REPUBLIC OF TURKEY
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CORE-CROSSLINKED AMPHIPHILIC BLOCK COPOLYMER MICELLES FOR ANTIBACTERIAL APPLICATIONS

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A thesis submitted by Melike Şeyma KADAYIFÇI in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** is approved by the committee on 27.04.2018 in Department of Bioengineering.

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Melike Şeyma KADAYIFÇI

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LIST OF SYMBOLS

^{o}C	The degree celsius
Mm	Micrometer
$[CTA]_0$	Initial concentrations of RAFT agent
$[I]_0$	Initial concentration of initiator
$I\cdot$	Initiator derived radical
K_p	Chain propagation rate constant
$[M]_0$	Initial concentration of monomer
$M_{n,theorecthical}$	Theoretical number average molecular weight
M^m	The molecular weight of the micelle
$M_{w,monomer}$	Molecular weight of the monomer
$M_{w,CTA}$	Molecular weight of the RAFT agent
nm	Nanometer
P_n	Polymeric radical
R_g	The radius of gyration of the micelle
Z	The aggregation or association number
R_h	The total hydrodynamic radius of the micelle
R_c	The micellar core radius

LIST OF ABBREVIATIONS

ACVA	4,4'-Azobis (4-cyanovaleric acid)
ADIm	1-allyl-3-decylimidazolium bromide
AIBN	Azobisisobutyronitrile
AMP	Antibacterial mimic peptides
Am-BCPs	Amphiphilic block copolymers
ATRP	Atom transfer radical polymerization
BCPs	Block copolymers
CCPMs	Core crosslinked polymeric micelles
CM	Cytoplasmic membrane
CMC	Critical micelle concentration
CMT	Critical micelle temperature
CRP	Controlled radical polymerization
CTA	Chain transfer agents
CRP	Living/controlled radical polymerization
CTA	4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid
Da	Dalton
DNA	Deoxyribonucleic acid
DMF	*N*, *N*-Dimethylformamide
FRP	Free radical polymerization
FTIR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
HEMA	2-hydroxyethyl methacrylate
IEP	Isoelectric point
MIC	Minimum inhibitory concentration
MMA	Methyl methacrylate
NIPAm	N-isopropylacrylamide
NMP	Nitroxide-mediated polymerization
NMR	Nuclear magnetic resonance spectroscopy
NP	Nanoparticle
OEGMA	Oligo (ethylene glycol) methyl ether methacrylate
PAMAM	Poly(amidoamine)
PEI	Polyethyleneimine
PEG	Polyethylene glycol
PEGMA	Poly (ethylene glycol methacrylate)
PRE	Persistent radical effect
PDI	Polydispersity index
PLL	Poly-L-lysine

PMs	Polymeric micelles
RAFT	Reversible addition-fragmentation chain transfer polymerization
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
St	Styrene
QAC	Quaternary ammonium compounds
QAS	Quaternary ammonium salts
Q-LPEI	Quaternized linear polyethyleneimine
Q-PVP	Quaternized Poly (4-Vinyl pyridine)
TiO2	Titanium dioxide
TMC	N, N, N-trimethyl chitosan
UPy	2-ureido-4[1H]-pyrimidinone
VP	4-Vinylpyridine

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ABSTRACT

CORE-CROSSLINKED AMPHIPHILIC BLOCK COPOLYMER MICELLES FOR ANTIBACTERIAL APPLICATIONS

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MSc. Thesis

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The aim of this study is to synthesize and characterize positively charged core crosslinked polymeric micelles for antibacterial applications. To achieve this aim, at first, the homopolymer of POEGMA (Poly (Oligo Ethylene Glycol Methyl Ether Methacrylate) was synthesized via RAFT polymerization (Reversible Addition-Fragmentation Chain Transfer Polymerization) and then it was used as a macro-RAFT agent to enable the synthesis of Poly (4-Vinyl pyridine) (PVP)-POEGMA block copolymer.

When POEGMA-*b*-PVP amphiphilic copolymer was dissolved in water with bifunctional quaternization agent and heated, the copolymer self-assembled into core-crosslinked micelles. This study is first to report such core-crosslinked POEGMA-*b*-PVP copolymer-based micelles. The crosslinking and the quaternization agent 1, 6-Dibromohexane was used to quaternize PVP segment of the micelles. To investigate the effect of quaternization degrees on the antibacterial characteristics of the micelles, different amounts of 1, 6-Dibromohexane were used. Moreover, to clarify the effect of POEGMA on the antibacterial properties of the samples, quaternized PVP nanoparticle and POEGMA-*b*-PVP copolymer were also prepared and characterized. Gel Permeation Chromatography (GPC), Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (Zeta Sizer), Scanning Electron Microscopy (SEM), Nuclear Magnetic Resonance Spectroscopy (NMR) and antibacterial assays were performed to characterize the obtained materials.

The results revealed that POEGMA-*b*-PVP block copolymer was successfully synthesized by RAFT method in a controlled manner. The prepared core crosslinked micelles have an average particle size of 100 nm and zeta potential values within the range of (+25) - (+45) mV. To determine the antibacterial activity of the micelles, *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* were chosen as model

microorganisms and three different methods were used namely, broth microdilution, disc diffusion and, agar diffusion. These micelles exhibited antibacterial activity against *E. coli* better than *S. aureus* after overnight incubation. Based on our findings, it can be concluded that the obtained positively charged core-crosslinked micelles can be used for antibacterial applications.

Keywords: Micelles, Antibacterial, Block copolymer, RAFT Polymerization, Quaternization

ÖZET

ÇEKİRDEKTEN ÇAPRAZ BAĞLI AMFİFİLİK BLOK KOPOLİMER MİSELLERİN ANTİBAKTERİYEL UYGULAMALARI

Melike Şeyma KADAYIFÇI

Biyomühendislik Anabilim Dalı

Yüksek Lisans Tezi

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Bu tezin amacı, pozitif yüklü çekirdekten çapraz bağlı polimerik misellerin antibakteriyel uygulamalar için sentezlenmesi ve karakterize edilmesidir. Bu amacı gerçekleştirmek için, öncelikle POEGMA (Poli (Oligo Etilen Glikol Metil Eter Metakrilat) homopolimeri, RAFT polimerizasyonu (Tersinir Katılma-Parçalanma Zinciri Transfer Polimerizasyonu) yöntemi ile sentezlenerek Poli (4-Vinil piridin) (PVP)-POEGMA blok kopolimeri sentezi için bir makro-RAFT ajanı olarak kullanılmıştır.

POEGMA-*b*-PVP amfifilik kopolimeri su içinde bifonksiyonel kuarternizasyon ajanı ile çözündüğünde ve ısıtıldığında, kendiliğinden çekirdekten çapraz bağlı misel halini almıştır. Bu çalışma, literaturdeki çekirdekten çapraz bağlı POEGMA-*b*-PVP kopolimer esaslı misellerle alakali ilk çalışmadır. Çapraz bağlama ve kuaternizasyon ajanı olan 1,6-Dibromohekzan, misellerin PVP segmentini kuaternize etmek amacıyla kullanılmıştır. Kuarternizasyon derecelerinin, misellerin antibakteriyel özelliklerine etkisini araştırmak için 1,6-Dibromohekzan farklı miktarlarda kullanılmıştır. Buna ek olarak, örneklerin antibakteriyel özellikleri üzerinde POEGMA'nın etkisini açıklığa kavuşturabilmek için kuaternize PVP nanopartikülü ve POEGMA-*b*-PVP kopolimeri de hazırlanarak karakterize edilmiştir. Elde edilen malzemelerin karakterizasyonu için Jel Geçirgenlik Kromatografisi (GPC), Fourier Transform Kızılötesi Spektroskopi (FTIR), Dinamik Işık Saçılması (Zeta Sizer), Taramalı Elektron Mikroskopisi (SEM), Nükleer Manyetik Rezonans Spektroskopisi (NMR) yöntemleri kullanılmış ve antibakteriyel analizler yapılmıştır.

Elde edilen sonuçlar, POEGMA-*b*-PVP blok kopolimerinin, kontrollü bir şekilde RAFT yöntemiyle başarıyla sentezlendiğini ortaya koyulmuştur. Hazırlanan çekirdekten çapraz bağlı misellerin 100 nm ortalama tanecik boyutunda ve (+25) - (+45) mV aralığında zeta

potansiyel değerlerine sahip olduğu bulunmuştur. Misellerin antibakteriyel aktivitesini belirlemek için broth mikrodilüsyon, disk kuyucuk ve agar kuyucuk metotları kullanılarak, *Escherichia coli (E. coli)* ve *Staphylococcus aureus (S. aureus)* model mikroorganizmalar olarak seçilmiştir. Antibakteriyel çalışma sonuçları, misellerin, *E.coli* üzerinde daha iyi antibakteriyel aktiviteye sahip olduğunu göstermiştir. Bulgularımıza dayanarak, elde edilen pozitif yüklü çekirdekten çapraz bağlı misellerin antibakteriyel uygulamalar için kullanılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Misel, Antibakteriyel, Blok kopolimer, RAFT Polimerizasyonu, Kuaternizasyon

CHAPTER 1

INTRODUCTION

1.1 Literature Review

Bacterial infections are a global problem that causes many types of diseases which are difficult to treat and result in a serious threat to public health [1]. Antibiotics are naturally available and/or synthetically producible compounds which inhibit microorganisms especially bacteria [2]. Although antibiotics have been used as a bactericidal for many decades, bacteria-killing mechanisms of the antibiotics cause bacterial resistance [3].

To prevent bacterial resistance, materials having antibacterial effect including silver [4], copper [5], zinc [6], titanium dioxide (TiO_2) [7] have been studied as alternative treatments in the literature. However, these materials have caused quite serious problems such as high toxicity and environmental hazards [6], [8]. Besides, cationic polymers, quaternary ammonium salts (QAS), antibacterial mimic peptides (AMP) and, antibacterial nano-materials have also been investigated extensively [9]. Among these materials, cationic polymers have been one of the most promising materials to overcome bacterial resistance. Cationic polymers are polyelectrolytes which have positive charges in their chains and used to destroy the bacteria cell walls [10]. These polymers can be prepared in various forms including block copolymers (BCPs), star polymers, dendrimer structures and so forth [11].

Recently, copolymers have been synthesized by controlled/living polymerization techniques namely, atom-transfer radical-polymerization (ATRP), and Reversible addition−fragmentation chain-transfer polymerization (RAFT) [12]. These techniques are advantageous since they provide copolymers with desired molecular weight, chain length, and polymers which have functional groups in the chain end [13]. For instance, RAFT

polymerization is based on a reversible addition-fragmentation reaction mediated by thiocarbonylthio compounds used as chain transfer agent (CTA) [14]. The CTA structure and the experimental conditions (temperature, pressure, initiation, CTA/initiator ratio, concentration) are important factors for optimizing the kinetics and the efficiency of the molecular-weight-distribution control [15].

Amphiphilic block copolymers (Am-BCPs) have both hydrophobic and hydrophilic properties [16]. Amphiphilic is a term used to describe molecules which have both hydrophobic (water repelling) and hydrophilic (water-loving) groups [17]. Amphiphilic molecules (amphiphiles) can be block copolymers which show a variety of self-assembled nanostructures, including spherical micelles, cylinders, nanotubes, bilayers, vesicle and gel-like phases [18]. Am-BCPs can constitute micelle formation spontaneously in suitable solvents where the hydrophobic block forms a core surrounded by chains of the hydrophilic block extending into the solvent phase [19]. The amphiphilic copolymers self-assemble due to the decrease in free energy of the system as a result of the removal of the hydrophobic fragments from the aqueous environment [20].

Polymeric micelles (PMs) are self-assembly organized amphiphilic molecules which have many significant applications including solubilizers, emulsifiers, antibacterial agent and drug/gene delivery systems [21], [22]. Core crosslinked polymeric micelles (CCPMs) are core-shell structures consist of both hydrophobic and hydrophilic blocks. While the core part is made of hydrophobic blocks which are chemically and physically stabilized, [23] the mycelium shell part is made of hydrophilic blocks which dissolve in the water [24]. CCPMs require biocompatible block or blocks to be used in biological systems such as gene and drug delivery or biomedical devices such as wound dressings [2]. These segments can be polymers such as Poly(ethylene glycol) (PEG), Poly(ethylene glycol methacrylate) (PEGMA), (2-hydroxyethyl methacrylate) (HEMA) etc. [25].

Cationic copolymers can be prepared using a well-known strategy known as quaternization [26]. In the literature, cationic polymers with quaternary ammonium salts are one of the most used antibacterial materials [27]. It was reported that incorporation of hydrophilic functional segments with quaternary-PVP (Q-PVP) enhances bactericidal activity [28]. It is harmful to use PVP in biological systems because of its toxicity and its hydrophobic property. Therefore, PVP should be coated with biocompatible polymers such as PEG, PEGMA and, HEMA which decrease the cytotoxicity of the copolymer [29]. Previously, PVP-*b*-PEG copolymer and PVP-*b*-PEG micelles [30],and PVP-*co*-

PEGMA copolymers have been published. [31]. Moreover, PVP-*b*-POEGMA copolymers have also been reported by Topuzogullari et al.[32], [33]. However, although PVP, PEG and PVP-*b*-PEG based micelles were already prepared in the literature, as far as we are aware, there is no study on the antibacterial activity of these types of micelles. Furthermore, a core-crosslinked form of these micelles has not been prepared yet.

In this thesis, it occurred to us that by preparing micelles based on POEGMA-*b*-PVP amphiphilic block copolymers and then positively charging them, we could achieve producing novel materials having antibacterial activity. For this purpose, the POEGMA-*b*-PVP copolymer was synthesized by RAFT polymerization and then bifunctional quaternization agent (1, 6-dibromo hexane) was used to crosslink the hydrophobically associated VP based blocks to form CCPMs. To the best of our knowledge, this study is first to report core-crosslinked POEGMA-*b*-PVP copolymer-based micelles and their antibacterial activity. To characterize the micelles FTIR, NMR, GPC, DLS, SEM and antibacterial assays were used. The above-mentioned POEGMA-*b*-PVP copolymer was quaternized using monofunctional quaternization agent (1-bromo hexane) to obtain quaternized copolymer. The antibacterial efficiency of the core crosslinked micelles was compared with this copolymer. Furthermore, to clarify the effect of POEGMA shell, crosslinked PVP nanoparticles were also synthesized and characterized for comparison.

1.2 Objective of the Thesis

This study aims to synthesize and characterize antibacterial amphiphilic core crosslinked POEGMA-*b*-PVP micelles for antibacterial applications. To achieve this aim, Am-BCP based on POEGMA as hydrophilic block and PVP as the hydrophobic block was prepared via RAFT polymerization. The Am-BCP was self-assembled into micelles where hydrophobic PVP blocks were crosslinked by quaternization using bifunctional quaternization agent. Herein, our goal is to optimize the antibacterial properties while improving the solubility and biocompatibility of these novel micelles which have various degree of quaternization. Moreover, the antibacterial properties of the micelles were also being compared with the quaternized POEGMA-*b*-PVP copolymer and PVP nanoparticles.

1.3 Hypothesis

As it is known in the literature, PVP and POEGMA copolymer can be quaternized bearing two bromines in the end groups, and thus give a positively charged, crosslinked and a highly biocompatible copolymer. In this study, it is expected that when the POEGMA-*b*-PVP copolymer is dissolved in water in the presence of a quaternization (crosslinking) agent, it would create CCPMs which have potential antibacterial applications.

CHAPTER 2

GENERAL INFORMATION

2.1 Antibacterial Polymers

An antibacterial polymer can be defined as an agent that kills bacteria or inhibits bacterial growth [34]. Antibacterial polymers were synthesized using 2-methacryloxytroponones by Cornell and Dunraruma in 1965 where they were first described as bactericidal polymers and copolymers which kill the bacteria [35], [36]. Then, polymers with quaternary ammonium groups were synthesized in 1971 by Panarin et al [37]. Recently, new structures and engineering designs of polymers with antibacterial properties made a great advance. Antibacterial polymers can be applied to biomedical devices, renewable energy, food markets, water purification, textiles, agriculture and hygienic applications [38]. Besides, they are great candidates preventing the bacterial resistance associated with antibiotics use. Mostly, the activity of these antibacterial agents is directly proportional to their toxicity on humans [39]. Therefore, the development of antibacterial polymers with high bactericidal activity as well as less toxicity is strongly needed.

2.1.1 Classification of Antibacterial Polymers

Polymers that possess antibacterial properties can be classified as naturally and synthetically derived cationic polymers. [40]. The most common natural-derived cationic polymers are chitosan, cellulose derivatives, and ε-Poly-L-lysine (EPL) [41] whereas the most known synthetic cationic polymers are poly-4-vinyl pyridine (PVP), poly(amidoamine) (PAMAM) and Polyethyleneimine (PEI) [42].

2.1.1.1 Naturally Derived Cationic Polymers

Natural cationic polymers are generally biodegradable, biocompatible, bactericidal and non-toxic materials. Due to these advantageous properties, they have gained an increasing interest. Recently, they have been extensively used in drug and gene delivery, antibacterial applications, tissue engineering and water treatment [43]. In the following paragraphs, the most well-known natural cationic polymers will be shortly reviewed.

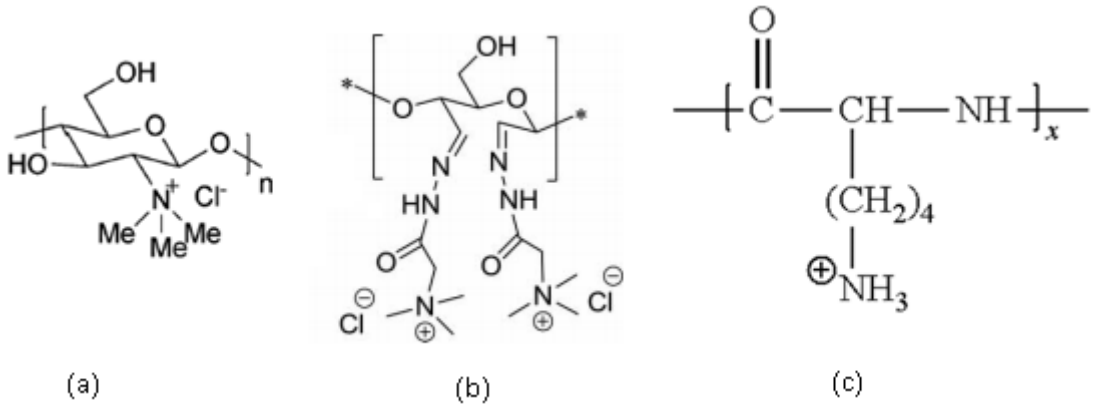


Figure 2. 1 Naturally derived cationic polymers; chitosan (a) [44], cellulose derivative [45] (b), EPL (c)

Chitosan is a natural polycation (cationic polymer) obtained from chitin by N-deacetylation reaction with alkali [46]. Chitosan is extensively used as an antibacterial agent due to its nontoxicity, high biodegradability, and bactericidal characteristics. [47].

Cellulose is the most abundant natural polymer in the world. Cationic cellulose and its derivatives are used in several therapeutic applications such as drug delivery and cosmetic [48] for their water solubility, biodegradability, inexpensive and bactericidal properties [49].

Poly-L-lysine (PLL) is a polypeptide containing a group of cationic polymers that are produced by bacterial fermentation [50]. EPL has an antibacterial activity that disrupts the bacterial outer membrane by adsorption on its cell surface causing the death of microorganism by stripping [51].

2.1.1.2 Synthetically Derived Cationic Polymers

The major drawbacks of natural cationic polymers can be overcome using synthetic cationic polymers. They have unique properties due to their easy chemical modification [44]. Polymers with quaternary ammonium compounds (QAC) are one of the most

studied classes of synthetic cationic polymers that are able to destroy bacteria. These polymers supply wide structural versatility by changing the molecular weight, hydrophobicity, and surface charge [52]. Among QAC polymers, PVP, PAMAM [42], PEI, polymethacrylate (PMMA) and, *N*-halamines are the most common and significant ones, therefore, they will be briefly mentioned below.

Poly(4-vinyl pyridine)

PVP is a neutral polymer and has reactive pendant pyridine ring that can lead to pyridinium-type cationic polymers [52]. PVP attracts considerable attention because of its unique properties, including the amphoteric characteristics of the pyridine rings, hydrophilic-hydrophobic balance and the modifiability of the nitrogen atoms through numerous chemistries [53]. The positively charged PVP can be obtained via quaternization reaction by modifying with different agents or end groups [33], [54]. Alkylated PVP (Figure 2.2) is one of the most investigated cationic polymers that exhibit antibacterial activity [55]. Various alkyl halides (RX) have been used to alkylate the nitrogen atoms of the pyridine ring of PVP to form QAC moieties [56].

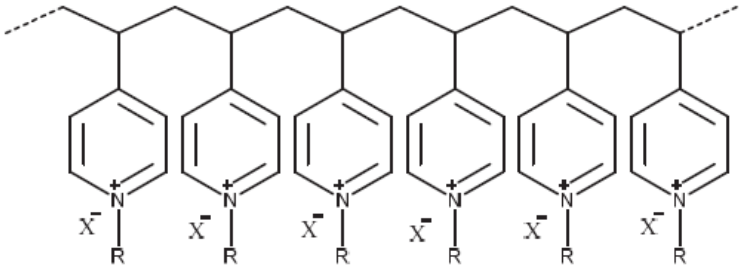


Figure 2. 2 Structural representation of alkylated poly(vinyl)pyridine. X^- = counterion, R = alkyl group [56]

The most accepted antibacterial mechanism is an electrostatic interaction between positively charged PVP and negatively charged bacterial cell wall that causes the disruption of the bacterial cell wall, thus leading to cell death [57]. The bactericidal activity of quaternized PVP can change depending on charge density. Bactericidal activity has been determined by previous studies for a wide spectrum of bacteria such as *Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa* and, *Streptococcus pneumonia* [58].

