Mechanisms of Action and Chemical Origins of Biologically Active Antimicrobial Polymers

Jeff Shen, Geoffrey C. Gurtner, Lynette Cegelski, and Yunzhi Peter Yang

Abstract The creation of synthetic tissues for patients with traumatic or debilitating injuries and diseases has proven to be a rapidly growing field. Scaffold design plays a crucial role in determining the biocompatibility, function and longevity of these engineered tissues. Biodegradable polymers with high levels of biocompatibility and functional flexibility are currently the primary choice for scaffold construction. Due to the fiscal and healthcare-related costs of replacing scaffolds during the healing process, manufacturing transplants with the ability to withstand foreign infection is tantamount to the success of the field. Antimicrobial polymers (AMPs) can serve as materials for such synthetic transplants. A variety of AMPs bearing different chemical motifs and biological effects have been studied with regard to their viability as biocompatible engineering materials. This review discusses the merits and faults of AMPs in their potential applications toward tissue scaffold design.

Keywords Antimicrobial · Polymer · Scaffold · Tissue engineering · Synthetic · Biocompatible · Transplant · Chitosan · Quaternized ammonium

J. Shen
Department of Chemistry, Stanford University, Stanford, CA, USA

Genentech, San Francisco, CA, USA
e-mail: shenj45@gene.com

G. C. Gurtner
Departments of Surgery and Materials Science and Engineering, Stanford University School of Medicine, Stanford, CA, USA
e-mail: ggurtner@stanford.edu

L. Cegelski
Department of Chemistry, Stanford University, Stanford, CA, USA
e-mail: cegelski@stanford.edu

Y. P. Yang (✉)
Departments of Orthopaedic Surgery, Materials Science and Engineering, and Bioengineering, Stanford University School of Medicine, Stanford, CA, USA
e-mail: ypyang@stanford.edu

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Introduction

In recent years, the engineering of synthetic tissues, including tendon and bone transplants, has reached stages where it is now feasible to create highly biomimetic and biocompatible synthetic implants. However, successful clinical translation of these developing technologies hinges upon infection-free incorporation of the implants, as well as maintaining an antimicrobial environment in the absence of a traditional immune response, as is often the case with these transplants.

Due to the circumstances surrounding the cases where transplants are required, such as in trauma or disease-induced organ and tissue loss, the immune system is incapable of handling microbial infections at the site of the transplants. Microorganism-related infections provide the most serious complications in the healthcare industry, particularly when medical devices and hospital equipment are involved [1]. For example, contamination of catheters can lead to high levels of discomfort and illness within medical patients. Furthermore, treatment of these infections often requires complete removal of the implants, which prove to be costly and inconvenient procedures for both the patient and hospital. More serious infections can occur when the microbes migrate from the infection site to the spinal fluid or the brain, as traditional antibiotics cannot treat infections in those locations. Therefore, prevention of these microbial infections is a high priority and mandatory step to ensure safe, long-lasting transplants, especially when using synthetic materials.

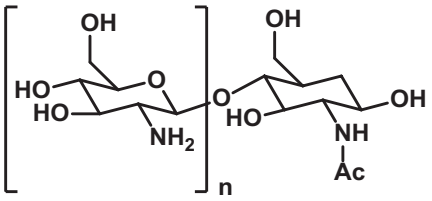
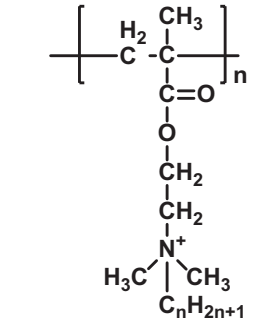
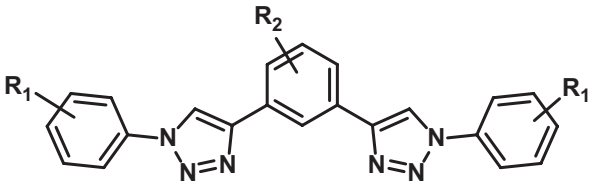
Many research groups have dedicated their efforts toward synthesizing biocompatible materials that provide their host with antibiotic activity. One strategy has been to employ traditional antibiotics in a sustained release fashion, generally through encapsulation of the antibiotics in materials that slowly degrade over a long time scale [2]. By incorporating this into synthetic transplants, the transplant itself will gradually release antibiotics into the surrounding area, keeping the microbial infections at bay. The major disadvantage to this is that most antibiotics bind to their targets with very high affinity, but a single point mutation in the gene that encodes for the antibiotics' target could lead to resistance against the drug [3]. This is exacerbated when there are large and diverse populations of microbes incubating over a long period of time, as is the case when transplants are involved.

