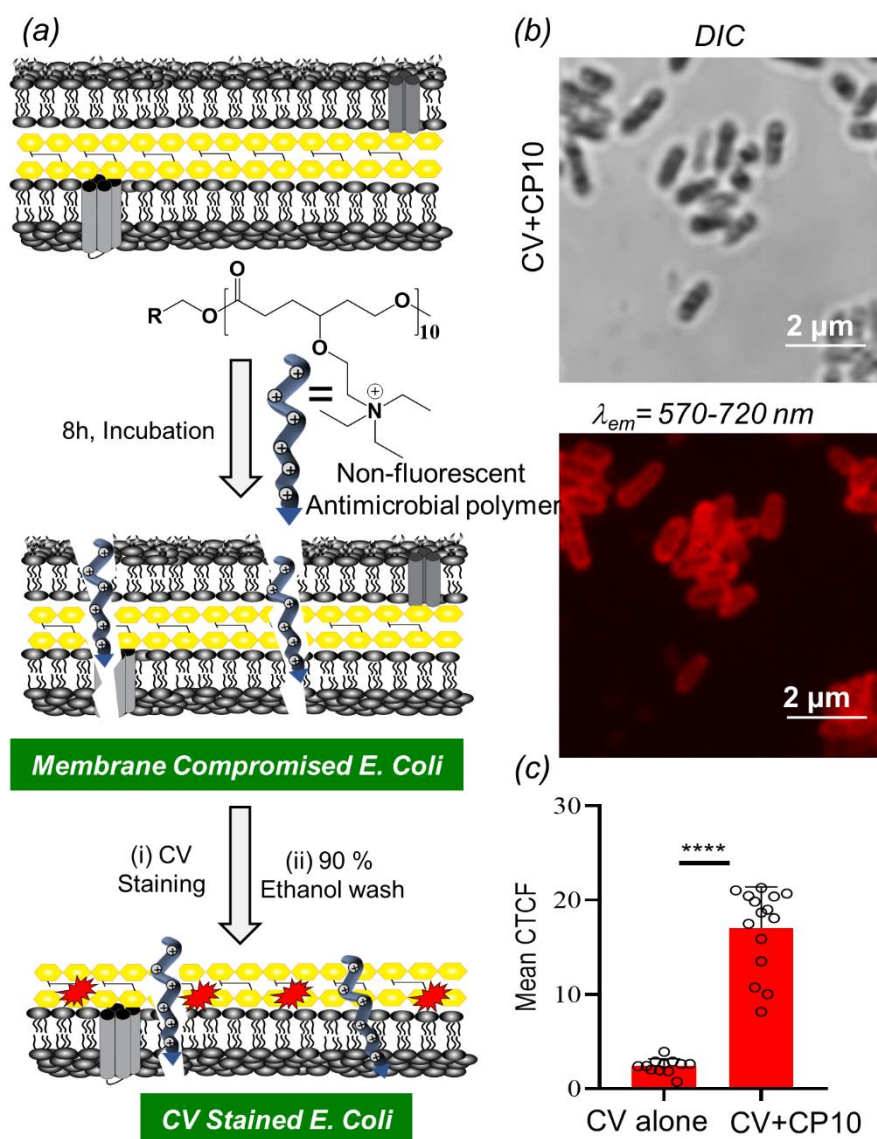


Figure 3.25. (a) Schematic representation of CV staining on *E. coli* membrane after incubation with antibacterial polymer CP-10 (non-luminescent mentioned in Chapter-2) treatment and remove the unbounded CV by 90 % ethanol wash. (b) Confocal microscopy images *E. coli* bacterial cells (1×10^8 CFU/mL) stained with CV after treatment with non-fluorescent polymer. (c) Plot showing mean CTCF values for CV alone and CV+CP10 samples, $\lambda_{exc} = 561 \text{ nm}$, $\lambda_{em} = 570 - 720 \text{ nm}$ (**** $P < 0.0001$). For CTCF measurement, each point is mean $\pm$ SEM ($n = 15$).

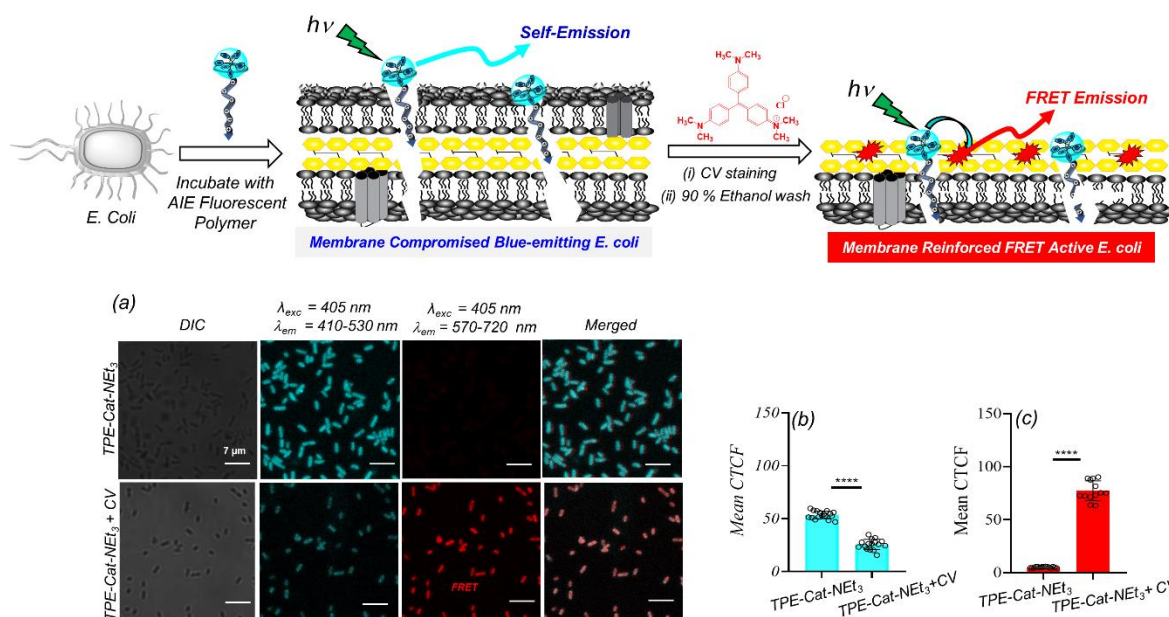


Figure 3.26. (a) Schematic representation of interaction between a fluorescent antibacterial polymer (TPE-Cat-NEt₃) with *E. coli* followed by CV staining. (b) Confocal microscopy images of polymer-stained *E. coli* bacterial cells (1×10^8 CFU/mL) without CV (first panel) and with CV staining (second panel). Plot showing mean CTCF values for TPE emission (c) and CV FRET emission (d) at $\lambda_{exc} = 405\text{ nm}$, $\lambda_{em} = 410 - 530\text{ nm}$ and $\lambda_{em} = 570 - 720\text{ nm}$ (**** $P < 0.0001$). For CTCF measurement, each point is mean $\pm$ SEM ($n = 15$).

The CV image based assay in Figure 3.25 could indirectly account for the antibacterial activity of the non-luminescent polymers; however, it does not provide conclusive evidence for the actual location of polymer on the membrane surface. This emphasized the need for the development of bi-molecular interaction reflecting assay such as FRET-between a fluorescent antibacterial donor polymer (TPE cationic polymer in the present case) and membrane bound acceptor biomarker like CV as an acceptor. The present investigation is emphasized on the development of this new FRET probe-concept. TPE containing antibacterial polymer is exploited as a FRET donor along with CV to understand the polymeric membrane breaching mechanism in *E. coli*. TPE-Cat-NEt₃ polymer treatment for 8 h followed by staining with CV, washed with ethanol, and imaged as shown in Figure 3.26. The cells were excited at TPE utilizing 405 nm laser and the emission signals were collected at two well-separated emission channels: $\lambda_{em} = 410 - 530\text{ nm}$ (TPE self-emission, blue channel) and $\lambda_{em} = 570 - 720\text{ nm}$ (CV FRET emission, red-channel). Upon 405 nm excitation, the self-emission from the TPE was reduced in the blue-channel and simultaneous enhancement in CV FRET emission was observed in the red-channel in Figure 3.26a. It is

classic evidence of the photoexcitation energy transfer from TPE to CV for the occurrence of FRET on the membrane reinforced donor-acceptor arrangements on *E. coli*. The fluorescence intensity of the cells was further quantified by CTCF calculations which clearly showed a qualitative decrease in average magnitude of blue channel emission in the presence of CV acceptor with concomitant increase in red channel emission at $\lambda_{\text{exc}} = 405 \text{ nm}$ (see Figures 3.26b and 3.26c). In order to determine the percentage FRET energy transfer following equation was used: $1 - \{F_{\text{DA}}/F_{\text{D}}\}$, where F_{DA} = fluorescence intensity of the donor in presence of acceptor while, F_{D} = fluorescence intensity of donor alone.²⁴⁹ Upon substituting the CTCF values of F_{DA} and F_{D} from average CTCF values in Figure 3.26b, clearly signifies nearly 50 % energy transfer from TPE to CV on the *E. coli* membrane surface. Hence, the development of FRET based bio-imaging assay directly correlate the involvement of the TPE-polymer on membrane disruption process.

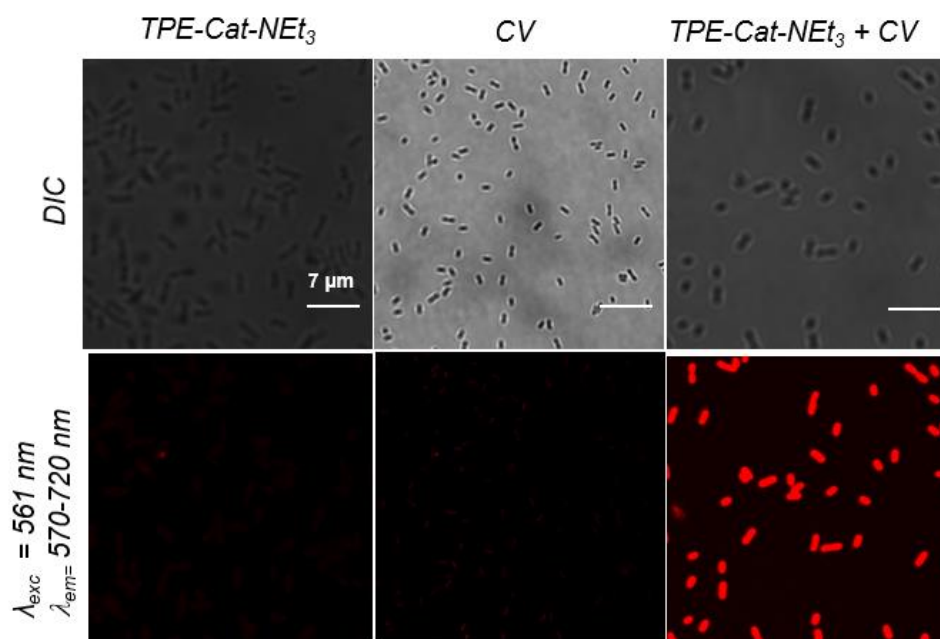


Figure 3.27. Confocal microscopy images of *TPE-Cat-NEt₃* incubated *E. coli* bacterial cells ($1 \times 10^8 \text{ CFU/mL}$) without CV (first panel) and with CV staining (second panel), alone CV (third panel) and CP10+CV (fourth panel). For $\lambda_{\text{exc}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 410 - 530 \text{ nm}$ and $\lambda_{\text{em}} = 570 - 720 \text{ nm}$ were used and for $\lambda_{\text{exc}} = 561 \text{ nm}$, $\lambda_{\text{em}} = 570 - 720 \text{ nm}$.

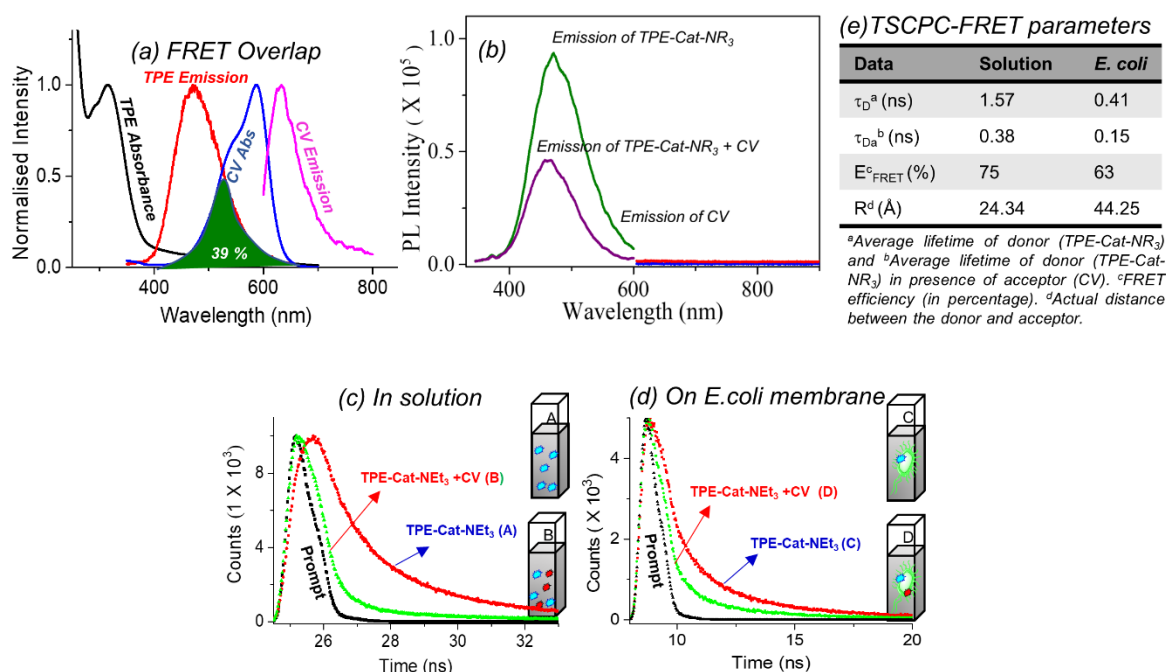


Figure 3.28. (a) Normalized absorbance and emission spectra of TPE-Cat-NEt₃ polymer and Crystal violet in glycerol where the shaded portion (green in color) shows the energy overlap between emission spectrum of TPE and absorbance spectrum of CV along with TCSPC-FRET parameters. (b) Fluorescence spectrum of TPE-Cat-NEt₃ and TPE-Cat-NEt₃ + CV at $\lambda_{exc} = 330$ nm and only CV and TPE-Cat-NEt₃ + CV at $\lambda_{exc} = 580$ nm in PBS. TCSPC decay profiles for (c) TPE-Cat-NEt₃ polymer, and along with CV as acceptor in the absence of *E. coli* (schematic representation of two cuvettes showing TPE fluorophore (A) and TPE fluorophore along with CV (B)). (d) TPE-Cat-NEt₃ polymer, and along with CV as acceptor in the presence of *E. coli* (schematic representation of two cuvettes showing TPE fluorophore (C) and TPE fluorophore along with CV (D)). (e) Table enlisting all the TCSPC FRET parameters.

At 560 nm excitation (with respect to CV self-excitation), the *E. coli* treated with TPE-Cat-NEt₃ did not show any signals from 570-720 nm (as shown in Figure 3.27). *E. coli* without polymer treatment did not show signal since the cells cannot be stained by CV. On the other hand, the *E. coli* treated with polymer and co-stained CV showed strong CV self-emission from 570-720 nm. This further validate that the FRET CV signals and CV self-emission could be distinguished by the selective photoexcitation of TPE and CV at 405 and 560 nm, respectively. To estimate the FRET overlap between TPE donor and CV acceptor, absorbance and emission spectra were plotted together and shown in Figure 3.28a. The emission spectrum of TPE showed about 39 % overlap with the CV absorbance spectrum and confirming their appropriate energy levels for efficient photoexcitation energy transfer.

To measure the energy transfer process from *TPE-Cat-NEt₃* to CV, the emission spectrum was recorded by $\lambda_{\text{exc}} = 330$ nm in PBS and shown in Figure 3.28b. The self-emission of TPE was significantly reduced (~ 50 %) in presence of CV confirming the excitation energy transfer from TPE donor to CV acceptor. However, CV did not show FRET emission at 580 nm in PBS due to the lack of AIE phenomena in CV by free rotation of molecules in PBS. To determine the FRET parameters, time-correlated single photon counting (TCSPC) technique was utilized to study fluorescence lifetime decay profile of *TPE-Cat-NEt₃* in water, *TPE-Cat-NEt₃* + CV in water, *TPE-Cat-NEt₃* incubated with *E. coli* for 4h in water, and *TPE-Cat-NEt₃* incubated with *E. coli* for 4h and stained with CV in water. The fluorescence lifetime of TPE-donor polymer alone (τ_D , sample A) or in presence of CV acceptor (τ_{DA} , sample B) in water is shown in Figure 3.28c. The lifetime of the donor polymer (*TPE-Cat-NEt₃*) was found to be 1.57 ns and it has been observed to reduce to 0.38 ns in presence of crystal violet. The FRET efficiency, $E_{\text{FRET}} = 1 - (\tau_{DA}/\tau_D)$ was estimated to be 75% which is very good for any probe development. TCSPC data for TPE-polymer adhered on the *E. coli* membrane alone (by incubation with *TPE-Cat-NEt₃* for 4 h, sample-C) and subjected to further CV staining (sample D) are shown in Figure 3.28d. The lifetime of *TPE-Cat-NEt₃* polymer in the free state was 1.57 ns which reduced to 0.41 ns upon binding on the *E. coli* membrane. The reduction in the life time values of the probe is direct indication for the TPE-polymer bound on the *E. coli* membrane. In the presence of the acceptor CV molecules, the donor polymer life time further reduced from 0.41 ns to 0.15 ns confirming the donor-acceptor bimolecular interaction on the *E. coli* membrane. The FRET efficiency in the *E. coli* bound state (Figure 3.28e) was estimated as 63 % which is also comparable to that of their free state in solution. It confirms that FRET energy transfer has been observed in both the cases with good FRET efficiency between the two fluorophores.

The set of non-antibacterial and antibacterial polymers shown in Scheme-3.3 were further differentiated based on their ability to form a FRET pair with CV as shown in Figure 3.29. To demonstrate this molecular interaction between the newly synthesized TPE-conjugated polymer and CV, *E. coli* was incubated with all the TPE-based PCL polymers for 4 h and the cells were stained with CV followed by 90 % ethanol to remove surface adsorbed CV prior to confocal imaging as shown in Figure 3.29a. The treatment of antibacterial TPE-polymers make the *E. coli* into blue-fluorescent, and the subsequent CV

staining make them further into a dual emitting FRET substrate having both TPE donor and CV acceptor together on the membrane surface. Non-antibacterial TPE-polymers do not disrupt the membrane; thus, CV cannot be stained, and the resultant *E. coli* membrane could not be used for FRET studies (see Figure 3.29a). Confocal microscope images corresponding to *E. coli* cells followed by treatment with non-antibacterial polymers: *TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NR₂-NH₂*, and *TPE-Cat-NMe-Imz*, and antibacterial polymers: *TPE-Cat-NEt₃* and *TPE-Cat-NH₂* are shown in Figures 3.29b and 3.29c, respectively. The cells were excited at TPE utilizing 405 nm laser and the emission signals were collected at two well-separated emission channels: $\lambda_{em} = 410 - 530$ nm (TPE self-emission, second row) and $\lambda_{em} = 570 - 720$ nm (CV FRET emission, third row). Self-emission of CV was collected by 561 nm excitation and collection at $\lambda_{em} = 570 - 720$ nm (fourth row). The corrected total cell fluorescence (CTCF) from these images are calculated and plotted for various TPE polymers in Figures 3.29d to 3.29f.

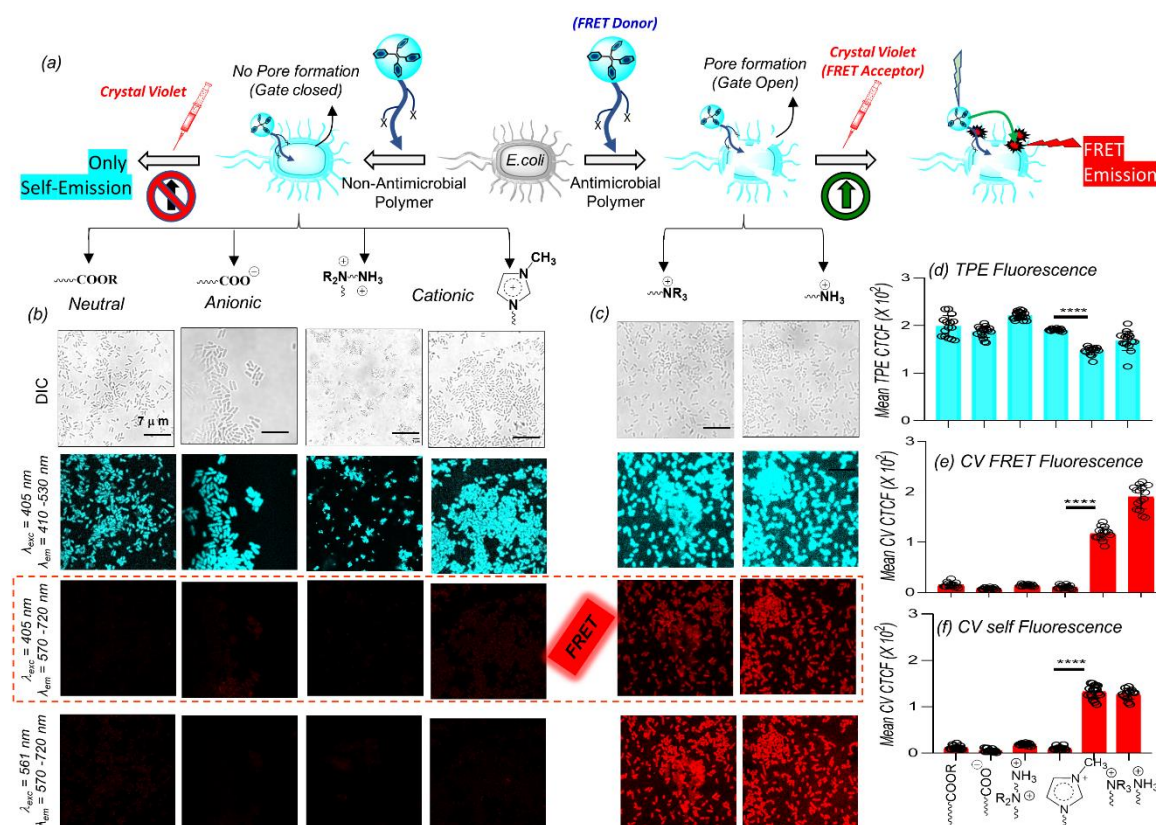


Figure 3.29. (a) Schematic representation of the FRET imaging protocol for *E. coli* bioimaging followed by the treatment of antibacterial and non-antibacterial polymers. (b) Confocal microscopy images of *E. coli* treated with non-antibacterial polymers (*TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NR₂-NH₂* and *TPE-Cat-NMe-Imz*), stained with CV and washed with 90 % ethanol. (c) Confocal

microscopy images of *E. coli* treated with antibacterial polymers (*TPE-Cat-NEt₃* and *TPE-Cat-NH₂*), stained with CV and washed with 90 % ethanol. (d) Comparative Mean CTCF values for TPE self-emission ($\lambda_{exc} = 405 \text{ nm}$, $\lambda_{em} = 410 - 530 \text{ nm}$). (e) Comparative Mean CTCF values for CV FRET emission ($\lambda_{exc} = 405 \text{ nm}$ and $\lambda_{em} = 570 - 720 \text{ nm}$). (f) Comparative Mean CTCF values for CV self-emission ($\lambda_{exc} = 561 \text{ nm}$ and $\lambda_{em} = 570 - 720 \text{ nm}$). (**** $P < 0.0001$). For CTCF measurement, each point is mean $\pm$ SEM ($n = 15$).

In the case of non-antibacterial polymers (*TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NR₂-NH₂*, and *TPE-Cat-NMe-Imz*), TPE self-emission in the blue channel was observed in Figure 3.28b suggesting that the *E. coli* membrane was adhered by the polymers irrespective of their charges-cationic, neutral and anionic. This suggests that both the PCL backbone and TPE chromophores facilitated the membrane adherence. On the other hand, the subsequent CV staining was not facilitated by these polymers due to the gate-closed as shown in Figure 3.3. Therefore, no CV emission was observed in the FRET channel nor in CV self-emission channels. Interestingly, the antibacterial polymers *TPE-Cat-NEt₃* and *TPE-Cat-NH₂* breach the membranes efficiently and gate-opened for the CV staining (see Figure 3.3). In Figure 3.28c, the *E. coli* cells incubated with the antibacterial polymers exhibited bright illumination from the red channel corresponding to CV FRET emission and CV self-emission. This directly evident for *E. coli* membrane compromised by the antibacterial cationic polymers and their function as a gate-keeper for CV staining in the peptidoglycan layer as shown in Figure 3.3. Ethanol wash is a crucial factor to remove the unbound CV in the gram-staining protocol and building neat FRET probes based on TPE-polymers. More description regarding this protocol is discussed in Figures 3.30 and 3.31. The Mean CTCF plots for *E. coli* incubated with six different polymers for three different emission channels (TPE fluorescence, CV FRET fluorescence and CV self fluorescence) are shown in Figures 3.29d, 3.29e and 3.29f. A considerable amount of TPE emission can be observed from various polymer incubated *E. coli* cells suggesting successful binding of TPE-conjugated polymers on the surface of *E. coli* as shown in Figure 3.29d. However, CTCF values for CV FRET and self emissions for antibacterial (*TPE-Cat-NEt₃* and *TPE-Cat-NH₂*) and non-antibacterial polymers (*TPE-Neutral*, *TPE-Anionic*, *TPE-Cat-NR₂-NH₂* and *TPE-Cat-NMe-Imz*) were completely different. In case of non-antibacterial polymers, CV FRET and self emission was observed to be minimalistic as compared to that of antibacterial polymers (Figure 3.29e and 3.29f). This confirms the membrane disruptive action of antibacterial polymers is very crucial factor to stain CV in the peptidoglycan layer and successful

construction of *E. coli* membrane re-inforced FRET probe. These experiments validated the gate-opening for FRET probe function as shown in Figure 3.3. The significance of ethanol wash has been demonstrated in Figures 3.30 and 3.31. *E. coli* was incubated with the polymer sample (*TPE-Neutral* and *TPE-Cat-NEt₃*) (without ethanol wash) stained with CV and directly fixed for confocal microscope imaging. In case of non-antibacterial polymers, as it has been shown in the diagrammatic representation (Figure 3.30a), there was an initial physical adsorption of polymers (*TPE-Neutral*) on the surface of *E. coli* and then simultaneous adsorption of CV on the non-compromised *E. coli*.

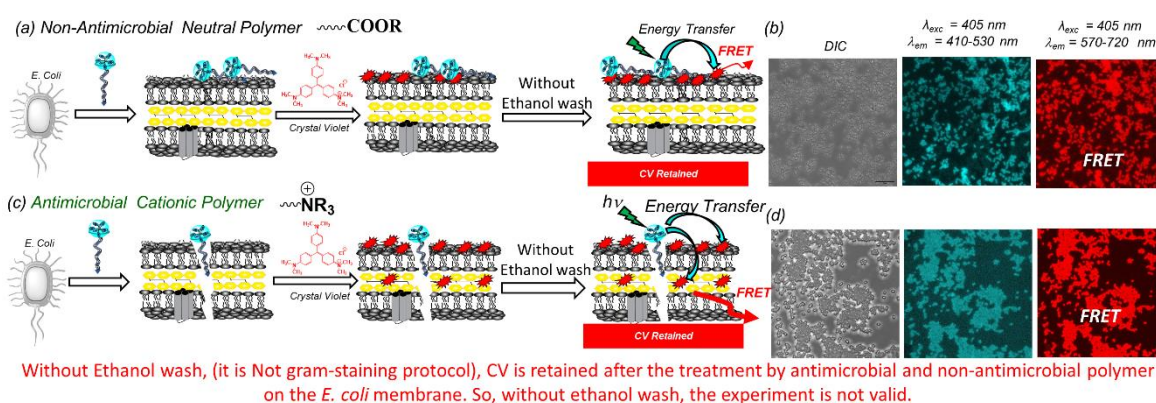


Figure 3.30. (a) Schematic representation of the imaging protocol for non-antibacterial and its (b) confocal microscopy images of polymer incubated bacterial cells (1×10^8 CFU/mL) for 4 h. (c) Schematic representation of the imaging protocol for antibacterial and its (d) confocal microscopy images of polymer incubated bacterial cells (1×10^8 CFU/mL) for 4 h. Laser source: 405 nm excitation, two well-defined emission channels, $\lambda_{em} = 410 - 530$ nm and $\lambda_{em} = 570 - 720$ nm were imaged.

Due to the appropriate distance and dipole-dipole coupling between the two fluorophores, FRET emission could also be observed from *E. coli* surface. Cells were excited at TPE excitation wavelength ($\lambda_{exc} = 330$ nm) and collected at TPE emission channel ($\lambda_{em} = 410 - 530$ nm) and CV FRET channel ($\lambda_{em} = 570 - 720$ nm). Confocal microscopy images show that signals were obtained for both TPE self-emission as well as for CV FRET emission which confirms the presence of both the fluorophores on *E. coli*. (See Figure 3.30b). For antibacterial polymer (*TPE-Cat-NEt₃*), since the membrane is compromised there must be dual retainment of CV at two different locations i. e on the surface of *E. coli* as well as in the peptidoglycan layer as shown in Figure 3.30c. In this case as well, emission from both the fluorophores confirms that both TPE and CV are retained on *E. coli* and devised as a FRET pair (see Figure 3.30d).

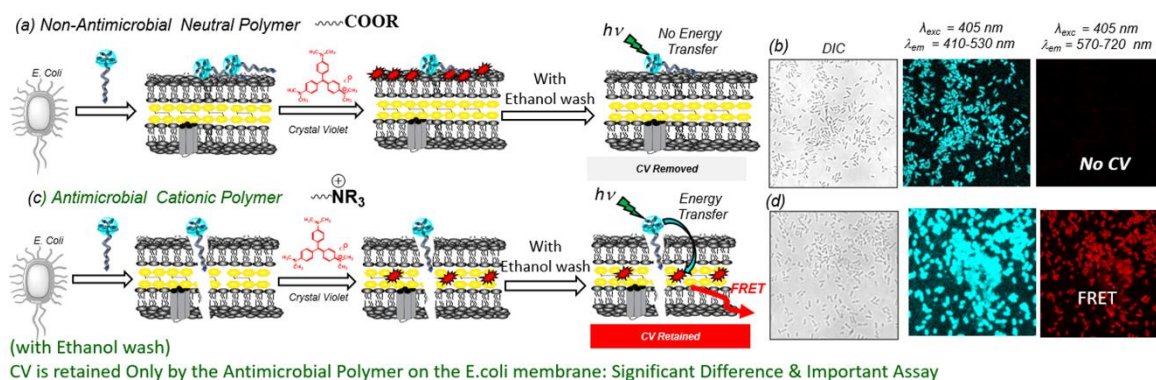


Figure 3.30. (a) Schematic representation of the imaging protocol for non-antibacterial with ethanol-wash and its (b) confocal microscopy images of polymer incubated bacterial cells (1×10^8 CFU/mL) for 4 h. (c) Schematic representation of the imaging protocol for antibacterial with ethanol-wash and its (d) confocal microscopy images of polymer incubated bacterial cells (1×10^8 CFU/mL) for 4 h. Laser source: 405 nm excitation, two well-defined emission channels, $\lambda_{em} = 410 - 530$ nm and $\lambda_{em} = 570 - 720$ nm were imaged.

For non-antibacterial polymer, physically adsorbed crystal violet on the surface of *E. coli* gets washed away along with lipid component upon ethanol treatment (see Figure 3.31a). This has been observed through confocal imaging where TPE emission is retained (due to its membrane submerged state) but CV FRET emission was absent as it was present only on the outer membrane lipid surface (Figure 3.31b). But, for antibacterial polymer CV emission was observed due to the membrane compromised state and successful FRET emission could be observed and simultaneously confirmed by imaging as shown in Figures 3.31c and 3.31d. These observations prove membrane “slit-through” induced FRET pair consisting of TPE-conjugated cationic antibacterial NP and CV is stable on the surface of *E. coli*, suggesting successful bacterial detection through photoinduced energy transfer between TPE and CV. In the present investigation, the TPE antibacterial polymers initially breach the bacterial membranes and gate-opened for CV staining. Therefore, the possibility for photoexcitation energy transfer induced reactive oxygen species (ROS) generation by CV and its subsequent antibacterial activity is insignificant.²⁴² To study ROS effect from CV, control experiments were carried out and the details are shown in Figure 3.32. TPE-Cat-NEt₃ and CV were administrated to 1×10^6 *E. coli* cells in media, immediately irradiated with blue-LED light source (400 nm, Osram 58 W/865 LUMILUX T8) for 1 minute.

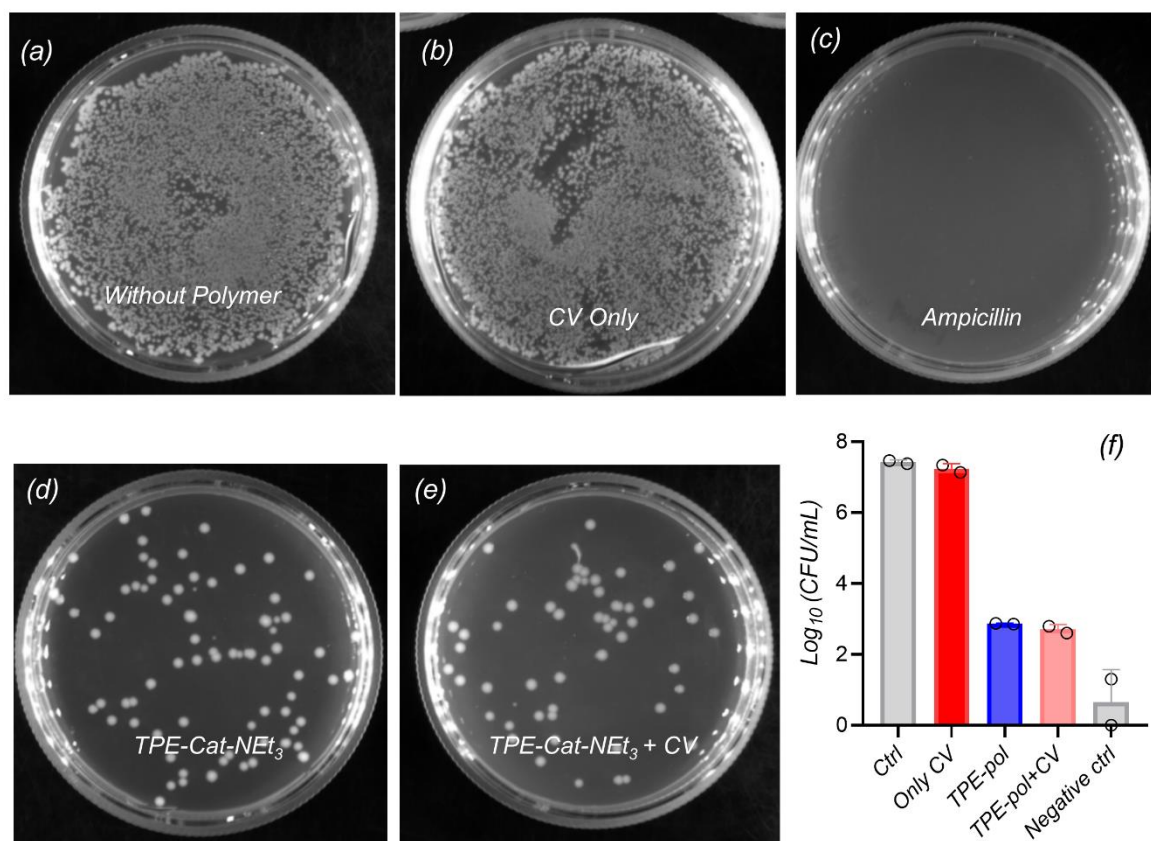


Figure 3.32. Photographs of *E. coli* smeared plates for (a) control (no treatment), (b) CV dye ($100\mu\text{g/mL}$), (c) Ampicillin treated ($16\mu\text{g/mL}$), (d) TPE-CPCL polymer ($10^3\mu\text{g/mL}$) and (e) TPE-CPCL+CV ($10^3\mu\text{g/mL} + 100\mu\text{g/mL}$) after 18 h incubation at 37°C . (f) histogram showing $\log_{10}$ (CFU/mL) for various samples.

E. coli cells were incubated for 18h, then smeared in agar plate and incubated for additional 18-20 h at 37°C . Control experiments were done under identical conditions by treating *E. coli* with TPE-Cat-NEt₃ (alone) CV alone, without polymer treatment (positive control) and treatment with ampicillin (as negative control). As it can be observed in Figure 3.32, a larger number of bacterial colonies were observed in the positive control as well as CV alone treated samples (Figures 3.32a and 3.32b). *E. coli* growth was completely inhibited by Ampicillin (Figure 3.32c). A significant reduction in number of cells was observed in the samples treated with TPE-polymer sample (Figure 3.32d). Comparison of Figures 3.32e and 3.32f suggests that the number of colonies seems to be almost the same for the polymer treated plates irrespective of CV staining. Colony forming unit (CFU/mL) estimated from each of these plates in Figure 3.32f supported the above observation. Based on this evidence, the ROS generation through the photoexcitation process is ruled out. This further supported

the gate-opening action of the TPE-polymer exclusively by the membrane breaching action as shown in Figure 3.3. Time dependent FRET probe action was studied on *E. coli* cells upon incubation with TPE-Cat-NEt₃ polymer at various time intervals from 2 h to 8 h followed by staining with CV. The deconvoluted confocal microscopy images are shown for better resolution in Figure 3.33a (other images are shown in Appendix Figure 8.17). The first row corresponds to 2h of bacterial incubation with polymer where a considerable red emission is observed when the sample is excited at TPE excitation source ($\lambda_{exc} = 405$ nm) which can be corroborated to occurrence of FRET energy transfer between TPE and CV (see Figure 3.33a). Similar trend for FRET energy transfer has been observed for other timepoints as well.

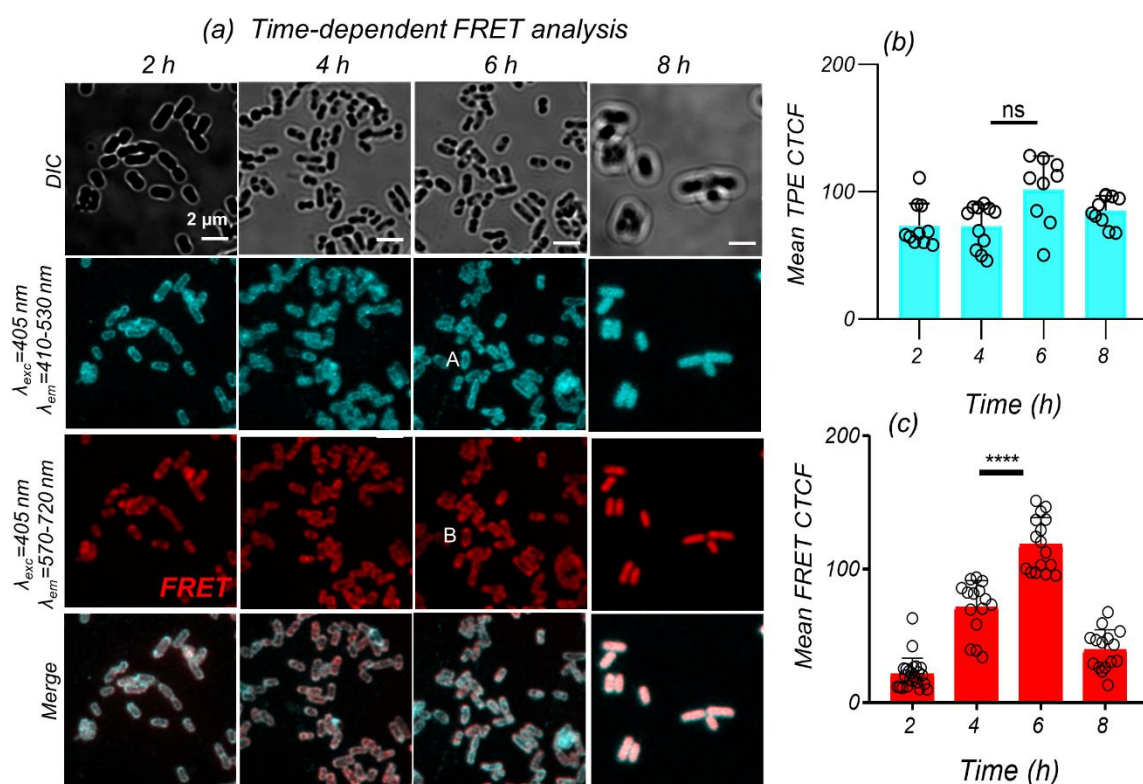


Figure 3.33. (a) Deconvoluted confocal microscopy images of TPE-Cat-NEt₃ incubated and CV-stained *E. coli* at various incubation times. Bar diagram showing mean CTCF value quantified for (b) TPE self-emission at $\lambda_{exc} = 405$ nm (**** $P < 0.0001$) (c) FRET emission for CV by selective photoexcitation of TPE using 405 nm laser (**** $P < 0.0001$). (d) Plot showing mean CTCF values for TPE-Cat-NEt₃ incubated *E. coli* for $\lambda_{exc} = 561$ nm, $\lambda_{em} = 570 - 720$ nm (**** $P < 0.0001$). The quantification was done from the digital images at lower magnification. Concentration of polymer was maintained at 10^3 μ g/mL and number of bacterial cells were 10^8 CFU/mL. Here, values represent mean $\pm$ SEM ($n = 10$).

A residual TPE self-emission (after the energy transfer to CV) in the blue channel was preserved on *E. coli* bacteria at all time points suggesting the stability of *TPE-Cat-NEt₃* polymeric nanoparticles on the surface of *E. coli*. CV self-emission at $\lambda_{\text{exc}} = 561 \text{ nm}$ markedly increases with time due to increase in CV uptake on outer-membrane compromised *E. coli* cells due to time dependent antibacterial action of *TPE-Cat-NEt₃* polymer as shown in Figure 3.33a. The CTCF value of TPE self-emission was observed to be stable and did not alter with increase in time due to successful adherence of *TPE-Cat-NEt₃* polymer on the surface of *E. coli* (see Figure 3.33b). A significant increase in FRET emission was observed from 2 h to 6 h as shown in the CTCF values in Figure 3.33c. As anticipated, self-emission of crystal violet at various time-points was observed to be more than the FRET emission (see Figure 3.33d).

