PREPARATION AND CHARACTERIZATION OF ANTIBACTERIAL GUANIDINE-BASED POLYMERIC MATERIAL

ANTİBAKTERİYEL GUANİDİN BAZLI POLİMERİK MALZEME HAZIRLANMASI VE KARAKTERİZASYONU

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ÖZET

ANTİBAKTERİYEL GUANİDİN BAZLI POLİMERİK MALZEME HAZIRLANMASI VE KARAKTERİZASYONU

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Bu tez çalışması kapsamında, antibakteriyel özelliğe sahip guanidin bazlı pamuk kumaşlar gıda ambalajları, tekstil ve medikal alandaki uygulamalara yönelik olarak hazırlanmıştır. Pamuk kumaşlara antibakteriyel özellik kazandırılması amacıyla antibakteriyel poli(hekzametilen guanidin) hidroklorür (PHMG) polimeri, hekzametilen diamin ve guanidin hidroklorür monomerlerinin polikondenzasyon reaksiyonu ile sentezlenmiş ve daha sonra kumaşlar PHMG ile modifiye edilmiştir.

Pamuk kumaşların antibakteriyel modifikasyonunda iki farklı yaklaşım çalışılmıştır. İlk yaklaşımda, PHMG polimerinin pamuk kumaşlara aşılanması amacıyla öncelikle birlikte ışınlama ile aşılama ve peroksidasyon ile başlatılan aşılama yöntemleri ek bir kimyasal malzeme kullanılmadan çalışılmış, ancak bu yöntemlerle modifikasyon elde edilememiştir.

İkinci yaklaşımda, pamuk kumaşların PHMG ile daha sonra modifiye edilmek üzere fonksiyonel hale getirilmesi için farklı radyasyon ile başlatılan aşı polimerizasyonu metotları kullanılarak glisidil metakrilat (GMA) ve akrilik asit (AAc) monomerleri ile aşılama çalışmaları gerçekleştirilmiştir. Denemelerde kullanılan koşullarda GMA aşılama çalışmalarından uygun sonuç elde edilemediğinden, pamuk kumaşın fonksiyonel hale getirilmesi için gerçekleştirilen çalışmalara AAc monomeri ile devam edilmiştir. Pamuk kumaşlar oksijen varlığında, gama radyasyon kaynağı kullanılarak, 30 kGy dozda ışınlanmış ve daha sonra AAc monomerinin sulu çözeltileri ile muamele edilmiştir. AAc aşılama çalışmaları, %20, %30 ve %40 (v/v) derişimde AAc sulu çözeltileri ile, 65 °C sıcaklıkta ve 3 saat reaksiyon süresinde gerçekleştirilmiştir.

Antibakteriyel etkinliğe sahip pamuk kumaşlar, AAc ile aşılanarak fonksiyonel hale getirilen pamuk kumaşların yapısında bulunan karboksil grupları ve PHMG polimerinin sahip olduğu amino fonksiyonel grupları aracılığı ile amid bağları üzerinden kimyasal bir reaksiyon ile hazırlanmıştır. Öncelikle AAc aşılı pamuk kumaşlardaki karboksil grupları DMTMM reaktif ajanı kullanılarak aktifleştirilmiş, daha sonra kumaşların oda sıcaklığında, yaklaşık 18 saat reaksiyon süresi boyunca, %10 (w/v) PHMG sulu çözeltisi ile bir araya getirilmesi sonucunda PHMG polimeri modifiye edilmiş pamuk kumaşlar elde edilmiştir.

Yapılan çalışmalar sonucunda, optimum aşılama koşulları %30 (v/v) AAc sulu çözeltisi derişimi, 65 °C reaksiyon sıcaklığı ve 3 saat reaksiyon süresi olarak belirlenmiştir. Bu koşullarda hazırlanan kumaşlar için ortalama aşılama verimi %13,8 olarak ve PHMG ile modifikasyon sonucunda ortalama modifikasyon verimi %19,6 olarak belirlenmiştir.

Sentezlenen PHMG polimerinin kimyasal yapısı, elemental bileşimi ve molekül ağırlığı FTIR, NMR, elemental analiz ve MALDI-MS ile incelenmiş, antibakteriyel özellikleri ise agar kuyu difüzyon ve broth dilüsyon yöntemleri ile araştırılmıştır. Broth dilüsyon testi sonucunda test tüplerinden elde edilen MİK değerleri *E. coli* için 8 mg/L, *S. aureus* için 4 mg/L olarak belirlenmiştir. Agar plaklarından elde edilen MİK değerleri ise *E. coli* için 64 mg/L, *S. aureus* için 16 mg/L olarak belirlenmiştir.

Antibakteriyel PHMG polimeri ile modifikasyon sonrası pamuk kumaşların yapısında oluşan kovalent amid bağları FTIR spektrumunda 1633 cm^{-1} ve 1550 cm^{-1}'de gözlemlenen pikler aracılığıyla FTIR analizi ile doğrulanmıştır. Modifikasyon sonrası yapıdaki elementlerin bileşimi elemental analiz çalışmaları ile belirlenmiştir. Hazırlanan

kumaşlardaki fiberlerin yüzey morfolojileri SEM analizi ve antibakteriyel özellikleri agar difüzyon testi ile araştırılmıştır. Agar difüzyon testi sonucunda, PHMG ile modifiye edilmiş pamuk kumaşların antibakteriyel aktivitesi doğrulanmıştır.

Anahtar Kelimeler: Pamuk kumaş, radyasyonla başlatılan aşı polimerizasyonu, akrilik asit, poli(hekzametilen guanidin) hidroklorür, antibakteriyel özellik.

ABSTRACT

PREPARATION AND CHARACTERIZATION OF ANTIBACTERIAL GUANIDINE-BASED POLYMERIC MATERIAL

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Within the scope of this thesis study, guanidine-based cotton fabrics with antibacterial properties have been prepared for applications in food packaging, textiles, and the medical field. To impart antibacterial properties to the cotton fabrics, the antibacterial polymer poly(hexamethylene guanidine) hydrochloride (PHMG) was synthesized through the polycondensation reaction of hexamethylenediamine and guanidine hydrochloride monomers, and then the fabrics were modified with PHMG.

Two different approaches were studied for the antibacterial modification of cotton fabrics. In the first approach, the direct and peroxide grafting techniques were initially performed to graft PHMG polymer onto the cotton fabrics without using additional chemical materials. However, these methods did not provide the desired modification.

In the second approach, grafting studies were conducted using different radiation-induced graft polymerization methods with glycidyl methacrylate (GMA) and acrylic acid (AAc)

monomers to functionalize the cotton fabrics for subsequent modification with PHMG. Since convenient results were not obtained from the GMA grafting studies with the conditions applied during trials, the studies to functionalize the cotton fabrics were continued with AAc monomer. Cotton fabrics were irradiated at a dose of 30 kGy using a gamma radiation source in the presence of oxygen, and then treated with aqueous solutions of AAc monomer. AAc grafting studies were performed with AAc aqueous solutions at concentrations of 20%, 30%, and 40% (v/v), at a reaction temperature of 65°C and a reaction time of 3 hours.

Cotton fabrics with antibacterial activity were prepared by a chemical coupling reaction of the carboxyl groups present in cotton fabrics grafted and functionalized with AAc and the amino functional groups of the PHMG polymer through amide bonds. Firstly, the carboxyl groups in the AAc-grafted cotton fabrics were activated using the DMTMM coupling reagent. Then, the fabrics were combined with a 10% (w/v) aqueous solution of PHMG and allowed to react at room temperature for approximately 18 hours. As a result, cotton fabrics modified with PHMG polymer were obtained.

Based on the conducted studies, the optimum grafting conditions were determined as 30% (v/v) concentration of AAc aqueous solution, 65°C reaction temperature, and 3 hours reaction time. The average grafting yield for fabrics prepared under these conditions was determined as 13.8%, while the corresponding average modification efficiency (coupling yield) of PHMG modification was 19.6%.

The chemical structure, elemental composition, and molecular weight of the synthesized PHMG polymer were investigated using FTIR, NMR, elemental analysis, and MALDI-MS. The antibacterial properties were examined using agar well diffusion and broth dilution methods. As a result of the broth dilution test, the MIC values obtained from the test tubes were 8 mg/L for *E. coli* and 4 mg/L for *S. aureus*. On the other hand, the MIC values determined from the agar plates were 64 mg/L for *E. coli* and 16 mg/L for *S. aureus*.

For the final materials, PHMG-modified cotton fabrics, the presence of covalent amide bonds in the structure upon PHMG modification was confirmed by FTIR analysis via the peaks observed at 1633 cm^{-1} and 1550 cm^{-1} on the FTIR spectrum. The elemental analysis was carried out to obtain the final composition of elements within the cotton fabrics after PHMG modification. The surface morphology of the fibers in the prepared fabrics was

observed using SEM, and the antibacterial properties were investigated using the agar diffusion test. The antibacterial activity of the PHMG-modified cotton fabrics was confirmed through the qualitative agar diffusion test.

Keywords: Cotton fabric, radiation-induced graft polymerization, acrylic acid, poly(hexamethylene guanidine) hydrochloride, antibacterial property.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

PHMG	Poly(hexamethylene guanidine) hydrochloride
AAc	Acrylic acid
GMA	Glycidyl methacrylate
AGU	β-D-anhydroglucopyranose
DP	Degree of polymerization
CI	Crystallinity index
XRD	X-ray diffraction
NMR	Nuclear Magnetic Resonance
FTIR	Fourier-transform Infrared Spectroscopy
RIG	Radiation-induced grafting
GY	Grafting yield
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
MES	2-(N-morpholino)ethanesulfonic acid
SA	Sodium alginate
CDA	Cellulose diacetate
PPGDE	Poly(propylene glycol) diglycidyl ether
SEM	Scanning Electron Microscope
MALDI-MS	Matrix-assisted Laser Desorption Ionization Mass Spectrometry
PBS	Phosphate-buffered saline
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
M_W	Mass average molecular weight
M_n	Number average molecular weight
w/v	Mass/Volume
v/v	Volume/Volume

1. INTRODUCTION

The control and prevention of infections caused by pathogenic microorganisms have been more crucial in recent years, considering the ongoing harmful consequences of coronavirus disease (COVID-19) on public health, economic conditions, and social subjects all over the world. In general, antimicrobial drugs have been employed to overcome such infections, yet this method is becoming less efficient due to the increasing drug resistance by microorganisms [1]. From this point of view, developing novel material systems with antimicrobial efficiency may facilitate the combat and mitigation of infectious diseases.

The polymer-based material systems displaying antimicrobial properties have been an attractive and significant research topic, especially in textile, medical, and food packaging applications since the activity of pathogenic microorganisms leads to concerns in these fields [2, 3]. The relevant systems can be prepared from cellulose and an antimicrobial agent, such as poly(hexamethylene guanidine) hydrochloride (PHMG), by combining the advantages of a natural polymer and synthetic bioactive component.

Among widely studied antimicrobial agents, cationic polymers presenting broad antibacterial activity and low potential for resistance development appear as up-and-coming alternatives [4, 5]. Poly(hexamethylene guanidine) hydrochloride is a polymer with a cationic character, and excellent antimicrobial activity against various microorganisms including bacteria (with or without antibiotic resistance), fungi, and viruses [6, 7]. Besides its commercial use in diverse applications in medicine, food, agriculture, and textile industry-related products [6, 8], PHMG has emerged as an essential component of antimicrobial material systems in the literature developed from synthetic and natural polymers. Cellulose [9, 10, 11], chitosan [12], starch [13], sodium alginate [14] and polyurethane [7, 15], polyacrylonitrile [16], poly(vinylidene fluoride) [17] are some of the natural and synthetic polymers studied in combination with PHMG, respectively.

Cotton is a cellulose-derived natural plant fiber with remarkable features like renewability, air permeability, biodegradability, and comfort due to its compatibility with human skin [8, 18]. However, cotton presents a porous and hydrophilic structure, which

may provide convenient conditions for the adhesion and growth of microorganisms [18, 19]. Consequently, the proliferation of bacteria on cotton fabrics causes discomfort in the personal use of the materials and may also lead to bacterial infections in public [8, 10, 18]. The modifications for rendering the cotton fabrics antibacterial are quite important to eliminate these concerns and support the safe use of the materials produced from them. In that context, combining the active polymer with cotton material to prepare the fabric with antimicrobial properties seems to be an interesting approach.

The surface of cellulose-based materials could be modified by adding antimicrobial agents via physical or chemical interactions [20]. The systems prepared by simply mixing the cellulose and the agent can lead to uncontrolled leaching of the antimicrobial agent out of the system over time due to the weak interactions between them. Therefore, the efficiency of the systems against microorganisms and the safety of the user may decrease in time [8, 11]. Besides, environmental problems related to the agent's toxicity may be a concern [20]. Thus, the development of novel material systems presenting durable antimicrobial activity via strong interactions has great importance.

In this study, the trials to prepare antibacterial material systems composed of PHMG and cotton fabrics for medical, textile, and food packaging areas were performed by applying various approaches. The guanidine-containing antibacterial polymer, PHMG, was synthesized by a polycondensation reaction in the first step. Afterward, direct grafting (simultaneous grafting) and peroxide grafting radiation methods were studied to graft the PHMG polymer on the cotton fabrics without additional chemical substances.

In addition, the post-effect irradiation method was carried out to functionalize the surface of the cotton fabrics with glycidyl methacrylate (GMA) monomer by graft polymerization. However, favorable results could not be obtained with the applied grafting conditions, and experiments to functionalize the surface of the fabrics proceeded on another monomer, acrylic acid (AAc). The guanidine-based antibacterial cotton fabrics were prepared in two steps. In the first step, the surface of the cotton fabrics was functionalized by grafting AAc monomer via the peroxide grafting radiation technique, and this was followed by the modification of AAc-grafted cotton fabrics with PHMG by a chemical coupling method. The proposed method distinguishes among the similar studies offering methods for antibacterial modification of the cellulose-based materials

with PHMG via covalent interactions since it presents a simple application without including complex steps and enables to use of a mild solvent in each step.

2. GENERAL INFORMATION

2.1. Cellulose Chemistry

Cellulose is the most abundant polymer in nature and belongs to the class of polysaccharides. The polymer has common attractive features with other polysaccharides, such as biodegradability, biocompatibility, and acquisition from renewable sources; therefore, the systems that contain cellulose or its derivatives have developed for a diverse range of applications from the field of paper to food [20].

2.1.1. Chemical Structure and Characteristics

Cellulose is a homopolymer consists of monomer units called β-D-anhydroglucopyranose, AGU (Figure 2.1). These units are bonded covalently via β-1, 4-glycosidic bonds formed between the C4 carbon atom of one glucose ring and the C1 carbon atom of the neighboring one by resulting the linear polymer chains [20, 21]. The number of AGU in a cellulose polymer chain represents the chain length that also defines as the degree of polymerization (DP) [21, 22]. DP could change between hundreds to several tens of thousands depending on the polymer's source and its isolation method. The literature includes examples of DP of the cellulose in cotton in the range of 800-10,000 based on the source and treatment conditions [22, 23].

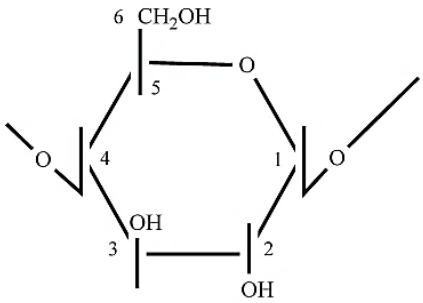


Figure 2.1. The carbon atoms on the anhydroglucose unit of cellulose [21].

The three hydroxyl groups (-OH) located at C2, C3, and C6 positions on an anhydroglucose unit are associated with the chemical reactions of cellulose (Figure 2.1) [22]. The relative reactivity of three -OH groups is expressed as OH-C_6 ≫ OH-C_2 > OH-C_3 [21].

According to the fringed-micelle model, cellulose fibers comprise microfibrils consisting of amorphous and crystalline regions in their structure [21, 23, 24]. The amorphous area includes short, separate, twisted, and unorganized polymer chains, yet the longer chains of the polymer are found aligned and packed in parallel to each other in the crystalline parts (Figure 2.2.a) [25]. The hydroxyl groups on the polymer's chains are connected via hydrogen bonds, which could be formed between the neighboring glucose units of a polymer chain (intramolecular) and between the neighboring chains (intermolecular). The intramolecular bonds give stability to crystalline conformation by obstructing the free rotation of the glucose rings. Besides, the intermolecular hydrogen bonds enable the polymer chains to get closer together, creating well-organized crystalline regions in the structure [22]. The structure of cellulose fibers is expressed as a supramolecular structure which has a significant impact on the physical and chemical properties of the polymer such as solubility, degradation, and reactivity [21].

Crystallinity of cellulose influences the various features of the polymer and may present essential information for developing the approaches regarding its mechanical (tensile strength, hardness), degradation (enzymatic hydrolysis), chemical (reactivity) characteristics [21, 26, 27].

Cellulose is found in the polymorphic crystalline structures called Cellulose I, II, III, and IV. Cellulose I is the form occurred in nature, while Cellulose II, III, and IV could be obtained by using one of the other forms via some treatments [27, 28].

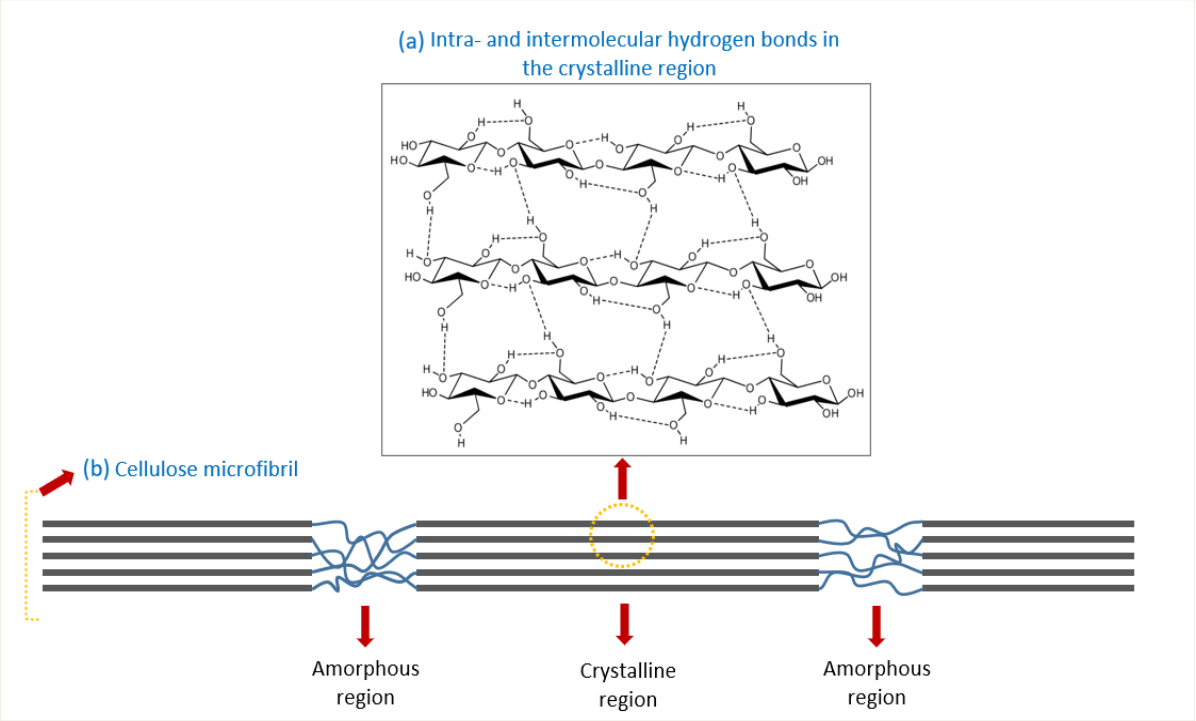


Figure 2.2. The hydrogen bond network in the crystalline region (a) [22], Structure of cellulose microfibril (b).

The crystallinity index (CI) is a parameter that defines the relative percentage ratio of the crystalline content in cellulose. The index could be determined by applying different techniques such as X-ray diffraction (XRD), solid-state ^{13}C NMR, Raman spectroscopy, and Fourier-transform infrared spectroscopy (FTIR) [28, 29]. The result may differ depending on the measurement technique, yet the XRD method is the one that is commonly used in a wide range of studies and is simple to interpret by enabling the comparison of the CI of different cellulose samples. CI value can be basically calculated via peak height method which requires the intensity values of lattice and amorphous regions from the XRD diffractogram [29, 30, 31]. Some studies include the crystallinity index of different cellulose samples determined via XRD analysis by using the peak height method. CIs were indicated as 81 % and 77% for microcrystalline cellulose [32] and cotton cellulose [30], respectively. Furthermore, literature covers some examples regarding CI that are used to compare and evaluate the difference within the structures before and after physical [33], chemical [31], and mechanical [29] treatments applied for modifying the cellulose's structure.

The solubility tendency of cellulose could be associated with its amorphous and crystalline nature. In the crystalline region, the packing of the chains due to the hydrogen bond network (Figure 2.2.b) does not allow the solvent molecules to pass into or access the cellulose fibrils leading to the insolubility of the polymer in water and commonly used organic solvents [20, 22, 34]. However, cellulose is known as highly hydrophilic since the amorphous parts contain space between the chains that allow the structure to absorb large amounts of water via hydrogen bonds formed between the chains and water molecules. As a result of the binary structure of the polymer, cellulose does not dissolve in water but swells by absorbing the solvent [24]. In addition to the solubility, the enzymatic hydrolysis of the polymer is affected by the accessibility of the polymer chains [34].

Besides the drawback in the solubility, cellulose is poorly compatible with the hydrophobic polymer matrix, has poor dimensional stability, and lacks thermoplasticity and antibacterial properties [21, 24]. Therefore, the desired properties regarding the aimed applications are usually imparted to the cellulose structure via physical and/or chemical modification.

2.1.2. Sources, Derivatives, and Area of Application

Cellulose is a constituent of various plant-based renewable sources such as cotton, wood, olive, sugarcane, and sunflower [26]. Besides its plant origin, the polymer is produced by bacteria, and this type of cellulose is called bacterial cellulose or microbial cellulose [35]. *Acetobacter xylinus*, and *Gluconacetobacter* are prominent strains that provide bacteria-based cellulose [21, 24, 35, 36].

The polymer has a few handicaps that restrict its utilization, like the weak solubility in mainly used solvents. A diverse range of cellulose derivatives is used to cope with such limitations by meeting the needs for aimed applications [21, 37]. Carboxymethyl cellulose and cellulose acetate are the commonly studied cellulose derivatives belonging to cellulose ethers and esters groups, respectively.

Table 2.1 displays some examples of cellulose-containing systems' application areas according to the types or derivatives of the polymer. The nano-forms of cellulose also attracted attention due to their promising characteristics. The plant-based cellulose nanocrystals and nanofibrils cellulose are known to be used as reinforcing agents for polymers and nanocomposites to improve the mechanical properties of the systems [37].

Table 2.1. Examples of application area according to the type or derivative of cellulose.

Type/derivative of Cellulose	Aimed Area of Application
• Sugarcane bagasse cellulose	Antimicrobial hydrogels for wound dressing [11]
• Cotton cellulose fabrics	Adsorbent for heavy metal ions in water [38, 39] Antibacterial materials for applications in surgical equipment, hospitals, hotels [18], and clothing, medical gauze [10]
• Wood - derived cellulose fibers	Paper packaging and medical paper products [19]
• Bacterial cellulose	Antimicrobial wound dressings [9] Fabrics, and garments in textile, and additives in food [35]
• Carboxymethyl cellulose	Drug delivery systems in biomedical applications, stabilizers or thickeners in food products, food packaging, adsorbent in water treatment [40]
• Cellulose acetate	Blood filtration devices, tissue engineering scaffolds [21]

2.2. Cotton Fabric

Cotton is a natural plant fiber that majorly consists of cellulose. The approximate cellulose content of the cotton fiber was indicated as around 88-96% [30], 90% [41], and 94% [42] in different studies. The remaining small portion of the fiber contains materials including proteins, pectin, organic acids, minerals, waxes, etc. [30, 42]. Although cotton fibers are composed of almost pure cellulose, refinement is necessary to eliminate the non-cellulosic content of fibers [22]. Scouring enables the removal of the impurities that may affect the processing, increases the cellulose content in the fibers, and endows a highly hydrophilic feature [30, 42, 43].