Another antibacterial design approach relies on selecting and tuning the chemical and biochemical properties of the materials used to construct or coat a transplant, as certain materials exhibit bactericidal properties due to electronics and sterics [4]. Because the mechanisms of action of such antimicrobial polymers rely on general properties, particularly with regard to the microbial cell surface and cell membrane, resistance is often avoided, even after long exposure to the same antimicrobial materials [5]. This is attributed to the difficulty inherent in microbes enacting large scale changes in biochemical properties to alter a target such as the cell wall or cell membrane, as single mutations would not be sufficient to generate resistance. This chapter will focus primarily on polymers of this type with inherent antimicrobial properties and will include a discussion of their proposed mechanisms of action against microorganisms and activity with human cells, a perspective of current limitations in the field, and future opportunities.

Overview of Different Types of Antimicrobial Polymers

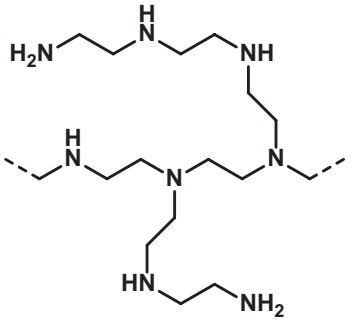
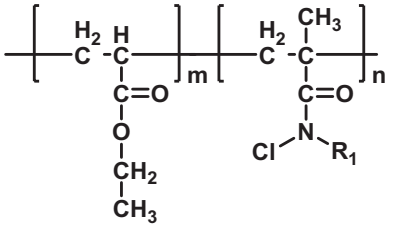
Currently, there are a wide variety of antimicrobial polymers (AMPs) that have demonstrated high selectivity and strong fungicidal, bactericidal, and antiviral capabilities. They are derived from diverse chemical origins and rely on particular chemical functionality for their mechanisms of action. Table 1 provides a summary of

Table 1 Chemical structures and general mechanisms of AMPs

Polymer	Mechanism of action
 Chitosan	Interaction between positively charged chitosan and negatively charged microbial membranes leads to perforations in the membrane. Activity is pH dependent
 Quaternary Ammonium-containing Compounds	Integration of hydrophobic tail into microbial membrane impairs membrane integrity. Leads to leakage and cell lysis
 Synthetic Protein Mimics	Mimicry of antimicrobial peptides, often designed to exhibit enhanced stability and microbial targeting, serving to penetrate or impair cell barriers

(continued)

Table 1 (continued)

Polymer	Mechanism of action
 Polyethylenimines	Inhibits attachment of viral and bacterial organisms to host cells, thus preventing microbial biofilm formation on host cells and tissue. Additionally, electrostatic interactions impair bacterial membrane integrity
 N-Halamines	Oxidative halogens can target specific thiols or amino groups on proteins, leading to inactivation and eventual cell death

representative polymers, their notable chemical moieties that contribute to their biological activity, and the generally understood basis for their antimicrobial properties.

Overall, it can be noted that the majority of the AMPs rely on charge interactions between the AMPs and their target microbes. Generally, positively charged AMPs are attracted toward negatively charged microbes and this interaction is the basis of many antimicrobial activities. However, there are certain types of AMPs that derive their activity from biomimetic properties, such as the synthetic mimics of antimicrobial peptides (SMAMPs), and halogen-based polymers, which serve to transfer halogens such as chlorine through direct contact to microbial membranes or proteins at the cell surface.

Chitosan-Based Polymers

Chitosan is a well-known natural polymer that consists of acetylated and non-acetylated glucosamines that are attached linearly with an ether bond. It is a hetero polysaccharide with polycationic character and derives its chemical flexibility mostly from its amine groups, which can be functionalized to provide further versatility and utility to this polymer. It can be found naturally in the cell walls of fungi,

green algae, or in insect cuticles [6]. It is generally produced through the deacetylation of chitin, which is the fully acetylated form of chitosan that is found commonly in many living organisms. For the production of chitosan, chitin is generally submerged in a basic solution and the degree of deacetylation (DDA) is determined via UV spectroscopy [7, 8].

