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Infectious Disease: Connecting Innate Immunity to Biocidal Polymers

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Abstract

Infectious disease is a critically important global healthcare issue. In the U.S. alone there are 2 million new cases of hospital-acquired infections annually leading to 90,000 deaths and 5 billion dollars of added healthcare costs. Couple these numbers with the appearance of new antibiotic resistant bacterial strains and the increasing occurrences of community-type outbreaks, and clearly this is an important problem. Our review attempts to bridge the research areas of natural host defense peptides (HDPs), a component of the innate immune system, and biocidal cationic polymers. Recently discovered peptidomimetics and other synthetic mimics of HDPs, that can be short oligomers as well as polymeric macromolecules, provide a unique link between these two areas. An emerging class of these mimics are the facially amphiphilic polymers that aim to emulate the physicochemical properties of HDPs but take advantage of the synthetic ease of polymers. These mimics have been designed with antimicrobial activity and, importantly, selectivity that rivals natural HDPs. In addition to providing some perspective on HDPs, selective mimics, and biocidal polymers, focus is given to the arsenal of biophysical techniques available to study their mode of action and interactions with phospholipid membranes. The issue of lipid type is highlighted and the important role of negative curvature lipids is illustrated. Finally, materials applications (for instance, in the development of permanently antibacterial surfaces) are discussed as this is an important part of controlling the spread of infectious disease.

1. Introduction

Infectious disease remains a critically important global healthcare issue. The rapid development of bacterial resistance, to even our most powerful antibiotics is increasing at an alarming rate. About 2 million people acquire bacterial infections in U.S. hospitals each year, and 90,000 die as a result [1,2]. Also 50,000 deaths per year are attributed to catheter infections in the U.S. alone. Resistant pathogens often require extended hospital stays leading to higher healthcare costs of nearly 5 billion dollars annually in the U.S. Additionally, escalating occurrences of community-acquired outbreaks beyond hospitals, from elementary schools to restaurants and athletic arenas, have been in the news recently.

In general, this problem is multifold. At one end of the spectrum, a person who acquires a microbial infection is treated through a course of antibiotics. Antibiotics, also known as antimicrobial drugs, are compounds that fight infections by destroying or inhibiting growth of bacteria and other microorganisms. After their discovery in the 1940's they transformed medical care and dramatically reduced illness and death from infectious diseases. However,

over the decades, bacteria have developed resistance to these drugs. The tremendous success of early antibiotics, led by a rush to discover novel antimicrobial agents, has lulled researchers into a false sense of security and shockingly only two new classes of antibiotics have been introduced since 1968 according to the Food and Drug Administration (FDA) (List of abbreviations can be found in the Appendix) [2]. Today, virtually all important bacterial infections in the U.S. and throughout the world are becoming antibiotic resistant and some would pose that the current drug pipeline is woefully weak to address these problems. Consequently, antibiotic resistance has been called one of the world's most pressing public health problems [1,3].

At the other end of the spectrum, increased effort has focused on materials that limit bacteria colonization, in other words, biofilm formation, on their surfaces in an effort to prevent the rampant growth of bacteria and the transmission of infectious organisms. This approach has made it into our everyday lives as a trip to the supermarket will show with the ever increasing number of "antimicrobial" products. These two fields, pharmaceutical antibiotics and antiseptic materials, have traditionally existed completely independent of each other but the development of antimicrobial macromolecules (AMMs) offers one bridge between these two disparate areas of important research. These novel AMMs can mimic the biological activity of the natural host-defense peptides (HDPs) and hold promise for novel therapeutics and new materials to prevent the spread of infectious disease. AMMs (both polymers and oligomers) that mimic the functions of proteins may fill a critical need for both 1) new classes of antibiotics that do not elicit drug-resistant bacterial strains and 2) novel materials that possess antimicrobial activities yet are benign to human cells. So while the antibacterial properties of cationic poly-amino acids [4,5] and cationic polyelectrolytes (polymer biocides) [6–10] have been studied since the early 1950's and mid-1980's, respectively, *selective* antimicrobial polymers, in other words antimicrobial polymers that are non-toxic to mammalian cells, have not been as well explored. Interest in the development of novel selective antimicrobial polymers is growing and many potential products using these types of molecules can be imagined. A short list includes sterile clothing, biocompatible medical materials (catheters, sutures, indwelling devices, prosthetics, etc.), air filters, and coatings that resist biofouling.

Interest in the HDPs and their mimics has grown as increasingly more sophisticated biophysical instrumentation and techniques have advanced the understanding of the mechanisms by which these bioactive molecules kill bacteria. What has been revealed time and time again is that many HDPs and their mimics act by general disruption of the cell membrane. It should also be mentioned that other intracellular targets may be important; however both are non-specific targets compared to classical antibiotics. As stated above, the problem of resistance (how a microorganism undermines the killing mechanisms of antimicrobial agents) is of grave concern and resistance evaluation will no doubt be a standard assay for novel AMMs coming down the drug and materials pipeline. In contrast to the direct membrane disrupting action of AMMs, prototypical small molecule antibiotics such as the penicillins and vancomycin, commonly used as the drug of last resort, actually transverse the cell membrane and operate via very specific intracellular targets. Unfortunately, resistant strains have emerged and these strains now commonly plague hospitals. For instance methicillin-resistant *Staphyloccus aureus* (MRSA) have acquired a gene that produces a neutralizing penicillin binding protein and vancomycin-resistant *Enterococci* (VRE) have mutated NAM/NAG peptide subunits, the main target of vancomycin. The HDPs, and particularly non-peptidic HDP mimics, with their general mode of membrane disruption, may provide an answer to the resistance problem since in these cases a particular molecule is not targeted; whereas it has been shown that bacteria can easily effect a slight change in a drug's specific target via gene acquisition or protein mutation as observed with MRSA and VRE. More on this topic will be discussed in section 4 and in particular sections *4.1.3. Antibacterial mechanism* and *4.1.4. Antimicrobial agent resistance by pathogens*.

Due to the many groups now conducting research in antimicrobial polymers and the quick expansion of this field, certain terms have seen their definition evolve over the last decade. Therefore it would be useful to clear up several definitions used for the purpose of this review. First, the terms “antimicrobial agent” and “antibacterial agent” are rarely distinguished in the literature and will be used interchangeably in this review. These terms will mean any agent that suppresses growth or kills pathogenic microorganisms. Some AMMs discussed in this review will have activity against non-bacterial entities such as fungi, viruses, and yeasts, as well, and these examples will be pointed out. Second, it appears that “disinfectant” and “biocide” are also used interchangeably in the literature and here we will follow whatever term is used by a particularly cited article when applicable. In general, polymer biocides are considered to be polymers that kill all living organisms and often the main motivation to make polymer biocides is to create highly sterile materials or surfaces. Third, the ability of antimicrobial agents to selectively target bacteria cell membranes over mammalian cells may, as stated previously, be advantageous for materials that have intimate and continuous contact with mammalian cells. So in these cases “selective” or “non-toxic” antimicrobial agents are desired. It seems though that once a biocide’s toxicity (to mammalian cells) is evaluated, it can be deemed selective if it meets certain criteria. For instance, the concentration at which 50% of red blood cells (RBCs) lyse, or hemolytic concentration (HC_{50} or just HC) is a standard measure of toxicity and the ratio between the minimum inhibitory concentration of bacteria (MIC) and HC can be a measure of selectivity. (MIC is the minimum concentration, many times expressed in μg/mL, of compound required to inhibit bacteria growth by, typically, 90 – 100%. Although some studies report MICs at 50% inhibition) There are other methods available to show non-toxicity including testing on different mammalian cells, such as hepatocytes and fibroblasts, or directly conducting allergy experiments on mammals such as mice or guinea pigs. In this review certain AMMs will be characterized as selective antimicrobial agents while other AMMs will be considered as polymer biocides. Once again, both classes are important research areas and the pursuit of either hinge on the researchers’ envisioned final application. Nevertheless, it appears the community would be well served by the careful use of terms “antimicrobial” and “biocidal” or “disinfectant”.

Fig. 1 below, using representative compounds from several classes, tries to illustrate the, admittedly hazy, distinction between selective antimicrobial molecules and biocides. This figure was compiled with some trepidation because, although a popular technique, not all research groups use MIC experiments to demonstrate antimicrobial activity and not all AMMs were evaluated via the MIC/HC ratio for selectivity. Further complicating such a comparison is that MIC experiments from different groups often reveal the use of various bacterial strains, incubation times, and viable cell counting techniques among the many available protocols. However, Fig. 1 serves as an opportunity to introduce the variety of structures in the field of AMMs and as a flexible example of how one can view these AMMs; it is not intended as a strict classification system.

One strategy that has been successful in developing selective AMMs is to design polymers and oligomers that are facially amphiphilic (FA) [11]. A goal in this emerging field is to mimic the architecture and more importantly the activities of host-defense peptides HDPs [12,13]. HDPs are natural antimicrobial peptides (AMPs), many of which display a novel FA conformation in which non-polar and charged groups extend from opposite sides of the final folded conformation. This FA conformation is believed to be central to the activity of HDPs in which bacteria are killed rapidly by membrane disruption yet there is no toxicity against the host. Also this FA conformation may be achieved by AMMs that are relatively flexible in solution but become organized via self-assembly and/or binding to the lipid membrane. These situations will be discussed in the context of the wide array of biophysical techniques used to study the dynamic behavior of AMMs and the membranes they perturb.

This review will highlight AMMs whose putative FA architecture (whether in solution or at membrane interfaces) drives their antimicrobial activity. It will not, however, discuss HDPs in any detail since these important peptides have been routinely reviewed [12,13]. This is not to diminish the historically important work on HDPs, or the current efforts in this field. Quite the contrary, the fantastic work on HDPs has provided much of the inspiration for AMMs. However, bridging the areas of HDPs, biomimetic oligomers, FA polymers, and biocidal polymers is an important goal of this review. Many of the AMM systems have been synthesized and/or studied since 2000 and the state-of-the-art biophysical techniques applied to these AMMs along with the reported antimicrobial effectiveness of new materials incorporating AMMs warrants a review of the field. Based on the groundbreaking work of early synthetic AMMs and the current state of understanding, it is our opinion that the fully rational design of highly potent and selective AMMs from practically any desirable polymeric backbone will be achieved in the near future for use as antimicrobial drugs and in materials. As new antibiotics, the future looks extremely promising. These oligomers lend themselves to the diversity of organic chemistry so that pharmacological problems *in vivo* can quickly be remedied. The materials area has a whole separate list of requirements; yet coatings which prevent bacterial growth have been demonstrated.