Among all plant fibers containing cellulose, cotton fibers have the cellulose with the highest molecular weight and structural order [41]. The cellulose chains within the cotton

are found in fringed fibril morphology (crystalline and amorphous regions together) with a pretty ordered crystalline part at a ratio over 60% [43].

Cotton fibers present renewability, biodegradability, comfort, compatibility with human skin, and air permeability [8, 18], which make them one of the major raw materials used in the textile industry and in the production of medical products such as sutures, and absorbent pads [10, 30, 43]. On the other hand, cotton's porous and hydrophilic structure may provide convenient conditions for the adhesion and growth of the microorganisms [19]. The proliferation of bacteria on cotton fabrics causes discomfort in the personal use of the materials and may also lead to bacterial infections in public [8, 10, 18]. The modifications for rendering the cotton fabrics antibacterial are quite important to eliminate these concerns. The antibacterial property could be imparted to the cotton fabrics via antibacterial structures such as quaternary ammonium compounds, chitosan, metals and metal salts, and guanidine compounds [18].

2.3. Poly(hexamethylene guanidine) Hydrochloride

Poly(hexamethylene guanidine) hydrochloride (PHMG) is a guanidine-based synthetic polymer with a cationic character, which presents a broad spectrum and efficient antimicrobial activity against microorganisms [44]. This polymeric biocide has odorless, colorless, non-corrosive, and highly water-soluble features [45, 46]. Its activity against pathogenic microorganisms includes Gram-positive, Gram-negative, antibiotic-resistant bacteria [45, 47], fungi [46], and viruses [6]. PHMG demonstrates rapid activity against microorganisms even when it is used at low concentrations [44, 45] and reveals as a crucial substance in combatting pathogens.

2.3.1. Chemical Structure, Characteristics, and Antimicrobial Action

PHMG is a copolymer that can be synthesized by the melt polycondensation reaction of hexamethylenediamine and guanidine hydrochloride monomers [6, 48] and is also expressed as an oligomer, oligo guanidine [49, 50], due to the relatively low molecular weight of its chains (Figure 2.3).

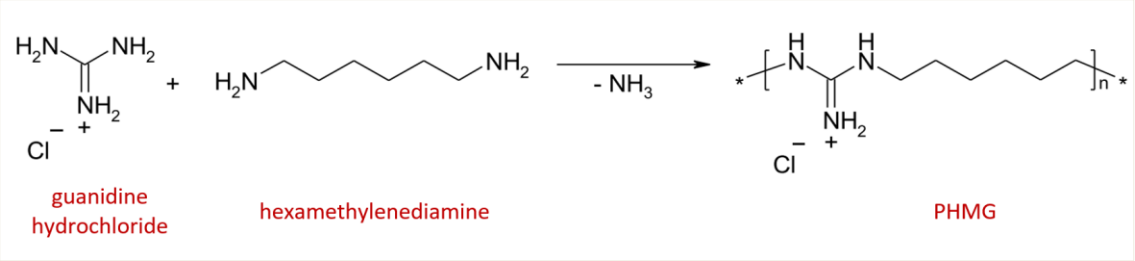


Figure 2.3. Reaction scheme for the synthesis of PHMG [7].

Table 2.2 displays some examples from the literature studies regarding the molecular weight of PHMG both obtained via synthesis or commercially. The molecular weight of guanidine oligomers is an important characteristic since it may have influence on the antimicrobial activity of the structure [49, 50], and it differs depending on the conditions of the synthesis reaction and the measurement technique.

Table 2.2. Examples of molecular weight of PHMG from literature.

Source	**M_w / M_n**	**Value or Range (g / mol)**	**Ref.**
Commercial	M_n	2100	[10]
Commercial	M_n	800	[16]
Synthesis	M_n	600	[17]
Synthesis	M_w / M_n	576 / 481	[47]
Synthesis	M_w	650 – 960	[49]
Synthesis	M_w	408 – 956	[50]
Synthesis	M_w / M_n	1008 / 771	[51]
Synthesis	M_w / M_n	1600 / 1300	[52]
Synthesis	M_n	720	[53]

Mass spectrometry is the generally preferred technique in the literature to acquire information about PHMG's characteristics such as repeating unit, end groups of the chains, polydispersity, average molecular weights (M_W and M_n), and types of the molecules contained within the structure [50, 54, 55].

PHMG is composed of various molecules with different chain lengths and structures. The molecules are indicated in seven types of structures; linear (type A, B, and C), branched (type D), and cyclic (type E, F, and G) shapes [50].

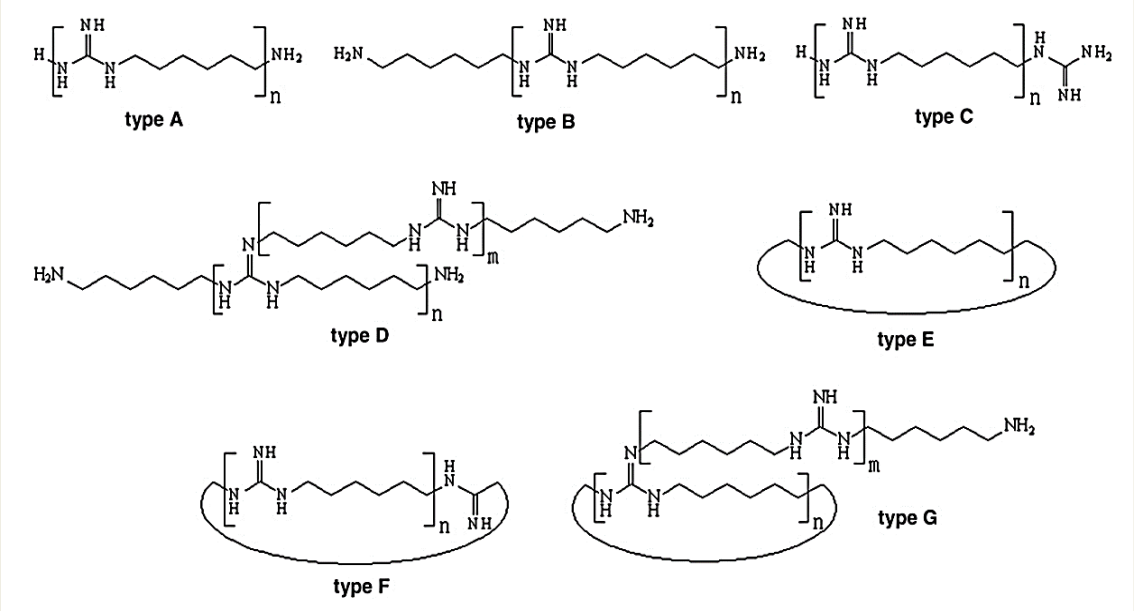


Figure 2.4. Seven types of molecular structures (types A–G) in PHMG [50].

The cationic nature of PHMG can be associated with the guanidine groups in the structure. These guanidine groups turn into guanidinium cations when they are protonated and present strong basic features (pKb=0.4) [6]. Since the guanidine groups are found in the form of guanidinium cations in a wide pH range [18], PHMG polycation can demonstrate antimicrobial action in that broad region. Besides, the guanidine polymers are suitable for electrostatic interactions with negatively charged particles, and structures thanks to their positive charges [56].

The hypothesis regarding the polymer's antimicrobial mechanism of action is explained by its cationic character. The positively charged polymer molecules can interact with negatively charged cell surfaces of bacteria. This electrostatic interaction enables the polymer molecules' adhesion to bacterial cell wall, followed by the initiation of wall disruption. Once the wall that protects the bacterial cell is damaged, the inner cytoplasmic membrane weakens and becomes more permeable due to the occurrence of the pores. The components, cytoplasmic fluid, within the membrane leak through the spoiled areas causing the loss of function and inactivation of the bacterial cell [5, 11, 19, 57, 58].

2.3.2. Drawbacks

The polymer has been known to have relatively lower toxic effects on human cells than the ordinarily used disinfectants or antimicrobial agents [45, 46, 59]. However, the harmful side effects regarding its use as an ingredient of the disinfectants of household humidifiers were revealed in South Korea [60]. According to the relevant literature, the polymer in commercial disinfectants applied to prevent the growth of microorganisms within the water tanks of humidifiers caused fatal consequences such as lung injuries via the emission of disinfectant droplets into the air and their repetitive inhalation by people [54, 55]. Recent studies have shown that long-term exposure to PHMG, as for instance additive in disinfectants, can have quite toxic effects on human health [61, 62].

The low molecular weight of the oligomeric structure may be considered as another drawback since it may lead leaching problems when PHMG is integrated into material systems directly by mixing [48]. This results in the loss of antimicrobial efficiency of PHMG incorporated material applications [13].

The polymer's incorporation into the material systems through strong interactions or bonds by non-leaching approaches instead of using it as an ingredient in some disinfectant solutions is quite important for eliminating its free form’s toxic effects, and migration possibility from the systems, while benefiting from its excellent antimicrobial properties.

2.3.3. Area of Application

The polymer has been used as a component of various commercial goods including personal hygiene products, contact lens solutions, and hand cleaners [6]. Besides these, it has been used in the disinfectant form for sterilization of swimming pools, surfaces and equipment in hospitals, in textiles, food packaging, and in household humidifiers [7, 10, 60, 63]. Thanks to its colorless and odorless features it has also found applications in the disinfectants of food and drug industries [59]. Table 3 demonstrates some examples of material systems containing PHMG polymer from the literature and aimed applications of the corresponding study.

Table 2.3. Examples of PHMG containing material systems and their aimed area of the application.

Material System	Aimed Area of Application
• Commercial thermoplastic polyurethane - PHMG composite	Medical applications; wound dressing, surgical drapes [7]
• Poly(lactic acid) matrix embedded with starch – PHMG microparticles	Medical devices and food packaging applications [13]
• Thermoplastic polyurethane membrane incorporated with PHMG - grafted graphene oxide	Wound dressing [15]
• PHMG - functionalized polyacrylonitrile nanofibrous membranes	Water filtration systems, medical devices and protective textiles [16]
• Poly(vinylidene fluoride) - PHMG membrane	Water treatment [17]
• Chitosan - polyvinyl alcohol / PHMG sponge	Wound healing [58]
• PHMG - based disinfectant	Food preservation (cocoa beans), agriculture [64]
• PHMG solution as disinfectant	Dental applications [65]
• PHMG	Food disinfection, vegetable sanitizer [66]

2.4. Radiation Grafting Technique

2.4.1. Ionizing Radiation

Ionizing radiation can create reactive species on the matter to induce chemical interactions and is utilized for many different purposes, including grafting modification, cross-linking, and chain scission in polymeric systems, sterilization of medical devices, and curing of coatings. The main sources of ionizing radiation are considered electron accelerators producing electron beams, and radioactive sources, ^{60}Co in general, providing gamma rays. Using radiation provides certain advantages in application since

it enables to process of the materials in different physical forms, at ambient temperature and in a short time, and is a clean tool by not requiring additional chemicals or producing waste [67].

2.4.2. Radiation-induced Grafting (RIG) Methods

The desired characteristics for the aimed applications can be endowed to polymers by modifying their surface with various monomers via the radiation-induced grafting technique. The technique can be applied using forms of ionizing radiation such as electron beams and gamma rays, and the obtained systems are called graft copolymers that possess a branched structure with a polymer backbone and monomer side chains bonded covalently to the polymer as branches (Figure 2.5) [68].

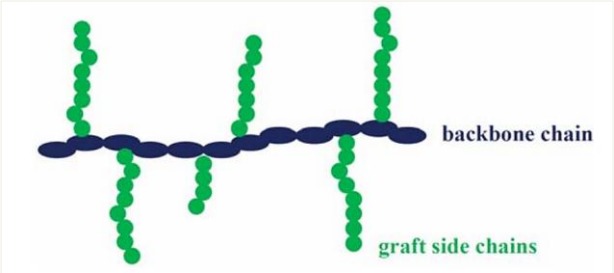


Figure 2.5. Simplified structure of graft copolymer [68].

The radiation-induced grafting approach can be divided into three main methods: direct, peroxide, and pre-irradiation grafting [69]. The explanations regarding the mechanism of the grafting methods are as follows.

2.4.2.1. Direct Grafting Method

The method is based on ionizing radiation exposure (generally gamma source) of the polymer substrate and the monomer to be grafted simultaneously. When the polymer substrate in the monomer solution is irradiated, free radicals formed on the polymer backbone start a graft polymerization between the substrate and a monomer molecule. Then, the propagation step occurs, during which the graft monomer chain grows by the addition of the monomers to the macroradicals. Consequently, the chain growth ends with the growing macroradicals' recombination (Figure 2.6).

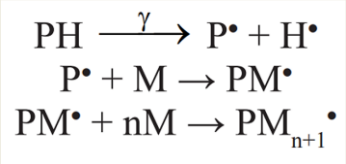


Figure 2.6. The mechanism of the simultaneous grafting, where PH, M, PM•n+1 represent the polymer substrate, monomer, and macroradicals, respectively [68].

Upon the simultaneous irradiation application, free radicals are created on the monomer besides the ones formed on the polymer backbone. The monomers can undergo a homopolymerization reaction by connecting via these radicals. Since the homopolymer formation limits the grafting yield, some inhibitors, such as inorganic salts, are added to the grafting media to manage it [68, 69].

2.4.2.2. Peroxide Grafting Method

In this method, the polymer substrate is irradiated under oxygen or an air atmosphere, and resulting alkyl radicals are oxidated to form peroxide radicals. The peroxide radicals generate peroxides (R-O-O-R) and hydroperoxides (R-O-O-H) gathering with the polymer substrate. These species decompose when exposed to high temperatures, and the breakdown of their bonds emerges alkoxy and hydroxy radicals. The radicals initiate the copolymerization reaction with the monomer [68, 69]. This method is also called the oxidative pre-irradiation method [70]. Since the polymer substrate is pre-irradiated before the addition of the monomer to be grafted, the homopolymer formation of the used monomer is relatively negligible [69]. Besides, the method enables obtaining peroxides, which can be kept stable on the polymer substrate when stored at low temperatures (0° or below). This allows performing the grafting a while after the irradiation is applied to the material [68].

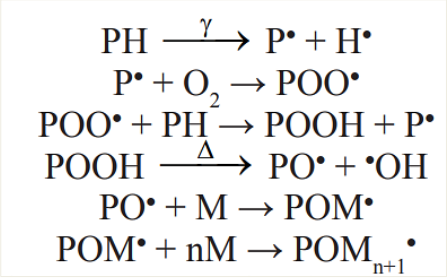


Figure 2.7. The mechanism of the peroxide grafting, where PH, P•, POO•, POOH, PO•, and M represent the polymer substrate, alkyl radicals, peroxide radicals, hydroperoxides, alkoxy radicals, and monomer, respectively [68].

2.4.2.3. Pre-irradiation Grafting Method

In the pre-irradiation method without oxygen, the polymer substrate is irradiated under a vacuum or inert atmosphere to create reactive alkyl radicals. The method requires a high radical concentration for the initiation of polymerization; accordingly, it is necessary to irradiate the substrate at high dose rates. Just after irradiating the substrate, a de-oxygenated monomer solution is added for grafting, and the reaction proceeded under an inert atmosphere to prevent the probable interactions between oxygen and radicals. A reaction temperature may be needed depending on the reactivity of the monomer to be grafted. Similarly to the peroxide method, since the monomer is not irradiated and therefore does not contain radicals, the homopolymer formation can be considered negligible [68, 69].

2.4.3. Parameters Affecting the Radiation-induced Grafting

The grafting yield (GY) is the main parameter to evaluate the efficiency of the obtained grafting and can be determined according to Equation 2.1 below.

$$GY\ (\%) = \left(\frac{Wf - Wi}{Wi}\right) \times 100 \qquad \textbf{(2.1)}$$

In the equation, W_i represents the initial weight of polymer substrate, while W_f is the weight of grafted polymer.

There are various parameters that affect the radiation-induced grafting, and therefore the GY (Figure 2.8) [68, 69].

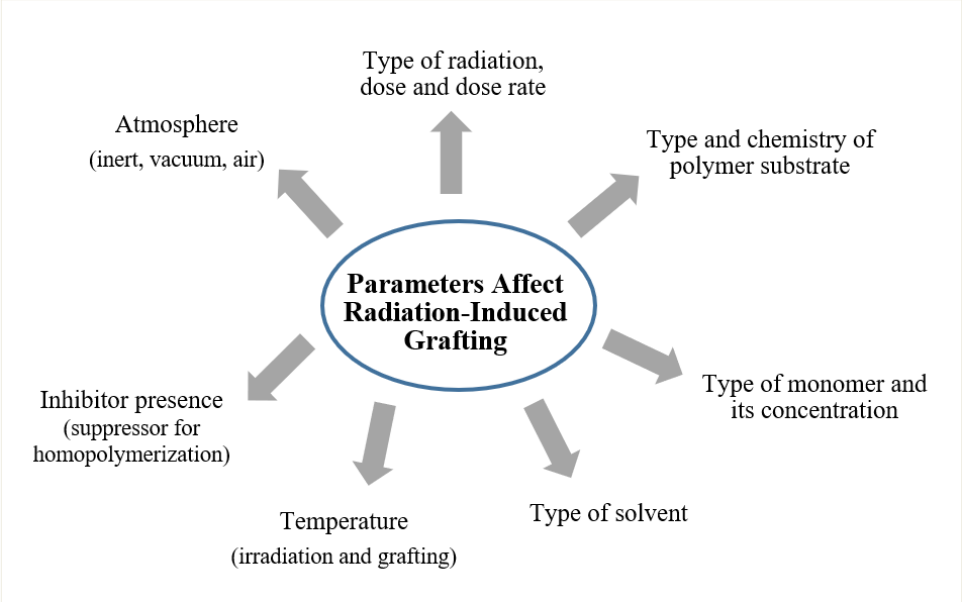


Figure 2.8. Parameters in radiation-induced grafting.

2.4.4. Monomers Used in Radiation-induced Grafting

2.4.4.1. Glycidyl Methacrylate

Glycidyl methacrylate (GMA) ($C_7H_{10}O_3$) is a monomer that possesses a non-toxic, hydrophobic, and highly reactive structure [71] (Figure 2.9). The methacrylic and epoxy groups within the GMA molecules endow the monomer with dual functionality [72], render the monomer available for copolymerization with other monomers, and in the preparation of functional polymeric material systems as a precursor [71]. In the case of GMA being used as a precursor in the preparation of material systems, it can be grafted on a trunk polymer, e.g. cellulose, by various methods, including chemical, plasma, and radiation techniques [26, 73], and the grafted structure can be functionalized or modified via the reaction between the reactive epoxy groups of GMA and the functional groups such as amino, carboxylic acids, etc. [71].

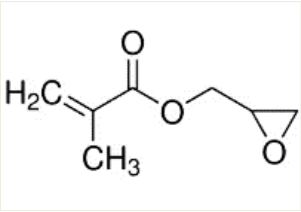


Figure 2.9. Chemical structure of glycidyl methacrylate.

In the literature, GMA-grafted cellulose-based materials such as cotton fabrics and gauzes have been used to prepare the adsorbents in water treatment [72] and to develop antimicrobial agents delivering-releasing systems for preventing wound infections [74]. Another study includes the modification of an antibacterial polymer, PHMG, with GMA by a ring-opening reaction between the epoxy groups of GMA and the amino groups of the polymer. The study aimed to introduce permanent antimicrobial properties to material systems via carbon-carbon double bonds, which were imparted to the antibacterial polymer via modification. An acrylonitrile copolymer was synthesized from the GMA-modified PHMG and acrylonitrile monomers, and the copolymer was indicated to be exhibited excellent antimicrobial activity [51].

2.4.4.2. Acrylic Acid

Acrylic acid (AAc) ($C_3H_4O_2$) is one of the most often preferred monomers to functionalize material surfaces with graft polymerization [68] (Figure 2.10). This organic molecule, also known as prop-2-enoic acid, is the simplest form of unsaturated carboxylic acids. The chemical is found in colorless liquid form at room temperature, has a pungent odor, is corrosive, and can irritate the skin at the contacting site [75]. Besides, it is miscible with various solvents such as water, alcohols, ethers, and chloroform. AAc takes part in polymerization reactions via the double bond (C=C) and the carboxyl group (-COOH) it holds [76], and is used in the applications, including the production of medical products, detergents, wastewater treatment chemicals, plastics, coatings, adhesives, and elastomers [75].

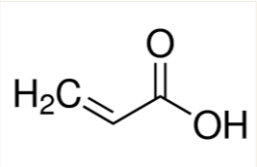


Figure 2.10. Chemical structure of acrylic acid.

In the literature, rice straw cellulose was modified with acrylic acid via radiation graft copolymerization method by aiming the applications as heavy metal adsorbent and ion exchanger [77]. In another study, acrylic acid-grafted polypropylene nonwoven fabric was modified with chitosan to investigate the wettability and antibacterial properties of the materials [78].

2.5. Chemical Coupling

2.5.1. Amide Coupling Reaction

Amide bonds are the chemical bonds that connect the amino acid monomers, the building blocks of protein molecules, and are also found in the structure of a wide variety of drugs produced in the pharmaceutical industry. These bonds are ordinarily obtained from carboxylic acids and amines via amide coupling reactions. The synthesis reaction usually requires the activation of the carboxylic acid by using coupling reagents in the first place. In general, activating the carboxylic acid groups is applied before including the amines and occurs based on the release of the acids' hydroxyl group (-OH) via a good leaving group (Figure 2.11). Carbodiimides, phosphonium salts, and triazine-based reagents are some of the groups of amide coupling reagents [79].

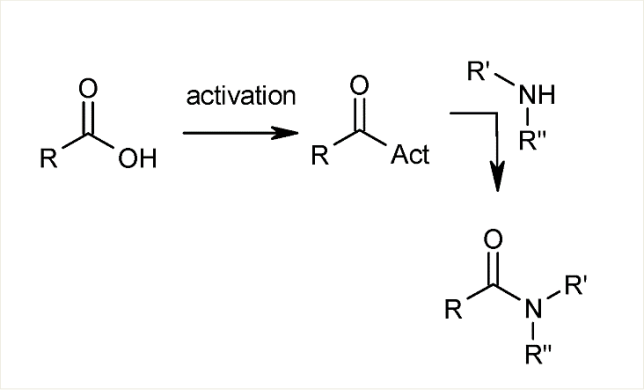


Figure 2.11. Principle of the activation process for amide-bond formation [79].

There is a study that covered the acquisition of the covalent bonds via an amine reaction of carbodiimide chemistry using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and N-Hydroxysuccinimide between PHMG and carboxylated cellulose diacetate [80].

2.5.2. DMTMM Reagent and Its Function in Amide Coupling Reaction

4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride or DMTMM ($C_{10}H_{17}ClN_4O_3$) belongs to the group of triazine-based coupling reagents and is used for activating carboxylic acids in the synthesis of amides. DMTMM-promoted amide coupling can be indicated to be take place in two steps. The acid activation step occurs providing an activated ester from the reaction between DMTMM and the acid. Then, the formation of the amide bonds is achieved via the interaction of the activated ester with

the amine. The produced water-soluble byproducts of those steps during the amide coupling reaction, N-Methylmorpholine hydrochloride and 1-hydroxy-3,5-dimethoxytriazine, respectively, can be removed by washing in aqueous media. DMTMM reagent is solid with stability in air and water and can assist the amide synthesis in the solvents such as alcohols or water, without ester formation or hydrolysis product [81].