Quaternized PVP containing both block and random copolymers also displayed good antibacterial activity [59]. For instance, Medjahed et al. reported the synthesis of PVP

containing quaternary alkyl bromides to obtain cationic copolymer poly[N-isopentyl(4-vinyl pyridinium)] bromide (P4VPIPBr) (Figure 2.3) [60].

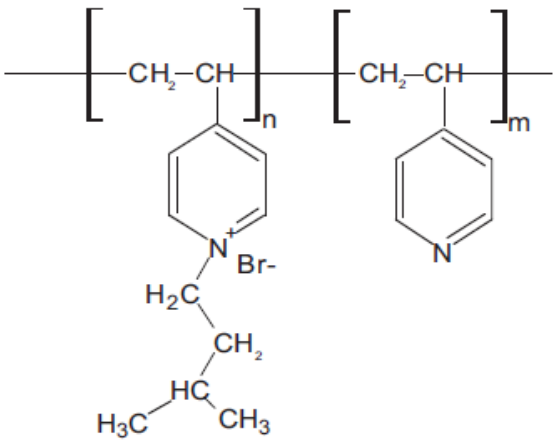


Figure 2. 3 Structural representation of quaternized P4VPIPBr [60]

Polyethyleneimine

PEI is one of the most widely used cationic polymers as antibacterial agent containing reactive amino groups offering several types of chemical modifications. It can be synthesized in both linear and branched structure [61]. The cationic group of PEI tends to cling to the bacterial cell membrane, causing the cell membrane to break. The antibacterial effect of PEI has been proven on both gram-positive and gram-negative bacteria species [62]. Quaternized PEI can be prepared by both tertiary amination reaction and quaternization (Figure 2.4) [63].

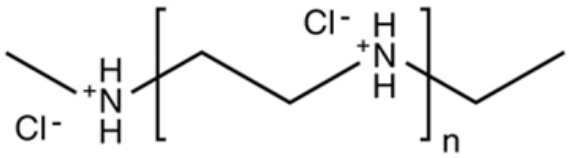


Figure 2. 4 Chemical structure of quaternized linear PEI (Q-LPEI) [63]

Poly (amidoamine)

PAMAMs are a group of synthetic cationic polymers with numerous desirable characteristics including biodegradability, biocompatibility, hydrophilicity and a lower toxicity compared to other cationic polymers [64]. PAMAMs can be synthesized by Michael-type polyaddition of primary amines and obtained as both linear and dendrimer structure [65].

Linear PAMAMs have a good DNA binding and gene transfer characteristics [66]. In spite of PAMAM dendrimers displaying high antibacterial effect, they exhibit high toxicity [67]. The dendrimers increase exponentially in functional groups with generations and they can be obtained as macromolecules with a regular, narrow dispersity and highly branched three-dimensional structure. Each layer of the dendrimer is called single generation [68]. S. Charles et al. reported an elegance study about PAMAM (Generation 3, G3) dendrimers which is modified into QAS using tertiary amines (Figure 2.5). Disk diffusion method was used as an antibacterial assay and the modified amphiphilic quaternized dendrimers displayed antibacterial activity against *E. coli* and.*S. aureus* [69].

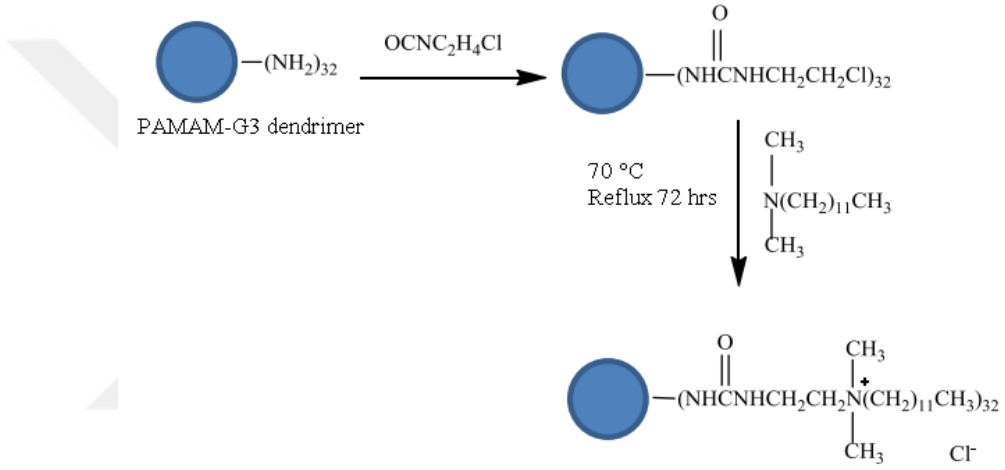


Figure 2. 5 Schematic representation of the synthesis of PAMAM-G3 quaternary dendrimer ammonium salt [69]

N –halamine Polymers

N-halamines can be defined as compounds containing one or more halogen atoms covalently binding to the nitrogen atoms [70]. Recently, *N*- halamines have gained great interest as antibacterial agent [71]. *N*-halamines and polymers with *N*-halamines functional groups have been studied by Sun et al. to stabilize the antibacterial properties of halogens (chlorine or bromine). The major antibacterial activity of the *N*-halamines relates to the specific influence of oxidative halogen (Br+ or Cl+) targeted at amino groups or thiol groups in proteins receptor on to direct contact with a cell which arrives at cell deactivation or cell inhibition [72]. In addition, after extensive usage of *N*-halamines, the antibacterial activity can be lost. However, the antibacterial activity could be regenerated by treating halogen-release agents again. Therefore, *N*-halamines are

strong and refreshable antibacterial agents against a broad spectrum of gram-positive and gram-negative bacteria [71].

Polymeric *N*-halamines are advantageous materials over monomeric ones in numerous applications such as wound dressing, water treatment, and textile industries. To prepare polymeric *N*-halamines, several approaches have been developed [73]. Considering the great antibacterial mechanism of *N*-halamines, combining them with QAS was used as one of the best approaches to synthesize stronger biocides [74]. Figure 2.6 shows the synthesis of 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC) compound by halogenation of its precursor 2,2,5,5-tetramethyl-1,3-imidazolidinone (TMIO).

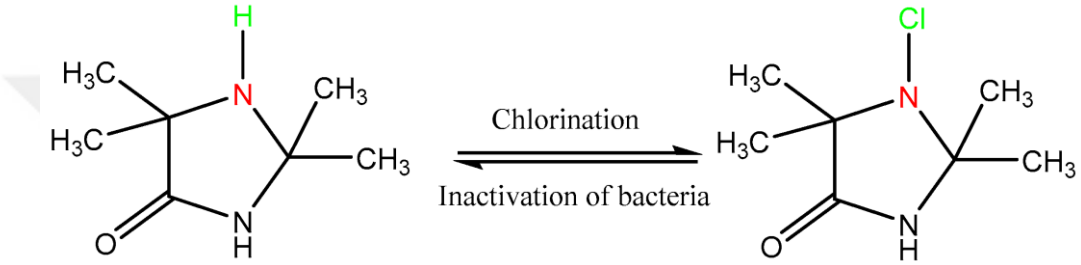


Figure 2. 6 Schematic illustration of MC synthesis process by chlorination of TMIO precursor [75]

Other Cationic Polymers Containing Active Pendant Groups

These polymers do not possess inherently antibacterial activity, however, they acquire antibacterial effect by adhering to different components or active groups using chemical modification techniques [76].

Most common cationic quaternary polyelectrolytes used as antibacterial polymers are acrylic or methacrylic derivatives, and many of them are provided by methacrylic monomers which are commercially available such as 2-(dimethylamino) ethyl methacrylate. These polymers supply wide structural versatility by the change of molecular weight, hydrophobicity, surface charge and other parameters [34]. A large number of amphiphilic copolymer series with polymethacrylate and polymethacrylamide groups which has hydrophobic and cationic side chains were studied by Kuroda et al. This investigation revealed that the characteristic of amine side chains and also the hydrophobic characteristic of polymers are key factors in optimal antibacterial activity [77].Phenol, benzoic acid, and p-hydroxybenzoate esters are the most extensively used disinfectants due to their antibacterial activity. Synthesizing new antibacterial polymers with enhanced activity was attempted by integrating them with some polymer backbone

[78]. Benzaldehyde is another important compound of this group that is known for its antibacterial effect. Benzaldehyde with methyl methacrylate polymers have been synthesized and its bactericidal activity against *Bacillus macroides*, *P. aeruginosa* and *Dunaliella tertiolecte* exhibited [79].

Antibacterial polymers containing quaternary phosphonium or sulfonium groups exhibit mechanisms similar to the quaternary ammonium groups. Polymers containing phosphonium are more effective than quaternary ammonium salt polymers with regard to antimicrobial activity [80].

2.1.2 Killing and Repelling Mechanisms of Antibacterial Polymers

In order to kill or inhibit the growth of bacteria, various polymeric materials have been produced (i.e., polymers, copolymers, hydrogels [81], cryogels [82], micelles [83]) and, their bacterial killing mechanisms were investigated. This section of the thesis describes the various mechanisms according to the activity of antibacterial polymers. These mechanisms can be divided into either repelling (passive action) or killing (active action) the bacterial cells.

Repelling bacteria was realized with polymeric material coatings mostly based on PEG or similar polymers, by ultra-hydrophobic modifications (low surface energy, exclusion steric repulsion) or highly negatively charged polymers (low surface energy) (Figure 2.7) [84], [85].

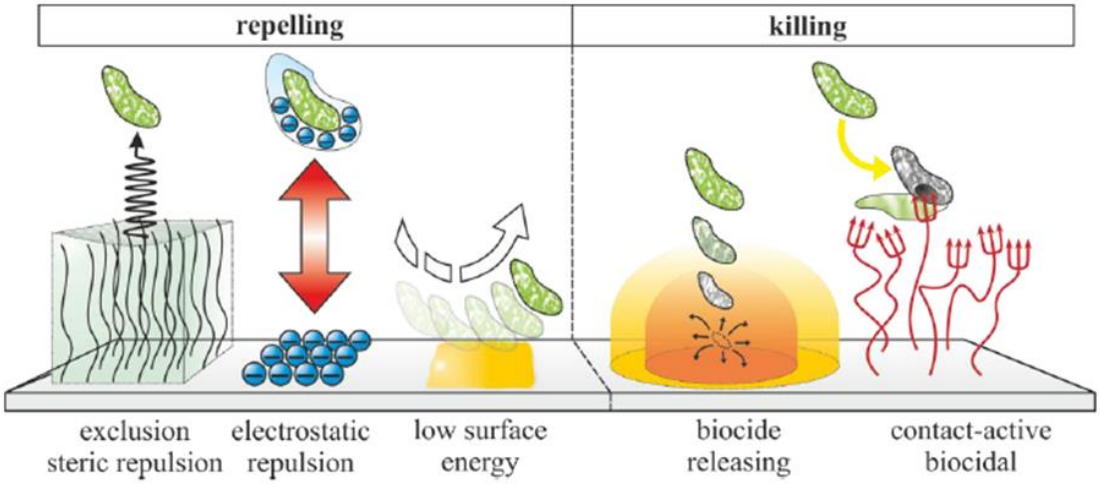


Figure 2. 7 The repelling and killing mechanisms of the antibacterial polymers [36]

The killing of bacteria can be executed by either a biocide releasing from a polymeric matrix [86] or by contact-killing cationic polymers such as QAS, alkyl pyridinium, or quaternary phosphonium [87]. Several antibacterial mechanisms are reported to demonstrate how these cationic polymers are capable of disruption of the bacterial cell membrane via contacting the bacteria cell wall depending on the type of bacteria.

The general bacteria killing mechanism on contact is indicated as follows (Figure.2.8):

(i) At first, the cationic polymer must cling to the bacterial cell wall.

(ii) The most bacterial cell walls are negatively charged; therefore, the adsorption of cationic polymers has demonstrated to be more effective than adsorption of anionic polymers.

(iii) It has been proved that the high levels of cations are able to confer antibacterial characteristics by ion exchange mechanism (electrostatic interactions) between the charged surface and the bacterial membrane.

(iv) The cationic chain of the polymer must then diffuse through the cell wall and cling to the cytoplasmic membrane.

(v) The destruction of the cytoplasmic membrane and subsequent leakage of cytoplasmic components causes the cell death [88].

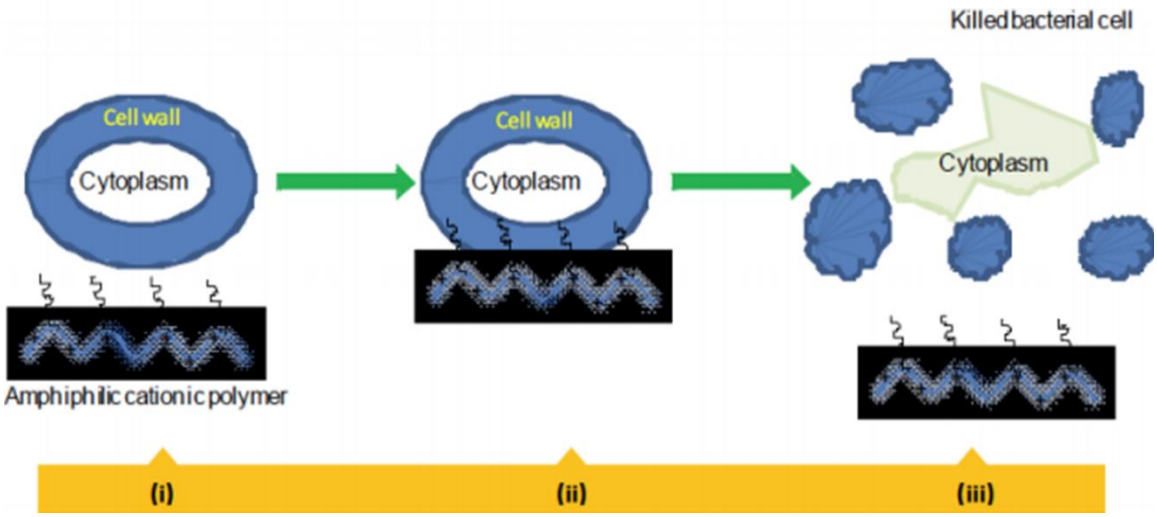


Figure 2. 8 The general bacterial cell killing of an amphiphilic cationic polymer on contact (i). Interaction of amphiphilic cationic polymer with bacteria cell wall, (ii) diffusion to interact with cytoplasmic membrane, and (iii) destruction of bacteria cell upon contact [43]

Song et al. produced series of cationic polymers with random, alternating, uniform backbones. They suggested the antibacterial mechanism of these polymers are causing

potassium efflux, damaging the bacterial cytoplasmic membrane and breaking down the membrane potential [89].

Besides the bacteria-killing mechanisms of cationic polymers depending on electrostatic interactions, there are also some other killing mechanism approaches reported in the literature. Wu et al. reported the killing mechanism of cationic micelles with bearing amide moieties (trimeric, tetrameric and hexameric surfactants) which they produced: the cationic micelles integrate with the cell membrane of E. coli through two steps. Firstly, the unity of outer membrane of E. coli is destroyed by the electrostatic interaction of the positively charged ammonium groups of the micelles with negative charged groups of E. coli, causing in loss of the barrier function of the outer membrane. Secondly, the inner membrane is split by the hydrophobic interaction of the hydrocarbon chains of micelles with the hydrophobic domains of the inner membrane, resulting in the cytoplast leakage (Figure 2.9) [90].

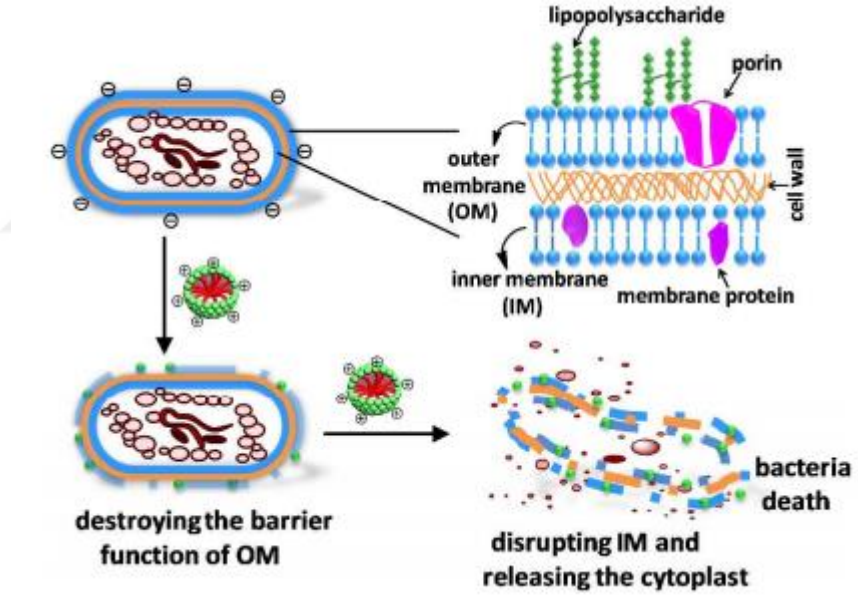


Figure 2. 9 The schematic representation of antibacterial mechanism of the cationic surfactant micelles to *E. coli* [90]

The bactericidal mechanism of EPL/poly (ε-caprolactone) (PCL) copolymer self-assembled into NPs was reported by Zhao et al. The self-assembled NPs have been explored that they can destroy bacterial walls/membranes and induce the increase in reactive oxygen species (ROS) and alkaline phosphatase levels. Moreover, the NPs induced the alterations in bacterial osmotic pressure, leading in cell invagination to form holes and result in the leakage of cytoplasm [91].

Chindera et al. reported a new bacteria-killing mechanism of polyhexamethylene biguanide (PHMB) contrary to previously accepted general killing mechanism. It was suggested that PHMB is able to pass into the bacterial cells, block cell division and condense chromosomes. These lead to intracellular foci of DNA. It was assumed that DNA binding is an alternative killing mechanism. This mechanism was confirmed by observations that PHMP NPs effects on bacterial growth suppressed by DNA binding ligand Hoechst 33258 with the pairwise combination [92].

Zhi et al.reported the design of a two-level dual-functional (release-killing and contact-killing) antibacterial coating with both QAS and silver. These dual-functional antibacterial coatings demonstrated strong bacteria-killing activity due to the release of Ag^{+} and after the depletion of Ag antibacterial activity persisted because of the immobilized QAS [93].

2.1.2.1 Factors Effecting Killing Mechanisms of Antibacterial Polymers

The main approach for designing new antibacterial polymers has been determined by the common structural traits of the outer envelope of different bacteria. The important property of the outer envelope of bacterial cells is a negatively charged; hence most antibacterial polymers are positively charged [94]. The negative load of the bacterial cell wall is caused by lipoteichoic acid molecules of gram-positive bacteria, the lipopolysaccharides, and phospholipids of the gram-negative bacterial outer membrane and phospholipid bilayer of cytoplasmic membrane [95]. Considering features of cell wall/outer membrane of the bacterial cell, most parts of antibacterial polymers were decorated with cationic amphiphilic molecular systems targeting cytoplasmic membrane [70]. Amphiphilic polymers contain hydrophilic polar segment bearing a positive charge and a non-polar hydrophobic segment. Such copolymer structures provide adsorption/absorption capacity, surface-activity characteristics and high binding affinity for bacterial cells [70]. They are enhanced by high lipophilicity to damage structure of cell membranes, followed by cell membrane disruption, leakage of cytoplasmic contents, and cell lysis [96].

The bactericidal activity of antibacterial polymers is regarded as a role of balance between various factors. One of these factors are polymer associated; molecular weight, hydrophilicity, charge density, counterion, beside and environmental factors such as pH,

temperature, etc [76]. Some main factors affecting antibacterial activity are reviewed in the following sections.

Molecular Weight

The molecular weight (Mw) is important to modulate physicochemical characteristics of polymers. Mw is the major factor in controlling bactericidal activity for polyacrylates and polymethyl acrylates with side-chain biguanide groups. Ikea et al. reported that the optimum range of Mw is between 5×10^4 and 1.2×10^5 Da for antibacterial effect when above or below this range, bactericidal activity decreased [97]. Likewise, another study indicates that optimum Mw range of poly (tributyl 4-vinyl benzyl phosphonium chloride) is between 1.6×10^4 to 9.4×10^4 Da [98].

Charge Density

Polymers with high positive charge density develop better electrostatic interaction with the bacterial cell wall. For instance, in the case of chitosan, electrostatic interaction enhances with increasing charge density. It was reported by Takahashi et al. that higher degree of deacetylation results in increasing charge density, hence leading to a higher antibacterial effect activity of chitosan towards *S. aureus* [99].

Yin et al. reported a series of polymeric films using 1-allyl-3-decylimidazolium bromide (ADIm) as antibacterial agent and N,N-diallylimidazolium (DIm) bromide as a crosslinking agent. The ratio of hydrophobicity and the charge density group of the polymer was varied and prepared via thiol–ene photopolymerization. The resulting cationic films exhibited a significant antibacterial effect against both *S. aureus* and *E. coli*, which correlated with the charge density [100].