2. Scope of the review

In sum, the scope of this review will cover *antimicrobial macromolecules whose biological activities are influenced by the amphiphilicity of the polymer or oligomer as a whole rather than the activity of an antimicrobial moiety either embedded or covalently attached.* Three notable systems beyond the scope of this review are 1) polymers embedded with silver ions [14,15] for the generation of fibers [16,17] and medical implants [18–20], 2) polymers with known antibiotics either embedded or covalently attached to the backbone or termini [21–23], and 3) chlorine releasing polymers, for example the *N*-halamines, commonly used in disinfectants or fibers that can be “recharged” to be sterile [24–35]. Many of the systems that will be considered here have a proven mode of action involving membrane disruption and not interaction with a specific cellular target essential for bacteria survival, which is common to many antibiotics and other pharmaceuticals. Much of the research reviewed utilizes structurally diverse sets of AMMs in order to investigate closely the effect of amphiphilicity on biological activity. In many reports, active polymers and their oligomeric analogues were proven to be membrane-interacting agents and their activities can indeed be traced to a careful balance of hydrophilic and hydrophobic moieties. With that said, striking this balance remains tricky especially when pursuing antimicrobial polymers and materials with strong propensities to kill bacteria but not mammalian cells. Obtaining a better understanding of this delicate balance will be an important goal for future research with one ideal objective being the ability to quantify this balance in a useful physicochemical parameter analogous to log p, log K_{OW}, or the hydrophobic moment, which is known for small peptides.

As stated above there are three notable antimicrobial polymer systems beyond the scope of this review. In each of these cases the bactericidal action stems from the antimicrobial agent rather than the polymer itself, which functions mainly as a mode of support and delivery. Therefore it can be thought that these polymers are “endowed” with antimicrobial activity whereas the AMMs, focused on in this review, have “inherent” antimicrobial activities based on their physicochemical properties and how their amphiphilicity affects their interactions with the membrane.

The next section “Antimicrobial macromolecules arranged by chemical structure” will survey the literature and give a brief overview of each molecular system divided into three classes, antimicrobial peptidomimetics, facially amphiphilic antimicrobial polymers, and biocidal cationic polymers. Following this part, “Membrane perturbation and biophysical techniques”

will summarize several exciting biophysical techniques that have been recently employed to characterize the interactions of AMMs with phospholipids bilayers. Close attention will be paid to work investigating the emerging importance of lipid composition and AMM ordering within lipid bilayers when elucidating the mode of action. The section on "Applications in materials" will report on some of the current pioneering efforts in generating and evaluating new sterile and antibacterial materials using AMMs.

Our goals for this review are 1) to bridge the currently disparate research areas of natural HDPs with selective AMMs and biocidal polymers, 2) to highlight the emerging concept that lipid type is an important means of selectivity for AMMs, 3) to highlight the role of peptide to lipid ratios (*P/L*) in which biophysical studies are usually conducted at *P/L* ratios lower than the MIC values suggesting these studies can capture the physical mechanisms involved in cell death, 4) to illustrate how AMMs work in solution, at the membrane interface, and as materials, and 5) to show secondary and tertiary structure is not necessary for selective biological activity.

3. Antimicrobial macromolecules arranged by chemical structure

This section will briefly characterize many of the main AMMs shown in Table 1. In all cases the efficiency of a compound's ability to halt bacteria growth (bacteriostatic activity) or kill bacteria (bactericidal activity) is used to characterize a compound's "antimicrobial activity". MIC (minimum inhibitory concentration) studies, which entails monitoring bacteria growth is by far the most popular method to screen potentially new AMMs. On the other hand, studying the biological activity of materials often involves quantifying bacteria colonies directly on the polymer or polymer treated surface to determine bactericidal efficiency and the prevention of biofilm growth. Due to the variability of protocols and criteria that exist to determine antimicrobial activity (different bacteria strains, growth medias, incubation times, and so on), caution has to be taken before comparing one system to another.

3.1. Antimicrobial peptidomimetics

Most antimicrobial peptidomimetics are typically discrete non-natural oligomers whose units are in many cases, connected via amide bonds. Much of the synthetic interest in peptidomimetics comes from the fact that these oligomers can present a wide variety of side chains which could be chemically identical to those found in natural peptides, but along an artificial backbone. The consequence of this hybrid structure is that peptidomimetics can mimic the conformation and functionality of biopolymers yet are not limited by the side chains of the main twenty naturally occurring α-amino acid building blocks. Also the artificial backbone makes most peptidomimetics resistant to degradation enzymes thus increasing the stability of peptidomimetic drugs in the body. The β-peptides are by far the most well-studied peptidomimetics and a review focused on their antibacterial peptide mimicry was published in 2002 [36]. Finally, since antimicrobial peptidomimetics have been studied for nearly a decade now, and HDPs for even longer, there exists a collection of biophysical techniques in the literature that can be applied to the study of newer bioactive agents such as the amphiphilic AMMs.

3.1.1. β-peptides—DeGrado and coworkers reported in 1999 the *de novo* design of antibacterial β-peptides that were highly active against *E. coli* but were also hemolytic (lysis of RBCs occurs) [37]. Generally, compounds that are not hemolytic at relative concentrations versus their antimicrobial activity are typically deemed "selective" and have the potential for promising therapeutics that may kill bacteria but not mammalian cells. Once again, it is worth mentioning that caution has to be taken since the criteria for selectivity can vary from paper to paper. In addition, hemolysis, by far the most common measure of mammalian toxicity, may not be the best predictor [38]. Extension of this original work did result in antimicrobial and significantly non-hemolytic analogues such as **1** in Fig. 2 [39]. The authors posited that the

lack of selectivity of their original β-peptides arose from excessive hydrophobicity. Seebach and coworkers have also extensively studied β-peptides for antimicrobial and hemolytic activity and demonstrated that "β^2/β^3" type peptides, such as **2**, can also display selectivity [40,41].

In 2000, Gellman and coworkers reported work on antimicrobial 17-mer β-peptide, **3**, that formed helices just like the class of HDPs known as the magainins, which have 20 – 30 residues [42]. This particular β-peptide, termed "β-17" was reported to be effective against two pathogens that are resistant to common antibiotics plus they are not hemolytic. Interesting differences in the mode of action between β-17 and magainin-II with various lipids were observed [43]. Close analogues of β-17 were studied where the oligomers still contained cyclic side groups but the position of the secondary amine, the charge ratio, and the monomer sequence were systematically varied and overall the authors found that a 40% "cationic face" along the helical cylinder was best for activity [44]. Other work included a series of β-peptides with the incorporation of flexible acyclic residues which revealed that variation in helical propensity does not lead to significant changes in antibiotic activity for this system [45]. The most recent work from Gellman and coworkers investigated oligomers containing a 1:1 pattern of α- and β-amino acids supporting the hypothesis that having a globally amphiphilic helical conformation is *not* a prerequisite for selective antibacterial activity [46,47]. This hypothesis, that a secondary or tertiary structure is not necessary for selectivity, has been demonstrated with Shai's D/L peptides [48] and by Tew's report on phenylene ethynylene oligomers [49]. One interesting observation related to interaction with membranes showed phase segregation of anionic and zwitterionic lipids induced by one of these α/β-peptides but not a close structural analogue [50].

3.1.2. Peptoids—Peptoids, also known as oligo-*N*-substituted glycines, have garnered interest and the group from Chiron Corporation reported a combinatorial process for antimicrobial peptoids that resulted in 65 pools of 13 compounds [51]. Compound **4**, an example of a small non-folding oligomer, in Fig. 3, was subjected to thorough analysis of its antimicrobial activity against a broad spectrum of microorganisms, its membrane disruption activity, and its *in vivo* effectiveness in mice [52]. In the exploration of longer peptoids, Barron and Patch synthesized selective peptoid mimics of magainin-II, such as **5**, which represented in general the first report of a folded, bioactive peptoid [53]. More recently, another peptoid, **6**, was shown to maintain fouling resistance of Ti surfaces for several months [54].

3.1.3. Cyclic peptides—Cyclic peptides studied for antimicrobial activity were constructed with six or eight residues of D (rare isomer) and L (naturally abundant isomer) α-amino acids. These peptidomimetics caused rapid cell death of bacteria compared to mammalian cells apparently by increasing membrane permeability and disrupting membrane ion potentials [55]. Since nanotube formation was proposed as a possible step in membrane disruption, the surface of the bioactive peptide nanotube aggregate could be diversified just by mixing and matching different cyclic peptides. The intravenous efficacy of several derivatives were tested in mouse thigh infection models with promising results [56]. Also bacteria were unable to easily develop spontaneous resistance upon prolonged exposure to the peptides at sublethal concentrations, a feature expected for AMMs acting via membrane permeabilization.

3.2. Facially amphiphilic antimicrobial polymers and oligomers

The widespread success of peptidomimetics is well-documented. In general, they are small molecules of well-defined shape and size which are very familiar to the pharmaceutical industry. The antimicrobial peptidomimetics just discussed fall more or less into this category. One important distinction is that their sizes extend beyond those of typical peptidomimetics. For example, depending on the counter ion weight, β-peptides, **1** and **3** are ~ 1700 and ~ 2000

g/mol, respectively, peptoid, **5**, is ~ 2000 g/mol, and magainin-II, is ~ 2500 g/mol. Of course, the many diverse biological functions of proteins are well-known; At the same time proteins are even larger structures. Therefore it became clear that developing polymers with biological activity arising from the inherent functionality of the repeating group chemistries and macromolecular connectivity could be extremely powerful. This area has been mostly overlooked but is ripe for success as demonstrated by the ability to afford polymeric backbones with antimicrobial yet non-toxic properties as will be illustrated next.

Polymers in general may be well-suited for the discovery of antimicrobial agents due to their relative ease of synthesis and accessibility to a wide range of molecular weights (MWs) compared to peptidomimetics, which are synthesized typically in a step-wise fashion. Easily available AMMs as permanently sterile materials can benefit from the available literature on polymer processing and the blending of different synthetic polymers. Some of the AMMs with conformationally restrictive backbones were proven, via X-ray for example, to adopt a FA conformation. On the other hand, it is difficult to predict the conformation of other AMMs, for example, random copolymers of non-polar and charged monomers along a more flexible backbone. In all backbone types though, selective derivatives have been successfully identified and this fact distinguishes the FA polymers from most of the biocidal cationic polymers, discussed later in section 3.3., which are typically developed to have strong disinfectant activities although they are not more active than the AMMs discussed in this section. Therefore, besides materials application, some of the FA antimicrobial oligomers are potential antibiotics due to their excellent selectivities.