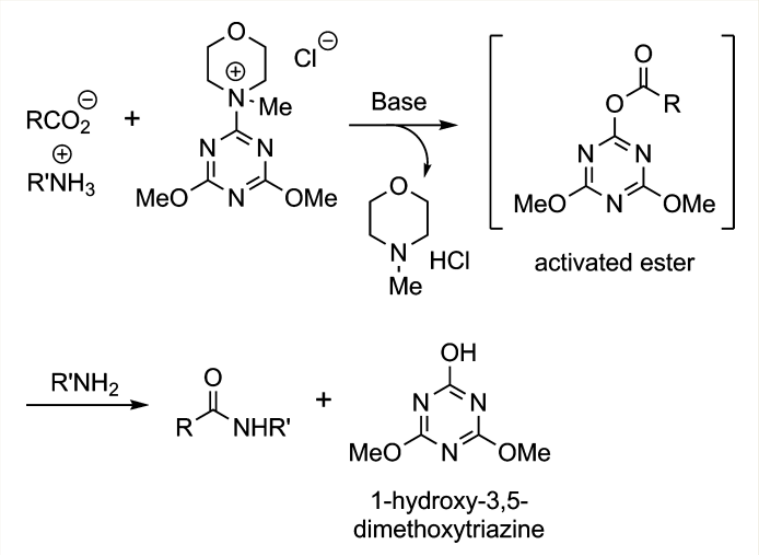


Figure 2.12. Acid activation mechanism with DMTMM via activated ester [81].

2.5.3. MES Hydrate Buffer

2-(N-morpholino)ethanesulfonic acid (MES) hydrate ($C_6H_{13}NO_4S \times H_2O$) is a buffer widely used to regulate and retain a stable pH value for biological applications such as plant culture medium and physiological experiments [82]. The compound is also known as one of the Good's buffers, is soluble in water, and has a pKa value of 6.15 at 20 °C [83].

2.6. Summary of Literature Studies

The relevant example studies regarding the modification approaches of various cellulose types and polysaccharides by PHMG or similar structures have been summarized as follows.

In the study of Kukharenko and colleagues (2014), a material system with antimicrobial properties was prepared from the bacterial cellulose and polymeric biocide, PHMG, for potential wound healing applications. The systems were obtained by dipping the bacterial cellulose membranes into the aqueous PHMG solutions. It was indicated that the

interactions within the system were created via the hydrogen bond formation between the biocide's chloride ions and the hydroxyl groups of cellulose. According to microbiological testing, the developed structures displayed great antimicrobial activity against yeast, various multidrug-resistant strains, and biofilm formation. As a conclusion, the prepared membranes presented high biocide release rate from the structure, and some new approaches may be applied to prolong the time of release [9].

Cai and colleagues (2018) proposed a method for the grafting of commercially available PHMG on cotton cellulose fibers by covalent bonds for obtaining long-lasting antibacterial activity. The approach consisted of the oxidization of the fibers and following two-step chemical reactions; obtaining the multiple aldehyde groups on the fiber surface via oxidizing them, the formation of Schiff bases between the terminal amine groups of PHMG and aldehyde groups of oxidized cotton fibers, and the reduction of the unstable Schiff base carbon−nitrogen double bonds into stable sigma bonds in the last step. The resulting antibacterial polymer-coated fibers were reported to be demonstrated durable activity against Gram-positive and Gram-negative bacteria after repetitive 1000 cycles of washing [10].

Pan and colleagues (2019) aimed to develop an antimicrobial hydrogel based on sugarcane bagasse cellulose and PHMG as wound dressing materials for wound healing applications. The materials were prepared in three steps: the epoxidation modification of the cellulose by a coupling agent, the grafting of PHMG on the epoxidized cellulose, and, obtaining hydrogels using sugarcane bagasse cellulose, PHMG-grafted cellulose, and a crosslinker in the last step. It was concluded that the covalent bond formation between PHMG and cellulose rendered the materials with durable antimicrobial properties, and the hydrogels displayed non-leaching antibacterial efficacy against *Escherichia coli (E. coli)* [11].

In a similar approach proposed by Yang and colleagues (2022), a bi-functional medical dressing material for chronic wounds was prepared by functionalizing the cotton gauze surface via chemical grafting of PHMG and physical adsorption of a natural polymer, chitosan. The grafting of PHMG on the oxidized cotton surface was achieved via the Schiff base reaction, and then the Schiff base bonds were reduced to stable sigma bonds by a chemical operation. In the last step, chitosan’s physical adsorption on the modified cotton was shown to be obtained via hydrogen bonds between amino groups of chitosan and hydroxyl groups of cotton cellulose by a dipping process. The presence of PHMG

provided efficient antibacterial activity against both gram-positive and gram-negative bacteria, and chitosan integration promoted biocompatibility and brought significant characteristics to the cotton-based materials regarding wound healing [12].

Ojogbo and colleagues (2020) developed poly(lactic acid) films embedded with the microparticles from PHMG-modified starch as contact-active surfaces for medical devices and food packaging applications. The study contained the chemical grafting of PHMG on the surface of starch by using isophorone isocynate as a coupling agent at the first step, and it was followed by the introduction of prepared antimicrobial microparticles into the poly(lactic acid) polymers via a solvent casting method. Consequently, it was pointed out that the final materials exhibited activity against Gram-positive and Gram-negative bacteria upon contact of the bacteria to the surface of the materials without the release of the antimicrobial agent since the agent was covalently immobilized on the starch surface found in the films [13].

In a recent study by Zhang and colleagues (2022), a dressing system with antibacterial and hemostatic features was prepared from sodium alginate (SA) nonwoven and PHMG for wound healing applications. The materials were obtained by dipping the SA fibers into the aqueous PHMG solution, and antibacterial PHMG coating on the SA fiber's surface was suggested to be created via the adsorption of the positively charged polymer on the negatively charged surface of SA by the electrostatic interactions. According to the results, SA-PHMG dressings displayed decent antibacterial action against various bacteria by the contact of the materials and the bacteria without releasing PHMG after repetitive use or washing [14].

Sun and colleagues (2020) developed material systems with antibacterial activity from cellulose fibers and PHMG via a chemical method that aimed to be used for paper packaging and medical paper products' potential applications. Glycidyl propargyl ether was grafted on the chemically modified cellulose fibers by a reaction to endow epoxy groups on the fiber surface. Then, it was followed by the covalent grafting of PHMG on the cellulose fiber surface through the epoxy ring-opening click reaction between the polymer's amino groups and the epoxy groups of functionalized fibers. According to the results, the systems provided contact-active antibacterial property, and preserved the activity at a ratio of ~ 99 % against two months of air-exposed storage by promising long-term effectiveness [19].

Xiao and colleagues (2022) developed a versatile wound dressing system from cellulose diacetate (CDA) and PHMG. The dressings were prepared in three steps, carboxylation of CDA, obtaining a nanofiber form by electrospinning of carboxylated CDA, and the covalent grafting of antimicrobial PHMG onto the CDA dressing surface by amide reaction in the last step. It was concluded that PHMG-grafted CDA-based dressing, presenting attractive characteristics to support the healing process of wounds such as excellent antibacterial activity, rapid hemostasis, and biocompatibility, is promising for clinical applications [80].

In a recent study by Dong and colleagues (2022), an adsorbent material was developed from guanidine-functionalized microcrystalline cellulose microspheres using a radiation technique to remove hazardous dyes from wastewater. The adsorbent was prepared in two steps; the radiation grafting of glycidyl methacrylate (GMA) on cellulose to obtain epoxy groups on its surface, and the addition of guanidine hydrochloride to the modified cellulose by a ring-opening reaction between epoxy groups of GMA and amino groups of the monomer. The final guanidine-functionalized cellulose material was reported to be presented excellent adsorption performance for the tested dyes and reusability [84].

Besides the functionalization of cellulose-based materials or polysaccharides in the first step, the literature demonstrates works when the modifications of PHMG polymer were done initially to facilitate further coupling of the polymer with a substrate surface.

One of such studies conducted by Guan and colleagues (2007) displayed the in situ free-radical polymerization method regarding the grafting of cellulose fibers' surface with PHMG via covalent interactions to obtain materials with antimicrobial properties. Firstly, PHMG was chemically modified with GMA to impart the unsaturated double bonds to the polymer's structure. Afterward, the modified polymer was grafted on the fibers via the interactions of radicals created by a chemical initiator on the cellulose backbone and the polymer's carbon-carbon double bonds. It was concluded that the bacterial growth was inhibited over 99% when the fibers with 1.0% (wt) grafted PHMG was tested [48].

The study of Wei and colleagues (2017) demonstrated a method for the preparation of antimicrobial paper by dip-coating process. In that approach, a copolymer, prepared by the chemical reaction of PHMG's amino groups and poly(propylene glycol) diglycidyl ether (PPGDE)'s epoxy groups, was used for the functionalization of cellulose surface via physical adsorption and chemical bonding. The chemical interactions were suggested

to be created between the hydroxyl groups of cellulose and epoxy groups of the copolymer. The antimicrobial-coated paper presented non-leaching property, and inhibitory activity against *E. coli* [57].

In a similar approach studied by Li and colleagues (2018), a copolymer prepared by the chemical reaction of PHMG and PPGDE was applied for the antimicrobial functionalization of cotton fabric's surface via physical adsorption and chemical bonding. The materials were prepared by dipping the cotton fabrics into aqueous PHMG-PPGDE dispersions and drying them under high temperature. The chemical interactions were assumed to be created between PPGDE's epoxy groups and PHMG's amino groups during the copolymer synthesis, and they might have been formed between the hydroxyl groups of cotton cellulose and epoxy groups of copolymer along the final materials' preparation. According to the results, the functionalized cotton fabrics displayed broad-spectrum, excellent antimicrobial properties, and resistance against different laundering processes were suggested to be promising as materials with non-leaching characteristics for industrial applications [8].

2.7. Characterization and Evaluation of Antimicrobial Activity

2.7.1. Antimicrobial Agents

Antimicrobial agents are substances that can kill microorganisms or inhibit microbial growth. These are called antibacterial, antifungal, and antiviral, depending on the type of microorganism they display activity against, and are also expressed with other terms such as disinfectants, antiseptics, antibiotics, etc. [85].

The material systems with antimicrobial activity are developed from various material groups like polymers, ceramics, metals, and composites [1]. Among these, the polymer-based systems appear as promising materials for combating pathogenic microorganisms. The synthetic polymers that possess antimicrobial properties can be classified according to chemical structure as polymers with quaternary nitrogen atoms, polymers that mimic natural peptides, halogen polymers, polymers with phospho and sulfo derivatives, and guanidine-containing polymers (polyguanidines and polybiguanides) [2].

2.7.2. Testing of Antimicrobial Substances

Diffusion and dilution methods are widely used for the evaluation and screening the antibacterial activities of substances being developed for antibacterial applications.

2.7.2.1. Diffusion Methods

The Agar disc diffusion and Agar well diffusion are the commonly used diffusion tests. Both methods work according to the diffusion mechanism in which the antimicrobial agent is expected to diffuse into the agar medium and inhibit the microorganism's growth throughout the incubation [86].

In these methods, the standardized inoculum of the test microorganism is added to the agar plates as the first step. Then it is followed by the placement of filter paper discs with certain concentrations of antimicrobial agent on the agar medium for the Agar disc-diffusion test. On the other hand, the solutions at desired concentrations of the antimicrobial agent are poured into the wells (holes) that are punched in the agar medium for Agar well diffusion test. The agar plates are incubated at the proper conditions for the microorganisms used for tests. Consequently, the diffusion of the tested agent into the medium results in the formation of clear zones around the discs or wells called inhibition zone. The tests are concluded by measuring the diameters of the zone of inhibition [86]. Figure 2.13 displays the operation flow of agar diffusion method's variants.

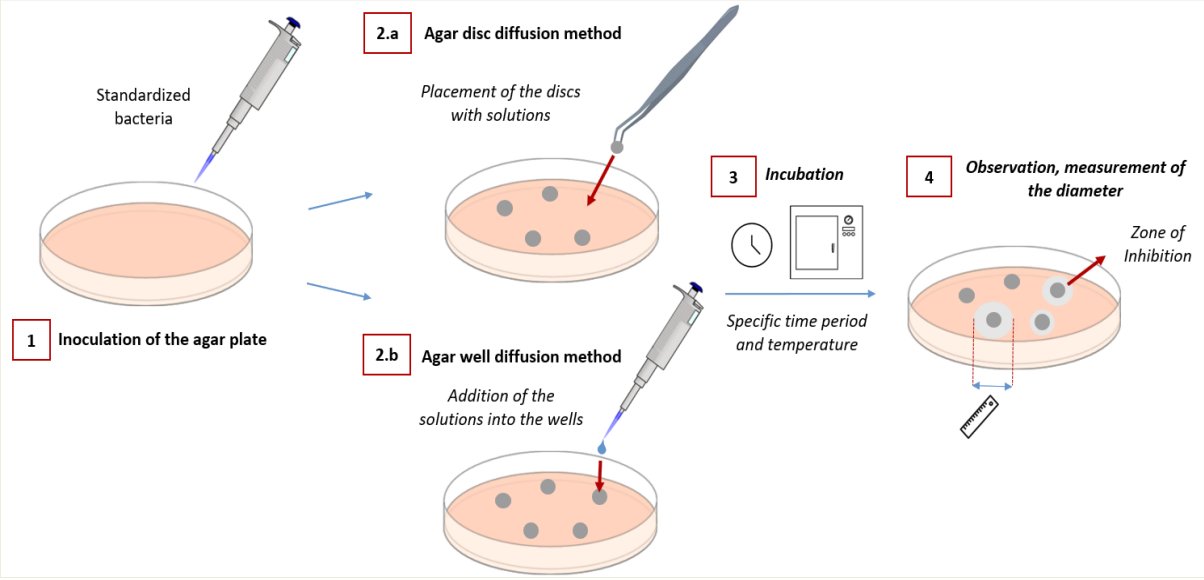


Figure 2.13. Illustration of the agar diffusion method's variants.

Diffusion methods are convenient to apply simply in laboratories by not requiring special equipment, the tests provide easily assessable results for various antimicrobial agents' activity on a wide range of microorganisms. These methods do not enable obtaining the exact amount of tested antimicrobial agent diffused into the agar medium [86]. However, the methods generally provide information regarding the presence or absence of

antimicrobial activity [87] and provide comparable results between the two variants of the agar diffusion method [88].

In the literature, Agar diffusion methods are generally applied to test and confirm the non-leaching properties of antibacterial agent-modified material systems, which means the agent is not released from the system over time [11, 17, 57, 63]. Therefore, the following studies related to investigating the antibacterial activity of a substance alone, such as oligomers, and disinfectants, via Agar diffusion methods were considered while evaluating the developed antibacterial agent, poly(hexamethylene guanidine) hydrochloride, within the studies of this work.

In a recent study, guanidinium oligomers' antibacterial and fungicidal activity were investigated. The oligomers' bactericidal activity was tested via the standard disc-diffusion method, while their fungicidal activity was studied by applying the well's method in agar. It was indicated that the compounds with 1–3 % concentrations inhibited the growth of gram-negative and gram-positive bacteria, and a 1 % concentration of the oligomers displayed fungicidal activity against fungal isolates. The diameters of the inhibition zones obtained from the tests were classified according to the sensitivity of the microorganisms against the tested compounds. As for the disc-diffusion test classification, the diameter of the inhibition zone was categorized into three ranges 0-10 mm, 11-25 mm, and above 25 mm, which means no sensitivity, sensitivity, and high sensitivity to the antibacterial agent, respectively [89].

Another recent study presented information regarding the bactericidal, fungicidal, and sporicidal activities of disinfectants derived from poly(hexamethylene guanidine) hydrochloride. The sporicidal properties of the disinfectants were investigated by applying the radial diffusion method in Hottinger's agar medium against *Bacillus cereus*. The method was quite similar to the other diffusion techniques since it contained dropping the disinfectant solutions with various concentrations of the active substance ranging from 2% to 0.05 % on the agar surface, and the lysed zone of microbial growth was determined after incubation. It was concluded that the growth of the *Bacillus cereus* spores was suppressed by the test solutions resulting in the formation of lysed zones ranging from 4.78 ± 0.46 to 1.69 ± 0.08 cm^2 depending on the applied concentration [90].

2.7.2.2. Dilution Methods

Dilution methods enable testing *in vitro* activity of novel developing agents against diverse microorganisms and provide quantitative results like minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). These outputs of the dilution tests are defined as follows.

MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of tested microorganisms under specific in vitro conditions and is generally given in mg/L (or μg/ml) [86, 91].

MBC is the lowest concentration of an antimicrobial agent required to kill 99.9% of the inoculum compared with the control sample after 24 h incubation under standardized conditions [86].

The dilution tests could be conducted on agar or in a broth medium. Both methods include examining the serially diluted solutions of an antimicrobial agent inoculated with microorganisms. The aim is to determine the agar or broth with the lowest agent concentration in which the antimicrobial activity of the tested substance prevents the tested microorganisms' visible growth upon incubation. Examination of the agar plates or broth containing tubes could be carried out by visual control (naked eye). The lowest concentration is determined by ignoring the single colonies on the plates for agar dilution and comparing the growth in the tubes with the one in the control sample (displays the ultimate bacterial growth) for broth dilution [91, 92].

The literature summary in which the systems containing poly(hexamethylene guanidine) hydrochloride polymer were studied with the dilution methods to define a MIC value is as follows.

In a study, the *in vitro* antimicrobial activity of poly(hexamethylene guanidine) hydrochloride and its three analogs were evaluated for the applications such as non-leaching sterile-surface materials and novel disinfectant improvement for hospital infection control. The study was carried out against various clinical strains, including antibiotics-resistant isolates. The Muller Hinton broth microdilution method was used to determine the MIC values of polymers by preparing two-fold serial dilutions between 0.5 and 2048 mg/L. Macroscopically defined MIC values of poly(hexamethylene guanidine) hydrochloride against antibiotics-susceptible and -resistant Gram-negative and Gram-positive clinical bacteria ranged from 1 to 64 mg/L. It was also indicated that the polymer

was less active against Gram-negative bacteria (4 – 64 mg/L) than Gram-positive bacteria (1 – 32 mg/L) [47].

A recent study covered the development of antibacterial dressing systems containing chitosan-polyvinyl alcohol/poly(hexamethylene guanidine) hydrochloride for wound healing applications. The antibacterial testing of the materials included MIC determination of poly(hexamethylene guanidine) hydrochloride solution against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)*. The polymer concentrations of the dilutions in the Luria-Bertani medium ranged between 0.05 and 4%, and it was concluded that the visible growth of both bacteria was inhibited at 0.1% polymer concentration [58].

2.7.3. Testing of Textiles' Antimicrobial Activity

The antimicrobial efficacy of the fabrics can be tested and evaluated via several standard methods. AATCC 147 (American Association of Textile Chemists and Colorists) and JIS L 1902 (Japanese Industrial Standards) are some common qualitative standard methods that can be applied to screen the antimicrobial activity of the fabrics against microorganisms [93, 94].

2.7.3.1. Agar Diffusion Test

The agar diffusion test is a qualitative and basic technique to evaluate the activity of textiles that are modified to endow antimicrobial properties. The method consists of four main steps; inoculation of agar plates, placement of the textile samples on the agar plates inoculated with bacterial cells, incubation of the plates, and examination of the bacterial growth around and underneath the fabric samples after incubation. The evaluation step reveals whether the tested sample displays antimicrobial activity and can provide information about the mechanism of action of the antimicrobial agent contained in the textile's structure [93]. The fabric can have leaching or non-leaching antimicrobial characteristics based on the treatment method and applied antimicrobial agent [95]. In terms of the diffusible antimicrobial action (i.e., leaching type characteristic), the antimicrobial agent diffuses into the agar, a zone of inhibition around the samples is expected to be obtained, and the zone size points out some further information like the release rate of the agent and its activity's potency. On the other hand, when the antimicrobial agent is incorporated into the textile by strong interactions such as covalent bonds, it cannot diffuse into the agar, yet it demonstrates antimicrobial action via the

contact mechanism with its non-leaching characteristic. In such case, the agent prevents bacterial growth by creating a clear zone under the fabric along the contact area between the fabric and agar [93]. Figure 2.14 demonstrates the preparation method of the agar plates for the diffusion test. Besides, it provides an idea regarding the final status of the agar plates, on which antimicrobial textiles with leaching or non-leaching properties are tested.

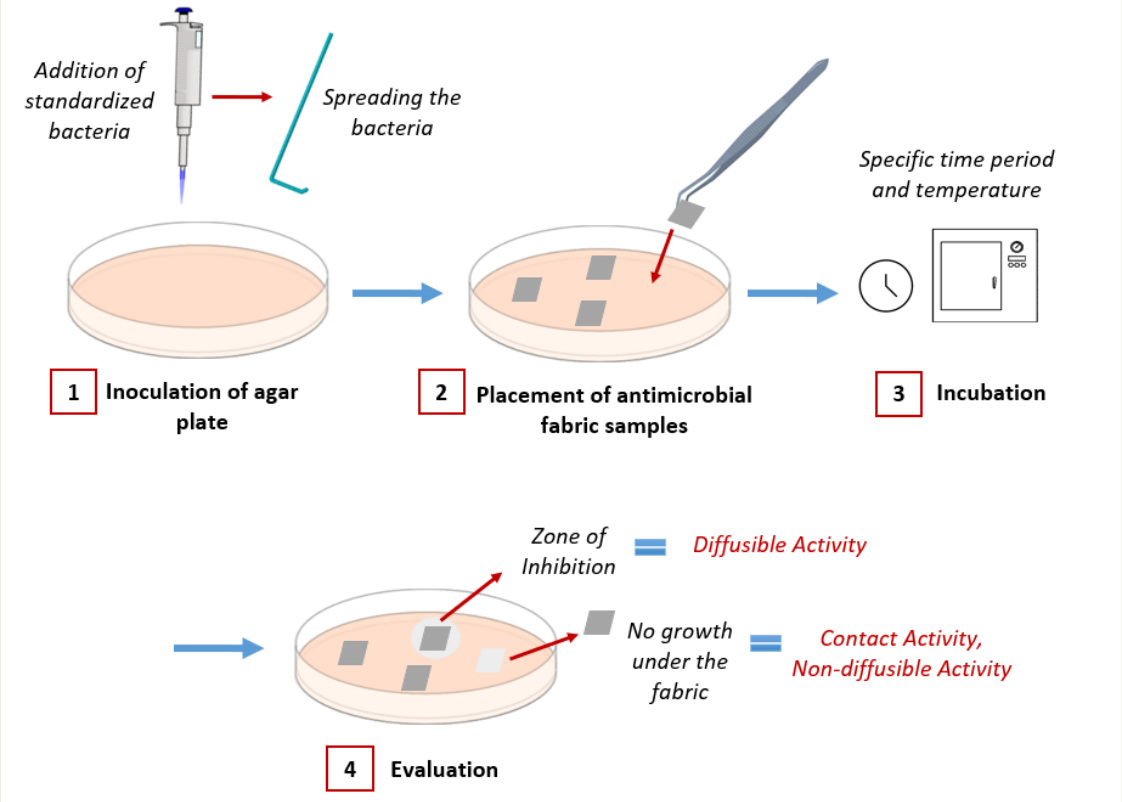


Figure 2.14. Illustration of the agar diffusion method for fabrics.

3. EXPERIMENTAL STUDIES

3.1. Materials

Hexamethylenediamine ($C_6H_{16}N_2$) and guanidine hydrochloride ($CH_5N_3 \cdot HCl$) monomers used in the PHMG polymer's synthesis were supplied from Sigma-Aldrich (France and Germany, respectively). Ethanol (C_2H_6O, ≥99.9%) used in the precipitation of the synthesized polymer was obtained from Isolab (Germany).

The nonwoven cotton fabric used as the trunk polymer in all grafting experiments was obtained from Marusan Industry Co., Ltd (Japan). Glycidyl methacrylate (GMA) ($C_7H_{10}O_3$) and Tween 20 (surfactant) ($C_{58}H_{114}O_{26}$) used in the graft polymerization trials were obtained from Sigma-Aldrich (Japan and France, respectively). The acrylic acid (AAc) ($C_3H_4O_2$) monomer used in the graft polymerization was acquired from Sigma-Aldrich (Czech Republic).