Hydrophilicity and Hydrophobicity

Hydrophilicity is a substantial necessity for the activity of any antibacterial agent. Amphiphilic polymethacrylates are designed by alternating the content of hydrophobic groups, exhibiting better antibacterial functions [101]. Likewise, chitosan derivatives are obtained by quaternization, alkylation, and acylation resulting in a higher bactericidal activity than in their natural form [102].

The structure-bactericidal activity relationship of polycations, in the form of branched PEI modified with various QACs and alkyl groups, with bacteria was studied by Kiss et

al [103]. The results of antibacterial assays revealed the connection between bactericidal behavior and polymer hydrophobicity which is the bactericidal activity and membrane binding affinity of the functionalized PEIs decreased with increasing hydrophobicity. This result was ascribed to a high tendency for micellization for the PEIs that were more hydrophobic. Thus, the hydrophobic parts required for bactericidal membrane interactions were shielded within the core, therefore obstructive binding between the cationic polymer and membrane lipid layers.

Counter Ions

The bactericidal effect of counterions has been noticed towards polymers with quaternary ammonium/phosphonium groups. The strong binding affinity of counter ions due to quaternary groups results in less bactericidal activity because of slow and less release of free ions during the interaction. Among quaternary ammonium groups, bromide and chloride provide the highest antibacterial effect [104].

Flemming et al. reported polyurethanes modified with methyl and ethyl quaternary ammonium chloride groups exhibited antibacterial activity. However, the same polymer with iodine counterion displayed more activity toward S. aureus [105]. This observation was attributed to counter ions have a high impact on antibacterial efficacy.

pH

The pH effect of polymers which have amphoteric property (can react with both acids and bases) has been investigated. For instance, chitosan exhibits pH-dependent bactericidal effect which is at a highest at acidic pH due to its good solubility and also cationic charge. However, there are not any reports of materials displaying bactericidal effect at basic pH [106].

2.1.3 Applications of Antibacterial Polymers

2.1.3.1 Medicine and Healthcare Products

The surfaces of biomedical devices provide an inhabitant for bacterial growth and cause bacterial infections. Although improvements in biomaterials and methods, generally hospital-acquired infections derive from biomedical devices [107]. Therefore, new approaches for the improvement of polymer conjugates used to coat implantable devices

carry out an opportunity to apply antibacterial agents directly to the device surface. Thus, these advanced conjugates prevent bacterial colonization on the medical devices and inhibiting device-associated infection [108].

Polyacrylate derivatives are one of the most studied materials because of their availability, low toxicity, wide alteration of functionalized monomers, and simple processing. Moreover, in biocidal coatings, the potential of various *N*-halamine siloxane and quaternary ammonium salt siloxane copolymers are used by Liang et al. Both *N* - halamine and quaternary groups displayed activity against *S. aureus*. However, the *N* - halamine groups are only effective against *E. coli* [109].

For polymeric drugs, antibacterial polymers are strong candidates with high activities, which can be attributed to their characteristics nature on carrying high charge density of the active pendant groups of the polymer chains. For instance, electrospun fibers including tetracycline hydrochloride based on poly (ethylene-*co*-vinyl acetate) and poly (lactic acid), were prepared to use as an antibacterial wound dressing [110]. Furthermore, gentamycin loaded poly (methyl methacrylate) beads form an effective drug delivery system for local antibiotic therapy in bone and soft tissue infections [111].

Antibacterial sutures were prepared by the grafting of 2-hydroxyethyl methacrylate monomer (HEMA) on polypropylene monofilament by radiation. The modified sutures showed an antimicrobial activity effect against *S. aureus* [88].

Antibacterial peptides and antibacterial peptide mimetics are a new generation of antibacterial agents with high bactericidal, wide spectrum activity against a variety of microorganisms and modulation of the immune response [112]. Antibacterial wound-dressings comprising of cotton gauze containing antibacterial peptides conjugated with polyelectrolytes (chitosan, alginic acid sodium salt) exhibits a high bactericidal effect against *Staphylococcus aureus* and *Klebsiella pneumonia*. It has been proven that these wound-dressings do not demonstrate cytotoxicity to human dermal fibroblasts [113].
In cosmetics, chitosan, and its derivatives are used as setting agents and hair conditioners, whereas quaternary ammonium cellulose derivatives are commonly used as skin and hair moisturizer [114].

2.1.3.2 Water Treatment

Antibacterial polymers have extensive application in water treatment systems, such as hand-held water filters, fibrous disinfectants, and surface coating. Chlorination is regarded as a classic and important part of the disinfection of drinking water and, wastewater treatment [115]. However, these disinfectants have some drawbacks like short-term stability in aqueous solution, creating chlorine-resistant microbial species and, releasing residual toxic products from degradation of chlorination process such as carcinogenic trichloromethanes [116]. This has increased the safety concerns of such disinfectants and led to the development of alternative, new and safe disinfectants.

A new sand filtration water polymeric disinfection is developed based on the antibacterial properties of hydrophobic polycations (N-hexylated PEI) covalently attached to the sand's surface. These polymers have the ability to be renewed by simple washing steps. They are especially effective against chlorine-resistant bacteria [117]. Polystyrene copolymer beads supported dendrimers were synthesized and investigated as a water treatment system. For use in drinking water disinfection, *N* -halamine polymers in the form of highly cross-linked porous beads have been studied. It was found that *N* -halamines displayed strong, and durable bactericidal activities against E. coli and S. aureus [118].

A representative example of the application of a water-insoluble copolymer using methyl methacrylate (MMA) and N-vinyl-2-pyrrolidone polymers was reported by Tyagi et al. for water treatment purposes[119]. The copolymers and refill cartridge were iodinated to provide antibacterial activity as depicted in Figure 2.10.

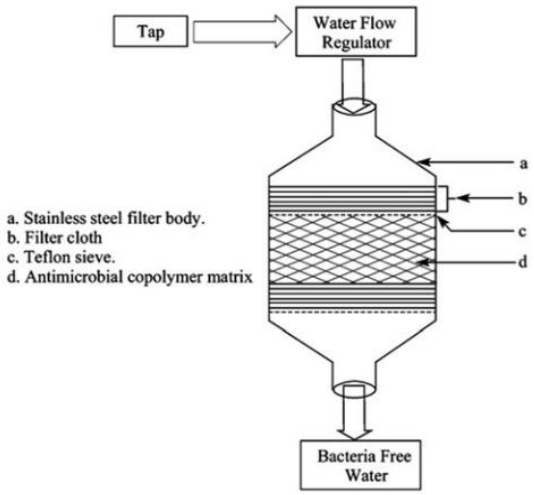


Figure 2. 10 Water purification system in antibacterial copolymer matrix (MMA-*c*-N-vinyl-2-pyrrolidone) provides antibacterial activity [119]

2.1.3.3 Food Applications

Bacterial contamination of food has two crucial consequences, namely reducing the shelf life of food and increasing the risk of food-borne disease [115]. Antibacterial agent included in packaging materials can control microbial contamination by decreasing the growth rate and maximum growth population and/or expanding the lag phase of the target microorganisms or by deactivating the microorganisms by contact. Antibacterial polymers can be utilized in several food-related applications involving packaging [120].

Polymer samples with high antimicrobial activity in growth medium and low activity in foods consist of triclosan in plastics. Some polymers that are naturally antimicrobial are used in films and layers. Chitosan has been used as a layering and preserves fresh vegetables and fruits without harming the fungi [121].

There are several alternatives where antibacterial polymers can participate in the design of antibacterial packaging such as:

- Integrating into the final polymeric food package containing the volatile antimicrobial agents.
- Doping the antibacterial agents into the polymer.
- Surface modifications by attaching antibacterial polymers via covalent linkages or by coatings.
- Using inherently antibacterial polymers or polymers with QAC [122].

2.1.3.4 Fibers and Textiles

Antibacterial polymers are quickly becoming a standard for some textile products such as for medical, institutional, and hygienic usage [123]. To provide anti-odor or biostatic properties, antibacterial sportswear has lately become popular in women's dress and aesthetic clothes [124].

Recently, various developments of antibacterial polymers are taking place in the field of textile coating and finishing. Many options are available in the antibacterial textiles market: Immobilization of the antibacterial agents in the fiber; treating fibers with resins or crosslinking agents; Encapsulation of antibacterial agents; surface-coating of the fibers; chemical modification with covalent linkages using of homopolymers, graft polymers, and blending with the fiber [125].

Antibacterial activity of polyamide fibers was provided by graft polymerization of acrylic acid. The final fibers which have carboxylic groups in their chemical structure were also modified with neomycin and, gentamycin to get antibacterial fibers. The bactericidal activity was tested against *E. coli*, *S. aureus*, and *P. aeruginosa*, and the fibers showed high antibacterial effects [88], [126].

To improve the shrinkage and antibacterial features of woolen fabrics, polyurethane-chitosan blend polymer is used [127]. Moreover, Polymers with *N*-halamine functional groups are widely used to make antibacterial textile materials [128].

2.2 Antibacterial Polymer Preparation

Antibacterial polymers can be obtained in various ways based on their structure such as homopolymers, random copolymers, alternating copolymers and, block copolymers (Figure 2.11). In previous studies, the antibacterial activity of polymers was investigated in terms of their monomeric compositions and structures. For instance, Takahashi et al. studied antibacterial activity of methacrylate-based homopolymer (PE_0) and random copolymer (PE_{31}) and reported that PE_0 was the more effective towards cariogenic bacterium *S. mutants* and removing biofilm compared to PE_{31} [129]. Since block copolymers are the subject of this thesis, they will be discussed in more detail in the next sections.

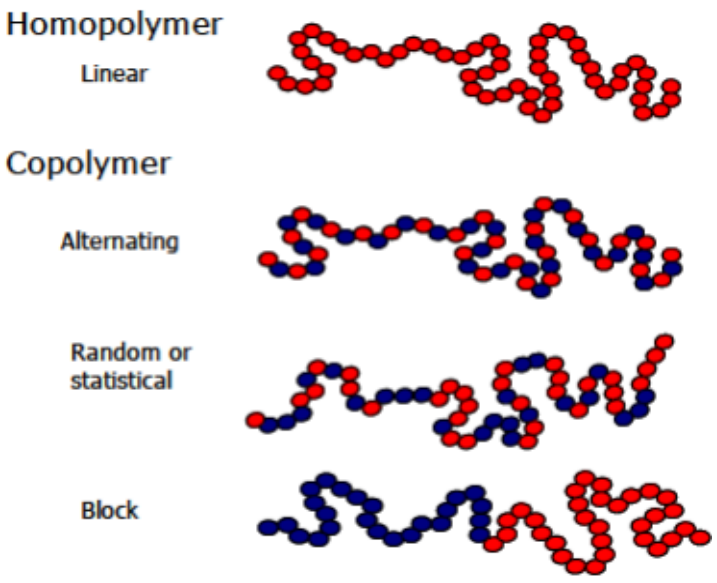


Figure 2. 11 Structural representation of homopolymer and copolymers (alternating, random and block) [130]

2.2.1 Block Copolymers

Block copolymers (BCPs) are a specific group of copolymers comprised of a substantial sequence of each repeat unit. BCPs are normally synthesized by controlled/living polymerization and their sequential formation can vary from A-B diblock structure to A-B-A triblock structure and to multiblock A-B-C systems with many segments (Figure 2.12) [131].

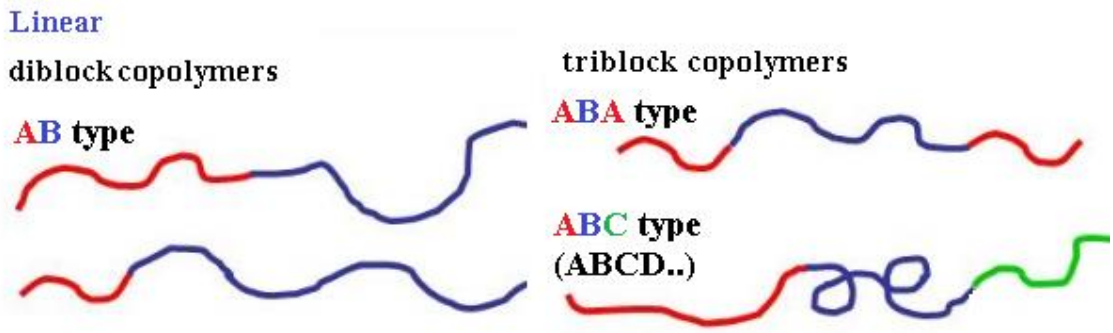


Figure 2. 12 Representative architectures of diblock(AB),triblock(ABA), multiblock(ABCD..) of block copolymers [131]

2.2.1.1 Synthesis of Block Copolymer

In polymer chemistry, controlled/living radical polymerization (CRP) is a technique of chain growth polymerization where the ability of an extended polymer chain to terminate has been removed. This is a form of addition polymerization in living polymerization or controlled polymerization [132]. Furthermore, the rate of chain initiation is much larger than the rate of chain propagation. This is contributed to the polymer chains growth at a more constant rate than in tradition chain polymerization, this results in polymer chain lengths being equal (i.e., they are monodispersed) [133].

Over the past years, the limitations of conventional free radical polymerization (FRP) have been overcome as many methods for CRP have evolved considering the understanding of chemistry developed in the areas of conventional radical and controlled ionic polymerizations (Figure 2.13). There are several CRP techniques that have been developed and the most used CRP techniques are NMP (nitroxide-mediated polymerization), ATRP (atom transfer radical polymerization) and RAFT (reversible addition fragmentation chain transfer polymerization) [134]. These techniques are commercially important and promising methods. For the extending lifetime of the propagating chains, each of these techniques relies on creating a dynamic equilibrium

between a low concentration of active propagating chains and amount of dormant chains which are not able to terminate [135].

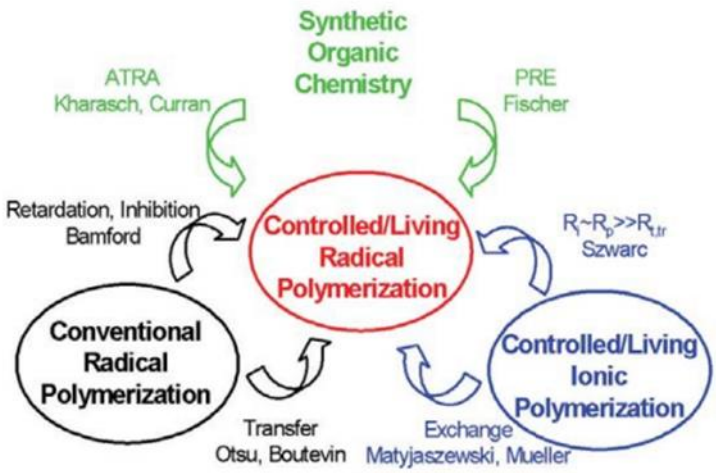


Figure 2. 13 Development of CRP by integrating into many areas of chemistry [135]

CRP is a popular technique for synthesizing block copolymers. It has a great advantage including predetermining molar mass and control over end groups. CRP forms new block polymers including, linear multi-block terpolymer, comb, tri- and mikto-arm star copolymer, cyclic block terpolymer, and graft copolymers (i.e., comb, centipede, barbwire) (Figure 2.14) [136].

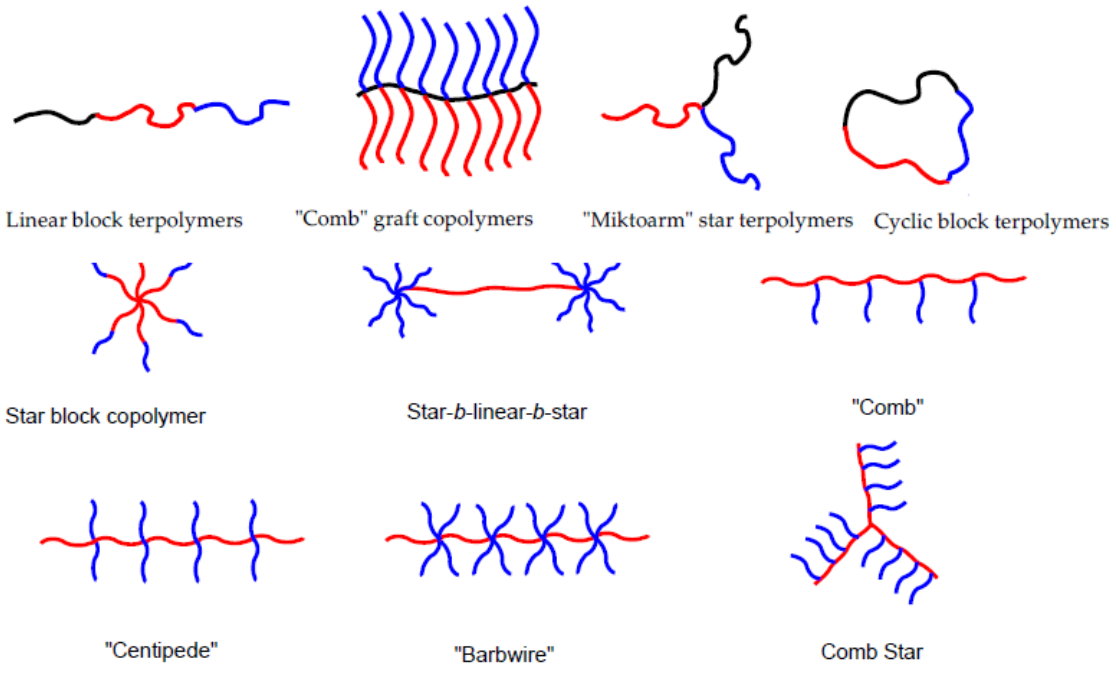


Figure 2. 14 Block copolymer architectures obtained from the living/controlled radical polymerization [136]

Among CRP techniques, RAFT polymerization is one of the most suitable methods for the synthesis of block copolymers. [137]. RAFT, on the other hand, has seen rapid growth due to its superior compatibility with a broader range of functionalities and high tolerance of impurities.

Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization

The RAFT polymerization technique was first discovered in 1998 by a CSIRO (Commonwealth Scientific and Industrial Research Organization) group in Australia [138] and, until today it has been the subject of thousands of studies [139]. The RAFT process progresses differently than the NMP and ATRP methods. This method relies on degenerative chain transfer instead of persistent radical effect (PRE) to provide the control over polymerization [140]. In a degenerative transfer system, the overall number of radicals do not change during the activation-deactivation process (Figure 2.15), therefore a source of radicals is needed, typically a radical initiator [139].

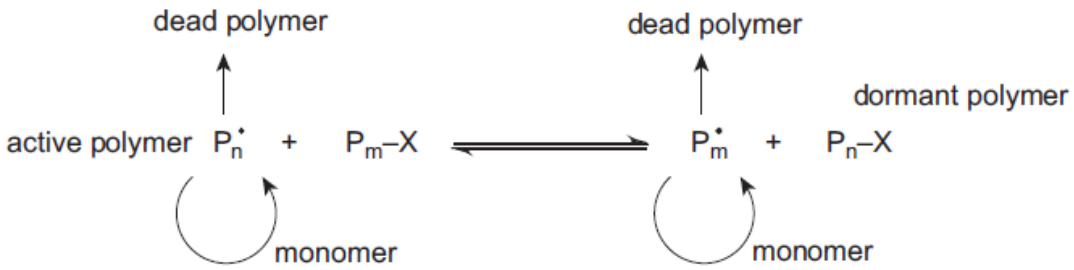


Figure 2. 15 RAFT polymerization by degenerate chain transfer [141]

RAFT polymerization is facilitated in the presence of the thiocarbonylthio chain transfer (or RAFT) agents ([ZC (=S)SR](Figure 2.16) but, requires being selected in accordance with the monomer(s) used [142]. This agent is a molecule containing a thiocarbonylthio group and being active against radicals. The generic structure R–S–C(=S)–Z of RAFT agents containing, the R and the Z groups (called the *reinitiating* group and the *activating* group, respectively), carry out different functions. Hence, the Z group firstly controls which free-radical groups bearing to the C=S bond and stabilizing carbonylthio radical intermediate. Besides, the R group must be a good homolytic reinitiating group, having the ability to initiate new polymer chains [143].