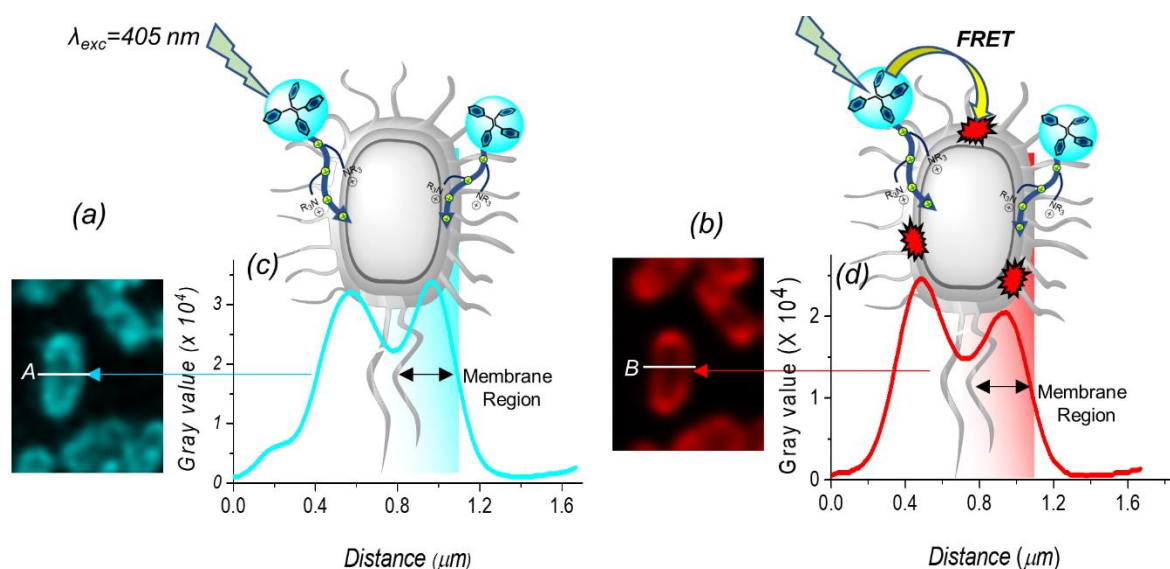


Figure 3.34. (a, b) Zoomed in images of *E. coli* cells at 6 h timepoint where region- A and B signifies the single bacterium across which energy profile curve has been generated for TPE self-emission and CV FRET emission respectively. (c, d) Energy profile curve for TPE self-emission and CV FRET emission for 6 h data along with an illustrative *E. coli* diagram.

A closer examination of the zoomed images (for 6h data) revealed colocalization of TPE self-emission and CV FRET emission from the bacterial membrane. In order to quantify the same, a horizontal line was drawn across a single bacterium (marked as point A) in TPE self-emission image (Figure 3.34a) and CV FRET emission image (Figure 3.34b). A distance dependent fluorescence intensity profile plots for both TPE and CV fluorophores was plotted as shown in Figure 3.34c and 3.34d. The maxima of intensity profile curves for TPE self-

emission and CV FRET emission colocalized at the same position inside the bacterium which clearly accounts for maximum accumulation of TPE and CV fluorophores at the bacterial membrane. For clear demonstration, a diagrammatic representation of *E. coli* is shown where the emission from the membrane region is marked with a double-sided arrow (see Figure 3.34c and 3.34d). Enclosing all the above detailed photophysical studies and imaging quantification, it can be concluded that the newly-designed TPE-tagged cationic polymer nanoparticle fulfilled the necessary objectives for development of FRET probe based on TPE and crystal violet donor-acceptor pair for quantitative estimation of antibacterial activity of the cationic amphiphilic polymer through membrane-disruption mechanism.

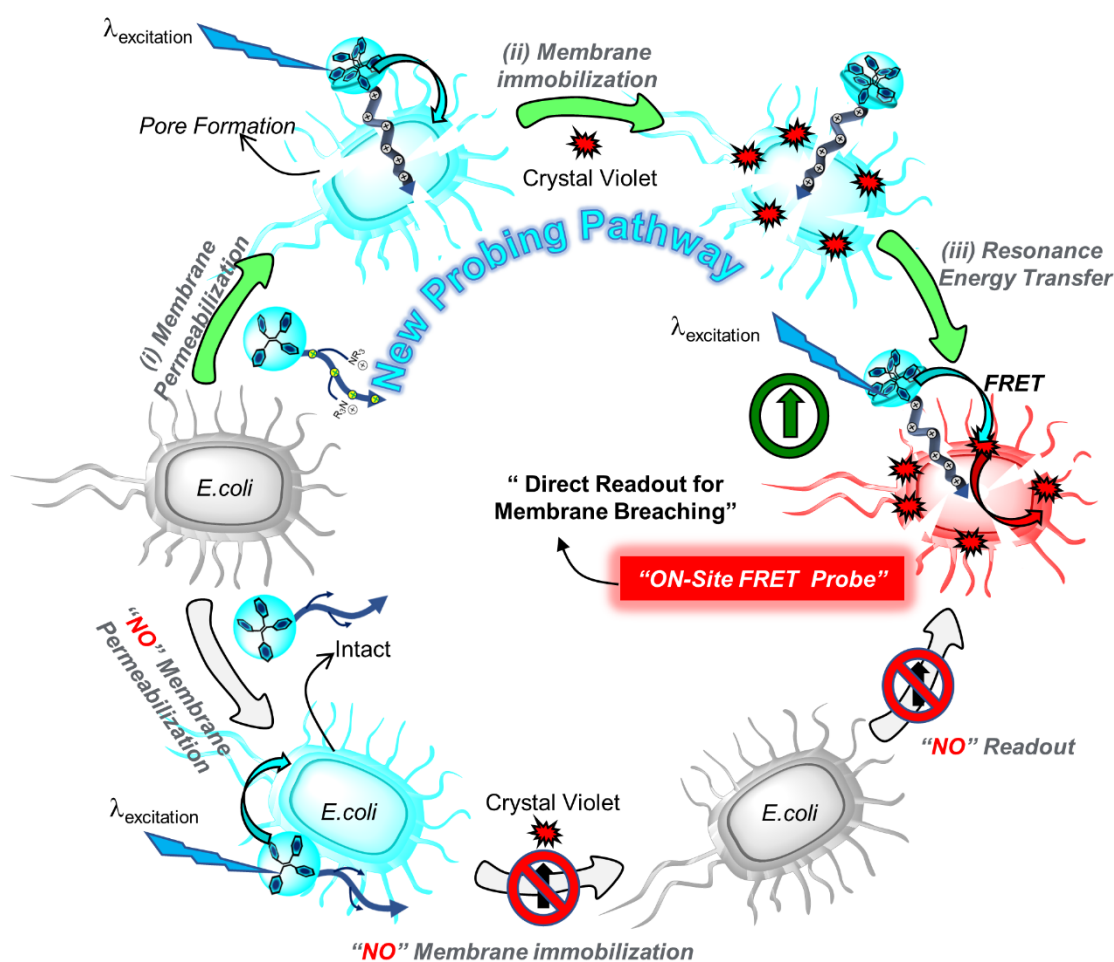


Figure 3.35. Proposed mechanism for FRET probe as a direct readout for membrane breaching.

Based on the above experimental evidence, a mechanism for the membrane-reinforced FRET probe design is proposed in Figure 3.35. It provides a detailed overview of the consecutive steps required for the successful observance of FRET on the surface of *E. coli*. The lower half circle of the plausible mechanism describes the limitation in the development of TPE-CV based FRET probe in case of non-antibacterial polymers due to the inherent impermeable property of CV in viable *E. coli* by gram-staining procedure. A new probing pathway has been developed based on the antibacterial action of the polymer (donor) to induce membrane disruption, which would lead to crystal violet adherence onto *E. coli* membrane and its successful utilization as a FRET acceptor along with TPE-based cationic antibacterial polymers as FRET donor. It is composed of three consecutive steps, wherein the first step involves the utilization TPE cationic NP as a membrane-permeable antibacterial agent for disrupting the *E. coli* membrane and providing necessary pores for the entry of CV. The second step proposes the successful immobilization of CV in the peptidoglycan layer in the membrane-compromised cells. Ultimately, the close vicinity and optimal energy gaps between the two fluorophores in the *E. coli* membrane layer permits efficient energy transfer from TPE to CV as shown in step third, Figure 3.35. This on-site FRET probe has been utilized as a direct readout for membrane breaching mechanism of TPE-conjugated cationic polymer on *E. coli*. This investigation opens new avenues for the development of AIE-FRET theranostic probes for both therapeutics and diagnostics together in a single polymer platform, and the proof-of-concept is demonstrated successfully in gram-negative *E. coli* bacterial membrane.

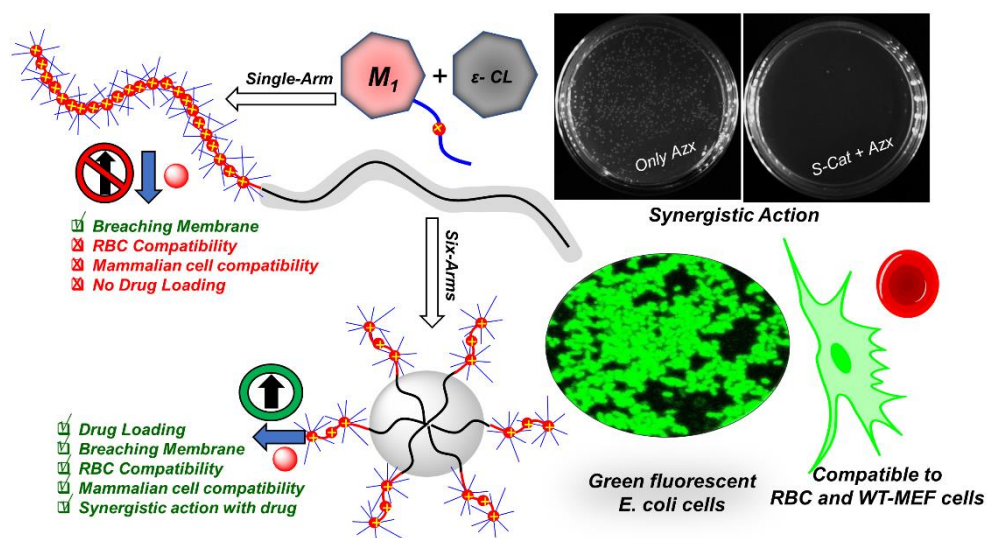
3.4. Conclusion

In summary, a new on-site membrane active FRET probe was developed employing a set of antibacterial and non-antibacterial polymers as an antibacterial switch where FRET energy transfer was feasible only when the polymers were antibacterial in nature. For this purpose, TPE-derived blue-luminescent PCL antibacterial polymers (donor) and peptidoglycan-adherent crystal violet (CV) (acceptor) fluorophores were employed to study the membrane breaching mechanistic aspects. The polymeric synthesis was attained by utilizing TPE derived initiator for solution-phase ring-opening polymerization of chlorine-substituted caprolactone and tert-butyl ester substituted monomers and transmuted to their respective neutral, anionic and cationic polymers. Two cationic polymers *TPE-Cat-NEt₃* and

TPE-Cat-NH₂ exhibited antibacterial activity ($MIC_{90} = 1 \times 10^2 \mu\text{g/mL}$) against *E. coli* by broth microdilution method which was further corroborated by charge neutralization assay by measuring zeta potential and electron microscopic images of polymer treated *E. coli* samples. The *E. coli* membrane functions as a “*FRET gate*” which upon polymeric membrane slit-through action “*Gate-Opened*” for CV to permeabilize at the peptidoglycan layer. It has also been shown that in the case of non-antibacterial polymers, the membrane remained intact i.e., “*Gate-Closed*” for the permeabilization of CV. TCSPC analysis further confirmed energy transfer between the two fluorophores in solution and in presence of *E. coli*. Time-dependent FRET analysis affirmed the stability of TPE-CV FRET probe at various time-points of incubation. The intensity profile curve clearly confirms the nano-confinement of TPE and CV fluorophores in *E. coli* membrane as evident from the colocalization of their respective emission intensities inside a single *E. coli*. The bacterial-membrane reinforced FRET probe development, in principle could be expanded to study other biological pathways for both fundamental understanding as well as making therapeutic formulation in improving the treatment of infectious diseases. Currently efforts are taken in this direction to exploit the full potential of the membrane-reinforced FRET probe strategy in biomedical field.

Chapter 4

Tweaking Polymer Topology for Combination Therapy in Bacterial Research



Abstract

Polymeric topology is one of the important aspects towards development of bactericidal polymers with low therapeutic indices along with antibiotic drug loading capability to accomplish synergistic combination therapy. The role of polymer topology control is investigated in detail by carefully designed 1D rod-shape (linear) and 3D star-shaped diblock (non-linear) core-shell cationic polymers of similar molecular weights, repeating units, size, morphology, and hydrophilic/hydrophobic content. These two different polymeric topologies were explored to understand the effect of chain architecture on infiltrating the biological membranes. Even though, minimum inhibitory action (MIC_{98}) for both the architectures were similar with a MIC_{98} value of 500 $\mu\text{g/mL}$, star architecture was found to be less toxic to WT-MEF cell lines (mammalian cells) and RBCs. Moreover, effective loading of a hydrophilic bioprobe (HPTS) facilitated direct imaging of *E.coli* cells along with PI as a membrane-impermeable dye. This helped us to visualize and quantify the membrane breaching mechanism of star polymer. Flow cytometry analysis further showed enhanced uptake of HPTS loaded star nanoparticle in *E. coli* cells than HPTS alone. Furthermore, encapsulation of a hydrophobic clinical broad-spectrum bacteriostatic macrolide antibiotic (azithromycin) maximized the antibacterial action impact via synergistic combination therapy. There has been an observance of 50 times reduction in MIC concentration of azithromycin when it is encapsulated in the polymeric nano-assemblies. Disk diffusion assay also showed a greater zone of inhibition in case of polymer + drug sample as compared to free drug. The development of such polytherapy treatment builds the foundation for substantial enhancement of drug efficacy along with reduced drug resistance by reducing their systemic dose amount.

4.1 Introduction

The upsurge in antibacterial resistance has become a serious public health concern due to the emerging rise of multi-drug resistant ‘superbugs’ at an alarming rate.^{250,251} Drug combinations are under investigation to fight against these multi-drug resistant bacterial infections but their systematic exploration in bacteria is under process.^{252, 253} Recently, polymeric biomaterials such as hydrogels and nanoparticles assisted delivery of antibiotics is explored for treating bacterial infections.²⁵⁴ These delivery systems can protect the cargo (antibiotic) from degrading enzymes, facilitate simultaneous delivery of more than one drugs and thus improving the efficacy of therapeutic drugs by decreasing their systemic dose amount.^{88,255, 256} Along with being a conveyor of drugs, polymeric macromolecular antibacterial agents exhibit sustained inhibitory effect, broad spectrum activity and scope of action tunability through covalent and non-covalent functionalization.²⁵⁷ Antibacterial platforms have been developed based on essential oils⁵⁵, nitric oxide release²⁵⁸, and potent antibiotics.²⁵⁹⁻²⁶² A synergistic antibacterial action between cationic antibacterial polymer and doxycycline is shown in Figure 4.1.⁸⁸

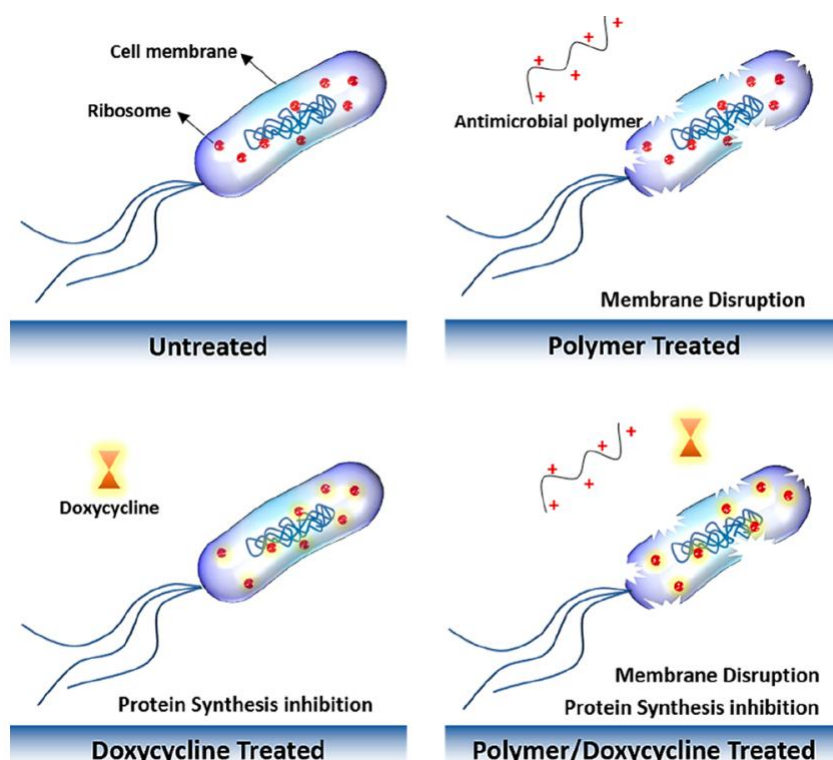


Figure 4.1. Synergistic antibacterial activity of synthetic antibacterial polymer and doxycycline. (adapted from ACS Infect. Dis. 2019, 5, 1357–1365)

The cationic charge on polymers interacts with bacterial negatively charged cell membrane through electrostatic interaction while the hydrophobic component allows the polymer to permeate through the membrane for membrane disruption. A proper hydrophilic/hydrophobic balance is essential for its antibacterial action. In addition, overall molecular weight, hydrophobicity content, and most importantly polymeric architecture/structure greatly affects polymeric antibacterial efficiency and drug loading. It has been observed that non-linear architectures are more effective in mitigating antibacterial resistance than linear architecture due to increased drug's susceptibility as well as non-toxicity (as shown in Figure 4.2).²⁶³⁻²⁶⁵ Polymeric films-based m-PEG(PMMA-ran-PDMAEMA)₂ dendritic copolymers were found to be more antibacterial as compared to linear architecture.²⁶⁶ Different non-linear architectures were also used as a template to load Ag nanoparticle²⁶⁷⁻²⁶⁹, nitric oxide^{55, 270,271}, macrolides such as azithromycin²⁷² and as semipermanent antibacterial coating.²⁷³ Few more examples include studies on polyacrylic acid based organic–inorganic hybrid brush²⁷⁴ and cyclodextrin/cationic polymers^{275,276} etc.

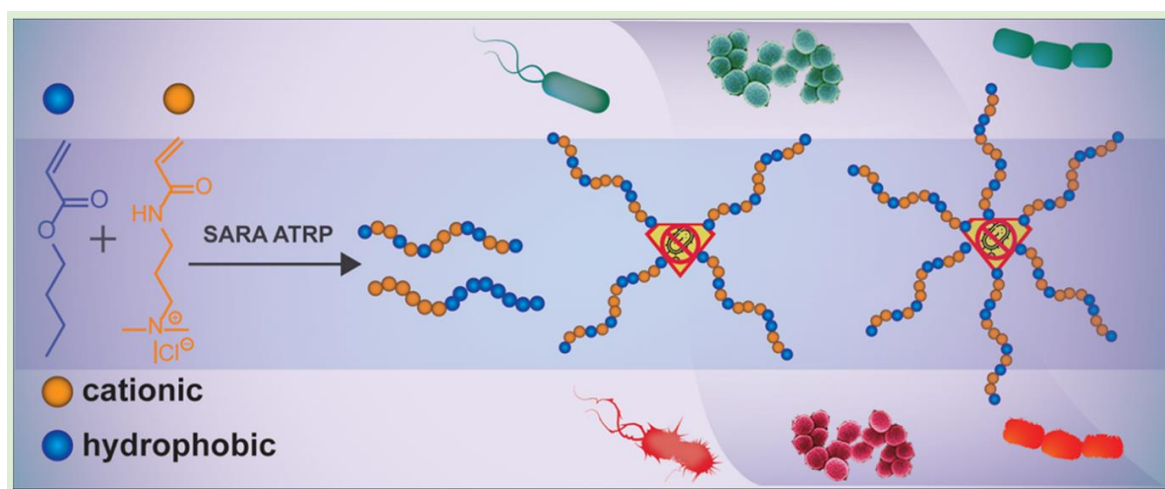


Figure 4.2. Schematic representation of amphoteric cationic copolymers which shows an increase in antibacterial activity in higher molecular weight of non-linear architectures. (Adapted from *Biomacromolecules* **2019**, 20, 1146–1156)

Star polymers represent a broad spectrum of three-dimensional architectures which consists of a central core and polymeric branches radiating out of the central “core.”^{277, 278} Due to their unique topological structures and encapsulation capabilities, they have been widely employed for biomedical applications. Although the three-dimensional globular structure of star polymers seems to be like dendritic architecture but due to presence of linear arms as compared to branched arms of dendrimers, they are less viscous and more

flexible.^{279, 280} Few classifications of star polymer based on their architecture is shown in Figure 4.3.²⁷⁷

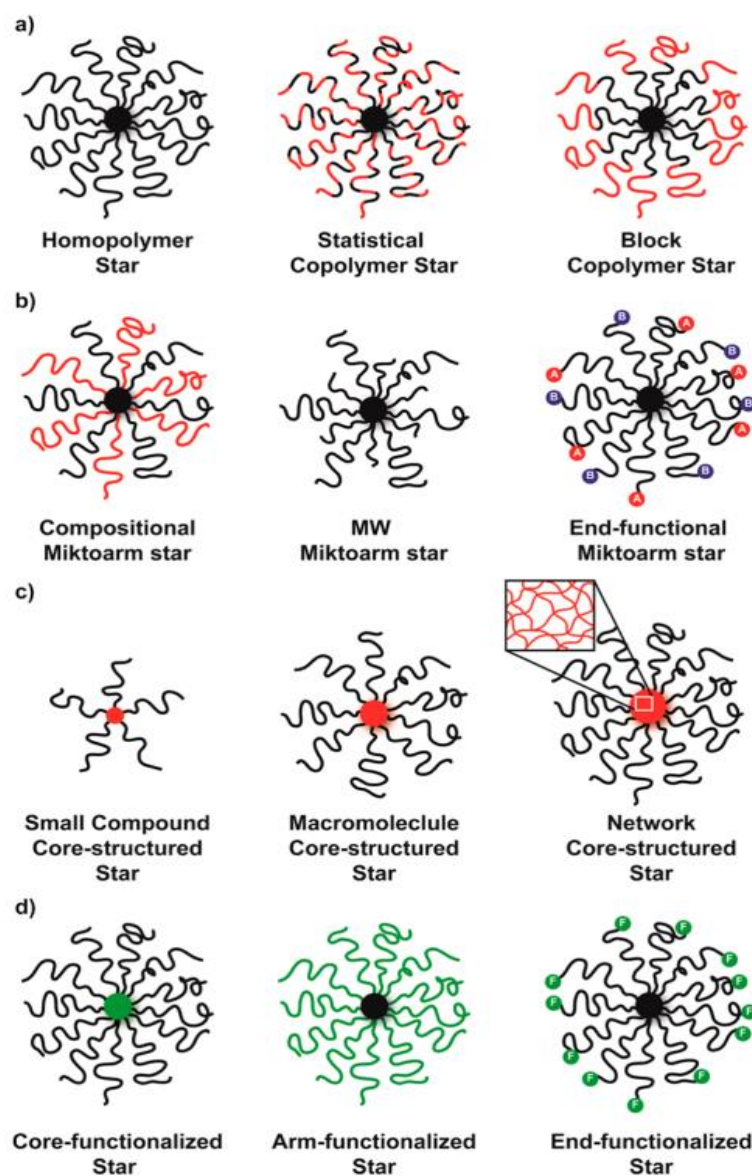


Figure 4.3. Illustration of various types of star polymers classified by (a) composition and sequence distribution of the arm polymer, (b) difference in arm species, (c) core structure, and (d) functional placement. (adapted from Chem. Rev. **2016**, 116, 6743–6836)

They have been greatly explored in the development of antibacterial polymers as well. Star-shaped poly[2-(dimethylamino)ethyl methacrylate]-based zwitterionic copolymers with poly (sulfo-betaine) were observed to be non-toxic to RBCs²⁸¹ while maintaining their antibacterial activity, fluorogenic/magnetic probe conjugated star-shaped

polymers has been employed for bacterial inhibition²⁸². Moreover, different architectures of non-biodegradable polymers comprising hydrophobic poly(butyl acrylate) and cationic poly((3-acrylamidopropyl)trimethyl ammonium chloride) (PAMPTMA) segments are utilized to understand the effect of MW, hydrophobicity content and architecture (linear, 4-arm stars, 6-arm stars) against representative gram-positive and gram-negative bacterial strains²⁸³. Although, these polymeric systems are widely explored, presence of non-biodegradable backbone is a major challenge for *in vivo* applications.^{284,285} Out of the various biodegradable polymers, 3D-architectures of peptide polymer nanoparticles based on copolymers of lysine and valine²⁸⁶⁻²⁸⁸, arginine and glycine²⁸⁹, poly(l-ornithine)^{290, 291}, PEI-g-PLL²⁹², L-lysine²⁹³ and γ -functionalised L-glutamate²⁹⁴ are widely explored for their broad-spectrum antibacterial activity. Their hybrid polymeric architectures based on α -poly-L-lysine and glycopolymer²⁹⁵, PCL-b-AMP⁴⁹, indole grafted poly lysine (PLL-g-indo)²⁹⁶, etc. enhanced bacterial affinity and mammalian cell biocompatibility.²⁹⁴ Non-linear architectures of polycarbonates like mannose functionalised cationic star polycarbonates are also reported which are found to target BCG mycobacteria inside immune cells without causing toxicity to mammalian cells.²⁹⁷ Apart from these two classes of biodegradable polymers, star-shaped polyesters based on poly lactic acid (PLA/ β -CD-based fibres)²⁹⁸, polycaprolactones¹³⁹, PEO-b-PCL etc are only utilized as delivery vehicles to enhance the accumulation of antibiotics like ofloxacin, ciprofloxacin, vancomycin, octenidine hydrochloride etc.^{139,140} In the field of biodegradable macromolecular architectures, influence of polymer topology on antibacterial efficacy, therapeutic indices and combination therapy with other therapeutic agents has been least explored. Recently, from our group Pranav et. al. demonstrated structurally engineered unimolecular star block copolymers as anticancer drug delivery systems.¹⁴⁸ These PCL based star block copolymers were developed with various arm numbers to understand the influence of degree of branching on unimolecular nature of star polymers. The anticancer drug loading content in these unimolecular micelles were found to be 3-4 times higher than aggregated micellar assembly. They were found to be extremely efficient drug loading carriers which can be used for next generation drug delivery agent.

In this work, topological influence on polymeric bactericidal activity has been recognized by exploring two different architectures in order to develop polycaprolactone (PCL) based linear di-block copolymer and star nano-architectures of similar molecular weight and composition of hydrophobic and hydrophilic content in the polymeric chains (As

shown in Figure 4.4). The main objective of this work was (i) to gain structural understanding of cationic linear diblock copolymer and star diblock copolymer, (ii) to probe the influence of different polymer topologies towards antibacterial activity, (iii) to investigate the toxicity in mammalian cells and mammalian RBCs and lastly, (iv) their drug loading capability to deliver clinical antibacterial drug (azithromycin) for synergistic combination therapy etc.. Herein, the hydrophobic block comprised of polycaprolactone units while the hydrophilic block was composed of PCL functionalized n-dodecyl projecting imidazolium units. Their self-assembled nano-assemblies exhibited aggregated nanoparticles in the similar size range along with identical surface charge potential. Although, their topological influence was not observed in their overall macromolecular nano-assemblies (size and surface charge), but it played a major role in their biological action. It was observed that both cationic nano-assemblies were bactericidal towards *E. coli* but the therapeutic selectivity (selectivity towards bacterial cells as compared to RBCs and mammalian cells, denoted as, HC_{50}/MIC_{50} or IC_{50}/MIC_{50} respectively) demonstrated by linear polymer was far lower than star architecture which provides a rational understanding of topology control in antibacterial therapy.

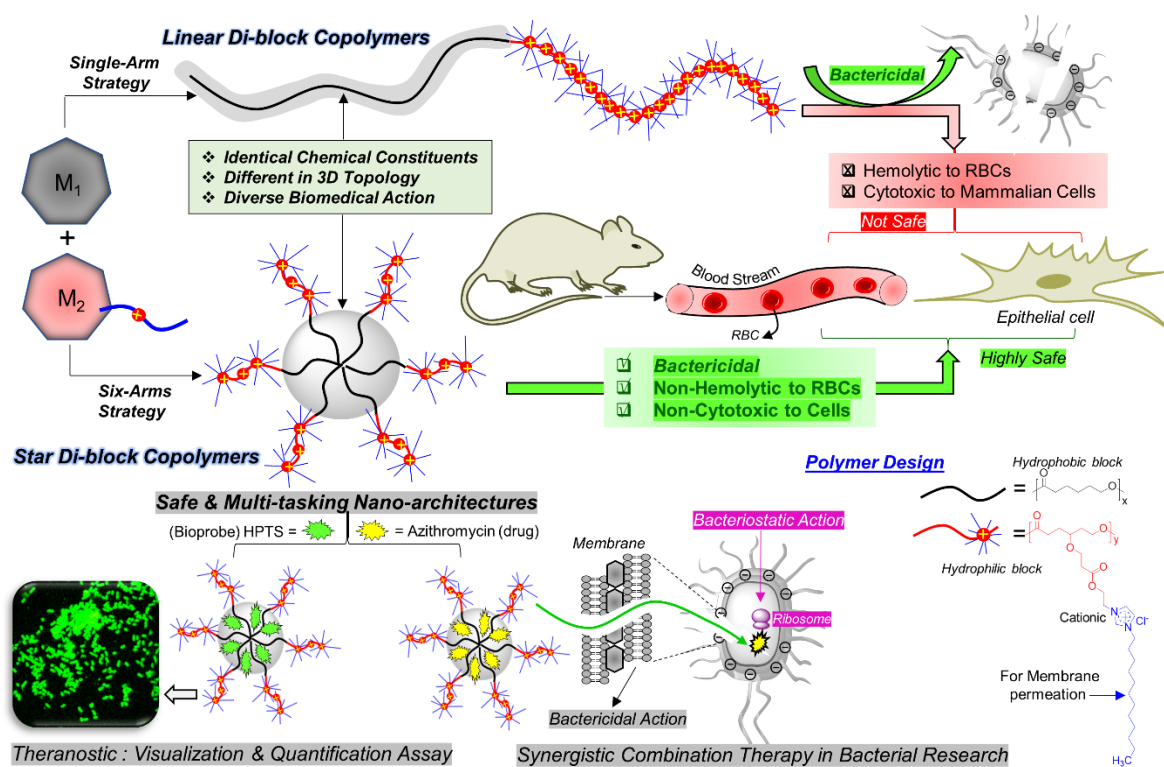


Figure 4.4: Development of linear and star polymeric architecture for antibacterial activity testing along with combination therapy with azithromycin (clinical drug)

Furthermore, star di-block nano-architecture was further utilized for encapsulating (a) hydrophilic dye (HPTS) to substantiate theranostic approach towards visualization and quantification of dead/alive *E. coli*, as well as (b) hydrophobic semi-synthetic macrolide antibiotic (azithromycin) for simultaneous dual action towards bacterial infection via bactericidal membrane breaching as well as bacteriostatic inhibition of protein synthesis (see Figure 4.4). Overall, two polymeric systems with similar chemical compositions were studied based on their topological variation, antibacterial activity, cytotoxicity, hemolytic activity as well as combination therapy along with an encapsulated drug. It has been postulated that such an amalgamation would hinder bacterial resistance development and thus could be a promising candidate for potent antibacterial strategies. Overall, this study yields valuable information related to structure–activity correlation of PCL based cationic antibacterial copolymers.

4.2 Experimental Section

4.2.1 Materials and Methods: Imidazole, 1-bromo dodecane diol were purchased from Aldrich chemicals and used without further purification. Analysis was carried out by Bruker 400 MHz spectrophotometer, Gel Permeation Chromatography, HRMS-ESI-Q-TOF LC-MS, MALDI TOF/TOF Analyzer, Perkin-Elmer thermal analyzer STA 6000 model, UV-Visible Spectrophotometer, SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer, DLS instrument, Agilent ZnSe ATR system. Syngene G:Box Chemi XX6 was utilized for high resolution images for disk diffusion assay. FE-SEM analysis require sample drop cast on silicon wafer utilizing Zeiss Ultra Plus Field Emission Scanning Electron Microscope and Agilent instruments respectively. Flow cytometry was performed using a *BDFACS Celesta* cell analyzer. The substituted caprolactone monomer 2-chloroethyl 3-((7-oxooxepan-4-yl) oxy) propanoate (monomer M1) was reported in Chapter 2. **Note:** Animal ethics committee approval number for this project is IISER-Pune/IAEC/2021-01/10/A1.

4.2.2 Synthesis of Cationic unit 1-dodecyl-1H-imidazole (N-alkyl Imidazole)(1): Imidazole (3.00g, 0.04 mol) and powdered potassium hydroxide (4.50 g, 0.08 mol) in DMSO (15 mL) were stirred at 25 °C for 30 minutes. The reaction mixture was immersed in water bath at 25 °C and 1-bromo dodecane (10.00g, 0.04 mol) was added dropwise. The reaction was continued at 25 °C for 12 h, distilled water (200.00 mL) was added to quench the reaction and product was extracted in diethyl ether (40.00 mL) twice. Solvent was evaporated and

crystalline solid as a product. Yield = 9.00 g (94%). ^1H NMR (400 MHz, CDCl_3) δ ppm: 7.42 (s, 1H), 7.01 (s, 1H), 6.99 (s, 1H), 3.88 (t, 2H), 1.73 (m, 2H), 1.30 (m, 18H), 0.85 (t, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 137.34, 129.60, 119.06, 77.53, 47.35, 32.19, 31.37, 29.88, 29.81, 29.72, 29.62, 29.36, 26.84, 22.97 and 14.40. FT-IR (cm^{-1}): 3362, 2922, 2853, 1650, 1509, 1465, 1377, 1283, 1229, 1109, 1078, 1032, 907, 809, 727 and 664. HR-MS (ESI+): m/z [$M + H^+$] for $\text{C}_{38}\text{H}_{44}\text{O}_2$ Calculated = 237.23 and found = 237.23.

4.2.3 Synthesis of Macro-Initiator: Linear PCL Macroinitiator (PCL Macroinitiator): In a schlenck tube, triethylene glycol monomethyl ether (24.00 mg, 0.14 mmol) was taken in dry toluene (1.00 mL) under nitrogen atmosphere. Under nitrogen purge at 25 °C, $\text{Sn}(\text{Oct})_2$ (29.50 mg, 0.07 mmol) was added to the reaction mixture and stirring was continued for 15 min. ϵ -Caprolactone (CL) (1.00 g, 8.70 mmol, for $M/I = 60$) was dissolved in 1.00 mL dry toluene and added to the reaction mixture under nitrogen atmosphere, and the polymerization was continued at 110 °C for 48 h. The polymer was precipitated in methanol. Yield = 0.90 g (87 %). ^1H NMR (400 MHz, CDCl_3) δ ppm: 4.04 (t, 2H), 3.64 (m, 12H), 3.36 (s, 3H), 2.29 (t, 2H), 1.63 (m, 4H), 1.36 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 173.59, 64.17, 62.63, 34.13, 32.32, 28.36, 25.54, 24.59. FT-IR (cm^{-1}): 2944.24, 2865.81, 1723.03, 1470.85, 1418.70, 1397.26, 1366.55, 1294.54, 1242.33, 1189.28, 1107.71, 1046.54, 961.79, 933.87, 840.67 and 732.05. GPC molecular weights: $M_n = 8700$ g/mol, $M_w = 12900$ g/mol, and $M_w/M_n = 1.4$.

4.2.4. Synthesis of Linear PCL-block-chlorine PCL polymer (Linear PCL-block-ClPCL): PCL macroinitiator (470.00 mg, 0.08 mmol) was added in a schlenck tube followed by addition of 1.00 mL dry toluene and it was nitrogen purged for 15 min at 25 °C. $\text{Sn}(\text{Oct})_2$ (16.20 mg, 0.04 mmol) was added and nitrogen purged for an additional 15 min at 25 °C. Monomer 4 (1.00 g, 4.00 mmol, for $M/I = 50$) was dissolved in 1 mL Dry toluene and the solution was added to reaction mixture and polymerization was kept at 110 °C for 48 h under sealed condition. The polymer was then precipitated in 50 mL diethyl ether. Yield = 1.40 g (77 %). ^1H NMR (400 MHz, CDCl_3) δ ppm: 4.35 (m, 2H), 4.13 (m, 2H), 4.05 (t, 2H), 3.69 (m, 16H), 3.46 (m, 1H), 3.38 (s, 3H), 2.58 (t, 2H), 2.36 (t, 2H), 2.30 (t, 2H), 1.88-1.78 (m, 4H), 1.64 (m, 4H), 1.38 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 173.43, 75.47, 64.22, 63.98, 41.37, 34.93, 33.93, 29.49, 28.63, 28.14, 25.32, 24.37, 13.73 and 11.56. FT-IR (cm^{-1}): 2944.17, 2867.92, 1721.92, 1458.24, 1418.83, 1365.64, 1295.12, 1244.14, 1172.02,

1104.51, 1064.00, 1048.03, 962.64, 732.19 and 667.26. GPC molecular weights: $M_n = 3900$ g/mol, $M_w = 8500$ g/mol, and $M_w/M_n = 2.1$.

4.2.5 Synthesis of Block copolymer: Linear PCL-block-Imz substituted cationic PCL polymer (L-Cat): Polymer L-PCL₆₀-b-ClPCL₅₀ (200.00 mg, 0.70 mmol) was added in round bottom cylindrical reaction vessel followed by addition of **1** (1.700 g, 7.50 mmol) along with 1.00 mL Dry DMF (1 mL). The reaction mixture was kept at 85 °C for 48 h. The polymer was precipitated in 50.00 mL diethyl ether. Yield = 190.00 mg (54.20 %). ¹H -NMR (400 MHz, D₂O) δ ppm: 9.05-7.35 (m, 3H), 4.60-4.03 (m, 8H), 3.70 (m, 4H), 3.50 (m, 1H), 2.61 (m, 2H), 2.28 (m, 4H), 1.85 (m, 6H), 1.61 (m, 4H), 1.24 (bs, 20H), 0.84 (t, 3H). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 173.39, 77.05, 63.97, 33.94, 31.71, 30.72, 29.40, 29.15, 28.85, 28.16, 26.30, 25.35, 24.39, 22.49 and 13.94. FT-IR (cm⁻¹): 3414.57, 2925.37, 2855.70, 2310.79, 1725.91, 1565.13, 1513.01, 1467.92, 1418.69, 1367.52, 1295.52, 1244.91, 1172.28, 1106.05, 963.40, 841.27, 732.19 and 667.92.

4.2.6 Synthesis of Star PCL Macroinitiator (Star-PCL Macroinitiator): A custom-made melt reactor set up was utilized with a build-in overhead stirrer for ring-opening polymerization of ϵ -caprolactone utilizing dipentaerythritol as an initiator. Dipentaerythritol initiator (37.12 mg, 0.14 mmol) was added in schlenck tube along with ϵ -caprolactone (CL) (1.00 g, 8.70 mmol, for M/I = 60) as monomer (statistically 10 units in each arm). Sn (Oct)₂ (177.45 mg, 0.43 mmol) was added to the same and reaction was sealed under vacuum. The polymerization was continued for 6h at 110 °C. The polymer was precipitated in 50.00 mL methanol. Yield = 1.00 g (96.43 %). ¹H -NMR (400 MHz, CDCl₃) δ ppm: 4.05 (bs, 2H), 3.64 (bs, 2H), 3.39 (bs, 4H), 2.30 (bs, 2H), 1.64 (bs, 4H), 1.38 (bs, 2H). ¹³C NMR (100 MHz, CDCl₃) δ ppm: 173.39, 77.05, 63.98, 62.43, 33.94, 32.13, 28.16, 25.34, 24.39. FT-IR (cm⁻¹): 2943.79, 2865.33, 1720.72, 1470.67, 1418.32, 1396.84, 1365.55, 1293.62, 1239.31, 1165.79, 1106.42, 1064.92, 1044.22, 960.88, 933.68, 840.65, 803.33, 731.60 and 710.18. GPC molecular weights: $M_n = 5500$ g/mol, $M_w = 7000$ g/mol, and $M_w/M_n = 1.27$.

4.2.7 Star PCL-block-chlorine PCL polymer (Star PCL-block-ClPCL): In a Schlenk tube, macroinitiator S-PCL₆₀ (473.00 mg, 0.06 mmol) was dissolved in dry toluene (1 mL) and nitrogen purged for 15 min at 25 °C. Sn (Oct)₂ (81.00 mg, 0.20 mmol) was added to the reaction mixture while maintaining the nitrogen atmosphere and nitrogen purge was continued for another 15 min at 25 °C. Monomer **M1** (1.00 g, 4.00 mmol, for M/I = 60) was dissolved in 1.00 mL dry toluene and to the reaction mixture at 25 °C. Polymerization was

kept at 110 °C for 48 h under sealed conditions. The polymer was precipitated in diethyl ether (50.00 mL). For purification, it was redissolved in THF and re-precipitated in diethyl ether. Yield = 1.00 g (72 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 4.35 (t, 2H), 4.13 (t, 2H), 4.05 (t, 2H), 3.69 (m, 4H), 3.47 (m, 1H), 2.59 (m, 2H), 2.37 (m, 2H), 2.30 (t, 2H), 1.86-1.78 (m, 4H), 1.65 (m, 4H), 1.38 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 173.44, 77.04, 63.98, 33.94, 29.50, 28.48, 28.15, 25.34 and 24.39. FT-IR (cm^{-1}): 2945.28, 2868.04, 1729.20, 1553.42, 1455.65, 1418.41, 1359.31, 1165.54, 1103.01, 1064.16, 969.59, 805.01, 739.95 and 667.92. GPC molecular weights: M_n = 6800 g/mol, M_w = 8600 g/mol, and M_w/M_n = 1.26.