Chitosan's properties are heavily dependent on the degree of acetylation of the polymer, as this affects the viscosity, charge distribution and ultimately bactericidal abilities of chitosan [9]. The currently accepted DDA for chitosan requires at least a 40% deacetylation for the polymer to be considered chitosan. A key property of chitosan is its solubility in aqueous solutions. It is insoluble in water or alkaline media, but becomes soluble in solutions at and below pH 6.5 due to the pKa of the amines. Protonation of the amino groups enables the production of a cationic polysaccharide, while the overall balance of charge of the polymer is also dependent on the acetylation density. Thus, the fewer the acetyl groups on the polymer, the more cationic properties the chitosan will have. The bactericidal properties of chitosan are ascribed to its cationic character, enabling it to bind to negatively charged membranes and biomolecules such as phosphate-rich teichoic acids [10]. Chitosan is also an attractive polymer for its highly biodegradable and biocompatible properties. It is easily hydrolyzed by enzymes, such as lysozymes or cellulases. There are also enzymes specific to chitosan but not chitin called chitosanases that hydrolyze the glycosidic bonds between the deacetylated glucosamines [11, 12].

In terms of its antimicrobial activity, chitosan has been shown to be effective against yeast, bacteria, fungi, with more activity against gram-positive rather than gram-negative bacteria. It has been noted that chitosan primarily prevents bacterial growth rather than directly killing bacteria [13]. After being separated from the chitosan through membrane filtration, bacteria can continue to grow, demonstrating that chitosan does not permanently damage bacteria [14]. Furthermore, this suggests that chitosan is associated outside of bacteria and needs this association for its antimicrobial efficacy. Therefore, chitosan is mostly a bacteriostatic, although it exhibits bactericidal properties when it accesses the bacterial membrane, suffocating bacteria by physically blocking access to nutrients [15]. One curious observation is that the potency of chitosan is not dependent on molecular weight, as the minimum inhibitory concentrations (toward bacteria) of chitosan polymers ranging from 80 to 1500 kDa were all within one order of magnitude [16].

The mechanism of action of chitosan-based polymers is generally assumed to require interactions with the cell envelope, which alters cell surface properties, leading to disruption of cellular function and cell leakage. This is thought to be due to the protonated amino groups allowing chitosan to form strong electrostatic interactions with the negatively charged surface of most microorganisms. It has been shown that chitosan loses its antimicrobial activity above pH 7 and that its antimicrobial activity is dependent on acetylation. These factors determine how positively charged the chitosan polymer is, and both support the idea that the protonated amino groups are essential for chitosan to function as an antibacterial molecule [17].

Chitosan has been functionalized by many different research groups at its amino site in order to modify its biochemical properties. For example, a modified chitosan

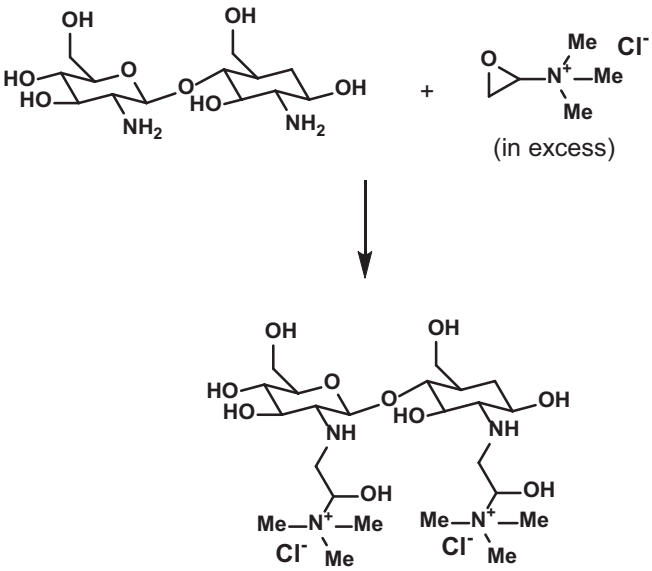


Fig. 1 The reaction scheme for synthesis of the modified chitosan, a one-step process. (Modified from [14])

was formed through the reaction of glycidyltrimethylammonium chloride with chitosan to generate an alkylated version of chitosan with extra quaternary ammonium groups, as seen in Fig. 1 [1]. This chitosan derivative exhibited enhanced antimicrobial activity over regular chitosan, due to the inclusion of the quaternary ammonium groups. Other modifications include the addition of a vinyl sulfonic acid sodium salt to the amino group to generate a zwitterionic structure that has optimal antibacterial properties at pH 5.75 but dropped significantly when the pH was increased to 6.2 [18]. It can be seen that chitosan's chemical structure lends itself readily to modifications that allow its antimicrobial properties to be hybridized with other chemical motifs. Chitosan also has the ability to chelate metal ions such as Cu^{2+}, which can also contribute to additional antimicrobial properties [19]. These options for modification lead to interesting combinations of antimicrobial activities that can have broad spectrum applications due to the diverse chemical groups that can be added to chitosan.