3.2.1. Arylamide oligomers and analogues—Perhaps the first report to branch outside of the peptidomimetics was from the groups of Tew, Klein, and DeGrado who reported a new class of FA arylamide polymers that utilize hydrogen-bonding to produce conformationally stiff backbones. For instance, compound **7** in Fig. 4, designed with the aid of density functional theory (DFT) computed torsional potentials, showed promising activity against a range of bacteria although they were hemolytic near the MIC and thus not very selective [11]. Nevertheless, this report represented an important expansion of the molecular structures. A set of arylamide oligomers with side groups of various hydrophobicities were later synthesized and highly selective compounds, such as **8**, were identified [57]. Computational investigations resulted in a new set of parameters (force constants and bond angle potentials) that accurately describe the dynamical behavior and a strong link was established between these functions and the conformation of the polymers at lipid bilayer interfaces [58]. Urea-linked versions of these oligoaryls also showed better antimicrobial activity than a magainin derivative [59].

Further FA design refinements included replacement of the center aryl moiety with a pyrimidine ring as shown by AMM **9**. This change led to additional hydrogen-bonding as evidenced by NMR and X-ray and this derivative displayed excellent antimicrobial properties with modest selectivities [60]. One biophysical study on these arylamides, that will be highlighted in the next section, includes sum frequency generation (SFG) vibrational spectroscopy which allowed real-time *in situ* monitoring of ordering changes in both leaflets of a single-substrate supported lipid bilayer in the presence of an antimicrobial pyrimidine derivative similar to **9** [61].

3.2.2. Phenylene ethynylenes—Tew and coworkers have elucidated the conformation of FA polymers, in particular a series of amphiphilic phenylene ethynylenes, such as **10** in Fig. 5, in solution and at the oil-water interface [62,63]. Based on the facial amphiphilicity of the extended conformation observed with Langmuir experiments [64], the authors envisioned a promising membrane-disrupting agent just like many of the facially amphiphilic natural HDPs [49]. These phenylene ethynylenes were the first polymeric AMMs reported with selectivity. In addition they were the first non-peptide system to show that antimicrobial activity was not

dependent on a folded helical or beta-sheet conformation. Most recently, phenylene ethynylene, **11** (m = 2), was shown to be extremely effective against a very large panel of microorganisms, including antibiotic-resistant strains, and importantly was found to be remarkably non-toxic to RBCs, fibroblasts, and liver cells [38]. Small angle X-ray scattering (SAXS) and vesicle leakage studies have thus far illustrated that lipid composition, and not just charge, is an important factor in selectivity; plus the overall concentration of a given lipid was shown to be another important factor [65]. SAXS and fluorescence microscopy experiments also indicated that this AMM induces pore formation with 3 nm holes. [66]. The section on biophysical techniques will elaborate more on these studies. Lastly, imaging of polymer-treated polyurethane samples showed strong prevention of bacteria growth on the surface in preliminary materials studies [67].

3.2.3. Polynorbornenes—While the most selective arylamide and phenylene ethynylene AMMs are low MW oligomers (< 3000 g/mol) rather than large polymers, the activities and selectivities of a set of polynorbornenes, such as **12** in Fig. 6, appeared to be unusually MW independent in a relatively wide MW range of 1600 – 25,000 g/mol [68]. This particular set of polynorbornenes also places a non-polar group and a charged group on every monomer so that the polymer, at least at the monomer level, is FA. Satisfyingly, copolymerizing 10% monomer of the most active but non-selective homopolymer, (**12b**), with 90% of the monomer from the most selective but only moderately active homopolymer, (**12a**), resulted in “the best of both worlds,” a non-hemolytic polymer that did not sacrifice antimicrobial activity. In other work, a guanidinium functionalized homopolymer, **13**, was found to have superior activities and selectivities over its primary amino counterpart and the effect of separating the charged and non-polar groups, as in **14**, is currently being investigated [69].

3.2.4. Polymethacrylates—Through the free radical random copolymerization of *N*-(*tert*-butoxycarbonyl)aminoethyl methacrylate and butyl methacrylate, over different composition ratios, DeGrado and coworkers have created antimicrobial polymethacrylates with a range of hydrophobicities [70]. Antimicrobial and hemolysis studies indicate that the properties of these types of polymers can be tailored. Plus, due to the flexibility of the backbone, the authors stated that a polymer interface can induce a FA conformation in a large enough population of the polymers to lead to antimicrobial activity, a similar conclusion arrived by others [46,47,71].

In recent work, probing the dynamic flexibility of polymethacrylates in aqueous solution and at water-lipid interfaces, thirty-four copolymer sequences were set up for molecular dynamics and details of the insertion process were reported [72]. The authors showed that selectivity could be fine-tuned by controlling the overall hydrophobicity, the chemical composition, and even the sequential order; most of these findings are in agreement with experimental observations.

3.3. Biocidal cationic polymers

A comprehensive 2001 review by Tashiro on antibacterial macromolecules focused mainly on biocidal cationic polymers containing functional groups such as biguanide, quaternary ammonium salts, quaternary pyridinium salts, and phosphonium salts [73]. Some of these systems will be considered briefly here while those that have found significant use in materials will be discussed in section 5.

3.3.1. Polymers with biguanides—Polyhexamethylene biguanides (PHMBs), **15**, in Fig. 7, is a widely used environmental biocide and contact lens disinfectant that has been demonstrated to have antifungal activity as well [74,75]. Several early antimicrobial studies on this cationic polymer appeared in the 1980’s. For instance, Gilbert and coworkers investigated PHMB of two to greater than ten units and showed that growth inhibition and

bactericidal activity increased with polymer length. The authors claimed that cytoplasmic membrane damage is a direct result of biocide action, rather than mediated through the induction of autolytic enzymes [10]. Early studies by Tazuke and coworkers showed that with the addition of PHMB, fluorescence polarization of diphenylhexatriene embedded in negatively charged bilayers was reduced to a great extent, especially in the gel phase [9]. The authors interpreted this result in terms of induced expansion and fluidization of the bilayer, which enables the probe molecule to undergo less-hindered torsional motion. They also drew similarities in the mode of action between PHMB and polymyxin B, a natural biocidal peptide-based molecule commonly used for comparison. Later, the same group investigated polymethacrylates having biguanides as a pendant group from monomer, **16**, and studied MW fractions obtained by gel filtration [7]. Based on their studies, the authors asserted that while increasing MW enhances cell adsorption, high MW polymers are impeded from diffusing through the cell wall, therefore an optimum MW exists.

3.3.2. Oligoguanidines—Polycondensation of guanidinium salts (chloride or carbonate) and diamines provided complex mixtures of linear and cyclic oligoguanidines between 540 and 1250 MW and their MICs were determined against several microorganisms [76,77]. An average MW of 800 afforded as good or better activities than Vantocil, a standard disinfectant, while lower MWs and the use of longer diamine chains or guanidinium carbonate salts resulted in decreased activity [76].

3.3.3. Polymers containing quaternary ammoniums or quaternary pyridiniums —A popular strategy for the development of antimicrobial polymers is to append quaternary ammonium or quaternary pyridinium groups to different polymer backbones, in particular to polymethacrylates and polystyrenes.

Mathias and coworkers have synthesized active methacrylate polymers containing DABCO, **17** in Fig. 8 [78]. Along with the MIC, the minimum bactericidal concentration (MBC) was also determined and showed that an increase in the alkyl chain length from four to six carbons significantly increased the antimicrobial activity. Post-polymerization functionalized methacrylate polymers, **18**, of around 32,500 MW and having a greater than 90% quaternized conversion were also studied [79]. Interestingly, while all polymers of this type were effective, the antibacterial activity against *S. aureus* improved with an increase in the alkyl chain length of the ammonium groups, whereas the activity against *E. coli* worsened with increasing alkyl chain length. In another example, photopolymerization of non-antimicrobial quaternary ammonium monomers can lead to crosslinked yet active polymers in some cases [80]. Lastly, antimicrobial copolymer, **19**, containing quaternized pyridinium groups and *N*-isopropylacrylamide (NIPAAm) have been reported [81]. Here, the quaternized copolymers with high NIPAAm content showed temperature responsive behavior including lower critical solution temperatures (LCSTs) and the effect of alkyl chain length was probed. The quaternized water-soluble copolymers showed very good antibacterial activities using both the broth dilution and spread plate methods. To our knowledge no selectivity data for the above polymers are available at this time.

Jérôme and coworkers have published a series of reports on antimicrobial particles, blends, and coatings [82–84]. In terms of the synthesis and biological evaluation of their antimicrobial polymers in solution, they have studied a block copolymer, **20**, in Fig. 9, (one of the few block copolymers in this field) of poly(ethylene-co-butylene) with poly(dimethylamino) ethylmethacrylate [85]. ATRP synthesis involved catalyst substitution of CuCl for CuBr and notably, addition of excess $CuCl_2$ as a deactivator to the catalyst system to afford these new antibacterial surfactants.

Kenawy and coworkers have also developed several routes to access quaternary ammonium or phosphonium salts on a polymethacrylate [86] or a polyamide, **21**, backbone [87]. For both series, the polymer with the tributyl phosphonium salt was observed to be the most effective against bacteria and fungi.

Polystyrenes derivatized with quaternary ammonium groups have been explored as well. Tazuke and coworkers looked at various poly(trialkylbenzylammonium chlorides) of type **22** and found those that possessed the dodecyl chain exhibited greater antimicrobial activity [8]. Gellman and coworkers later showed that a dimethylamino functionalized polystyrene has greater antimicrobial activity than the quaternary ammonium derivative suggesting reversible protonation has some impact on biocidal activity [88].

Several groups have explored the antibacterial properties of alkylated pyridine (also known as quaternary pyridinium) polymers afforded by the near quantitative quaternization of the pyridine pendant groups after polymerization. One of the earliest examples comes from Tazuke and coworkers who prepared polyesters and polyamides with pendant *N*-alkylpyridinium groups, such as **23** in Fig. 10 [89]. While polymers with longer pyridinium alkyl groups were generally more active, it was also observed that an intermediate backbone spacer length of 4 carbons between these pyridinium units was found to give the most antimicrobial activity. Another early study comes from Kawabata and Nishiguchi who probed linear versions of poly (benzylvinylalkyl pyridinium), **24**, with different degrees of polymerization and a quaternization yield of 99% [90]. The authors found better activity against Gram-positive than Gram-negative bacteria for their system.