4.2.8 Synthesis of Star PCL-block-Imz PCL cationic polymer (S-Cat): S-PCL₆₀-b-ClPCL₆₀ (100.00 mg, 0.30 mmol) was taken along with 1 (446.00 mg, 1.80 mmol) in a round bottom cylindrical reaction vessel. 1.00 mL Dry DMF was added to the reaction mixture and reaction was placed at 85 °C for 36 h. Polymer was precipitated in 50.00 mL diethyl ether. Yield = 159.00 mg (90 %). ^1H -NMR (400 MHz, CDCl_3) δ ppm: 7.61 (bs, 1H), 7.10-6.96 (m, 2H), 4.35-4.14 (m, 4H), 4.05 (t, 2H), 3.93 (m, 2H), 3.68 (m, 4H), 3.44 (m, 1H), 2.54 (m, 2H), 2.30 (m, 4H), 1.89 (m, 2H), 1.78 (m, 4H), 1.63 (m, 4H), 1.44 (m, 2H), 1.24 (bs, 18H), 0.87 (t, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ ppm: 173.71, 77.36, 64.29, 34.25, 32.02, 29.73, 29.46, 29.14, 28.47, 25.65, 24.70, 22.80 and 14.25. FT-IR (cm^{-1}): 3392.22, 2926.37, 2856.38, 2311.08, 1731.67, 1565.34, 1456.37, 1418.59, 1359.91, 1168.05, 1104.29, 1066.03, 971.11, 904.71 and 732.84.

4.2.9 Determination of Critical Micellar Concentration (CMC): Pyrene was utilized as a fluorescent probe to determine critical micellar concentration for linear and star architecture. Pyrene solution was prepared by dissolving 3.64 mg of pyrene in 10 mL of HPLC acetone. The solution was diluted 20 times (serially diluted twice by taking 1 mL of stock solution and adding 9 mL acetone) to prepare 3.64×10^{-6} g/mL concentration. 100 μL of resultant solution were taken in glass vials and acetone was evaporated to get final amount of 0.36×10^{-6} g which corresponds to 0.6 μM in 3mL solution. Different polymeric concentrations ranging from 0.5 mg/mL to 1 ng/mL were prepared. The resultant solution was sonicated for 4 h to induce encapsulation of pyrene into the nano-assemblies, then the solutions were equilibrated at 25 °C for 12 h. This was done to settle down unencapsulated pyrene at the bottom of the vials. Pyrene was excited at 334 nm and spectrum was collected from 350 nm to 500 nm with excitation and emission slits of 2 nm. The ratio of fluorescence emission

intensities corresponding to first ($\lambda = 372$ nm) and third vibrational peaks ($\lambda = 382$ nm) (I_I/I_{III}) was sensitive to probe's microenvironment and its ratiometric change upon changing the polymer/surfactant concentration led to the determination of CMC.

4.2.10 DLS size and Zeta Potential determination: 1 mg polymer was weighed in an eppendorf and directly dissolved in 1 mL milli pore water. 100 μ L of the polymeric solution was taken and 900 μ L of millipore water was added to make resultant concentration of 0.1 mg/mL. Hydrodynamic diameter was determined using DLS instrument along with zeta potential.

4.2.11 FE-SEM sample preparation for Polymers: 0.5 mg/mL solution of Linear and star polymer were prepared along with HPTS loaded polymeric samples. 5 μ L drop of all the four polymeric samples were added in silicon wafers and they were kept at room temperature overnight. After that, the samples were dried in vacuum oven at 37 °C for 48 h. Gold-coating was done and samples were analyzed using Zeiss Ultra Plus Field Emission Scanning Electron Microscope for FE-SEM imaging.

4.2.12 Hemolysis Assay: Hemolysis assay was determined by procuring blood from C57BL/6 mice. Blood was collected in 5 % EDTA coated 15 mL falcon tube and 5 % v/v RBC solution was prepared. 100 μ L of RBC solution was incubated with 100 μ L of linear and Star polymers at MIC concentration and incubated at 37 °C for 1 h along with PBS and 1 % Triton X-100 as negative and positive controls. Eventually, samples were centrifuged at 8000 rpm for 5min to obtain an undisturbed RBC pellet at the bottom of the eppendorfs in case of healthy erythrocytes. 100 μ L of supernatant were taken in 96 well plate and absorbance were measures at $\lambda_{\text{abs}} = 570$ nm and hemolysis were determined following earlier reports²⁴⁴.

4.2.13 Hydrophilic dye (HPTS) loading: 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) was encapsulated in the above-mentioned polymers using the following procedure. 5 mg polymer + 0.5 mg dye were dissolved in 1 mL HPLC grade DMSO. The polymers were completely solubilized in DMSO followed by dropwise addition of the polymeric solution in 14 mL milli-Q water. The resultant solution was stirred at 25 °C for 4 h followed by dialysis using 3.5kDa dialysis bag for 48 h. Water was replenished frequently to ensure complete removal of DMSO and unencapsulated dye. Drug loading content (DLC) and Drug loading efficiency (DLE) was determined as shown in Chapter-2.

4.2.14 Hydrophobic drug (Azithromycin) loading: Drug was encapsulated using double emulsion solvent evaporation technique.²⁹⁹ In the following technique, 2 mg of polymer was dissolved in 40 μL of THF. Similarly, 40 μL solution of azithromycin from 10mg/mL stock (in THF) was added to the same. The following polymer and drug solution was added to 1.5 mL water in a glass vial dropwise under vigorous stirring. The solution was kept under stirring for half an hour followed by evaporation of THF by placing it under heating stirrer at 50 $^{\circ}\text{C}$. Complete evaporation of volatile solvent took around 30 min followed by centrifugation at 14000 rpm for 5 min to separate unloaded insoluble drug molecules. The solution was decanted and absorbance of unloaded azithromycin was determined in 1:1 acetonitrile-water solvent composition by UV/vis spectrophotometry at 210 nm and referred to a standard calibration plot (appendix Figure 8.19). The amount of total drug loading was determined by referring to subtracting optical density value (O.D. at 210 nm) for unloaded drug molecules to total drug taken. DLC and DLE was determined similarly to that of dye loaded nanoparticles.

4.2.15 Transmission Electron Microscopy (TEM) imaging of *E. coli* samples: A single colony of *E. coli* was picked and it was allowed to grow for 8 h. Bacterial suspension was diluted to 1×10^5 CFU/mL which was then incubated with 250 $\mu\text{g/mL}$ (MIC) concentration of polymers (star and linear architectures) for 4 h. LB was removed by centrifugation at 8000 rpm for 2min and resultant samples were washed with an ascending order of ethanol gradients from 10% to 90% ethanol in water. Finally, they were stored at 4 $^{\circ}\text{C}$ in 2.5 % glutaraldehyde overnight. The samples were washed with water and dropcasted in TEM grids and dried at room temperature for 12 h followed by 37 $^{\circ}\text{C}$ for another 12 h.

4.2.16 Colony Suspension Method for Bacterial Inhibition Assay: A bacterial inoculum was prepared by suspending few well-isolated *E. coli* colonies in LB broth resulting in final turbidity of the suspension equivalent to 0.5 McFarland standard. The bacterial suspension was diluted to $\text{O.D}_{600 \text{ nm}} = 0.005$ which corresponds to 1×10^6 CFU/mL. 100 μL of the bacterial suspension was added into 96 well plate. 100 μL of serially diluted polymeric stock solutions (500 $\mu\text{g/mL}$ to 0.1 $\mu\text{g/mL}$) were also added to the *E. coli* cell suspension along with positive control (without polymer) and negative control (with Azithromycin, 50 $\mu\text{g/mL}$). Bacterial turbidity was measured at $\lambda_{\text{exc}} = 600 \text{ nm}$ for 18 h using Perkin Elmer Enspire Multimode Plate Reader.

4.2.17 Determination of Zeta Potential of polymer treated *E. coli* cells: PBS is prepared by dissolving NaCl (1.33 g, 45.7 mM), KCl (33.5 mg, 0.9 mM), NaH₂PO₄ (234 mg, 3.3 mM) and KH₂PO₄ (41 mg, 0.6 mM) in 500 mL distilled water and sonicated for 30 min. The resultant PBS was autoclaved and used for the experiment. One *E. coli* colony was picked up and inoculated in LB for 12 h. Cells were washed with PBS and 0.05 O.D. at 600 nm was maintained. 500 μ L of 0.05 O.D. of bacterial culture was incubated with 500 μ L of polymeric solution (250 μ g/mL) in PBS for 3 h. Zeta potential of all the polymer incubated *E. coli* samples were measured along with nascent *E. coli* and polymeric solutions using Nano ZS-90 Zetasizer.

4.2.18 Confocal Imaging for *E. coli* incubated with HPTS loaded *S-PCL*₆₀-*b-CatImzPCL*₆₀ polymer: 100 μ L of HPTS loaded star polymer (1 mg/mL) was incubated along with 100 μ L of *E. coli* suspension (1 X 10⁸ CFU/mL) for 8 h. Cells were given PBS washes (X 2) followed by PI (10 μ L, 56 μ g/mL in PBS) staining and incubating at dark for 15 min. After that, cells were washed to remove un-encapsulated PI and heat-fixed in glass-slide. Imaging was done under oil immersion objective (100 X) by using the Leica SP8 optical microscope employing $\lambda_{405\text{ nm}}$ (blue channel) and $\lambda_{561\text{ nm}}$ (red channel) lasers.

4.2.19 Flow cytometry: A single colony of *E. coli* was picked and inoculated in LB for 6 h. The cell suspension was then diluted to 0.5 O.D. HPTS loaded *S-PCL*₆₀-*b-CatImzPCL*₆₀ (100 μ L, 1 mg/mL) polymer and Free HPTS (100 μ L, 0.1 μ g/mL) were incubated with *E. coli* suspension (100 μ L, 0.5 O.D.) for 2 h followed by addition to 300 μ L 70 % ethanol (to fix the cells). Cell suspension was then kept at 4 °C. Solution was centrifuged and re-dissolved in 600 μ L PBS. Fluorescence reading in cells were analyzed with *BDFACS Celesta* cell analyzer utilizing a 405 nm laser for HPTS excitation and emission was collected using BV 510-A filter.

4.2.20 Disk-Diffusion Assay: LB agar was poured on 100 mm X 20 mm polystyrene petri dish and allowed to cool. Simultaneously, a single *E. coli* colony was picked up and incubated at 37 °C for 6 h. *E. coli* cell suspension was diluted to 0.1 O.D. at 600 nm and 100 μ L of the final inoculum was evenly spread on LB agar plate and allowed to dry. 20 μ L single drop of different samples (1mg/mL of only polymer, 25 μ g/mL of only azithromycin, 1 mg/mL of azithromycin loaded polymer) were added to whatmann filter paper disks of 6mm diameter and allowed to dry for 2 h inside hood at medium air flow. The disks were then

carefully placed on LB agar plate and kept at 37 °C for 18 h and images were taken under white light illumination using Syngene G: Box Chemi XX6 instrument.

Zone (area) of inhibition calculation: $\text{area} = \pi(R^2 - r^2)$,

Wherein, $R = D/2$ and $r = d/2$, D = Total diameter of disk and circular area of inhibition, d = diameter of circular disk alone

4.2.21 Colony Counting Experiment: A single colony was picked from an *E. coli*-streaked plate and grown in 3 mL of LB broth for 4 hours. This experiment was done in duplicate. *E. coli* concentration was reduced to 1×10^6 CFU/mL. 100 μL of *E. coli* was incubated with 100 μL of 1 X MIC concentration of linear and star polymeric samples for various time points. Incubation was done in 1.5 mL eppendorf with pores on its cap at 37 °C in shaking condition. After incubation, samples were 10 times diluted and 100 μL of the sample was smeared on LB agar plate and left to dry for 30 minutes. The plates were incubated at 37 °C for 18 h. Colonies were counted and absolute number of *E. coli* colonies were determined upon incubation with polymers using the following formula:

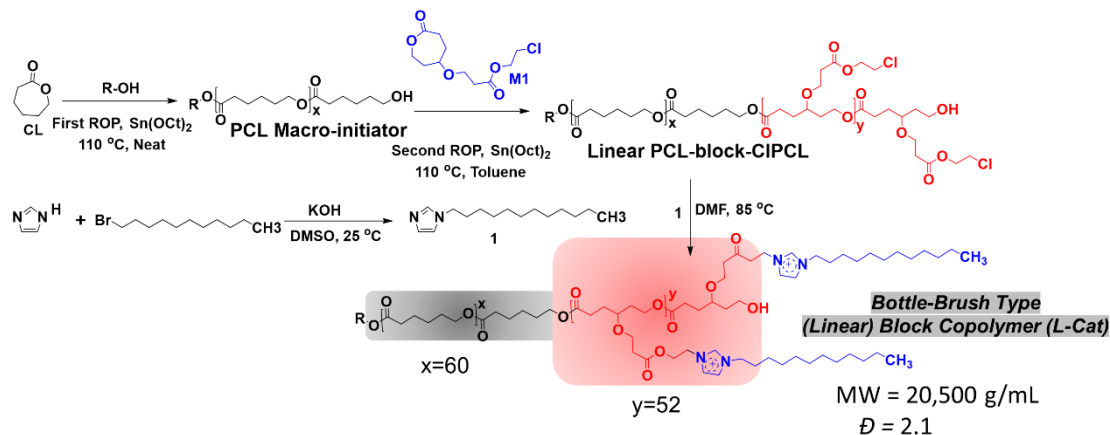
$$\text{CFU/mL} = \frac{\text{Total number of colonies} \times \text{dilution factor}}{\text{Volume of specimen spread on the solid culture}}$$

4.3 Result and Discussion

4.3.1 Synthesis and Characterization of Bottle-brush type linear cationic diblock copolymer

The synthetic scheme for one-dimensional linear di-block copolymer architecture is shown in Scheme 4.1. The linear architecture involves ring opening polymerisation of ϵ -caprolactone (CL) in toluene utilizing monofunctional initiator triethylene glycol monomethyl ether to develop a single arm PCL-based macroinitiator. This PCL based macroinitiator then underwent ring opening polymerisation of M1 monomer to yield amphiphilic diblock copolymer based on polycaprolactone and chlorine substituted polycaprolactone (PCL-block-ClPCL) as shown in Scheme 4.1. The chlorine units of the functionalised PCL block were substituted with N-dodecyl substituted imidazole units, to

yield bottle-brush type linear cationic block copolymer. This polymer is also referred as L-CAT. The degree of substitution for PCL-Macroinitiator and Linear PCL-block-ClPCL was maintained as 60 and 50 units respectively by varying the $[M]/[I]$ ratios.



Scheme 4.1. Synthetic Scheme showing the synthesis of cationic linear bottle brush type block copolymer.

^1H -NMR of the linear PCL Macro-initiator is shown in Figure 4.5a. In order to determine the number of repeating units, $-\text{OCH}_3$ protons of TEG initiator at 3.38 ppm (proton a) were compared to that of polymer ester peak at $-\text{OCH}_2$ at 4.06 ppm (proton b). The degree of polymerisation from ^1H -NMR (X_n) was found to be 64 ± 1 , for $[M]/[I] = 60$. The chain end $-\text{OH}$ group of the macroinitiator was then further utilized for second polymerisation of γ -Cl substituted caprolactone monomer²⁴⁴ (M1) utilizing tin-2-ethylhexanoate as catalyst at 110 °C in toluene, resulting in linear PCL-block-ClPCL copolymer (LB-ClPCL). The successful incorporation of monomer units in the growing polymeric chain was confirmed by ^1H -NMR by comparing TEG initiator protons at 3.38 ppm to that of newly appeared polymer peak $-\text{CH-O-}$ at 3.45 ppm (proton c) in Figure 4.5b. The degree of polymerization for the second block appeared to be 51 ± 1 for $[M]/[MI] = 50$. The GPC chromatogram of PCL Macro-initiator and L-PCL-b-ClPCL is shown in Figure 4.6. A slight shift in retention time is observed in case of block copolymer (L-PCL-b-ClPCL) as compared to macro-initiator (L-PCL). The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity ($\bar{D}$) obtained from NMR and GPC

analysis can be seen in Table 4.1 wherein the number average molecular weight was determined using the formula, $M_n = X_n \times M_0$ where M_0 is the mass of each repeating unit.

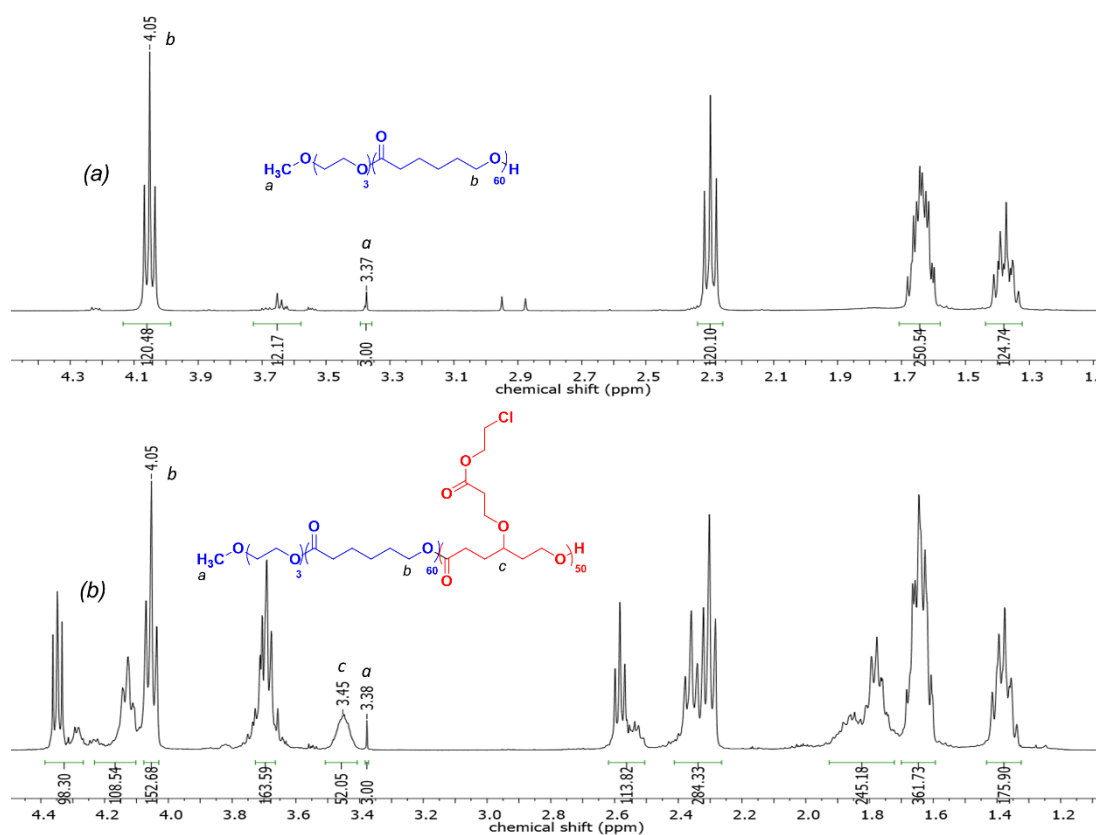


Figure 4.5. ^1H -NMR spectrum of (a) PCL Macroinitiator using triethylene glycol monomethyl ether as initiator and (b) Linear PCL-block-CIPCL polymer in CDCl_3 as solvent

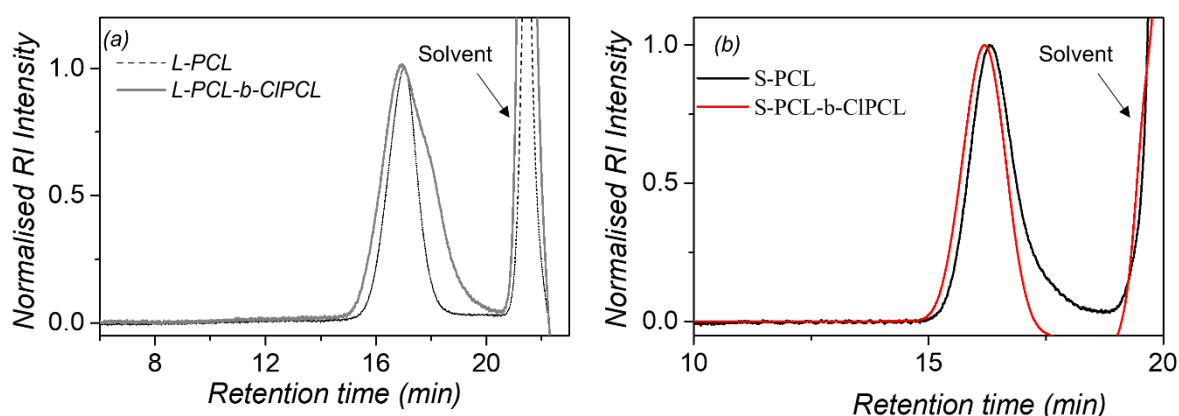
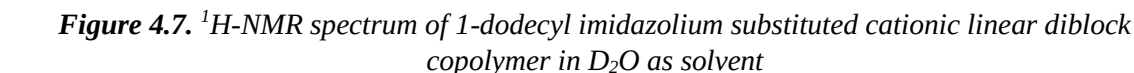


Figure 4.6. GPC chromatograms of (a) PCL macroinitiator (TEG-PCL₆₀) and LB-CIPCL (TEG-PCL₆₀-b-CIPCL₆₀) (b) Star-Macroinitiator (Star-PCL₆₀) and SB-CIPCL (Star-PCL₆₀-b-CIPCL₆₀), CHCl_3 as mobile solvent.

Table enlisting various properties of the polymers

^aMonomer/initiator feed ratio for each arm. ^bDegree of polymerization (*n*) and molecular weights were estimated by ¹H-NMR analysis. ^cMolecular weights were determined by GPC in THF solvent using polystyrene standards for calibration.



159

(C₁₂ hydrophobic unit) necessarily facilitates membrane insertion and simultaneous destabilization of the bacterial membrane.³⁰⁰ Keeping that analogy in mind, the present newly designed polymer comprises of an imidazolium cationic unit for electrostatic interaction with negatively charged bacterial cytoplasmic membrane, a hydrophobic PCL block for bacterial membrane disruption and an additional hydrophobic dodecyl aliphatic chain projecting outwards facilitating facile insertion into the *E. coli* lipid bilayer membrane.

4.3.2. Synthesis and Characterisation of Star cationic diblock copolymer

‘Core-first’ approach²⁷⁷ was employed for the synthesis of star-shaped diblock copolymer with identical molecular weight and composition to that of linear architecture. It involves dipentaerythritol as a multifunctional initiator for ring opening polymerisation of ϵ -caprolactone resulting in *Star PCL Macroinitiator* (see scheme 4.2), wherein all the six -OH initiating sites had equal reactivity and initiation efficiency. For feed $[M]/[I] = 60$, the incorporated number of monomeric units was determined from ¹H-NMR to be 60 ± 1 , suggesting the statistical distribution of 10 units per arm. The ¹H-NMR spectrum of *Star PCL Macroinitiator* is shown in Figure 4.8a wherein the number of repeating units was confirmed by comparing initiator protons -O-CH₂-C- at 3.38 ppm (proton a) to that of -CH₂-O- at 4.06 ppm (proton b) of the growing polycaprolactone units. The observance of protons (~ 11 protons) at 3.64 ppm corresponds to chain end -CH₂-OH units which confirmed the ability of growing polymer chain (*Star PCL Macroinitiator*) to initiate another block copolymer through living polymerisation. These “living” chain ends of the six arm macroinitiator was availed for the polymerisation of *M1* monomer yielding star shaped block copolymer (*Star PCL-block-ClPCL*) with $X_n = 56 \pm 3$, for $[M]/[MI] = 60$ and its ¹H-NMR spectrum is shown in Figure 4.8b. The Macroinitiator peaks at 1.38 ppm (proton e) corresponds to -CH₂- protons were taken into consideration for determining number of repeating units for Cl-substituted PCL block copolymer. (Note: -CH₂-O protons (proton a) at 4.05 ppm of macroinitiator was not considered due to its merging with new proton peak -CH₂-O- at 4.13 ppm of the growing polymer chain).

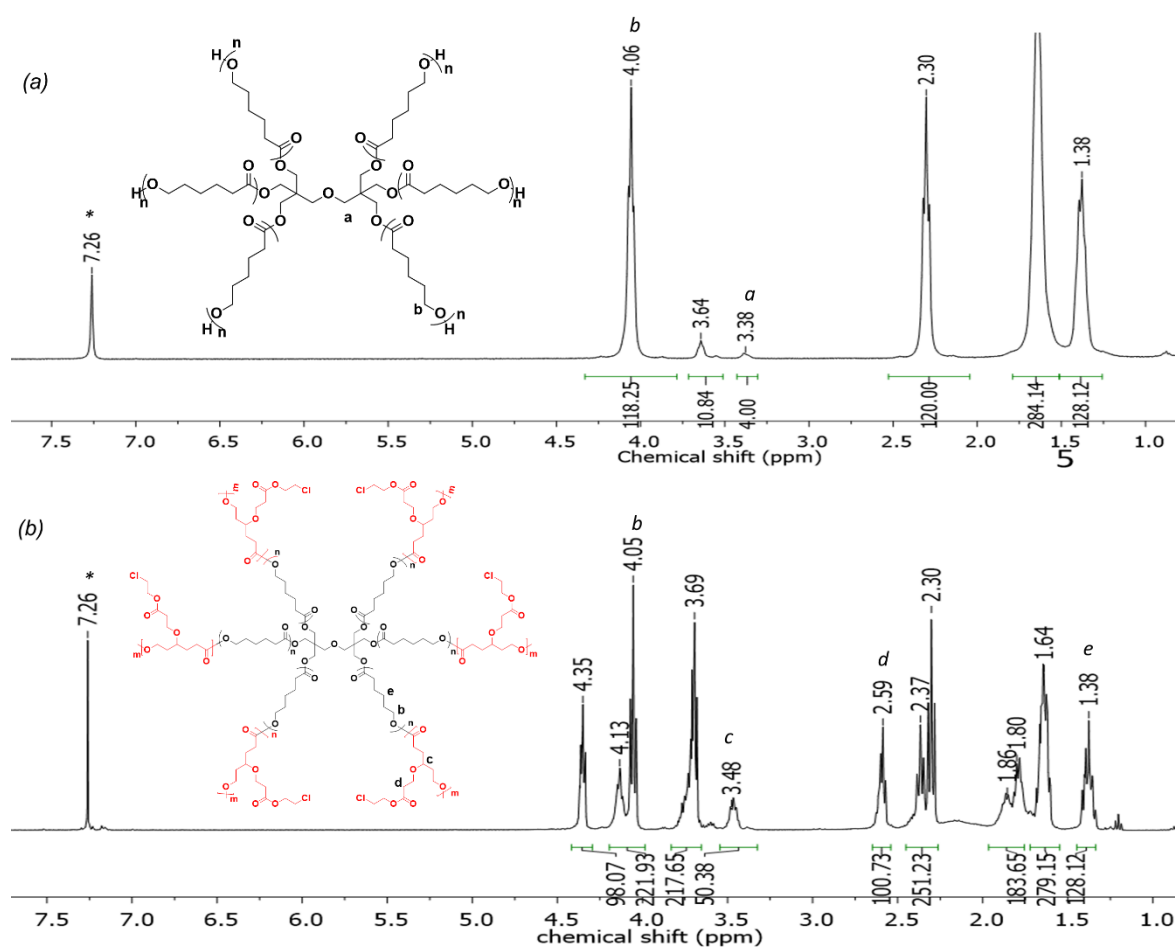


Figure 4.8. ^1H -NMR spectrum of (a) Star PCL Macroinitiator using dipentaerythritol as initiator and (b) Star PCL-block-ClPCL polymer in CDCl_3

The post-polymerization functionalization of star shaped block copolymer with 1-dodecyl-1H-imidazole yielded imidazolium ion containing cationic block copolymer (*S-cat*) with C_{12} chain projecting outwards. Polymer peak $-\text{CH}_2\text{CO}-$ at 2.57 ppm (proton d) was compared to chain end $-\text{CH}_3$ protons at 0.87 ppm (proton f) of imidazolium unit containing dodecyl chain to determine degree of quaternization. By integrating both protons, complete quaternization of chlorine functionalized PCL block was confirmed (see Figure 4.9). The above mentioned cationic diblock copolymers based on linear and star architectures were similar in terms of their molecular weight and ratio of hydrophobic/hydrophilic content in each polymer chain but only differ in their topology. Ultimately, these polymers were taken forward to understand polymeric topology influence on self-assembly aspects in aqueous environment.

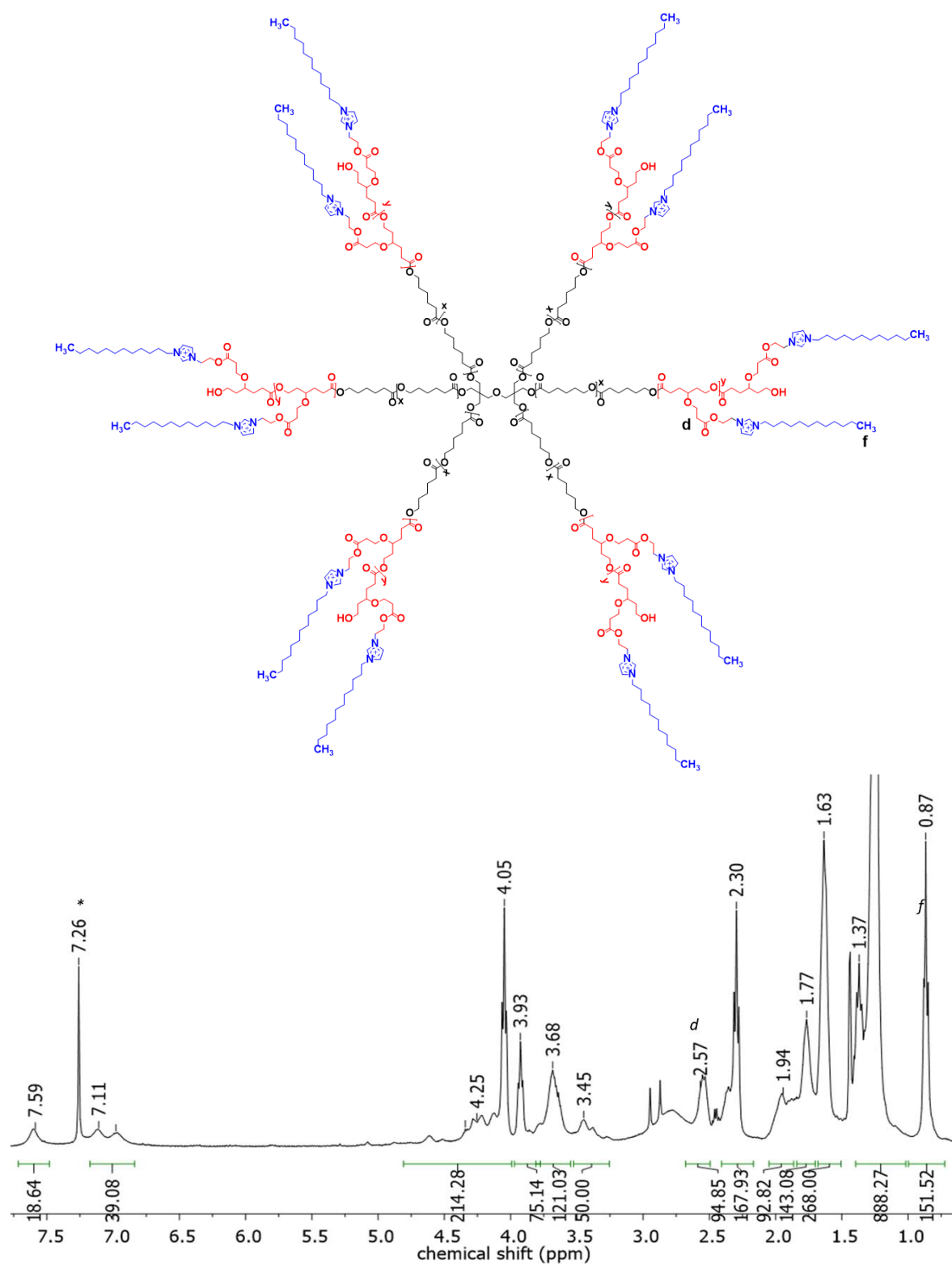


Figure 4.9. ^1H -NMR spectrum of 1-dodecyl imidazolium substituted cationic star diblock copolymer in CDCl_3

4.3.3. Self-assembly of Star and Linear Polymer.

The difference in self-assembled nanoparticles of star and linear polymer arose from contrasting molecular architecture of single polymeric chain. As compared to linear diblock copolymer, the star shaped polymer comprises of 6 arms of PCL hydrophobic block followed by 6 arms of cationic hydrophilic block (10 units each arm, statistically), facilitating inward collapsing of individual branched polymeric chains due to well-defined compartmentalization of hydrophobic and hydrophilic environment. This gives rise to well-defined core-shell architectures (as shown in Figure 4.10a). further aggregate in water to provide aggregated micelles of DLS size in the range of 250 ± 30 d. nm along with a positive zeta potential of $+28.4 \pm 0.2$ mV as shown in Figure 4.10b and 4.10c. The observance of such large sized nanoparticles (> 200 nm) is due to the decreased interaction between the polymeric chains which would limit chain entanglements due to densely grafted side chains of dodecyl aliphatic units.

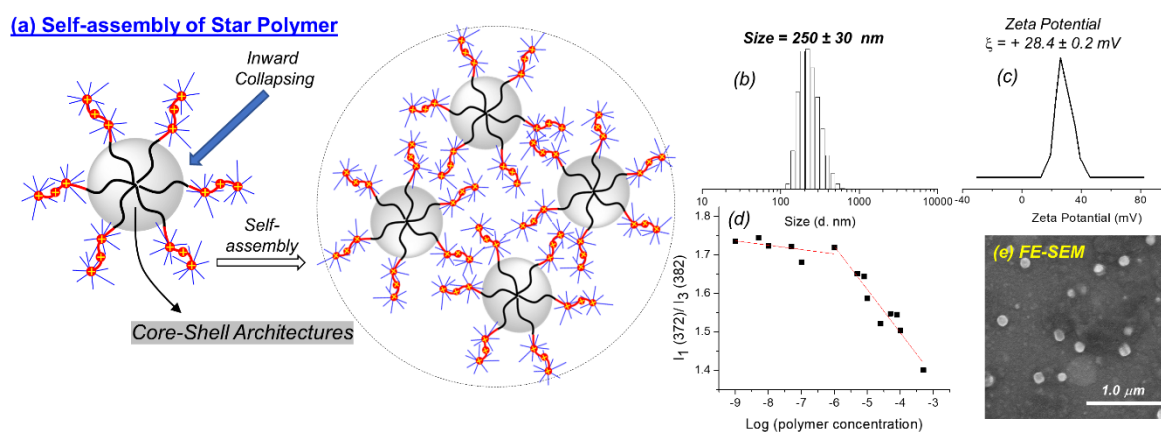


Figure 4.10. (a) Schematic representation of self-assembled nanoparticle of star polymer. (b, c) DLS size and zeta potential of star polymer architecture at 0.1 mg/mL concentration. (d) Plot of I_1/I_3 vibronic emission peaks of pyrene for CMC determination. (e) FE-SEM image of star polymer architecture at 0.1 mg/mL concentration.

CMC was determined by utilizing pyrene as a probe where different polymeric concentrations (5×10^{-4} to 1×10^{-9} g/mL) were incubated with fixed pyrene concentration to determine the absolute polymeric concentration at which pyrene would locate in the hydrophobic environment of the nano-cavity. It has been observed that CMC for star polymer was 1.5×10^{-6} g/mL which signifies a shift in pyrene's microenvironment from hydrophilic to hydrophobic environment (see Figure 4.10d). At concentrations above CMC, the polymeric nanoparticle transpired to remain in aggregated state. It has also been well

corroborated by FE-SEM images of star nanoparticle showing uniform distribution of particles in the size range of 230 ± 20 d. nm at 0.1mg/mL (Figure 4.10e).

In case of bottle brush type linear architecture, individual polymeric chains would phase segregate in water such that the particles would collapse projecting the branched hydrophilic polar head group towards aqueous layer and hydrophobic PCL block tightly packed away from the aqueous environment giving rise to highly compact micellar nanoparticles as shown in Figure 4.11a. These micellar nanoparticles were found to form stable aggregated micelles in aqueous environment in the size range of 200 ± 10 nm with a positive surface potential of $+ 25.6 \pm 0.7$ mV (Figure 4.11b and 4.11c). These micelles were subjected to critical micellar concentration (CMC) determination employing pyrene as a probe. A polymeric concentration dependent change in pyrene's fluorescence spectrum (ratio of first and third vibronic levels (I_1/I_3)) confirmed its encapsulation in polymeric nanoparticles as shown in Figure 4.11d. The CMC was observed to be 1×10^{-6} g/mL which reiterated that micellar nano-assembly of these polymeric nanoparticles are concentration dependent. FESEM images further showed formation of spherical nano-assemblies in the size range of 230 ± 20 d. nm (see Figure 4.11e).

(a) Self-assembly of Linear Polymer

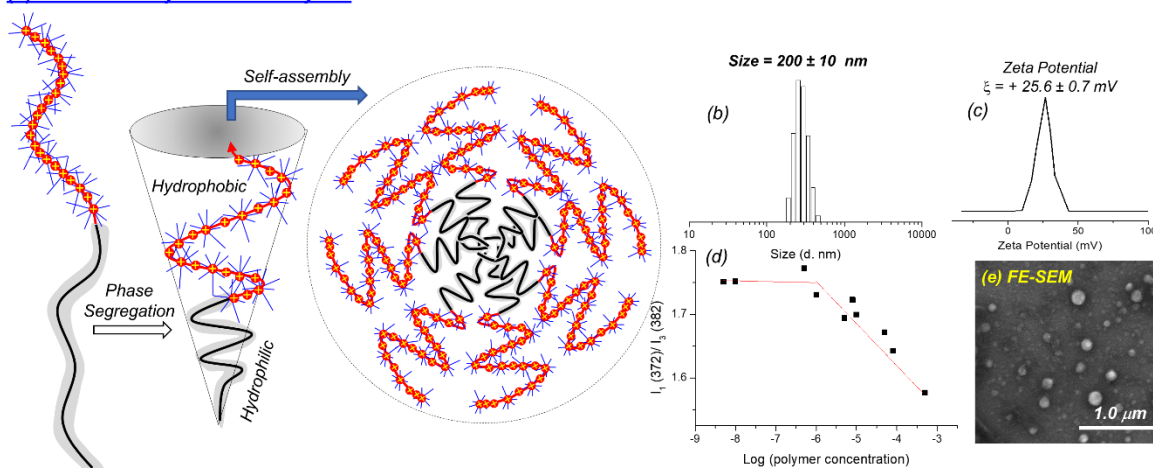


Figure 4.11. (a) Schematic representation of self-assembled nanoparticle of linear polymer. (b, c) DLS size and zeta potential of linear polymer architecture at 0.1 mg/mL concentration. (d) Plot of I_1/I_3 vibronic emission peaks of pyrene for CMC determination. (e) FE-SEM image of linear polymer architecture at 0.1 mg/mL concentration.

These observations further confirmed the formation of large number of multimolecular micelles in aqueous environment. Encompassing all these characteristic features, it can be strongly suggested that though star and linear polymers have different

topology with respect to their single chain architecture, but their macromolecular nano-assemblies are similar in terms of molecular weight, number of repeating units, hydrophobic/hydrophilic content, size, morphology, and zeta potential.

4.3.4. Antibacterial Susceptibility testing

In order to determine the antibacterial activity of star and linear polymers, a fixed concentration of *E. coli* (2×10^6 CFU/mL) was incubated with various concentration of polymer (0.1×10^{-6} g/mL to 5×10^{-4} g/mL) at 37 °C in shaking incubator for 18 h. The change in turbidity of bacterial suspension was monitored with time. It was observed that as time progresses, *E. coli* colonies continued to grow exponentially (log phase) and ultimately saturates in optical density (O.D. at 600 nm) after 15 h, while on the other hand, for ampicillin incubated bacterial cells, growth was completely retarded (see Figure 4.12a). A concentration dependent decrease in turbidity was observed in case of star and linear polymer sample incubated *E. coli* suspension as shown in Figure 4.12a and 4.12c.

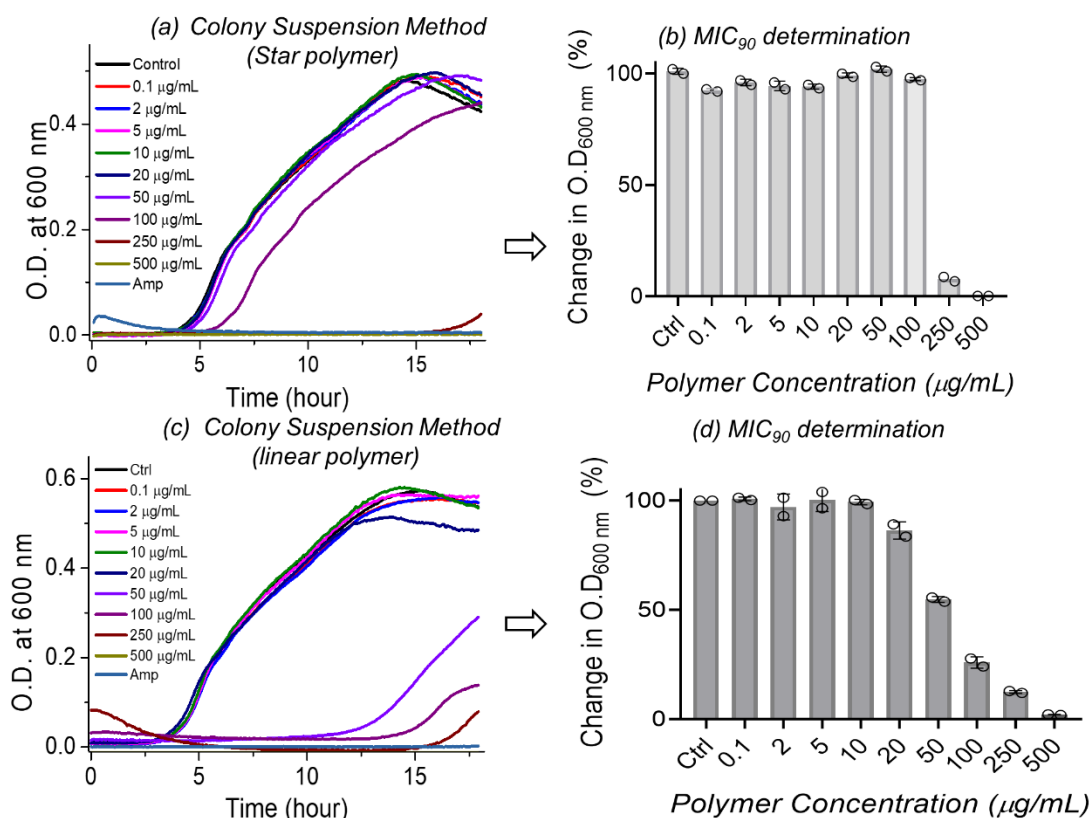


Figure 4.12. (a, c) Colony suspension method for MIC determination of Star and linear polymer (b,d) Determination of MIC₉₀ for star polymer.