Polymers Containing Quaternized Ammonium

Compounds that contain quaternized ammonium salts (QAS) are another widely studied set of polymers due to their strong antimicrobial properties. Unlike chitosan, these are generally not found naturally and are synthetic polymers that have biocompatible backbones with the quaternized salt attached as a pendant group. The

Environmental Protection Agency (EPA) has revealed that QAS polymers are the most popular when it comes to household disinfectants, as they are found in over 50% of commercially available products [20]. They are also found in cosmetic products, mouthwash, and surface finishings.

In making these polymers, backbones such as polyethylene glycol (PEG), polynorbornene, and poly(ε-caprolactone) (PCL), are used to render the material properties of the compounds. The QAS chains are then added to provide chemical and antimicrobial properties. Generally, the QAS chains that contain 8–18 carbons have the best antimicrobial properties, with shorter lengths being better against gram-positive bacteria and longer lengths better against gram-negative bacteria [21]. The QAS chains also protect polymers like PEG by imparting resistance to redox and acid–base reactions as it protects vulnerable groups on the base polymer. Lastly, due to the diverse structures that QAS polymers can take, they can be made water soluble or water insoluble, meaning that they can be used in a wide variety of medical devices and synthetic transplants.

The mechanism of action of QAS polymers is proposed to be through penetration of the cell membrane and cell wall, leading to eventual cell death [22]. Higher weight polymers have been shown to have higher positive charge densities, which strengthens the adsorption of the polymers onto the surface of microorganisms. Adsorption then facilitates the ability of the polymers to enter the cell membrane. The hole-boring mechanism of action for QAS polymers has been verified through atomic force microscopy and fluorescence correlation spectroscopy. Another factor affecting the activity of these polymers is the structure of the counter anions, with Cl^- being the most effective toward antimicrobial performance. It has been postulated that using the correct counter anion facilitates dissociation of the quaternary salts [23].

The synthesis of these QAS polymers varies greatly, due to the different backbones that can be used. Examples in recent literature include biodegradable versions of PCL that have been grafted with alkyne chains containing QAS motifs or poly(ethylene glycol) methacrylate (PEGMA) with pyridine groups. The addition of the QAS polymers greatly enhances the ability of the polymer to inhibit and kill bacteria, particularly gram-positive bacteria. Naturally occurring primary ammonium modified cellulose has also been discovered in certain bacterial cells as part of a phosphoethanolamine modification, although these polymers have the inverse effect of promoting adhesion to other bacterial fibers and do not impact viability of the producing organisms [24, 25]. However, this does open the door for considerations of generating biosynthetically modified cellulose as a feasible alternative to chemically produced polymers.

Values of minimum inhibitory concentrations (MICs) of different antimicrobial polymers enable quantitative comparisons of the ability of these polymers to prevent bacterial growth. Table 2 shows a comparison between alkyl group functionalized polymers with quaternized nitrogen and commonly used sources of antibacterial potential, such as silver and streptomycin. The MICs for QAS polymers are comparable to traditional antibiotics and silver, although cell lysis and undesired toxicity can occur, as will be discussed in section “Cytotoxicity of Polymers.”

Table 2 Comparison of minimum inhibitory concentrations (MICs) between antibacterial compounds and alkylated QAS polymers

Antibacterial material	MIC in *E. coli* (μg/mL)	MIC in *B. subtillis* (μg/mL)
Silver nanoparticles	12.5	>25
Streptomycin	12.5	25
Ethyl-QAS polymers	200	200
Butyl-QAS polymers	200	200
Hexyl-QAS polymers	12.5	4
Octyl-QAS polymers	4	4
Decyl-QAS polymers	12.5	6
Phenylethyl-QAS polymers	12.5	12.5

Synthetic Protein Mimics

Currently, antimicrobial peptides are being studied as a class of antibiotics that can be used in place of traditional drugs. The major advantage that peptides confer over traditional antibiotics is that they demonstrate broad-spectrum activity against pathogens instead of targeting specific epitopes or enzymes in the microorganisms. There are currently hundreds of antimicrobial peptides that have been studied and databases exist to keep track of them. However, these peptides have had little to no success in being developed as FDA-approved antibiotics. The major obstacle is currently the synthesis of these peptides, as protein synthesis methods are too costly to create industrial amounts of these macromolecules [26].