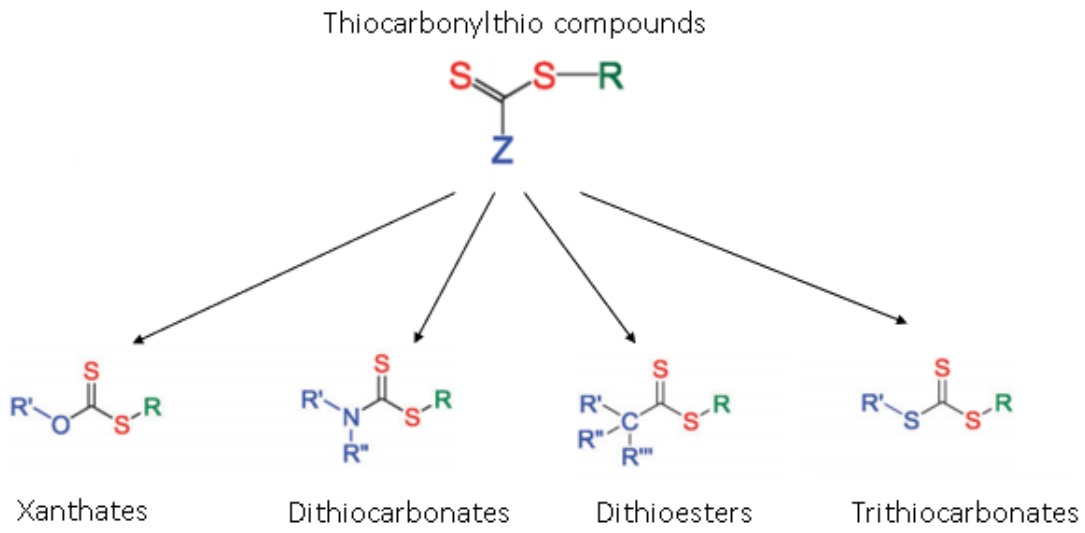


Figure 2. 16 General structures of RAFT agents belonging to four classes based on differences in functional groups at the Z position of thiocarbonylthio compounds [143]

The main characteristics of RAFT polymerization can be summarized as follows;

- RAFT polymerization can be carried out by adding a chosen amount of a suitable RAFT agent to a conventional free radical polymerization. Generally, the same monomers, solvents, initiators, and, temperatures are used [133].
- The Mw of the product obtained in RAFT polymerization is controlled by changing the ratio of the concentration of monomer to the concentration of CTA. The ratio $[M]_0/[CTA]_0$ required for targeted molecular weight values can be calculated using the equation 2.1 below as follows [24]:

$$M_{n,\ \text{theoretical}} = \frac{[M]_0 \times M_{\text{W}}\ \text{monomer} \times \rho}{[\text{CTA}]_0} + M_{\text{W}}\text{CTA} \tag{2.1}$$

  - $M_{n,\ \text{theoretical}}$: Theoretical number average molecular weight,
  - $[M]_0$ and $[CTA]_0$: Initial concentrations of monomer and RAFT agent,
  - $M_{w,\ \text{monomer}}$, and $M_{w,\ CTA}$: Molecular weights of monomer and RAFT agent (g/mole).
- A low molecular weight distribution is provided and generally, the polydispersity is <1.20.
- Polymers are generally pink or yellow color because they contain thiocarbonyl thio end groups. Loss of color indicates that the RAFT end group is diminishing [144].

- RAFT is most appropriate for the polymerization or copolymerizations of methacrylic monomers and other similar monomers [145].

RAFT Polymerization Mechanism

The mechanism of RAFT polymerization can be performed in five steps: initiation, initialization, reinitiation, chain equilibrium, and termination (Figure 2.17).

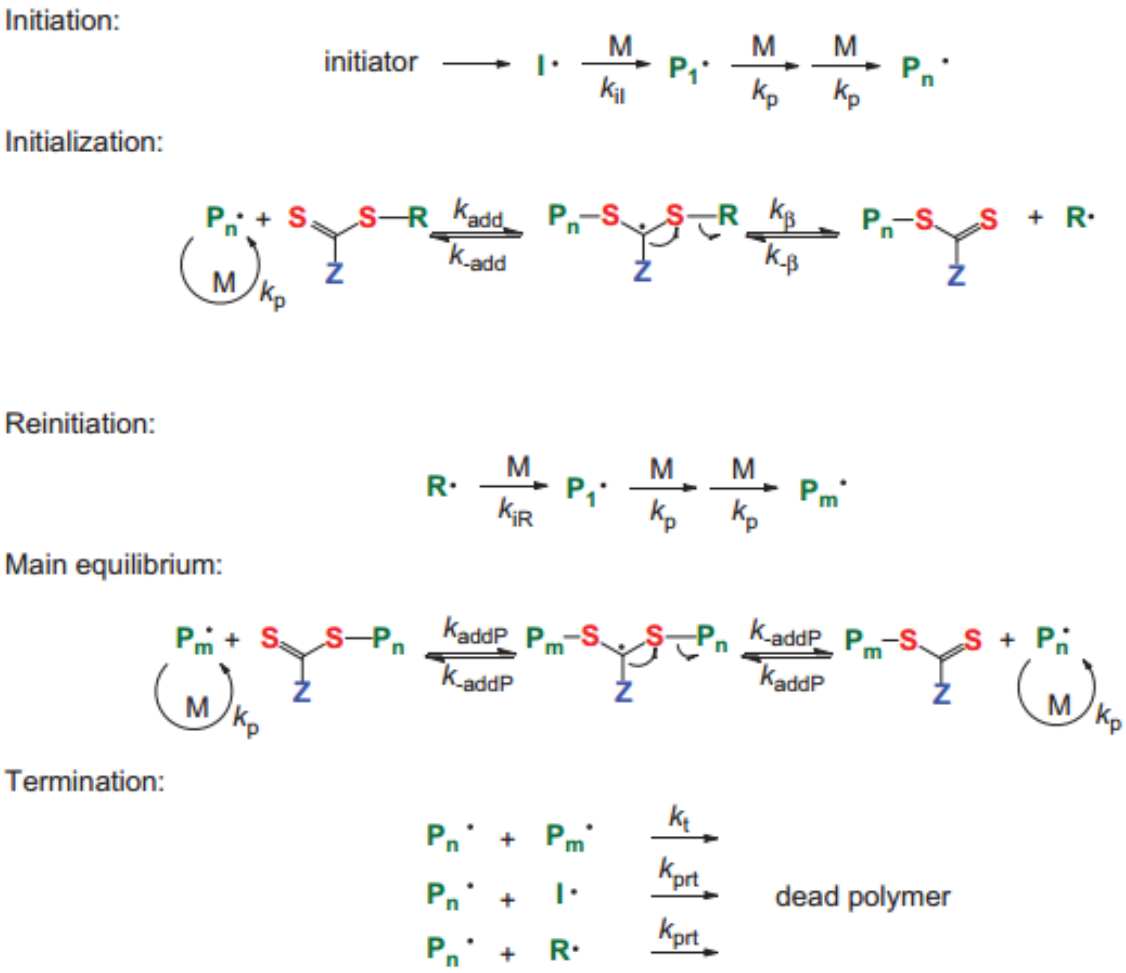


Figure 2. 17 The steps of RAFT polymerization where thiocarbonylthio compounds are used as chain transfer agents [141]

Initiation: The RAFT process is initiated by radical initiators, such as AIBN. Generally, radicals can be occurred by heat, light, laser, γ- radiation. The initiator (I) produces a radical species which initiates an active polymerizing chain (Pn·) by reacting with the monomer.

Initialization: The active polymer chain Pn· quickly adds to the reactive CS bond of the CTA (rate constant, k_{add}) fabricating an intermediate adduct radical. This radical bears

reversible fragmentation either toward the initial growing chain (rate constant, k_{add}) or to free the group R by B-scission (rate constant, k_b) and simultaneously produces a macro chain transfer agent (macro-CTA).

Re-initiation: The leaving (re-initiation) group radical (R) is capable of initiation and reacts with the monomer to initiate another active polymer chain ($Pm^{\cdot}$).

Main equilibrium (chain equilibrium): The active chain Pm goes through the addition-fragmentation process resulting in equilibrium between the active (radical) and dormant (bound to the CTA) states. This is the main equilibrium that represents the fundamental step in the RAFT process. It holds the majority of the active propagating species into the dormant CTA compound because of limiting the possibility of conventional radical-radical termination reactions. The other (Pn) is active in polymerization when one polymer chain (Pm) is in the dormant stage [146]. It is evident from the above mechanism shown in Figure 2.19 that the thiocarbonylthio group of the original RAFT agent is maintained in the polymeric product (via Pn-X and Pm-X). The thiocarbonylthio moiety is a key feature of the RAFT process and it is responsible for the living character of the RAFT polymer.

Termination: Possible termination reactions among propagating and intermediate radicals are shown in Figure 2.17. The fast interchange in the chain transfer process ensures that the concentration of growing radical chains is kept lower than that of the stabilized radical intermediates, limiting termination reactions. Consequently, limited termination reactions still occur via combination or disproportionation mechanisms [147].

Advantages of RAFT Polymerization

The most important property of RAFT polymerization is that it can be used for almost all of the polymerization of vinyl polymers including functional polymers such as vinyl acetate and acrylic acid [148]. The thiocarbonylthio end of the chain is protected in RAFT polymerization. That's why living chain end is formed and it can be obtained as macro-CTA [149].

The reaction can be determined by the desired Mw and length of the chain [13]. It allows obtaining monodisperse polymers [150]. In addition, different structural copolymers such as block, gradient, star, and graft copolymers can be easily synthesized via RAFT

polymerization [151]. Other advantages of the RAFT approach is the use of common radical initiators and the low toxicity of some RAFT agents [152].

Disadvantages of RAFT Polymerization

One of the major disadvantages of RAFT is that the resulting polymer products are usually pink or have various shades of this color. This is because of the fact that chain transfer agents are derived from derivatives such as dithioester, thiocarbamate, and xanthate. RAFT agents can have a pungent odor because of the gradual disintegration of the thiocarbonylthio moieties to yield small sulfur compounds [153].

The synthesis of a RAFT agent needs a multistep synthetic procedure and purification [154].

Block copolymerization via RAFT Technique:

The general and simplest method for the synthesis of BCPs using RAFT polymerization is through the integration of two (or more) monomers. For the preparation of an AB diblock copolymer, the first sequence (homopolymer) is synthesized via a RAFT process, followed by purification. The resulting end-reactive homopolymer acts as a macro-RAFT agent for the second step of polymerization. The final diblock product is obtained by adding the second monomer with an appropriate initiator (Figure 2.18) [155].

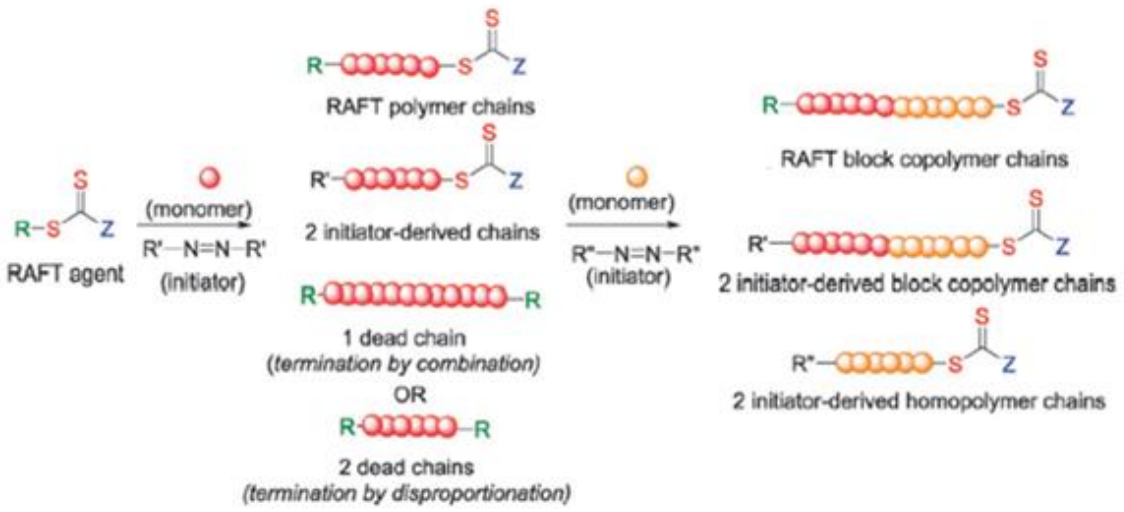


Figure 2. 18 The various steps of RAFT polymerization for the synthesis of block copolymers [137]

2.2.2 Self-Assembly of Block Copolymers

Amphiphilic block copolymers (Am-BCPs) are macromolecules composing covalently bonded segments of opposite water-compatibility. The amphiphilic nature of these block copolymers containing thermodynamically incompatible parts leads to their unique properties in selective solvents, at surfaces and in the bulk, due to microphase separated morphologies [156]. Their distinctive self-organization in the presence of selective media (solvent) often results in the creation of aggregates such as spherical micelles, vesicles, nanotubes, nanofibers and other colloidal size structures [157]. Those aggregations are driven by some interactions such as hydrogen bonds, steric effects, hydrophobic and, electrostatic interaction in accordance with their aspects of segments types [158].

Stability of the Am-BCPs within the aggregates in solution is given both to the hydration of the hydrophilic head groups and to the insertion of the hydrophobic tail(s) in the aqueous solution [159]. The first main driving force of self-assembly of Am-BCPs are an enthalpic gain in dissociation because of hydrogen bond formation and, the second is a gain in entropy of the bulk water, called the hydrophobic effect [160].

Hydrogen bonds are fundamental in biological systems as they can bind to biomolecules however their force is not enough to break into living cells. The hydrophobic effect is important not only in the development of amphiphilic micellar aggregates but also for a large variety of biological applications such as antibacterial, protein folding or drug delivery in bio-membranes [161].

Amphiphilic polyelectrolytes have another significant interaction that mainly relates to the ionic self-assembly and stability in solution which is represented by Overbeek et. al., called the theory of charged amphiphiles [162]. This theory expresses the headstones to rationalize forces between charged amphiphiles at interfaces and to elucidate their aggregation acting in a selective solvent. It assumes that the interaction in a solvent between charged amphiphiles is both the van der Waals and double layer interactions [163].

The van der Waals interaction is constituted by a short-range repulsive force and these interactions are vital in bio-membranes where the phospholipids are packed in an aqueous medium [164]. The double layer interaction occurs from the solvation of polyelectrolytes from the solution. As represented in Figure 2.19, the electrical double layer composes a

layer of ions strongly bound to the stern layer (charged surface) and a contiguous region of the loosely associated mobile ion [165].

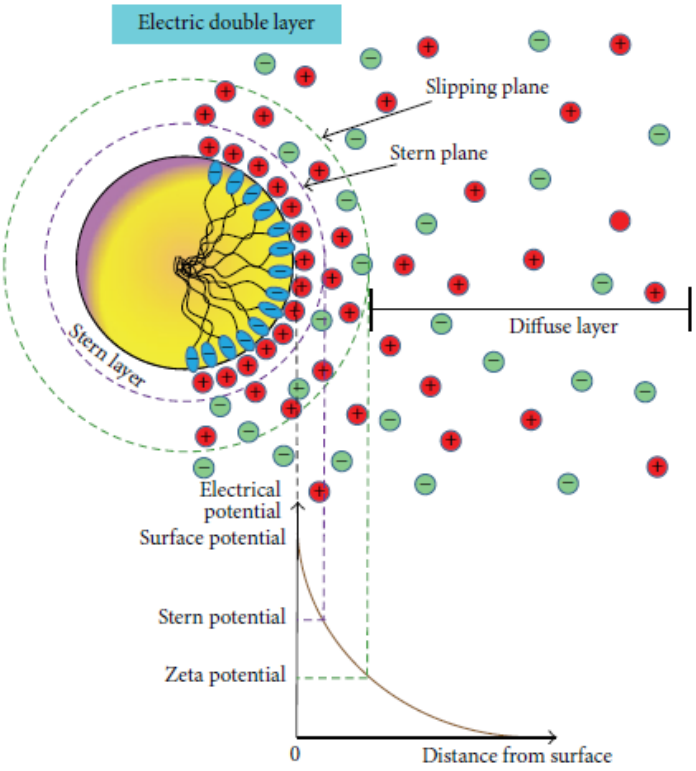


Figure 2. 19 An electrical double layer of self-assembled amphiphilic polyelectrolytes [161]

The shape and size of the micellar aggregates consist of the molecular geometry of its constituent molecules on the surface and the solution terms, for example, concentration, temperature, ionic strength, pH, and surfactant. Controlling the shapes of the micellar aggregates enables developing a wide range of nanostructures architecture [166].

2.2.3 Antibacterial Block Copolymer Preparation Through Quaternization

BCPs which in one of the blocks presenting basic tertiary amine moieties that can be transformed to quaternary ammonium salts by reaction with acids such as alkyl halides or HCI [167]. In this way, the PVP blocks of PS-PVP have been converted to quaternary ammonium salts in the presence of CH_3I, HCI or using other quaternization agents to produce cationic block polyelectrolytes [168]. The quaternized new copolymers could be dispersed in water in contrast with precursors that are insoluble in aqueous solution [169].

In case of poly(butyl methacrylate) (PBMA)-P(1-(2-(4-methylthiazol-5-yl) ethyl)-1H-1,2,3-triazol-4-yl) methyl methacrylate (PMTA) block copolymer were quaternized using

both alkyl chains, butyl and methyl, to amplify their antibacterial characteristics (Figure 2.20) [170].

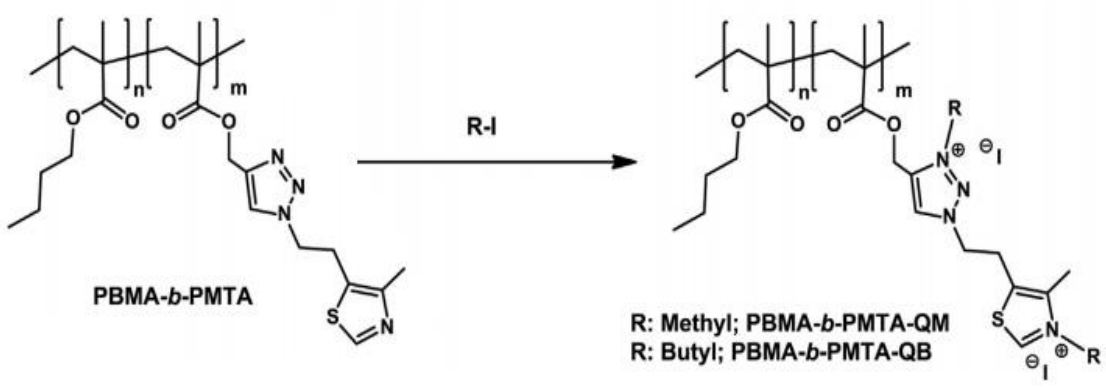


Figure 2. 20 Schematic representation of quaternized PBMA-*b*-PMTA block copolymers [170]

When the quaternization agent is a cycloaklylsultone or lactone, the conversion of the tertiary amine group to a zwitterionic group (sulfobetaine or carboxy betaine) is accomplished. BCPs containing a hydrophilic block with zwitterionic side groups and a hydrophobic block produced by the reaction of poly (dimethylaminoethylmethacrylate)-b-polymethacrylate blocks, prepared by guanosine triphosphate (GTP) with 1,3-propane sultone [171].

Tertiary amine groups can also be converted to nitric oxide groups, resulting in blocks from hydrophobic block precursors which are water-soluble [169].

2.3 Polymeric Micelles

As highlighted above, Am-BCPs with large solubility differences between the hydrophilic and hydrophobic blocks, self-assemble in a selective solvent into polymeric micelles. The hydrophobic blocks of the polymers are insoluble in water and combine with hydrophobic interaction to form the mycelial core [172]. Hydrophilic blocks also form the outer shell that allows mycelium to dissolve in water. The micelle formation occurs at concentrations higher than the critical micelle concentration (CMC) (Figure 2.21), and a balance is established between the resulting micelle and individual polymers (unimers) [173].

CMC is an important parameter for characterizing micellization process. The CMC is defined as the threshold polymer concentration for micellization [174]. At concentrations lower than the CMC, the copolymer exists in solution as free chains. Above the CMC,

micelles are present, and for monodisperse systems, the concentration of free chains remains constant upon further increase in the copolymer concentrations [175]. The CMC for a given copolymer depends on solution properties such as temperature and solvent nature. However, in a selected solvent and at a constant temperature, the CMC is a function of copolymer properties such as relative block length, molecular weight, and chain architecture [176].

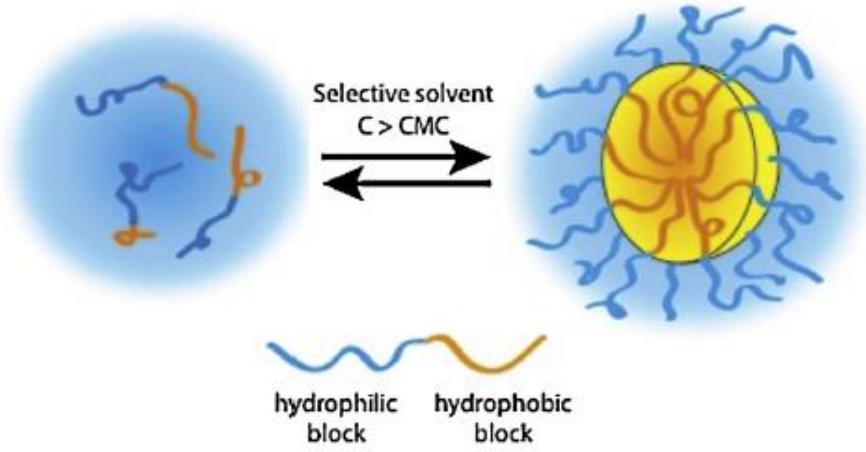


Figure 2. 21 Micellization of Am-BCPs when dissolved in selective solvents at C>CMC [23]

The micellar system can be characterized by;

- The molecular weight of the micelle *(M^m)*,
- The aggregation or association number (*Z)*, e.g. the average number of polymer chains in a micelle
- The radius of gyration of the micelle *(R_g)*,
- The total hydrodynamic radius of the micelle (*R_h)*,
- The micellar core radius *(R_c)*,
- The thickness of the shell (corona) formed by the soluble block (*L)*,
- The equilibrium constant unimers and micelles,
- The critical micelle concentration and the critical micelle temperature (the CMC and CMT, respectively),
- The morphology of micelle [177].

The particle morphologies of polymeric micelles can be varied as spherical micelles, cylindrical micelles (also called worm-like micelles), vesicles, rods have been reported [178]. For instance, Rodrigues et al. reported PEG-b-PVP based micelles from spherical to worm-like (Figure 2.22)[179].