More recently, Gao and coworkers synthesized random copolymers, **25**, of acrylamide and vinyl pyridine of varying MWs and pyridine content, which were subsequently quaternized [91]. The authors studied the mechanism of action using the method of measuring the activity of galactosidase and TTC-dehydrogenase present in living bacteria. They found that their polymer “antibacterial ratio” (number of original cells minus viable cells divided by number of original cells) reached 100% under the conditions of a concentration of 20 mg/L and a contact time of 5 min.

In other studies with polystyrene-random-pyridinium copolymers, Li and coworkers, showed that their polymers possessed a strong ability to kill Gram-positive and Gram-negative bacteria, and yeasts [92]. The toxicity had also been appraised and in acute stimulation and allergy experiments, allergic reactions on the skin were not observed in tested animals. Additionally, SEM photographs were taken of surfaces in contact with bacteria laying the ground work for antibacterial materials studies. These researchers also synthesized insoluble crosslinked pyridinium-type polymers, **26**, whose extent of quaternization was measured by a combination of nonaqueous titration and elemental analysis [93]. Block and random copolymer versions of styrene/quaternized vinyl pyridine were compared by Yoon and coworkers [94]. Interestingly, they observed that the block copolymer showed superior activity over the random analogue and posited that the content of quaternized vinyl pyridine units at the polymer/solution interface is higher for the block copolymer.

4. Membrane perturbation and biophysical techniques

Natural HDPs, which are an essential component of the innate immune system, have attracted increased attention due to their novel mechanisms of action. Despite significant research, these peptides have important liabilities associated with *in vivo* toxicity, poor tissue distribution, as well as difficulties and high cost of production. In the above section, it was discussed how different research groups sought to develop simpler synthetic scaffolds that capture the broad spectrum antibiotic activity of HDPs to reduce these liabilities. Understanding the *structure-function correlation* of HDPs should lead to improved synthetic scaffold designs with more

potent antibacterial activity and minimum toxicity. Because interactions of HDPs/AMMs with lipid membranes that compose the cell surface strongly influence the antibacterial activity of HDPs/AMMs, this section, will highlight notable biophysical techniques, which have provided significant insight into the fundaments of amphiphilic macromolecule/membrane interactions.

4.1. Lipid membrane and molecular mechanism

Lipids are one of the major components of biological membranes. In 1925, one of the earliest biophysical studies reported lipid extraction from erythrocyte membranes and measured the area covered by these lipids at the air-water interface [95]. Bilayer lipid organization, which provides a permeability barrier between the exterior and interior cell compartments, has remained a dominant theme in understanding the organization and function of biological interactions.

4.1.1. Bacterial and mammalian cell membranes—It is well known that most HDPs and AMMs act by disruptive interaction on the lipid interface, although the details of binding, insertion, and transport across the membrane are still vague and much remains to be learned. The most interesting feature of HDPs and AMMs is their ability to exhibit higher activity towards bacterial cells over mammalian cells. The origin of this selectivity is believed to rise from predominant interactions of antibacterial molecules with particular lipids depending on their presence and abundance in different membranes. Apart from their differing constituents such as membrane proteins, bacterial cell walls and eukaryotic cell membranes are very distinct with respect to their phospholipid composition (Table 2). Eukaryotic cells, for example human erythrocytes, exhibit a large difference in the lipid composition between the two monolayer leaflets of the cell membrane bilayer. The neutral (zwitterionic) outer leaflet of the asymmetric erythrocyte membrane bilayer is devoid of anionic lipids and composed of 25% cholesterol (CH), 33% phosphatidylcholine (PC), 18% sphingomyelin (SM), and a trace amount (9%) of phosphatidylethanolamine (PE) [96,97]. While the inner leaflet is composed of 10% negatively charged lipid, phosphatidylserine (PS), along with PE (~ 25%), PC (~ 10%) and SM (~ 5%) [95,97,98]. Beyond lipid type, another level of complexity is found in the Gram-negative bacteria cells that posses multiple membrane systems unlike Gram-positive bacteria having only one membrane bilayer (Fig. 11) [95]. Most Gram-negative bacterial membranes (e.g. in *E.coli*) lack cholesterol and have 70 – 80% PE as their most common zwitterionic lipid, but also contain 20 – 25% of negatively charged lipids such as phosphatidylglycerol (PG) and cardiolipin (CL) [98–100]. On the contrary, Gram-positive bacteria cell membranes (e.g. in *B. subtilis*) is mainly composed of anionic lipids, PG (70%) and CL (4%) and a minor amount of PE (12%) [50,101].

This larger percentage of negatively charged lipids on the outer leaflet makes bacterial cells more anionic than eukaryotic cells at the phospholipid level. Although bacterial cells are composed of more negatively charged lipids, they also contain a significantly greater majority of negative curvature lipid, which is another important difference in basic lipid chemistry between bacterial and eukaryotic cells.

4.1.2. General lipid chemical structure, geometry, and conformation in membranes—The ability of lipids to organize into the fundamental bilayer membrane is dictated by their amphiphilic character and intrinsic curvature (C_0), which is controlled by the size of the polar or hydrophilic head group, and the size of the nonpolar or hydrophobic acyl tail shown in Fig. 12(A). Membrane curvature is crucial to create membrane domains and to organize various membrane activities associated with the cells [105]. Membrane curvature dynamically depends upon the modulation or changes in lipid composition and the insertion of foreign molecules into the membrane. How and to what extent membrane curvature is affected by the volume ratio of the phosphate head group and acyl side chains of the single

lipid component, is depicted in Fig. 12(A) and 12(B) [106]. Zwitterionic lipids, PC and SM, prefer the lamellar phase because of their cylindrical shapes; whereas zwitterionic PE lipids prefer to form a hexagonal phase in the membrane at room temperature due to its comparatively smaller head group compare to the acyl tails. Nevertheless, the only difference between PE and PC is that the latter contains three methyl groups on the headgroup nitrogen, whereas the former contains hydrogen. The head group of PE is smaller, such that the width of the PE head group is less than that of the hydrophobic tails, hereby, PE is defined as a negative curvature lipid ($C_0 < 0$) (Fig. 12). The anionic phospholipids PS and PG (and CL when not bound to metal ions) prefer the lamellar phase at neutral pH and physiological ionic strength [107]. The diversity in chemical structure found in lipid type gives rise to differences in hydration, hydrogen-bonding, and charge as well as a geometrical difference in C_0. The corresponding intrinsic curvatures of different lipids are shown in Fig 12.

4.1.3. Antibacterial mechanism—Several models of membrane interaction, such as the *toroidal pore* (also known as wormhole), *barrel stave* (also known as helix bundle), and *carpet* (also known as detergent like) model have been extensively proposed and reviewed for HDPs [104,108–115]. (Excellent illustrated schemes of these three models can be found in a Nature Reviews Microbiology article by Brogden [108].) A less commonly discussed mechanism involves the favorable binding of cationic amphiphilic molecules to the outer layer of the membrane, creating an uneven membrane pressure between the outside and inside of the bilayer. The unequal pressure forces rearrangement to the inner membrane resulting in transient hole formation in the membrane [116,117]. A toroidal pore mechanism was proposed for magainin-2, protegrin-1, melittin, while ceropin and ovispirin exhibit a carpet like mechanism and alamethicin is well known to operate through a barrel stave mechanism [108]. In the toroidal-pore model, antimicrobial molecules insert into the membrane and induce the lipid monolayers to curve continuously forming the pore. According to this model, the antimicrobial molecules remain associated with the phospholipid head group regions of the bilayer. In the carpet model, antimicrobial molecules accumulate on the surface of the lipid membrane with a parallel (in-plane) orientation to the membrane surface. At some critical concentration of the bound antimicrobial, the surface oriented molecules penetrate into the bilayer membrane and solubilize it with the formation of micelles and transient toroidal holes. The barrel stave model is unique, in that antimicrobial molecules form a bundle in the membrane that lines the interior of the pore with their hydrophilic faces exposed to water and their hydrophobic faces toward the hydrophobic membrane. Since a large number of studies, based on HDPs have already been reported, it is hoped that this expansive body of knowledge offers a guide to understand the conformational properties and mechanism of newly discovered AMMs. The relatively few, but pioneering, examples of biophysical studies investigating newly discovered AMMs will be highlighted in this review.

Recently, Huang provided an excellent overview on the origin of cooperativity in terms of peptide/lipid (*P/L*) ratios and how this ratio is intimately tied to the type of mechanism by which the HDP operates [118]. A critical threshold *P/L* ratio (defined as *P/L**) is extremely important and is necessary for all the proposed models to execute membrane disruption or permeation. The significance of *P/L* ratios will be emphasized for many of the techniques described below when this data is available.

4.1.4. Antimicrobial agent resistance by pathogens—As an essential part of an organism's defense against pathogenic microorganisms, higher order animals and even plants have evolved HDPs with several types of membrane-disrupting mechanisms to combat persistent infection. However, infection-causing microbial pathogens themselves have not been passive to the evolutionary process. They have evolved multifaceted and effectual countermeasures to defend themselves against HDPs [113]. Such approaches include protease-mediated resistance, extracellular structural modifications, cytoplasmic membrane

modification, intracellular target modification, efflux dependent mechanism etc. A goal held by many groups is a clearer understanding of how antimicrobial peptides function in defense against infection. Furthermore, this understanding may provide new models and strategies for developing novel antimicrobial agents that may boost host immunity by minimizing antimicrobial resistance or even by synergistically amplifying the effectiveness of conventional antibiotics.

Because an essential step in every proposed mechanism of action for HDPs includes interaction with the outermost surface of the target pathogen, compositional changes to this membrane that limit HDP interactions seems like a reasonable choice. In fact, several studies have documented such changes. Certain *Staphylococcus* species express membranes with reduced negative charge. *Enterococcus* species exhibit broad resistance to a panel of cationic HDPs and heavy metal ions. Hajek and coworkers examined the phospholipid composition of a group of *Staphylococcus* species [119]. Most species examined display polar lipid profiles consisting predominantly of PG and CL. However, among the organisms tested, *S. aureus* was unique in having a lipid composition enriched in unsaturated menaquinones with eight isoprene units, and lysyl-PG, a derivative of PG that results in a considerably less anionic membrane. Various studies demonstrated that a antimicrobial protein tPMP-1-resistant *S. aureus* strain exhibits a significant increase in unsaturated membrane lipids, compared with its tPMP-1-susceptible counterpart. This resistant strain had correspondingly higher degrees of membrane fluidity as assessed by fluorescence polarization. This data suggest that constitutive alterations in the cytoplasmic membrane structure or function may be critical to why the antimicrobial peptide resistance observed in *S. aureus*. Importantly, Miller and coworkers also hypothesized analogous modifications in the outer membrane of some Gram-negative bacteria thus preserving the membrane integrity in the presence of HDPs [120].