A new strategy toward simplifying synthesis is to create chemically similar mimics that have the same functional groups as the peptides, whilst being readily accessible via straightforward chemistry like click chemistry. Figure 2 showcases a relatively short process used to create a synthetic mimic of an antimicrobial peptide, which was shown to have strong selectivity against *Staphylococcus aureus* and *Escherichia coli*, while requiring a 10–50 fold increase in concentration before lysing human red blood cells.

The mechanism of action of SMAMPs follows the trend of membrane interaction as seen with most antimicrobial polymers. Currently, there are a few different models that attempt to describe how SMAMPs interact with their target membranes. In the toroidal pore model, the SMAMPs bend the membrane of the target microorganism in order to form toroidal pores that lead to leakage of macromolecules. In the carpet model, the SMAMPs act as a detergent by covering the surface of the membranes, eventually dissolving the membrane and leading to large lesions on the cell surface [27]. Furthermore, there is evidence that SMAMPs target intracellular DNA and RNA, and that they inhibit cell-wall synthesis and nucleic acid synthesis.

SMAMPs have low frequency in selecting for resistant strains while maintaining high target selectivity and fast acting permeabilization of bacterial membranes, making them naturally potent against biofilms [28]. Biofilms derive antibiotic resistance, in part, from their low growth and metabolic rates, which are overcome by SMAMPs. Toward this, it has been shown by Barron et al. that antimicrobial peptoids have strong activities against Pseudomonas biofilms and *Mycobacterium tuberculosis* [29].

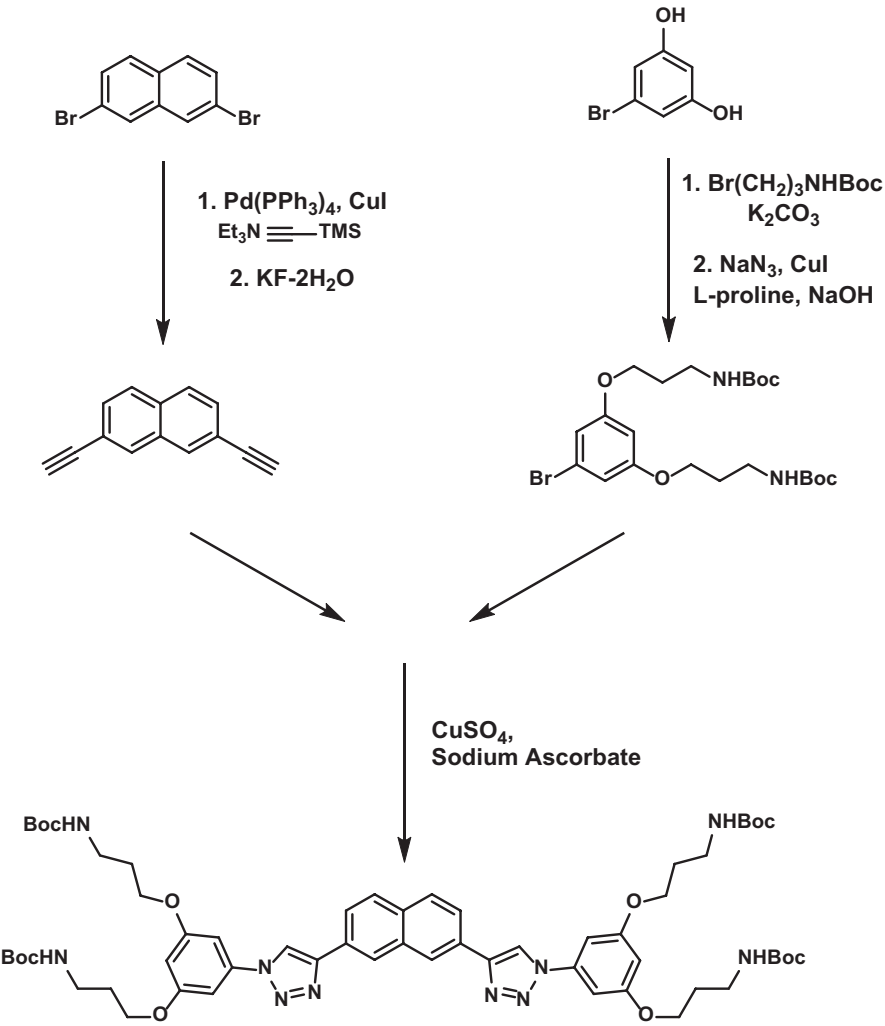


Fig. 2 Standard representative scheme of click chemistry being used to synthesize SMAMPs containing triazoles. (Modified from [18])