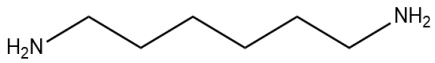
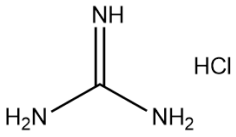
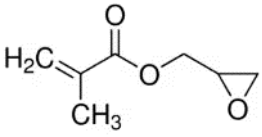
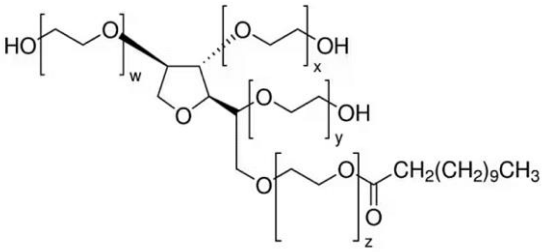
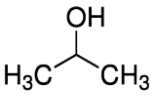
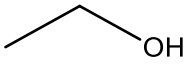
For the coupling reaction of AAc-grafted cotton fabrics with the synthesized PHMG polymer, 2-(N-morpholino)ethanesulfonic acid (MES) hydrate ($C_6H_{13}NO_4S \times H_2O$) used as a buffer in the reaction environment, and sodium carbonate (Na_2CO_3) used to adjust the pH of buffer solution were obtained from Sigma-Aldrich (Hungary and Germany), respectively. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) ($C_{10}H_{17}ClN_4O_3$), used as a coupling reagent for the reaction was acquired from Sigma-Aldrich (Japan).

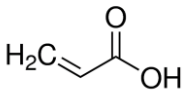
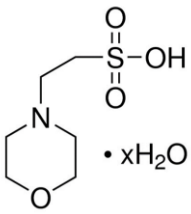
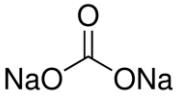
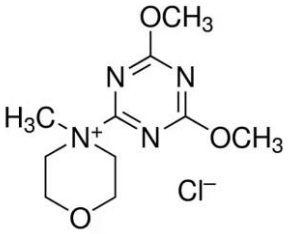
For the antibacterial tests of the synthesized PHMG polymer, Gram-negative bacteria *Escherichia coli (E. coli)* ATCC 8739 and Gram-positive bacteria *Staphylococcus aureus (S. aureus)* ATCC 6538 were used. Mueller Hinton agar, phosphate-buffered saline (PBS), and antibiotics solution (penicillin-streptomycin) (contained 10,000 units/mL and 10,000 µg/mL, respectively) were supplied from BD BBL™ (USA), Gibco™ (UK), and Gibco™ (USA), respectively. Besides, the Mueller Hinton broth was obtained from BD BBL™ (France).

In the antibacterial test of the PHMG polymer-modified cotton fabrics, Nutrient agar used to prepare the agar plates was acquired from BTL Ltd. (Poland).

All chemicals are shown in Table 3.1 and were used in the experiments as received without further purification.

Table 3.1. The chemical compounds used in the experiments and their molecular structures.

Name of Chemical Compound	**Structure**
Hexamethylenediamine	
Guanidine hydrochloride	
Glycidyl methacrylate	
Tween 20	
Isopropyl alcohol	
Ethanol	

Acrylic acid	
MES hydrate	
Sodium carbonate	
DMTMM	

3.2. Synthesis of PHMG Polymer

Poly(hexamethylene guanidine) hydrochloride was synthesized by the polycondensation reaction of guanidine hydrochloride and hexamethylenediamine monomers. The method used in the synthesis was a combination of the methods indicated in several different references [7, 11, 50].

In this process, equimolar amounts of guanidine hydrochloride (Mw: 95.53 g/mol, 0.1 mol) and hexamethylenediamine (Mw: 116.20 g/mol, 0.1 mol) were added in a round-bottomed two-necked flask and mixed at 100 °C for one hour under nitrogen atmosphere with a mechanical stirrer at 300 rpm speed. The reaction mixture was then heated to 140 °C, stirred for one hour at this temperature, and afterward for three hours at 170 °C. The transparent and highly viscous reaction product obtained after the reaction took a glassy form when cooled to room temperature. A total of 12 mL of distilled water and ethanol mixture (6 mL of distilled water and 6 mL of ethanol) was added to the reaction vessel to

obtain the pure polymer from the reaction product and to separate the unreacted monomers within the product. The solution was kept for a day for the polymer's precipitation, and the polymer was obtained as white solid. The solution over the precipitated polymer was removed, and the polymer was dried at room temperature.

The yield of the polymerization reaction (PY) was calculated as described in Equation 3.1 by dividing the mass of the polymer obtained by the initial total mass of the monomers added to the reaction vessel.

$$\text{PY (\%)} = \left(\frac{Polymer\ weight}{\text{Initial total monomer weight}}\right) \times 100 \quad \textbf{(3.1)}$$

3.3. Grafting Trials of PHMG onto the Cotton Fabrics by Radiation Technique

In this part of the study, the grafting trials of the synthesized PHMG polymer on the cotton fabrics were performed without any additional chemicals. Two different grafting methods, direct and peroxide grafting, were applied. The electron beam (EB) irradiation was employed for the initiation of grafting. Irradiation was performed using a Linear Accelerator (Linac, ELU-6) located at the Institute of Applied Radiation Chemistry of Lodz University of Technology, Poland.

3.3.1. Direct (Simultaneous) Grafting Method

Cotton fabric samples were prepared as 1.0×1.0 cm^2 square pieces. Every piece of cotton fabric was put individually into a 5 mL ampoule, and 3 mL of aqueous solution of the synthesized PHMG polymer (1 % w/v) was added to the ampoules. Air in the ampoules was replaced with inert argon gas via purging for 20 minutes. Then the ampoules were tightly closed and irradiated with electron beam at specific doses. Besides the fabric samples irradiated in polymer solutions, the cotton fabrics dipped in and taken out of the polymer solution were also irradiated in a solid state at different doses. Figure 3.1 displays the details about the samples and applied doses of irradiation. After irradiation, the fabric samples were washed with deionized water for three cycles of 1 minute for each cycle and dried in an oven at 37 °C. After oven drying, the samples were transferred to the vacuum oven at 40 °C for further drying. Two control samples were used in the experiment. The first was the original cotton fabric, and the second was a cotton fabric dipped into the polymer solution and dried without washing.

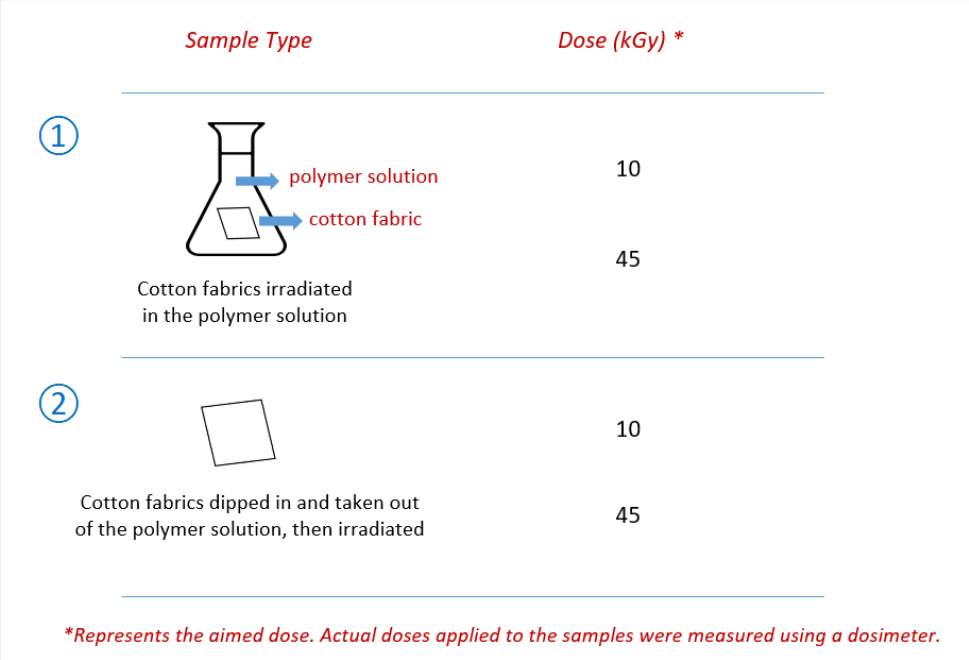


Figure 3.1. Details of the irradiated samples.

3.3.2. Peroxide Grafting Method

Cotton fabric samples were prepared cut in the same dimensions as for the previous experiment, transferred to open-ended glass ampoules, and exposed to electron beam irradiation at 40 kGy under air atmosphere. Then the air in the ampoules containing irradiated cotton fabrics was substituted with inert gas, and 3 mL of the deoxygenated aqueous solution of the synthesized PHMG polymer (2 % w/v) was introduced to the ampoules. The ampoules containing the final samples were then placed in an oven at 50˚C and kept for two hours for grafting. Afterward, the fabrics were washed with deionized water for three cycles of 1 minute for each cycle to remove the unreacted polymer. The last step included the drying operation of the samples. They were dried in the oven at 37 °C and then moved to a vacuum oven (at 50 °C) for further drying. A cotton fabric sample as a control was irradiated at the same dose without applying further operations.

3.4. Grafting Monomers onto the Cotton Fabrics

The grafting trials, which were carried out to functionalize the cotton fabrics for further modifications with PHMG polymer, were performed using glycidyl methacrylate (GMA) and acrylic acid (AAc) monomers. The methods applied are as follows.

3.4.1. Grafting GMA onto the Cotton Fabrics by Radiation Technique

The grafting trials of GMA monomer on the cotton fabrics were carried out via the post-effect method without oxygen.

The electron beam (EB) irradiation was used for the grafting, and the Linear Accelerator employed in the previous grafting trials (direct and peroxide grafting trials) was used for this method.

To graft glycidyl methacrylate (GMA) on the cotton fabrics, a procedure similar to those described in the literature was followed with some modifications [73, 84].

Cotton fabric samples were prepared cut in the same dimensions as for the previous grafting experiments and transferred into glass ampoules. The air in the ampoules containing fabric samples was substituted with inert gas for 20 minutes, and the ampoules were tightly closed; then, they were exposed to electron beam irradiation.

5 mL of deoxygenated aqueous GMA emulsion with or without a surfactant (Tween 20), which is used for the stabilization of GMA micelle in water [73], was added to the previously irradiated cotton. The ampoules containing the final samples were then placed in an oven with a maintained temperature and kept for a certain time for GMA grafting. After grafting time, the cotton samples were washed with deionized water a few times to remove the unreacted content and dried in the oven at 37 °C.

In this section, the cotton fabrics were exposed to radiation doses of 10, 20, and 40 kGy. The concentrations of deoxygenated aqueous GMA/Tween 20 monomer/surfactant emulsion solutions used were 2% (v/v) GMA, 2% / 0.05% (v/v) GMA/Tween 20, and 5% / 0.125% (v/v) GMA/Tween 20. Grafting temperatures of 40 °C and 50 °C, and grafting times of 1 and 2 hours, were studied for the grafting reaction. Grafting yields obtained from grafting experiments conducted under different conditions were evaluated using gravimetric analysis via Equation 2.1.

3.4.2. Grafting AAc onto the Cotton Fabrics by Radiation Technique

The grafting trials of AAc monomer on the cotton fabrics were performed via oxidative pre-irradiation technique (i.e., peroxide grafting).

The irradiation application was performed in the radiation chamber at the Institute of Applied Radiation Chemistry of Lodz University of Technology, Poland, using an Ob-Servo-D panoramic gamma irradiator (Izotop, Hungary) containing ^{60}Co sources emitting gamma rays of an average quantum energy of 1.25 MeV. Samples were irradiated at a dose rate of 4.5 kGy/h, determined by the film (B3WINdose dosimeter, GEX Corporation) and Alanine pellet (ES 200-2106, Bruker Biospin) dosimeter, respectively.

A procedure in the literature studies was applied with minor modifications to graft the acrylic acid on the cotton fabrics via oxidative pre-irradiation technique [70, 77].

Cotton fabric samples were prepared as 10.0×1.0 cm^2 pieces, and their masses were measured. Afterward, the fabrics were exposed to gamma radiation in open perforated bags under an air atmosphere at a 30 kGy dose. The irradiated cotton fabrics were then transferred to glass vials containing 10 mL of deoxygenated aqueous acrylic acid solutions with 20 %, 30 %, and 40 % (v/v) concentrations. After immersing the fabrics into the vials, oxygen was replaced with argon gas for ten minutes, and the caps of the vials were tightly closed. The vials were placed in an oven with a temperature of 65˚C and kept there for three hours for grafting reaction. After grafting, the fabrics were washed with deionized water for four cycles (two minutes per cycle) to remove the unreacted monomer and the homopolymer that may have formed. In the last step, the fabrics were dried in the oven at 37 °C overnight and then moved to a vacuum oven (at 40 °C) for further drying for one day. The fabrics were weighed after they were dried completely. The grafting yield was obtained via gravimetric analysis according to Equation 2.1 and is presented in the results part by taking the average of the three data points.

3.5. Chemical Coupling of Grafted Cotton Fabrics with PHMG Polymer

In this part of the study, the AAc-grafted cotton fabrics obtained at the optimum grafting conditions were used for the chemical coupling reaction to modify the fabrics with PHMG polymer. A procedure found in the literature used for chemical coupling of the structures that possess amino and carboxyl functional groups was employed with small modifications [96].

The buffer solution, a medium maintaining constant pH conditions during the coupling reaction, was prepared by dissolving 1950 mg of MES hydrate in 20 mL of distilled water. The sodium carbonate solution was prepared by dissolving 1060 mg of the material in 4 mL of distilled water and used for adjusting the pH of buffer solution to 5.5. To determine the amount of required coupling reagent (DMTMM) for the carboxyl groups' activation, the amount of the grafted AAc (mmol) on the cotton fabrics was calculated. The excess amount of DMTMM, 1.5 times with respect to the AAc amount (carboxyl groups), was used by adding to the pH-adjusted buffer solution. The final solution with buffer and coupling reagent was poured on AAc-grafted cotton fabrics to activate the carboxyl groups. The reaction vessel was placed at a laboratory rocker and gently agitated there for about two hours for activation.

Based on the assumption that each carboxyl group on the AAc-grafted cotton fabric would be coupled with an amino group of the polymer molecule, the number of amino groups that correspond to the number of carboxyl groups on the AAc-grafted cotton fabrics was calculated. The excess amount of the polymer, 1.5 times with respect to the AAc amount (carboxyl groups), was determined and used considering that every polymer molecule contains one or two amino groups at the chain ends depending on the molecular structures of type A, B and C [50]. The aqueous polymer solution was prepared by dissolving the calculated amount of the synthesized PHMG polymer in distilled water to obtain a 10 % (w/v) concentration. After the activation, the solution was poured into the reaction vessel that contains the fabrics for the coupling reaction. The conjugation reaction was carried out overnight (for about 18 hours) at room temperature under gentle agitation. Afterward, the reaction solution was discarded, and the polymer-modified cotton fabrics were washed three times (five minutes per cycle) with distilled water to remove the unreacted polymer and residues of the coupling reagent. After washing, the fabrics were dried in an oven at 37 °C overnight and then moved to a vacuum oven (at 40 °C) for further drying for one day. The fabrics were weighed after they were dried completely. The polymer coupling yield (CY) obtained via the coupling was calculated by Equation 3.2.

$$\text{CY (\%)} = \left(\frac{\text{Wm} - \text{Wg}}{\text{Wg}}\right) \times 100 \qquad \textbf{(3.2)}$$

In the equation, W_g represents the weight of the AAc-grafted cotton fabric, while W_m is the weight of the polymer-modified cotton fabric obtained in this step.

3.6. Characterization of Synthesized PHMG Polymer and PHMG-modified Cotton Fabrics

3.6.1. Fourier Transform Infrared Spectroscopy (FTIR)

Investigation of the chemical structure of the synthesized PHMG polymer was carried out via FTIR using the Thermo Scientific Nicolet iS10 model FTIR spectrometer. The FTIR spectra of the polymer sample were obtained by scanning in the wavelength range of 4000-400 cm^{-1} with a resolution 4 cm^{-1}. The number of scans was 64.

FTIR was also used to investigate and confirm the chemical structures of the materials prepared in the monomer grafting trials onto the cotton fabrics and in the chemical coupling reaction steps. The measurements were carried out in mid-infrared (MIR) 4000–500 cm^{-1} region using an FTIR spectrometer Nicolet iS50 (Thermo Scientific) equipped with attenuated total reflection accessory (ATR) and with DTGs detector. Acquisition parameters were chosen as follows: resolution: 4 cm^{-1}, number of averaged scans: 128.

3.6.2. Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectroscopy (MS)

The molecular weight of the synthesized PHMG polymer was determined by MALDI-MS analysis. Mass spectra were acquired using the Rapiflex MALDI mass spectrometer (Bruker, Germany) equipped with a smartbeam™ 3D laser in positive ion mode. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix, the matrix: polymer ratio was prepared as 10:1, and the analysis was performed with the On Spot technique. The concentration of polymer solutions used in the analysis was 1 mg polymer/mL water. Some other characteristic properties of the polymer; number-average molecular mass (Mn), mass-average molecular mass (Mw), and polydispersity (PD) or heterogeneity index (HI) were determined via MS data using FlexAnalysis 4.0 software. The types of chemical structures (linear, branched, or cyclic form) in the synthesized polymer were also revealed according to the spectra.

3.6.3. Elemental Analysis

The elemental content of the synthesized PHMG polymer, pure cotton fabric, AAc-grafted cotton fabric, and PHMG polymer-modified cotton fabric was determined by a CHNS Vario MICRO CUBE (Elementar Analysensysteme GmbH, Germany) elemental analyzer. The analysis was repeated twice, and the given values represent the average weight percentages (%) of C, H, and N elements.

3.6.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

The proton NMR (^{1}H-NMR) and carbon NMR (^{13}C-NMR) spectrometry techniques were applied to investigate the chemical structure of the synthesized PHMG polymer. The NMR spectra were recorded with a Bruker AM 400 (Bruker Corporation, Billerica, MA, USA) spectrometer, in Deuterated DMSO (dimethyl sulfoxide-d6) solvent, at 400 MHz.

3.6.5. Scanning Electron Microscope (SEM) Analysis

Scanning electron microscopy was used to examine the surfaces of fibers in the cotton fabrics. The SEM images of pure, AAc-grafted and PHMG polymer-modified cotton fabrics were obtained by a Hitachi Tabletop Microscope (TM-1000) under vacuum. The fabric samples were prepared by coating their surfaces with gold to impart electrical conductivity. The pictures of samples were obtained at magnifications of 500 x and 1000 x.

3.6.6. Antibacterial Testing

The antibacterial activity of the synthesized PHMG polymer was investigated via Agar-well diffusion and Broth dilution methods. The activity of PHMG-modified cotton fabrics was investigated via agar diffusion test. All antibacterial tests were performed with two bacteria, *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)*. The details of these test methods are as follows.

3.6.6.1. Agar-well Diffusion Test of PHMG Polymer

A similar procedure to the studies in the literature was followed with some modifications [86, 87, 88].

Inoculum Preparation

The inoculum densities of bacterial suspensions of *E. coli* and *S. aureus* grown in nutrient broth in the incubator at 33°C, which is the suitable temperature for the growth of the bacteria in the test laboratory, were identified via the turbidity measurement. In the first step, the turbidity of McFarland barium sulfate standard 0.5 (corresponds to 1.5×10^{8} CFU/mL bacterial density) was measured with the Densitometer. Then, the turbidities of the suspensions of test bacteria were measured. The inoculum densities were determined using the turbidity and the bacterial density values of McFarland barium sulfate standard 0.5, and measured turbidity values of test bacteria via a direct proportion calculation. In the next step, the suspensions were diluted with PBS to reach a final bacteria

concentration of 2.5 - 5 × 10^5 CFU/mL, the appropriate density of the bacteria determined based on the previous tests conducted in the test laboratory. Before adding to the Petri dishes, the actual densities of the bacteria after dilution were 4.12 × 10^5 CFU/mL and 5 × 10^5 CFU/mL for *E. coli* and *S. aureus*, respectively.

Agar Plate Preparation

1 mL of bacterial suspension (2.5 - 5 × 10^5 CFU/mL) was added to the polystyrene petri dish (⌀ 90 mm), and 15 mL of melted Mueller Hinton Agar was spread on the bacteria by moving the dish circularly. Then, the plates were kept without moving for about 1 minute for the agar to be cooled down and solidified. After the agar plates with bacteria were obtained, 5 wells (holes) with a diameter of 5 mm were punched in the medium with a sterile cork borer. 25 µL of the aqueous PHMG solutions with 2%, 1%, and 0.5% (w/v) concentrations were introduced into the respective wells. 25 µL of PBS was added to a separate well, and the same volume of antibiotics solution (penicillin-streptomycin) (in concentration 100 units/mL and 100 µg/mL, respectively) was added to another well, as control samples. The placement of the polymer solutions and the controls in the agar plate is given in Figure 3.2. Three agar plates were prepared for each bacteria as repetition samples. Then, all agar plates were incubated at 33°C for 24 h.

Interpretation of the Agar Plates

The antibacterial activity of the polymer was evaluated by measuring the diameters of the inhibition zones. The measurements were done on three repetition plates for each bacteria, and the diameter of the zone of inhibition was given as average values in mm.

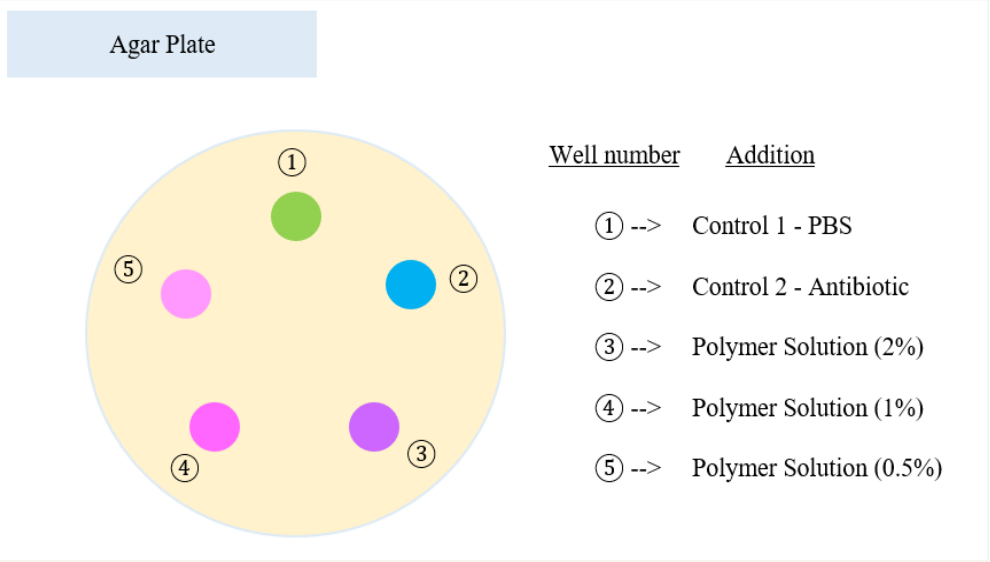


Figure 3.2. Placement of the tested specimens in wells on the agar plate.

3.6.6.2. Broth Dilution Test of PHMG Polymer

The broth dilution method was applied to perform a quantitative analysis regarding the polymer's antibacterial activity. A similar procedure to the studies in the literature was followed with some modifications [63, 91, 97]. The broth dilution method was completed at 8 steps and the procedure is as follows.

Step 1. Preparation of Polymer's Stock Solution

The stock solution of the PHMG polymer was prepared at 1024 mg/L concentration by dissolving the required amount of the synthesized polymer in distilled water.

Step 2. Preparation of Dilution series of the Polymer

The dilution series of the polymer was prepared in Mueller Hinton broth by carrying out two-fold dilution method which means that the solution is diluted to half concentration in the next tube after each dilution. 1 mL of the sterile broth (growth medium) was added to each tube in aseptic conditions. Then, 1 mL of testing agent (the polymer solution) was poured into the tube 1 from undiluted stock solution and the tube was mixed well. After mixing, the serial two-fold dilutions were prepared by adding 1 mL of the contents of each test tube to the next one and mixing well after each addition. 1 mL was poured out from the last tube (tube 10), so the volume of all tubes was adjusted to 1 mL. Consequently, the dilutions of the polymer with the concentrations in Figure 3.3 were obtained.