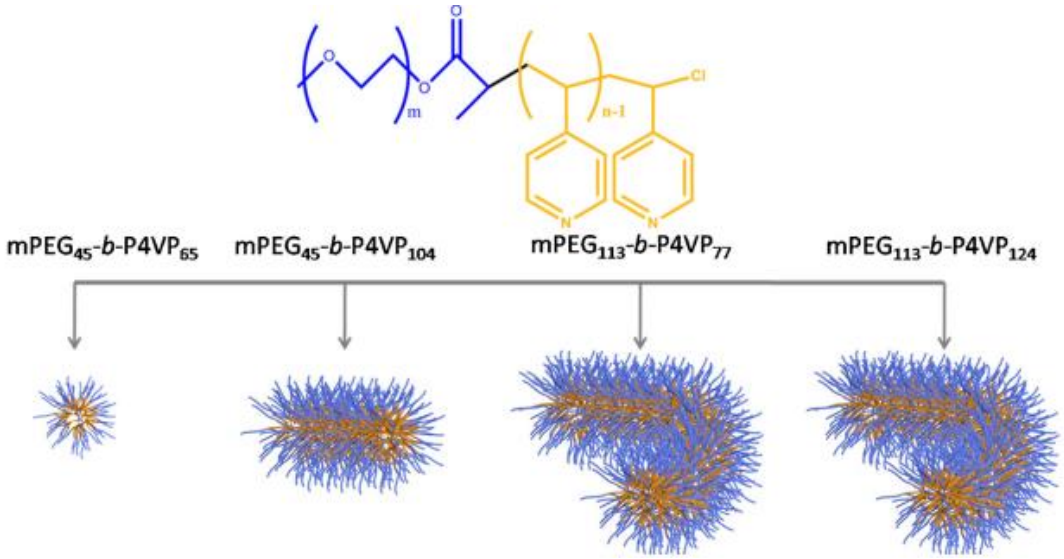


Figure 2. 22 Morphologies of micelles: (a) Sphere-like micelle; (b) rod-like micelle ; (c) worm-like micelle [179]

The major disadvantages of the micellar structures are that they become unstable and separated into their unimers at various temperatures below the critical micelle temperature or at concentrations below the critical micelle concentration or in the solution composition. The most important method used to maintain the stability of the micelle structure is crosslinking. In this respect, the micelle structure does not dissociate under environmental conditions [180]. Another reason for crosslinking is to provide time-dependent control over the structure. The crosslinked polymeric grains can be separated into two as block copolymer grains with crosslinking in the shell or core (Figure 2.23) [181].

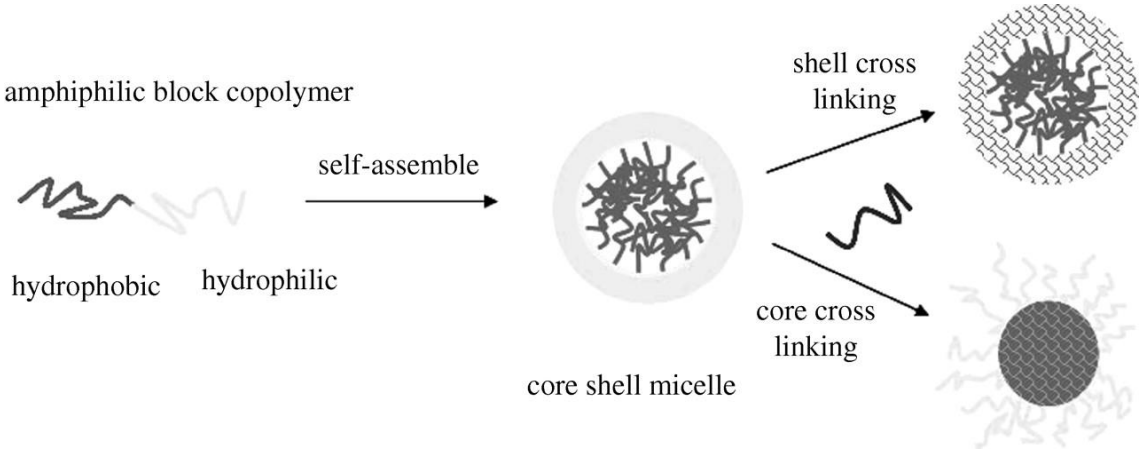


Figure 2. 23 Self-assembling of Am-BCPs in a selective solvent into spherical polymeric micelles and stabilization to provide either shell- or core-crosslinked polymer micelles [182]

Since core-crosslinked spherical polymeric micelles are the subject of this study, they will be reviewed in the next paragraphs.

As mentioned above, dynamic nature of polymeric micelles which leads to instabilities at high temperature, low concentrations, and under certain changes in solvent conditions. To stabilize polymeric micelles for in vivo applications block copolymers can be cross-linked in the micellar core [183]. It was proved that CCPMs have enhanced therapeutic efficacy, increased circulation time and increased target site accumulation [184], [185]. The example of a schematic representation for the formation of CCPMs (Poly (ethylene glycol)-*b*-poly(N-isopropylacrylamide-co-2-ureido-4[1H]-pyrimidinone) (PEG-*b*-P (NIPAm -*co*-UPy)) is shown in Figure 2.24.

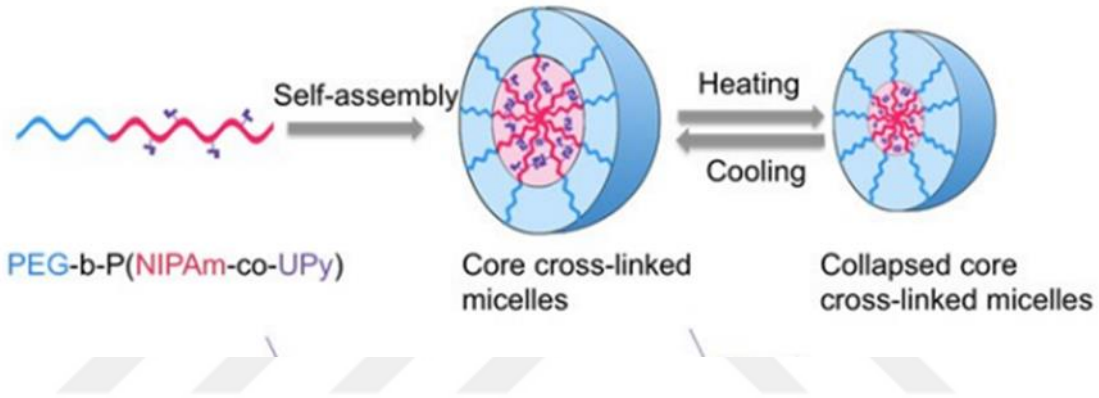


Figure 2. 24 Schematic representation of the formation of CCPMs (PEG-*b*-P(NIPAm-*co*-UPy)) with thermo-responsive cores from UPy containing DHBCs through hydrogen-bonding interactions of UPy groups [186]

The most extensively used methods for synthesizing CCPMs are:

(i) Radical polymerization used with micelles consisting of polymers containing polymerizable groups,

(ii) The addition of a bifunctional crosslinker, (R—R), and

(iii) Disulfide bridges in the event of PM containing thiol groups [23], [172].

2.3.1 Antibacterial Polymeric Micelles

Polymeric core-shell micelle nanoparticles are regarded as one of the most comprehensively studied nanostructures for antibacterial applications. This is attributed to the fact that such nanoparticles have been interested in a great number of studies in drug delivery for the past three decades [187]. Interestingly, there have been debates mostly about the role of micellization in bactericidal activity with respect to polymeric

micelles that are inherently antibacterial. This discussion can be categorized into two parts; the role of micellization dismissing and promoting the antibacterial efficacy.

The polymeric systems such as polyacrylates [188], polymethacrylates [189], and polycarbonates [190] where the synthesized polymers were able to form into micelles, the notion of self-assembly as a prerequisite for bactericidal activity was dismissed.

The positive contributory role of PMs formation on antibacterial effect was first suggested by Lenoir et al. through self-assembled micelles formed by poly(ethylene-co-butylene)-b-poly[2-(dimethylamino)ethylmethacrylate] (PEB-*b*-PDMAEMA) block copolymers that were quaternized via octyl bromide [191]. Antibacterial activity against *E. coli* that resulted in a (99.99%) reduction in cell counts within 30 min incubation at a polymer concentration of 100 μg/mL was demonstrated.

In another study, Yuan et al reported antibacterial and biodegradable poly(ethylene oxide)-b-poly(ε-caprolactone)-b-poly[(2-tert-butylaminoethyl) methacrylate] (PEO-b-PCL-b-PTBAM) micelles (Figure 2.25) [192]. PCL block was developed to self-assembly and impart biodegradability, while PEO was provided the biocompatibility and colloidal stability. This study relied on quaternization for antibacterial activity, the micelles reported as inherently antibacterial and membrane-active due to the PTBAM block contained secondary amine that is positively charged under physiological conditions.

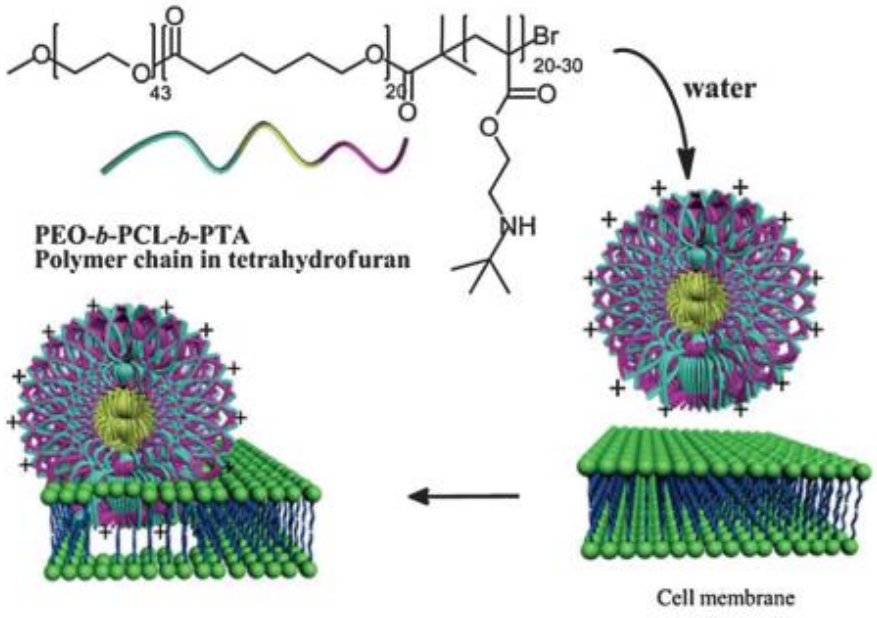


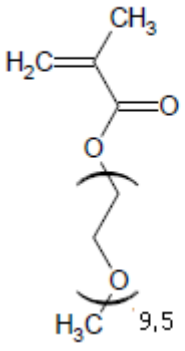
Figure 2. 25 Micellization of PEO-*b*-PCL-*b*-PTA triblock copolymer and the antibacterial mechanism of the micelle [192]

CHAPTER 3

EXPERIMENTAL SECTION

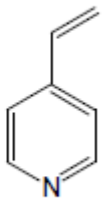
3.1 Materials

Oligo (ethylene glycolmethacrylate) 475 ($OEGMA_{475}$, monomer) (Sigma-Aldrich) 4-Vinylpyridine (4-VP, monomer) (Figure 3.1) (Sigma-Aldrich), Azobisisobutyronitrile (AIBN solution, radical initiator), (Sigma-Aldrich) 4,4'-Azobis (4-cyanovaleric acid) (Sigma-Aldrich) (ACVA, initiator), 1,6-Dibromohexane **(**Sigma-Aldrich) (crosslinker), Dichloromethane (Sigma-Aldrich), 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CTA, RAFT Agent) (Strem Inc.), *N*,*N*-Dimethylformamide (DMF, solvent) (Carlo Erba), sodium chloride (Carlo Erba), diethyl ether (Merck), and acetic acid (Riedel-de Haë) were used as received. Ultrapure water was obtained from the Millipore Milli-Q water purification system.



Oligo (ethylene glycol) methacrylate $OEGMA_{475}$

(Mn= 475 Da)



4-Vinilpiridin

4-VP

(Mn=105,14 Da)

Figure 3. 1 OEGMA and 4-VP monomers used for the block copolymer preparation.

3.2 Methods

3.2.1 Synthesis of Polymers

3.2.1.1 Synthesis of POEGMA Homopolymer via RAFT Polymerization

The synthesis reaction of the targeted homopolymer is shown in Figure 3.2. $[M]_0/[CTA]_0/[I]_0$=75/1/0.2 ratio was utilized for POEGMA synthesis reaction [148]. The initial concentration of the monomer was 0.5 M and the total solution volume was 10 ml. 2.375 g of $OEGMA_{475}$ was dissolved in 4,75 ml DMF in a penicillin vial using magnetic stirrer followed by addition of 9.31 µg CTA. 1.09 mg AIBN in 0,25 ml DMF solution was prepared in another vial and nitrogen (N_2) gas was exposed to the OEGMA and AIBN solutions for 30 min. The OEGMA solution was then placed in a temperature-controlled oil bath at 70 °C under magnetic stirring (1000 rpm) for 5 min, and polymerization was initiated by the slow addition of the AIBN solution. The reaction proceeded under magnetic mixing for 2 hours. Then, the excessive amount of the polymerization solution was precipitated using cold diethyl ether and centrifuged. The ether was removed by decantation and the obtained pink viscous polymer was dissolved in dichloromethane and precipitated with cold ether. The sample was dried in vacuum incubator overnight at 50 ^{0}C. The molecular weight of the homopolymer (Macro CTA) was determined by GPC.

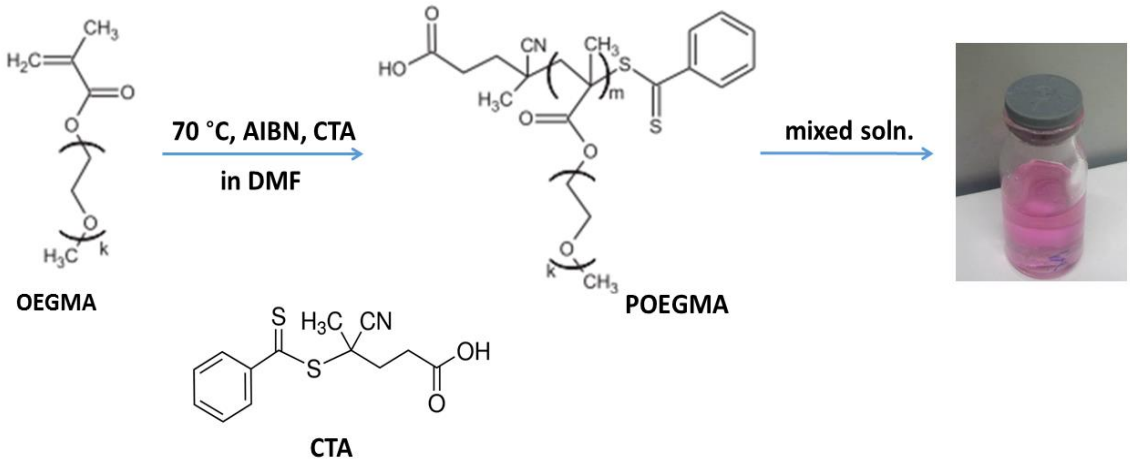


Figure 3. 2 Schematic representation of the synthesis of POEGMA475 homopolymer (MacroCTA) via RAFT polymerization

3.2.1.2 Synthesis of Amphiphilic Block Copolymer POEGMA-b-PVP via Raft Polymerization

The synthesized POEGMA was used as the RAFT agent (macroCTA) and AIBN was used as the initiator in block copolymer synthesis. The extended block copolymer synthesis of the PVP block from MacroCTA is schematically shown in Figure 3.3. Block copolymer synthesis was carried out using $[M]_0/[CTA]_0/[I]_0$=200/1/0.5 ratio. 950 mg of POEGMA was dissolved in 2.7 ml of DMF. Then, 556 µl of 4-VP was added to the solution. The mixture was exposed to N_2 gas for 30 minutes and then placed in a temperature-controlled oil bath at 70 °C under magnetic stirring (1000 rpm). The N_2 gas was exposed to 2 mg AIBN in 0.2 ml of DMF. After the increase of the temperature of the reaction system, the AIBN solution was dropped into the mixture to initiate the copolymerization. After 24 h, the solution was precipitated with cold diethyl ether and centrifuged. Then after, the ether was removed by decantation thus, the orange viscous copolymer was obtained, and it was dissolved in dichloromethane and precipitated with cold ether. The sample was dried in vacuum incubator overnight at 50 °C.

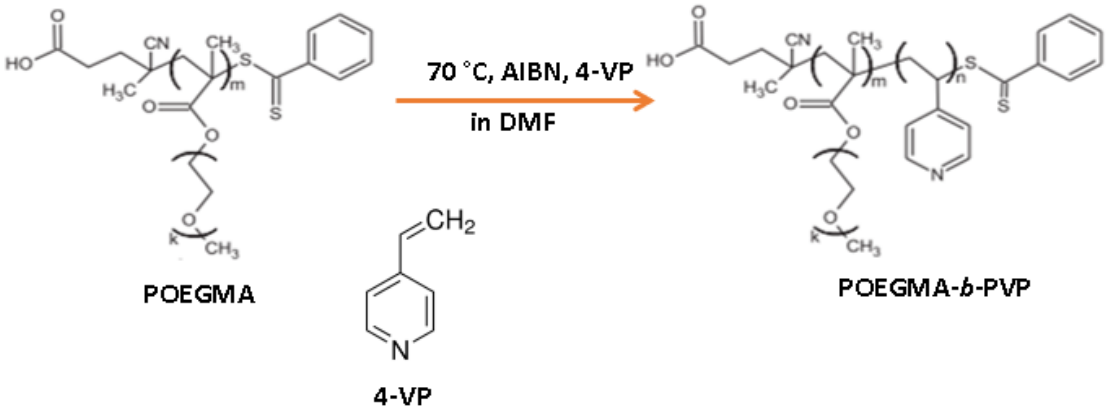


Figure 3. 3 Schematic representation of the synthesis of POEGMA-*b*-PVP block copolymer via RAFT polymerization

3.2.1.3 Synthesis of Core-crosslinked Micelles via Quaternization of Block Copolymers

Preparation of core-crosslinked micelles can be divided into two parts: While the first part is self- assembly of micelles in solution, the second part is the quaternization of the micelles. At first, the previously prepared 8 mg of POEGMA-*b*-PVP copolymer was dissolved in 1 ml of dH_20 by varying the concentrations of 1, 6- Dibromo hexane (1, 6-DBH) for each micelle. The copolymer was partially dissolved in water to self-assemble into micelles whereby the PVP and 1, 6-DBH form the core part of the micelle (Figure

3.4). The amount of the 1,6-DBH was varied to change the quaternization degrees by 40 %, 25 % and 10 % where the obtained micelles were designated as Micelle 1, Micelle 2 and Micelle 3, respectively.

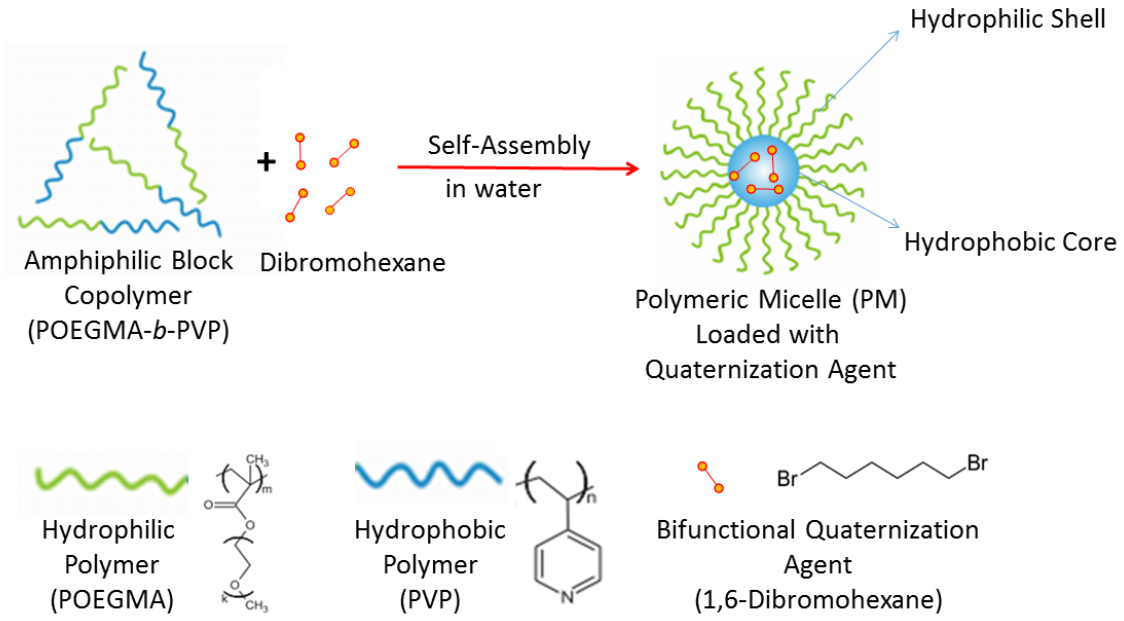


Figure 3. 4 Self-assembly of POEGMA-*b* PVP diblock copolymer into micelle in water

The prepared micelle solutions were stirred in an oil bath at 70 °C for overnight to get core-crosslinked and quaternized micelle forms (Figure 3.5). Finally, the mixtures were dialyzed against water to remove the unreacted crosslinking agent.