Polyethylenimines

Polyethylenimine (PEI) is a synthetic, cationic polymer that is not biodegradable but contains multiple, differently functionalized nitrogens. This allows for a wide variety of chemical modifications toward these amino groups, as the variable substitution levels on the amines of this polymer have different reactivity profiles. For example, alkylation of these polymers was shown to greatly increase the bactericidal activity against *S. aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *E. coli* by over 30% [30]. This strong bactericidal activity is due to the polycationic nature of PEI, as well as the ability of the alkyl groups to greatly

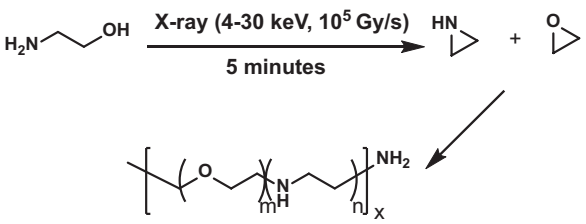


Fig. 3 Synthesis of linear-like PEI-co-PEG via X-ray irradiation. (Modified from [23])

increase association with bacterial membranes. Another property of the polymer is that it can be synthesized as either a branched or linear form, again allowing for flexibility in its functionalization and material properties.

Functionalized PEI polymers have been shown to be potent transfection agents in addition to exhibiting antibacterial properties [31]. This is likely due to its ability to bind to DNA, thus helping gene transfection as PEI-DNA complexes help open up the DNA to gene therapy agents. This property has been translated toward antiviral properties, as it has been shown that PEI can be used to inhibit the activities of papillomaviruses and cytomegaloviruses. Incubation of cells with PEI caused the virus to be unable to bind to the cells, and PEI was also shown to lack cytotoxic effects at the relevant concentrations required for viral inhibition [32]. It is thought that PEI inhibits the viral ability to bind to heparin sulfate proteoglycans that most strains of human papillomavirus rely on.

Synthesis of PEI polymers is relatively straightforward, as it generally involves acid-catalyzed polymerization of aziridines, followed by hydrolysis. Furthermore, it can also be synthesized in conjunction with other polymers, such as PEG, to form copolymers in an efficient and quick manner, as shown in Fig. 3 [33]. This method yields the copolymer with a mono-dispersive molecular weight and can be performed in an aqueous solution, ideal for translation into biological purposes.

Halamines

There are two types of halogen containing polymers that are being studied with respect to antimicrobial properties. The first category is halamines, which are molecules that contain nitrogen-halogen covalent bonds. These are the more common type of halogenated polymers, and the second type refers to polymers with halogens attached to other atoms. Halamines are highly stable in both aqueous and dry conditions, environmentally friendly, and have shown stability over long periods of time. These polymers provide a source of slowly released, active halogen species that inhibit the activity of many types of microbial organisms. They are commonly used as coatings made via electrogeneration or polymerization on the surfaces of textiles and healthcare products [34].

Halamines have been shown to have broad-spectrum activities against microorganisms, and are considered safe for human health [35]. They are also used for their unique ability to recharge halogens, as they can be reacted with halogen donors such as sodium hypochlorite. This gives halamines their renewable nature, meaning that

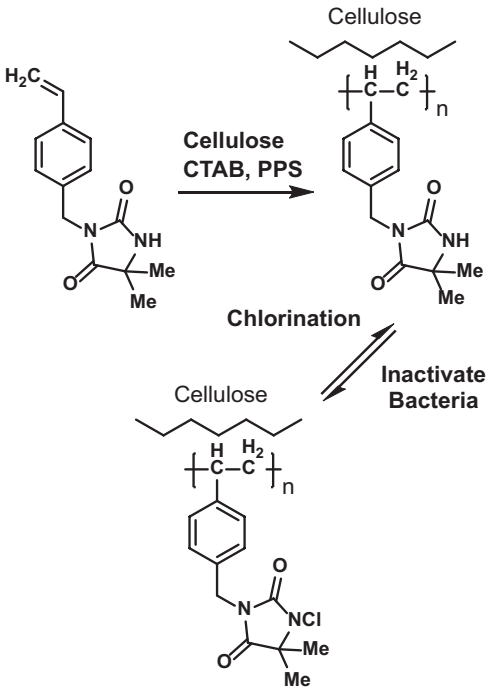


Fig. 4 Synthesis of *N*-halamine biocidal cellulose. Cetrimonium bromide (CTAB), polyphenylene sulfide (PPS)