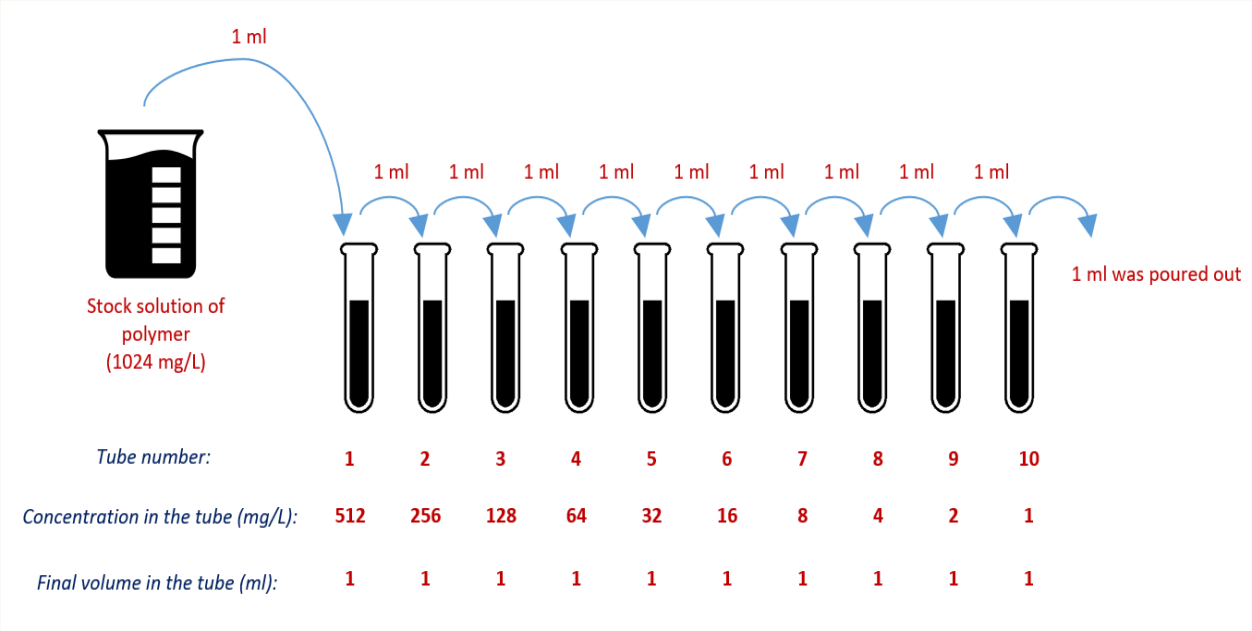


Figure 3.3. Preparation of the serial dilutions of the polymer.

Step 3. Preparation of Inoculum

The inoculum was obtained by diluting the broth cultures of *E. coli* and *S. aureus* that were preserved in the incubator at 33°C. Firstly, the inoculum densities of bacterial suspensions in nutrient broth were identified via the turbidity measurement according to the McFarland barium sulfate standard of 0.5 (corresponds 1.5×10^8 CFU/mL) for both species. Then, the suspensions were diluted in Mueller Hinton broth to adjust them to a final bacteria concentration of approximately 1×10^6 CFU/mL.

According to the reference method, the final bacterial density should be 5×10^5 CFU/mL after inoculation in the next step [91]. Therefore, since the density of the bacteria in each tube would decrease to half of the preceding inoculum concentration at the next step, the starting densities were adjusted to 1×10^6 CFU/mL.

Before adding to the test tubes, the approximate density of the adjusted inoculum was measured as 1.14×10^6 CFU/mL for *E. coli* and *S. aureus*.

Step 4. Inoculation of the Test Tubes

The inoculation step included the addition of bacterial suspension on the serially diluted polymer solutions prepared in the 2nd step. 1 mL of the adjusted inoculum was added to each test tube containing 1 mL of dilution series of the polymer. Two control samples,

C1 and C2, were prepared. C1 was the positive control containing 1 mL bacterial inoculum and 1 mL Mueller Hinton broth to check the viability of the bacteria, while C2 was the negative control that had 1 mL bacterial inoculum and 1 mL antibiotics solution (penicillin-streptomycin) (in concentration 100 units/mL and 100 µg/mL, respectively). This step results in a 1:2 dilution of each polymer concentration and a 1:2 dilution of the inoculum densities (final density: 5×10^5 CFU/mL). The final polymer concentrations in the test tubes are given in Figure 3.4.

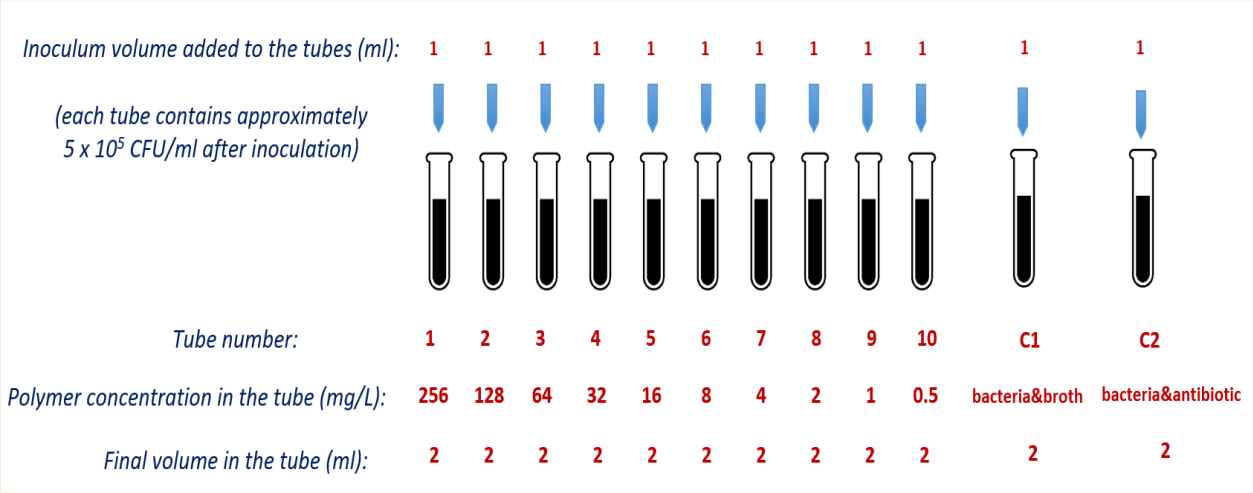


Figure 3.4. Inoculation of the test tubes and sample preparation.

Step 5. Incubation

Test tubes were closed with the caps and incubated at 33 °C for 20 h.

Step 6. Interpretation of the Test Tubes Results

The test tubes were checked visually regarding the turbidity or clarity of the tube content after incubation in comparison with the content of C1 control that ideally displays the growth of bacteria without inhibition by showing the maximum turbidity [91, 98].

The minimum inhibitory concentration (MIC) value was predetermined by recording the tube number with the lowest polymer concentration in which there is a clear liquid content and so no visible growth of bacteria. Because the visual control on the tubes might be misleading, agar plates were prepared to analyze the results deeper and obtain the MIC value precisely.

Step 7. Preparation and Incubation of Agar Plates

15 mL of melted Mueller Hinton agar was spread on the petri dish and kept for about 1 minute until it was completely solidified. 100 µl of the components from the test tubes was dropped in the middle of the agar plate and spread on the agar surface by using an L-shape spreader. Two replicate agar plates (A and B) were prepared from each test tube including the control samples, and the plates were incubated at 33 °C for about 20 h.

Step 8. Interpretation of the Agar Plates Results

The antibacterial activity of the different concentrations of the polymer was evaluated by comparing the colony formation on the agar surface after the incubation. The MIC value was determined considering the agar plate with the lowest polymer concentration that completely inhibits visible growth when detected by a naked eye, ignoring a single colony or a thin haze within the area of the inoculated spot [92].

In addition, the MIC values obtained from the agar plates were compared to the ones from the visual examination of the incubated test tubes in the previous steps.

3.6.6.3. Agar Diffusion Test of PHMG-modified Cotton Fabrics

A procedure given in the literature was followed with minor modifications for the antibacterial testing of the final materials, PHMG-modified cotton fabrics [57, 94].

Inoculum Preparation

The inoculum densities of bacterial suspensions of *E. coli* and *S. aureus* (in nutrient broth) preserved in the incubator at 33°C, which is the suitable temperature for the growth of the bacteria in the test laboratory, were identified via the turbidity measurement. In the first step, the turbidity of McFarland barium sulfate standard 0.5 (corresponds to 1.5×10^8 CFU/mL bacterial density) was measured with the Densitometer. Then, the turbidities of the suspensions of test bacteria were measured. The inoculum densities of the test bacteria were calculated via a direct proportion calculation using the turbidity and the bacterial density values of McFarland barium sulfate standard 0.5 and measured turbidity values of test bacteria's suspensions. In the next step, the suspensions of *E. coli* and *S. aureus* were diluted with PBS from the calculated density to reach a final bacteria concentration of 1×10^6 CFU/mL, the recommended density in the reference article [57].

Sample Preparation

Six cotton fabric samples with a square shape (1.0 × 1.0 cm^2) were prepared for the test as follows:

Sample 1: antibiotics control, the pure cotton fabric samples were dipped in the antibiotics solution (penicillin-streptomycin) (in concentration 100 units/mL and 100 µg/mL, respectively), kept there for five minutes with gentle shaking, taken out, and dried in an oven at 37°C for a day. *Sample 2:* pure cotton fabric control. *Sample 3:* releasing control (with expected diffusion of the PHMG polymer), the pure cotton fabrics were coated with the polymer in two steps. Firstly, the fabric samples were dipped in the aqueous solution of the synthesized PHMG polymer with a concentration of 1 % (w/v), kept there for five minutes with gentle shaking, taken out, and dried in an oven at 37°C for a day. In the second step, the coated cotton fabrics were dipped in the polymer solution again, kept there for one minute, taken out, and dried in an oven at 37°C for another day. *Sample 4:* AAc-grafted cotton fabrics (grafting conditions: AAc concentration: 20% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h). *Sample 5:* PHMG-modified cotton fabrics 1st (coupling yield: 17.5 %). *Sample 6:* PHMG-modified cotton fabrics 2nd (coupling yield: 23.8 %).

Agar Plate Preparation

15 mL of melted Nutrient agar was spread on the polystyrene Petri dish (⌀ 90 mm) and kept without moving for about one minute until it was completely solidified. 100 µl of bacterial suspension (1 × 10^6 CFU/mL), prepared in the previous step, was dropped in the middle of the agar plate, and spread on the agar surface using an L-shape spreader. After the agar plates with bacteria were obtained, square cotton fabric samples were placed on the agar surface. Since the fabric samples were dry, there was a folding possibility of the fabrics during the test preventing their contact with the bacteria-inoculated agar surface. Therefore, the fabric samples were wetted by dropping PBS on their surface to ensure full and constant contact between the samples and the agar surface. 30 µl of PBS was applied to the first three samples, while 60 µl was required to wet the rest of the samples. The placement of the samples on the agar plate is given in Figure 3.5. The agar plates were prepared in duplicate for both bacteria and incubated at 33°C for 24 h.

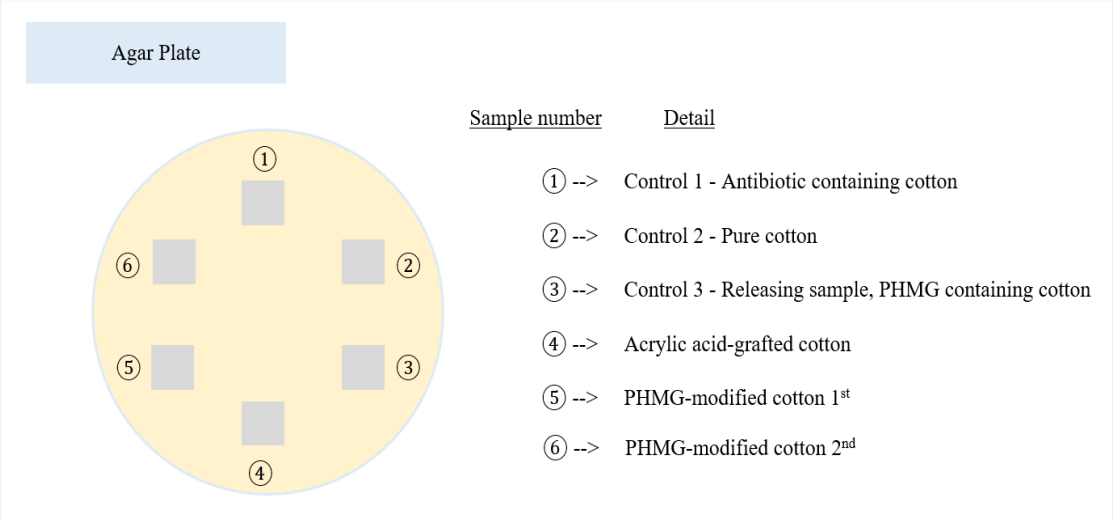


Figure 3.5. Placement of the fabric samples on the agar plate.

Interpretation of the Agar Plates Test Results

Antibacterial activity was evaluated qualitatively by observing bacterial growth around and underneath the fabric samples at the end of the incubation time. The formation of a clear area, the zone of inhibition, around the fabric sample meant that the antibacterial activity was displayed via the diffusion of the substance within the fabric into the agar. On the other hand, although there was no inhibition zone occurrence around, a clear zone underneath the sample implied that the antibacterial agent was strongly attached to the fabrics and demonstrated antibacterial activity without being diffused but inhibiting bacterial growth along the contact area via contact mechanism [93].

4. EXPERIMENTAL RESULTS AND DISCUSSION

In this study, cotton fabrics with antibacterial properties were prepared for medical, textile, and food packaging areas. An antimicrobial poly(hexamethylene guanidine) hydrochloride polymer was synthesized and employed to impart the antibacterial feature to the cotton fabrics.

4.1. Synthesis of PHMG Polymer

Poly(hexamethylene guanidine) hydrochloride was synthesized by the polycondensation reaction of guanidine hydrochloride and hexamethylenediamine monomers (Figure 2.3). The synthesis was carried out under nitrogen atmosphere by mixing the reaction mixture consistently and increasing the temperature gradually. The yield of the synthesis was determined to be approximately 14% by using Equation 3.1. The low yield of the synthesis reaction (condensation polymerization) may be attributed to impurities in the reaction environment, which results from using the monomers without prior purification.

The obtained PHMG polymer was characterized by FTIR, NMR, elemental analysis, and MALDI-MS techniques, and its antibacterial properties were investigated. The results of the characterization studies are given in the following parts. The synthesized PHMG polymer was used in the further parts of this study to modify the cotton fabrics.

4.2. Grafting Trials of PHMG onto the Cotton Fabrics by Radiation Technique

In the initial approaches, the PHMG polymer's grafting onto the cotton fabrics via irradiation was investigated using two different methods, direct and peroxide grafting. The literature search conducted did not provide any study where PHMG was grafted onto cotton fabric using the performed methods in this part of the research.

4.2.1. Direct (Simultaneous) Grafting Method

Experimental results demonstrated that the attempts to graft the PHMG polymer on cotton fabrics by direct radiation could not be achieved. The proposed grafting method presented unfavorable results upon exposing the cotton fabrics to irradiation in the PHMG polymer solution or at solid-state (fabrics covered with the PHMG solution by dipping). If any grafting took place, the grafting degree was below the detection limit of the FTIR technique and gravimetric analysis. No visual changes were observed on the fabrics after the grafting attempts.

4.2.2. Peroxide Grafting Method

Similar to the results of the direct grafting trial, the grafting of the PHMG polymer on the fabrics with the peroxide technique could not be confirmed. Besides, no visual changes were observed on the cotton fabrics after the peroxide grafting method was carried out.

4.3. Grafting Monomers onto the Cotton Fabrics

Monomer grafting or graft polymerization is applied to functionalize the material surfaces by introducing the functional groups to change the material's surface properties, for instance, to ensure the suitability of the materials for further modifications or specific applications. In the second approach, the grafting of GMA and AAc monomers on the cotton fabrics' surface was performed to achieve functionalized cotton fabrics for further reactions with PHMG antimicrobial polymer.

4.3.1. Grafting GMA onto the Cotton Fabrics by Radiation Technique

The grafting trials of GMA monomer on the cotton fabric surface were performed using the post-effect method without oxygen. Although grafting was detectable on specific fabric samples using FTIR, it was concluded that grafting yield may have been too low to be detected by gravimetric analysis. The attempted GMA grafting under the applied irradiation and reaction conditions did not present convenient results for this study.

Since this approach appeared impractical for the aim of this study, the experiments to functionalize cotton fabrics were continued using another monomer, AAc.

4.3.2. Grafting AAc onto the Cotton Fabrics by Radiation Technique

In this step, the acrylic acid monomer was grafted on the trunk polymer, cotton fabrics, to obtain the carboxyl functional groups (-COOH) on the materials' surface. The monomer grafting on the fabric surface was carried out using the oxidative pre-irradiation technique. The fabrics were exposed to gamma radiation under the air atmosphere to create free radicals, which subsequently reacted with oxygen from the air and form peroxide (ROOR) and hydroperoxide (ROOH) functional groups, typically on the material surface. In the next step of the synthesis these groups decompose and break down upon heating, providing oxyl-type free radicals that react with the acrylic acid monomer to polymerize AAc from the surface [70].

The cotton fabrics were exposed to gamma radiation at a 30 kGy dose under the air atmosphere, and the grafting reaction was obtained at 65˚C for three hours using three

different aqueous AAc solutions with 20 %, 30 %, and 40 % (v/v) concentrations. The grafting was confirmed by gravimetric analysis, and the resulting grafting yields obtained by Equation 2.1 versus the used concentration of AAc monomer solution are given in Figure 4.1.

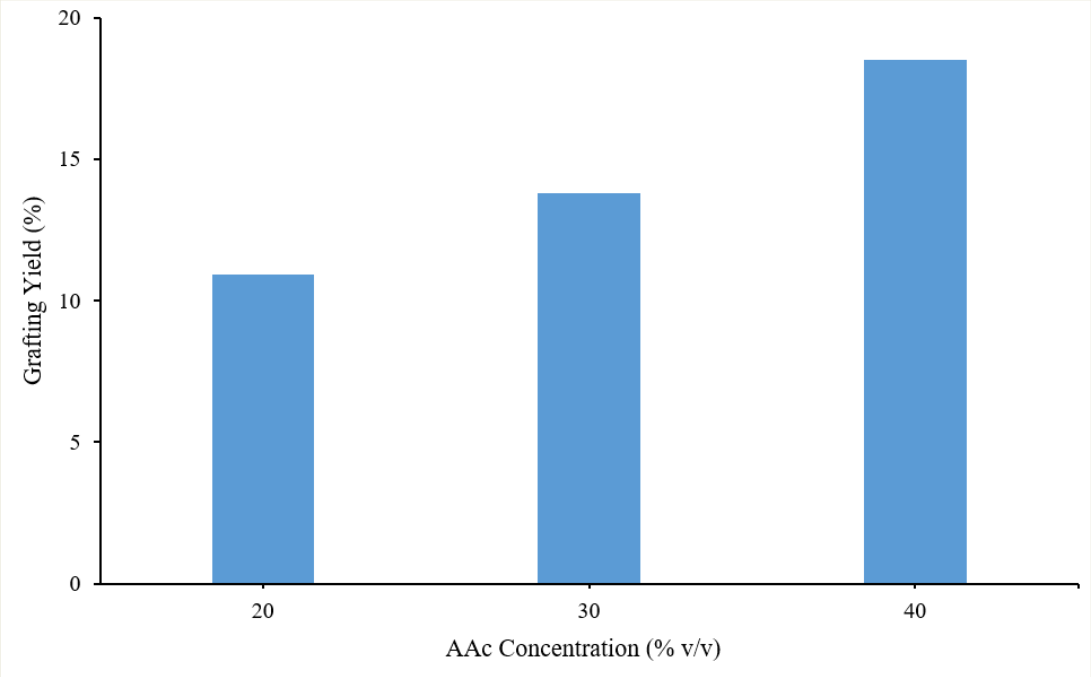


Figure 4.1. AAc monomer concentration versus obtained grafting yield (absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h).

4.4. Chemical Coupling of Grafted Cotton Fabrics with PHMG Polymer

Amide coupling reactions allow obtaining chemical amide bonds through the reactions between carboxylic acids and amines [79].

In this step, the synthesized PHMG polymer was chemically bounded to the AAc-grafted cotton fabrics. Firstly, the carboxyl functional groups on the AAc-grafted cotton fabrics were activated using a coupling reagent, DMTMM, at 5.5 pH. Afterward, the aqueous PHMG polymer solution with 10 % (w/v) concentration and the fabrics were brought together for a coupling reaction between the amino functional groups (-NH_2) of the polymer and the activated carboxyl groups of the acrylic acid on the fabrics. The reaction was performed for about 18 hours at room temperature under gentle agitation. The resulting interaction was expected to be obtained via amide bond formation.

In the grafting section, AAc-grafted cotton fabrics prepared using aqueous AAc solutions with different concentrations (20 %, 30 %, and 40 % (v/v)) were modified with PHMG in this section. The coupling reaction (i.e., modification) was confirmed by gravimetric analysis via the weight measurement of the fabrics before and after the reaction. The coupling yield (%) of the PHMG polymer based on the calculation carried out via Equation 3.2 is demonstrated for AAc-grafted cotton fabrics obtained at different grafting conditions in Figure 4.2.

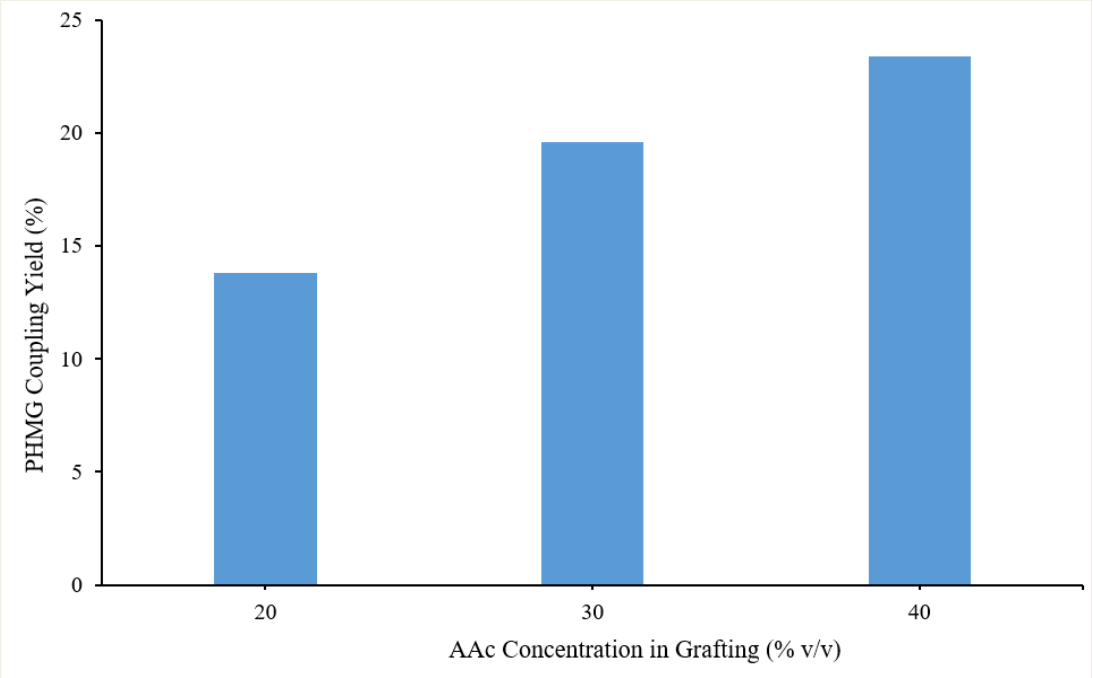


Figure 4.2. The yield of chemical coupling reaction of AAc-grafted cotton fabrics with PHMG polymer (modification concentration: 10 % (w/v) aqueous PHMG, pH: 5.5, time: 18 h, room temperature).