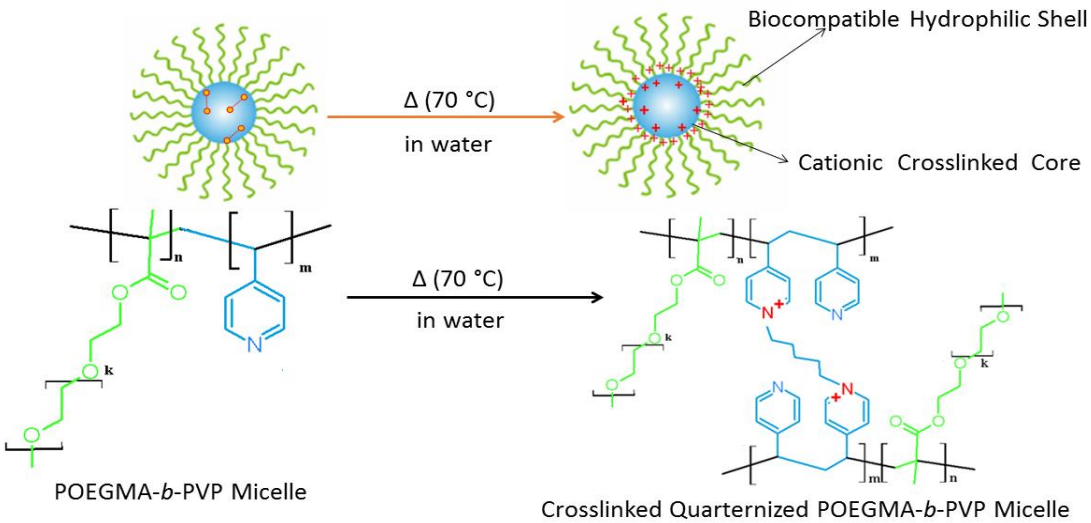


Figure 3. 5 The quaternization reaction of the POEGMA-*b*-PVP copolymer which yields core-crosslinked micelles

3.2.2 Synthesis of 4-VP Crosslinked Nanoparticle

PVP crosslinked nanoparticles were prepared as a control group. Briefly, 11.5 ml of dH_20 was added to 108 µl of the 4-VP solution. 22.8 µl of 1, 6-DBH was added as a bifunctional agent (quaternization and cross-linker agent). The mixture was exposed to N_2 gas for an hour and then placed in a temperature-controlled oil bath at 70 °C under magnetic stirring (1000 rpm). After 5 min of stirring, 2.64 mg/ 0.5 ml ACVA in DMF was added to initiate the reaction. The reaction was completed in 24 hours. The mixture solution was dialyzed against water to expel the unreacted crosslinking agent.

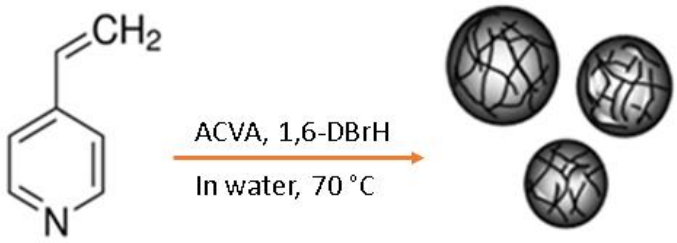


Figure 3. 6 Schematic representation of the synthesis of quaternized and cross-linked PVP nanoparticles

3.2.3 Quaternization of POEGMA-*b*-PVP Copolymer

The POEGMA-*b*-PVP copolymer was quaternized to be used as another control group. 8 mg of POEGMA-*b*-PVP, 5 µl of 1- Bromo hexane and 160 µl methanol was mixed in a 1 ml vial. 1- Bromohexane was used as the quaternization agent. Then the mixture was placed in a temperature-controlled oil bath at 70 °C under magnetic stirring (1000 rpm) for 24 hours.

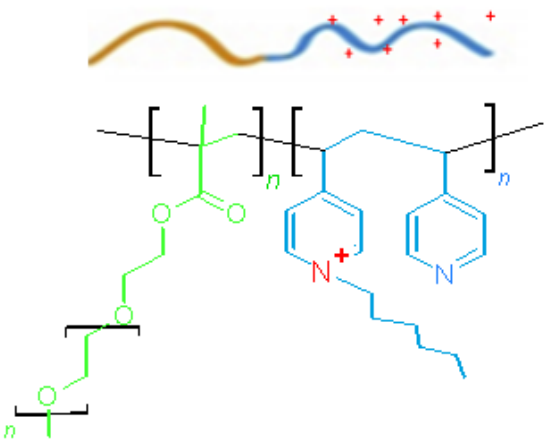


Figure 3. 7 Quaternized POEGMA-*b*-PVP copolymer

3.3 Characterization Techniques

3.3.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy analyses were carried out by Bruker Avance III 500 MHz NMR instrument. The compositional ratios of the synthesized copolymer were calculated and thereby the structure of the polymer was determined by NMR.

3.3.2 Gel Permeation Chromatography (GPC)

Gel Permeation Chromography analyses were carried out by Viscotek TDA 302 GPC instrument having refractive index and light scattering detectors. The column model was Eprogen Catsec 300. The sample injection volume was 100 uL. The mobile phase was consisting of 0.1 M acetic acid and 0.15 M sodium chloride and the flow rate was 0.4 mL/min at room temperature. Polyethylene oxide (PEO) (250 kDa) was used as the calibration standard. The prepared samples were filtered through a 0.45 μm injector filter prior to measurement.

3.3.3 Fourier-Transform Infrared Spectroscopy (FTIR)

IR spectra of the homopolymer, copolymers and micelles were obtained by Shimadzu IR-Prestige 21 model FTIR spectrophotometer. Measurements were carried out at an interval of 4000-650 cm-1 using ATR apparatus.

3.3.4 ZetaSizer

The sizes and zeta potentials of the micelles were measured by Malvern, Zetasizer NanoZS model photon correlation spectrometer.

3.3.5 Scanning Electron Microscopy (SEM)

The topography and compositions of the micelles were examined by Scanning Electron Microscope (SEM) (Model Zeiss EVO LS 10). Samples were prepared by adding a drop of micelle solution on a SEM sample stub followed by drying of this solution in an oven at 600C. Then after, the samples were sputter coated by gold-palladium prior to the measurements.

3.3.6 Antibacterial Assays

The antibacterial activity of the prepared polymeric materials was evaluated by three different methods including broth microdilution, disk diffusion, and agar well diffusion. These methods were carried out separately for model *E. Coli* (gram-negative) and *S. aureus* bacteria (gram-positive) since they have different bacteria cell wall structure; (*E. coli* (ATCC number:25922), *S. aureus* (ATCC number: 25923), respectively.

Preparation of bacterial suspensions

The bacterial cultures used in this thesis were prepared by suspending isolated colonies from Mueller Hilton Agar. It was incubated at 37°C and 200 rpm in an incubator overnight. Then, until the optical density was reached to 0.1-0.2 at 600 nm, the bacterial suspensions were diluted with sterile buffer solution. This measured value assigns the concentration of 10^8 colony forming-unit/ml (CFU ml^{-1}) which is used as a bacterial working dilution in the antibacterial methods applied for this study.

Broth Microdilution Method

To screen the in vitro antibacterial activities of M1, M2, M3 micelles, quaternized nanoparticle and the quaternized block copolymer, broth microdilution method was chosen as a quantitative method. Broth Microdilution Method was carried out in accordance with the CLSI standard, January 2012, 'Methods for dilution antibacterial susceptibility tests for bacteria that grow aerobically; approved standard'[193]. This method was used to determine MIC (minimum inhibitory concentration) value of the prepared polymeric materials. Shortly, 2 mg of the samples were dissolved in 1 ml water (stock solutions). To determine MIC of the materials, serial dilutions of the stock solutions were prepared in Mueller Hilton broth using a 96-well plate (12,5, 25, 50, 100, and 200 µg/ml). Each well was inoculated with a microbial inoculum (turbidity adjusted to 0.5 McFarland standard). The microorganisms in 96-well plate were incubated at 37°C for 24h. The negative control was only broth and bacteria without any material. The blank control was broth which was not inoculated with bacteria. The bacterial concentration was determined by optical density measurement (OD_{600}) and MIC values were confirmed by standard plate counts.

Disk and agar well diffusion method

Disc diffusion and agar well diffusion methods were chosen as qualitative methods to assess the antimicrobial activity of the prepared materials. Disc diffusion study was

carried out based on the EUCAST standard (April 2013, ‘Antimicrobial susceptibility testing EUCAST disk diffusion method’) [194]. A bacteria culture (which has been adjusted to 0.5 McFarland standard) was used to lawn agar plates evenly using a sterile swab. Different concentrations of stock solutions (2 mg/1 ml) was prepared (10 µg/mL and 20 µg/mL) and loaded over sterile blank discs. The loaded discs with different concentrations of agents were placed on agar plates. The agar well diffusion test was performed as described by Arasoglu et al. [195]. Bacterial culture was plated onto Mueller–Hinton agar. Agar wells punched on agar plates. Two different concentrations of agents (100 and 200 µg /ml) introduced into the wells. Agar plates were incubated at 37 °C for 24 hours. Next day the antibacterial activity was evaluated according to the appearance of inhibition zone around the discs and wells.

CHAPTER 4

RESULTS AND DISCUSSION

The main objective of this thesis is to prepare antibacterial cationic polymeric materials. To achieve this aim, initially, POEGMA homopolymer was prepared via RAFT polymerization. Then after, an amphiphilic block copolymer of POEGMA-*b*-PVP was synthesized via RAFT polymerization as well and used to obtain the novel core-crosslinked type of micelles. As will be seen in the following paragraphs, both the prepared polymers and micelles were characterized by various methods including the antibacterial assays which resulted in the antibacterial efficacy of the materials.

4.1 POEGMA Homopolymer Preparation via RAFT Polymerization

In the first step of polymerization, OEGMA was synthesized via RAFT polymerization as a precursor to obtain a POEGMA-*b*-PVP block copolymer. The POEGMA homopolymer ($M_{n,\,GPC}$ = 15.715 Da, Table 4.1) was obtained as a pink color and viscous liquid with RAFT polymerization.

Figure 4.1 represents the FTIR spectrum of POEGMA; the band at 2850 cm^{-1} indicates the -CH2- and -CH3 groups of the polymer. The strong band at 1748 cm^{-1} corresponds to the C=O group of the polymer and the band at 1095 cm^{-1} belongs to group C-O-C.

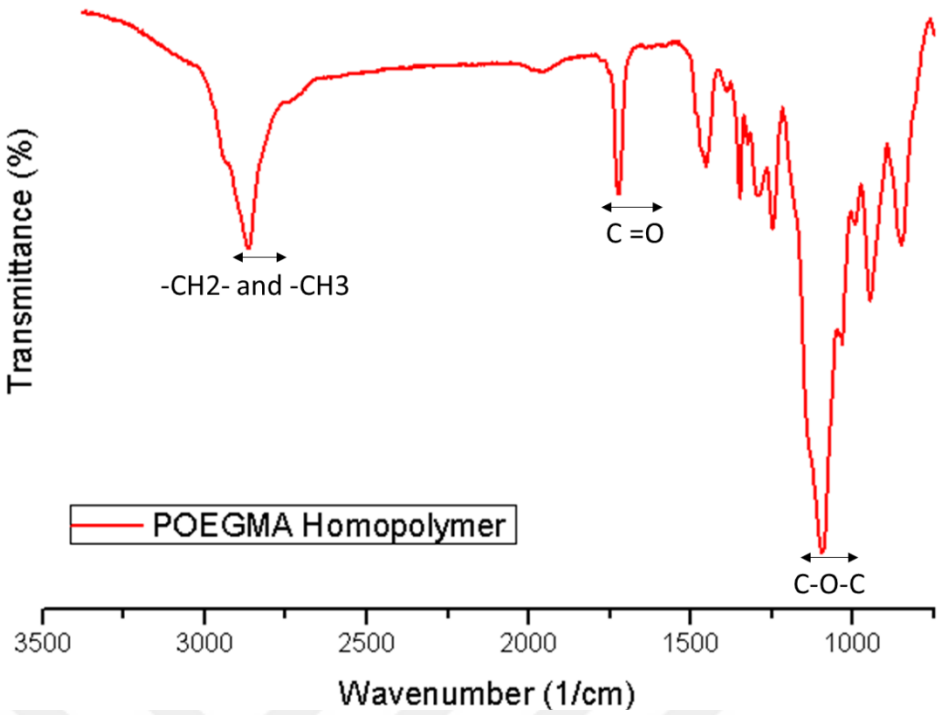


Figure 4. 1 FTIR Spectra of POEGMA Homopolymer

Figure 4.2 exhibits the GPC chromatogram of POEGMA homopolymer where the peak is quite narrow indicating the monodispersity of POEGMA.

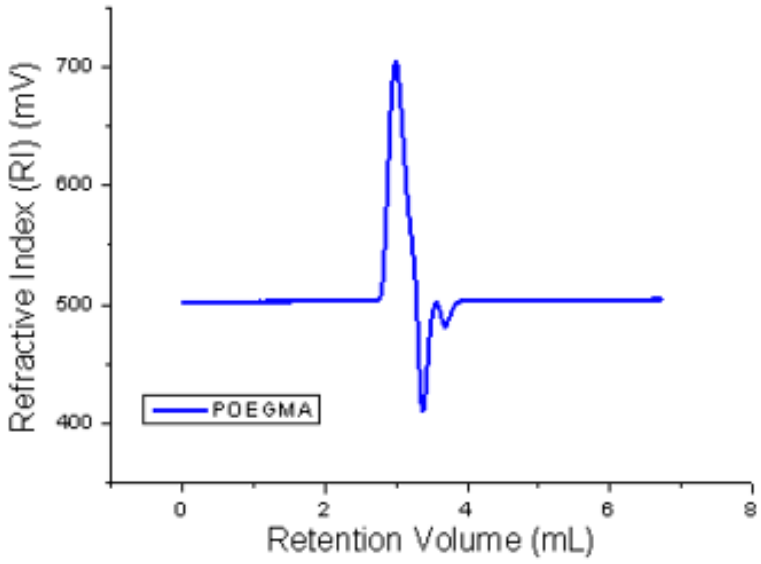


Figure 4. 2 GPC chromatogram of POEGMA acquired by Refractive Index (RI) detector

The average molecular weights of the polymer together with the Polydispersity Index (PDI) (Mw/Mn) are shown in Table 4.1. PDI was found close to 1.0 as 1.094 which also indicates that the homopolymer is monodisperse.

Table 4. 1 The average molecular weights and PDI of the homopolymer POEGMA obtained using GPC

POEGMA	**GPC Results**
Peak RV- (ml)	2,987
Mn- (Daltons)	15.715
Mw- (Daltons)	17.192
Mz- (Daltons)	21.263
Mw/Mn	1,094

As will be seen in the following Section 4.2, subsequently, utilization of POEGMA as macroCTA formed a block copolymer.

4.2 Amphiphilic Block Copolymer POEGMA-*b*-PVP via Raft Polymerization

The previously prepared POEGMA homopolymer was used as macro chain transfer agent to synthesize amphiphilic block copolymer via RAFT polymerization. The amphiphilic block copolymer was obtained by extending hydrophobic PVP block on hydrophilic POEGMA block. The POEGMA-*b*-PVP copolymer was characterized by FTIR, NMR, and GPC. The structure of POEGMA-b-PVP block copolymer was confirmed by FTIR spectra. As it is depicted in Figure 4.3, the characteristics bands of POEGMA and PVP blocks were obtained where the band at 1735 cm^{-1} belongs to C=O groups of POEGMA and at 1600 cm^{-1} belongs to C=C groups of pyridine ring of PVP. Thus, the FTIR result clearly demonstrates that the copolymerization was successfully carried out.

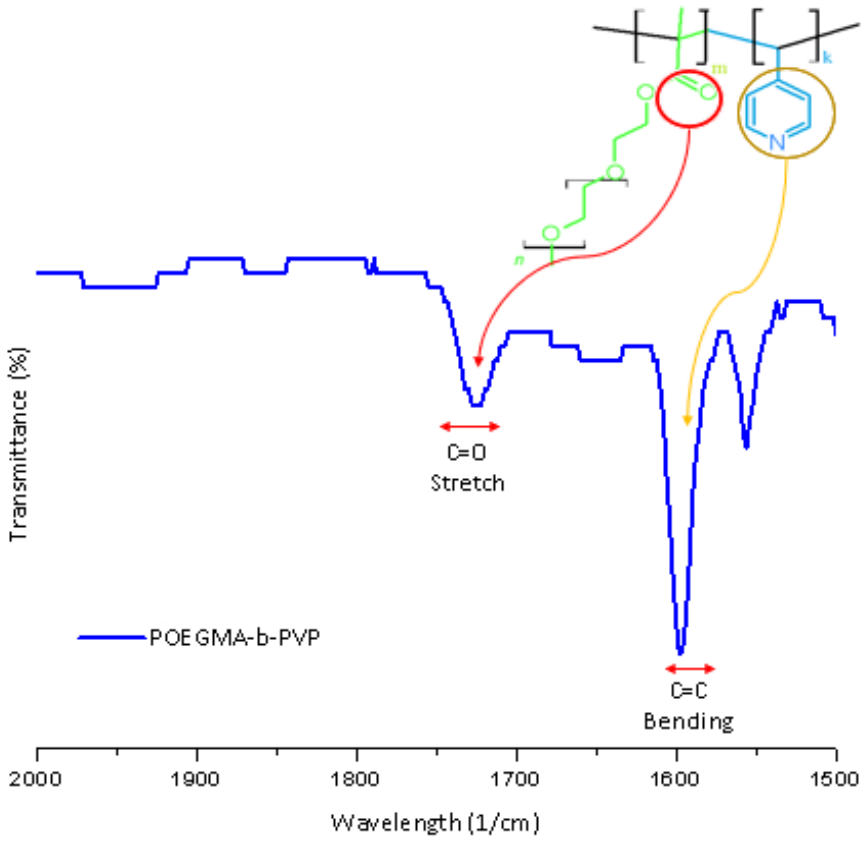


Figure 4. 3 FTIR spectra of the POEGMA-*b*-PVP block copolymer

Figure 4.4 represents the ^{1}H-NMR spectrum of POEGMA-*b*-PVP copolymer in DMSO. The copolymer units were characterized by proton signals. The $POEGMA_{475}$ spectrum at 4.02 ppm (c) was attributed to the protons of methylene groups which linked to the methacrylate groups of $POEGMA_{475}$. The protons of the main chains of $POEGMA_{475}$ polymer was ascribed to methylene at 2.5 ppm (b), methyl protons of methacrylate at 0,76 ppm (a), and side chains of methylene protons (-O-CH2- CH2-) (d) methyl protons (-O-CH3) (e) of POEGMA475 at 3.67 ppm and at 3.46 ppm, respectively. Besides the spectra of $POEGMA_{475}$, the signals at 8.35 ppm and 6.40 were assigned to the aromatic protons of the pyridine rings (h, i) respectively. The signals at 1.4 and 1.7 ppm were attributed to the protons of vinyl chain (f, g) of PVP block, respectively. The band at 2.5 ppm indicates the solvent (DMSO) residue.

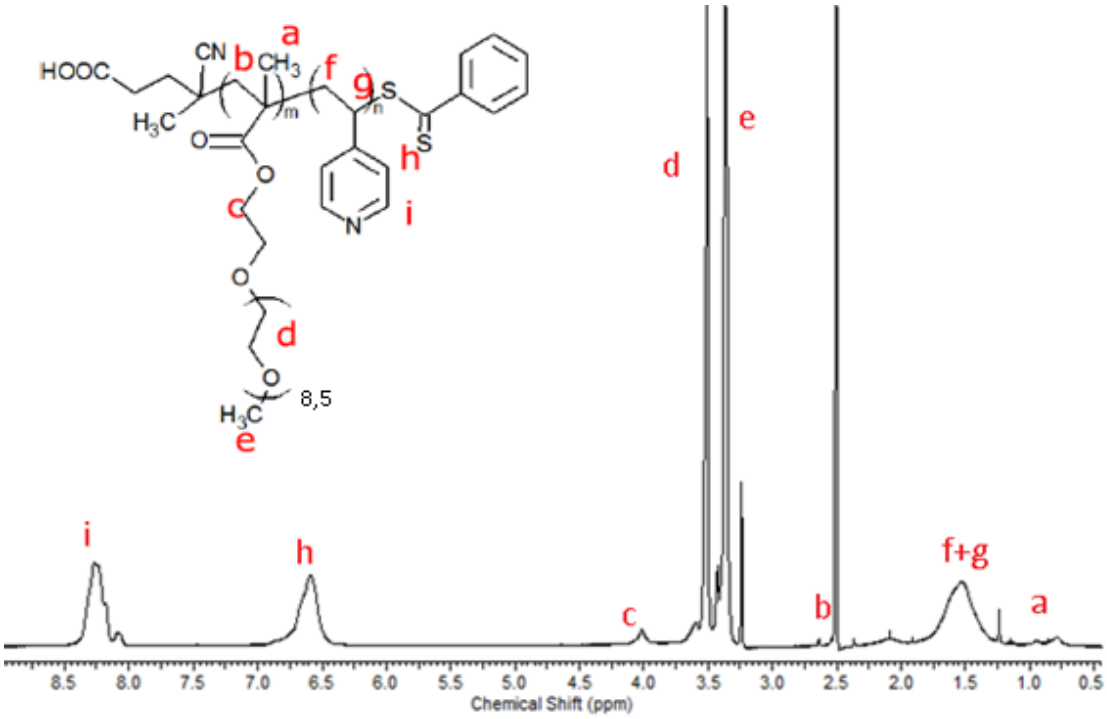


Figure 4. 4 ^{1}H-NMR spectra of the POEGMA-*b*-PVP copolymer in DMSO

To confirm the polymers were formed as diblock copolymers, GPC analyses were carried out after completing each polymer block. The number average of molecular weight (Mn) of polymers, weight average of molecular weight (Mw) and PDI values of POEGMA-*b*-PVP copolymer were determined by GPC using dual detectors, refractive index (RI) and light scattering (LS). GPC analyses indicated a controlled polymerization with low dispersity (Mw/Mn=1,33, Table 4.2) Furthermore, Figure 4.5 shows the comparison of the GPC chromatograms of the POEGMA homopolymer and POEGMA-*b*-PVP copolymer using refractive index detector. The copolymer's molecular weight is higher than the homopolymer's molecular weight. Therefore, as expected the retention volume of the copolymer is less than the homopolymer.