Modifications of lipid A and Lipopolysaccharide (LPS) in Gram-negative *Enterobacteriaceae* have also been identified as a common mechanism of HDP resistance. These inducible responses include lipid A acylation [120], 4-amino-4-deoxy-L-arabinose and palmitate derivation of lipid A in *E. coli* similar to that seen in *Salmonella* [121], aminoarabinose versions of LPS in *Pseudomonas* strains associated with cystic fibrosis, and myristylation of LPS [122,123]. These mechanisms of resistance and regulation are topics of several excellent reviews [124,125]. Therefore, it seems clear that the intrinsic characteristics of microbial phospholipid membranes are likely inseparable from HDP resistance. Taken together, these observations emphasize the numerous ways in which microbial pathogens may vary their membrane surfaces chemistries and thus properties to subvert cationic HDP binding and their lethal action. As a result, this is yet another reason that a clearer understanding of how HDPs/AMMs interaction with phospholipid membranes is critical.

4.2. Biophysical techniques

The mode of action of the AMMs in disrupting cell membranes is of fundamental importance in understanding the efficiencies of different antimicrobials and if they behave like true mimics. In addition, this knowledge may help scientists define essential design elements for antibiotics with improved properties. It is believed that antibacterial molecules can differentiate mammalian cell membranes from bacteria cell membranes through several mechanisms and thus selectively kill bacteria without harming host cells. The antibacterial mechanism usually acts by disrupting cell membranes rather than by targeting specific receptors inside the cell or on the cell surface. A variety of analytical techniques has been employed to probe the interactions between antimicrobials and model bilayer membranes. However, ambiguities still abound in the molecular mechanisms involved in these interactions. In addition, it is likely that HDPs and AMMs change mechanism at high concentration and so the importance of this factor in the experimental studies cannot be overstated. Further, diverse sample preparations

including monolayer, bilayers, and multilayers are found in the literature. These analytical and biophysical techniques include X-ray (SAXS, GIXD), other spectroscopic techniques (solid state NMR and isotope labeling, vibrational spectroscopy, oriented circular dichroism, fluorescence, neutron reflection), calorimetry (ITC, DSC), microscopy (AFM, TEM, confocal, fluorescence), vesicle leakage and flip-flop assays, as well as molecular dynamic simulations. In this section, we attempt to summarize the use of various analytical techniques to elucidate the interaction of antimicrobial molecules with lipids, including the scope and limitation of the each technique; we apologize for any omissions, which are likely in such a large and important field.

4.2.1. Fluorescence spectroscopy—Several different types of fluorescence studies have been widely utilized to detect vesicle-peptide interactions [39,64,65,68,99,102,126–138]. Many HDPs, ceropin A, magainin-2, indolicidin, defensins and their synthetic mimic oligomers and polymers are able to insert and penetrate membrane vesicles releasing fluorescence dyes such as dextran, calcein, carboxyfluorescein and other probes. Dye leakage assays from model vesicles provide valuable information regarding antibacterial mechanism as well as membrane selectivity and the role of specific lipid type in membrane activity. Vesicles having different lipid compositions can be easily prepared and the corresponding leakage of the trapped fluorescence dye monitored to determine the affinity of HDPs towards a broad spectrum of single or mixed lipid types.

Flip-flop assays are another useful method to monitor lipid movement upon addition of HDPs, where asymmetrically labeled vesicles are prepared so that the fluorescent label (e.g. NBD) is located at the inner surface of the bilayer [65,110]. Addition of HDP causes a reduction of the fluorescence intensity due to the movement of the labeled lipid to the outer surface of the bilayer followed by irreversible quenching from sodium bisulfite present in the surrounding solutions.

The mode of interaction and membrane-permeating properties of peptides labelled with fluorescent probes such as 7-nitrobenz-2-oxa-1,3-diazole-4-y1, rhodamine, or fluorescein have also been examined with zwitterionic or acidic phospholipid bilayers [139,140]. Labeling of the peptide with the fluorophore allows examination of the location and the binding state of the peptide in phospholipid bilayers and calculation of its surface partition coefficients. Hoekstra and coworkers showed that an amphipathic net-negatively charged peptide strongly promotes fusion of LUVs using fluorescence techniques [141]. The intrinsic tryptophan (Trp) fluorescence and iodide quenching experiments were carried out and revealed the absence of migration of the Trp residue to a hydrophobic environment, upon their interaction with the target membranes. These results suggested that peptide folding occurred along the vesicle surface. The depth of peptide insertion into model bacteria membranes can also be estimated by Trp fluorescence quenching using doxyl groups variably positioned along the phospholipid acyl chains.

4.2.2. Solid state NMR spectroscopy—NMR has become an important tool to measure the orientation and penetration of antimicrobial molecules into lipid bilayers with several groups providing important insight [142–155]. Transmembrane (for barrel stave mechanism) or in-plane (for carpet mechanism) orientation information can be readily determined using solid state NMR techniques, because nuclear spin frequencies are inherently dependent on molecular orientation relative to the magnetic field. In one example, Bechinger and coworkers utilized ^{31}P and ^{15}N NMR to demonstrate how the insertion of alamethicin and the related HDPs, peptaibol and zervamicin, depends on the hydrophobic thickness of the lipid bilayer relative to the peptide length [151,156]. Hong and coworkers demonstrated the insertion of HDP Protegrin-1 (PG-1) in DLPC and POPC (abbreviations for the lipids can be found in the Appendix) bilayers in presence of paramagnetic Mn^{2+}, which binds to the surface of the bilayer and induces distance-dependent dipolar relaxation of the nuclear spin [157,158]. Ulrich and

coworkers looked into the orientation and orientational changes of the HDPs, PGLa and gramicidin S (GS), in DMPC bilayers by highly sensitive solid-state NMR measurements of ^{19}F dipolar couplings on CF_3-labeled side chains, and supported their findings with ^{15}N label experiments [159,160]. At a low *P/L* ratio of 1/200, the HDP resides on the membrane surface in the so-called S-state. However, at high peptide concentration (*P/L* = 1/50) the helix axis changes its tilt angle from 90° to 120° into a tilted "T-state" which represents a novel feature of HDPs, which is distinct from a membrane-inserted I-state. Solid state proton-decoupled ^{31}P NMR of the lipid head group shows a typical signature for the lamellar (L_a) and hexagonal ($H_{??}$) phase of the lipid array, which is very efficient at determining the phase change of the lipid membrane upon insertion of HDPs [153]. This phase change depends strongly on temperature, hydration, lipid types and *P/L* ratios.

As can be seen, solid state NMR is a versatile and powerful technique for determining the dynamic structure of membrane active molecules with high resolution [142]. This tool provides useful information about the orientation and depth of insertion for HDPs and AMMs that spontaneously insert into lipid membranes, and this knowledge is particularly useful for determining the mechanism of membrane disruption. The major disadvantage of this technique is that it is time consuming, requires extensive expertise, and a relatively large amount of sample (up to 100 mg of lipid and HDP). Lastly, solid state NMR data needs very careful interpretation in order to relate it to the solution (*in vivo* or *in vitro*) behavior.

4.2.3. Attenuated total reflectance Fourier transform infrared spectroscopy— Polarized attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy experiments reveal HDP orientation to the membrane surface. Coupled with spin-label EPR, ATR-FTIR results indicated that low melittin concentrations bind at the interface with the helix approximately parallel to the plane of the membrane without deeply penetrating into the hydrophobic acyl-chain region [48]. FTIR spectroscopy can also be used to investigate the conformational disposition of the peptide in solution and upon interacting with lipids. Lohner and coworkers showed that interaction of d-lysin with DMPC caused a net increase in the population of the amide protons that are shielded from the aqueous phase [144]. Aliphatic C-H stretching bands at 2800 and 3000 cm^{-1} were also used to characterize the thermotropic phase behavior of the DMPC/d-lysin mixture. Band maxima of the lipid CH_2 stretching occurred at slightly higher frequencies for peptide rich versus peptide free lipids. The results indicated incorporation of d-lysin into DMPC bilayers due to an overall increase in hydrocarbon chain disorder in both the gel and liquid crystalline (LC) state. Ghadiri and coworkers used this method to prove that cyclic peptides can self-assemble to form nanotubes in synthetic lipid membranes [55]. The infrared spectra displayed tightly hydrogen-bonded amide-A (NH stretch) that supports a tight ring-to-ring network of hydrogen bonding and amide-I and amide-II bands which are characteristic of antiparallel, β-sheet structure.

4.2.4. Sum frequency generation vibrational spectroscopy—Chen and Tew utilized sum frequency generation (SFG) vibrational spectroscopy as a powerful and unique method to investigate the interactions of a derivative of AMM **7** with a single substrate-supported, asymmetrically deuterated lipid bilayer composed of DPPG (DPPG/d-DPPG). It was observed that the distal leaflets were disrupted at very low *P/L*, while the proximal leaflets remained intact below a threshold concentration very close to the MIC (0.8 μg/mL) value [61]. The vibrational spectra can be related to both the lipid bilayer integrity and the peptide structure at the molecular level. Chen expanded these studies and observed different modes of action for melittin, tachyplesin-1, magainin-II, MSI-843, gramicidin and synthetic antimicrobial oligomers [61,161,162]. As demonstrated, SFG is very useful in studying the kinetics of HDP/AMM-lipid interactions, and has superb surface/interface sensitivity to observe changes in either proximal or distal (or both) leaflets of the bilayer. Sample preparation requires expensive deuterated lipids, equipment setup is expensive, and skillful expertise is required.