A relative percentage decrease in optical density with respect to control was determined by using the formula: $(\text{O.D.}_{\text{at } 600 \text{ nm of sample}} / \text{O.D.}_{\text{at } 600 \text{ nm of control}}) \times 100$ and bar graph representations are shown in Figure 4.12b and 4.12d. MIC_{90} was determined as the concentration at which 90% inhibition of bacterial cells was achieved. For both star and linear polymers, MIC_{90} was observed to be 5×10^{-4} g/mL. It can be observed that through colony suspension method, both the linear and star polymer appear to be inhibiting the growth of *E. coli* at the same rate.

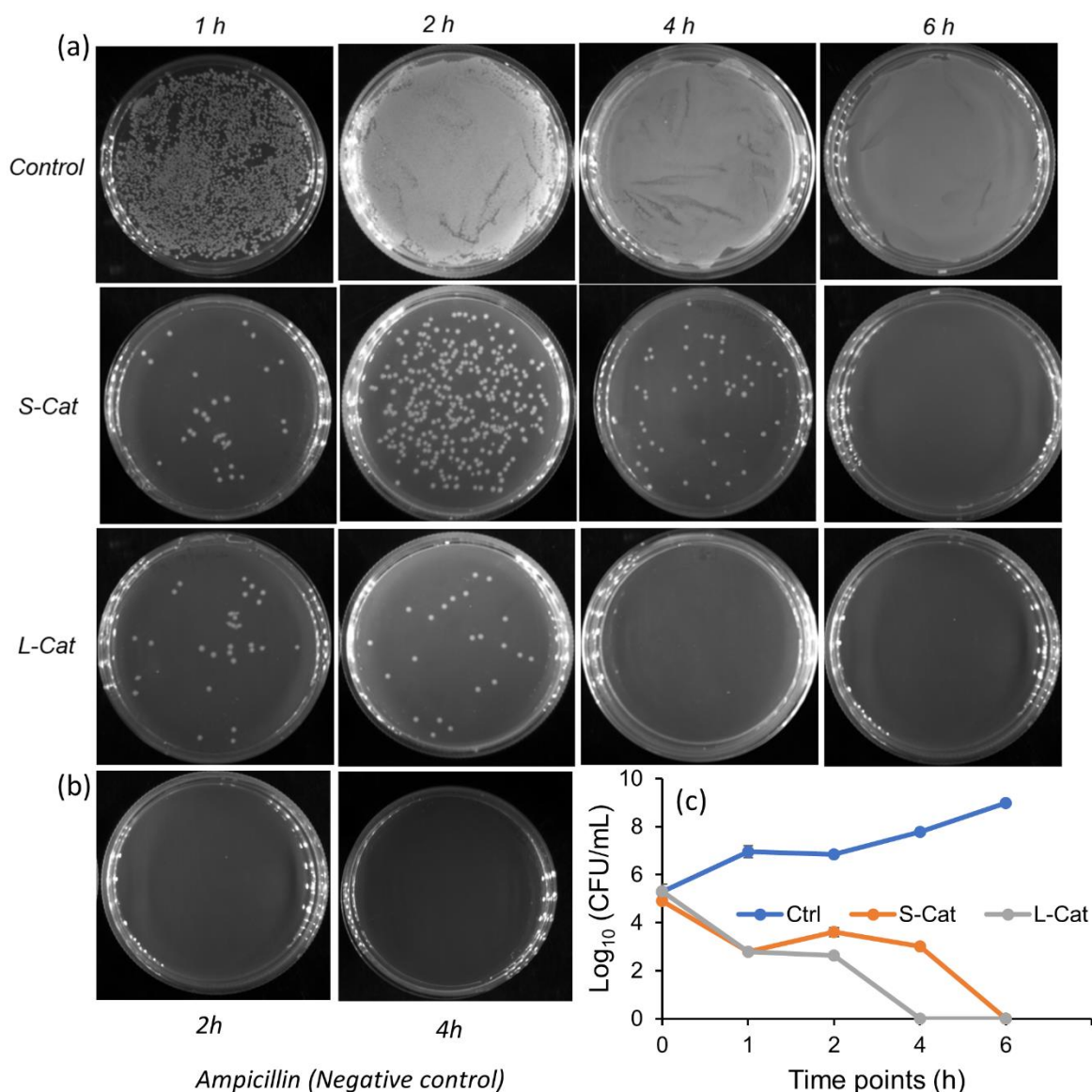


Figure 4.13. (a) Spread plate method plate photographs for determination of Colony forming units (CFU/mL) upon *E. coli* incubation with S-Cat and L-Cat along with positive control (no polymer) for various time points. (b) Photograph of plate showing *E. coli* growth after incubation with ampicillin (negative control) for 2h incubation period. (c) Plot showing effect of polymeric incubation on *E. coli* colonies with time.

To further probe the influence of the polymeric topology on bacterial inhibition, spread plate technique was employed. In this method, a small volume (100 μL) of bacterial suspension was spread evenly on the surface of agar plate so that exact number of isolated bacterial colonies can be enumerated at a particular interval of time. In this method, equal volumes of 2×10^6 *E. coli* cells are incubated with MIC concentration (0.5 mg/mL) of star and linear polymers along with positive control (no treatment) and negative control containing ampicillin (at 1 X MIC concentration). After incubation at various time points (1h, 2h, 4h and 6h), bacterial suspension was diluted 10 times and evenly spread on a solidified culture media (LB agar plate) and incubated in 37 °C for 18 h. Each of the viable bacteria would multiply to form a separate colony and by counting the visible colonies, CFU/mL was calculated. The photographs of the final plates after 1 h, 2 h, 4h and 6h incubation time is shown in Figure 4.13a wherein the first panel corresponds to positive control, second and third panels correspond to S-Cat and L-Cat incubated *E. coli* cells for various time-points respectively. A plate of ampicillin incubated bacterial cells for 2 h is also shown in Figure 4.13b. In case of positive control, a dense population growth in *E. coli* cells were observed at all time points (see Figure 4.13, first panel) whereas for negative control, no viable colonies were observed which commented on ampicillin's bactericidal action (Figure 4.13b). A distinguished demarcation in the number of viable colonies can be observed for star and linear polymer at lower time-points which suggested that linear diblock copolymer was more effective in abolishing bacterial cells rather than star diblock architecture till 4 h incubation period (Figure 4.13a) after which they both are equally active towards bacterial inhibition.

In order to understand the kinetics of antibacterial effect, polymers were incubated with fixed *E. coli* suspension for various time points and then CFU/mL was determined. The plot for log (CFU/mL) versus incubation time for star and linear polymers along with positive control (no polymer) is shown in Figure 4.13c. In case of control, as the time progresses, there has been an enhancement in number of viable colonies which corroborates to the growth of *E.coli* in favorable physiological conditions. For polymeric systems, at initial timepoints (1 h and 2 h) the number of viable colonies were similar for both linear and star polymers but as the time progresses, we observe a growing gap in the rate of bactericidal action. After 4h of incubation, no colonies were observed for linear polymer but star polymer showed comparatively larger number of colonies which suggests that relatively linear polymeric architecture was quicker than star non-linear architecture for membrane

permeabilization and ultimately 6 h onwards, they both were equally active. This can be further correlated to the observance of their similar MIC concentration (0.5 mg/mL) after 18 h of incubation by colony suspension method (broth microdilution assay).

4.3.5. Mechanism of Membrane Disruption

The antibacterial action of synthetic cationic antibacterial polymers is via membrane breaching due to optimal balance of cationic and hydrophobic units. It is a two-step process which involves (a) physical adsorption of polymer on bacterial surface and ultimately masks *E. coli* surface charge potential and (b) membrane permeabilization by hydrophobic residue of the polymer chain (Figure 4.14a). In order to authenticate these two major steps of membrane disruptive action of polymers, two independent experiments were performed.

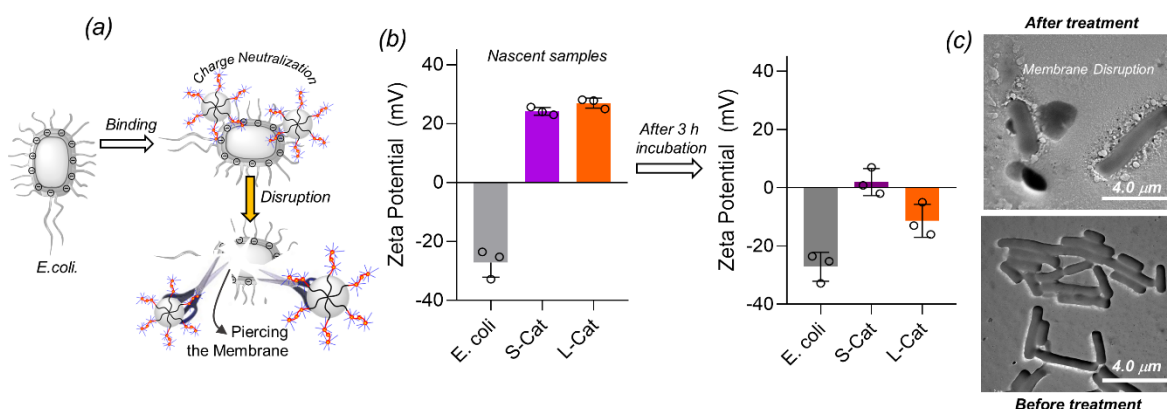


Figure 4.14. (a) Schematic representation showing the mechanism of membrane disruption in *E. coli*. (b) Charge neutralization assay showing the zeta potential of *E. coli*, star, and linear polymers before (left) and after incubation (right). (c) FE-SEM images of *E. coli* before (below image) and after treatment (above image).

Firstly, change in surface charge potential was determined for *E. coli* in free state as well as upon incubation with star and linear polymers (see Figure 4.14b). Initially, *E. coli* has a negative membrane potential (-25 mV) while the cationic polymers showed positive zeta potential of +22 to +25 mV, while upon 4 h incubation, it was observed that there has been a reduction in magnitude of zeta potential of *E. coli* and it becomes almost neutral upon polymeric action. This observation confirms the adhesion of polymer on the surface of *E. coli*. Secondly, membrane permeabilization mechanism of polymer was authenticated by visualizing the changes in *E. coli* morphology upon polymer interaction. To understand the same, star polymer (at MIC concentration) was incubated with *E. coli* for 6 h along with control (no polymer) and fixed for TEM imaging. It was observed that in case of control, the

visual features of *E. coli* remain unaltered and intact while upon polymeric incubation, the membrane appeared to be disturbed and imperfect (Figure 4.14c). Small circular particles could be observed stuck to the surface of *E. coli* which resembles polymeric nanoparticles. This clearly signifies the membrane permeabilization activity of polymer on the surface of *E. coli*.

4.3.6. Theranostic activity of star antibacterial polymer

In order to visualize the action of star and linear polymers on the surface of *E. coli* (diagnostic probe) along with its inbuilt antibacterial action (therapeutic action), a known bioprobe (HPTS dye) was attempted to load in polymer NP (shown diagrammatically in Figure 4.15a). It was observed that for star polymer, drug loading content (DLC) for HPTS dye was 1 % wherein DLC for linear polymer was 0.04 % (Figure 4.15b).

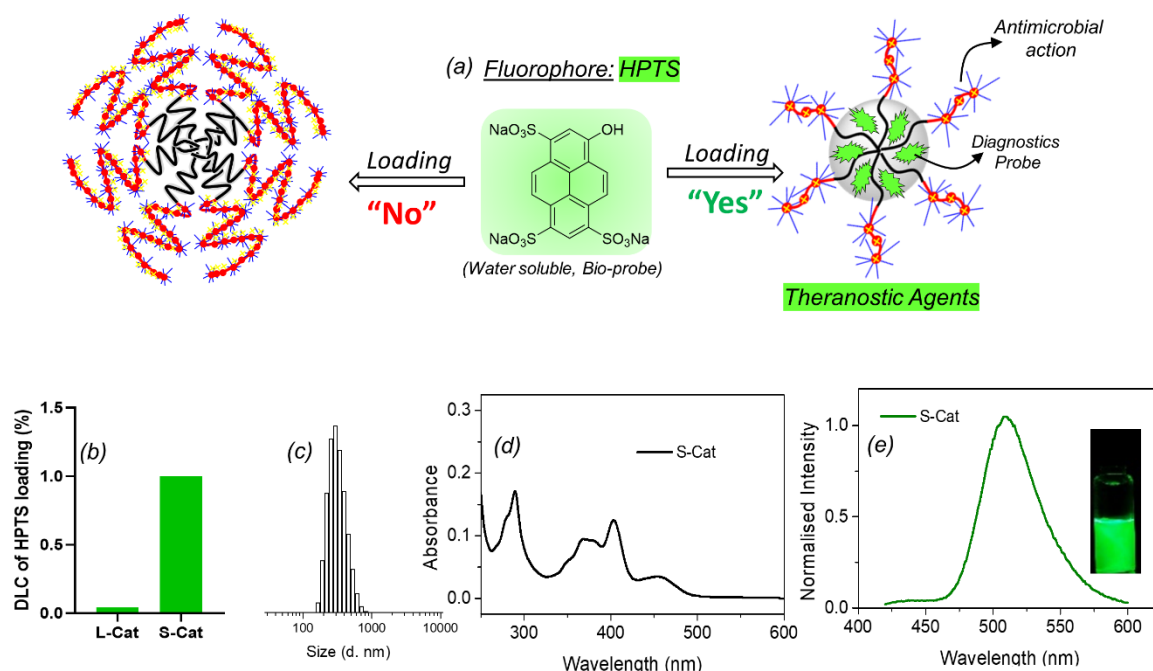


Figure 4.15. (a) Schematic representation of HPTS loaded star shaped nanoparticle. (b, c) DLS size and zeta potential of HPTS loaded nanoparticle.

As it has been already discussed in Figure 4.11, linear polymers would self-assemble to give highly compact micellar nanoparticles with a positive zeta potential. It is plausible that such tightly packed nano-assemblies are unable to confine a water-soluble dye which leads to extremely low loading capability. The drug loading capability was profoundly enhanced for star polymer architecture which might be due to the inbuilt compartmentalization of hydrophobic and hydrophilic arms in a single polymeric chain. The

DLS plot for HPTS loaded star polymer showed monomodal distribution of spherical nanoparticles in the size range of 250 ± 4 d. nm (Figure 4.15c). A photograph of a vial containing HPTS loaded star polymer is also shown for demonstration of green fluorescence emission. The absorbance and fluorescence spectra of S-Cat + HPTS (HPTS loaded star polymer) are shown in Figures 4.15 d and 4.15e. This confirms that the fluorescence characteristics of the fluorophore is intact and these HPTS loaded nanoparticles can be used for bio-imaging. The absorbance and fluorescence property of HPTS dye remained unaltered upon encapsulation in a polymeric NP which established its usage as a nanoprobe to study membrane disruptive action of polymer on *E. coli*.

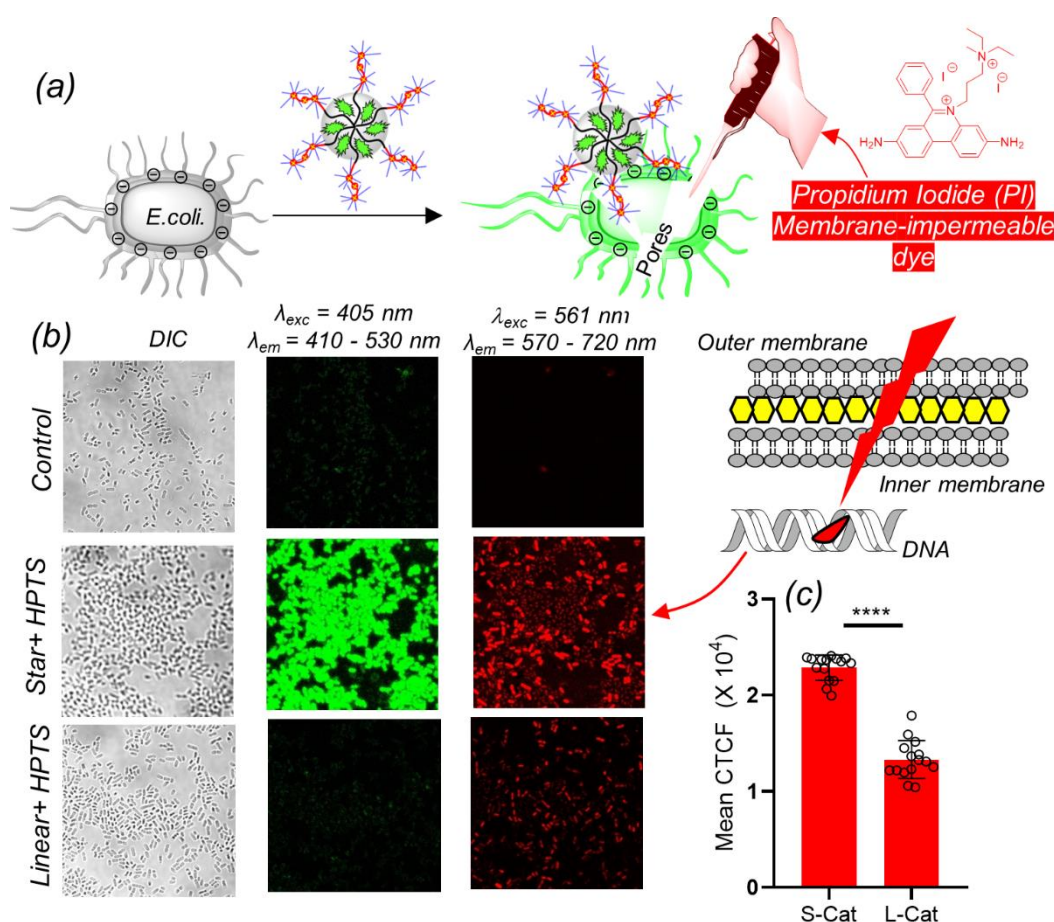


Figure 4.16. a) Diagram showing theranostic effect of HPTS loaded star NP and simultaneous facilitating PI staining. (b) Confocal microscopy imaging of control (without polymer), Star + HPTS, Linear + HPTS after 8 h incubation with 1×10^8 *E. coli* bacterial cells (**** $P < 0.0001$). (f) Mean CTCF values for star and linear polymers. Each point represents mean $\pm$ SEM ($n = 15$).

The design strategy is clearly illustrated in Figure 4.16a which shows that initially *E. coli* cells were incubated with HPTS loaded star and linear polymer (1 X MIC) for 8 h followed by addition of propidium iodide (PI) as a membrane impermeable dye. The ability of propidium iodide to translocate only in membrane compromised cells would differentiate between dead and alive *E. coli* cells. The confocal microscopy images for control (without polymer), S-Cat + HPTS (HPTS loaded star polymer), L-Cat + HPTS (HPTS loaded linear polymer) are shown in Figure 4.16b. The first fluorescent row corresponds to excitation of HPTS fluorophore ($\lambda_{ab} = 405$ nm and $\lambda_{em} = 410 - 530$ nm) while the second fluorescent row corresponds to collection of PI fluorescence ($\lambda_{ab} = 561$ nm and $\lambda_{em} = 570 - 720$ nm). It was observed that star polymer was brightly illuminated in both green and red emission channels while due to low loading content, linear polymer at similar polymeric content only showed bright emission in red channel. Interestingly, PI intensity for star polymer was higher than linear polymer which suggest a better intake of PI upon membrane breakage in case of star shaped polymer as shown in Figure 4.16c.

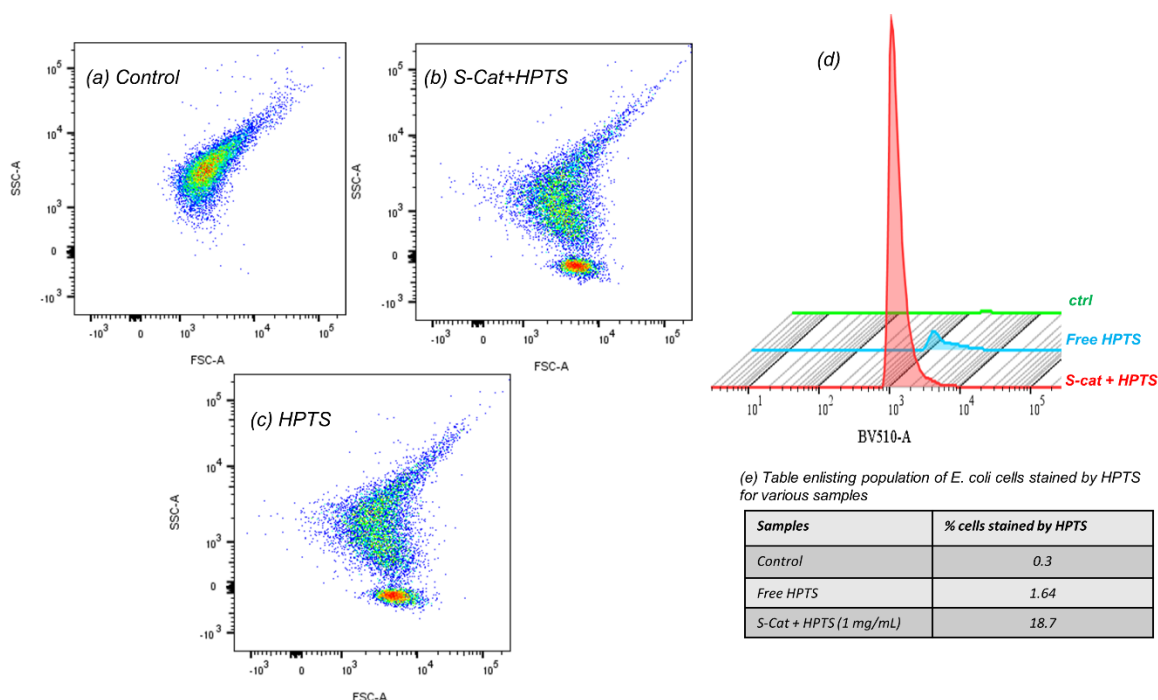


Figure 4.17. The dot plot showing the cell density in SSC and FSC axes for (a) Control (without treatment), (b) S-Cat + HPTS and (c) HPTS alone. (d) Graph showing relative uptake of HPTS by three sample, namely; control, Free HPTS and S-Cat + HPTS after 2h incubation. (e) Table containing percentage cells stained by HPTS for the three above mentioned samples.

In order to further validate the uptake of HPTS loaded star nanoparticles in *E. coli*, flow cytometry experiment was performed. It is a laser-based technique which differentiates

cell population based on their size and granularity. Moreover, it can also account for the fluorescent characteristics of a cell population by employing different laser beam sources and filters. In the present case, the relative uptake of control sample (without treatment), free HPTS and HPTS loaded star nanoparticle is determined by employing 405 nm laser for HPTS excitation and emission was collected using BV 510-A filter. The cell population density for all the three cases is shown in Figure 4.17a, 4.17b and 4.17c. In case of control, no emission was observed in the HPTS channel as *E. coli* cells were not incubated with HPTS fluorophore (see Figure 4.17d). The relative uptake of HPTS loaded star nanoparticle (S-Cat + HPTS) by *E. coli* cells was higher than free HPTS. The statistical percentage of cells stained by HPTS was 0.3 % for control, 1.64 % for Free HPTS while 18.7 % for HPTS loaded star polymers (see Figure 4.17e).

4.3.7. Cytotoxicity and Hemolytic Activity

In order to evaluate the biocompatibility of star and linear polymers, their interaction has been studied with mice RBCs and *WT-MEF* cell lines as shown in Figure 4.18a and 4.19a. In general, animal cell membranes are zwitterionic in nature (~ 0 mV) while membrane potential for RBCs are slightly negative (~ -15 mV). Hemolytic effect of polymeric nanoparticles wherein erythrocytes were incubated with star and linear polymeric architecture at MIC concentration and star polymeric architecture was observed to be completely non-hemolytic to cells as compared to linear polymeric architecture as shown in Figure 4.18b and 4.18c.

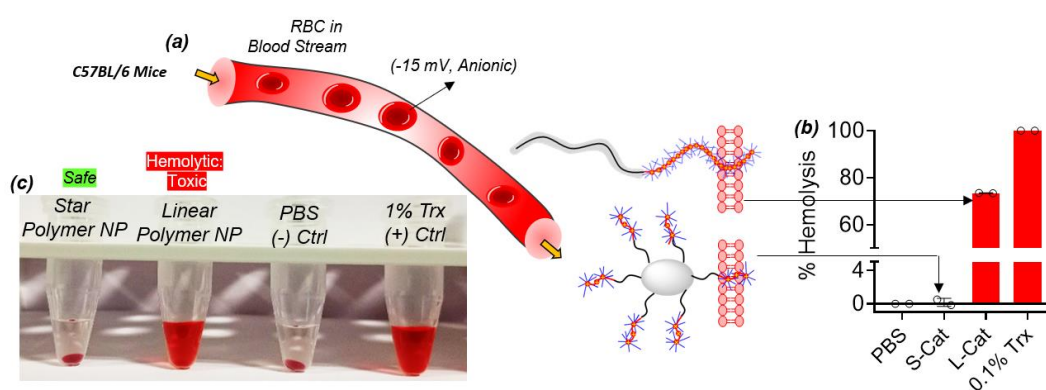


Figure 4.18. (a) Diagrammatic representation of interaction of star and linear polymers with RBCs derived from C57/B1 mice blood. (b) Hemolysis data of star and linear polymers with RBCs. (c) Photograph of eppendorfs with RBC solution after polymeric interaction along with PBS and 1% Trx.

Percentage hemolysis was calculated with PBS as negative control (no lysis) and 0.1 % Triton-x as positive control (100 % lysis), and relatively for star polymer hemolysis was observed to be $< 1\%$ ($0.33 \pm 0.17\%$) while strikingly in case of linear polymeric architecture with similar monomeric composition hemolysis was observed to be $73.44 \pm 0.08\%$ at MIC concentration.

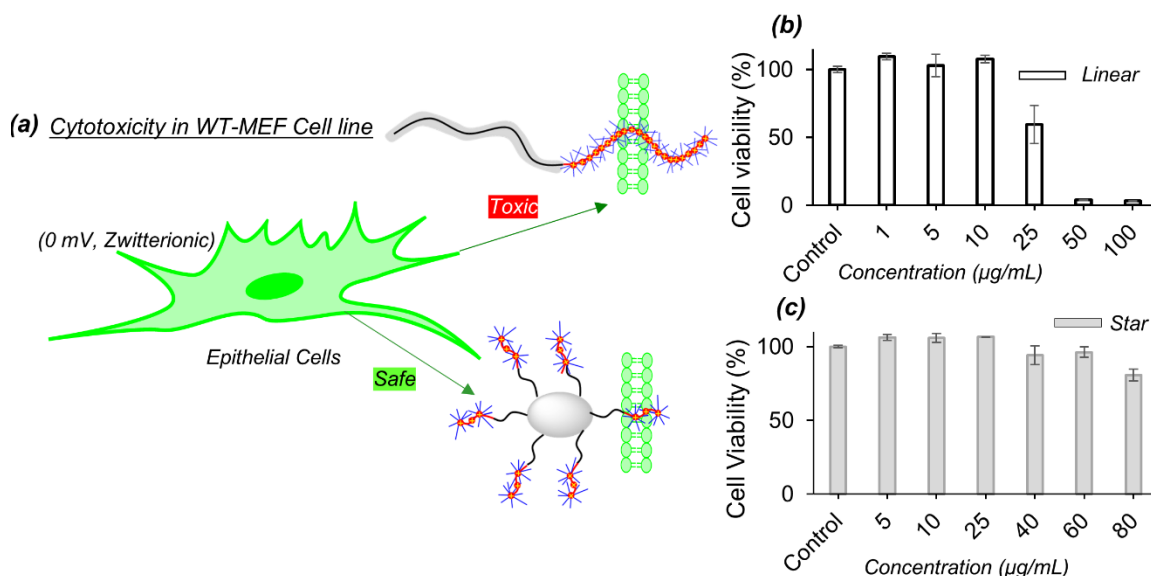


Figure 4.19. (a) Diagrammatic representation of interaction of star and linear polymers with, mice epithelial cells. (b, c) MTT assay to measure cytotoxicity of linear and star polymers upon interaction with WT-MEF cell lines.

This observation was further supported by MTT assay has been used to determine cellular metabolic activity which could be translated as an indicator for cell viability, proliferation and cytotoxicity (see Figure 4.19a). The cell viability for linear and star polymers at various concentrations are shown in Figure 4.19b and 4.19c. Unlike their bactericidal activity, the two polymeric architecture showed contrasting difference in their interaction with WT-MEF cell lines. For linear polymer, IC_{50} value was observed to be 25 $\mu\text{g/mL}$ while in the case of star polymer, $\text{IC}_{50} > 80 \mu\text{g/mL}$ which demonstrates that linear architecture is toxic due to membrane disintegration while star architecture is safe to interact with mammalian cells. Even though both the polymers were composed of similar compositions of PCL and imidazolium ion substituted cationic PCL counterparts, the striking difference in their interaction with animal cell line, strongly suggests that polymer topology is an important factor for understanding membrane-macromolecule interaction. Bactericidal and toxic properties of an antibacterial polymer is affected by various factors like cationic and hydrophobic components, charge type, molecular weight, morphology,

polymeric topology, position of cationic charge²⁶³. In the present investigation, all these physical characteristics were kept comparable except for their polymeric topology. Moreover, it has also been established that selectivity of an antibacterial polymer is compromised upon induction of high hydrophobic residues in a polymeric chain²⁵⁵. So, it can be postulated that the non-specific interaction of cationic linear polymer to bacterial membrane (*E. coli*), mammalian membrane (WT-MEF) as well as RBCs which leads to cellular toxicity might be due to equal proportions of PCL (hydrophobic unit) and imidazolium ion functionalised (cationic and hydrophilic) unit. Interestingly, star polymeric architecture was observed to be extremely selective towards *E. coli* cell as compared to WT-MEF cell lines and RBCs irrespective of the presence of similar hydrophobic composition to that of linear architecture (overall 60 PCL units). This could be understood due to the 3D network of star architecture where presence of 6 individual arms containing 10, 10 units of PCL and cationic polymer segment would dilute the overall impact of membrane perturbation as compared to 60 units of continuous PCL chain interacting altogether.

4.3.8. Combination Therapy with Azithromycin

Macrolide Antibiotics

Bacteriostatic hydrophobic drug
Inhibits protein synthesis
Easy to develop resistance

Mechanism:

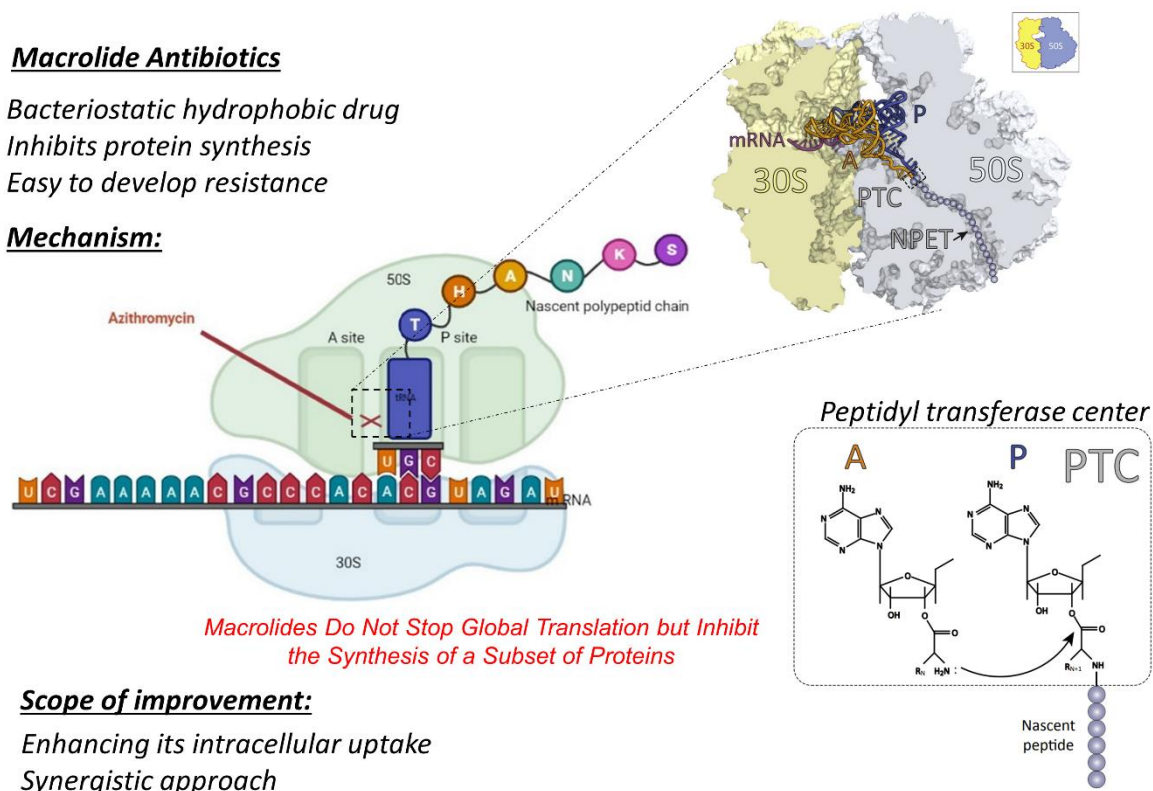


Figure 4.20. Mechanism of Action of Macrolide Antibiotics

Azithromycin falls under the class of macrolide antibiotics which shows broad-spectrum antibacterial activity due to inhibition of protein synthesis by targeting bacterial ribosomes.^{301,302} Azithromycin (Azx) is a known macrolide based antibiotic. They work by targeting bacterial ribosomes and inhibiting protein synthesis mechanism. This is initiated by blocking nascent peptide exit tunnel (NPET) which is a pathway through which the synthesized polypeptides leave ribosomes as shown in Figure 4.20. It has been recently understood that they don't stop the global translation of proteins by blocking the peptidyl transferase centre (PTC) but retards the growth of few subsets of proteins³⁰¹. Even though, it is effective in eradicating antibacterial infections, its potency is restricted mainly due to two factors, firstly due to reduced intracellular accumulation inside bacterium due to its hydrophobic nature. Gram-negative bacteria like *E. coli* have intrinsic resistance to various hydrophobic (water-insoluble) antibiotics such as outer membrane permeability, highly efficient efflux pumps etc. which compromises the drug accumulation (see Figure 4.21). Secondly, azithromycin is a bacteriostatic drug due to fast dissociation kinetics from bacterial ribosomes and thus limits its efficacy. So, in order to circumvent these challenges, nanodrug formulation of azithromycin was prepared with star polymer architecture to produce azithromycin loaded star polymer nano-assemblies which ultimately led to synergistic combination therapy to treat bacterial infection as shown in Figure 4.21. The polymeric nanoparticles would act as potentiators to enhance the activity of azithromycin by increasing its bio-accumulation inside the *E. coli* cells.

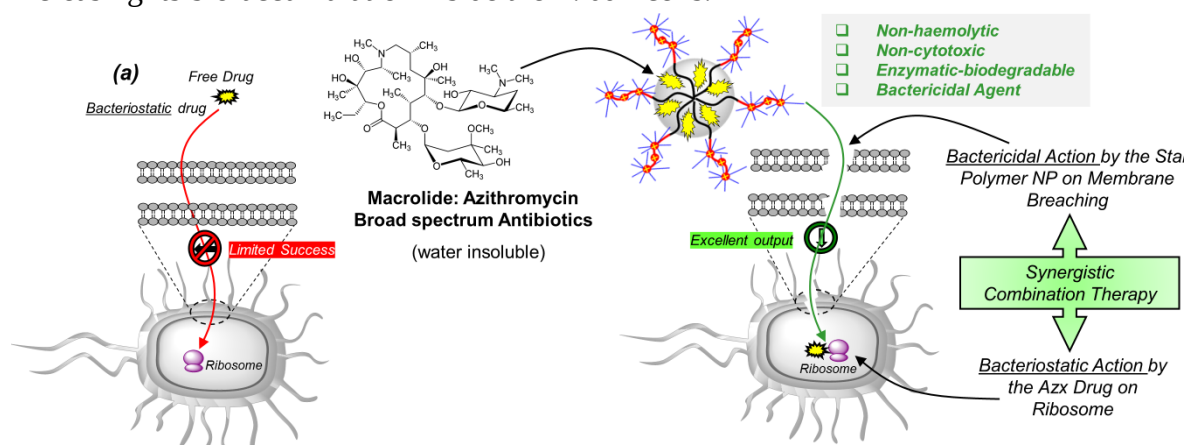


Figure 4.21. (a) Diagrammatic representation of free azithromycin drug acting on bacterial cells along with Azithromycin loaded nanoparticles inside the *E. coli* cells and observance synergism between polymer and drug.

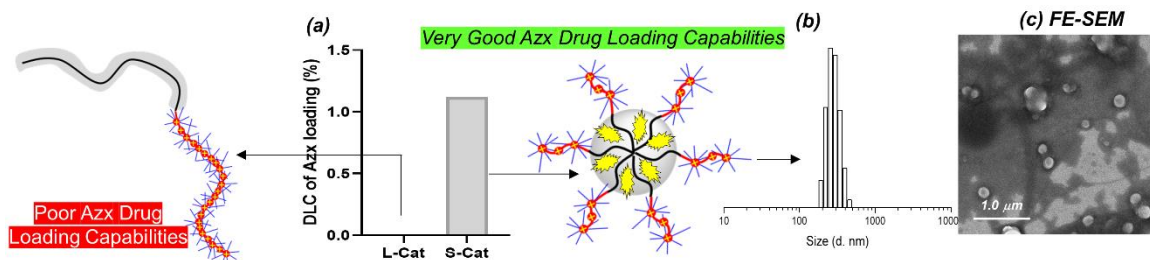


Figure 4.22. (a) Comparative DLC of linear and star architecture. (b, c) DLS histogram and FESEM image of azithromycin loaded nanoparticles.

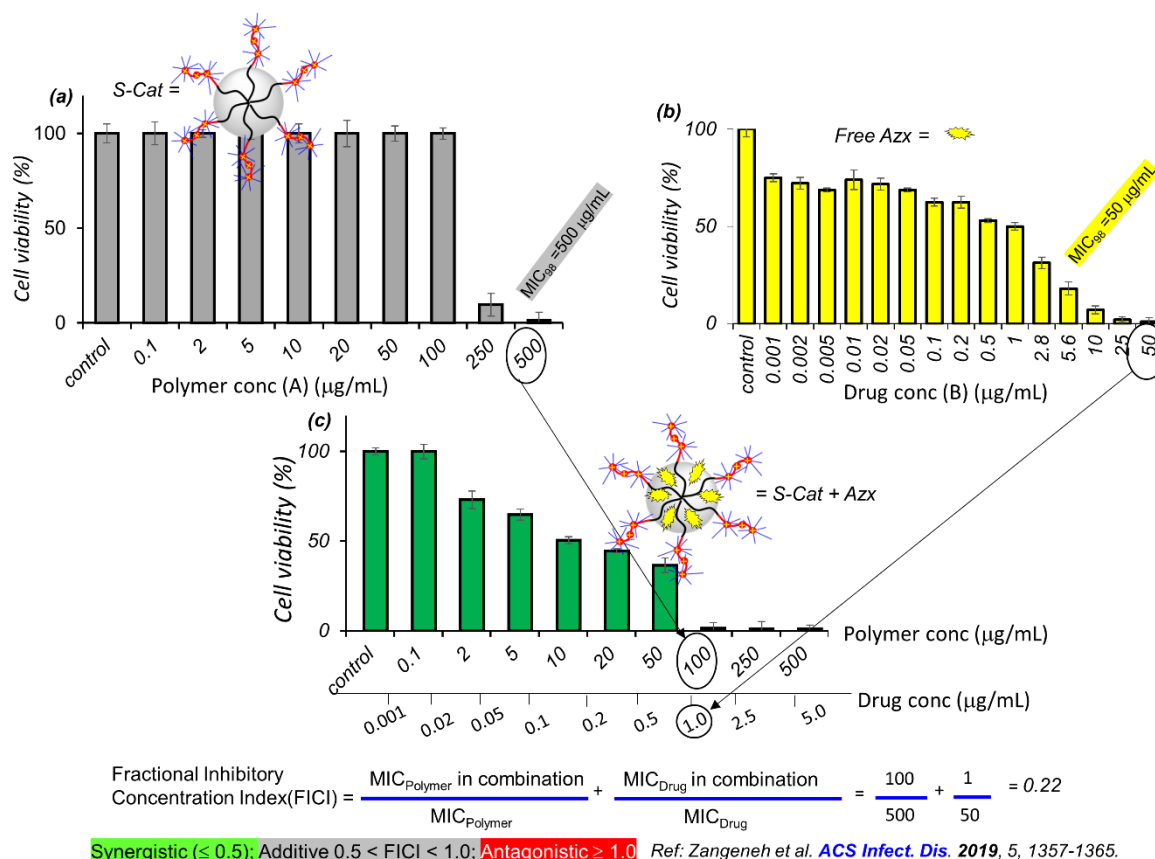


Figure 4.23. Plots of *E. coli* viability deduced from bacterial optical density has been shown for (a) Star polymer (b) Azithromycin (c) Azithromycin loaded star polymer architecture for different concentrations with *E. coli* cells along with the determination of fractional inhibitory concentration index (FICI) for azithromycin loaded polymeric nano-assemblies.

The drug loading content (DLC) for star architecture was found to be 1.1 % with a drug loading efficiency of 2.8 % while in case of linear architectures no loading was observed (Figure 4.22a). This might be due to their discrete chain-folding induced self-assembly which favors star architecture to form colloiddally stable drug loaded nanoparticles. The DLS and FE-SEM image is shown in Figure 4.22b and 4.22c respectively which

authenticate the formation of stable nanoparticles. Azithromycin loaded star polymeric nano-assemblies were further explored for combination therapy. Here, broth microdilution assay was performed to determine minimum inhibitory concentration for drug encapsulated polymeric samples, free polymeric sample, and free antibiotic. For drug loaded polymeric samples, screening was done at similar concentrations of polymer to that of free polymeric sample and relatively drug concentration was determined for individual polymeric concentrations. Cell viability is defined as the percentage of *E. coli* cells which are viable after 18 h of incubation time. The cell viability plot for free polymer, free azithromycin and drug encapsulated polymeric sample is shown in Figure 4.23. The minimum concentration for 98 % inhibition of *E. coli* was observed to be 500 $\mu\text{g/mL}$ for free polymer (see Figure 4.23a) while for free azithromycin MIC_{98} was found to be 50 $\mu\text{g/mL}$ (Figure 4.23b). Interestingly, for drug loaded polymeric samples, a reduction in MIC values for both polymer and drug were observed. Overall, 98 % inhibition of bacterial colonies was observed at 100 $\mu\text{g/mL}$ polymeric concentration containing 1 μg of azithromycin (Figure 4.23c). This has been an extremely important observation because drug concentration has been reduced 50 times upon encapsulating inside a bactericidal polymer.