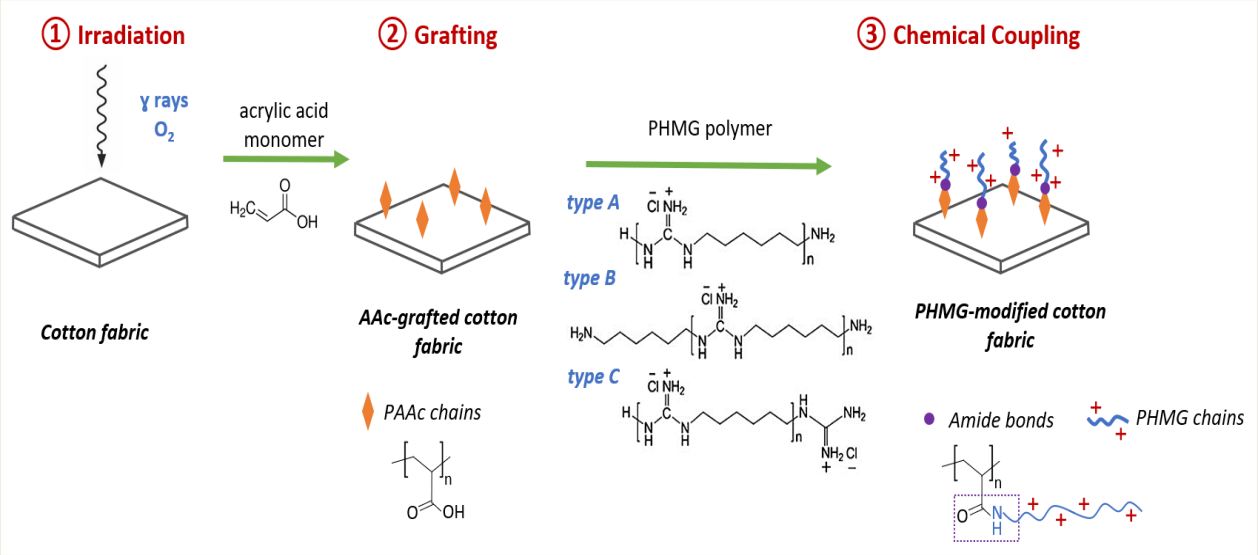


Figure 4.3. Scheme of the preparation of guanidine-based antibacterial cotton fabrics.

The method followed to prepare guanidine-based antibacterial cotton fabrics is described in Figure 4.3, which demonstrates each step applied during the studies.

Based on the series of optimization grafting and chemical coupling reaction experiments, the optimum conditions for preparing the guanidine-based antibacterial cotton fabrics were obtained. The optimum conditions for AAc grafting were determined as 30 kGy absorbed dose, the aqueous acrylic acid solution with 30% (v/v) concentration, 65 °C reaction temperature, and three hours reaction time. The average AAc grafting yield on the cotton fabrics prepared at the optimum conditions was obtained via gravimetric analysis using Equation 2.1 as 13.8%. The average coupling yield of the modification reaction between PHMG polymer and the AAc-grafted cotton fabrics prepared at the optimum grafting conditions was determined by gravimetric analysis via Equation 3.2 as 19.6%.

The cotton fabrics prepared by applying the optimum conditions were investigated for characterization studies in the next sections.

4.5. Characterization of Synthesized PHMG Polymer and PHMG-modified Cotton Fabrics

4.5.1. FTIR Results

The FTIR spectrum of the synthesized PHMG polymer is displayed in Figure 4.4. The obtained spectrum was compared with the FTIR spectra found in the literature to confirm the chemical structure of the polymer. The characteristic bands belonging to the characteristic chemical groups of the PHMG structure of the polymer were observed in the spectrum. The distinct band, between 1527 cm^{-1} and 1740 cm^{-1}, peaking at 1621 cm^{-1} mainly corresponds to the C=N vibration. The broad bands peaking at approximately 3149 cm^{-1} and 3255 cm^{-1} wavelengths correspond to the N-H stretching vibration. Two distinct peaks observed at 2930 cm^{-1} and 2856 cm^{-1} correspond to asymmetric and symmetric stretching vibrations of methylene ($-CH_2-$) groups, respectively. Also, the peak at 1460 cm^{-1} shows the bending vibration of CH_2. Consequently, the results complying with the previous studies described in the literature were reached, and the chemical structure of the PHMG polymer was confirmed [7, 9, 99].

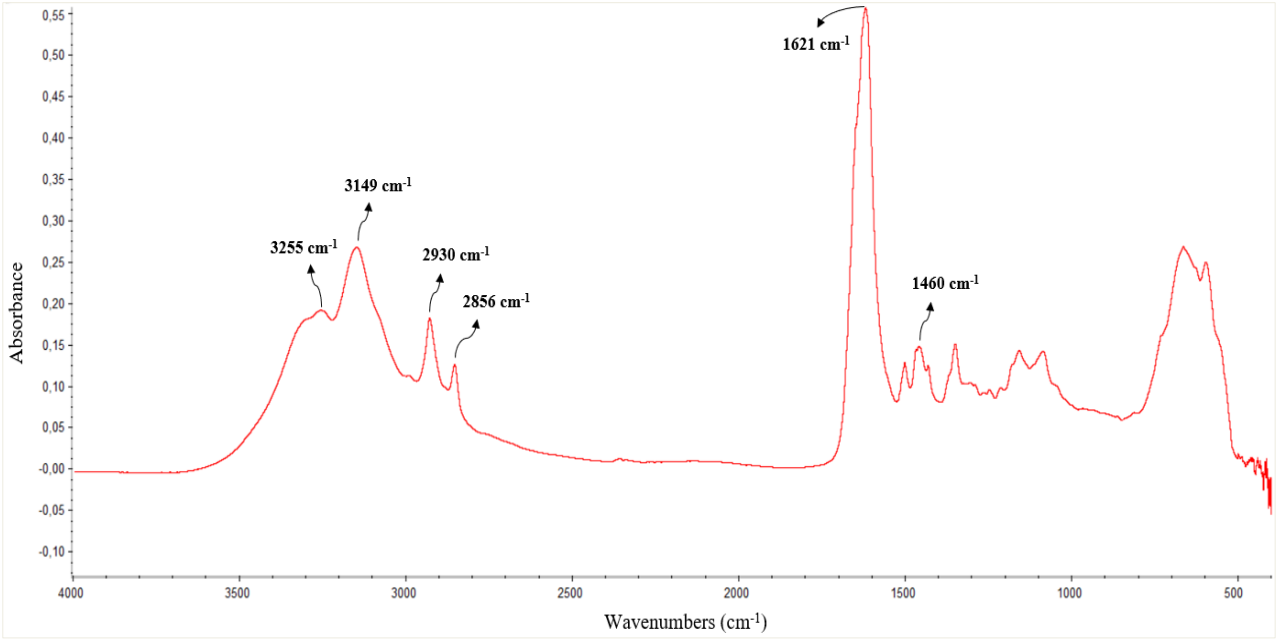


Figure 4.4. FTIR spectrum of synthesized PHMG polymer.

The acrylic acid grafting on the cotton fabrics was confirmed via FTIR analysis by comparing the spectra obtained from pure, irradiated (as control), and AAc-grafted cotton fabrics (Figure 4.5).

The FTIR spectrum of the pure cotton fabric was interpreted by examining the bacterial cellulose, sugarcane bagasse cellulose, and cotton fabric's spectra from the literature. In Figure 4.5.b, bands between 3600 and 3000 cm^{-1} correspond to O–H stretching vibration. It is stated in the literature that the vibrational modes of the band with a significant peak at 3333 cm^{-1} in this range is caused by the intramolecular bonds and hydrogen bonds contained in cellulose [9]. C-H stretching vibration is observed at 2898 cm^{-1}, and the peak arising from aromatic skeletal vibrations is observed at 1635 cm^{-1} [11]. 1160 cm^{-1}, 1029 cm^{-1}, and 897 cm^{-1} correspond to asymmetrical C–O–C, C–O stretching vibration and asymmetric out-of-phase ring stretching vibration (C1-O-C4; β glycosidic bond), respectively [100]. According to the spectrum of irradiated cotton fabric given in Figure 4.5.a, the irradiation exposure of the cotton fabric at 30 kGy did not result in any noticeable difference in its structure compared to the pure cotton fabric.

The FTIR spectrum of the AAc-grafted cotton fabric sample, which was prepared in 30 %, (v/v) concentration aqueous acrylic acid solution, at 30 kGy absorbed dose, 65 °C reaction temperature, and 3 h reaction time, is shown in Figure 4.5.c. Regarding AAc grafting, a distinctive peak that corresponds to the stretching vibration of the carbonyl group (C=O) from the carboxyl group of acrylic acid [70] was observed at 1708 cm^{-1} on the spectra of AAc-grafted cotton fabric. Consequently, the results complied with the literature studies, and the AAc grafting onto the cotton fabrics was verified by FTIR analysis [70, 77].

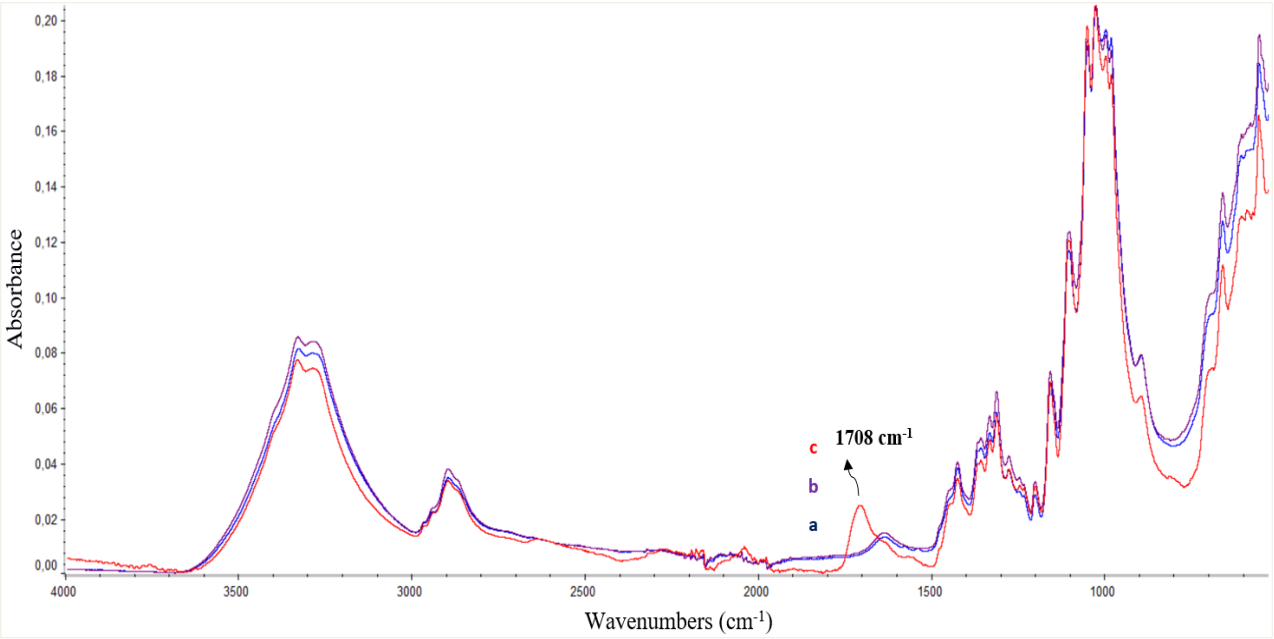


Figure 4.5. FTIR spectra of (a) irradiated cotton fabric (control), (b) pure cotton fabric (control), (c) AAc-grafted cotton fabric (grafting conditions: AAc concentration: 30% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h).

The PHMG polymer modification on the AAc-grafted cotton fabrics was confirmed via FTIR analysis by comparing the spectra of pure cotton fabric, AAc-grafted cotton fabric, and PHMG-modified cotton fabric shown in Figure 4.6. As a result of the chemical coupling of PHMG and AAc-grafted cotton fabrics, two distinct peaks appeared in the spectra of the final materials. The peaks observed at 1633 cm^{-1} and 1550 cm^{-1} represent the amide bond formation (–CONH–) and correspond to the –C=O stretching and –NH bending vibration bands, respectively. It can be concluded that the chemical coupling reaction between PHMG's amino end groups and AAc-grafted cotton fabrics' carboxyl groups was confirmed by observing the characteristic bands of the resulting covalent amide bonds on the FTIR spectra [101, 102].

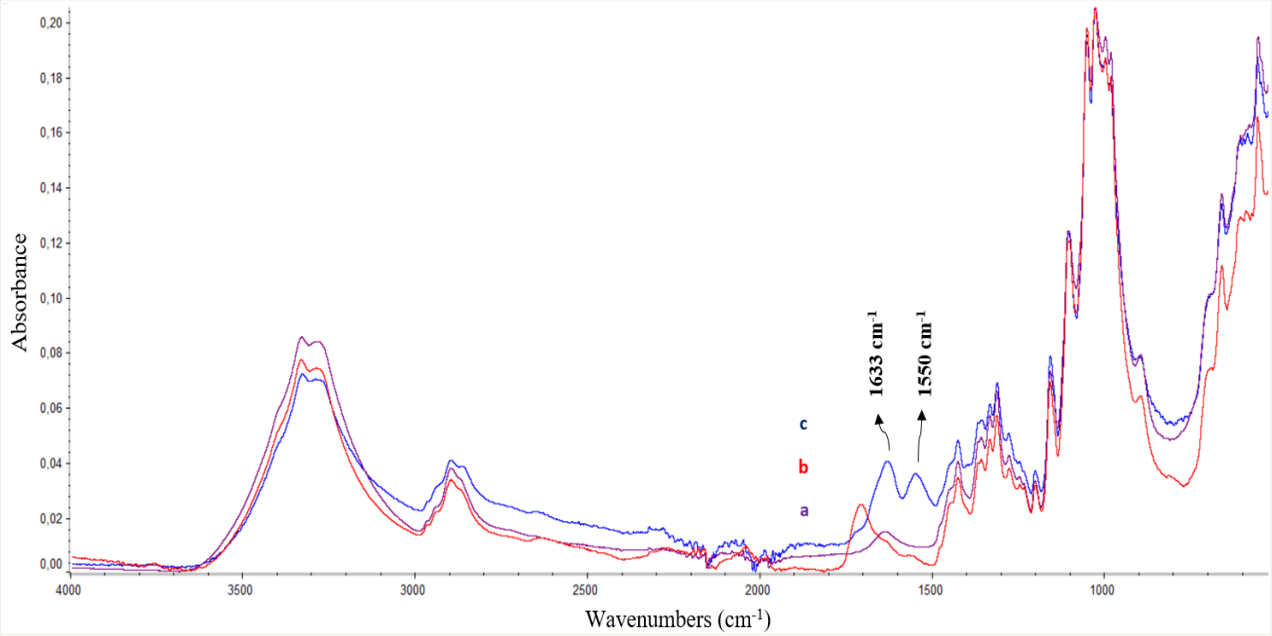


Figure 4.6. FTIR spectra of (a) pure cotton fabric (control), (b) AAc-grafted cotton fabric (grafting conditions: AAc concentration: 30% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h), (c) PHMG-modified cotton fabric (coupling yield: 20.5 %).

4.5.2. MALDI-MS Results

The MALDI mass spectrum of the synthesized PHMG polymer is displayed in Figure 4.7. PHMG can be found in forms with different molecular structures. A, B, C are linear, D is branched, F and G are cyclic types (Figure 2.4) [50]. The mass spectrum can be used to determine the chemical forms found in the polymer sample.

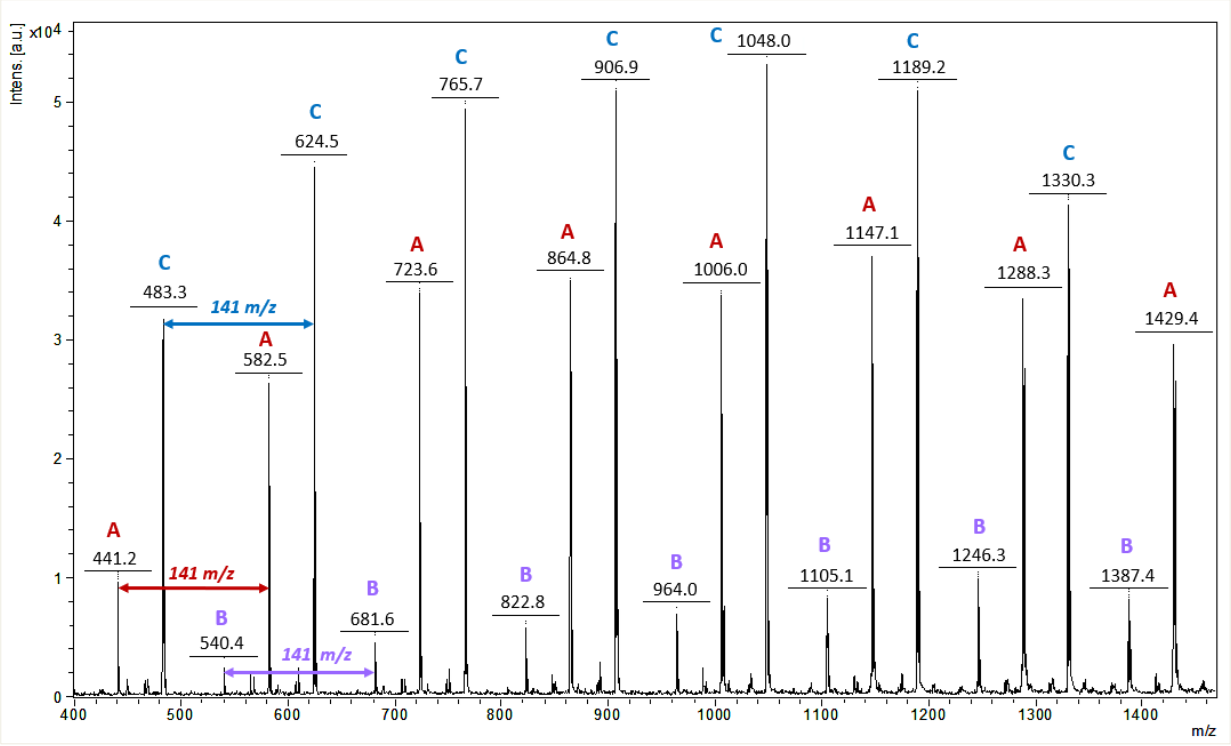


Figure 4.7. Mass spectrum of the synthesized PHMG polymer.

To identify the molecular structures, the peak series with an equal mass difference, as much as a repeating unit (141 m/z), were chosen and labeled on the mass spectrum (Figure 4.7). Then, these series of peaks were matched to the linear, branched, or cyclic chemical structures considering the end group masses and using the following calculation method.

The Calculation Method

$[M+H]^+$ Ion mass (signal in mass spectrum), protonated oligomer signal

$[M+H]^+ - H = M$ Ion mass - Hydrogen atom mass = Oligomer chain mass

$M / 141 = Z$ Oligomer chain mass / Repeating unit mass = Number of repeating units and the number to be used to determine the end group

After subtracting the integer part (number of repeating units) from the Z number, the remaining number is multiplied by the repeating unit mass until one of the end group masses of the chemical structures is reached. Besides using the calculation method, the specific signals belonging to the molecular structures can be found in the literature [50, 55]. According to the mass spectrum obtained by the analysis, it was determined that the polymer contains mainly its linear forms, A, B, and C. Molecular weights and other

characteristic properties such as polydispersity index (pd) and degree of polymerization (DP) obtained using Polytools software over the mass spectrum are given in Table 4.1.

Table 4.1. Characteristic properties of the synthesized PHMG polymer determined with MALDI-MS as referred to structures depicted in Figure 2.4.

Type of Polymer	End Group Mass (u)	M_n (g/mol)	M_w (g/mol)	pd (M_w/ M_n)	DP
A	17.91	1207	1365	1.13	8.55
B	117.02	1356	1505	1.11	9.60
C	59.93	1097	1268	1.16	7.77

4.3.3. Elemental Analysis

The weight percentages of C, H, and N elements in the synthesized PHMG polymer were obtained by the analysis to investigate the chemical structure. The experimental values were compared to the theoretical ones calculated based on the monomers feed in the synthesis. The calculation method used for acquiring the theoretical values is as follows.

Calculation of theoretical weight percentages based on the monomer unit

1. Molecular weight calculation via the atomic weights

The molecular weight was calculated by multiplying the atomic weight of each element by the number of atoms within a molecule and summing the contributions.

The molecular weight considering the molecular formula ($C_7H_{16}N_3Cl$) of the monomer unit (Figure 4.8) of PHMG polymer was calculated as 177.679 (Table 4.2).

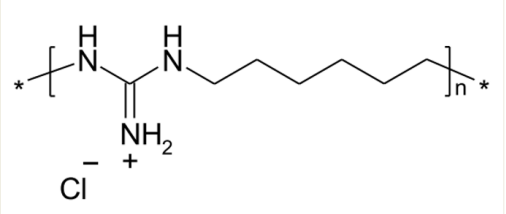


Figure 4.8. The monomer or repeating unit of PHMG.

Table 4.2. The atomic weights and weight contributions of the elements in the structure.

Element	Atomic weight (u)	Calculation of weight contribution (u)
C	12.011	7 × C = 84.077
H	1.008	16 × H = 16.128
N	14.007	3 × N = 42.021
Cl	35.453	1 × Cl = 35.453

2. Determination of the weight percentages

The weight percentage of the elements was calculated using the ratio of the weight contribution of each element in the structure to the molecular weight obtained in the previous step (Table 4.3).

Table 4.3. The weight percentages of the elements.

Element	Calculation of weight percentage	Weight percentage (%)
C	(84.077 / 177.679) × 100	47.32
H	(16.128 / 177.679) × 100	9.08
N	(42.021 / 177.679) × 100	23.65
Cl	(35.453 / 177.679) × 100	19.95

It is essential to consider the calculation method of theoretical values while comparing the experimental and theoretical percentages of the elements. The theoretical values are generally calculated via the monomer unit (repeating unit) in the literature [7, 9].

Table 4.4. The elemental content of PHMG polymer.

Element	% Weight Content	
	E	*T*
C	42.32	47.32
H	12.27	9.08
N	24.24	23.65
Cl	-	19.95

**E: experimental value, T: theoretical value*

According to the comparison done on the Table 4.4, N element's theoretical and experimental percentages provided consistent results, while the C percentage obtained from the analysis was lower than its theoretical value. The polymer sample may have contained some moisture that caused the difference between the experimental and theoretical values. The moisture could explain the higher value of H obtained from the analysis compared to its theoretical value and may mean the existence of oxygen. It should be considered that the sample is supposed to contain Cl, yet the analysis does not provide this kind of result. Therefore, the sum of the resulted elemental weight percentages could not be completed to 100 %. Besides, the sample requires further drying to obtain results that are not affected by moisture.

The elemental compositions of the prepared materials at every step of the studies were obtained with the analysis, and the experimentally acquired C, H, and N contents of the synthesized PHMG polymer, pure, AAc-grafted, and polymer-modified cotton fabrics are demonstrated in Table 4.5. The data represents weight percentages (%) of the elements in the structures. Since the results obtained in the PHMG polymer's elemental characterization part (Table 4.4) implied the presence of moisture in the polymer sample, the elemental analysis of the PHMG was repeated after drying the sample in a vacuum oven at 40 °C for two days. After the drying operation, it was observed that the H content decreased from 12.27 % to 8.69 %, which is quite closer to the theoretical value (9.08 %).

The results displayed the presence of the N element in the PHMG polymer-modified cotton fabric sample, which confirmed that the synthesized PHMG polymer was attached to the AAc-grafted cotton fabrics via the chemical coupling method.

Table 4.5. Results of elemental analysis.

Sample Number	Sample	Element		
		C (%)	H (%)	N (%)
1	PHMG polymer	43.47	8.69	24.77
2	Pure cotton fabric	42.84	5.86	< 0.3
3	AAc-grafted cotton fabric (grafting conditions: AAc concentration: 30% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h)	44.08	5.82	< 0.3
4	PHMG polymer-modified cotton fabric (coupling yield: 20.5 %)	45.72	6.47	5.12

4.3.4. NMR Spectroscopy Results

^{1}H-NMR and ^{13}C-NMR spectra of the synthesized PHMG polymer are shown in Figures 4.9 and 4.10, respectively. Depending on the analysis, the letters from (a) to (d) on the spectra represent hydrogen (H) or carbon (C) atoms in the structure.