they can retain their antimicrobial properties indefinitely, even after their initial dose of active halogens has been released [36]. These polymers have been shown to be effective against *S. aureus* and *E. coli* in cotton swatch tests and could regenerate up to 70% of the chlorine lost after washing [37]. The activity of halamines is attributed to the released halogens being active radicals that inhibit or inactivate microbes. This is attributed to the radicals' ability to rapidly penetrate membranes of microorganisms and attack key proteins, as well as DNA. This is seen in iodine, chlorine and bromine, and they are all strongly active against fungi, bacteria, spores and viruses.

The synthesis of halamines generally involves the formation of a covalent bond of an *N*-halamine precursor with the target polymer. For example, cellulose is chlorinated and converted into biocidal cellulose in Fig. 4 [38]. Another common precursor for N-halamines is hydantoin, as it contains two secondary amines that are readily available for reaction, which can then be halogenated with ease. N-halamines can also be copolymerized with monomers such as siloxane, which can then be coated onto cotton fabrics and are highly potent against both gram-negative and gram-positive bacteria [39].

Cytotoxicity of Polymers

As previously mentioned, an important drawback to using antimicrobial polymers often lies in the mechanism of their unique potency, which serves as a double-edged sword. Although these polymers can provide nearly indefinite resistance toward

Table 3 Comparison of DOBAB activity against different human and bacterial cells

Cell type	Cell count	DOBAB conc. at 50% survival (μg/mL)
Kidney epithelial cells	10^5	3400
3T3(cloneA31) fibroblasts	10^4	631
SV40-SVT2 fibroblasts	10^4	631
E. coli	2×10^7	17.7
S. typhimurium	2×10^7	6.3
P. aeruginosa	3×10^7	3.2

growth of microorganisms on materials used for transplants and tissue growth, they also act indiscriminately, thereby targeting and lysing human cells as well. Therefore, when evaluating the effectiveness of certain polymeric materials, selectivity of potency toward human and foreign cells and organisms is a crucial component of determining the usefulness of a polymer.

In terms of selectivity, a key value to look at is the hemolytic capability of the polymers. Due to the negatively charged surface of red blood cells (RBCs), care must be taken when designing polymers that have polycationic charges. It is often seen that the polymers not only target bacterial cell surfaces, but RBCs as well. An example of this can be seen in a brominated ammonium compound, dimethyldioctadecylammonium bromide (DODAB), as shown in Table 3 [40]. As expected, the fibroblasts required roughly five times less material to fall below a 50% survival in comparison to kidney epithelial cells, but the material still shows promising selectivity against multiple strains of bacteria.

Furthermore, it is fairly common to see alkyl chain lengths and molecular weight strongly affect the hemolytic capabilities of the polymers. Figure 5 shows a study conducted on QAS polymers of different alkyl chain lengths and different molecular weights [41]. The general trend is that the more hydrophobic the molecules are, the more hemolytic activity they show, but increasing the molecular weight of the polymer offsets this to a degree.

Future Directions

Tissue engineering is an emerging interdisciplinary field that combines various disciplines, including chemistry, biology, and material science [42]. Although the current approach toward scaffold design principally utilizes polyhydroxyl acids due to their degradation profile for controlled drug release, the combination of drugs and scaffold material is a promising direction for the field [43]. The long-term goal would be to create scaffolds that have minimal infection risks without relying on the degradation of the scaffold itself for timed release. Toward this endeavor, the field has yet to design polymers with sufficient material and antibiotic properties that pose no significant threat to the surrounding cells and tissues.

At the present, a viable subset of AMPs is quaternary ammonium salts due to their broad spectrum antimicrobial activity and long-term biocidal efficiency [44].