4.2.5. Oriented circular dichroism—Huang and coworkers developed an oriented circular dichroism (OCD) method which is a very fast technique to measure the orientation of peptides in membranes [163–165]. OCD spectra of the multilayer sample are acquired at the normal as well as oblique incident angles with respect to the bilayer planes. Interactions of helical peptides [164,165], β-sheet peptides [166], and cyclic peptides [167] with bilayer membranes have been evaluated using OCD. It was observed that at low *P/L* the peptide porientation indicates surface binding (S state). For alamethicin [168], melittin [169], magainin [170], and protegrin [166], at conditions above the *P/L** concentration, an increasing fraction of the individual peptides change to another orientation (I state). The *P/L** value depends on the HDP as well as the lipids (e.g., DPhPC or DOPC) present in the bilayer. A sample of fixed *P/L* can exist in either S or I state upon fluctuating the hydration or the temperature. An important advantage of OCD is that the same sample can be used for neutron scattering experiment as well [118]. At the same time, it is limited to the study of chiral structures with known CD signals.

4.2.6. Neutron scattering—Neutron in-plane scattering is a useful technique to detect HDP induced pore formation in the membrane. Two different neutron scattering-length densities are the signature for the membrane with and without HDPs [154,171]. Neutron off-plane scattering records the diffraction patterns of HDP induced pores within membranes in oriented multilayers or liquids [172]. Deuterium labeling provides contrast, which allows observation of water filled pores in the bilayers against a lipid background. By examining the contrast variations, the pore diameters can be accurately measured. Even if the in-plane scattering curves of two HDPs are similar, the differences in the off-plane scattering curves can differentiate pore sizes in membranes [172]. Huang and coworkers found an important correlation between OCD and neutron scattering experiments [169]. When OCD shows all the HDPs in the S state, neutron scattering shows no pores present in the membrane. On the contrary, when neutron scattering shows the presence of pores in the membrane, OCD exhibits a detectable amount of HDPs in the I state. This scattering method, one of the earliest employed in this field, will continue to be important for the characterization of HDP/AMM-membrane interactions.

4.2.7. Langmuir monolayer, X-ray reflectivity and grazing incidence X-ray diffraction—The effect of chemical and physical properties on membrane selectivity of the HDP, PG-1, was nicely demonstrated by Langmuir monolayer experiments showing area expansion as a result of peptide insertion followed by an increase of the surface pressure [173]. Grazing incidence X-ray diffraction (GIXD) coupled with X-ray reflectivity (XR) and Langmuir monolayer insertion assays provide a very useful tool to understand the detailed interactions of antimicrobial molecules with lipid monolayers. Lee and Tew, for the first time, combined these three techniques and successfully elucidated the interaction of phenylene ethynylene AMMs **10** and **11** with DPPC and DPPG lipid layers [174]. XR and GIXD experiments illustrated insertion of these molecules partially into the tail group region and eventual perturbation of the lateral packing of lipids. Small oligomer **11** (m = 2) was found to be more actively inserted into the lipid layer than the polymer **10**, which correlates with their MIC values. While both the polymer and the small molecule exhibited greater affinity towards anionic lipid DPPG than the zwitterionic lipid DPPC consistent with their greater antimicrobial activity than hemolysis.

The disadvantage for this technique is the requirement of an expensive and sophisticated synchrotron and the use of monolayer membranes instead of bilayer membranes. On the other hand, the ability to maintain constant pressure during the experiment and to screen a wide range of lipid types are major advantages.

4.2.8. X-ray scattering—X-ray scattering methods precisely measure the membrane thickness or, more specifically, the distance between the two phosphate groups across the

bilayer [118]. Oriented X-ray scattering perpendicular to the bilayer membrane allows measurement of the electron density profile across the membrane. Using this method, a reduction of the membrane thickness has been observed for HDPs, alamethicin [175], magainin [176], protegrin [177], and melittin [178]. More interestingly, membrane thickness reduces linearly with *P/L* until it reaches a threshold ratio, afterwards the thickness remains constant with further increases of *P/L* [118]. Small and wide angle X-ray diffraction experiments show that gramicidin promotes the formation of a bicontinuous inverted cubic phase in model *E.coli* membranes at a *P/L* of 1/25 [179]. Andrä and coworkers developed a novel a-helical peptide antibiotic, NK-2, and utilized SAXS to understand the mechanisms of selectivity and membrane destruction for bacteria and RBC membranes [180]. At a varied temperature range (10 °C to 80 °C, with 2 °C/min) SAXS experiments were done at different *P/L* ratios (1/3000, 1/1000, 1/300 and 1/100). No influences on the phase transition temperatures (T_{pre}, T_m) were observed for DPPC vesicles. DSC data for the same peptide, at the same *P/L*, as used for the SAXS experiments revealed that this peptide has no effect on the phase behavior of DPPC vesicles. X-ray diffraction experiments of pure POPE showed a typical Bragg diffraction pattern with a first order diffraction peak for lamellar L_β (gel) and L_a (liquid crystalline, LC) phases with a T_m of 25 °C. Above 70 °C reflections for a typical inverted hexagonal phase (H_{II}) were observed. The most dramatic effect was observed for the lamellar/inverted hexagonal transition of PE which was reduced by more than 10 °C, and lead to the conclusion that NK-2 promotes a negative membrane curvature leading to the collapse of the PE-rich bacteria membrane [180]. Willumeit and Tew demonstrated by SAXS experiment the lipid SOPS forms vesicles with a repeat distance of 5.3 nm at 20 °C and switches from the gel to LC phase at 17 °C. Upon the addition of AMM, **11** (m = 2), the shape of the scattering curves changed dramatically, suggesting that AMM induces highly ordered lamellar lipid structures. In addition, SOPS vesicles in the absence of AMM complete the phase transition within a 5 °C temperature window, in sharp contrast to the sample containing AMM, in which this transition is prolonged up to nearly 30 °C. This suggests that the presence of AMM almost completely hinders the phase transition from the gel to LC phase. The authors concluded that lipid composition is more important for selectivity than the overall net charge [65].

Wong and Tew investigated interactions and self-assembly of this same phenylene ethynylene family, **11**, with model membrane vesicles using synchrotron SAXS [66]. AMM, **11**, induced a regular hexagonal array of 3-nm water channels formed at a threshold *P/L* of 1/30. This study indicates that different AMMs (**11** having m = 1, 2, or 3) require different minimum threshold concentrations of negative-curvature lipids (e.g. DOPE) in order to form inverted hexagonal pores in the target membrane. This study is a clear example of how subtle changes in the chemical structure of AMMs can unexpectedly give dramatically different behaviors associated with bilayer reorganization.

The major disadvantage of such scattering techniques is its limitation to ordered structures. In other words, it does not characterize the disordered regions. However, fine structure details obtained by X-ray scattering provide extremely important insight into the HDP and AMM interactions with lipids.

4.2.9. Differential scanning calorimetry—Differential scanning calorimetry (DSC) studies have yielded a wealth of quantitative information on the influence of HDPs on the phase transition properties of membrane system. The effect of peptides on membranes can be evaluated on the basis of their effect on the lamellar (L_a) to inverse hexagonal ($H_{??}$) phase transition temperature as detected by DSC [181,182]. The molar ratio of different lipids in the bilayer is important in determining microscopic differences in their lateral organization, packing and/or mobility, which can be amplified by the interaction with other membrane constituents and in particular by interaction with membrane-active solutes in the environment. McElhaney, Gellman, and coworkers recently studied the effects of the HDPs on the

thermotropic phase behavior and organization of lipid vesicles by high sensitivity DSC [50, 129,143,144,183–185]. DSC heating scans, as shown in Fig. 13, illustrated the effect of *P/L* on the thermotropic phase behavior of multilamellar vesicles (MLV).

The arrangement of the HDP, alamethicin, was also studied by DSC [149]. This study showed slight shifting of the pretransition and broadening of the main transition suggesting that alamethicin induces a disordering effect on DHPC, which is a result of membrane-thinning at high alamethicin concentrations. DSC and X-ray techniques can provide complimentary information as shown earlier when X-ray was used to monitor lipid transitions. Both techniques detect phase changes, for example lamellar gel to liquid crystalline [144,181]. These techniques have provided evidence that peptides can show preferential interaction with different classes of phospholipids.

4.2.10. Isothermal titration calorimetry—ITC can provide a comprehensive thermodynamic description of the entire binding process of peptide to lipid vesicles. Binding parameters for structurally different membranes is essential for understanding the permeabilization mechanism and the membrane selectivity. In particular, an accurate determination of the association constant, permits a quantitative determination of the partition coefficients and relative degree of phospholipid binding specificity [186]. ITC studies of gramicidin S (GS) binding to phospholipid bilayer membranes indicated that GS is bound with higher affinity to anionic POPG than zwitterionic POPC vesicles due to electrostatic interactions in the former system. Additionally, Fig. 14 shows that the presence of cholesterol reduced binding only slightly, and the authors claim that the binding of GS is not highly sensitive to the order of the phospholipid bilayer system [186].

The binding of HDPs to neutral and negatively charged model membranes was studied in detail by Seelig and coworkers [150,187–189]. ITC of PGLa solutions with PC/PG (3:1) vesicles gave rise to two processes: 1) an exothermic binding of PGLa to the membrane followed by 2) a slower endothermic process. The latter was only detected at *P/L* ratios of 1/50 which was also shown to be the ratio that induced membrane leakage. The endothermic process was assigned to peptide pore formation and/or lipid perturbation [150]. Interactions of β-17, a potent AMM, with phospholipids vesicles showed stronger binding to anionic membranes (PG) than to the zwitterionic membrane (PE) [43]. Therefore, its microbial specificity was attributed to improved electrostatic interactions with microbial vs. eukaryotic membranes.

ITC binding studies can also be performed with either sonicated lipid vesicles (forming small unilamellar vesicles, SUVs, of ~ 30 nm diameter) or extruded vesicles (forming large unilamellar vesicles, LUVs, with diameters of ~ 100 nm or larger). Due to the high sensitivity of ITC, differences in the isotherms are usually observed when using SUVs versus LUVs and one has to be cautious comparing binding constants and enthalpies. In other words, large differences in the thermodynamic parameters of binding can exist from experiments using different vesicle constructs [188]. Another complication that can occur is that the heat signals can be a result of processes such as dilution or conformational changes of the vesicles rather than the binding processes. In addition, ITC measures the whole system and so caution needs to be used when assigning the specific energies to a molecular interaction or mechanism.

4.2.11. Micropipette aspiration technique—The micropipette aspiration technique is used to characterize the elastic moduli and critical tensions of lipid vesicles with varying lipid composition. It has not been used often to study HDPs but appears to be an important method with significant opportunities. Micropipette aspiration uses vesicles that are sufficiently large (25 μm in diameter). Changes of the projected area aspirated into the micropipette can be monitored by optical microscopy. For example a single vesicle can be exposed to different solutions and easily monitored to detect the change in membrane surface area [127,190]. In

another study, Tew and Santore have found a correlation between vesicle elastic modulus and the concentration of AMMs exposed to the vesicle [191]. The micropipette method can be used to test the expansion of a single bilayer with a resolution of better than 0.1% relative change in area stretching properties. The major drawback to the micropipette method is the long time period that is needed to obtain statistically significant amounts of data which limits its suitability to test large numbers of experimental conditions.