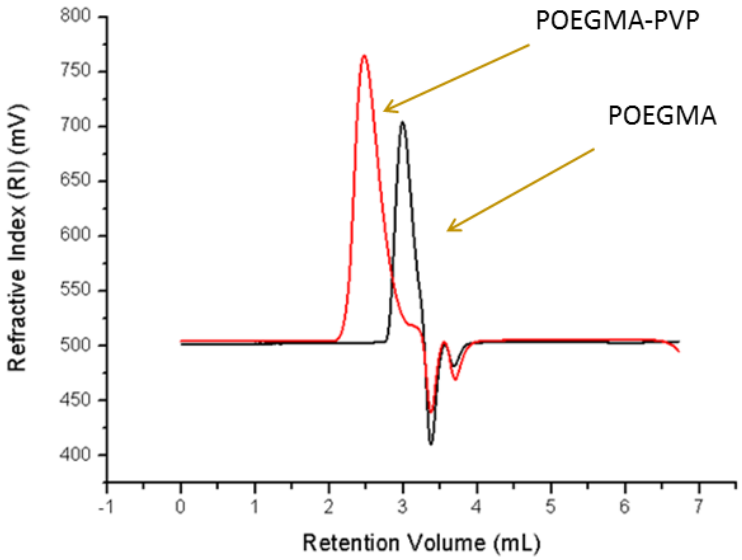


Figure 4. 5 GPC chromatograms of POEGMA and POEGMA-*b*-PVP acquired by RI detector

Figure 4.6 represents the GPC chromatograms of the homopolymer and copolymer which were obtained using the light scattering detector. Signals are directly related to the concentration of the polymers and molecular weight. In Figure 4.5, it is seen that the retention volume of the POEGMA-*b*-PVP peak appeared first and POEGMA appeared later. So, it can be concluded that the GPC results obtained using the refractive index and light scattering detectors were in accordance with each other.

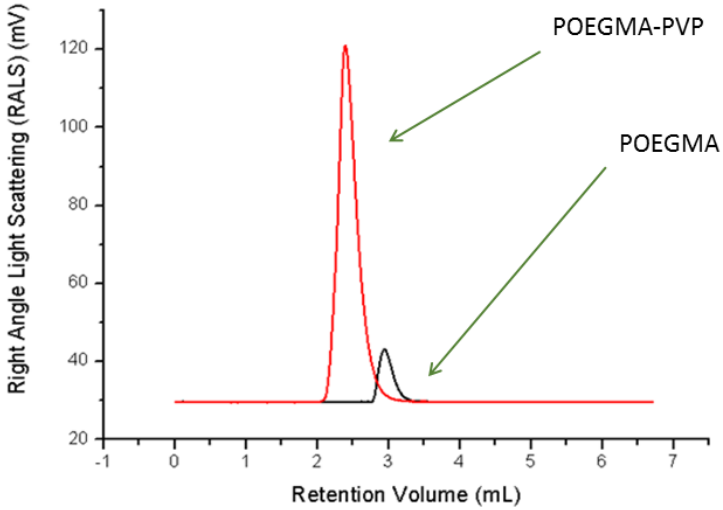


Figure 4. 6 GPC chromatograms of POEGMA and POEGMA-*b*-PVP acquired by Right Angle Light scattering (RALS)

Table 4. 2 The copolymer (POEGMA-*b*-PVP) results obtained from GPC.

POEGMA-*b*-PVP	**GPC Results**
Peak RV- (ml)	2,476
Mn- (Daltons)	28.998
Mw- (Daltons)	38.668
Mz- (Daltons)	50.569
Mp- (Daltons)	41.292
Mw / Mn	1,333

4.3 Core-crosslinked Micelles via Quaternization of Block Copolymers

In order to produce micelles, the prepared POEGMA-*b*-PVP copolymer was dissolved in water. The amphiphilic structure of the block copolymer cause to form micelles in water due to the hydrophobic effect [196]. PVP block comprised the core part of the micelles and dibromo hexane has also hydrophobic property so that, it was loaded into core part during micelle assembly. POEGMA block which interacts with water was comprised of the shell part of the micelles because of its hydrophilicity. Then, when the system was heated up, the bifunctional quaternization agent reacted with the micelles and the micelles were quaternized (positively charged) and core-crosslinked.

The structure of the prepared micelles (Micelle 1, Micelle 2, Micelle 3) were confirmed by FTIR spectra. Micelles were quaternized by 1, 6-DBH with various ratios. FTIR was used to determine quaternization degrees as well. POEGMA displays a band at 1735 cm^{-1} correspond to C=O stretching vibration. The pyridine ring of PVP displays a band at 1600 cm^{-1} correspond to C=C bending vibration. Besides, the FTIR spectra of the block copolymer, the quaternized pyridine ring of PVP band displays band at 1639,5 cm^{-1} which correspond to C=C stretching vibration [197]. Nevertheless, the existence of the quaternized pyridine ring of PVP in each of the three micelles verified the quaternization reaction. The intensities of these bands values were measured to determine the quaternization degrees by FTIR. The quaternization ratios were obtained using the Equation 4.1.

$$\%Q = A_{1640} / ((A_{1600} * 1{,}68) + A_{1640}) \quad (4.1)$$

Quaternization degrees of the micelles were calculated using Eq. 4.1 by FTIR analysis (Figure 4.7). The quaternization degree of the micelles was determined in a range between 30-40 %.

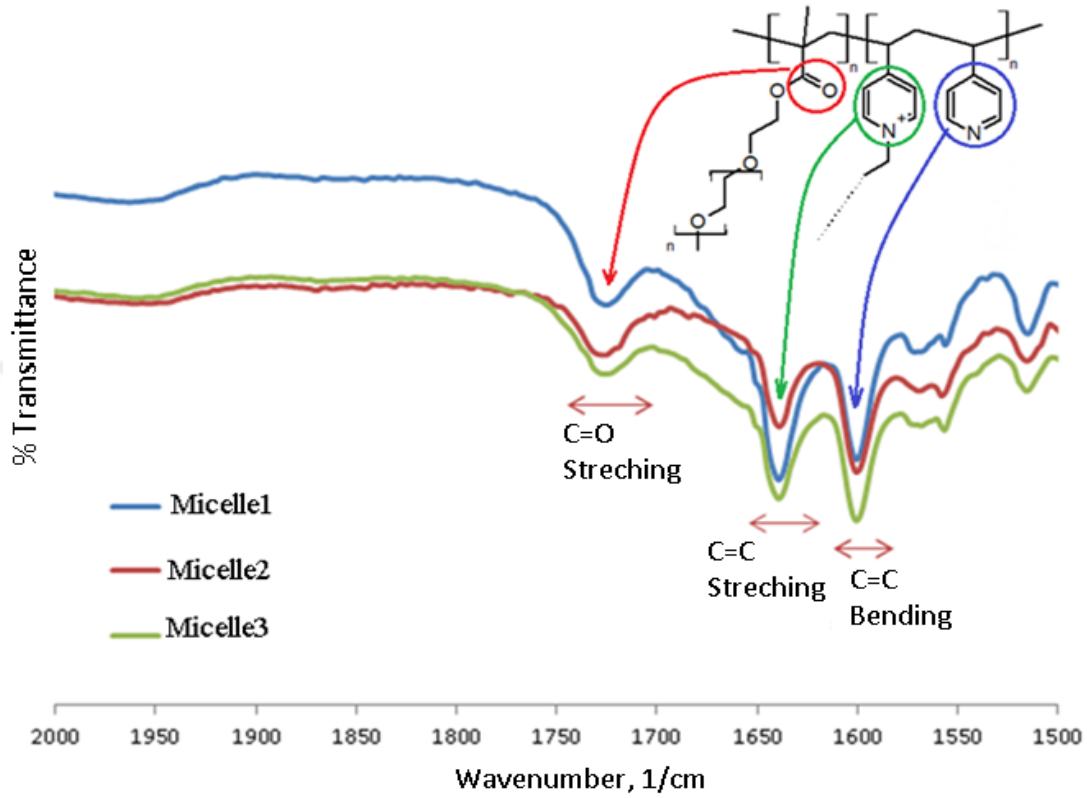


Figure 4. 7 FTIR spectra of the POEGMA-*b*-PVP micelles after quaternization

Table 4.3 exhibits the calculated quaternization degrees of the micelles. The quaternization degree of the micelle 2 is lower than micelle 3. It was assumed that the micelle 2 might have crosslinked one another and could not further quaternized because of the steric effect.

Table 4. 3 Quaternization percentages of the micelles

Sample No	**Quaternization Degrees**
Micelle1	39,79%
Micelle2	29,69%
Micelle3	32,92%

Figure 4.8 reveals the size distribution of the micelles by intensity where the plots for 3 type of micelles are overlapped since the diameter of micelles varies from 113 to 116 nm (Table 4.4).

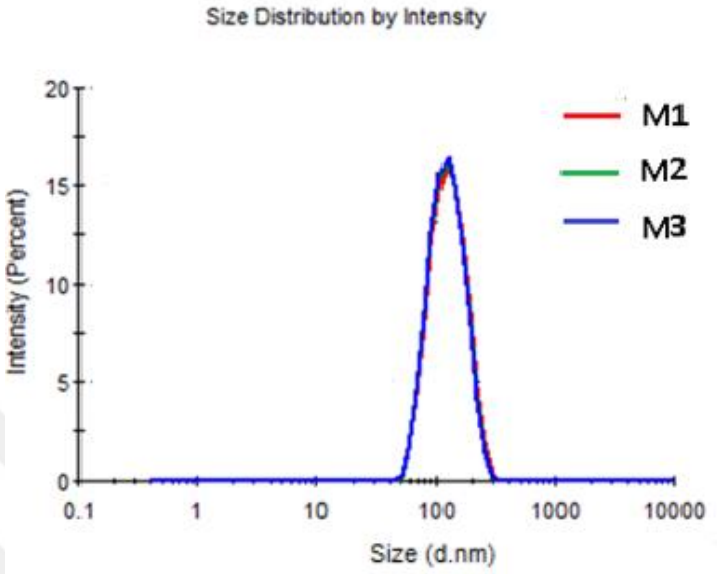


Figure 4. 8 Size distribution by %-Intensity of micelle 1 (M1), micelle 2 (M2), micelle 3 (M3)

Table 4.4 represents the size, PDI and zeta potentials of the three micelles. While the sizes of the micelles and PDI are close to one another, the zeta potential of micelles varies from +38 to +63 mV.

The spherical morphologies of the dried micelles were confirmed by SEM images and their size obtained as~120 nm in diameter for Micelle 1 and Micelle 2. On the other hand, it was suggested that some of the Micelle 3 particles had secondary aggregates thus its particle size is~210 nm.

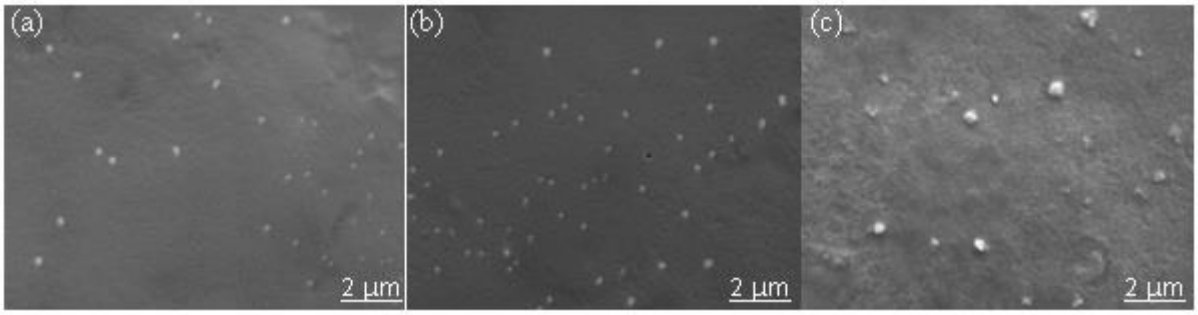


Figure 4. 9 SEM images of micelle 1(a) and, micelle 2(b) showed spherical morphologies with 120 ± 10 nm diameters and, micelle 3(c) with 210 ± 10 nm

Approximately same sized micelles facilitated the effect of the zeta potential of micelles on their antibacterial activities PDI value of the micelles is around 0.1 indicating the monodispersity of the micelles.

Table 4. 4 Size (d. nm), PDI and zeta potentials (mV) of the micelles

No	Z-Ave (d.nm)	PDI	Zeta Potentials (mV)
M1	116,1	0,105	+ 63
M2	113,4	0,114	+ 51.4
M3	113,2	0,114	+ 38.2

Moreover, the zeta potential results given in Table 4.4 indicate that all the micelles were positively charged by quaternization. Indeed, our results were in accordance with previously reported results as it known that increasing the quaternization agent amount increases the zeta potentials of the micelles [198], [199].

Antibacterial activity tests were carried out using Broth Microdilution, Disc and Agar well diffusion methods. To obtain reliable MIC values in Broth Microdilution Method, spectrophotometric measurements at an OD of 600 nm (ELISA reader) were performed. OD values of the micelles were obtained from the reader and are shown in Figure 4.10. Each concentration of the micelles was studied duplicately in wells. As shown in Figure 4.10A, the wells in the A row have the highest micelle concentration in which the micelle concentrations were diluted from A to E (200, 100, 50, 25, 12,5 µg/ml, respectively). MIC values of three micelles were measured at highest concentration against *E. coli* colored pink in Figure 4.10A. Yellow wells represent the negative control (bacteria and growth medium). In Figure 4.10B, MIC values of three micelles were not be able to be measured, therefore any well colored pink against *S. aureus*.

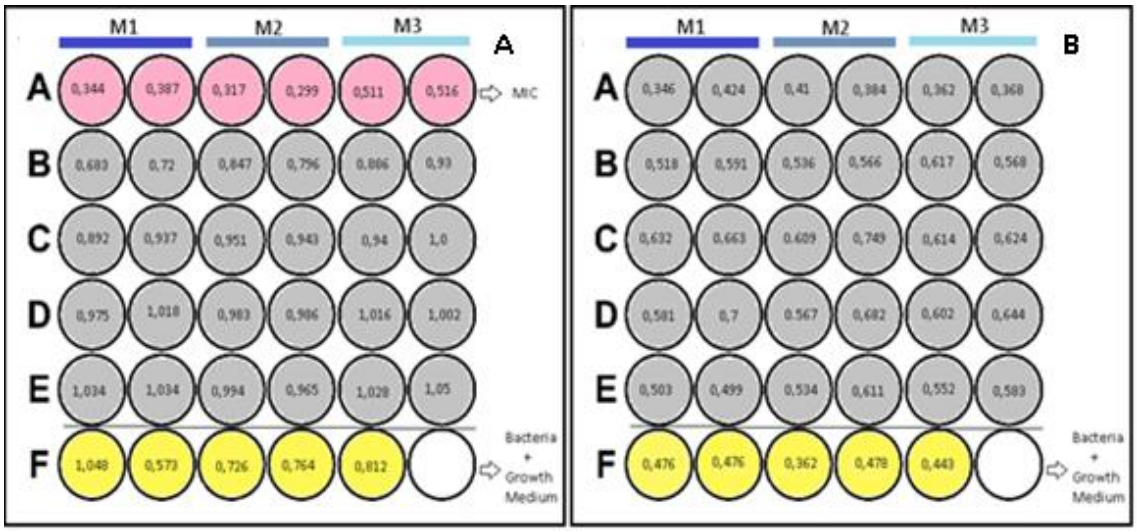


Figure 4. 10 OD at 600 nm values of broth microdilution assay of the micelles for *E. coli* (A), *S. aureus* (B)

The MIC values were also confirmed by standard plate count agar. As it is revealed in Figure 4.11A, two-fold dilution petri dishes were obtained from the negative control wells containing the *E. coli* bacteria (direct, 10^{-2}, 10^{-4}, 10^{-6}) after 24 h of incubation. In Figure 4.11B, petri images of the three micelle samples diluted in 10^{2} fold are displayed. When the control group is compared to a 10^{2} fold diluted petri dish, the reduction in colony count is clearly visible for the micelles. Therefore, it can be concluded that the standard plate count agar showed compatible results with the OD values of broth microdilution method.

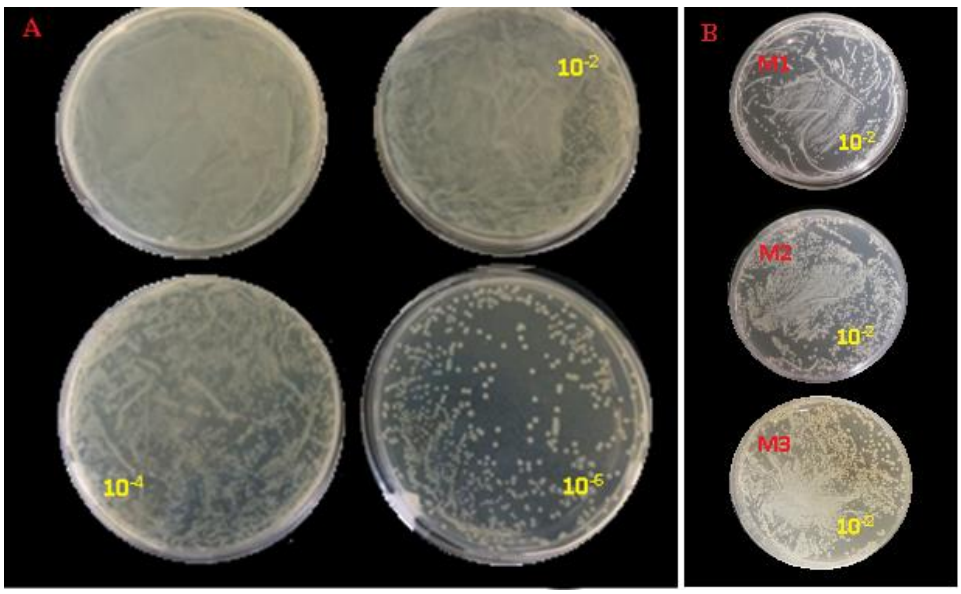


Figure 4. 11 The image of standard plate agar results. (A) The two-fold diluted plates of the *E. coli* control group, (B) The diluted bacteria 10^{2} fold results for M1, M2, M3, respectively

The determined MIC values of M1, M2, M3 are presented in Table 4.5. Three micelles showed similar antibacterial activity against *E. coli*. It can be assessed that different quaternization degrees did not have a high impact on antibacterial activity results. In the *E. coli* study, the micelles showed inhibitory activity at highest concentration level, while the activity is disappearing in serial dilutions. Additionally, for *S. aureus,* the cell growth inhibition was not observed for micelles at applied concentrations. Therefore, the standard plate count agar results of the micelles against *S. aureus* were not included in the thesis. The difference in antibacterial activities of micelles against gram-positive and gram-negative bacteria can be correlated with the differences in the cell wall structures of these bacteria.

Table 4. 5 MIC values of micelles samples (M1, M2, M3) against *S. aureus* and *E. coli* through broth microdilution method for the micelles

	MIC values (µg/mL)		
Species	M1	M2	M3
S. aureus	>666	>666	>666
E. coli	666	666	666

Although there are no reported works about the antibacterial studies of core-crosslinked micelles, our samples can be compared to antibacterial studies of other micelles with different forms. For instance, Yuan et al. reported amphiphilic ABC (PEO-*b*-PCL-*b*-PTA) three block micelle with positively charged shell part [192]. The MICs of the polymeric micelles were determined using a broth microdilution method against *E. coli* and *S. aureus*. The ABC micelle has MIC values of 0.28 and 0.13 mM against *E. coli* and *S. aureus*, respectively. While our micelles showed better antibacterial activity against *E. coli*, ABC block micelle exhibited better antibacterial activity against *S. aureus*. The reason for that is ascribed to a different type of polymers used and the amount of the positive charge. Moreover, while their positive charges of the micelles are in the shell part, positive charges of our micelles are in the core part. Thus, this shows that the location of the positive charge on the micelles has a significant effect on the antibacterial activity. Compared to the many reported micelles in the literature, the main advantage of the micelles prepared in this thesis is that since they are crosslinked they are stable enough not disassociating by changing the environmental conditions e.g. pH, temperature.

In the disc diffusion and agar well diffusion methods both strains did not exhibit a considerable growth inhibition zone (diameter ⩾15 mm) with materials as represented in Figure 4.12 The prepared micelles did not diffuse neither in disc diffusion system nor in well diffusion system due to their size.