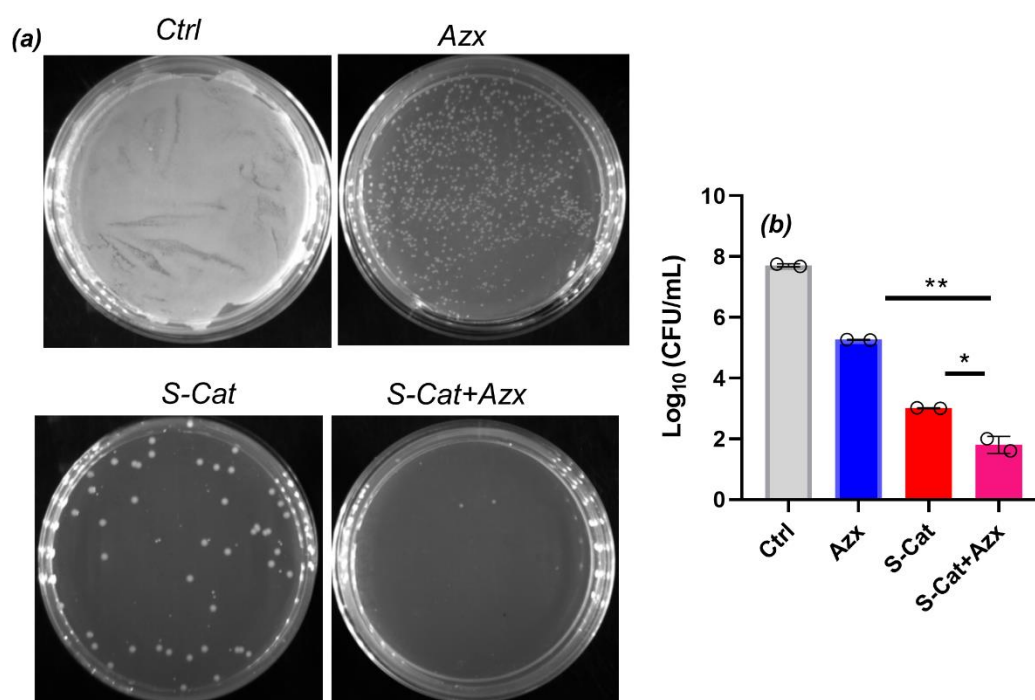
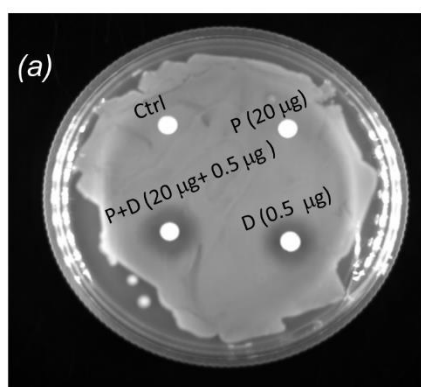


Figure 4.24. Photographs of plates containing *E. coli* suspension incubated with control, only Azithromycin (Azx, 5 $\mu\text{g/mL}$), S-Cat (500 $\mu\text{g/mL}$) and polymer loaded drug sample (S-Cat+Azx) at polymeric conc. of 500 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$ drug loading content for 4 h incubation period at 37 °C. (** $P < 0.01$ and * $P < 0.1$), each point represents mean $\pm$ SEM ($n = 2$).

In order to understand the type of interaction between drug and polymer, Fractional Inhibitory Concentration Index (FICI) has been determined. For a synergistic interaction between two component system, FICI should be ≤ 0.5 , while for additive effect, $0.5 < \text{FICI} < 1$ and ultimately for antagonistic behavior $\text{FICI} \geq 1$ ²⁵⁷. For the present system, FICI value was observed to be 0.22 which is less than 0.5, confirming synergistic interaction between the two components. This has been further illustrated by spread plate method where four different samples including control (no treatment), Only drug (Azx, at 5 $\mu\text{g/mL}$), Only S-Cat (at 500 $\mu\text{g/mL}$) and S-Cat + Azx (at 500 $\mu\text{g/mL}$ + 5 $\mu\text{g/mL}$) were incubated with *E. coli* cells for 4 h followed by spreading in agar plate to observe the number of viable colonies as shown in Figure 4.24 a. As we can observe, for control, plate is completely submerged with overlapping *E. coli* growth. Similarly, for Azithromycin also many countable viable colonies can be observed but for S-Cat sample, we observe a huge decrease in the number of viable colonies (as also seen in Figure 4.13a). Interestingly, in the drug loaded polymer (S-Cat + Azx) sample, least number of colonies were observed as shown in Figure 4.23a. A relative decrease in the number of viable *E. coli* cells is plotted and shown in Figure 4.24b. Upon comparing free Azx and S-Cat sample incubated cells, it is observed that free drug is unable to completely inhibit the growth as concentration of drug used in the following experiment is lower than its MIC concentration (50 $\mu\text{g/mL}$) and time of incubation is also only 4 h. But at the same point of time, drug + polymer sample was able to show synergistic effect by working together towards *E. coli* growth inhibition.



(b) Table enlisting zone of inhibition for various samples

Samples	Zone of inhibition (cm^2)
Only Polymer (1mg/mL)	--
Drug (25 $\mu\text{g/mL}$)	2.3
Polymer + Drug (1 mg/mL)	4.1

Figure 4.25. (a) Photograph of plate with different disks impregnated by (a) Control, (b) Only polymer, (c) Only Drug and (d) Polymer + drug placed on 0.1 O.D. of *E. coli* suspension and incubated at 37 °C for 24 h. Table enlisting the calculated area of inhibition for various samples in cm^2 .

Moreover, disk diffusion method was further employed to determine the susceptibility of *E.coli* cells to star polymer, azithromycin alone and azithromycin loaded polymeric scaffolds. In this method, an antibacterial sample impregnated disk is placed over an agar plate containing bacterial solution and incubated at 37 °C for 18 h. The radially outward movement of antibiotic is facilitated by plate's moisture as the mobile phase. The concentration of antibiotic diminishes as the distance is increased from the circular disk which leads to development of zone or ring formation around the antibiotic disk. Greater the area of the inhibiting zone, higher is the antibacterial action. In the following experiment, it can be observed that in case of control (no antibacterial agent treatment), no inhibition ring is observed which suggests no depletion in bacterial colonies around control sample (see Figure 4.25a). Star polymer impregnated disk also did not show any visible depletion in bacterial colonies. This was because the polymeric amount added to the disk (20 µg) was lower than its MIC concentration. As shown in photograph in Figure 4.25a, azithromycin alone showed a small inhibiting zone but interestingly for polymer+drug sample, a larger inhibition zone could be observed. The tabulated values of zone of inhibition is shown in Table 4.25b, wherein we can easily observe that in case of polymer+drug sample, the zone of inhibition was almost double in magnitude (4.1 cm²) as compared to only drug (2.3 cm²). This result also comments on the dual action of polymer and azithromycin together in a single scaffold for synergistic antibacterial action. As we can note that, polymer alone did not show any visible inhibition of bacterial growth but perturbs the gram-negative outer-membrane for enhanced uptake of azithromycin. Note: Further analysis is under progress where the adsorbed polymer amount is maintained at MIC concentration (500 µg/mL), in order to determine the polymer susceptibility to larger bacterial population (0.1 O.D. or $\sim 1 \times 10^8$ cfu/mL).

Combination (Synergistic) Mechanism

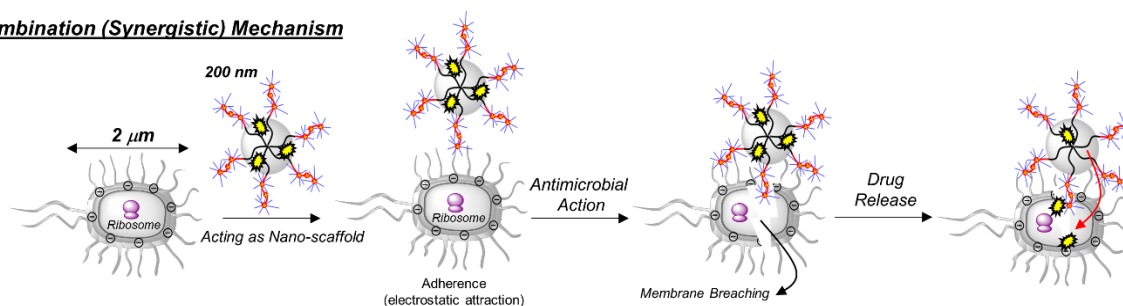


Figure 4.26. Mechanism of bactericidal action of azithromycin loaded polymeric nano-assemblies.

The synergistic action of drug loaded polymeric nanoparticle on *E.coli* is shown in Figure 4.26. It is composed of three concerted steps which primarily involves adherence of star cationic polymer on the surface of *E. coli* by electrostatic interaction due to presence of anionic phospholipids on cytoplasmic membrane. This is followed by bacterial membrane disruption through disruptive intercalation of hydrophobic component into the bilayer membrane and thus inducing release of cytoplasmic content outside the cell. Simultaneously, upon membrane breakage, enzymes released from *E. coli* cells would cleave the self-assembled nanoparticles and ultimately lead to the release of azithromycin from the nano-assemblies. Then, free azithromycin would also show bacterial inhibition by targeting bacterial ribosome and inhibiting the protein synthesis. The development of such polytherapy treatment builds the foundation for substantial enhancement of drug efficacy along with reduced drug resistance by reducing their systemic dose amount. This study will be of interest to wide range of researchers working in polymer chemistry, and chemistry-biology interface.

4.4 Conclusion

In the present investigation, a new class of PCL based cation diblock copolymers were prepared based on two distinctive topologies; 1D rod-shape and 3D star-shape. These two polymer topologies resulted in core-shell cationic polymers with sizes in the range of 250 nm and surface charge +25 mV. An interesting fact about these two cationic polymers was even though their single chain architecture was completely different, their physical characteristics like size, morphology and zeta potential were in the similar range. These cationic nanoparticles were found to be antibacterial in nature with MIC₉₈ value to be 500 µg/mL for both star and linear polymer. Even though they showed similar antibacterial activity but star polymer was found to be more selective towards bacterial cells as compared to mammalian cells and RBCs while linear diblock copolymer was found to be toxic towards *WT-MEF* cell lines (IC₅₀ value as 25µg/mL) and RBCs (73.44 ± 0.08 % , at MIC concentration). The polymers were observed to show difference in rate of inhibition of *E. coli* based on their topology where the linear architecture was more active at initial timepoints as compared to star architecture. Successful anionic fluorophore (HPTS) encapsulation was feasible to only star architecture as compared to linear architecture which was then utilized to determine polymer uptake by confocal microscopy imaging and flow cytometry. A clinical broad-spectrum bacteriostatic drug (Azithromycin) was encapsulated

in star polymer architectures to develop topology-directed drug delivery vehicles. A synergistic antibacterial action between star polymer and azithromycin was observed which led to enhancement of drug efficacy 50 folds higher than free drug due to enhanced accumulation and dual action of *E. coli* inhibition. The development of polytherapy treatment builds the foundation for substantial enhancement of drug efficacy along with reduced drug resistance by reducing their systemic dose amount. It has been postulated that such an amalgamation would hinder bacterial resistance development and thus could be a promising candidate for potent antibacterial strategies.

5. Overall Conclusion and Future Direction

In summary, this work is dedicated towards designing new biodegradable polymer based antibacterial agents and understanding their interaction with gram-negative bacterium *E. coli*. In the first chapter, new class of amphiphilic biodegradable polycaprolactone (PCL) based cationic antibacterial homopolymers were synthesized of different molecular weights. These nano-assemblies were characterized by dynamic light scattering, pyrene probing, FESEM, HR-TEM, and AFM microscopic analysis, etc. They encapsulated HPTS in its hydrophobic core and along with PI, they were imaged to directly visualize and quantify dead/alive bacterial cells at a particular interval of time. Furthermore, aggregated-induced emission (AIE) chromophore TPE was chemically tagged to these polymers so that polymeric adherence on the bacterial membrane could be directly visualized by image-based antimicrobial quantification assay. These TPE-conjugated polymers were expanded to develop bacterial membrane reinforced on-site demand FRET probe along with peptidoglycan adherent dye, Crystal violet (CV) for direct visualization and quantification of antimicrobial action. This helped us to differentiate between antimicrobial and non-antimicrobial polymers based on their membrane-bifurcating property. Furthermore, the significance of polymer topology for the development of efficient antimicrobial agent was explored by comparing linear and star di-block copolymers with identical chemical composition. Even though, the minimum inhibitory concentration (MIC) for both the polymers were in the similar range, the star-shaped polymer was less toxic to RBCs and mammalian cell lines. Azithromycin- a ribosome inhibiting clinical antibody was encapsulated in star-polymeric nano-assemblies to study the combination effect of membrane disruption mechanism via polymer and enzyme-inhibiting pathway via antibody together in a single nanoplatform. The star-polymer and drug together exhibited excellent synergistic antimicrobial activity leading to 50 times reduction in azithromycin's antibacterial concentration. This work provides an overview of polymeric biomaterials in antimicrobial field and focusses on the factors which can be responsible to enhance their selectivity towards bacterial infection.

This thesis work highlights the importance of understanding the structural complexities of antimicrobial polymers and its influence in enhancing their antimicrobial activity. The role of polymeric hydrophobicity and cationic charge has been understood during its interaction with bacterial membrane. Though these polymers were found to be extremely non-toxic to RBCs and mammalian cells via *in vitro* application, understanding their interaction with various organs and tissues under physiological condition is also extremely important (*in vivo* application). For any drug/antibiotic formulation, it is extremely important to understand two processes, firstly, its action on the body i.e., the study of its pharmacodynamics and secondly, how the body interacts to the drug i.e., the study of its pharmacokinetics. When a drug interacts with a cellular receptor, it leads to formation of signal, which ultimately leads to some biological response. These receptors can be divided broadly into four categories: (i) Ligand-gated ion channels, (ii) G-protein coupled receptors, (iii) Enzyme linked receptors and (iv) Intra-cellular receptors. These receptors are responsible for inducing a change in a particular cellular machinery and leads to generation of a response. Along with that, it is also important to understand the uptake of these therapeutic agents along with their distribution/pharmacokinetics. It involves four important processes, (i) absorption, (ii) distribution, (iii) metabolism and (iv) elimination, or ADME.

In vivo Application: NIR dye (IR-780 or cardiogreen) loaded star antibacterial polymers is needed to be considered for *in vivo* application (as shown in Figure 5.1). Cardiogreen (or indocyanine green) is an FDA approved near Infra-red contrasting agent which is widely used in medical diagnostics. It has a peak absorption maximum (λ_{max}) at about 800 nm, allowing successful penetration of tissue layers for *in vivo* imaging. They will be injected intraperitoneally to female balb/c mice along with free dye, nascent polymer. One mouse will be injected with phosphate buffer saline only without any compound; the organs and plasma procured from this mouse served as the control (for 24 h time-point). The experiment will be performed in a set of three as biological replicates. Organs and plasma will be procured at 24 h time point for histological analysis and *in vivo* full body imaging will be done using IVIS imaging technique. Moreover, serum compatibility will also be assessed to determine the half lifetime of nanoparticles in blood plasma. These are major two experiments planned in the future towards completion of the project.

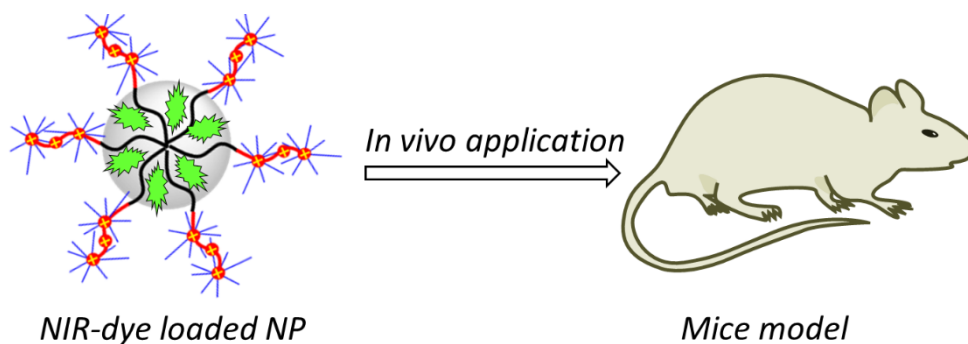
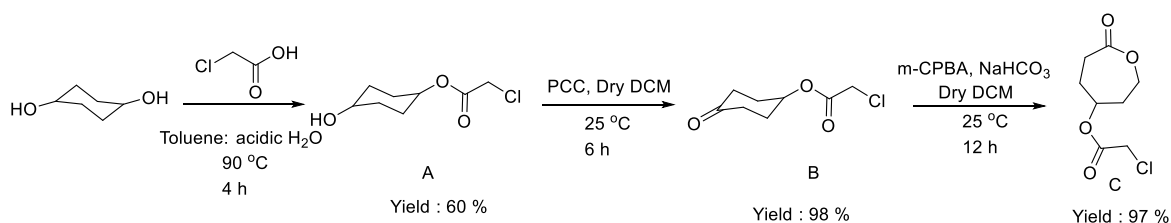


Figure 5.1. Schematic representation of in vivo applications of NIR-dye loaded NPs.

Bioconjugate Chemistry: One of the major challenges in this work was low drug/dye loading capacity of cationic polymers. One way to remove this limitation is by covalently conjugating a drug/dye molecule to the polymer for enhanced uptake. As these polymers are chlorine substituted, they can be easily substituted by azide units via reaction with NaN_3 to develop azide functionalised polymers. These polymers can be tagged with alkyne functionalised small molecules to develop fluorophore/drug conjugated polymeric systems. Synthetic scheme for a new chlorine substituted monomer is shown in Scheme 5.1.



Scheme 5.1. Synthetic scheme for developing a chlorine substituted monomer in three consecutive steps.

The first step involves the reaction of cyclohexane diol with chloroacetic acid to directly synthesize chlorine functionalised monomer (compound A). The mono-substituted product is purified and undergoes PCC oxidation reaction to develop 4-oxocyclohexyl 2-chloroacetate (compound B). Baeyer villiger reaction was performed on compound B with mCPBA and NaHCO_3 to yield ultimately 7-oxooxepan-4-yl 2-chloroacetate (compound c).

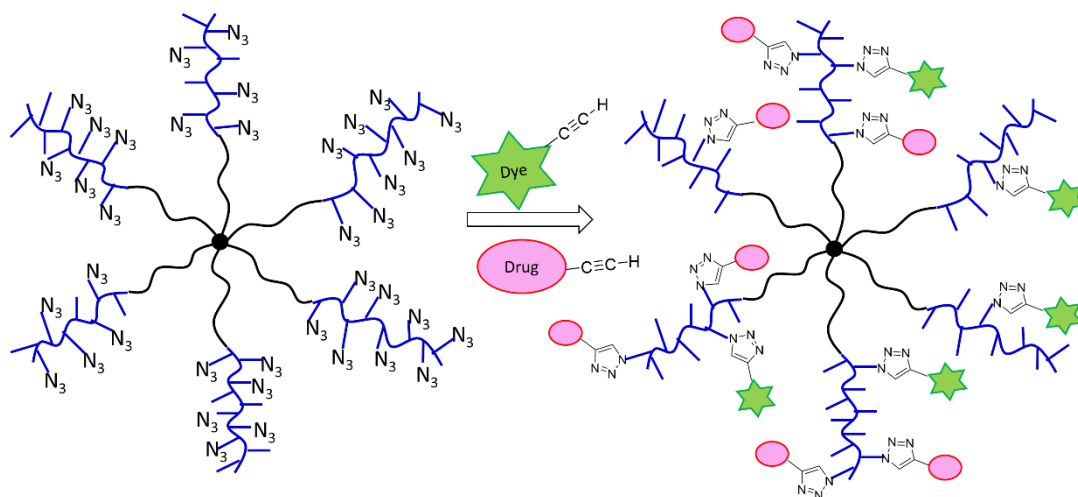


Figure 5.2. Schematic representation of different bio-conjugation on star architecture

The ring opening polymerisation of this monomer to develop chloring functionalised star shaped polymers can be converted into azide functionalised polymers via post polymer functionalisation. These azide functionalised polymers can be utilized for conjugation of dye or drug via azide-alkyne click reaction (see Figure 5.2). This would lead to the development of smart multi-functional polymers in a single nano-scaffold. Similar approach can also be extended to conjugate polyethylene glycol chains of various molecular weights (PEG-350, PEG-750, PEG-2K or PEG-5K) to develop hydrophilic chain containing cationic antimicrobial polymers. Polyethylene glycol chains are known to provide shielding to the nanoparticle from unwanted protein interaction and would also be helpful to increase the half-life time of the polymeric nanoparticles. These are few of the approached which can be used for development of next generation antimicrobial polymers.

6. List of Publications

1. **Ruma Ghosh**, Manickam Jayakannan, Theranostic FRET Gate to Visualize and Quantify Bacterial Membrane Breaching. [Biomacromolecules](#), 2022, *ASAP*, DOI: [10.1021/acs.biomac.2c01202](#)
2. **Ruma Ghosh**, Mehak Malhotra, Rupali Ravindra Madhuri Sathe, Manickam Jayakannan, Biodegradable Polymer Theranostic Fluorescent Nanoprobe for Direct Visualization and Quantitative Determination of Antimicrobial Activity. [Biomacromolecules](#) (2020), 21(7), 2896-2912. DOI:10.1021/acs.biomac.0c00653
3. Devesh Maurya,[†] Rahul Nisal,[†] **Ruma Ghosh**,[†] Parshuram Kambale, Mehak Malhotra, and Manickam Jayakannan, Fluorophore-Tagged Poly(L-Lysine) Block copolymer Nano-assemblies for Real-time Visualization and Antimicrobial Activity. [European Polymer Journal](#), 2023, 183, 111754. DOI: 10.1016/j.eurpolymj.2022.111754 (not included in the thesis, [†]Equal Contribution)
4. **Ruma Ghosh**, Manickam Jayakannan, Polymer Topology control towards Antimicrobial Activity and Synergistic combination Therapy in Bacteria. [Manuscript Submitted](#)
5. **Ruma Ghosh**, Manickam Jayakannan, AIE-based Lighting-up Polymer Probe for Real-time Monitoring of Bactericidal Activity. Manuscript Under Preparation
6. Rahul Nisal, **Ruma Ghosh**, Parshuram Kambale, Manickam Jayakannan. The Role of α -Helical and β -sheet Polypeptides Secondary Structures on the Bacterial Membrane Breaching Activity. [Manuscript Under Preparation](#) (not part of thesis work)

7. References

- 1) Giovannetti, L.; Viti, C. Editorial for the Special Issue: Macro and Microorganism Interactions. *Microorganisms* **2020**, *8*, 1751.
- 2) Mallon, C. A.; Elsas, J. D. V.; Salles, J. F. Microbial Invasions: The Process, Patterns, and Mechanisms. *Trends Microbiol.* **2015**, *23*, 719-729.
- 3) O'Neill, J. Tackling drug-resistant infections globally: final report and recommendations. London: *Review on Antimicrobial Resistance*, **2016**.
- 4) O'Neill J. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. London: *Review on Antimicrobial Resistance*, **2014**.
- 5) de Kraker MEA, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med* **2016**, *13*: e1002184.
- 6) Prestinaci, F.; Pezzotti, P.; Pantosti, A. Antimicrobial resistance: a global multifaceted phenomenon. *Pathog. Glob. Health* **2015**, *109*, 309–18.
- 7) WHO. Global action plan on antimicrobial resistance. Geneva: *World Health Organization* **2015**.
- 8) Naylor, N.R.; Atun, R.; Zhu, N. Estimating the burden of antimicrobial resistance: a systematic literature review. *Antimicrob. Resist. Infect. Control* **2018**, *7*, 58.
- 9) Cassini, A.; Högberg, L. D.; Plachouras, D. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* **2019**, *19*, 56–66.
- 10) Laws, M.; Shaaban, A.; Rahman, K. M. Antibiotic resistance breakers: current approaches and future directions. *FEMS Microbiology Reviews* **2019**, *43*, 490–516.
- 11) Yoneyama, H.; Katsumata, R. Antibiotic Resistance in Bacteria, and Its Future for Novel Antibiotic Development. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1060-1075.
- 12) David, M. Z.; Daum, R. S. Inherently Antimicrobial Biodegradable Polymers in Tissue Engineering of an Emerging Epidemic. *Clin. Microbiol. Rev.* **2010**, *23*, 616.
- 13) Dunachie, S. J.; Day, N. P.; Dolecek, C. The challenges of estimating the human global burden of disease of antimicrobial resistant bacteria. *Curr. Opin. Microbiol.* **2020**, *57*, 95–101.
- 14) Limmathurotsakul, D.; Dunachie, S.; Fukuda, K. Improving the estimation of the global burden of antimicrobial resistant infections. *Lancet Infect. Dis.* **2019**, *19*, 392–398.
- 15) Kapoor, G.; Saigal, S.; Elongavan, A. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J. Anaesthesiol. Clin. Pharmacol.* **2017**, *33*, 300-305.
- 16) Bell, B. G.; Schellevis, F.; Stobberingh, E.; Goossens, H.; Pringle, M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. *BMC Infect. Dis.* **2014**, *14*, 13.
- 17) Blair, J. M. A.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **2015**, *13*, 42-51.
- 18) Hiramatsu, K.; Hanaki, H.; Ino, T.; Yabuta, K.; Oguri, T.; Tenover, F. C. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.* **1997**, *40*, 135–136.
- 19) Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S. & Vester, B. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides,

- oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob. Agents Chemother.* **2006**, *50*, 2500–2505.
- 20) Correia, S.; Poeta, P.; Ebraud, M. H.; Luis Capelo, J. & Igrejas, G. Mechanisms of quinolone action and resistance: where do we stand? *J. Med. Microbiol.* **2017**, *66*, 551–559.
 - 21) Aldred, K. J.; Kerns, R. J.; Osherof, N. Mechanism of Quinolone Action and Resistance. *Biochemistry* **2014**, *53*, 1565–1574.
 - 22) Adams, R. A.; Leon, G.; Miller, N. M.; Reyes, S. P.; Thantrong, C. H.; Thokkadam, A. M.; Lemma, A. S.; Sivaloganathan, D. M.; Wan, X.; Brynildsen, M. P. Rifamycin antibiotics and the mechanisms of their failure. *J. Antibiot.* **2021**, *74*, 786–798.
 - 23) Ovung, A.; Bhattacharyya, J. Sulfonamide drugs: structure, antibacterial property, toxicity, and biophysical interactions. *Biophys Rev.* **2021**, *13*, 259–272.
 - 24) Corcione, S.; Lupia, T.; Rosa, F. G. D. Novel Cephalosporins in Septic Subjects and Severe Infections: Present Findings and Future Perspective. *Front. Med.* **2021**, *8*, 1-9.
 - 25) Velkov, T.; Thompson, P. E.; Nation, R. L.; Li, J. Structure-Activity Relationships of Polymyxin Antibiotics. *J. Med. Chem.* **2010**, *53*, 1898–1916.
 - 26) Cho, H.; Uehara, T.; Bernhardt, T. G. Beta-Lactam Antibiotics Induce a Lethal Malfunctioning of the Bacterial Cell Wall Synthesis Machinery. *Cell* **2014**, *159*, 1300–1311.
 - 27) Manioglu, S.; Modaresi, S. M.; Ritzmann, N.; Thoma, J.; Overall, S. A.; Harms, A.; Upert, G.; Luther, A.; Barnes, A. B.; Obrecht, D.; Müller, D. J.; Hiller, S. Antibiotic polymyxin arranges lipopolysaccharide into crystalline structures to solidify the bacterial membrane. *Nat. Commun.* **2022**, *13*, 6195.
 - 28) Stone, M. R. L.; Butler, M. S.; Phetsang, W.; Cooper, M. A.; Blaskovich, M. A. T. Fluorescent Antibiotics: New Research Tools to Fight Antibiotic Resistance. *Trends Biotechnol.* **2018**, *36*, 523–536.
 - 29) Phetsang, W.; Pelingon, R.; Butler, M. S.; KC, S.; Pitt, M. E.; Kaeslin, G.; Cooper, M. A.; Blaskovich, M. A. T. Fluorescent Trimethoprim Conjugate Probes to Assess Drug Accumulation in Wild Type and Mutant Escherichia coli. *ACS Infect. Dis.* **2016**, *2*, 688–701.
 - 30) Matijašić, M.; Kos, V. M.; Nujic, K.; Čučić, S.; Padovan, J.; Kragol, G.; Alihodžić, S.; Mildner, B.; Verbanac, D.; Haber, V. E. Fluorescently labeled macrolides as a tool for monitoring cellular and tissue distribution of azithromycin. *Pharmacol. Res.* **2012**, *66*, 332–342.
 - 31) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389–395.
 - 32) Steiner, H.; Hultmark, D.; Engstrom, A.; Bennich, H.; Boman, H. G. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **1981**, *292*, 246–268.
 - 33) Simmaco, M.; Mignogna, G.; Barra, D. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* **1998**, *47*, 435–450.
 - 34) Shai, Y. Mechanism of the binding, insertion, and destabilization of phospholipid bilayer membranes by α -helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim. Biophys. Acta* **1999**, *1462*, 55–70.

- 35) Bechinger, B.; Zasloff, M.; Opella, S. J. Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. *Protein Sci.* **1993**, *2*, 2077±2084.
- 36) Zasloff, M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl Acad. Sci. USA* **1987**, *84*, 5449-5453.
- 37) Mahlapuu, M.; Håkansson, J.; Ringstad, L.; Björn, C. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. *Front. Cell. Infect. Microbiol.* **2016**, *6*, 1-12.
- 38) Hancock, R. E. W.; Sahl, H-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotech.* **2006**, *24*, 1551-1557.
- 39) Meng, H.; Kumar, K. Antimicrobial Activity and Protease Stability of Peptides Containing Fluorinated Amino Acids. *J. Am. Chem. Soc.* **2007**, *129*, 15615-15622.
- 40) Ghosh, C.; Sarkar, P.; Issa, R.; Halder, J. Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. *Trends Microbiol.* **2019**, *27*, 323-338.
- 41) Pranantyo, D.; Zhang, K.; Si, Z.; Hou, Z.; Chan-Park, M. B. Smart Multifunctional Polymer Systems as Alternatives or Supplements of Antibiotics to Overcome Bacterial Resistance. *Biomacromolecules* **2022**, *23*, 1873–1891.
- 42) Si, Z.; Zheng, W.; Pranantyo, D.; Li, J.; Koh, C. H.; Kang, E-T.; Pethe, K.; Chan-Park, M. B. Polymers as advanced antibacterial and antibiofilm agents for direct and combination therapies. *Chem. Sci.* **2022**, *13*, 345–364.
- 43) Ergene, C.; Yasuhara, K.; Palermo, E. F. Biomimetic antimicrobial polymers: recent advances in molecular design. *Polym. Chem.* **2018**, *9*, 2407–2427.
- 44) Jing, Z-W.; Jia, Y-Y.; Wan, N.; Luo, M.; Huan, M-L.; Kang, T-B.; Zhou, S-Y.; Zhang, B-L. Design and evaluation of novel pH-sensitive ureido-conjugated chitosan/TPP nanoparticles targeted to *Helicobacter pylori*. *Biomaterials* **2016**, *84*, 276-285.
- 45) Ghosh, S.; Mukherjee, S.; Patra, D.; Halder, J. Polymeric Biomaterials for Prevention and Therapeutic Intervention of Microbial Infections. *Biomacromolecules* **2022**, *23*, 592–608.
- 46) Erathodiyil, N.; Chan, H.-M.; Wu, H.; Ying, J. Y. Zwitterionic Polymers and Hydrogels for Antibiofouling Applications in Implantable Devices. *Mater. Today* **2020**, *38*, 84–98.
- 47) Su, Y.; Feng, T.; Feng, W.; Pei, Y.; Li, Z.; Huo, J.; Xie, C.; Qu, X.; Li, P.; Huang, W. Mussel-Inspired, Surface-Attachable Initiator for Grafting of Antimicrobial and Antifouling Hydrogels. *Macromol. Rapid Commun.* **2019**, *40*, 1900268.
- 48) Yu, L.; Li, K.; Zhang, J.; Jin, H.; Saleem, A.; Song, Q.; Jia, Q.; Li, P. Antimicrobial Peptides and Macromolecules for Combating Microbial Infections: From Agents to Interfaces. *ACS Appl. Bio Mater.* **2022**, *5*, 366–393
- 49) Wang, H.; Chen, Z.; Cheng, S.; Li, R.; Pan, X.; Zhang, C.; Gu, H.; Xie, A.; Dong, W. Synthesis of cationic hydrogels with tunable physicochemical properties for antibacterial applications. *Eur. Polym. J.* **2022**, *173*, 111228.
- 50) Gao, J.; White, E. M.; Liu, Q.; Locklin, J. Evidence for the Phospholipid Sponge Effect as the Biocidal Mechanism in Surface-Bound Polyquaternary Ammonium Coatings with Variable Cross-Linking Density. *ACS Appl. Mater. Interfaces* **2017**, *9*, 7745–7751.

- 51) Takahashi, H.; Nadres, E. T.; Kuroda, K. Cationic Amphiphilic Polymers with Antimicrobial Activity for Oral Care Applications: Eradication of *S. mutans* Biofilm. *Biomacromolecules* **2017**, *18*, 257–265.
- 52) Xu, G.; Liu, X.; Liu, P.; Pranantyo, D.; Neoh, K-G.; Kang, E-T. Arginine-Based Polymer Brush Coatings with Hydrolysis-Triggered Switchable Functionalities from Antimicrobial (Cationic) to Antifouling (Zwitterionic). *Langmuir* **2017**, *33*, 6925–6936.
- 53) Santos, M. R. E.; Mendonca, P. V.; Branco, R.; Sousa, R.; Dias, C.; Serra, A. C.; Fernandes, J. R.; Magalhães, F. D.; Morais, P. V.; Coelho, J. F. J. Light-Activated Antimicrobial Surfaces Using Industrial Varnish Formulations to Mitigate the Incidence of Nosocomial Infections. *ACS Appl. Mater. Interfaces* **2021**, *13*, 7567–7579.
- 54) Chen, Z.; Zhang, W.; Chen, Y.; Wang, Y.; Bai, S.; Cai, Q.; Pu, H.; Wang, Z.; Feng, X.; Bai, Y. Alternatingly Amphiphilic Antimicrobial Oligoguanidines: Structure–Property Relationship and Usage as the Coating Material with Unprecedented Hemocompatibility. *Chem. Mater.* **2022**, *34*, 3670–3682.
- 55) Namivandi-Zangeneh, R.; Yang, Y.; Xu, S.; Wong, E. H. H.; Boyer, C. Antibiofilm Platform based on the Combination of Antimicrobial Polymers and Essential Oils. *Biomacromolecules* **2020**, *21*, 262–272.
- 56) Thappeta, K. R. V.; Vikhe, Y. S.; Yong, A. M. H.; Chan-Park, M. B.; Kline, K. A. Combined Efficacy of an Antimicrobial Cationic Peptide Polymer with Conventional Antibiotics to Combat Multidrug-Resistant Pathogens. *ACS Infect. Dis.* **2020**, *6*, 1228–1237.
- 57) Si, Z.; Hou, Z.; Vikhe, Y. S.; Thappeta, K. R. V.; Marimuthu, K.; De, P. P.; Ng, O. T.; Li, P.; Zhu, Y.; Pethe, K.; Chan-Park, M. B. Antimicrobial Effect of a Novel Chitosan Derivative and Its Synergistic Effect with Antibiotics. *ACS Appl. Mater. Interfaces* **2021**, *13*, 3237–3245.
- 58) Pranantyo, D.; Zhang, K.; Si, Z.; Hou, Z.; Chan-Park, M. B. Smart Multifunctional Polymer Systems as Alternatives or Supplements of Antibiotics to Overcome Bacterial Resistance. *Biomacromolecules* **2022**, *23*, 1873–1891.
- 59) Zhen, J. B.; Yi, J.; Ding, H. H.; Yang, K-W. Self-Assembled Cationic Nanoparticles Combined with Curcumin against Multidrug-Resistant Bacteria. *ACS Omega* **2022**, *7*, 29909–29922.
- 60) Perez-Soto, N.; Moule, L.; Crisan, D. N.; Insua, I.; Taylor-Smith, L. M.; Voelz, K.; Fernandez-Trillo, F.; Krachler, A. M. Engineering microbial physiology with synthetic polymers: cationic polymers induce biofilm formation in *Vibrio cholerae* and downregulate the expression of virulence genes. *Chem. Sci.* **2017**, *8*, 5291.
- 61) Namivandi-Zangeneh, R.; Sadrearhami, Z.; Bagheri, A.; Sauvage-Nguyen, M.; Ho, K. K. K.; Kumar, N.; Wong, E. H. H.; Boyer, C. Nitric Oxide-Loaded Antimicrobial Polymer for the Synergistic Eradication of Bacterial Biofilm. *ACS Macro Lett.* **2018**, *7*, 592–597.
- 62) Gupta, A.; Landis, R. F.; Li, C-H.; Schnurr, M.; Das, R.; Lee, Y-W.; Yazdani, M.; Liu, Y.; Kozlova, A.; Rotello, V. M. Engineered Polymer Nanoparticles with Unprecedented Antimicrobial Efficacy and Therapeutic Indices against Multidrug Resistant Bacteria and Biofilms. *J. Am. Chem. Soc.* **2018**, *140*, 12137–12143.