4.2.12. Fluorescence and confocal microscopy—Fluorescence dye leakage experiments from phospholipid unilamellar vesicles have been used to study the disruption of these model membranes by HDPs as discussed above. The leakage of vesicle contents to the external media can be monitored by the release of self-quenched calcein encapsulated in LUVs by monitoring via microscopy as well [138]. Yamazaki and coworkers investigated the interaction of magainin-II with single giant unilamellar vesicles (GUVs) composed of DOPG/DOPC lipids containing calcein [192]. Low concentrations of magainin-II caused the rapid leakage of calcein from single GUVs but did not disrupt the liposomes or change the membrane structure, suggesting that magainin-II forms membrane pores through which calcein leaked. Although this is a useful technique it must be employed with care if valid quantitative results are to be obtained because the amount of calcein in vesicles and even the amount of vesicles can vary wildly from experiment to experiment. One of the drawbacks is the rate of dye release may not always be a linear function of peptide concentrations thus making kinetics interpretation difficult [129].

Bagatolli and coworkers investigated the lytic mechanism of HDPs by performing single vesicle experiments using confocal fluorescence microscopy [193]. In this experiment, the time course of leakage for different MW, water soluble fluorescent markers incorporated inside of single GUVs was determined. Membrane lysis caused by HDPs was then rationalized by means of the carpet or pore forming model. McLaughlin and coworkers indicated that monovalent acidic lipids are not sequestered by membrane-bound basic peptides and the binding of basic peptides to vesicles produces no self-quenching of fluorescent monovalent acidic lipids [132]. Aggregation of fluorescent labeled hydrophobic peptides and the lateral diffusion of the resulting species in GUVs can be followed using confocal microscopy with photobleaching methods as well [194].

Klibanov and coworkers investigated the mode and time scale of action for N-alkylated polyethylenimine immobilized onto surfaces by fluorescence spectroscopy [195,196]. A fluorescein bandpass filter was used for visualization of live bacteria and a rhodamine bandpass filter was used for dead cells. Epand and coworkers showed aggregation of LUVs as a function of peptide concentration and time by absorbance at 436 nm using confocal fluorescence microscopy [50]. Vesicle aggregation was required for leakage due to formation of inverted hexagonal phases, but this is not required for other mechanisms of peptide-induced leakage, such as pore formation or the carpet mechanism. The nature of the lipid was crucial for aggregation. Aggregation of LUVs composed of DOPE:DOPG (2:1) occurred very rapidly versus DOPC:DOPG (2:1) and little aggregation was seen with DOPC upon addition of peptide [50].

4.2.13. AFM, SEM, and TEM—Interactions of polycationic polymers with DMPC lipid bilayers and live cell membranes have been investigated using atomic force microscopy (AFM). The addition of polymer into the AFM liquid chamber can form defects in the bilayer and the depth of these defects in the membrane can be measured [197]. AFM imaging of cells on quaternary amine modified glass surfaces suggested cell death by disrupting cell membranes and allowing release of the intracellular contents [198]. *E. coli* was imaged on both unmodified and quaternized glass. These images were then compared to a sample of quaternized glass that had not been exposed to any bacteria. When the height mode images were compared, it was

proposed that some cellular material had accumulated on the quaternized glass accounting for the observed results.

Transmission electron microscopy (TEM) revealed that melittin formed pores via peptide oligomerization consistant with toroidal model [199]. In the search for clues to possible alternative mechanisms of action on Gram-positive bacteria, TEM was performed on thin sections of bacteria that had been treated with the peptide for 30 min [200]. Laminar mesosomes (cytoplasmic invaginations) were seen arising from the septa and cell wall. Although, electron microscopy is an excellent method for visualizing the action of peptides against bacteria, the tendency has been to utilize concentrations well above the MIC for periods of 30 min or 1 h to observe the effects more obviously. Given the importance of *P/L* ratio on interaction mechanisms, one must interpret such results with caution [110].

Russell and coworkers described the use of TEM to study the interaction of their biocidal nanotubes with bacteria. [201]. TEM showed both nanotubes fused with the outer surface of bacteria cells and cells that were completely enveloped by nanotubes. Based on their images it was claimed that the antibacterial mechanism does not involve a complete disruption of the cell wall of the bacteria since the rodlike structure of the *E. coli* cell remains intact.

Morphological changes of *E. coli* in contact with modified low density polyethylene, LDPE, was observed by TEM and SEM. Release of fibrous and granular material, presumably cell contents through damaged membranes, was interpreted as evidence for the destruction of the bacteria membrane [202]. The surface morphology of *E. coli* remained unchanged in the presence of neat LDPE even after 60 min of contact (Fig. 15). The surface of *E. coli* showed steadily more pronounced wrinkles and blebs upon exposure to the modified LDPE for increasing periods of time (up to 60 min).

4.2.14. Computational studies—As increasingly complex biophysical systems are used to more closely mimic biological cells, theoretical and computational insight will continue to be essential. A theoretical description of the electrostatic interaction of cationic peptides with anionic lipids combines the Gouy-Chapman Stern theory of the electrostatic potential adjacent to a charged membrane and the Boltzmann's mass action equations.[128,145,189,203–205] Each model predicted reasonably how the binding energy depends on the number of basic residues on the peptide, the ionic strength of the solution, and the fraction of acidic lipids in the membrane [204].

Molecular dynamics (MD) simulations with defined *P/L* ratios have been used to model systems which can be compared with experimental results [206]. Simulations allow exploration of the interactions of such peptides with lipid bilayers, and the understanding of the effects of such interactions on the conformational dynamics of the peptides [207–211]. Unfortunately, good models for simple lipid bilayers are still needed. Nevertheless, simulations will continue to be important as the parameters and molecular details are refined. Monte Carlo (MC) simulations of latarcins, a linear peptide, in a water–octanol slab revealed a peripheral mode of its membrane binding. The results of modeling and experimental techniques suggested the peptide acts by the carpet mechanism [209]. Ding and coworkers investigated the detailed structural information of different peptides interacting with lipid molecules [211]. Peptides with identical polar faces and variable hydrophobic faces were tested and the presence of smaller, aliphatic hydrophobic residues resulted in stronger binding than bulkier aromatic residues. Computational models studying membranes and adsorbed basic peptides provided insight into the lateral organization of these molecules by quantifying the role of electrostatics [203].

Recently, Ivanov, DeGrado, and Klein used MD to investigate the structural properties and activity of AMMs. [58,72,212] The system differed by composition (i.e. the ratio of hydrophobic to charge units), length (8, 10, or 20 monomer units) and sequence (alternating vs. block copolymers). The polymer molecules were either simulated in aqueous solution or inserted in the aqueous phase above a pre-equilibrated DOPC bilayer patch. Molecular simulation findings were in agreement with experimental observation in that hydrophobicity is the primary determinant for activity, whereas the presence of charged amine groups is important for selectivity [72]. Computational studies on the interaction of known membrane-active arylamide based AMMs with phospholipid bilayers revealed spontaneous membrane insertion and cooperative action at low and high concentrations, respectively. In late-stage attack, antimicrobials cross the membrane core and occasionally align to provide a stepping-stone pathway for water permeation. This is consistent with the mechanism described earlier suggesting a possible new mode of action that does not depend on pore formation for transport to and across the inner leaflet [212].

4.2.15. Miscellaneous techniques—Temperature scanning densitometry (TSD) allows determination of specific volumes in dilute systems and their changes associated with thermotropic transitions. TSD showed that melittin affected the phase of DPPC at very low peptide concentration (*P/L* ratio of 1/1000) [213]. Turbidity is another technique to understand the fusion process of peptides with lipid vesicles [214]. The temperature induced variation in absorbance at 440 nm for DPPC vesicles interacting with low concentration of melittin (1 mol %) indicated changes both pretransition and broadening of the main phase transition of the lipid bilayer. Dynamic light scattering (DLS) can be used to measure fusion of LUVs in the presence of the antimicrobial cyclic peptide [215]. The average hydrodynamic diameter of pure and peptide bound LUVs can be evaluated and use to determine whether vesicle fusion occurs. Zeta potential, an indicator of surface charge, can be used to observe peptide interactions with a lipid matrix [180]. Andrä and coworkers indicated that the addition of α-helical peptide, NK-2, had no influence on the Zeta potential of DPPC suggesting that no interaction with the lipid bilayer occurred, whereas a charge neutralization was observed for DPPE and DPPG.

4.3. Lipid selection in biophysical studies

As one can see from the preceding discussion, the choice of lipids varies greatly in the preparation of “model” membranes. The choice of lipids can be a matter of convenience as dictated by the compatibility to a particular technique or ease of mono- or bilayer preparation. Many times though, deviations from known bacteria or mammalian cell lipid compositions may be a matter of the research isolating certain properties, such as charge or intrinsic curvature. Therefore, in studies that use synthetic bilayers as convenient models of cell membranes, careful lipid selection is extremely important in order to reasonably interpret the activity and selectivity of HDPs and AMMs. The model membrane design for the biophysical studies needs to be rational, otherwise misleading information regarding the molecular mechanism may be acquired. In section 4.1.1. the major lipid contents of the bacterial and mammalian cell membranes are discussed (see Table 2). Hereby, in order to mimic bacteria cell membranes the choice of a PE:PG lipid composition would be reasonable while a PE:PG:CL system would be an even closer mimic of Gram-negative bacteria cells. To mimic the outer surface of the RBC the lipid choice of PC or PC:SM would be more appropriate than PC:PS, which is commonly used. Examples in the literature where PC:PG lipids were used to investigate antibacterial activity of HDPs/AMMs, seems curious and potentially a completely wrong direction for investigating lipid mediated antimicrobial mechanistic studies. Nonetheless, thoughtful use of the lipids not exactly matching those found in bacteria cells may be helpful to determine the role or importance of a particular lipid in mediating lipid-HDP/AMM interactions. It is also likely that more complex lipid mixtures (and perhaps even membrane proteins) that more closely capture true cell membrane will provide new insight.