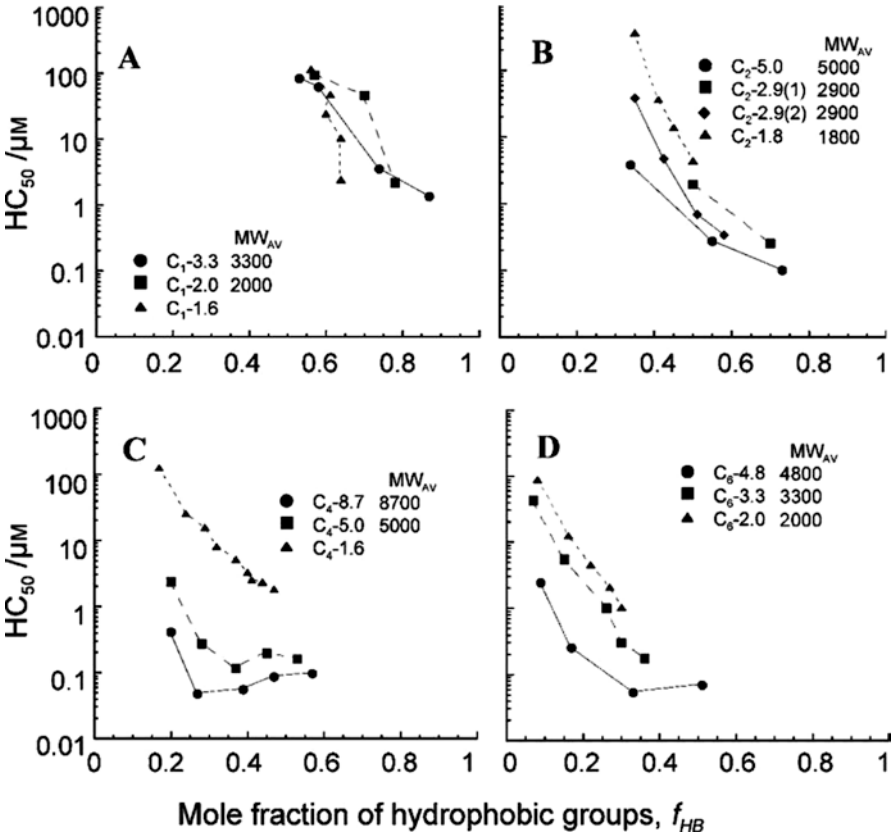


Fig. 5 Comparison of alkyl chain lengths and molecular weights to hemolytic ability (HC_{50}). (Modified from [29])

Much effort has been directed toward improving the biocompatibility of such AMPs, as they have generally proven toxic at high concentrations to nearby erythrocytes. Chitosan derivatives containing QAS moieties have been explored for their relatively low toxicity and flexible material properties [45]. Although these polymers have been tested in vitro, many of the synthesized materials have not been implemented or characterized in vivo. In addition, the long-term viability of these materials as scaffolds has been largely untouched, despite their long-lived antimicrobial properties.

Another subset of antimicrobial materials involves the application of peptides or peptoids due to their antibiofilm properties. These molecules exert substantial effects toward biofilm prevention and dispersal, as well as direct killing of biofilm cells [46–48]. Their biocompatibility is also not of concern as these peptides often have human origins [49]. However, these molecules are not suitable toward scaffold construction and instead could serve as material coatings, for example, which have a propensity to require maintenance over time. Lastly, the cost-effectiveness of bulk manufacturing of these peptides is of concern, causing these coatings to remain elusive in practical applications [50].

Conclusion

There are a wide variety of polymers and molecules that have been synthesized over recent years to exhibit strongly antimicrobial properties. The biggest setbacks toward application of these polymers are cost of production (generally for protein mimics), renewability and degradation rate, and cytotoxicity. However, there are promising polymers that have been functionalized to ameliorate these concerns, and both engineering and chemical advances have pushed our ability to generate these molecules with both financial and temporal expediency.

The direction of the AMP field seems to be trending toward protein mimics or antimicrobial peptides, as well as a hybrid of multiple subsets of AMPs. These types of polymers are naturally biocompatible and biodegradable, and generally avoid the cytotoxic pitfall that other types of polymers fall into. The major issue for protein-inspired antimicrobial molecules is their production costs. However, due to the rapid rate of advancement in biological sciences, it is only a matter of time before affordable synthetic paths or large-scale bio-production of these molecules becomes feasible, thus facilitating the route toward commercial application of these polymers. At the same time, the need is great and many opportunities exist for the entry of alternative and creative solutions to identify and develop antimicrobial treatment and prevention strategies.

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