- 63) Zhao, S.; Huang, W.; Wang, C.; Wang, Y.; Zhang, Y.; Ye, Z.; Zhang, J.; Deng, L.; Dong, A. Screening and Matching Amphiphilic Cationic Polymers for Efficient Antibiosis. *Biomacromolecules* **2020**, *21*, 5269–5281.
- 64) Lv, Y.; Wang, J.; Gao, H.; Wang, Z.; Dong, N.; Ma, Q.; Shan, A. Antimicrobial Properties and Membrane-Active Mechanism of a Potential α -Helical Antimicrobial Derived from Cathelicidin PMAP-36. *PLoS One* **2014**, *9*, e86364.
- 65) Lombardi, L.; Shi, Y.; Falanga, A.; Galdiero, E.; Alteriis, E. D.; Franci, G.; Chourpa, I.; Azevedo, H. S.; Galdiero, S. Enhancing the Potency of Antimicrobial Peptides through Molecular Engineering and Self-Assembly. *Biomacromolecules* **2019**, *20*, 1362–1374.
- 66) Nathalia Rodrigues de Almeida,[†] Han, Y.; Perez, J.; Kirkpatrick, S.; Wang, Y.; Sheridan, M. C. Design, Synthesis, and Nanostructure-Dependent Antibacterial Activity of Cationic Peptide Amphiphiles. *ACS Appl. Mater. Interfaces* **2019**, *11*, 2790–2801.
- 67) She, F.; Nimmagadda, A.; Teng, P.; Su, M.; Zuo, X.; Cai, J. Helical 1:1 α /Sulfon- γ -AA Heterogeneous Peptides with Antibacterial Activity. *Biomacromolecules* **2016**, *17*, 1854–1859.
- 68) Barras, A.; Martin, F. A.; Bande, O.; Baumann, J. S.; Ghigo, J. M.; Boukherroub, R.; Beloin, C.; Siriwardena, A.; Szunerits, S. Glycan functionalized diamond nanoparticles as potent *E. coli* anti-adhesives. *Nanoscale* **2013**, *5*, 2307–2316.
- 69) Kuhaudomlarp, S.; Siebs, E.; Shanina, E.; Topin, J.; Joachim, I.; da Silva Figueiredo Celestino Gomes, P.; Varrot, A.; Rognan, D.; Rademacher, C.; Imberty, A.; Titz, A. Non-Carbohydrate Glycomimetics as Inhibitors of Calcium(II)-Binding Lectins. *Angew. Chem., Int. Ed.* **2021**, *60*, 8104–8114.
- 70) Mitra, M.; Asad, M.; Kumar, S.; Yadav, K.; Chaudhary, S.; Bhavesh, N. S.; Khalid, S.; Thukral, L.; Bajaj, A. Distinct Intramolecular Hydrogen Bonding Dictates Antimicrobial Action of Membrane Targeting Amphiphiles. *J. Phys. Chem. Lett.* **2019**, *10*, 754–760.
- 71) Singh, S.; Papareddy, P.; Mörgelin, M.; Schmidtchen, A.; Malmsten, M. Effects of PEGylation on Membrane and Lipopolysaccharide Interactions of Host Defense Peptides. *Biomacromolecules* **2014**, *15*, 1337–1345.
- 72) Waxman, D. J.; Strominger, J. L. Penicillin-Binding Proteins and the Mechanism of Action of Beta-Lactam Antibiotics. *Annu. Rev. Biochem.* **1983**, *52*, 825–869.
- 73) Liang, H.; DeMeester, K. E.; Hou, C. W.; Parent, M. A.; Caplan, J. L.; Grimes, C. L. Metabolic labelling of the carbohydrate core in bacterial peptidoglycan and its applications. *Nat. Commun.* **2017**, *8*, 15015.
- 74) Li, P.; Zhou, C.; Rayatpisheh, S.; Ye, K.; Poon, Y. F.; Hammond, P. T.; Duan, H. W.; Chan-Park, M. B. Cationic Peptidopolysaccharides Show Excellent Broad-Spectrum Antimicrobial Activities and High Selectivity. *Adv. Mater.* **2012**, *24*, 4130–4137.
- 75) He, J.-X.; Le Mai Hoang, K.; Kho, S. H.; Guo, Z.; Zhong, W.; Venkata Thappeta, K. R.; Zamudio-Vazquez, R.; Hoo, S. N.; Xiong, Q.; Duan, H.; et al. Synthetic biohybrid peptidoglycan oligomers enable pan-bacteria-specific labeling and imaging: in vitro and in vivo. *Chem. Sci.* **2020**, *11*, 3171–3179.
- 76) Su, Y.-R.; Yu, S.-H.; Chao, A.-C.; Wu, J.-Y.; Lin, Y.-F.; Lu, K.-Y.; Mi, F.-L. Preparation and properties of pH-responsive, self-assembled colloidal nanoparticles

- from guanidine-containing polypeptide and chitosan for antibiotic delivery. *Colloids Surf.* **2016**, 494, 9–20.
- 77) Lu, S.; Bi, W.; Du, Q.; Sinha, S.; Wu, X.; Subrata, A.; Bhattacharjya, S.; Xing, B.; Yeow, E. K. Lipopolysaccharide-affinity copolymer senses the rapid motility of swarmer bacteria to trigger antimicrobial drug release. *Nat. Commun.* **2018**, 9, 4277.
 - 78) Cavaillon, J.-M. Exotoxins and endotoxins: Inducers of inflammatory cytokines. *Toxicon* **2018**, 149, 45–53.
 - 79) Addis, M. F.; Pisanu, S.; Monistero, V.; Gazzola, A.; Penati, M.; Filipe, J.; Di Mauro, S.; Cremonesi, P.; Castiglioni, B.; Moroni, P.; Pagnozzi, D.; Tola, S.; Piccinini, R. Comparative secretome analysis of *Staphylococcus aureus* strains with different within-herd intramammary infection prevalence. *Virulence* **2022**, 13, 174–190.
 - 80) Qiu, L.; Wang, C.; Lei, X.; Du, X.; Guo, Q.; Zhou, S.; Cui, P.; Hong, T.; Jiang, P.; Wang, J.; Li, Y.-Q.; Xia, J. Gelatinase-responsive release of an antibacterial photodynamic peptide against *Staphylococcus aureus*. *Biomater. Sci.* **2021**, 9, 3433–3444.
 - 81) Foster, T. J.; Geoghegan, J. A.; Ganesh, V. K.; Höök, M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol.* **2014**, 12, 49–62.
 - 82) Tam, K.; Torres, V. J. *Staphylococcus aureus* secreted toxins and extracellular enzymes. *Gram-Positive Pathogens* **2019**, 640–668.
 - 83) Holmes, A. H.; Moore, L. S. P.; Sundsfjord, A.; Steinbakk, M.; Regmi, S.; Karkey, A.; Guerin, P. J.; Piddock, L. J. V. Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet* **2016**, 387, 176–187.
 - 84) Webber, M.; Piddock, L. The importance of efflux pumps in bacterial antibiotic resistance. *J. Antimicrob. Chemother.* **2003**, 51, 9–11.
 - 85) Sun, J.; Deng, Z.; Yan, A. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem. Biophys. Res. Commun.* **2014**, 453, 254–267.
 - 86) Si, Z.; Lim, H. W.; Tay, M. Y.; Du, Y.; Ruan, L.; Qiu, H.; Zamudio-Vazquez, R.; Reghu, S.; Chen, Y.; Tiong, W. S. A Glycosylated Cationic Block Poly(β -peptide) Reverses Intrinsic Antibiotic Resistance in All ESKAPE Gram-Negative Bacteria. *Angew. Chem.* **2020**, 132, 6886–6893.
 - 87) Barman, S.; Mukherjee, S.; Ghosh, S.; Halder, J. Amino-Acid-Conjugated Polymer-Rifampicin Combination: Effective at Tackling Drug-Resistant Gram-Negative Clinical Isolates. *ACS Appl. Bio Mater.* **2019**, 2, 5404–5414.
 - 88) Namivandi-Zangeneh, R.; Sadrearhami, Z.; Dutta, D.; Willcox, M.; Wong, E. H.; Boyer, C. Synergy between Synthetic Antimicrobial Polymer and Antibiotics: A Promising Platform To Combat Multidrug-Resistant Bacteria. *ACS Infect. Dis.* **2019**, 5, 1357–1365.
 - 89) Si, Z.; Hou, Z.; Vikhe, Y. S.; Thappeta, K. R. V.; Marimuthu, K.; De, P. P.; Ng, O. T.; Li, P.; Zhu, Y.; Pethe, K. Antimicrobial Effect of a Novel Chitosan Derivative and Its Synergistic Effect with Antibiotics. *ACS Appl. Mater. Interfaces* **2021**, 13, 3237–3245.
 - 90) Nakae, T.; Nakajima, A.; Ono, T.; Saito, K.; Yoneyama, H. Resistance to β -Lactam Antibiotics in *Pseudomonas aeruginosa* Due to Interplay between the MexAB-

- OprM Efflux Pump and β -Lactamase. *Antimicrob. Agents Chemother.* **1999**, *43*, 1301–1303.
- 91) Zhong, P.; Shortridge, V. D. The role of efflux in macrolide resistance. *Drug Resist. Updates* **2000**, *3*, 325–329.
 - 92) Li, X.-Z.; Livermore, D. M.; Nikaido, H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob. Agents Chemother.* **1994**, *38*, 1732–1741.
 - 93) Mahamoud, A.; Chevalier, J.; Alibert-Franco, S.; Kern, W. V.; Pages, J.-M.; Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J. Antimicrob. Chemother.* **2007**, *59*, 1223–1229.
 - 94) Köhler, T.; Kok, M.; Michea-Hamzehpour, M.; Plesiat, P.; Gotoh, N.; Nishino, T.; Curty, L. K.; Pechere, J.-C. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **1996**, *40*, 2288–2290.
 - 95) Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 382–388.
 - 96) Goldberg, K.; Sarig, H.; Zaknoon, F.; Epand, R. F.; Epand, R. M.; Mor, A. Sensitization of gram-negative bacteria by targeting the membrane potential. *FASEB J.* **2013**, *27*, 3818–3826.
 - 97) Flemming, H.-C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S. A.; Kjelleberg, S. Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **2016**, *14*, 563.
 - 98) Blackman, L. D.; Qu, Y.; Cass, P.; Locock, K. E. Approaches for the inhibition and elimination of microbial biofilms using macromolecular agents. *Chem. Soc. Rev.* **2021**, *50*, 1587–1616.
 - 99) Houry, A.; Gohar, M.; Deschamps, J.; Tischenko, E.; Aymerich, S.; Gruss, A.; Briandet, R. Bacterial swimmers that infiltrate and take over the biofilm matrix. *Proc. Natl. Acad. Sci.* **2012**, *109*, 13088–13093.
 - 100) Sharma, D.; Misba, L.; Khan, A. U. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrob. Resist. Infect. Control.* **2019**, *8*, 1–10.
 - 101) Srinivasan, R.; Santhakumari, S.; Poonguzhali, P.; Geetha, M.; Dyavaiah, M.; Xiangmin, L. Bacterial Biofilm Inhibition: A Focused Review on Recent Therapeutic Strategies for Combating the Biofilm Mediated Infections. *Front. Microb.* **2021**, *12*, 1106.
 - 102) Sahariah, P.; Masson, M.; Meyer, R. L. Quaternary Ammoniumyl Chitosan Derivatives for Eradication of *Staphylococcus aureus* Biofilms. *Biomacromolecules* **2018**, *19*, 3649–3658.
 - 103) Takahashi, H.; Nadres, E. T.; Kuroda, K. Cationic Amphiphilic Polymers with Antimicrobial Activity for Oral Care Applications: Eradication of *S. mutans* Biofilm. *Biomacromolecules* **2016**, *18*, 257–265.
 - 104) Nguyen, T.-K.; Lam, S. J.; Ho, K. K. K.; Kumar, N.; Qiao, G. G.; Egan, S.; Boyer, C.; Wong, E. H. H. Rational Design of Single-Chain Polymeric Nanoparticles That Kill Planktonic and Biofilm Bacteria. *ACS Infect. Dis.* **2017**, *3*, 237–248.
 - 105) Li, J.; Zhang, K.; Ruan, L.; Chin, S. F.; Wickramasinghe, N.; Liu, H.; Ravikumar, V.; Ren, J.; Duan, H.; Yang, L.; Chan-Park, M. B. Block Copolymer Nanoparticles

- Remove Biofilms of Drug-Resistant Gram-Positive Bacteria by Nanoscale Bacterial Debridement. *Nano Lett.* **2018**, *18*, 4180–4187.
- 106) Vishwakarma, A.; Dang, F.; Ferrell, A.; Barton, H. A.; Joy, A. Peptidomimetic Polyurethanes Inhibit Bacterial Biofilm Formation and Disrupt Surface Established Biofilms. *J. Am. Chem. Soc.* **2021**, *143*, 9440–9449.
 - 107) Zhong, Y.; Godwin, P.; Jin, Y.; Xiao, H. Biodegradable polymers and green-based antimicrobial packaging materials: A mini-review. *Adv. Ind. Manuf.* **2020**, *3*, 27-35.
 - 108) Watson, E.; Tataru, A. M.; Kontoyiannis, D. P.; Mikos, A. G. A Macromolecular Approach to Eradicate Multidrug Resistant Bacterial Infections While Mitigating. *ACS Biomater. Sci. Eng.* **2017**, *3*, 1207–1220.
 - 109) Su, L-C.; Xie, Z.; Zhang, Y.; Nguyen, K. T.; Yang, J. Study on the antimicrobial properties of citrate-based biodegradable polymers. *Front. bioeng. biotechnol.* **2014**, *2*, 1-9.
 - 110) Mohanan, N.; Montazer, Z.; Sharma, P. K.; Levin, D. B. Microbial and Enzymatic Degradation of Synthetic Plastics. *Front. Microbiol.* **2020**, *11*, 1-22.
 - 111) Bano, R. K.; Kuddus, M.; Zaheer, M. R.; Zia, Q.; Khan, M. F.; Ashraf, G. M.; Gupta, A.; Aliev, G. *Curr. Pharm. Biotechnol.* **2017**, *18*, 429-440.
 - 112) Li, Y.; Liu, G.; Wang, X.; Hu, J.; Liu, S. Enzyme Responsive Polymeric Vesicles for Bacterial-Strain-Selective Delivery of Antimicrobial Agents. *Angew. Chem. Int. Ed.* **2016**, *55*, 1760–1764.
 - 113) Pathak, V. M.; Navneet. Review on the current status of polymer degradation: a microbial approach. *Bioresour. Bioprocess.* **2017**, *4*, 1-31.
 - 114) Deming, T. J. Synthesis of Side-Chain Modified Polypeptides. *Chem. Rev.* **2016**, *116*, 786–808.
 - 115) Wickremasinghe, H.; Yu, H. H.; Azad, M. A. K.; Zhao, J.; Bergen, P. J.; Velkov, T.; Zhou, Q. T.; Zhu, Y.; Li, J. Clinically Relevant Concentrations of Polymyxin B and Meropenem Synergistically Kill Multidrug-Resistant *Pseudomonas aeruginosa* and Minimize Biofilm Formation. *Antibiotics* **2021**, *10*, 405.
 - 116) Gupta, S.; Kapoor, P.; Chaudhary, K.; Gautam, A.; Kumar, R.; Raghava, G. P. S. In Silico Approach for Predicting Toxicity of Peptides and Proteins. *PLoS One.* **2013**, *8*, e73957.
 - 117) Hyldgaard, M.; Mygind, T.; Vad, B. S.; Stenvang, M.; Otzen, D. E.; Meyera, R. L. The Antimicrobial Mechanism of Action of Epsilon-Poly-L-Lysine. *Appl. Environ. Microbiol.* **2014**, *80*, 7758-7770.
 - 118) Zhao, R.; Wang, H.; Ji, T.; Anderson, G.; Nie, G.; Zhao, Y. Biodegradable cationic e-poly-L-lysine-conjugated polymeric nanoparticles as a new effective antibacterial agent. *Sci. Bull.* **2015**, *60*, 216–226.
 - 119) Zhou, C.; Li, P.; Qi, X.; Sharif, A. R. M.; Poon, Y. F.; Cao, Y.; Chang, M. W.; Leong, S. S. J.; Chan-Park, M. B. A photopolymerized antimicrobial hydrogel coating derived from epsilon-poly-L-lysine. *Biomaterials* **2011**, *32*, 2704e2712.
 - 120) Zhou, X.; He, J.; Zhou, C. Strategies from nature: polycaprolactone-based mimetic antimicrobial peptide block copolymers with low cytotoxicity and excellent antibacterial efficiency. *Polym. Chem.* **2019**, *10*, 945.
 - 121) Wang, M.; Zhou, C.; Chen, J.; Xiao, Y.; Du, J. Multifunctional Biocompatible and Biodegradable Folic Acid Conjugated Poly(ϵ -caprolactone)–Polypeptide Copolymer Vesicles with Excellent Antibacterial Activities. *Bioconjugate Chem.* **2015**, *26*, 4, 725–734.

- 122) Mutschler, A.; Tallet, L.; Rabineau, M.; Dollinger, C.; Metz-Boutigue, M-H.; Schneider, F.; Senger, B.; Vrana, N. E.; Schaaf, P.; Lavalley, P. Unexpected Bactericidal Activity of Poly(arginine)/Hyaluronan Nanolayered Coatings. *Chem. Mater.* **2016**, *28*, 8700–8709.
- 123) Li, J.; Koh, J-J.; Liu, S.; Lakshminarayanan, R.; Verma, C. S.; Beuerman, R. W.; Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. *Front. Neurosci.* **2017**, *11*, 1-18.
- 124) Artham, T.; Doble, M. Biodegradation of Aliphatic and Aromatic Polycarbonates. *Macromol. Biosci.* **2008**, *8*, 14–24.
- 125) Suyama, T.; Tokiwa, Y. Enzymatic degradation of an aliphatic polycarbonate, poly(tetramethylene carbonate). *Enzyme Microb. Technol.* **1997**, *20*, 122-126.
- 126) Nederberg, F.; Zhang, Y.; Tan, J. P. K.; Xu, K.; Wang, H.; Yang, C.; Gao, S.; Guo, X. D.; Fukushima, K.; Li, L.; Hedrick, J. L.; Yang, Y-Y. Biodegradable nanostructures with selective lysis of microbial membranes. *Nat. Chem.* **2011**, *3*, 409-414.
- 127) Chin, W.; Zhong, G.; Pu, Q.; Yang, C.; Lou, W.; De Sessions, P. F.; Periaswamy, B.; Lee, A.; Liang, Z. C.; Ding, X.; Gao, S.; Chu, C. W.; Bianco, S.; Bao, C.; Tong, Y. W.; Fan, W.; Wu, M.; Hedrick, J. L.; Yang, Y. Y. A Macromolecular Approach to Eradicate Multidrug Resistant Bacterial Infections While Mitigating Drug Resistance Onset. Synthesis of Antibacterial Glycosylated Polycaprolactones Bearing Imidazoliums with Reduced. *Nat. Commun.* **2018**, *9*, 917.
- 128) Makadia, H. K.; Siegel, S. J. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers* **2011**, *3*, 1377-1397.
- 129) Zhang, Z.; Tang, J.; Wang, H.; Xia, Q.; Xu, S.; Han, C. C. Controlled Antibiotics Release System through Simple Blended Electrospun Fibers for Sustained Antibacterial Effects. *ACS Appl. Mater. Interfaces* **2015**, *7*, 48, 26400–26404.
- 130) Xi, Y.; Song, T.; Tang, S.; Wang, N.; Du, J. Preparation and Antibacterial Mechanism Insight of Polypeptide-Based Micelles with Excellent Antibacterial activities. *Biomacromolecules* **2016**, *17*, 3922–3930.
- 131) Reinbold, J.; Hierlemann, T.; Hinkel, H.; Müller, I.; Maier, M. E.; Weindl, T.; Schlensak, C.; Wendel, H. P.; Krajewski, S. Development and in vitro characterization of poly(lactide-co-glycolide) microspheres loaded with an antibacterial natural drug for the treatment of long-term bacterial infections. *Drug. Des. Devel. Ther.* **2016**, *10*, 2823–2832.
- 132) Sardo, C.; Nottelet, B.; Triolo, D.; Giammona, G.; Garric, X.; Lavigne, J-P.; Cavallaro, G.; Coudane, J. When Functionalization of PLA Surfaces Meets Thiol–Yne Photochemistry: Case Study with Antibacterial Polyaspartamide Derivatives. *Biomacromolecules* **2014**, *15*, 4351–4362.
- 133) Ulbrich, K.; Hola, K.; Šubr, V.; Bakandritsos, A.; Tucek, J.; Zboril, R. Targeted Drug Delivery with Polymers and Magnetic Nanoparticles: Covalent and Noncovalent Approaches, Release Control, and Clinical Studies. *Chem. Rev.* **2016**, *116*, 5338–5431.
- 134) Zhen, J-B.; Kang, P-W.; Zhao, M-H.; Yang, K-W. Silver Nanoparticle Conjugated Star PCL-b-AMPs Copolymer as Nanocomposite Exhibits Efficient Antibacterial Properties. *Bioconjugate Chem.* **2020**, *31*, 51–63.
- 135) Teo, E. Y.; Ong, S-Y.; Chong, M. S. K.; Zhang, Z.; Lu, J.; Moomhala, S.; Ho, B.; Teoh, S-H. Polycaprolactone-based fused deposition modeled mesh for delivery of antibacterial agents to infected wounds. *Biomaterials* **2011**, *32*, 279-287.

- 136) Woo, G. L. Y.; Mittelman, M. W.; Santerre, J. P. Synthesis and characterization of a novel biodegradable antimicrobial polymer. *Biomaterials* **2000**, *21*, 1235-1246.
- 137) Xu, Y.; Zhang, K.; Reghu, S.; Lin, Y.; Chan-Park, M. B.; Liu, X-W. Synthesis of Antibacterial Glycosylated Polycaprolactones Bearing Imidazoliums with Reduced Hemolytic Activity. *Biomacromolecules* **2019**, *20*, 949–958.
- 138) Grossen, P.; Witzigmann, D.; Sieber, S.; Huwyler, J. PEG-PCL-based nanomedicines: A biodegradable drug delivery system and its application. *J. Control Release* **2017**, *260*, 46-60.
- 139) Maraldi, M.; Guida, A.; Sponchioni, M.; Moscatelli, D. Influence of the Polymer Architecture and Functionalization with Carboxylate Groups over the Release of Octenidine Hydrochloride from Poly(lactic acid)-Based Nanoparticles. *Macromol. Mater. Eng.* **2020**, 2000592.
- 140) Omoloia, C. A.; Kalhapurea, R. S.; Agrawala, N.; Jadhava, M.; Rambharosea, S.; Mocktara, C.; Govendera, T. A hybrid of mPEG-b-PCL and G1-PEA dendrimer for enhancing delivery of antibiotics. *J. Control Release* **2018**, *290*, 112–128.
- 141) Talacua, H.; Söntjens, S. H. M.; Thakkar, S. H.; Brizard, A. M. A.; Herwerden, L. A. V.; Vink, A.; Almen, G. C. V.; Dankers, P. Y. W.; Bouten, C. V. C.; Budde, R. P. J.; Janssen, H. M.; Kluin, J. Imaging the In Vivo Degradation of Tissue Engineering Implants by Use of Supramolecular Radiopaque Biomaterials. *Macromol. Biosci.* **2020**, *20*, 2000024.
- 142) Surnar, B.; Jayakannan, M. Stimuli-Responsive Poly(caprolactone) Vesicles for Dual Drug Delivery under the Gastrointestinal Tract. *Biomacromolecules* **2013**, *14*, 4377–4387.
- 143) Surnar, B.; Sharma, K.; Jayakannan, M. Core–shell polymer nanoparticles for prevention of GSH drug detoxification and cisplatin delivery to breast cancer cells. *Nanoscale* **2015**, *7*, 17964.
- 144) Surnar, B.; Jayakannan, M. Triple Block Nanocarrier Platform for Synergistic Cancer Therapy of Antagonistic Drugs. *Biomacromolecules* **2016**, *17*, 4075–4085.
- 145) Kulkarni, B.; Surnar, B.; Jayakannan, M. Dual Functional Nanocarrier for Cellular Imaging and Drug Delivery in Cancer Cells Based on π -Conjugated Core and Biodegradable Polymer Arms. *Biomacromolecules* **2016**, *17*, 1004–1016.
- 146) Kulkarni, B. B.; Malhotra, M.; Jayakannan, M. Perylene-Tagged Polycaprolactone Block Copolymers and Their Enzyme-Biodegradable Fluorescent Nanoassemblies for Intracellular Bio-imaging in Cancer Cells. *ACS Appl. Polym. Mater.* **2019**, *1*, 12, 3375–3388.
- 147) Malhotra, M.; Surnar, B.; Jayakannan, M. Polymer Topology Driven Enzymatic Biodegradation in Polycaprolactone Block and Random Copolymer Architectures for Drug Delivery to Cancer Cells. *Macromolecules* **2016**, *49*, 8098–8112.
- 148) Pranav, U.; Malhotra, M.; Pathan, S.; Jayakannan, M. Structural Engineering of Star Block Biodegradable Polymer Unimolecular Micelles for Drug Delivery in Cancer Cells. ASAP article *ACS Biomater. Sci. Eng.* 2022, doi: <https://doi.org/10.1021/acsbiomaterials.2c01201>.
- 149) Spellberg, B.; Guidos, R.; Gilbert, D. Bradley, J.; Boucher, H. W.; Scheld, W. M.; Bartlett, J. G.; Edward, J, Jr. The Epidemic of Antibiotic-Resistant Infections: A Call to Action for the Medical Community for the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2008**, *46*, 155-164.

- 150) Yoneyama, H.; Katsumata, R. Antibiotic Resistance in Bacteria and its Future for Novel Antibiotic Development. *Biosci. Biotechno. Biochem.* **2006**, *70*, 1060-1075.
- 151) Nathan, C. Antibiotics at the Crossroads, *Nature* **2004**, *431*, 899-902.
- 152) Li, Y.-Q.; Zhu, B.; Li, Y.; Leow, W. R.; Goh, R.; Ma, B.; Fong, E.; Tang, M.; Chen, X. A Synergistic Capture Strategy for Enhanced Detection and Elimination of Bacteria. *Angew. Chem. Int. Ed.* **2014**, *53*, 5837-5841.
- 153) Kelkar, S. S.; Reineke, T. M. Theranostics: Combining Imaging and Therapy. *Bioconjugate Chem.* **2011**, *22*, 1879-1903.
- 154) Chen, S.; Li, Q.; Wang, X.; Yang, Y.-W.; Gao, H. Multifunctional Bacterial Imaging and Therapy Systems. *J. Mater. Chem. B* **2018**, *6*, 5198-5214.
- 155) Kim, T.; Zhang, Q.; Li, J.; Zhang, L.; Jokerst, J. V. A Gold/Silver Hybrid Nanoparticle for treatment and Photoacoustic Imaging of Bacterial Infection. *ACS Nano* **2018**, *12*, 5615-5625.
- 156) Dai, X.; Fan, Z.; Lu, Y.; Ray, P. C. Multifunctional Nanoplatfoms for Targeted Multidrug-Resistant-Bacteria Theranostic Applications. *ACS Appl. Mater. Interfaces* **2013**, *5*, 11348-11354.
- 157) Kim, I.; Jin, S.-M.; Hna, E. H.; Ko, E.; Ahn, M.; Bang, W.-Y.; Bang, J.-K.; Lee, E. Structure- Dependent Antimicrobial Theranostic Functions of Self-assembled Short Peptides Nanoagents. *Biomacromolecules* **2017**, *18*, 3600-3610.
- 158) Zhao, Z.; Yan, R.; Yi, X.; Li, J.; Rao, J.; Guo, Z.; Yang, Y.; Li, W.; Li, Y.-Q.; Chen, C. Bacteria-Activated Theranostic Nanoprobes against Methicillin-Resistant Staphylococcus Aureus Infection. *ACS Nano* **2017**, *11*, 4428-4438.
- 159) Wu, W.; Mao, D.; Cai, X.; Duan, Y.; Hu, F.; Kong, D.; Liu, B. ONOO- and ClO- Responsive Organic Nanoparticles for Specific in Vivo Image-Guided Photodynamic Bacterial Ablation. *Chem. Mater.* **2018**, *30*, 3867-3873.
- 160) Ferreira, K.; Hu, H.-Y.; Fetz, V.; Prochnow, H.; Rais, B.; Müller, P. P.; Brönstrup, M. Multivalent Siderophore-DOTAM Conjugates as Theranostics for Imaging and Treatment of Bacterial Infections. *Angew. Chemie - Int. Ed.* **2017**, *56*, 8272-8276.
- 161) Smitten, K. L.; Southam, H. M.; De La Serna, J. B.; Gill, M. R.; Jarman, P. J.; Smythe, C. G. W.; Poole, R. K.; Thomas, J. A. Using Nanoscopy to Probe the Biological Activity of Antimicrobial Leads That Display Potent Activity against Pathogenic, Multidrug Resistant, Gram-Negative Bacteria. *ACS Nano* **2019**, *13*, 5133-5146.
- 162) Huang, N.; Chen, X.; Zhu, X.; Xu, M.; Liu, J. Ruthenium Complexes/Polypeptide Self-Assembled Nanoparticles for Identification of Bacterial Infection and Targeted Antibacterial Research. *Biomaterials* **2017**, *141*, 296-313.
- 163) Kirar, S.; Thakur, N. S.; Laha, J. K.; Bhaumik, J.; Banerjee, U. C. Development of Gelatin Nanoparticle-Based Biodegradable Phototheranostic Agents: Advanced System to Treat Infectious Diseases. *ACS Biomater. Sci. Eng.* **2018**, *4*, 473-482.
- 164) Setyawati, M. I.; Kutty, R. V.; Tay, C. Y.; Yuan, X.; Xie, J.; Leong, D. T. Novel Theranostic DNA Nanoscaffolds for the Simultaneous Detection and Killing of *Escherichia coli* and *Staphylococcus aureus*. *ACS Appl. Mater. Interfaces* **2014**, *6*, 21822-21831.
- 165) Kang, M.; Kwok, R. T. K.; Wang, J.; Zhang, H.; Lam, J. W. Y.; Li, Y.; Zhang, P.; Zou, H.; Gu, X.; Li, F.; Tang, B. Z. A Multifunctional Luminogen with Aggregation-Induced Emission Characteristics for Selective Imaging and Photodynamic Killing of

- Both Cancer Cells and Gram-Positive Bacteria. *J. Mater. Chem. B* **2018**, *6*, 3894–3903.
- 166) Li, Y.; Yu, H.; Qian, Y.; Hu, J.; Liu, S. Amphiphilic Star Copolymer-Based Bimodal Fluorogenic/Magnetic Resonance Probes for Concomitant Bacteria Detection and Inhibition. *Adv. Mater.* **2014**, *26*, 6734–6741.
 - 167) Li, Y.; Hu, X.; Tian, S.; Li, Y.; Zhang, G.; Zhang, G.; Liu, S. Polyion Complex Micellar Nanoparticles for Integrated Fluorometric Detection and Bacteria Inhibition in Aqueous Media. *Biomaterials* **2014**, *35*, 1618–1626.
 - 168) Wang, M.; Shi, J.; Mao, H.; Sun, Z.; Guo, S.; Guo, J.; Yan, F. Fluorescent Imidazolium-Type Poly(Ionic Liquid)s for Bacterial Imaging and Biofilm Inhibition. *Biomacromolecules* **2019**, *20*, 3161–3170.
 - 169) Zhai, L.; Zhang, Z.; Zhao, Y.; Tang, Y. Efficient Antibacterial Performance and Effect of Structure on Property Based on Cationic Conjugated Polymers. *Macromolecules* **2018**, *51*, 7239–7247.
 - 170) Zehra, N.; Dutta, D.; Malik, A. H.; Ghosh, S. S.; Iyer, P. K. Fluorescence Resonance Energy Transfer-Based Wash-Free Bacterial Imaging and Antibacterial Application Using a Cationic Conjugated Polyelectrolyte. *ACS Appl. Mater. Interfaces* **2018**, *10*, 27603–27611.
 - 171) Wang, X.; Zhu, S.; Liu, L.; Li, L. Flexible Antibacterial Film Based on Conjugated Polyelectrolyte/Silver Nanocomposites. *ACS Appl. Mater. Interfaces* **2017**, *9*, 9051–9058.
 - 172) Gao, G.; Yu, K.; Kindrachuk, J.; Brooks, D. E.; Hancock, R. E. W.; Kizhakkedathu, J. N. Antibacterial Surfaces Based on Polymer Brushes: Investigation on the Influence of Brush Properties on Antimicrobial Peptide Immobilization and Antimicrobial Activity. *Biomacromolecules* **2011**, *12*, 3715–3727.
 - 173) Lam, S. J.; Wong, E. H. H.; Boyer, C.; Qiao, G. G. Antimicrobial Polymeric Nanoparticles. *Prog. Polym. Sci.* **2018**, *76*, 40–64.
 - 174) Jaeger, W.; Bohrisch, J.; Laschewsky, A. Synthetic Polymers with Quaternary Nitrogen Atoms-Synthesis and Structure of the Most Used Type of Cationic Polyelectrolytes. *Prog. Polym. Sci.* **2010**, *35*, 511–577.
 - 175) Nimmagadda, A.; Liu, X.; Teng, P.; Su, M.; Li, Y.; Qiao, Q.; Khadka, N. K.; Sun, X.; Pan, J.; Xu, H.; Li, Q.; Cai, J. Polycarbonates with Potent and Selective Antimicrobial Activity toward Gram-Positive Bacteria. *Biomacromolecules* **2017**, *18*, 87–95.
 - 176) Engler, A. C.; Tan, J. P. K.; Ong, Z. Y.; Coady, D. J.; Ng, V. W. L.; Yang, Y. Y.; Hedrick, J. L. Antimicrobial Polycarbonates: Investigating the Impact of Balancing Charge and Hydrophobicity Using a Same-Centered Polymer Approach. *Biomacromolecules* **2013**, *14*, 4331–4339.
 - 177) Santos, F. G.; Bonkovoski, L. C.; Garcia, F. P.; Cellet, T. S. P.; Witt, M. A.; Nakamura, C. V.; Rubira, A. F.; Muniz, E. C. Antibacterial Performance of a PCL-PDMAEMA Blend Nanofiber-Based Scaffold Enhanced with Immobilized Silver Nanoparticles. *ACS Appl. Mater. Interfaces* **2017**, *9*, 9304–9314.
 - 178) Yuan, W.; Wei, J.; Lu, H.; Fan, L.; Du, J. Water-Dispersible and Biodegradable Polymer Micelles with Good Antibacterial Efficacy. *Chem. Commun.* **2012**, *48*, 6857–6859.
 - 179) Sardo, C.; Nottelet, B.; Triolo, D.; Giammona, G.; Garric, X.; Lavigne, J.-P.; Cavallaro, G.; Coudane, J. When Functionalization of PLA Surfaces Meets Thiol-Yne

- Photochemistry: Case Study with Antibacterial Polyaspartamide Derivatives. *Biomacromolecules* **2014**, *15*, 4351–4362.
- 180) Wang, M.; Zhou, C.; Chen, J.; Xiao, Y.; Du, J. Multifunctional Biocompatible and Biodegradable Folic Acid Conjugated Poly(ϵ -Caprolactone)-Polypeptide Copolymer Vesicles with Excellent Antibacterial Activities. *Bioconjug. Chem.* **2015**, *26*, 725–734.
 - 181) Huang, F.; Gao, Y.; Zhang, Y.; Cheng, T.; Ou, H.; Yang, L.; Liu, J.; Shi, L.; Liu, J. Silver-Decorated Polymeric Micelles Combined with Curcumin for Enhanced Antibacterial Activity. *ACS Appl. Mater. Interfaces* **2017**, *9*, 16880–16889.
 - 182) Haas, S.; Hain, N.; Raoufi, M.; Handschuh-Wang, S.; Wang, T.; Jiang, X.; Schönherr, H. Enzyme Degradable Polymersomes from Hyaluronic Acid-Block-Poly(ϵ -Caprolactone) Copolymers for the Detection of Enzymes of Pathogenic Bacteria. *Biomacromolecules* **2015**, *16*, 832–841.
 - 183) Hisey, B.; Ragona, P. J.; Gillies, E. R. Phosphonium-Functionalized Polymer Micelles with Intrinsic Antibacterial Activity. *Biomacromolecules* **2017**, *18*, 914–923.
 - 184) Xiong, M.-H.; Bao, Y.; Yang, X.-Z.; Wang, Y.-C.; Sun, B.; Wang, J. Lipase-Sensitive Polymeric Triple-Layered Nanogel for “on-Demand” Drug Delivery. *J. Am. Chem. Soc.* **2012**, *134*, 4355–4362.
 - 185) Singh, H.; Li, W.; Kazemian, M. R.; Yang, R.; Yang, C.; Logsetty, S.; Liu, S. Lipase-Responsive Electrospun Theranostic Wound Dressing for Simultaneous Recognition and Treatment of Wound Infection. *ACS Appl. Bio Mater.* **2019**, *2*, 2028–2036.
 - 186) Pastorino, L.; Pioli, F.; Zilli, M.; Converti, A.; Nicolini, C. Lipase-Catalyzed Degradation of Poly(ϵ -Caprolactone). *Enzyme Microb. Technol.* **2004**, *35*, 321–326.
 - 187) Surnar, B.; Sharma, K.; Jayakannan, M. Core-Shell Polymer Nanoparticles for Prevention of GSH Drug Detoxification and Cisplatin Delivery to Breast Cancer Cells. *Nanoscale* **2015**, *7*, 17964–17979.
 - 188) Surnar, B.; Jayakannan, M. Structural Engineering of Biodegradable PCL Block Copolymer Nanoassemblies for Enzyme-Controlled Drug Delivery in Cancer Cells. *ACS Biomater. Sci. Eng.* **2016**, *2*, 1926–1941.
 - 189) Kulkarni, B.; Jayakannan, M. Fluorescent-Tagged Biodegradable Polycaprolactone Block Copolymer FRET Probe for Intracellular Bioimaging in Cancer Cells. *ACS Biomater. Sci. Eng.* **2017**, *3*, 2185–2197.
 - 190) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163–175.
 - 191) Pitruzzello, G.; Thorpe, S.; Johnson, S.; Evans, A.; Gadêlha, H.; Krauss, T. F. Multiparameter Antibiotic Resistance Detection Based on Hydrodynamic Trapping of Individual *E. coli*. *Lab Chip* **2019**, *19*, 1417–1426.
 - 192) Monod, J. The Growth of Bacterial Cultures. *Annu. Rev. Microbiol.* **1949**, *3*, 371–394.
 - 193) Sezonov, G.; Joseleau-Petit, D.; D’Ari, R. *Escherichia coli* Physiology in Luria-Bertani Broth. *J. Bacteriol.* **2007**, *189*, 8746–8749.
 - 194) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In Vitro Cytotoxicity Testing of Polycations: Influence of Polymer Structure on Cell Viability and Hemolysis. *Biomaterials* **2003**, *24*, 1121–1131.
 - 195) Som, A.; Tew, G. N. Influence of Lipid Composition on Membrane Activity of Antimicrobial Phenylene Ethynylene Oligomers. *J. Phys. Chem. B* **2008**, *112*, 3495–3502.

- 196) Kuroda, K.; Caputo, G. A.; DeGrado, W. F. The Role of Hydrophobicity in the Antimicrobial and Hemolytic Activities of Polymethacrylate Derivatives. *Chem. - A Eur. J.* **2009**, *15*, 1123–1133.
- 197) Tokumasu, F.; Osters, G. R.; Amaratunga, C.; Fairhurst, R. M. Modifications in Erythrocyte Membrane Zeta Potential by *Plasmodium falciparum* Infection. *Exp. Parasitol.* **2012**, *131*, 245–251.
- 198) Riedl, S.; Leber, R.; Rinner, B.; Schaidt, H.; Lohner, K.; Zweglick, D. Human Lactoferricin Derived Di-Peptides Deploying Loop Structures Induce Apoptosis Specifically in Cancer Cells through Targeting Membranous Phosphatidylserine. *BBA - Biomembr.* **2015**, *1848*, 2918–2931.
- 199) Chowdhury, R.; Saha, A.; Mandal, A. K.; Jana, B.; Ghosh, S.; Bhattacharyya, K. Excited State Proton Transfer in the Lysosome of Live Lung Cells: Normal and Cancer Cells. *J. Phys. Chem. B* **2015**, *119*, 2149–2156.
- 200) Chen, Y.; Liu, Y.; Yao, Y.; Zhang, S.; Gu, Z. Reverse micelle-based water-soluble nanoparticles for simultaneous bioimaging and drug delivery. *Org. Biomol. Chem.* **2017**, *15*, 3232–3238.
- 201) Zhang, J.; Zhu, M.; Cui, J.; Wang, C.; Zhou, Z.; Wang, T.; Gong, L.; Su, C.; Qi, D.; Bian, Y.; Du, H.; Jiang, J. A Porphyrin-Pyranine Dyad for Ratiometric Fluorescent Sensing of Intracellular pH. *J. Photochem. Photobiol. A Chem.* **2020**, *396*, 112524.
- 202) Catania, C.; Thomas, A. W.; Bazan, G. C. Tuning Cell Surface Charge in *E. coli* with Conjugated Oligoelectrolytes. *Chem. Sci.* **2016**, *7*, 2023–2029.
- 203) Gavin, D. P.; Murphy, E. J.; Foley, A. M.; Castilla, I. A.; Reen, F. J.; Woods, D. F.; Collins, S. G.; O' Gara, F.; Maguire, A. R. Identification of an Esterase Isolated Using Metagenomic Technology which Displays an Unusual Substrate Scope and its Characterisation as an Enantioselective Biocatalyst. *Adv. Synth. Catal.* **2019**, *361*, 2466– 2474.
- 204) Godinho, L. F.; Reis, C. R.; Tepper, P. G.; Poelarends, G. J.; Quax, W. J. Discovery of an *Escherichia coli* Esterase with High Activity and Enantioselectivity toward 1,2-O-Isopropylideneglycerol Esters. *Appl. Environ. Microbiol.* **2011**, *77*, 6094–6099.
- 205) Buruaga-Ramiro, C.; Valenzuela, S. V.; Pastor, F. I. J.; Martinez, J.; Diaz, P. Unexplored lipolytic activity of *Escherichia coli*: Implications for lipase cloning. *Enzyme Microb. Technol.* **2020**, 109590.
- 206) Ahn, J. M.; Wentworth Jr., P.; Janda, K. D. Probing lipase/esterase libraries for lipid A hydrolases—discovery of biocatalysts for the detoxification of bacterially-expressed recombinant protein. *Chem. Commun.* **2004**, 364–365.
- 207) Aluri, R.; Jayakannan, M. Development of L-Tyrosine based Enzyme Responsive Amphiphilic Poly(ester-urethane) Nano-carriers for Multiple Drug Delivery to Cancer cells. *Biomacromolecules* **2017**, *18*, 189–200.
- 208) Aluri, R.; Saxena, S.; Joshi, D. C.; Jayakannan, M. Multistimuli-Responsive Amphiphilic Poly(ester-urethane) Nanoassemblies Based on l-Tyrosine for Intracellular Drug Delivery to Cancer Cells. *Biomacromolecules* **2018**, *19*, 2166–2181.
- 209) Kohanski, M. A.; Dwyer, D. J.; Hayete, B.; Lawrence, C. A.; Collins, J. J. A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell* **2007**, *130*, 797–810.
- 210) Alekshun, M. N.; Levy, S. B. Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell* **2007**, *128*, 1037–1050.

- 211) Wright, G. D.; Sutherland, A. D. New strategies for combating multidrug-resistant bacteria. *Trends. Mol. Med.* **2007**, *13*, 260-267.
- 212) Pearce, A. K.; O'Reilly, R. K. Polymers for Biomedical Applications: The importance of Hydrophobicity in Directing Biological Interactions and Application Efficacy. *Biomacromolecules* **2021**, *22*, 4459–4469.
- 213) Li, Z.; Lu, W.; Jia, S.; Yuan, H.; Gao, L.-H. Design and Application of Conjugated Polymer Nanomaterials for Detection and Inactivation of Pathogenic Microbes. *ACS Appl. Bio. Mater.* **2021**, *4*, 370-386.
- 214) Rahman, M. A.; Bam, M.; Luat, E.; Jui, M. S.; Ganewatta, M. S.; Shokfai, T.; Nagarkatti, M.; Decho, A. W.; Tang, C. Macromolecular-clustered facial amphiphilic antimicrobials. *Nat. Commun.* **2018**, *9*, 5231-5241.
- 215) Xing, C.; Xu, Q.; Tang, H.; Liu, L.; Wang, S. Conjugated Polymer/Porphyrin Complexes for Efficient Energy Transfer and Improving Light-Activated Antibacterial Activity. *J. Am. Chem. Soc.* **2009**, *131*, 13117-13124.
- 216) Wu, J.; Zawistowski, A.; Ehrmann, M.; Yi, T.; Schmuck, C. Peptide Functionalized Polydiacetylene Liposomes Act as a Fluorescent Turn-On Sensor for Bacterial Lipopolysaccharide. *J. Am. Chem. Soc.* **2011**, *133*, 9720–9723.
- 217) Huang, S.-H.; Wu, W.-S.; Huang, L.-Y.; Huang, W.-F.; Fu, W.-C.; Chen, P.-T.; Fang, J.-M.; Cheng, W.-C.; Cheng, T.-J. R.; Wong, C.-H. New Continuous Fluorometric Assay for Bacterial Transglycosylase Using Förster Resonance Energy Transfer. *J. Am. Chem. Soc.* **2013**, *135*, 17078–17089.
- 218) Kitamura, S.; Owensby, A.; Wall, D.; Wolan, D. W. Lipoprotein Signal Peptidase Inhibitors with Antibiotic Properties Identified through Design of a Robust In Vitro HT Platform. *Cell Chemical Biology* **2018**, *25*, 301–308.
- 219) Zhu, C.; Yang, Q.; Liu, L.; Wang, S. Rapid, Simple, and High-Throughput Antimicrobial Susceptibility Testing and Antibiotics Screening. *Angew. Chem. Int. Ed.* **2011**, *50*, 9607–9610.
- 220) Chen, H.; Wang, B.; Zhang, J.; Nie, C.; Lv, F.; Liu, L.; Wang, S. Guanidinium-pendant oligofluorene for rapid and specific identification of antibiotics with membrane-disrupting ability. *Chem. Commun.* **2015**, *51*, 4036-4039.
- 221) Yuan, H.; Zhao, H.; Peng, K.; Qi, R.; Bai, H.; Zhang, P.; Huang, Y.; Lv, F.; Liu, L.; Bao, J.; Wang, S. Conjugated Polymer-Quantum Dot Hybrid Materials for Pathogen Discrimination and Disinfection. *ACS Appl. Mater. Interfaces* **2020**, *12*, 21263–21269.
- 222) Zehra, N.; Dutta, D.; Malik, A. H.; Ghosh, S. S.; Iyer, P. K. Fluorescence Resonance Energy Transfer Based Wash-Free Bacterial Imaging and Antibacterial Application Using Cationic Conjugated Polyelectrolyte. *ACS Appl. Mater. Interfaces* **2018**, *10*, 27603–27611.
- 223) Hussain, S.; Lv, F.; Qi, R.; Senthilkumar, T.; Zhao, H.; Chen, Y.; Liu, L.; Wang, S. Förster Resonance Energy Transfer Mediated Rapid and Synergistic Discrimination of Bacteria over Fungi Using a Cationic Conjugated Glycopolymers. *ACS Appl. Bio Mater.* **2020**, *3*, 20–28.
- 224) Wang, Q.; Wang, X.; Sun, Y.; Yang, X.; Zhang, L.; Zhang, Q.; Hu, Z.-Q.; Hu, H.-Y. Ratiometric fluorescent probes for selective and sensitive visualization of bacterial microenvironment protease activity. *Chem. Commun.* **2019**, *55*, 5064-5067.
- 225) Chan, H. L.; Lyu, L.; Aw, J.; Zhang, W.; Li, J.; Yang, H.-H.; Hayashi, H.; Chiba, S.; Xing, B. Unique Fluorescent Imaging Probe for Bacterial Surface Localization and Resistant Enzyme Imaging. *ACS Chem. Biol.* **2018**, *13*, 1890–1896.