Results of ^{1}H-NMR Analysis

^{1}H NMR spectrum displayed two distinctive peaks at 1.31 ppm (a) and 1.46 ppm (b) that are associated with the protons of the long-chain methylene groups (-CH_2-) [99, 103]. The peak at 3.13 ppm (c) corresponds to the protons of the methylene group between the carbon and nitrogen (C-CH_2-N) [7, 99, 103]. The broad peaks between 7.0 and 8.0 ppm (d) are assigned to the protons of -C-NH-C and -C=NH_2^+ components [99, 103]. The peak belonging to the DMSO solvent used for the analysis could be seen at 2.5 ppm. The peak that appeared at around 3.40 ppm may have resulted from the moisture (water) content within the sample since the value corresponds to residual moisture [13, 51, 103].

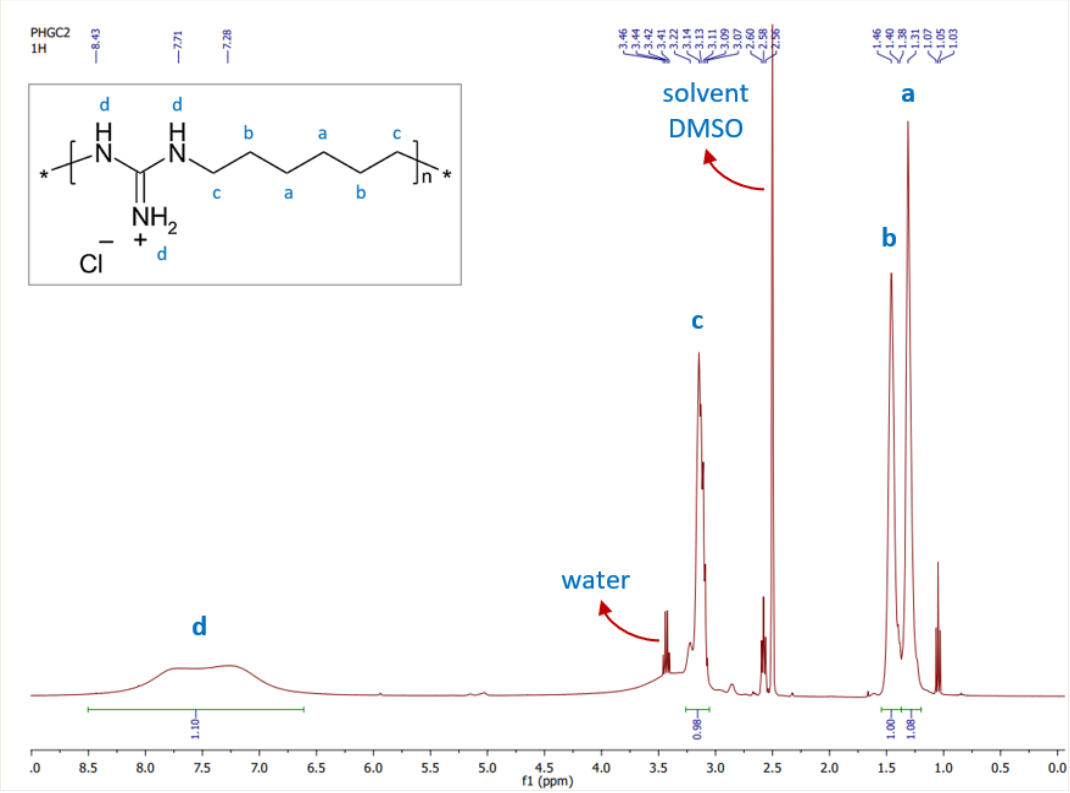


Figure 4.9. ^{1}H-NMR spectrum of synthesized PHMG polymer.

Results of ^{13}C-NMR Analysis

According to the relevant literature, ^{13}C-NMR was not a common technique to analyze the structure of the PHMG polymer, but the spectrum displayed consistent results with two studies that contained the method [51, 52]. The carbon atoms on the chemical structure and the corresponding peaks are shown in Figure 4.10. The chemical shifts were observed at around 26 (a), 28 (b), 31 (c), 155, and 157 (d) ppm. The shift at 40 ppm appears due to the DMSO solvent used for the analysis.

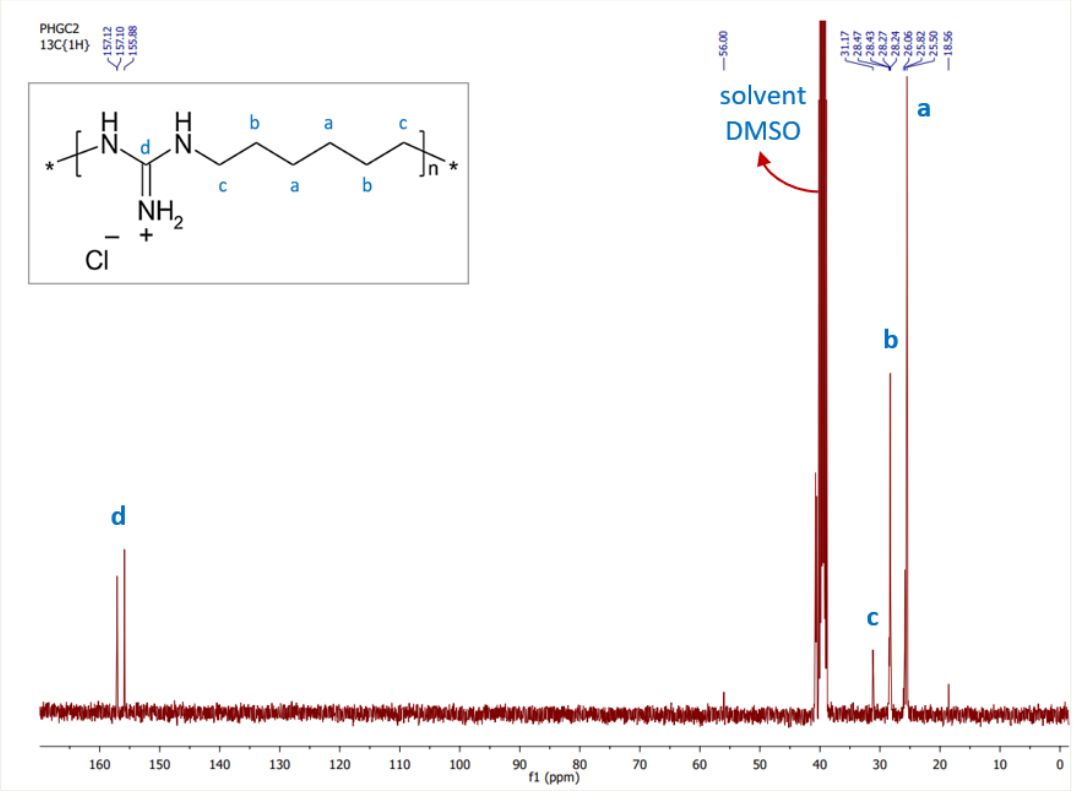


Figure 4.10. ^{13}C-NMR spectrum of synthesized PHMG polymer.

The obtained spectra from ^{1}H-NMR and ^{13}C-NMR both comply with those demonstrated in the literature. Consequently, the chemical structure of the synthesized PHMG polymer was confirmed via the applied NMR spectroscopy technique.

4.3.5. SEM Analysis Results

The SEM images of the pure, AAc-grafted and PHMG polymer-modified cotton fabrics are shown in Figures 4.11, 4.12, and 4.13, respectively. The images displayed that any significant difference did not occur in the fibers of the cotton fabrics upon grafting and following polymer modification applications. The small cotton particles observed on the fibers in Figure 4.13 were also detected for pure and AAc-grafted cotton fabrics yet have been more recognizable after the polymer modification step.

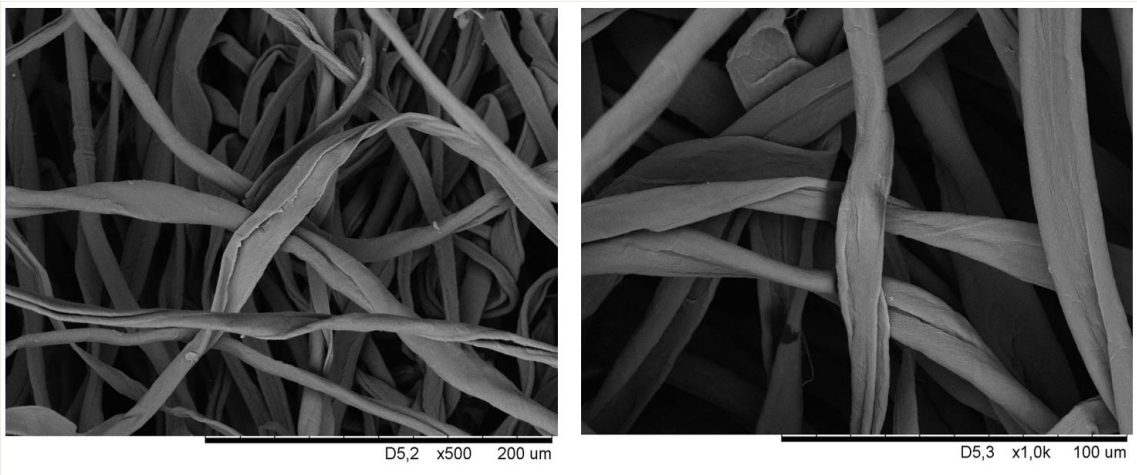


Figure 4.11. SEM images of pure cotton fabrics at 500x and 1000x magnification.

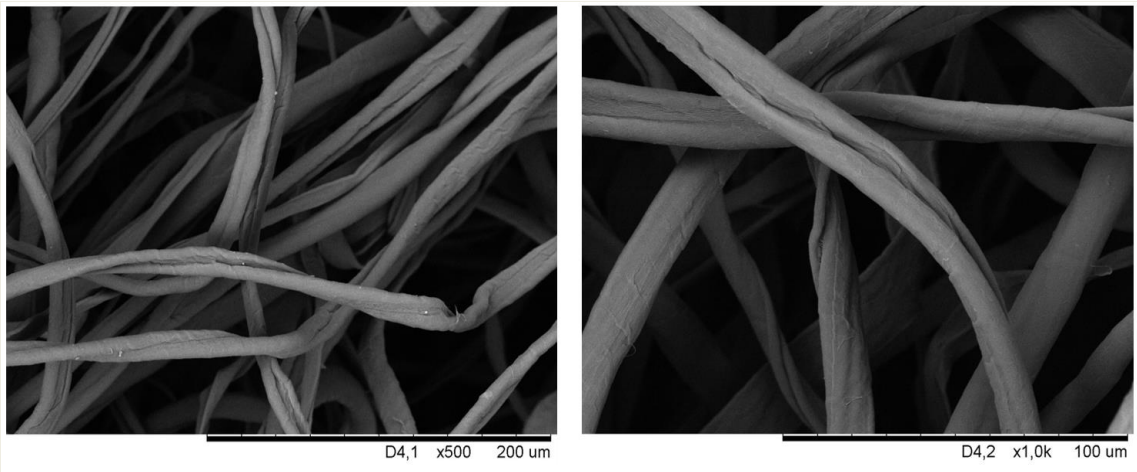


Figure 4.12. SEM images of AAc-grafted cotton fabrics at 500x and 1000x magnification (grafting conditions: AAc concentration: 30% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h).

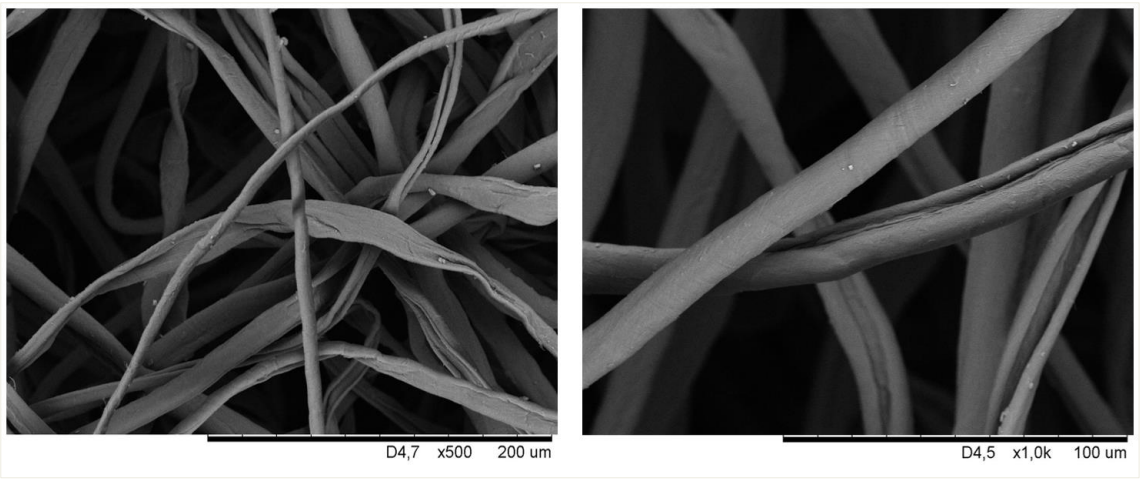


Figure 4.13. SEM images of PHMG polymer-modified cotton fabrics at 500x and 1000x magnification (coupling yield: 20.5 %).

4.3.6. Antibacterial Testing Results

4.3.6.1. Agar-well Diffusion Test Results of PHMG Polymer

Agar-well diffusion test was carried out to evaluate and screen the antibacterial activity of the synthesized PHMG polymer against *E. coli* and *S. aureus*. During the incubation time, the antibacterial agent in the wells diffused in the agar medium and inhibited the growth of the bacteria, creating a clear zone around the wells. The zones of inhibition that appeared for the tested bacteria are shown in Figure 4.14, and the measured diameters of these zones correspond to the wells' contents are expressed in Table 4.6.

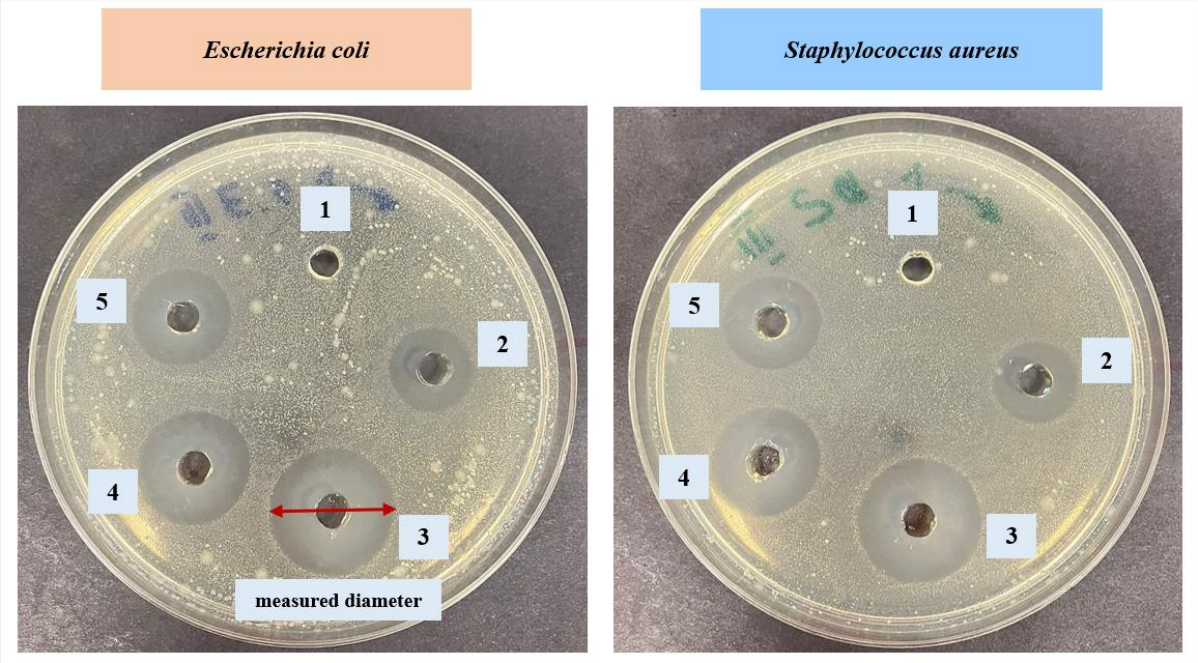


Figure 4.14. The inhibition zone on the agar plates with *E. coli* and *S. aureus* bacteria induced by the PHMG polymer compared to controls, refer to Table 4.6.

Table 4.6. The diameter of the inhibition zone (mm) in agar plate test of PHMG samples in solutions of three concentrations.

Well No & Content / Bacteria	1 PBS	2 Antibiotic	3 Pol. Soln. (2%)	4 Pol. Soln. (1%)	5 Pol. Soln. (0.5%)
E. coli	-	16.7	22.3	19.7	17.3
S. aureus	-	15.0	21.3	19.3	17.0

The PBS control did not create a clear zone around the well, while the second control well with antibiotics resulted in an inhibition zone. The controls validated the method by showing that the agar medium with microorganisms worked as it should have. The formation of inhibition zones around the wells containing polymer solutions proved that the PHMG polymer displays antibacterial properties against *E. coli* and *S. aureus* by diffusing through the agar and inhibiting the growth of the bacteria.

In addition, the diameters of the inhibition zones in agar plated inoculated with both bacteria tended to increase as the concentration of the polymer solution raised. The tested polymer created more extensive clear zones compared to the antibiotic control. Both antibiotic and the polymer can be assumed slightly more active against *E. coli* than *S. aureus* considering the inhibition zones (Table 4.6).

The results of this test have been compared with a study that indicated a sensitivity range according to the inhibition zone to classify the sensitivity of the bacteria against the antimicrobial agents, which have similar chemical structures to the subjected polymer. It could be concluded that *E. coli* and *S. aureus* bacteria have the sensitivity to the synthesized PHMG polymer since they created inhibition zones that fall into the sensitivity range between 11-25 mm mentioned in the study [89].

4.3.6.2. Broth Dilution Test Results of PHMG Polymer

The minimum inhibitory concentration (MIC) values of the PHMG polymer against *S. aureus* and *E. coli* were obtained in broth and on agar plates with the Broth dilution test. The results were analyzed and interpreted as follows.

Results of Staphylococcus aureus (S. aureus), Gram-positive bacteria

The final appearance of the test tubes after incubation is given in Figure 4.15. Both control samples displayed that the bacteria, broth, and antibiotic worked as expected for the experiments. The tube of the C1 control contained broth and bacteria and was turbid after incubation, representing the existence of visible bacterial growth. Besides, the C2 control tube with antibiotics and bacteria showed a transparent content by proving the antibiotic's activity in preventing bacterial growth. The agar plates of the control samples showed compatible results with the control tubes. A layer of bacteria was observed on the agar of both replicates of the C1 control. In contrast, 2 and 6 colonies of bacteria were counted on the agar surfaces of C2 control A and B replicates, respectively. The results of agar plates with antibiotics were acceptable since only a few single colonies were detected (Figure 4.16).

Figure 4.15. The test tubes of *S. aureus* after incubation in the presence of PHMG.

The test tubes in Figure 4.15 contained *S. aureus* bacteria and the serial two-fold dilutions of the PHMG polymer in sterile broth. The final concentration of the PHMG polymer in the test tubes numbered from 1 to 10 ranged from 256 to 0.5 mg/L. The incubation of the test tubes of *S. aureus* bacteria resulted in turbid contents in tubes 8, 9, and 10, but the rest of the tubes showed a transparent appearance. Tube 7 contained the lowest polymer concentration among the tubes appearing with a clear solution. Therefore, based on the visual control of the test tubes, the MIC value was obtained as 4 mg/L from tube 7 (Figure 4.15). However, after the contents of the test tubes were transferred to the agar and let the bacteria grow on the agar, bacteria were observed on the agar plates from plates 6 to 10. Consequently, plate 5 with 16 mg/L polymer concentration was determined as the lowest concentration that could inhibit bacterial growth according to the agar plate test (Figure 4.16).

It could be seen on the agar plates that the density of the colonies of the bacteria on agar surfaces increased from plate number 6 to 8. On plate 6, the colonies of the bacteria were discrete, and the space between the colonies was easily distinguishable. On the other hand, the colonies were closer and denser on plate 7, and a layer of bacteria was recognizable on plate 8. Even if the polymer concentrations of plates 6 and 7 did not completely inhibit the growth, they have limited the growth of bacteria compared to the rest of the plates from 8 to 10. The distinct bacterial layers on plates 8, 9, and 10 were similar to the C1 control emphasizing undisrupted bacterial growth (Figure 4.16).

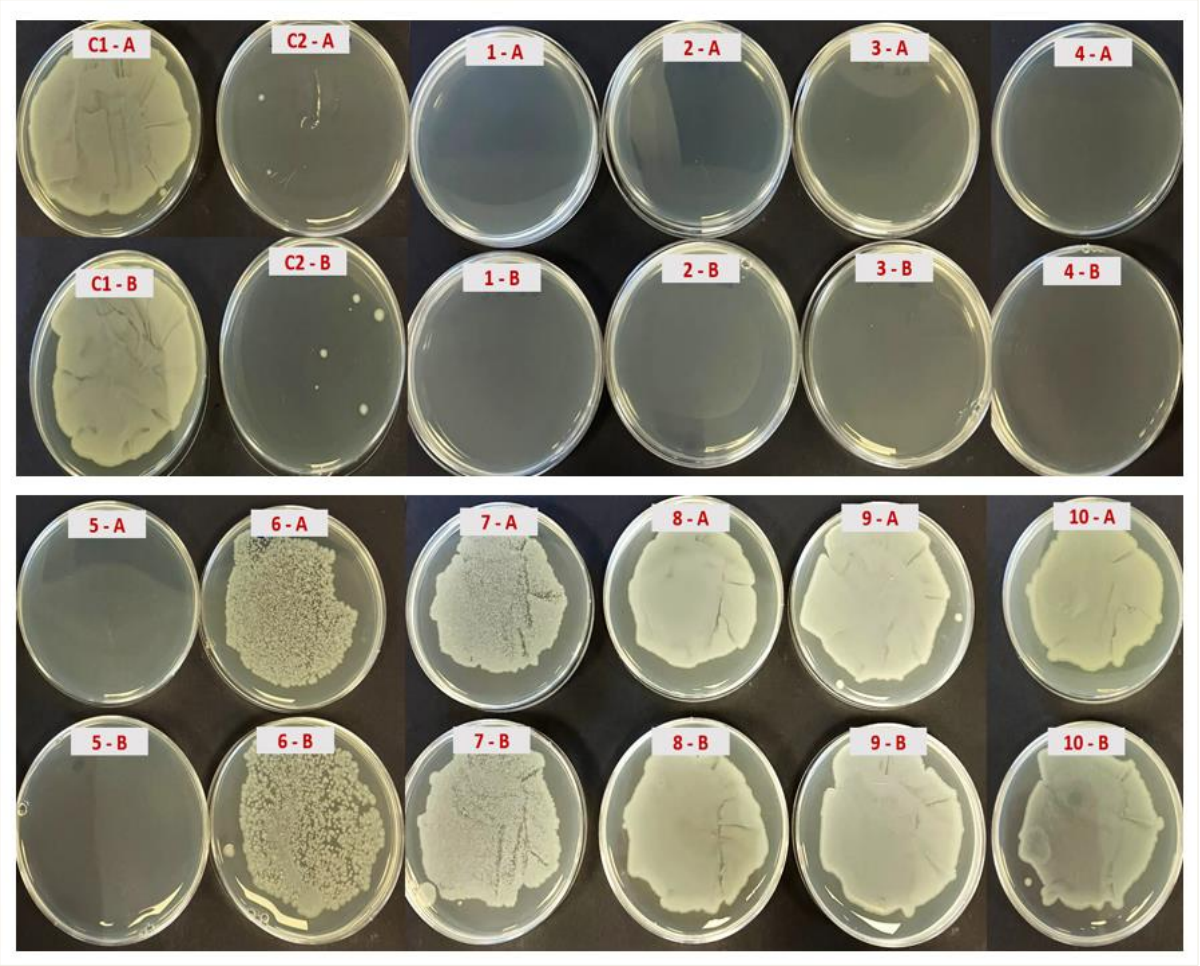


Figure 4.16. Agar plates of *S. aureus* after incubation in the presence of PHMG.