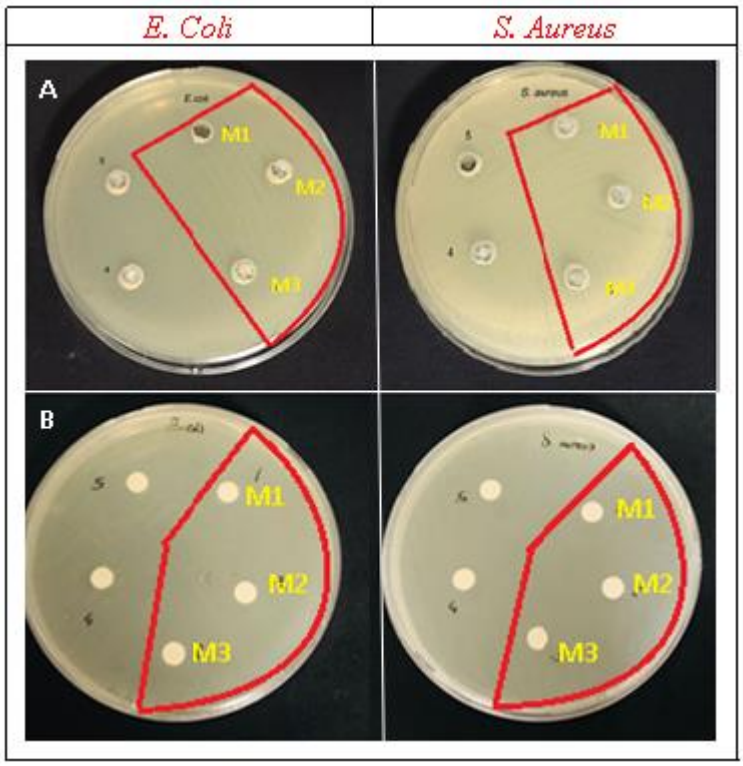


Figure 4. 12 The images obtained by agar well diffusion (A), and disk diffusion(B) methods of the micelles against both *E. coli* and *S. aureus*

4.4 PVP Crosslinked Nanoparticle

PVP crosslinked nanoparticles were synthesized to compare their properties with POEGMA-*b*-PVP micelles. Therefore, the antibacterial effect of hydrophilic POEGMA segment of the micelles would be clarified. Figure 4.13 demonstrates the FTIR spectra of quaternized PVP nanoparticle. Quaternized PVP has a band at 1650 cm^{-1} and non-quaternized PVP has a band at 1600 cm^{-1}. The quaternization degree of the PVP NP was calculated as % 19.75 by FTIR analyses.

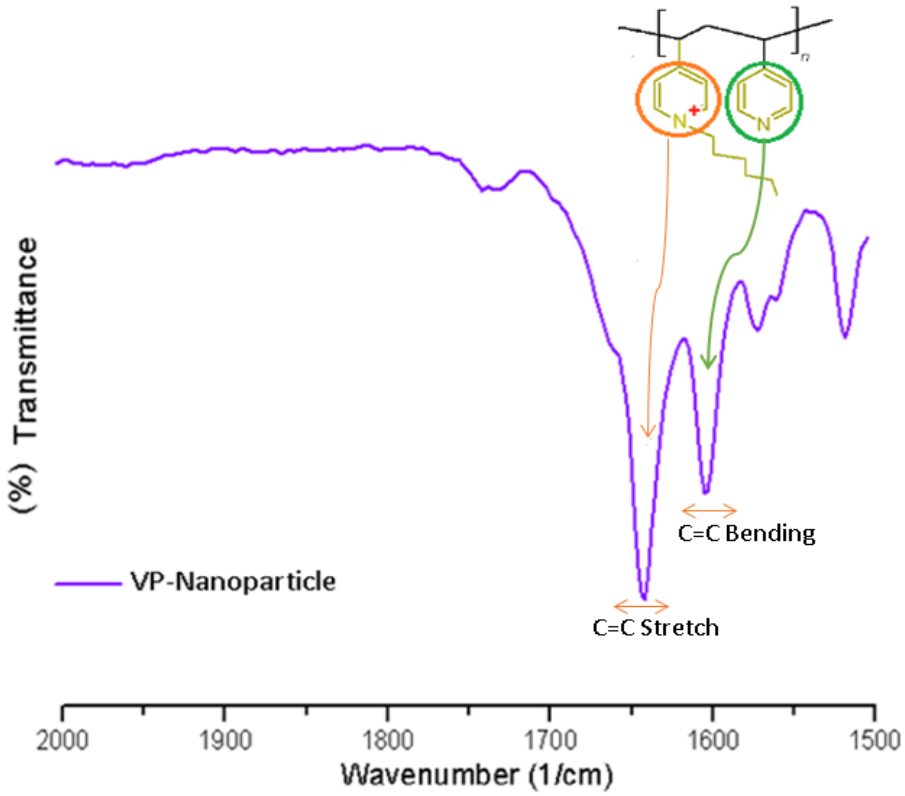


Figure 4. 13 FTIR spectra of 4-VP nanoparticle

While the average size of the PVP NP was determined to be 99 nm, the PDI and zeta potential values of the nanoparticle were 0,22 and +25 mV, respectively. Furthermore, the size of VP-NP was found to be close to the diameter of the micelles. Figure 4.14 represents the size distribution of the PVP-NP. The final NP exhibited a very narrow size distribution, thus it could be concluded that the VP-NP is monodisperse as well.

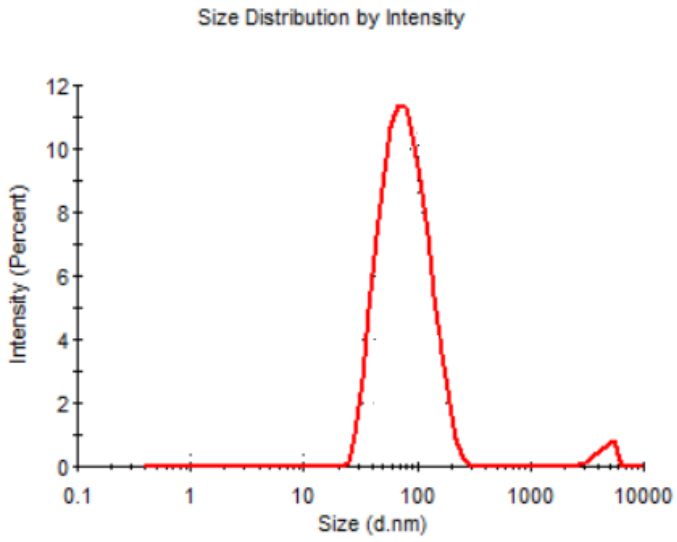


Figure 4. 14 Size distribution by %-Intensity of VP-Nanoparticle

Crosslinked quaternized PVP nanoparticle did not exhibit antibacterial activity against both two strains in three antibacterial methods used in this thesis. Similarly, Sahiner et al. studied quaternized PVP nanoparticles using a different quaternizing agent and they reported that all PVP nanoparticles were quaternized, however, all PVP nanoparticles did not display bactericidal effect [197]. Therefore, it was presumed that the type of the quaternization agent might have a great impact on the antibacterial activity of PVP nanoparticles. Both PVP and 1,6-Dibromo hexane are insoluble materials and, it was figured out that insoluble crosslinked polymeric materials do not show antibacterial activity [200].

4.5 Quaternized POEGMA-*b*-PVP Copolymer

In order to make a comparison of antibacterial activity, the quaternized POEGMA-*b*-PVP copolymer was also prepared and subjected to antibacterial tests. The quaternized POEGMA-*b*-PVP copolymer was characterized via FTIR and Zetasizer. FTIR spectra of the quaternized copolymer are displayed in Figure 4.15. The characteristic absorptions of C=O groups of POEGMA exhibit a band at 1735 cm^{-1} corresponding to stretching vibration. The band at 1600 cm^{-1} is ascribed to non-quaternized pyridine ring of PVP and the band at 1639,5 cm^{-1} is attributed to the quaternized pyridine ring of PVP. The quaternization degree of the quaternized copolymer was determined as %34,69.

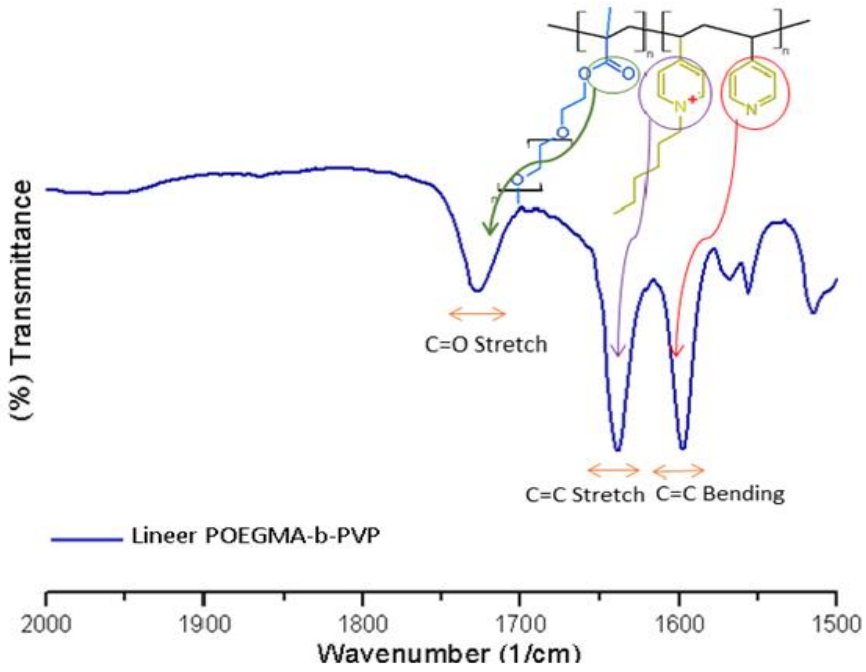


Figure 4. 15 FTIR spectra of quaternary POEGMA-*b*-PVP copolymer after quaternization

The zeta potential of the copolymer was determined to be +39 mV which is in the same range of the zeta potential of the micelles.

The MIC values of quaternized PVP-*b*-POEGMA copolymer was determined by broth microdilution method and the results are presented in Table 4.6. The MIC value of the quaternized copolymer was investigated against both *E. coli* and *S. aureus*.

Table 4. 6 The MIC value of agents against *S. aureus* and *E. coli* through broth microdilution method for quaternized PVP-*b*-POEGMA block copolymer

MIC Values (µg/mL)	
Species	Quaternary PVP-*b*-POEGMA copolymer
S. aureus	666
E. coli	333

The MIC value of quaternized PVP-*b*-POEGMA copolymer was determined as 666 µg/mL against *E. coli* and 333 µg/mL against *S. aureus.* The reason for the lower MIC values for *E. coli* was ascribed to variation in the cell wall structures of the bacteria species. Here, it can be argued that while the positive charges of the core-crosslinked micelles are hindered in the core of the samples, the more liberated positive charges on the quaternized linear copolymer caused enhanced antibacterial activity compared to the micelles.

The MIC values were also confirmed by standard plate count agar. Figure 4.16A shows the two-fold dilution petri dishes that are obtained from the negative control wells containing the only *E. coli* bacteria (direct, 10^{-2}, 10^{-4}, 10^{-6}) after 24 h of incubation. In Figure 4.16B, the petri image of the quaternized copolymer diluted in10^{2} fold are displayed. When the control group is compared to a 10^{2} fold diluted petri dish, the reduction in colony count is clearly visible for the quaternized block copolymer, thus indicating the improved antibacterial activity discussed above.

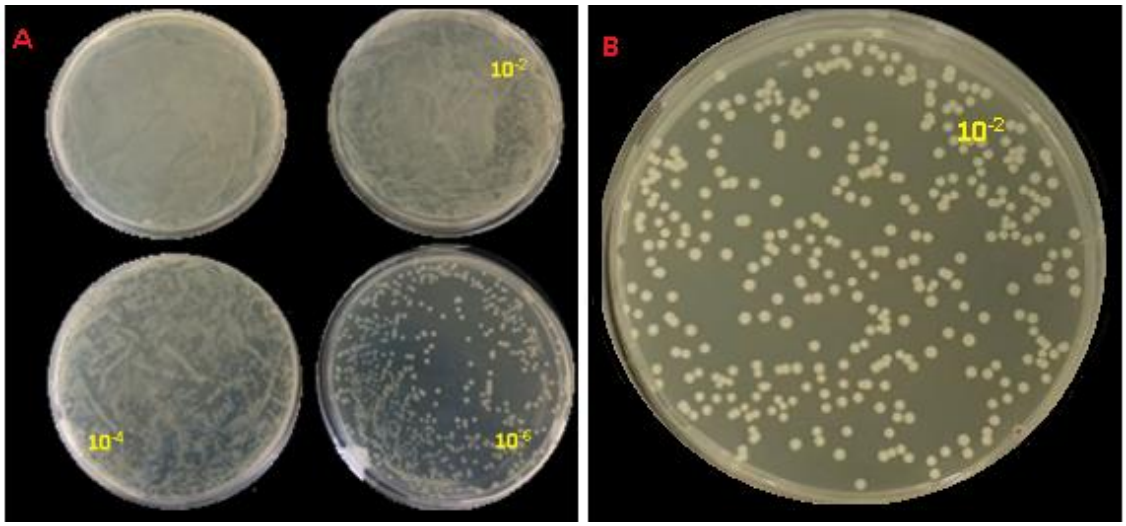


Figure 4. 16 The image of standard plate agar results. (A) The two-fold diluted plates of the *E. coli* control group, (B) The diluted bacteria 10^2 fold results for quaternized copolymer

Figure 4.17A reveals the two-fold dilution petri dishes obtained from the negative control wells containing the only *S. aureus* bacteria (direct, 10^{-2}, 10^{-4}, 10^{-6}) after 24 h of incubation. In Figure 4.17B, the petri images of the quaternized copolymer diluted in 10^2 fold are displayed. Similar to *E. coli* results, the antibacterial activity against *S. aureus* shown clearly for quaternized block copolymer compared with 10^2 fold diluted petri dish of the control group.

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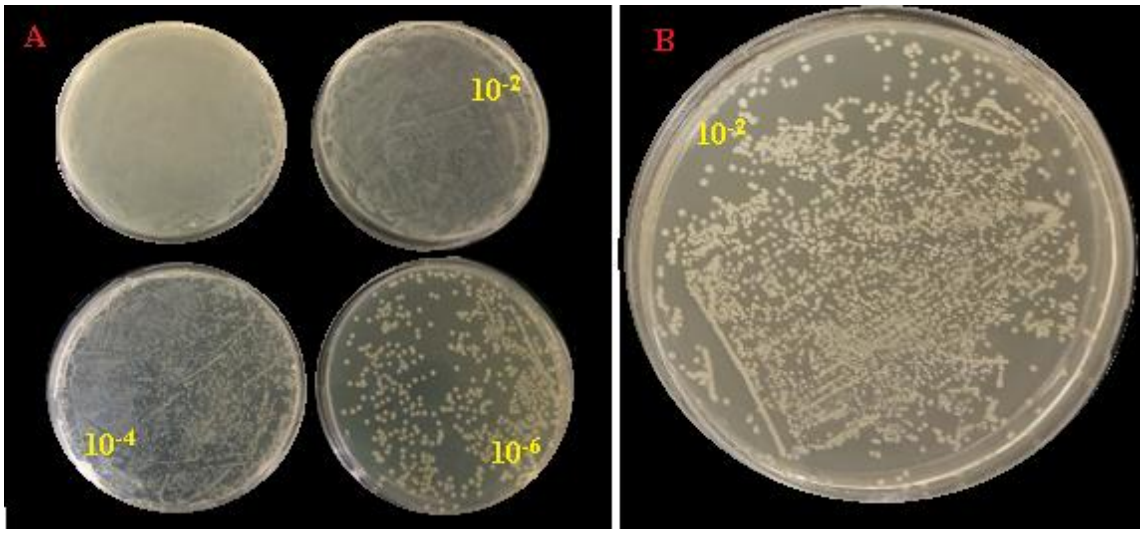

Figure 4. 17 The image of standard plate agar results. (A) The two-fold diluted plates of the *S. aureus* control group, (B) The diluted bacteria 10^2 fold results for quaternized copolymer

Youngblood et al. studied the random copolymer of quaternized PVP and POEGMA having different degrees of quaternization. [201]. They determined MBC (minimum bactericidal concentration) value which was obtained from broth microdilution method as well. MBC is the lowest concentration that bacteria viability can be reduced by more than 99%. In their study, 10% quaternized copolymer was found to be ineffective for MBC. The MBC value of 50% quaternized copolymer is 70 μg/mL. To compare these results with our 34,69% quaternized copolymer which is between 10-50%, it was found that our MIC result is in accordance with Youngblood et al. findings.

To compare the antibacterial activity of the micelles and qPVP-*b*-POEGMA copolymer, considering the presented results in Table 4.5 and Table 4.6, the MIC value of the micelles is higher than that of qPVP-*b*-POEGMA copolymer for both *E. coli* and *S. aureus*. The micelles showed inhibitory activity at highest concentration level for *E. coli*, while the activity disappeared in serial dilutions. This can be due to the fact that positive charges of the micelles are hindered by POEGMA shell; therefore, their antibacterial activity is less than the quaternized copolymer which has free positive charges.

In the disc diffusion and agar well diffusion methods both strains did not exhibit considerable growth inhibition (diameter $\geqslant$15 mm) with agents. This reason can be ascribed to synthesized block copolymer did not diffuse both in disc and well diffusion systems.

4.6 Conclusions

As a result, in this thesis, antibacterial cationic polymeric materials were prepared, and their bactericidal activity was investigated. Herein, a cationic POEGMA-*b*-PVP copolymer, core-crosslinked micelles formed by POEGMA-*b*-PVP copolymer and PVP nanoparticles were prepared successfully.

The conclusions that can be drawn from the experimental results of the study are as follows:

i. The FTIR, NMR and GPC results revealed the successful synthesis of the homopolymer POEGMA and the POEGMA-*b*-PVP copolymer.

ii. Three micelles were produced by quaternizing POEGMA-*b*-PVP copolymer with different amounts of bifunctional quaternizing agent 1,6-Dibromohexane. It was found that the zeta potentials of the micelles were increased by the increase of the

amount of 1,6-Dibromohexane. On the other hand, the average sizes of the three micelles were similar in diameters (114,2 ± 0.6 nm) indicating that the sizes of the micelles were not affected by the amount of 1,6-Dibromohexane.

iii. FTIR spectra of micelles revealed that there is no direct correlation between the 1,6-Dibromohexane amount and the quaternization degree.

iv. For comparison with micelles, the POEGMA-*b*-PVP copolymer was quaternized using a monofunctional quaternizing agent 1-Bromohexane. Since 1-Bromohexane bears only one bromine end-reactive group, the copolymer was not crosslinked. Instead, it only formed positively charged POEGMA-*b*-PVP copolymer. It was found that the zeta potential (+39 mV) and the quaternization degree (34,69%) of the copolymer were similar to the micelles.

v. Furthermore, PVP nanoparticles were also synthesized for comparison and to investigate the POEGMA effect on results. PVP nanoparticle was crosslinked and quartenized using 1,6-Dibromohexane as well. The zeta potential of PVP nanoparticle and its quaternization degree were found to be (+25 mV) and (19,75%), respectively.

vi. Both quantitative and qualitative analyses were performed against *E. Coli* and *S. aureus* to clarify antibacterial activity of the polymeric materials. Using qualitative methods, antibacterial effects were not observed since these polymeric materials did not diffuse to the assay systems. Despite that, the antibacterial effects were observed for the micelles and quaternized block copolymer using microdilution method. It was found that these three micelles and the quaternized block copolymer display inherent antibacterial activity against *E. Coli* after overnight incubation. Micelles didn't display antibacterial activity against *S.aureus* at applied concentration. However, the quaternized block copolymer displayed effect against *S.aureus*, too.

vii. It can be postulated that the hindered positive charges of the core-crosslinked micelles reduced the antibacterial activity. On the other hand, better antibacterial results were obtained against both bacteria for linear quaternized block copolymer due to its freer positive charges.

viii. PVP nanoparticle did not exhibit any bactericidal effect. Based on the previously reported results in the literature, it is speculated that the antibacterial activity is lost

when the cationic polymers are cross-linked or they are insoluble [200]. Here it can be concluded that the POEGMA segment of the POEGMA-*b*-PVP copolymer provides solubility to the micelles and the quaternized block copolymer, hence this increased solubility enabled the bactericidal effect.

The above results show the promise of the obtained cationic polymeric material for uses in antibacterial applications. In particular, quaternized POEGMA-*b*-PVP copolymer and micelles have great potential due to their biocompatibility, hydrophilicity and, bactericidal activity.

As future work, to improve the antibacterial activities of the core-crosslinked micelles, one of the strategies would be tailoring the ratio of PVP and POEGMA chain lengths to reduce the hindrance effect of POEGMA chains. Secondly, using OEGMA monomers having shorter side chains in synthesis of POEGMA block can also increase the interaction of positive charges with bacterial cell walls. Moreover, an alternative hydrophilic polymer block such as HEMA or PEG can be chosen as shell part of the micelles. For the PVP nanoparticles, another quaternization agent like dibromo PEG can be utilized to improve their antibacterial efficacy.

On the other hand, other antibacterial tests such as time-killing assays can be performed for better understanding the antibacterial properties of the produced polymeric materials. Furthermore, the antibacterial effect of the prepared samples can be tested against different bacterial species and, also micro fungi.

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PUBLISHMENTS

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