- 226) Nezakati, T.; Seifalian, A.; Tan, A.; Seifalian, A. M. Conductive Polymers: Opportunities and Challenges in Biomedical Applications. *Chem. Rev.* **2018**, *118*, 6766-6843.
- 227) Zhu, C.; Liu, L.; Yang, Q.; Lv, F.; Wang, S. Water-soluble Conjugated Polymers for Imaging, Diagnosis, and Therapy. *Chem. Rev.* **2012**, *112*, 4687-4735.
- 228) Würthner, F. Aggregation-Induced Emission (AIE): A Historical Perspective. *Angew. Chem. Int. Ed.* **2020**, *59*, 14192-14196.
- 229) Mei, J.; Leung, N. L. C.; Kwok, R. T. K.; Lam, J. W. Y.; Tang, B. Z. Aggregation-Induced Emission: Together We Shine, United We Soar! *Chem. Rev.* **2015**, *115*, 11718-11940.
- 230) Hong, Y.; Lam, J. W. Y.; Tang, B. Z. Aggregation-induced emission. *Chem. Soc. Rev.* **2011**, *40*, 5361-5388.
- 231) Leung, N. L. C.; Xie, N.; Yuan, W.; Liu, Y.; Wu, Q.; Peng, Q.; Miao, Q.; Lam, J. W. Y.; Tang, B. Z. Restriction of Intramolecular Motions: The General Mechanism behind Aggregation-Induced Emission. *Chem. Eur. J.* **2014**, *20*, 15349-15353.
- 232) Hu, F.; Qi, G.; Kenry; Mao, D.; Zhou, S.; Wu, M.; Wu, W.; Liu, B. Visualization and In Situ Ablation of Intracellular Bacterial Pathogens through Metabolic Labeling. *Angew. Chem. Int. Ed.* **2020**, *59*, 9288-9292.
- 233) Shi, Y.; Yin, G.; Yan, Z.; Sang, P.; Wang, M.; Brzozowski, R.; Eswara, P.; Wojtas, L.; Zheng, Y.; Li, X.; Cai, J. Helical Sulfono- γ -AApeptides with Aggregation-Induced Emission and Circularly Polarized Luminescence. *J. Am. Chem. Soc.* **2019**, *141*, 12697-12706.
- 234) Yu, Y.; Dai, X.; Wei, X.; Dai, X.; Yu, C.; Duan, X.; Zhang, X.; Li, C. Synthetic Fluorescent Nanoplatfrom Based on Benzoxaborole for Broad-Spectrum Inhibition of Bacterial Adhesion to Host Cells. *Chem. Mater.* **2018**, *30*, 8795-8803
- 235) Sapsford, K. E.; Bert, L.; Medintz, I. L. Materials for Fluorescence Resonance Energy Transfer Analysis: Beyond Traditional Donor-Acceptor Combinations. *Angew. Chem. Int. Ed.* **2006**, *45*, 4562-4588.
- 236) Deshpande, N. U.; Virmani, M.; Jayakannan, M. AIE-Driven Fluorescent Polysaccharide Polymersome as Enzyme-responsive FRET Nanoprobe to Study the Real-time Delivery Aspects in Live-Cells. *Polym. Chem.* **2021**, *12*, 1549.
- 237) Wilhelm, M. J.; Sheffield, J. B.; Gh., M. S.; Wu, Y.; Spahr, C.; Gonella, G.; Xu, B.; Dai, H-L. Gram's Stain Does Not Cross the Bacterial Cytoplasmic Membrane. *ACS Chem. Biol.* **2015**, *10*, 1711-1717.
- 238) Shuang Wang, S.; Zhao, J.; Lu, S.; Sun, J.; Yang, X. A duplex connection can further illuminate G-quadruplex/crystal violet complex. *Chem. Commun.* **2019**, *55*, 1911-1914.
- 239) Ma, D-L.; Kwan, M. H-T.; D. S-H.; Lee, P.; Yang, H.; Ma, V. P-Y.; Bai, L-P.; Jiang, Z-H.; Leung, C-H. Crystal violet as a fluorescent switch-on probe for i-motif: label-free DNA-based logic gate. *Analyst* **2011**, *136*, 2692.
- 240) Chen, Y.; Wang, J.; Zhang, Y.; Xu, L.; Gao, T.; Wang, B.; Pei, R. Selection and characterization of a DNA aptamer to crystal violet. *Photochem. Photobiol. Sci.* **2018**, *17*, 800.
- 241) Budin, G.; Chung, H. J.; Lee, H.; Weissleder, R. A Magnetic Gram Stain for Bacterial Detection. *Angew. Chem. Int. Ed.* **2012**, *51*, 7752-7755.
- 242) Owusu, E. G. A.; Yaghini, E.; Naasani, I.; Parkin, I. P.; Allan, E.; MacRobert, A. J. Synergistic Interactions of Cadmium-free Quantum Dots Embedded in a

- Photosensitised Polymer Surface: Efficient Killing of Multidrug-Resistant Strains at Low Ambient Light Levels. *Nanoscale* **2020**, *12*, 10609.
- 243) Owusu, E. G. A.; MacRobert, A. J.; Naasani, I.; Parkin, I. P.; Allan, E.; Yaghini, E. Photoactivable Polymers Embedded with Cadmium-Free Quantum Dots and Crystal Violet: Efficient Bactericidal Activity against Clinical Strains of Antibiotic-Resistant Bacteria. *ACS Appl. Mater. Interfaces* **2019**, *11*, 12367–12378.
 - 244) Ghosh, R.; Malhotra, M.; Sathe, R. R. M.; Jayakannan, M. Biodegradable Polymer Theranostic Fluorescent Nanoprobe for Direct Visualization and Quantitative Determination of Antimicrobial Activity. *Biomacromolecules* **2020**, *21*, 2896–2912.
 - 245) Saxena, S.; Jayakannan, M. π -Conjugate Fluorophore-Tagged and Enzyme-Responsive L-Amino Acid Polymer Nanocarrier and Their Color-Tunable Intracellular FRET Probe in Cancer Cells. *Biomacromolecules* **2017**, *18*, 2594–2609.
 - 246) Saxena, S.; Pradeep, A.; Jayakannan, M. Enzyme-Responsive Theranostic FRET Probe Based on L-Aspartic Amphiphilic Polyester Nanoassemblies for Intracellular Bioimaging in Cancer Cells. *ACS Appl. Bio Mater.* **2019**, *2*, 5245–5262.
 - 247) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163–175.
 - 248) Pelczar, M. J.; Chan, E. C. S.; Kreig, N. R. Microbiology, 5th ed.; *McGraw-Hill*, **1993**.
 - 249) Sahoo, H. Förster resonance energy transfer – A spectroscopic nanoruler: Principle and applications. *J. Photochem. Photobiol.* **2011**, *12*, 20–30.
 - 250) Brochado, A. R.; Telzerow, A.; , Bobonis, J.; , Banzhaf, M.; Mateus, A.; Selkrig, J.; Huth, E.; Bassler, S.; Beas, J. S.; Zietek, M.; Ng, N.; Foerster, S.; Ezraty, B.; Py, B.; Barras, F.; Savitski, M. M.; Bork, P.; Göttig, S.; Typas, A. Species-specific activity of antibacterial drug combinations. *Nature* **2018**, *559*, 259–263.
 - 251) Kalelkar, P. P.; Riddick, M.; García, A. J. Biomaterial-based antimicrobial therapies for the treatment of bacterial infections. *Nat. Rev. Mater.* **2022**, *7*, 39–54.
 - 252) Hancock, R. E. W. The end of an era? *Nat. Rev. Drug Discov.* **2007**, *6*, 28
 - 253) Silver, L. L. Multi-targeting by monotherapeutic antibacterials. *Nat. Rev. Drug Discov.* **2007**, *6*, 41–55.
 - 254) Brooks, B. D.; Brooks, A. E. Therapeutic strategies to combat antibiotic resistance. *Adv. Drug Deliv. Rev.* **2014**, *78*, 14–27.
 - 255) Hutnick, M. A.; Pokorski, J. K. Polymeric Interventions for Microbial Infections: A Review. *Mol. Pharm.* **2018**, *15*, 2910–2921.
 - 256) Shao, Z.; Wulandari, E.; Lin, R. C. Y.; Xu, J.; Liang, K.; Wong, E. H. H. Two plus One: Combination Therapy Tri-systems Involving Two Membrane-Disrupting Antimicrobial Macromolecules and Antibiotics. *ACS Infect. Dis.* **2022**, *8*, 1480–1490.
 - 257) Namivandi-Zangeneh, R.; Wong, E. H. H.; Boyer, C. Synthetic Antimicrobial Polymers in Combination Therapy: Tackling Antibiotic Resistance. *ACS Infect. Dis.* **2021**, *7*, 215–253.
 - 258) Worley, B. V.; Slomberg, D. L.; Schoenfisch, M. H. Nitric Oxide-Releasing Quaternary Ammonium-Modified Poly(amidoamine) Dendrimers as Dual Action Antibacterial Agents. *Bioconjugate Chem.* **2014**, *25*, 918–927.
 - 259) Tian, J.; Zhang, J.; Yang, J.; Du, L.; Geng, H.; Cheng, Y. Conjugated Polymers Act Synergistically with Antibiotics to Combat Bacterial Drug Resistance. *ACS Appl. Mater. Interfaces* **2017**, *9*, 18512–18520

- 260) Ding, X.; Yang, C.; Moreira, W.; Yuan, P.; Periaswamy, B.; Sessions, P. F. D.; Zhao, H.; Tan, J.; Lee, A.; Ong, K. X.; Park, N.; Liang, Z. C.; Hedrick, J. L.; Yang, Y. A Macromolecule Reversing Antibiotic Resistance Phenotype and Repurposing Drugs as Potent Antibiotics. *Adv. Sci.* **2020**, 7, 2001374.
- 261) Shaaban, M. I.; Shaker, M. A.; Mady, F. M. Imipenem/cilastatin encapsulated polymeric nanoparticles for destroying carbapenem-resistant bacterial isolates. *J Nanobiotechnology* **2017**, 15, 29.
- 262) Zhang, J.; Chen, Y. P.; Miller, K. P.; Ganewatta, M. S.; Bam, M.; Yan, Y.; Nagarkatti, M.; Decho, A. W.; Tang, C. Antimicrobial Metallopolymers and Their Bioconjugates with Conventional Antibiotics against Multidrug-Resistant Bacteria. *J. Am. Chem. Soc.* **2014**, 136, 4873–4876.
- 263) Namivandi-Zangeneh, R.; Kwan, R. J.; Nguyen, T-K.; Yeow, J.; Byrne, F. L.; b Oehlers, S. H.; Wong, E. H. H.; Boyer, C. The effects of polymer topology and chain length on the antimicrobial activity and hemocompatibility of amphiphilic ternary copolymers. *Polym. Chem.* **2018**, 9, 1735.
- 264) Teratanatorn, P.; Hoskins, R.; Swift, T.; Douglas, C. W. I.; Shepherd, J.; Rimmer, S. Binding of Bacteria to Poly(N-isopropylacrylamide) Modified with Vancomycin: Comparison of Behavior of Linear and Highly Branched Polymers. *Biomacromolecules* **2017**, 18, 2887–2899.
- 265) Shirbin, S. J.; Insua, I.; Holden, J. A.; Lenzo, J. C.; Reynolds, E. C.; O'Brien-Simpson, N. M.; Qiao, G. G. Architectural Effects of Star-Shaped “Structurally Nanoengineered Antimicrobial Peptide Polymers” (SNAPPs) on Their Biological Activity. *Adv. Healthcare Mater.* **2018**, 1800627.
- 266) Vigliotta, G.; Mella, M.; Rega, D.; Izzo, L. Modulating Antimicrobial Activity by Synthesis: Dendritic Copolymers Based on Nonquaternized 2-(Dimethylamino)ethyl Methacrylate by Cu-Mediated ATRP. *Biomacromolecules* **2012**, 13, 833–841.
- 267) Lin, F.; Qi, Q.; Zhang, J.; Zhou, W.; Zhang, J.; Fu, P.; Zhang, X.; Qiao, X.; Liu, M.; Pang, X.; Cui, Z. From Unimolecular Template to Silver Nanocrystal Clusters: An Effective Strategy to Balance Antibacterial Activity and Cytotoxicity. *ACS Appl. Mater. Interfaces* **2021**, 13, 39806–39818.
- 268) Dai, X.; Chen, X.; Zhao, J.; Zhao, Y.; Guo, Q.; Zhang, T.; Chu, C.; Zhang, X.; Li, C. Structure–Activity Relationship of Membrane-Targeting Cationic Ligands on a Silver Nanoparticle Surface in an Antibiotic-Resistant Antibacterial and Antibiofilm Activity Assay. *ACS Appl. Mater. Interfaces* **2017**, 9, 13837–13848.
- 269) Yang, Z.; Wu, Y.; Wang, J.; Cao, B.; Tang, C. Y. In Situ Reduction of Silver by Polydopamine: A Novel Antimicrobial Modification of a Thin-Film Composite Polyamide Membrane. *Environ. Sci. Technol.* **2016**, 50, 9543–9550.
- 270) Duong, H. T. T.; Jung, K.; Kutty, S. K.; Agustina, S.; Adnan, N. N. M.; Basuki, J. S.; Kumar, N.; Davis, T. P.; Barraud, N.; Boyer, C. Nanoparticle (Star Polymer) Delivery of Nitric Oxide Effectively Negates *Pseudomonas Aeruginosa* Biofilm Formation. *Biomacromolecules* **2014**, 15, 2583–2589.
- 271) Liu, T.; Li, G.; Wu, X.; Chen, S.; Zhang, S.; Han, H.; Zhang, H.; Luo, X.; Cai, X.; Ma, D. B-Cyclodextrin-graft-poly(amidoamine) dendrons as the nitric oxide deliver system for the chronic rhinosinusitis therapy. *Drug Deliv.* **2021**, 28, 306–318
- 272) Oelmann, S.; Travanut, A.; Barther, D.; Romero, M.; Howdle, S. M.; Alexander, C.; Meier, M. A. R. Biocompatible Unimolecular Micelles Obtained via the Passerini

- Reaction as Versatile Nanocarriers for Potential Medical Applications. *Biomacromolecules* **2019**, *20*, 90–101.
- 273) Siedenbiedel, F.; Fuchs, A.; Moll, T.; Weide, M.; Breves, R.; Tiller, J. C. Star-Shaped Poly(styrene)-block-Poly(4-vinyl-Nmethylpyridiniumiodide) for Semipermanent Antimicrobial Coatings. *Macromol. Biosci.* **2013**, *13*, 1447–1455.
- 274) Pu, Y.; Hou, Z.; Khin, M. M.; Zamudio-Vazquez, R.; Poon, K. L.; Duan, H.; Chan-Park, M. B. Synthesis and Antibacterial Study of Sulfobetaine/Quaternary Ammonium-Modified Star-Shaped Poly[2-(dimethylamino)ethyl methacrylate]-Based Copolymers with an Inorganic Core. *Biomacromolecules* **2017**, *18*, 44–55.
- 275) Liu, X.; Zhang, H.; Tian, Z.; Sen, A.; Allcock, H. R. Preparation of quaternized organic–inorganic hybrid brush polyphosphazene-co-poly[2-(dimethylamino)ethyl methacrylate] electrospun fibers and their antibacterial properties. *Polym. Chem.* **2012**, *3*, 2082.
- 276) Pan, Y.; Xue, Y.; Snow, J.; Xiao, H. Tailor-Made Antimicrobial/Antiviral Star Polymer via ATRP of Cyclodextrin and Guanidine-Based Macromonomer. *Macromol. Chem. Phys.* **2014**, *216*, 511–518.
- 277) Ren, J. M.; McKenzie, T. G.; Fu, Q.; Wong, E. H. H.; Xu, J.; An, Z.; Shanmugam, S.; Davis, T. P.; Boyer, C.; Qiao, G. G. Star Polymers. *Chem. Rev.* **2016**, *116*, 6743–6836.
- 278) Chen, G.; Wang, Y.; Xie, R.; Gong, S. A review on core–shell structured unimolecular nanoparticles for biomedical applications. *Adv. Drug Deliv. Rev.* **2018**, *130*, 58–72.
- 279) Wei Wua, Weigang Wanga, Jianshu Li. Star polymers: Advances in biomedical applications. *Prog. Polym. Sci.* **2015**, *46*, 55–85.
- 280) Zhou, C.; Wang, D.; Cao, M.; Chen, Y.; Liu, Z.; Wu, C.; Xu, H.; Wang, S.; Wang, Y. Self-Aggregation, Antibacterial Activity, and Mildness of Cyclodextrin/Cationic Trimeric Surfactant Complexes. *ACS Appl. Mater. Interfaces* **2016**, *8*, 30811–30823.
- 281) Li, Y.; Yu, H.; Qian, Y.; Hu, J.; Liu, S. Amphiphilic Star Copolymer-Based Bimodal Fluorogenic/ Magnetic Resonance Probes for Concomitant Bacteria Detection, and Inhibition. *Adv. Mater.* **2014**, *26*, 6734–6741.
- 282) Santos, M. R. E.; Mendonca, P. V.; Almeida, M. C.; Branco, R.; Serra, A. C.; Morais, P. V.; Coelho, J. F. J. Increasing the Antimicrobial Activity of Amphiphilic Cationic Copolymers by the Facile Synthesis of High Molecular Weight Stars by Supplemental Activator and Reducing Agent Atom Transfer Radical Polymerization. *Biomacromolecules* **2019**, *20*, 1146–1156.
- 283) Song, R.; Murphy, M.; Li, C.; Ting, K.; Soo, C.; Zheng, Z. Current development of biodegradable polymeric materials for biomedical applications. *Drug Des. Devel. Ther.* **2018**, *12*, 3117–3145.
- 284) Kamaly, N.; Yameen, B.; Wu, J.; Farokhzad, O. C. Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chem Rev.* **2016**, *116*, 2602–2663.
- 285) Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H. H.; Chen, Y.-Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C. Qiao, G. G. Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat. Microbiol.* **2016**, *1*, 16162.

- 286) Lam, S. J.; Wong, E. H. H.; O'Brien-Simpson, N. M.; Pantarat, N.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G. Bionano Interaction Study on Antimicrobial Star-Shaped Peptide Polymer Nanoparticles. *ACS Appl. Mater. Interfaces* **2016**, *8*, 33446–33456.
- 287) Zhang, Y.; Chen, T.; Pan, Z.; Sun, X.; Yin, X.; He, M.; Xiao, S.; Liang, H. Theoretical Insights into the Interactions between Star-Shaped Antimicrobial Polypeptides and Bacterial Membranes. *Langmuir* **2018**, *34*, 13438–13448.
- 288) Wang, J.; Lu, C.; Shi, Y.; Feng, X.; Wu, B.; Zhou, G.; Quan, G.; Pan, X.; Cai, J.; Wu, C. Structural Superiority of Guanidinium-Rich, Four-Armed Copolypeptides: Role of Multiple Peptide–Membrane Interactions in Enhancing Bacterial Membrane Perturbation and Permeability. *ACS Appl. Mater. Interfaces* **2020**, *12*, 18363–18374.
- 289) Zheng, M.; Lin, H.; Zhang, W.; Tang, S.; Liu, D.; Cai, J. Poly(L-ornithine)-Grafted Zinc Phthalocyanines as Dual-Functional Antimicrobial Agents with Intrinsic Membrane Damage and Photothermal Ablation Capacity. *ACS Infect. Dis.* **2021**, *7*, 2917–2929.
- 290) Pan, M.; Lu, C.; Zheng, M.; Zhou, W.; Song, F.; Chen, W.; Yao, F.; Liu, D.; Cai, J. Unnatural Amino-Acid-Based Star-Shaped Poly(l-Ornithine)s as Emerging Long-Term and Biofilm-Disrupting Antimicrobial Peptides to Treat *Pseudomonas Aeruginosa*-Infected Burn Wounds. *Adv. Healthcare Mater.* **2020**, 2000647.
- 291) Lu, C.; Quan, G.; Su, M.; Nimmagadda, A.; Chen, W.; Pan, M.; Teng, P.; Yu, F.; Liu, X.; Jiang, L.; Du, W.; Hu, W.; Yao, F.; Pan, X.; Wu, C.; Liu, D.; Cai, J. Molecular Architecture and Charging Effects Enhance the In Vitro and In Vivo Performance of Multi-Arm Antimicrobial Agents Based on Star-Shaped Poly(l-lysine). *Adv. Therap.* **2019**, 1900147.
- 292) Liu, H.; Zhang, X.; Zhao, Z.; Yang, F.; Xue, R.; Yin, L.; Song, Z.; Cheng, J.; Luan, S.; Tang, H. Efficient synthesis and excellent antimicrobial activity of star-shaped cationic polypeptides with improved biocompatibility. *Biomater. Sci.* **2021**, *9*, 2721.
- 293) Zhang, Y.; Song, W.; Li, S.; Kim, D-K.; Kim, J. H.; Kim, J. R.; Kim, I. Facile and scalable synthesis of topologically nanoengineered polypeptides with excellent antimicrobial activities. *Chem. Commun.* **2020**, 56, 356-359.
- 294) Pranantyo, D.; Xu, L. Q.; Hou, Z.; Kang, E-T.; Chan-Park, M. B. Increasing bacterial affinity and cytocompatibility with four-arm star glycopolymers and antimicrobial α -polylysine. *Polym. Chem* **2017**, *8*, 3364–3373.
- 295) Zhen, J-B.; Kang, P-W.; Zhao, M-H.; Yang, K-W. Silver Nanoparticle Conjugated Star PCL-b-AMPs Copolymer as Nanocomposite Exhibits Efficient Antibacterial Properties. *Bioconjugate Chem.* **2020**, *31*, 51–63.
- 296) Chen, Y-F.; Lai, Y-D.; Chang, C-H.; Tsai, Y-C.; Tanga, C-C.; Jan, J-S. Star-shaped polypeptides exhibit potent antibacterial activities. *Nanoscale* **2019**, *11*, 11696–11708.
- 297) Dongargaonkar, A. A.; Bowlin, G. L.; Yang, H. Electrospun Blends of Gelatin and Gelatin–Dendrimer Conjugates as a Wound-Dressing and Drug-Delivery Platform. *Biomacromolecules* **2013**, *14*, 4038–4045.
- 298) Yang, C.; Krishnamurthy, S.; Liu, J.; Liu, S.; Lu, X.; Coady, D. J.; Cheng, W.; Libero, G. D. Singhal, A.; Hedrick, J. L.; Yang, Y. Y. Broad-Spectrum Antimicrobial Star Polycarbonates Functionalized with Mannose for Targeting Bacteria Residing Inside Immune Cells. *Adv. Healthcare Mater.* **2016**, *5*, 1272–1281.
- 299) Iqbal, M.; Zafar, N.; Fessi, H.; Elaissari, A. Double emulsion solvent evaporation techniques used for drug encapsulation. *Int. J. Pharm.* **2015**, *496*, 173-90.

- 300) Pham, P.; Oliver, S.; Boyer, C. Design of Antimicrobial Polymers. *Macromol. Chem. Phys.* **2022**, 2200226.
- 301) Vázquez-Laslop, N.; Mankin, A. S. How Macrolide Antibiotics Work. *Trends Biochem. Sci.* **2018**, 43, 668-684.
- 302) Farha, M. A.; MacNair, C. R.; Carfrae, L. A.; Zahed, S. S. E.; Ellis, M. J.; Tran, H-K. R.; McArthur, A. G.; Brown, E. D. Overcoming Acquired and Native Macrolide Resistance with Bicarbonate. *ACS Infect. Dis.* **2020**, 6, 2709–2718.

Biodegradable Polymer Theranostic Fluorescent Nanoprobe for Direct Visualization and Quantitative Determination of Antimicrobial Activity



Author: Ruma Ghosh, Mehak Malhotra, Rupali Ravindra Madhuri Sathe, et al

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Jul 1, 2020

Copyright © 2020, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms and Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from {COMPLETE REFERENCE CITATION}. Copyright {YEAR} American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your RightsLink request. No additional uses are granted (such as derivative works or other editions). For any uses, please submit a new request.

If credit is given to another source for the material you requested from RightsLink, permission must be obtained from that source.

[BACK](#)

[CLOSE WINDOW](#)

Theranostic FRET Gate to Visualize and Quantify Bacterial Membrane Breaching



Author: Ruma Ghosh, Manickam Jayakannan

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Jan 1, 2023

Copyright © 2023, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms and Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from {COMPLETE REFERENCE CITATION}. Copyright {YEAR} American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your RightsLink request. No additional uses are granted (such as derivative works or other editions). For any uses, please submit a new request.

If credit is given to another source for the material you requested from RightsLink, permission must be obtained from that source.

[BACK](#)

[CLOSE WINDOW](#)



Indian Institute of Science Education and Research (IISER – PUNE)

Dr. Homi Bhabha Road, Pashan, Pune 411 008.

Certificate

This is to certify that the project “**Selective Killing of MDR (multi drug resistant) bacterial cells over mammalian cells by Cationic Amphiphilic Polymers (IISER/IAEC/2018-02/04)**” from **Prof. M. Jayakannan** has been approved by the IAEC.

Dr. Anjan Banerjee
Name of Chairperson/ Member Secretary IAEC:
Signature with date

Dr. Shital Suryavanshi
Name of CPCSEA nominee:
Signature with date

Chairman/ Member Secretary of IAEC:

Chairperson
Institutional Animal Ethics Committee
Indian Institute of Science Education & Research
Pune - 411 008, India

CPCSEA nominee:

Main Nominee CPCSEA
Indian Institute of Science Education & Research
Pune - 411 008, India

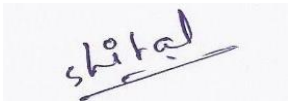


Indian Institute of Science Education and Research (IISER – PUNE)

Dr. Homi Bhabha Road, Pashan, Pune 411 008.

Certificate

This is to certify that the project “**Biodegradable Polymer Nano-assemblies for Antibacterial application**” (IISER_Pune/IAEC/2021_01/10) from **Prof. M. Jayakannan** has been approved by the IAEC.

Authorized by	Name	Signature	Date
Chairman:	Dr. Nixon Abraham		<u>18 Feb 2021</u>
Member Secretary:	Dr. Suraj Ingle		<u>18 Feb 2021</u>
Main Nominee of CPCSEA:	Dr. Shital Suryavanshi		<u>18 Feb 2021</u>

IAEC Protocol Amendment Form

(Change in procedure/ ✓ Personnel/ ✓ Protocol Extension/ ✓ Number of animals/ Change in Species/ Strain/ other)

Date: 22-02-2022

Current Protocol Number : IISER_PUNE/ IAEC/2021_01/10

Amended Protocol Number : IISER_PUNE/ IAEC/2021_01/10/A1

Project Title : Biodegradable Polymer Nano-assemblies for Antibacterial application

Amendment Details :

Sr. No.	Protocol Section No.	Details as per protocol	Amendment details
	Section-II Subsection: 2	List of all individuals authorized to conduct procedures under this proposal. a. Name : Ruma Ghosh b. Designation: Research Scholar c. Department : Chemistry d. Telephone No. e. E-mailId: ruma.ghosh@students.iiserpune.ac.in f. Experience in Lab animal experimentation	Student Details to be added current protocol: a. Name: Mr. Utreshwar Gavhane b. Address: IISER Pune (Department of Chemistry) c. Experience: To be trained under the supervision of Prof. M. Jayakannan, by Ms. Ruma Ghosh
	Section-II Subsection: 4	2. Duration of the animal experiment. 1 year a. Date of initiation (Proposed) IAEC committee approval b. Date of completion (Proposed) 1 year from the approval date	Project is needed to be extended for 1 more year .
	Section-II Subsection: 6	3. Animals required Eight (8)	Eight more mice are required.

Justification for Amendment:

Dear Committee members,

I sincerely thank you for extending ammendment opportunites in the approved protocols. I hereby request you to approve the above listed ammendments for the smooth functioning of the project. Due to the COVID-19 issues, the project was delayed by 6-8 months; and therefore, **I request you to give an extension for 1 more year (one year) and include one more student (Mr. Utreshwar Gavhane) for the following proposal.**



Principal Investigator

Amendment Approval:

Authorized by	Name	Signature	Date
Chairman:	Dr. Nixon Abraham		<u>04 Mar 2022</u>
Member Secretary:	Dr. Suraj Ingle		<u>04 Mar 2022</u>
Main Nominee of CPCSEA:	Dr. Shital Suryavanshi		<u>04 Mar 2022</u>

Biodegradable Polymer Theranostic Fluorescent Nanoprobe for Direct Visualization and Quantitative Determination of Antimicrobial Activity

Ruma Ghosh, Mehak Malhotra, Rupali Ravindra Madhuri Sathe, and Manickam Jayakannan*



Cite This: *Biomacromolecules* 2020, 21, 2896–2912



Read Online

ACCESS |



Metrics & More

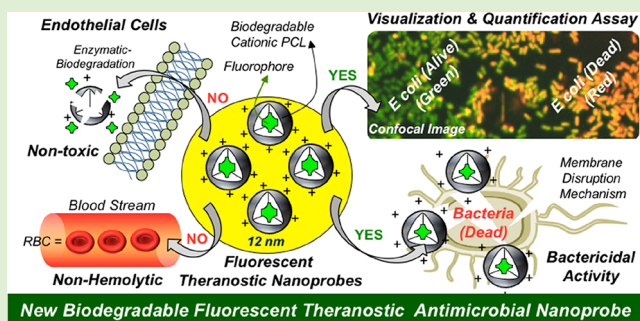


Article Recommendations



Supporting Information

ABSTRACT: We report a biodegradable fluorescent theranostic nanoprobe design strategy for simultaneous visualization and quantitative determination of antibacterial activity for the treatment of bacterial infections. Cationic-charged polycaprolactone (PCL) was tailor-made through ring-opening polymerization methodology, and it was self-assembled into well-defined tiny 5.0 ± 0.1 nm aqueous nanoparticles (NPs) having a zeta potential of +45 mV. Excellent bactericidal activity at 10.0 ng/mL concentration was accomplished in Gram-negative bacterium *Escherichia coli* (*E. coli*) while maintaining their nonhemolytic nature in mice red blood cells (RBC) and their nontoxic trend in wild-type mouse embryonic fibroblast cells with a selectivity index of $>10^4$. Electron microscopic studies are evident of the *E. coli* membrane disruption mechanism by the cationic NP with respect to their high selectivity for antibacterial activity. Anionic biomarker 8-hydroxy-pyrene-1,3,6-trisulfonic acid (HPTS) was loaded in the cationic PCL NP via electrostatic interaction to yield a new fluorescent theranostic nanoprobe to accomplish both therapeutics and diagnostics together in a single nanosystem. The theranostic NP was readily degradable by a bacteria-secreted lipase enzyme as well as by lysosomal esterase enzymes at the intracellular compartments in <12 h and support their suitability for biomedical application. In the absence of bactericidal activity, the theranostic nanoprobe functions exclusively as a biomarker to exhibit strong green-fluorescent signals in live *E. coli*. Once it became active, the theranostic probe induces membrane disruption on *E. coli*, which enabled the costaining of nuclei by red fluorescent propidium iodide. As a result, live and dead bacteria could be visualized via green and orange signals (merging of red+green), respectively, during the course of the antibacterial activity by the theranostic probe. This has enabled the development of a new image-based fluorescence assay to directly visualize and quantitatively estimate the real-time antibacterial activity. Time-dependent bactericidal activity was coupled with selective photoexcitation in a confocal microscope to demonstrate the proof-of-concept of the working principle of a theranostic probe in *E. coli*. This new theranostic nanoprobe creates a new platform for the simultaneous probing and treating of bacterial infections in a single nanodesign, which is very useful for a long-term impact in healthcare applications.



INTRODUCTION

Ever-increasing demand to treat the infectious diseases caused by bacteria, viruses, and other microorganisms in clinical contamination, wound healing, and respiratory chronic obstructive pulmonary diseases has become a major concern in healthcare and society.^{1,2} Multidrug resistance and steadfast mutations of microorganisms³ further obscure the treatment of the infectious diseases with existing drugs or antibiotics.⁴ Development of new diagnostic tools and methods are also equally important to accomplish timely required therapeutic efficacies.⁵ The next generation of antimicrobial candidates are essential to be smart multitasking probes having the capability to exhibit both therapeutics and diagnostics together (theranostics) in a single system so that maximum impact could be accomplished in a single dose of drug administration.^{6,7} Among the diverse diagnostic tools used include

fluorescent techniques that provide an excellent opportunity because they require very low probe concentrations and could also be employed under noninvasive protocols under physiological condition. Visible light to NIR-active fluorescent chromophores were encapsulated or tagged in antimicrobial polymers and antimicrobial peptides in the construction of fluorescent theranostic probes. Gold/silver nanoparticle-based photoacoustic⁸ and fluorescent imaging,^{9,10} NIR fluorophore-tagged silica–polyelectrolyte nanoparticles,¹¹ ROS-responsive

Received: April 27, 2020

Revised: June 12, 2020

Published: June 15, 2020



Theranostic FRET Gate to Visualize and Quantify Bacterial Membrane Breaching

Ruma Ghosh and Manickam Jayakannan*



Cite This: <https://doi.org/10.1021/acs.biomac.2c01202>



Read Online

ACCESS |



Metrics & More

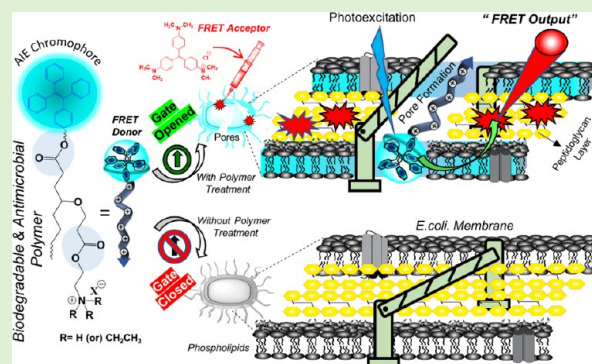


Article Recommendations



Supporting Information

ABSTRACT: Designing new antimicrobial-cum-probes to study real-time bacterial membrane breaching and concurrently developing inquisitorial image-based analytical tools is essential for the treatment of infectious diseases. An array of aggregation-induced emission (AIE) polymers (donor) consisting of neutral, anionic, and cationic charges were designed and employed as antimicrobial theranostic gatekeepers for the permeabilization of the peptidoglycan layer-adherable crystal violet (CV, acceptor). An AIE-active tetraphenylethylene (TPE)-tagged polycaprolactone biodegradable platform was chosen, and their self-assembled tiny amphiphilic nanoparticles were employed as a gatekeeper in the construction of bacterial membrane-reinforced fluorescent resonance energy transfer (FRET) probes. Electrostatic adhering of the cationic AIE polymer and subsequent gate opening aided fluorescent FRET probe activation on the membrane of Gram-negative bacteria, *Escherichia coli*. The selective photoexcitation energy transfer process in confocal microscopy experiments facilitated the building of a visualization-based FRET assay for the quantification of bactericidal activity. Nonantimicrobial AIE polymers (neutral and anionic) did not breach the bacterial membrane, resulting in no FRET signal. Detailed photophysical studies were done to establish the FRET probe mechanism, and a proof of concept was established.



INTRODUCTION

Bacterial microorganism mutations have continuously challenged humanity to find smart diagnostic tools for the early detection of infectious diseases and design efficient therapeutic drugs in the treatment and handling of public health care at large.^{1–3} Penicillin and other β -lactam antibiotic drugs are currently used for the treatment of bacterial infections; however, microbes are smart and quick in developing resistance against these clinical antibiotics and make them inefficient in the therapeutic regimens.⁴ Currently, fluorescent antibiotics are developed to study function at the cellular level, to rationalize the resistance mechanism, high-throughput screening, and so forth, and to improve therapeutic efficacies.^{5–7} Antimicrobial peptides (AMPs) are newly emerging second-generation therapeutic agents that are presently investigated for the treatment of bacterial infections.^{8,9} Since the past one decade, synthetic cationic polymers are emerging as alternative antimicrobial candidates for both Gram-negative and Gram-positive bacteria.^{10–13} Cationic antimicrobial polymers typically undergo nonspecific membrane disruption similar to AMPs in the inhibition of bacterial cell growth.^{14–17} Several small or polymeric fluorescent scaffolds are developed either based on steady-state fluorescence^{18,19} or FRET-based analysis to study bacterial enzyme responsiveness,^{20,21} antibiotic screening,^{22,23} pathogen discrimination,²⁴ wash-free bacterial detection,²⁵ differentiation of

bacteria over fungi,²⁶ probe localization,^{27,28} and so forth. Unlike in the area of antibiotics, in-depth molecular-level interaction between a polymer analyte and bacterial membrane substrate has been rarely understood. Furthermore, the majority of π -conjugated chromophores were found to undergo π -stack aggregation that caused quenching in an aqueous medium;²⁹ as a consequence, it was rather difficult to differentiate the fluorescent signals in Luria–Bertani (LB) broth (or medium in a free state) from the bacterial membrane-bound state.^{30,31} As an alternative, picking up aggregation-induced emission (AIE) π -conjugated fluorophores^{32–34} and building new AIE-assisted FRET probes is an elegant strategy to overcome the above limitations. Although the AIE concept has been known in the literature for bacterial imaging,^{35–38} AIE-based FRET probes are rarely reported. This is partially associated with the scarcity of low band-gap AIE-acceptor chromophores ($E_g \leq 2.0$ eV, red region) that could couple with the AIE donor chromophores

Received: October 5, 2022

Revised: November 26, 2022



ACS Publications

© XXXX American Chemical Society

A

<https://doi.org/10.1021/acs.biomac.2c01202>
Biomacromolecules XXXX, XXX, XXX–XXX



Fluorophore-tagged poly(L-Lysine) block copolymer nano-assemblies for real-time visualization and antimicrobial activity

Devesh Maurya¹, Rahul Nisal¹, Ruma Ghosh¹, Parshuram Kambale, Mehak Malhotra, Manickam Jayakannan^{*}

Department of Chemistry, Indian Institute of Science Education and Research (IISER Pune), Dr. Homi Bhabha Road, Pune 411008, Maharashtra, India

ARTICLE INFO

Keywords:

Antimicrobial Polypeptides
Fluorescent polymers
Theranostic probe
Poly(L-Lysine)
Ring opening polymerization (ROP)
Coumarin
Cytotoxicity

ABSTRACT

Development of self-reporting polymers having dual-action of membrane disruption for antimicrobial activity and simultaneous visualization and quantification by microscopic imaging techniques are urgently required for early detection and therapeutics. Here, we report a new series of self-reporting coumarin fluorophore-tagged polypeptides based on PEG-*block*-poly(L-lysine) copolymers via ring opening polymerization (ROP) strategy. For this purpose, NHC catalyzed controlled ROP was optimised for ϵ -Cbz-L-lysine NCA monomer to make desired chain length polypeptides. The resultant cationic polymer was self-assembled into 100 ± 15 nm nanoparticles (NP) having Zeta potentials of +25 to +44 mV associated with strong blue-emission in aqueous medium. The antimicrobial action of these polymers was tested in *E. coli* by colony suspension method and their MIC₉₀ values were obtained to be 1×10^{-4} to 3×10^{-4} g/mL. Real-time confocal microscopic imaging enabled the direct visualization of the fluorescent peptide NP action on the *E. coli* membrane, and propidium iodide co-staining assay further established the membrane pore formation mechanism for their antimicrobial activity. *In vitro* cytotoxicity and cellular uptake studies in MCF-7 breast cancer cell lines revealed that the fluorescent polypeptide NP were readily internalized at the cellular level, and they were also found to be nontoxic to cells up to 80 μ g/mL and showed minimal haemolysis to RBCs at their MIC concentration which further established their high biocompatibility as suitable biomaterials.

1. Introduction

Pathogenic micro-organisms are continuously throwing new challenges in biomedical field and escalates enormous uncertainty in handling life-threatening diseases and public health care at large [1]. The development of high-speed detection of pathogenic bacteria and virus at the early stage of infection is very crucial for the quick containment as well as for finding new drug formulation in the treatment of the infectious diseases. Some of the most advanced detection-techniques that are currently employed include fluorescent dye labelled antibody detection [2], polymerase chain reaction (PCR) [3], enzyme-linked immunosorbent assay (ELISA) [4], electrochemical methods [5–8] surface enhanced Raman scattering (SERS) [6,9,10], fluorescence confocal microscopic imaging [11–16], and fluorescence (or Förster) resonance energy transfer (FRET) assays [17], etc. Among them, fluorescence assay is most widely utilized non-invasive technique

due to its high specificity, sensitivity and real-time imaging in situ [18]. Fluorophore conjugated antibiotics are utilized to understand their intracellular accumulation, mechanism of action, and ultimately detection of resistant microbial infections [19]. In the last two decades, antimicrobial polymers are emerging as an alternative biomaterial due to their scope of substrate modification by functionalization and tunable material properties like hydrogels, polymeric micelles, polymeric vesicles, and so forth [20,21]. In this domain, cationic conjugated polymers have long been used as one of the effective signal amplifiers and non-invasive diagnostic agents [22]. They have been used as photodynamic antimicrobial chemotherapeutic agents (PACT) by enhancing ROS generating activity of photosensitizers [23]. Cationic π -conjugated polymers or oligomers were also employed as FRET probe along with fluorescein [24], green fluorescent protein [25], and porphyrin [26] for screening antibiotic action on *E. coli* and photodynamic therapy via singlet oxygen generation [27], etc. However, a major impediment

^{*} Corresponding author.

E-mail address: jayakannan@iiserpune.ac.in (M. Jayakannan).

¹ Equal contribution.