Results of Escherichia coli (E. coli), Gram-negative bacteria

Control samples resulted as anticipated according to the test tubes and agar plates' final appearance after incubation (Figure 4.17 and 4.18). A turbid content and a layer were observed in the C1 control's tube and plate, respectively, representing visible bacterial growth. For C2 control, the antibiotics' activity prevented the growth in the tube and the plate. The single colonies on the agar plates of C2 control were at an insignificant amount.

The test tubes in Figure 4.17 contained *E. coli* bacteria and the serial two-fold dilutions of the PHMG polymer in sterile broth. The final concentration of the PHMG polymer in the test tubes numbered from 1 to 10 ranged from 256 to 0.5 mg/L. Examination of the test tubes detected visible bacterial growth starting from tube 7 to tube 10; thus, the MIC value of the PHMG polymer against *E. coli* was determined as 8 mg/L at tube 6 (Figure 4.17).

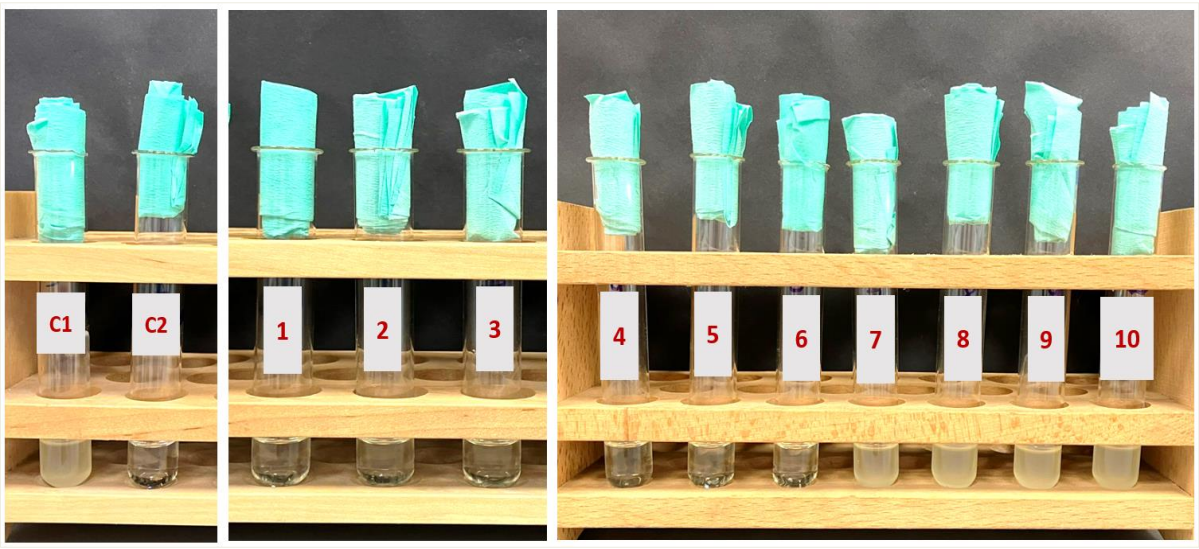


Figure 4.17. The test tubes of *E. coli* after incubation in the presence of PHMG.

The agar plate test provided more detailed information about the polymer's activity against the bacteria and the last status of the plates could be examined in Figure 4.18. The observed bacteria density on the agar plates naturally increased from plate 4 to 10 as the polymer concentration decreased. The lowest concentration of the polymer, which completely inhibited bacterial growth (MIC), was determined as 64 mg/L on plate 3. Besides, discrete colonies on the agar plates 4, 5, and 6 demonstrated that the growth of *E. coli* was limited by 32, 16, and 8 mg/L polymer concentrations, respectively.

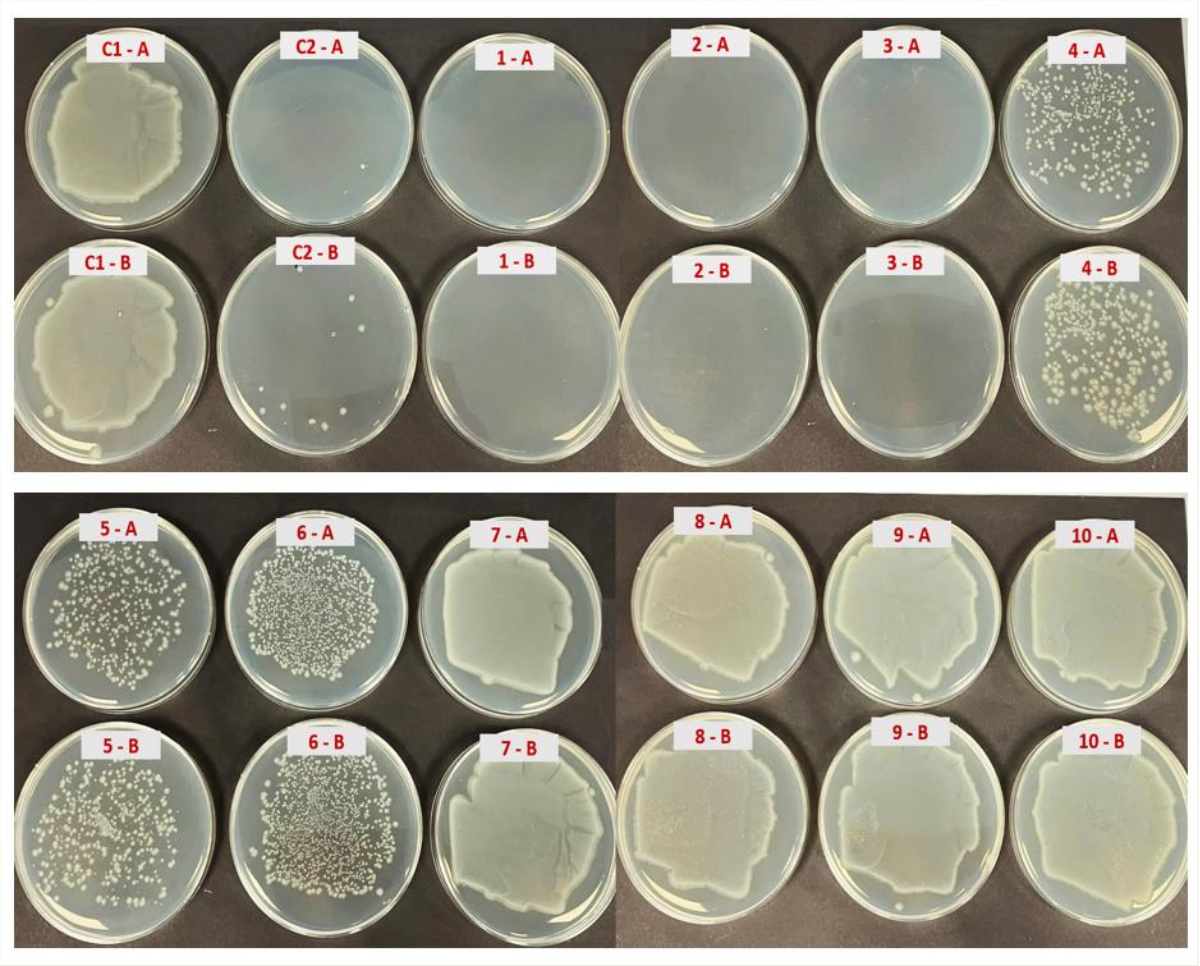


Figure 4.18. Agar plates of *E. coli* after incubation in the presence of PHMG.

The determined MIC values for the synthesized PHMG polymer in the thesis study were compared with those obtained from the antibacterial tests of the same polymer in the literature.

The MIC value obtained for *S. aureus* on visual evaluation of the test tubes is a consistent result compared to the values in the literature studied the same polymer [8, 47, 63]. On the other hand, the agar plate test resulted in a MIC value, higher than the lowest inhibitory polymer concentrations indicated in the literature. The difference may not show that the studied polymer is less active, but it could be associated with the evaluation technique used in the literature, as the MIC value was generally read by checking the visible bacterial growth in the tubes macroscopically. The agar plate test was additionally conducted by considering that the naked-eye controlling of the test tubes could not be adequate for precise detection of the growth.

In comparison with the studies that included the MIC values of the same polymer, the MIC value from the visual evaluation of the test tubes with *E. coli* was similar, while the agar plate test result was higher than the indicated values in the articles [8, 47, 52, 98]. Similar to the results of *S. aureus*, the MIC values obtained from broth and agar tests significantly differ for *E. coli*. This strengthens the idea that performing only visual control in broth for MIC determination is insufficient and misleading and requires analysis in an additional methodology, such as on the agar plate. As a result of considering the MIC values from agar plates, the PHMG was more active against *S. aureus* than *E. coli* since 16 mg/L polymer concentration was adequate for the complete inhibition of *S. aureus*. In contrast, the MIC value was found as 64 mg/L for *E. coli*.

The comparison of the MIC values of the PHMG polymer obtained against Gram-positive and Gram-negative bacteria can be examined in Table 4.7. Since it was indicated in the literature that the molecular weight of the polymer may affect the antimicrobial properties [50], the comparisons between the MIC values from different studies should be made considering the molecular weight of the polymers.

Table 4.7. MIC values of PHMG polymer from the literature.

Polymer's Molecular Weight (g/mol)	**Microorganisms**	**MIC (mg/L)**	**Reference Literature**
M_n : 720	*E. coli*	8	[8]
	S. aureus	4	
M_w / M_n : 576 / 481	*E. coli*	8	[47]
	S. aureus	4	
M_w / M_n : 1600 / 1300	*E. coli*	7.81	[52]
Mη (viscosity-average molecular weight) : 1317	*E. coli*	2	[63]
	S. aureus	4	
M_w : 2500	*E. coli*	8.2	[98]
M_w / M_n : 1365 / 1207 (A type) 1505 / 1356 (B type) 1268 / 1097 (C type)	*E. coli*	8	**This study - test tubes**
	S. aureus	4	
	E. coli	64	**This study – agar plates**
	S. aureus	16	

4.3.6.3. Agar Diffusion Test Results of PHMG-modified Cotton Fabrics

The agar diffusion test was carried out to evaluate the antibacterial activity of the final materials, PHMG-modified cotton fabrics, against *E. coli* and *S. aureus* bacteria. The cotton fabrics treated with antibiotics, pure cotton fabrics, and cotton fabrics treated with the PHMG polymer solution were used as controls for the experiments. AAc-grafted cotton fabrics and PHMG-modified cotton fabrics with different polymer coupling yields were the test samples. Figure 4.19 demonstrates the status of the agar plates with bacteria just after placing the fabrics and before incubation.

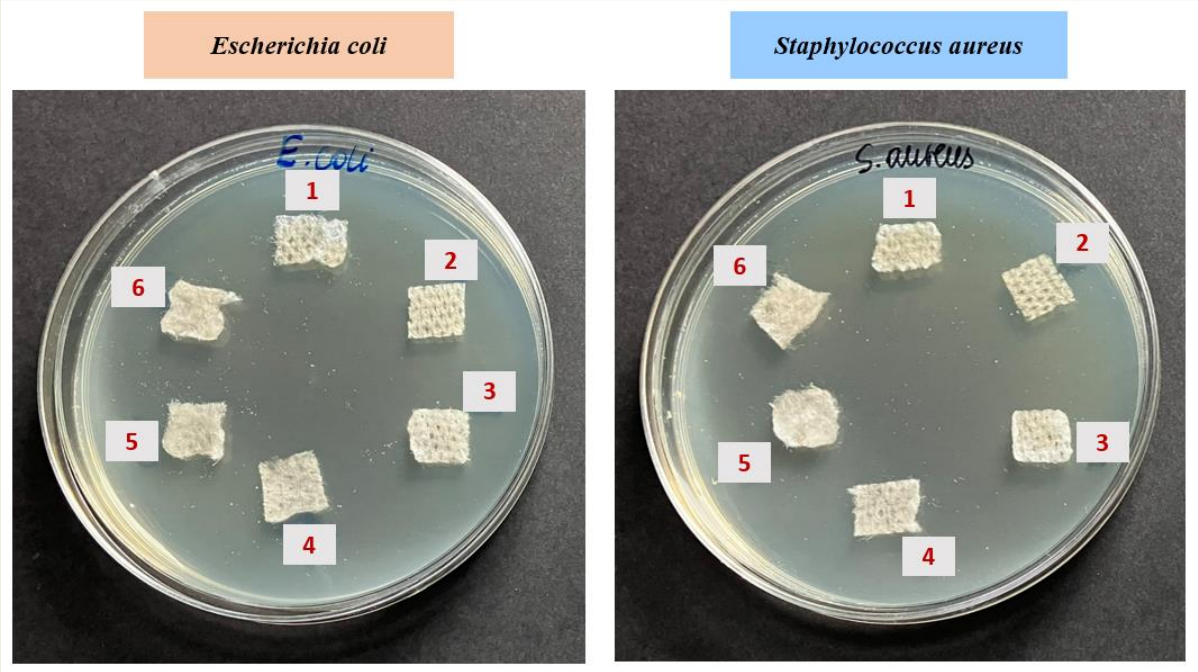


Figure 4.19. Agar plates with the fabric samples before incubation. Samples: 1. antibiotics control, 2. pure cotton fabric control, 3. releasing control (with expected diffusion of the PHMG polymer), 4. AAc-grafted cotton fabrics (grafting conditions: AAc concentration: 20% v/v, absorbed dose: 30 kGy, reaction temperature: 65 °C, reaction time: 3 h), 5. PHMG-modified cotton fabrics 1st (coupling yield: 17.5 %), 6. PHMG-modified cotton fabrics 2nd (coupling yield: 23.8 %).

The status of the agar plates after the incubation is displayed in Figures 4.20 and 4.21 for *E. coli*, and *S. aureus*, respectively. The results observed on the agar plates were almost the same for both tested bacteria. All control samples demonstrated the correct results. The first and third control samples with antibiotics and PHMG polymer, respectively, prevented the growth of the bacteria around and underneath the fabric by the diffusion of the substance into the agar, and inhibition zones were observed for the samples. No clear zones around or underneath the second control sample, pure cotton fabric, were detected, which meant that the bacterial growth occurred as expected. According to the results of these control samples, the agar medium with microorganisms worked as it should have during the test. Although there was no inhibition zone around the AAc-grafted cotton fabrics, it was observed upon removing the sample from the agar plate that bacterial growth was prevented along the contact surface between the agar and the sample. This

implied that the AAc-grafted cotton fabrics demonstrated antibacterial activity against the tested microorganisms via contact mechanism without diffusion of a substance due to the strong interactions between cotton and acrylic acid monomer. For the PHMG polymer-modified cotton fabrics, incubation concluded with the formation of thin clear frames along the edges of the samples. Under the samples, on the contact area of fabrics and agar, the growth of the bacteria was suppressed. The frame occurrence along the fabrics' edges may indicate the slight release and diffusion of PHMG into agar. However, it should be considered that even if there was PHMG diffusion from the PHMG polymer-modified fabric, it did not occur as it happened for antibiotic (Sample 1) or PHMG-releasing samples (Sample 3) with large inhibition zones. There was no apparent inhibition zone around samples 5 and 6, and PHMG was incorporated into fabrics with strong amide bonds. It can be concluded that the antibacterial activity was endowed to the cotton fabrics by grafting acrylic acid (Sample 4), yet the antibacterial efficiency may have been enhanced upon modification of the PHMG polymer (Samples 5 and 6).

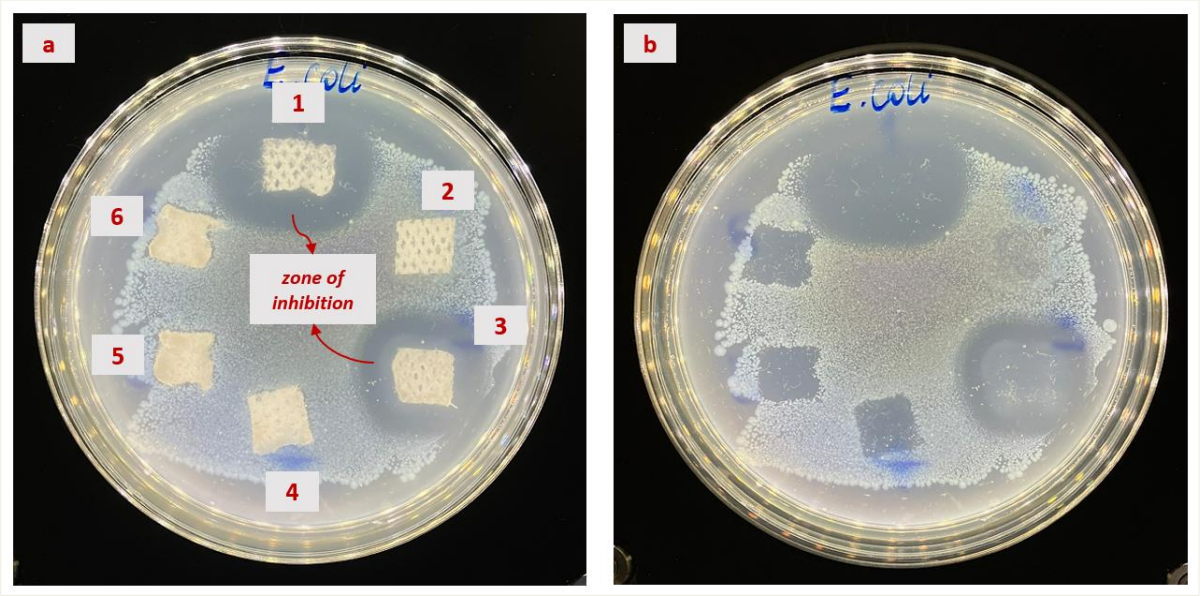


Figure 4.20. Agar plates of *E. coli* after incubation. (a) with fabric samples (b) after fabric samples were removed.

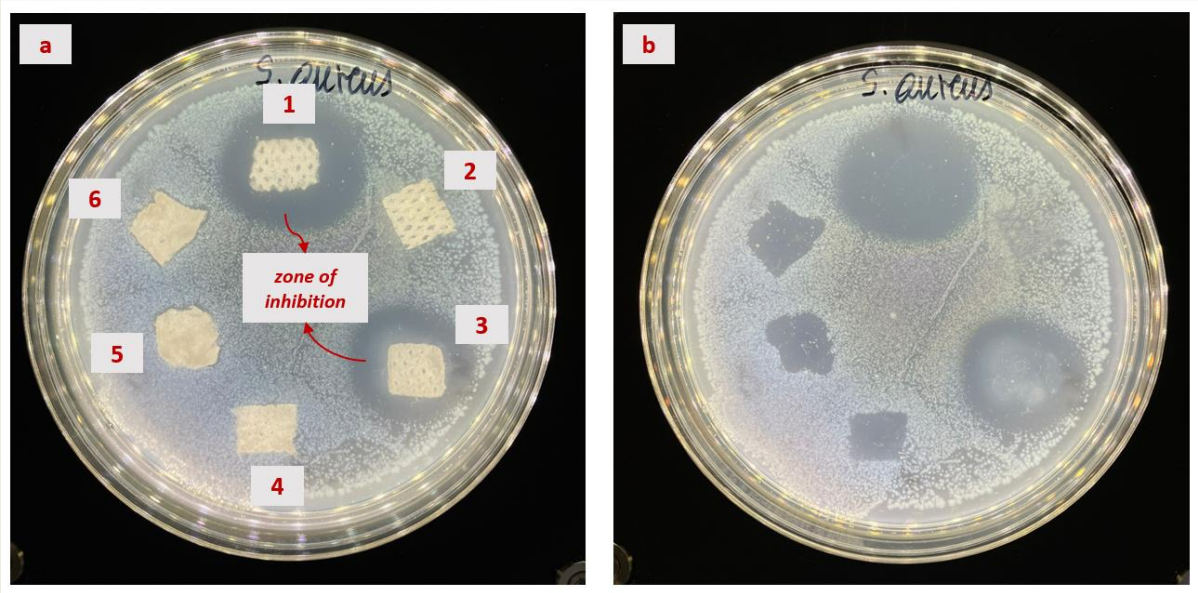


Figure 4.21. Agar plates of *S. aureus* after incubation. (a) with fabric samples (b) after fabric samples were removed.

The studies in the literature demonstrate that this test is used for the qualitative testing and evaluation of the antibacterial activity of fabrics [93, 94]. Furthermore, the test provides information regarding the mechanism of the antibacterial action. The fabrics can demonstrate leaching or non-leaching antimicrobial property depending on the treatment method and employed antimicrobial agent [95]. Similar tests have been conducted in various studies to examine the leaching characteristic of materials modified with PHMG. Examples of materials tested in such studies include PHMG-modified cotton fibers [10], poly(vinylidene fluoride) membranes [17], and paper [57].

As a result of the studies, cotton fabrics with the desired antibacterial properties based on guanidine were successfully obtained. These materials could be promising for applications in food packaging, textiles, and the medical field. Therefore, further research and development could be pursued to explore their full capabilities and potential benefits.

5. RESULTS

- In this thesis study, the cotton fabrics with antibacterial properties were obtained using antibacterial poly(hexamethylene guanidine) hydrochloride (PHMG) polymer. In the first step, cotton fabrics were subjected to radiation-induced graft polymerization to functionalize them with acrylic acid (AAc) monomer. Subsequently, the AAc-grafted cotton fabrics were modified through a chemical coupling reaction using the synthesized PHMG polymer.
- PHMG polymer was synthesized by the melt polycondensation reaction using equimolar amounts of guanidine hydrochloride and hexamethylenediamine monomers. The synthesis reaction was carried out under a nitrogen atmosphere with continuous stirring. The reaction mixture was gradually heated to temperatures of 100 °C, 140 °C, and 170 °C during five hours of total reaction time. Under the applied reaction conditions, the yield obtained from the synthesis of the PHMG polymer was calculated to be 14%.
- The grafting of AAc onto the cotton fabrics was performed using the oxidative pre-irradiation technique. The fabrics were exposed to gamma radiation under the air atmosphere and subsequently treated with AAc solutions under a certain temperature and time.
- The optimum conditions for grafting AAc onto cotton fabrics were determined as 30 kGy absorbed dose, 30% (v/v) aqueous solution of AAc at a reaction temperature of 65 °C, and a reaction time of three hours. Under these conditions, the average grafting yield on the prepared fabrics was calculated to be 13.8%.
- AAc-grafted cotton fabrics were modified with the synthesized PHMG polymer through an amide coupling reaction between the carboxyl groups endowed to cotton fabrics by grafting and the amino groups in the polymer. The average coupling yield of the modification reaction between PHMG polymer and the AAc-grafted cotton fabrics prepared at the optimum grafting conditions was determined as 19.6%.
- According to MALDI-MS analysis, the synthesized PHMG polymer majorly consists of types A, B, and C of the polymer. The average molecular weights of the respective types were determined using Polytools software. The results are as

follows: for type A, M_n=1207, M_w=1365; for type B, M_n=1356, M_w=1505; for type C, M_n=1097, M_w=1268.

- The minimum inhibitory concentration (MIC) values of the synthesized PHMG polymer against *S. aureus* and *E. coli* were determined in test tubes (in broth) and on agar plates. The MIC values obtained from the test tubes were 8 mg/L for *E. coli* and 4 mg/L for *S. aureus*, while the values obtained from the agar plates were 64 mg/L for *E. coli* and 16 mg/L for *S. aureus*.
- The chemical coupling reaction between PHMG's amino end groups and the carboxyl groups of AAc-grafted cotton fabrics was confirmed by observing the presence of characteristic bands corresponding to covalent amide bonds with FTIR analysis. These bands appeared at 1633 cm^{-1} and 1550 cm^{-1} on the FTIR spectrum of the PHMG-modified cotton fabrics.
- The PHMG-modified cotton fabrics were confirmed to exhibit antibacterial activity through a qualitative agar diffusion test.

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