9. *Appendix*

Chapter-2

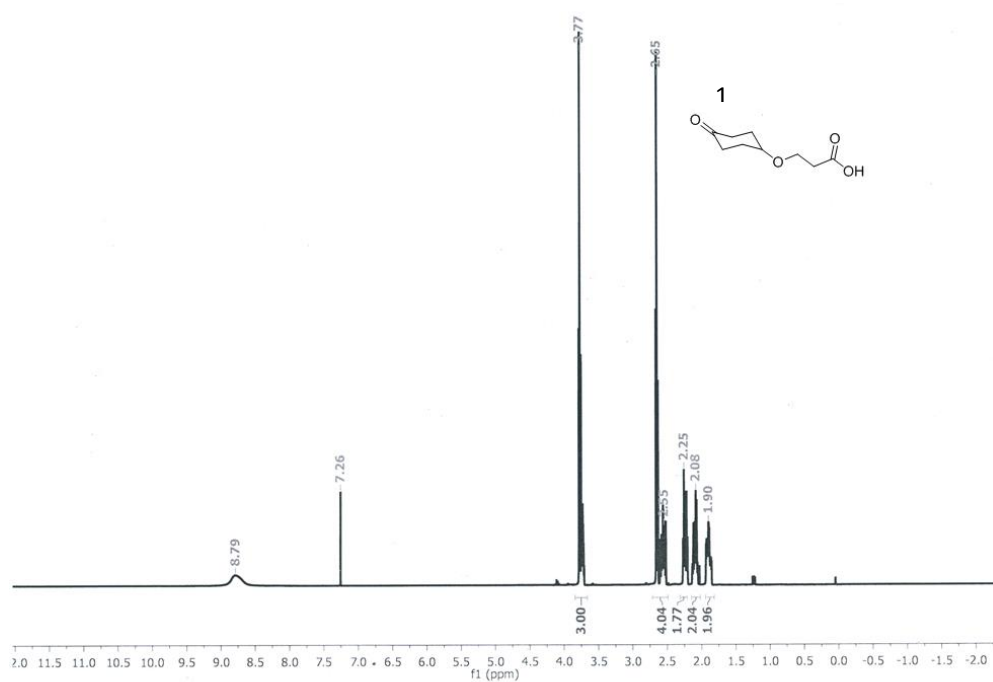


Figure 9.1. ¹H-NMR spectrum of compound 1 in CDCl₃.

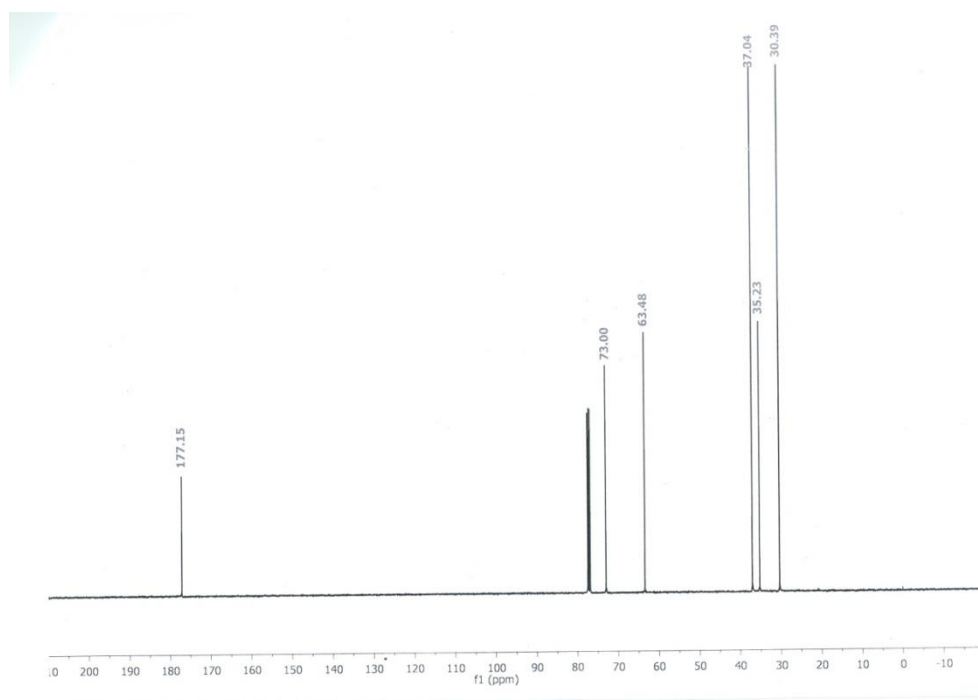


Figure 9.2. ¹³C- NMR spectrum of compound 1 in CDCl₃

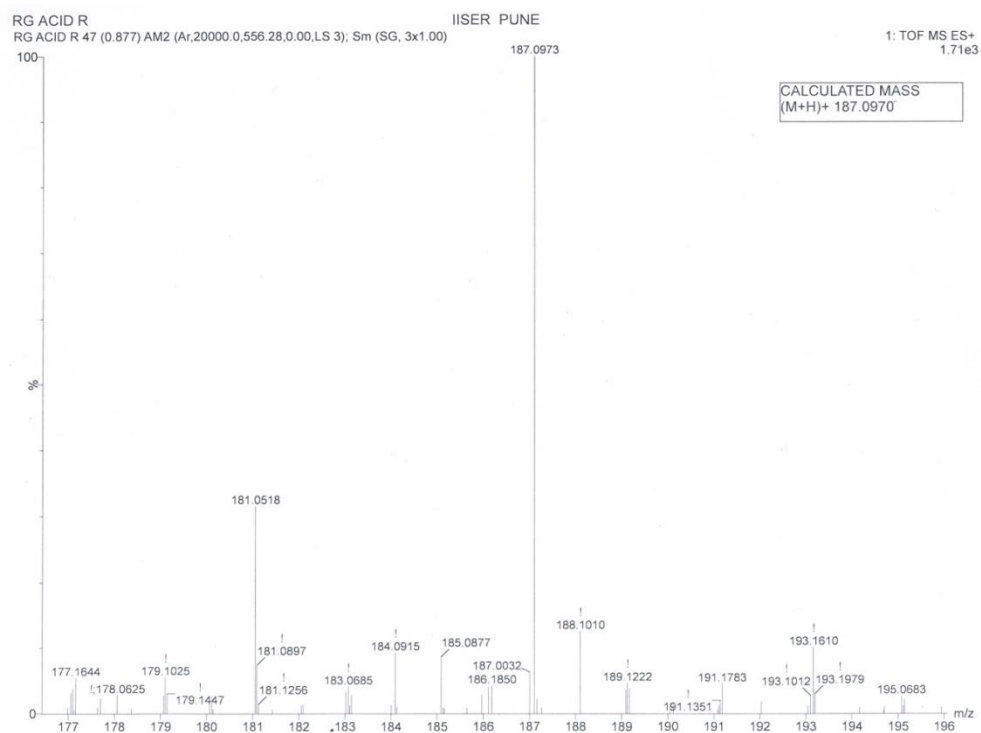


Figure 9.3. HRMS spectrum of compound 1 in CH_3OH

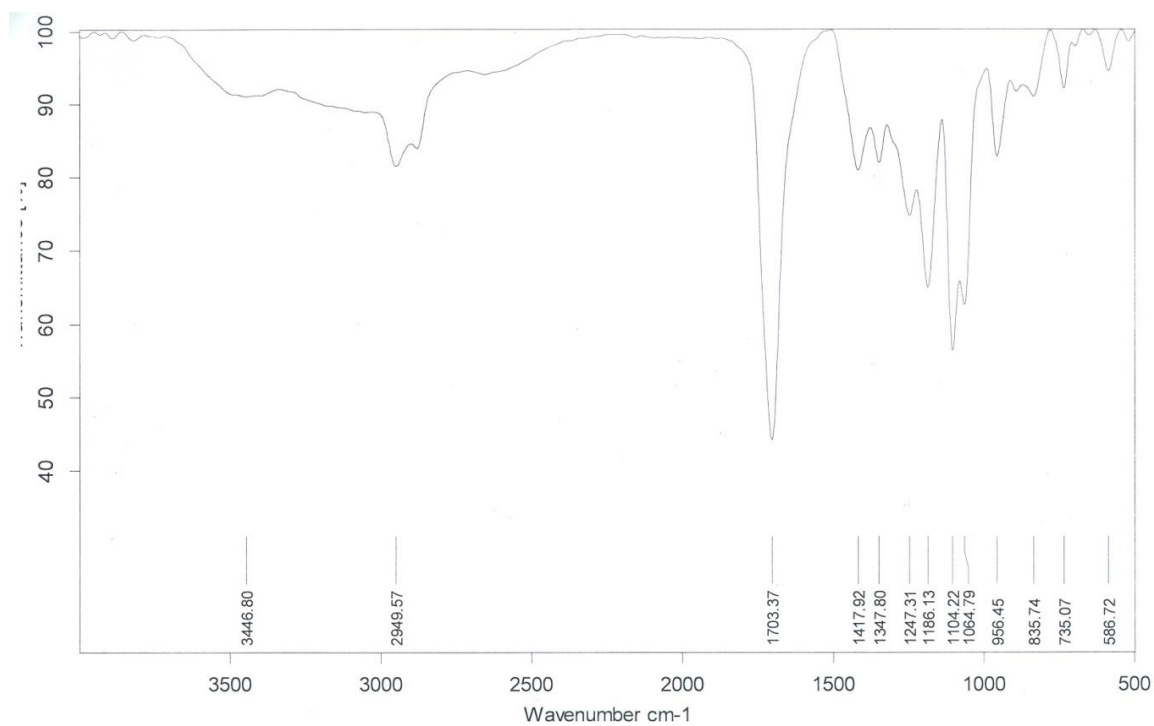


Figure 9.4. FTIR spectrum of compound 1 in $(\text{CH}_3)_2\text{O}$

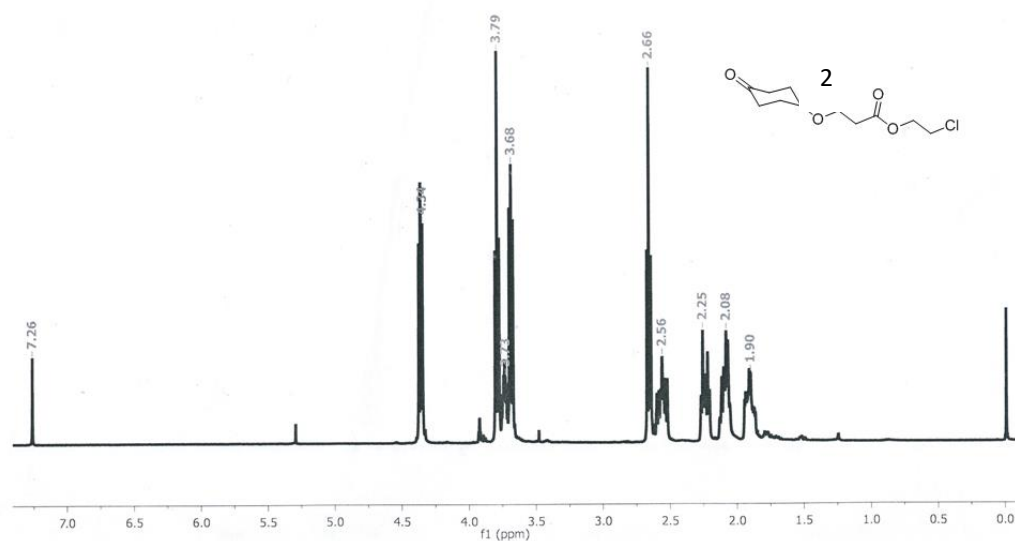


Figure 9.5. ^1H -NMR spectrum of compound 2 in CDCl_3

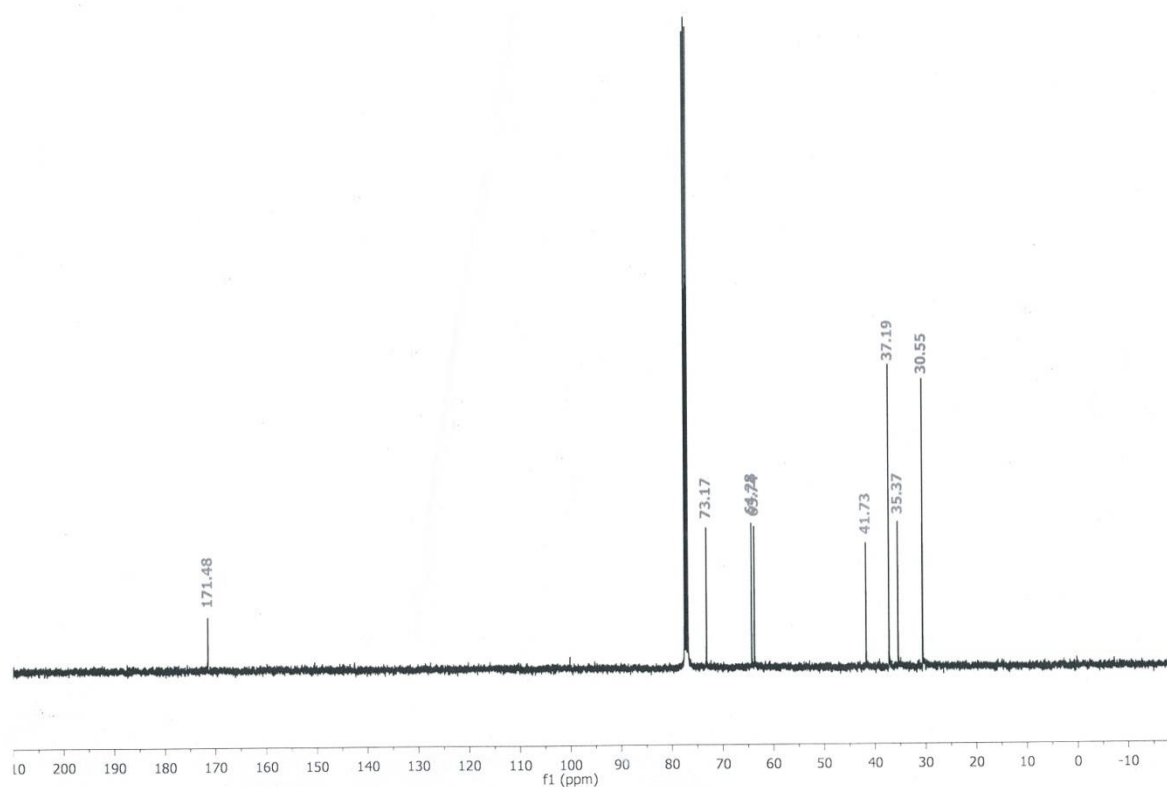


Figure 9.6. ^{13}C -NMR spectrum of compound 2 in CDCl_3

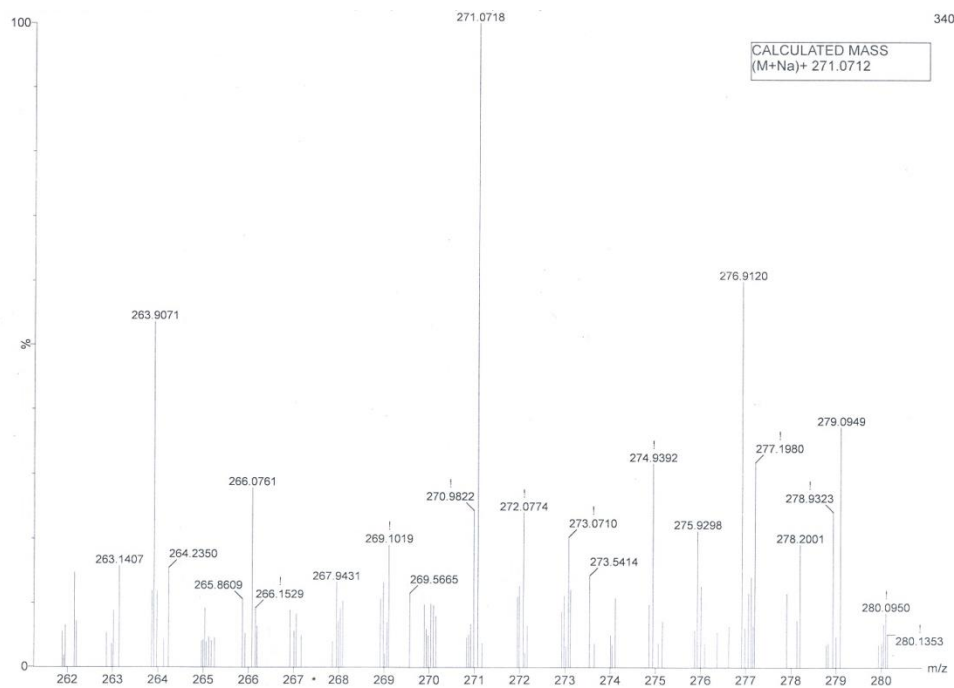


Figure 9.7. HRMS spectrum of compound 2 in CH_3OH

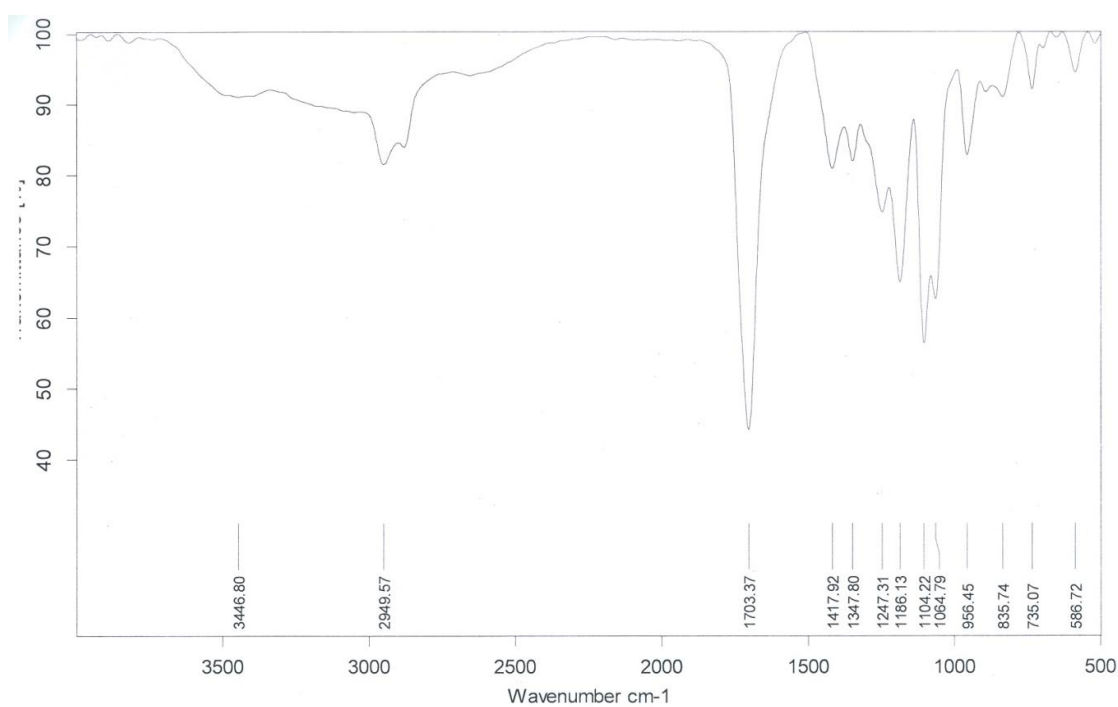


Figure 9.8. FTIR spectrum of compound 2 in $(\text{CH}_3)_2\text{O}$

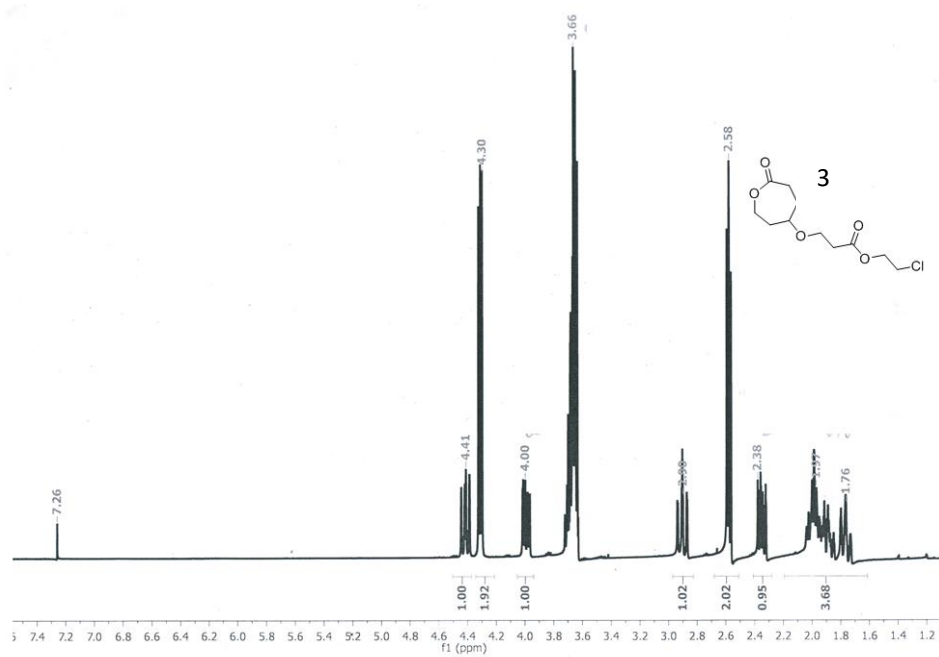


Figure 9.9. ¹H-NMR spectrum of compound 3 in CDCl₃

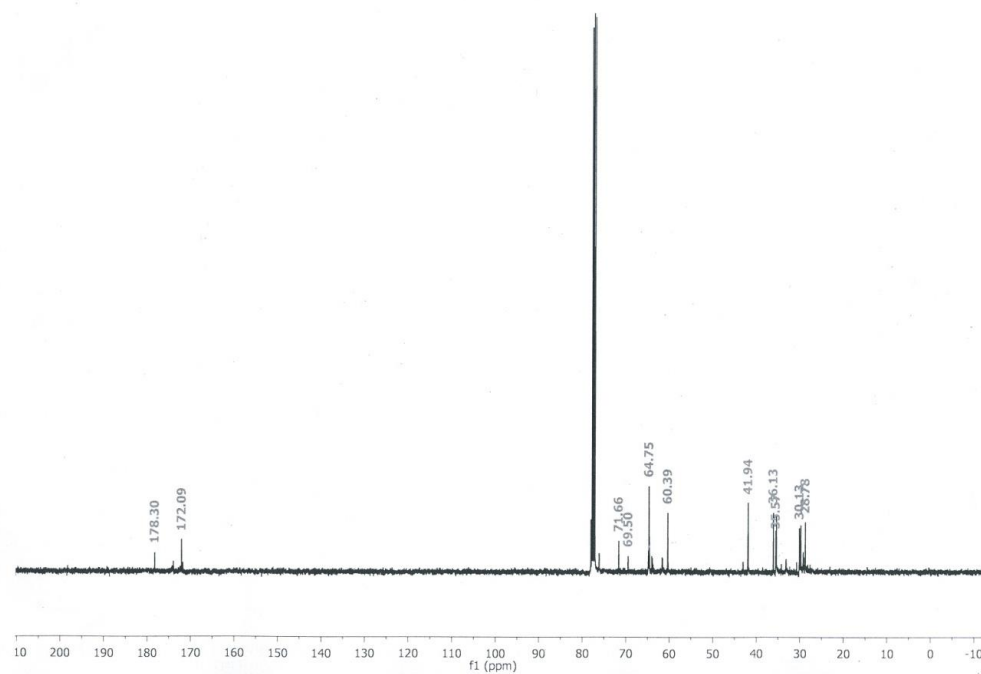


Figure 9.10. ¹³C-NMR spectrum of compound 3 in CDCl₃

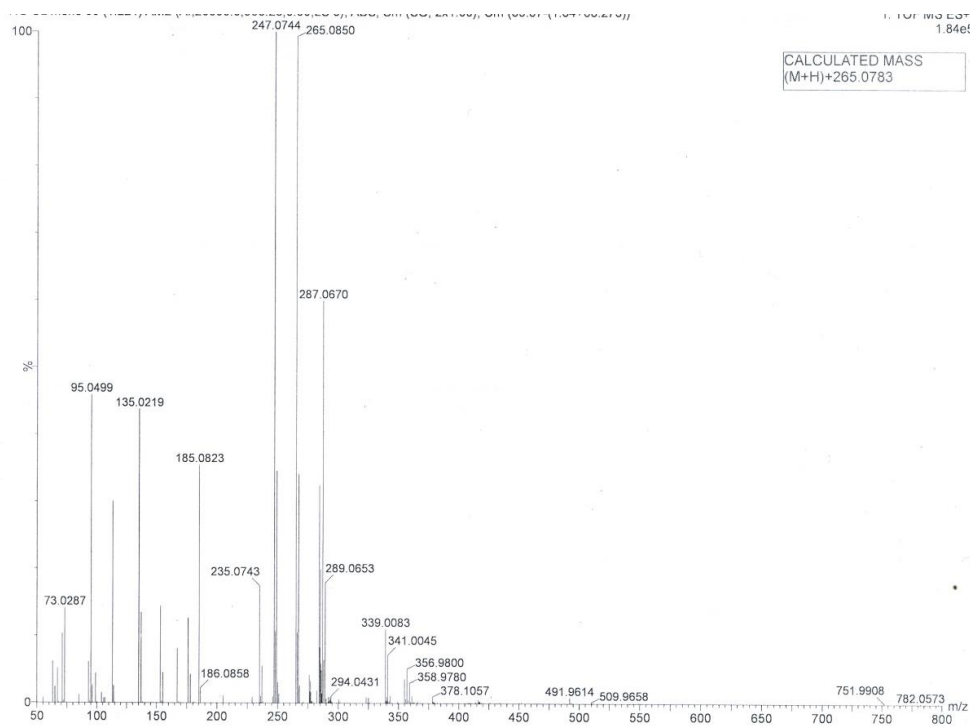


Figure 9.11. HRMS spectrum of compound 3 in CH_3OH

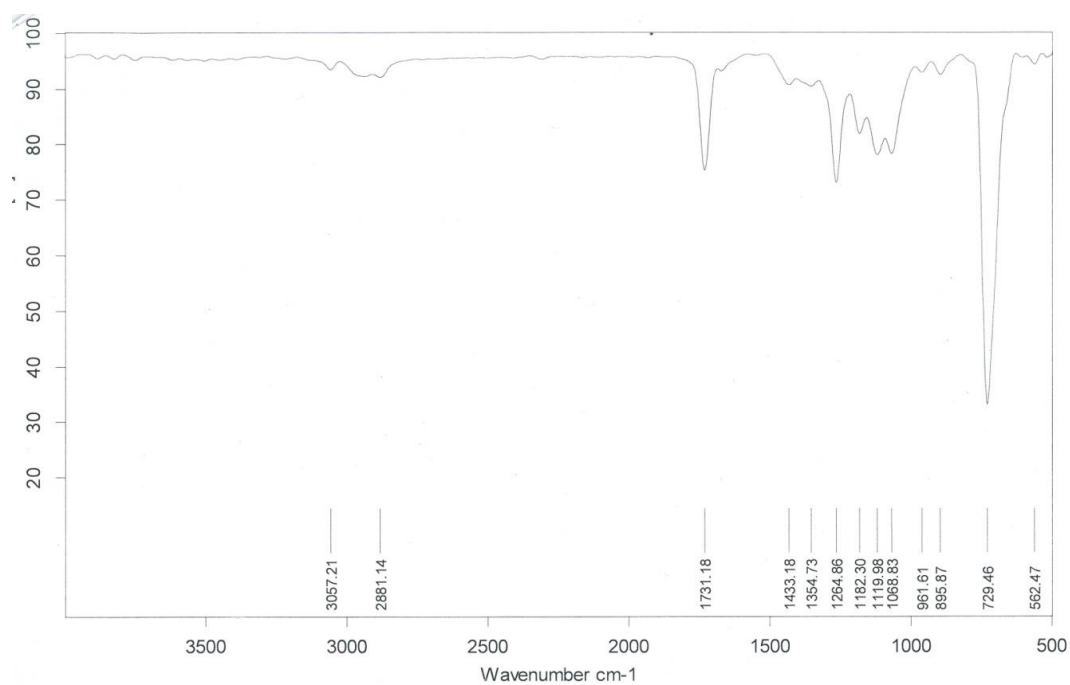


Figure 9.12. FTIR spectrum of compound 3 in $(\text{CH}_3)_2\text{O}$

Chapter-3

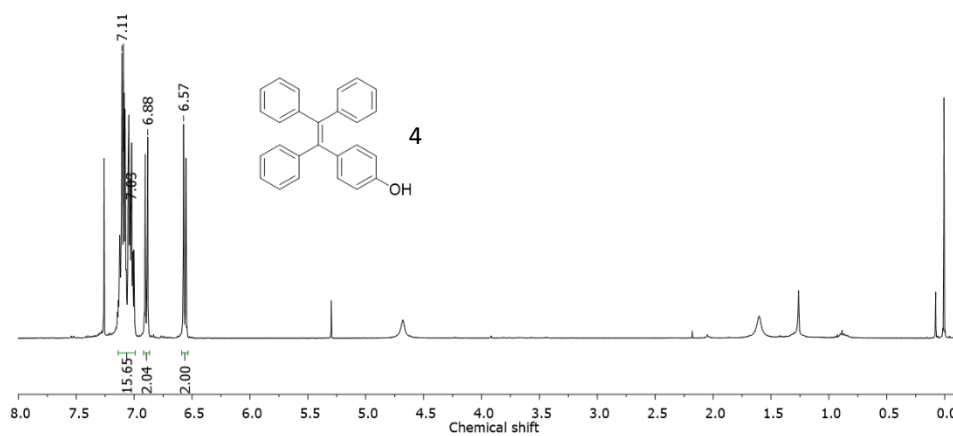


Figure 9.13. ^1H -NMR spectrum of compound 4 in CDCl_3

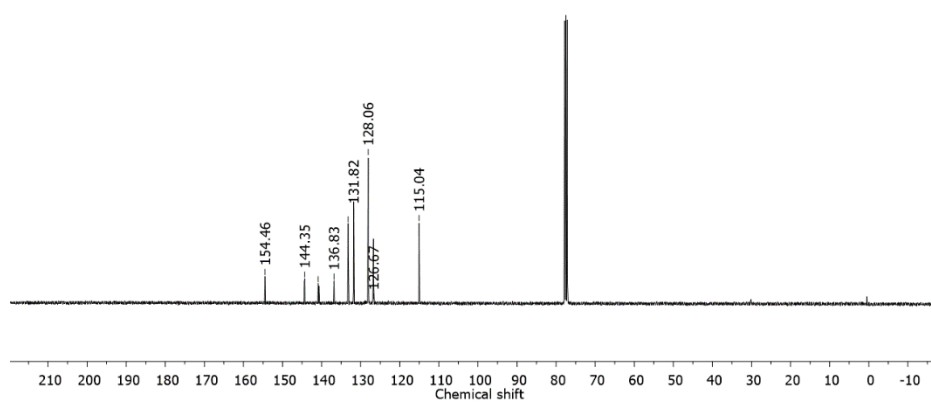


Figure 9.14. ^{13}C -NMR spectrum of compound 3 in CDCl_3

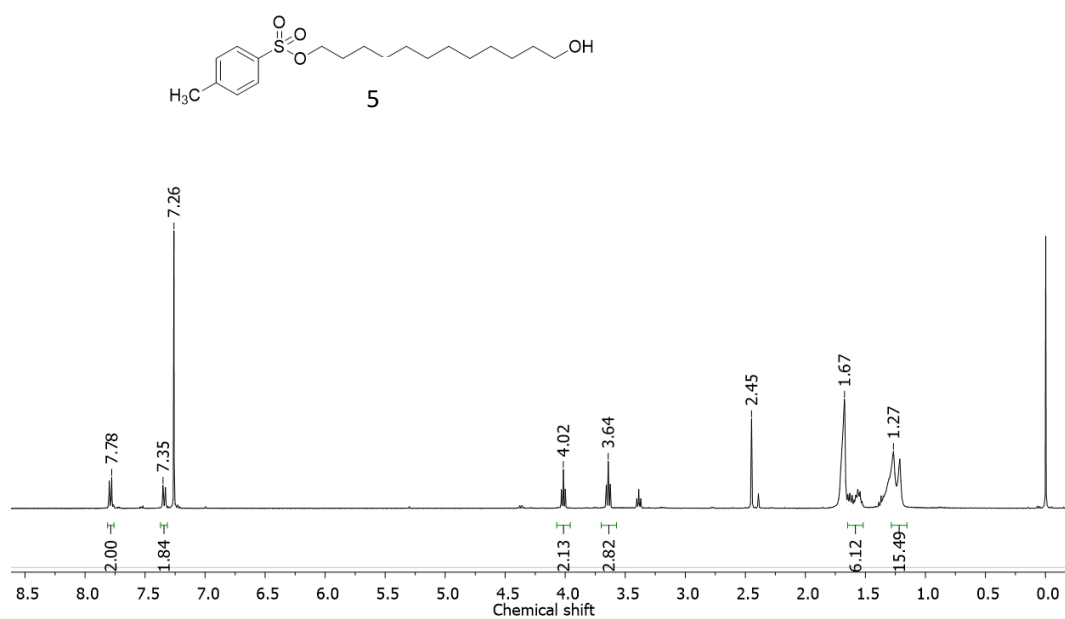


Figure 9.15. ^1H -NMR spectrum of compound 5 in CDCl_3

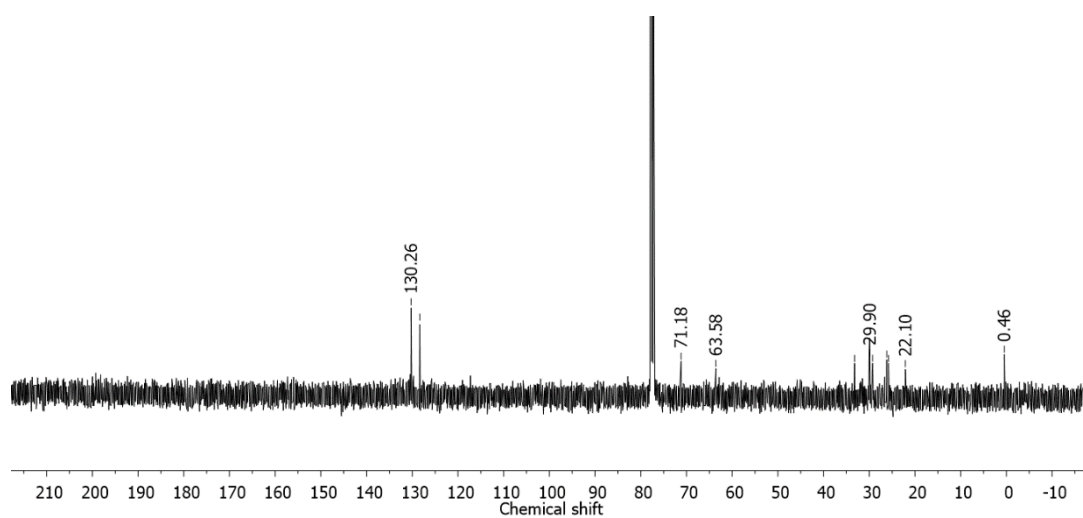


Figure 9.16. ^{13}C -NMR spectrum of compound 3 in CDCl_3

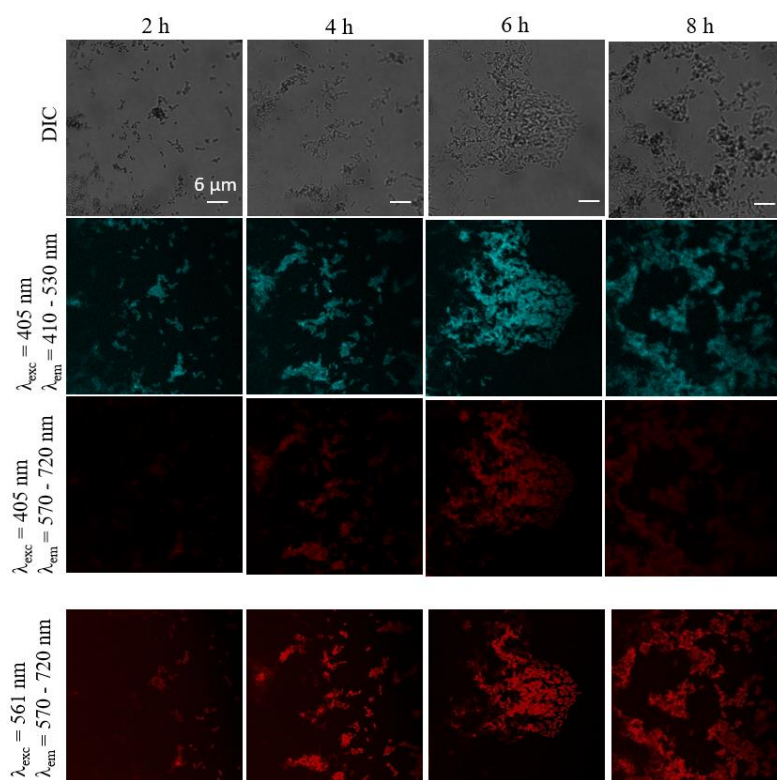


Figure 9.17. Confocal image of *E. coli* bacterial cells incubated with TPE-Cat-NEt₃ polymer for 2 h (first row), 4 h (second row), 6 h (third row) and 8 h (fourth row).

Chapter-4

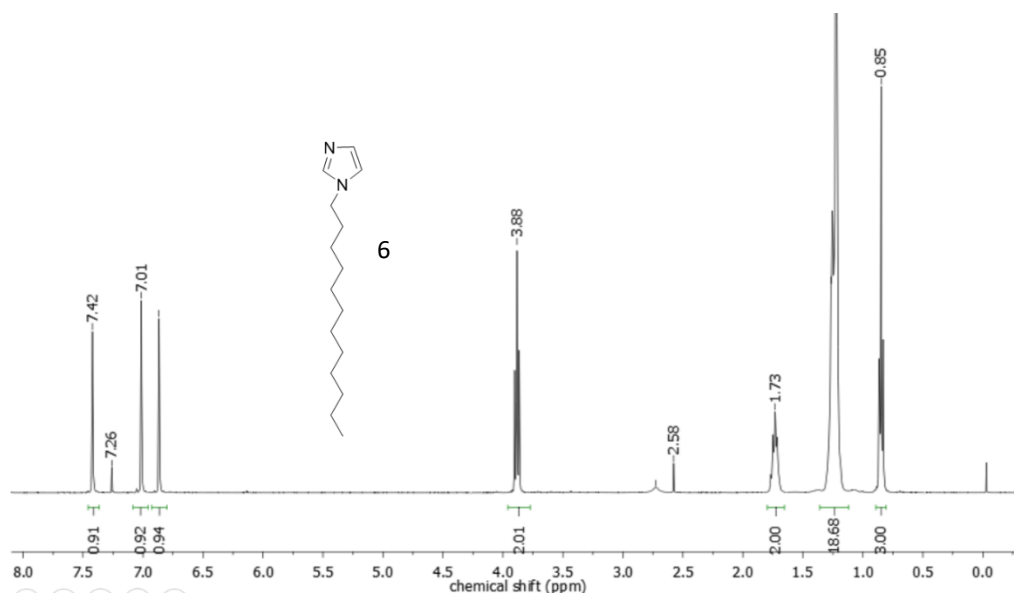


Figure 9.18. ¹H-NMR spectrum of compound 6 in CDCl₃

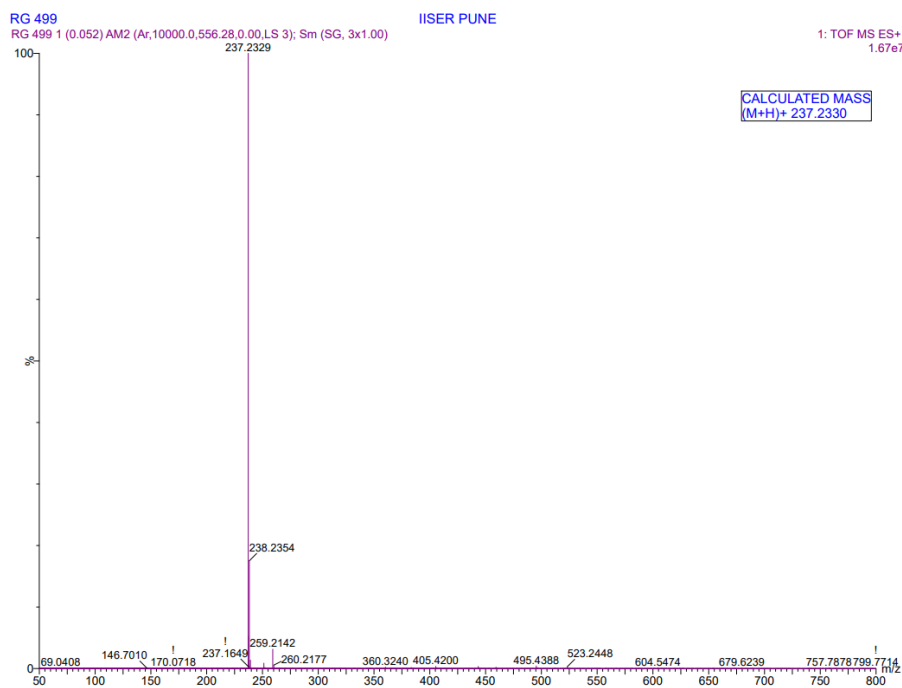


Figure 9.19. HRMS spectrum of compound 6 in CHCl₃

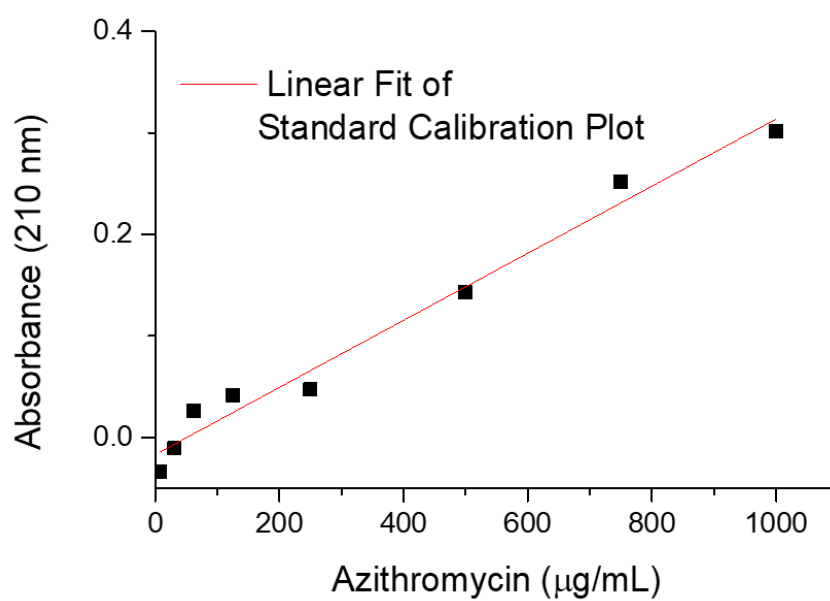


Figure 9.20. Standard calibration plot for absorbance at 210 nm for different drug concentration in acetonitrile : water mixture