The choice of the lipid acyl tail, designated by the first two letters as in POPG, representing palmitoyl and oleoyl tails on a phosphatidylglycerol head group for example, is wide and flexible as depicted in various examples. It is difficult to know what reason particular tails are chosen in particular studies (if a specific reason does exist) and often the choice of tails is just by convention across the same biophysical technique rather than for accurate structure mimicry. For instance, NMR experiments use dioleoyl lipid tails, assumedly to take advantage of the lipid symmetry, while in X-ray two unsaturated tails can lead to a desired intrinsic curvature. A fuller discussion about the choice of tails is beyond the scope of this review but abbreviations for the lipid acyl tails mentioned can be found ni the Appendix. The lack of discussion here does not imply this topic is unimportant; in fact the topic deserves fuller consideration in another report.

The above mentioned biophysical studies clearly demonstrated that HDPs and AMMs show preferential interactions with specific phospholipid classes. Furthermore, they revealed that in addition to charge-charge interactions, membrane curvature strain, and hydrophobic mismatch between AMMs/HDPs and lipids are important parameters in determining the mechanism of membrane perturbation. Hence, depending on the molecular properties of both lipid and peptide, creation of bilayer defects such as phase separation or membrane thinning, pore formation, promotion of nonlamellar lipid structures, or bilayer disruption may occur [181]. A better understanding of the mutual dependence of these parameters will help to elucidate the molecular mechanism of membrane damage by HDPs/AMMs and their target membrane specificity, keys for the rational design of novel types of antibiotics.

4.4. Significance of the P/L ratios

Most of the biophysical techniques observed a threshold *P/L* (*P/L**) that corresponds to a major change in HDP/AMM-membrane interaction. Huang and coworkers have examined the interaction of different HDPs (alamethicin, melittin, magainin, and protegrin) with a variety of model lipid compositions and found *P/L** varies from 1/200 to 1/10 [118]. The obvious question arises whether this *P/L* range (associated with model studies) is relevant to compare with the bioactivity (MIC, HC_{50}) of the HDPs associated with live cell studies. To the best of our knowledge, the *P/L* ratio of experiments *using real cells*, such as in MIC experiments, has not been previously considered. A sample calculation assumes that in an MIC experiment there are about 10^5 cells in 1 mL of media and the accepted approximate number of lipids per cell is 2.2×10^7 to 2.5×10^7. These values lead to a total lipid concentration of 3.65×10^{-3} μmol/L. Considering the MIC of magainin-II is ~ 3 μg/mL, this translates to a surprisingly high *P/L* ratio of 1/0.003 at this concentration. Even if the cell density is 10^7 or 10^9 per mL the *P/L* ratio is still 1/0.3 and 1/30, respectively. What this calculation suggests is that all biophysical studies using model membranes have a *P/L* ratio that always drastically underestimates the *P/L* ratio of a standard MIC experiment. Therefore, the *P/L* ratios used in biophysical studies are extremely conservative, in that very minute concentrations of peptide (well below the *P/L* ratio at the MIC) can cause significant membrane perturbation. It is likely that the perturbation occurring at these low *P/L* ratios will also occur at the MIC for biologically active HDPs/AMMs. Recalling the SAXS data in section 4.2.3., it was shown that AMM, **11**, caused hexagonal pore formation in model membranes at *P/L* 1/30. This is a common *P/L* ratio for many biophysical techniques. There were initial reservations that lipid reorientation was "forced" by using a supposedly high concentration of peptide. The fact though is that when we calculated the *P/L* ratio at the MIC of AMM **11** (m = 2, MIC 0.8 μg/mL; *P/L* 1/0.003) the SAXS experiment used concentration 10000 times lower than MIC experiment.

5. Application in materials

There is a great need to make antibacterial materials which are capable of preventing or limiting the spread of infectious microbes. Many indwelling medical devices can be easily colonized by bacteria (strong bacteria adhesion begins within 2 h of implantation) which lead to chronic bacterial infection through the formation of bacterial biofilms [18]. These biofilms are typically resistant to antibiotics and the host's own immune system. To decrease or prevent these infections, it is attractive to consider materials that do not support their growth or survival. Two general strategies have been taken to make antimicrobial materials. The most common method is the addition of a biocide to the polymer such as silver ions, quaternary ammonium salts, phenols and antibiotics [18–20]. These biocides are slowly leached to the surrounding environment killing the microorganisms. However, there are limitations of materials impregnated with a leaching antibacterial agent including contamination of the environment and short durations of antimicrobial action due to rapid leaching at the beginning of use. Consequently, several approaches have been used to make non-leaching biocidal materials to overcome these problems.

5.1. Method for making non-leaching biocidal materials

Non-leaching "permanently biocidal" materials can be made either by 1) covalently attaching an antimicrobial agent to its surface or by 2) blending with a non-leaching biocide. Several examples will be discussed.

5.1.1. Surface modification—In one of the earliest reports on surfaces modified to be permanently sterile, a group from Dow Corning, in 1972, reported the preparation of antibacterial glass by surface-bonded quaternary ammonium salts [216]. In their procedure, cleaned glass surfaces were treated with a 0.1% solution of 3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride followed by heating at 70 °C for 30 min. This protocol led to anchoring the reagent to the surface via covalent bonding. Their studies showed that these surfaces were very active in killing *S. faecalis* even after extensive rinsing with water. Kotek and coworkers applied the same reagent on poly(ethylene terephthalate) fibers reporting that treated fibers had excellent antibacterial effect against *E. coli* [217].

Klibanov and coworkers covalently attached poly(4-vinyl-*N*-alkylpyridinium bromides) to a glass surface either by "graft to" or "graft from" techniques [218]. Polymers were grafted from amino functionalized glass surfaces by treatment with acryloyl chloride, copolymerization with 4-vinylpyridine, and then *N*-alkylation with different alkyl bromides. Alternatively, using the "graft to" technique, poly(4-vinylpyridine) (PVP) was attached to glass slides and alkylated with hexyl bromide (Scheme 1). This study showed that the treated surfaces were able to kill up to 94 ± 4% of *S. aureus* cells sprayed on them; surfaces were even more effective towards *S. epidermidis*, *P. aeruginosa*, and *E. coli*.

Using the system above, Klibanov and coworkers conducted bacteria spraying studies. Fig. 16 shows that the untreated surface (left) has numerous colonies whereas the treated surface (right) killed almost all the bacteria (after spraying with bacteria and incubation under agar). The antibacterial activity of the polymers was strongly dependent on the alkyl chain length with surfaces containing hexyl chains. Glass surfaces containing polymers with decyl chains lost all antibacterial activity.

In another study, the same active alkylated PVP polymer was grafted to polymer surfaces such as nylon, HDPE, LDPE, PP, and PET after treatment to coat with silica [219]. Antibacterial studies showed that these surfaces were able to kill *S. aureus* and *E. coli*. Neoh and coworkers used a simpler technique to modify PET surfaces with the same polymer and these surfaces were able to kill *E. coli* as well [220].

Ober and coworkers studied the biocidal activity of polystyrene-*b*-poly(4-vinyl-*N*-alkylpyridinium bromides) copolymers (where alkyl is hexyl or 6-perfluorooctyl-1-hexyl) [221]. These polymers were sprayed on polystyrene-*b*-poly(ethylene-*ran*-butylene)-*b*-polystyrene coated glass slides then heated to 80 °C. Studies showed that the fluorinated pyridinium surfaces are more biocidal compared to their nonfluorinated analogues. The bactericidal effect was found to be related to the molecular composition and polymer organization in the top 2 – 3 nm of the surface and improved with increasing hydrophilicity and pyridinium concentration at this surface.

Alkylated polyethylenimines (PEI) attached to flat glass surfaces, also have high antibacterial activity [222]. Results show that theses surfaces have 90 – 99% bactericidal efficiency towards *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *E. coli.* PEI was also grafted to cotton, wool, polyester and nylon fabrics (after surface modification) and was found to render these fabrics antibacterial [223].

Following these results, Klibanov and coworkers prepared polymeric coatings that inactivate both influenza virus and pathogenic bacteria [224]. They “painted” a glass slide with a solution of branched *N*,*N*-dodecyl methyl-PEI in butanol and let the solvent evaporate. The formed surface killed influenza virus with 100% efficiency within minutes as well as *E. coli* and *S. aureus* [196].

Matyjaszewski and coworkers used ATRP to grow poly 2-(dimethylamino)ethyl methacrylate (DMAEMA) onto Whatman #1 filter paper or glass slides followed by quaternization using an alkyl halide (Scheme 2) [198]. This study showed that the treated surfaces were able to reduce the number of living bacteria substantially.

Tew and coworkers prepared poly(butyl methacrylate-*co*-aminoethylmethacrylate hydrochloride) on silicon wafers via “graft from” [225]. This polymer is known to have excellent antibacterial properties in solution [70] and their results show that this surface-bound polymer retained its antibacterial properties and kills *S. aureus* 100% by contact in less than 3 minutes.

Jérôme and coworkers reported a two-step “grafting from” method to prepare polymer brushes from stainless steel surfaces using cathodic electrografting of poly(2-phenyl-2-(2,2,6,6-tetramethyl-piperidin-1-yloxy)-ethylacrylate). This treatment was followed by nitroxide-mediated radical copolymerization of styrene (or n-butylacrylate) and 2-(dimethylaminoethyl) acrylate followed by quaternization of the resulting brushes [83]. Such quaternized copolymers are known to be biocidal. Using electrografting technique followed by ATRP, they grafted poly(TBAEMA-co-St), poly TBAEMA, and poly(TBAEMA-co-PEOMA) from stainless steel. They found that these brushes decrease *S. aureus* adhesion by 3 to 4 orders of magnitude compared to bare stainless steel [226].

5.1.2. Polymer blends—Antibacterial polyethylene was prepared by blending with biocidal polymers. Kern and coworkers compounded LLDPE with polymeric biocide poly(2-tert-butylaminoethyl) methacrylate (TBAM) at 1.5, 3.0 and 5.0 wt % of TBAM by extrusion at 215 °C [227]. Results indicated that these surfaces were very active towards *S. aureus* and reduced the number of colony forming units per mL (CFU/mL) to zero. However, the same surfaces were less effective towards *E. coli*. Only the material containing 5% TBAM reduced the amount of *E. coli* to zero CFU/mL. TEM studies on the treated LLDPE showed the presence of 0.05 to 0.5 μm particles of TBAM dispersed in the polyethylene matrix indicating phase separation.

To improve the solubility and permanency of TBAM in polyethylene, Jérôme and coworkers used poly(ethylene-*co*-butylene)-*b*-poly diblock copolymer (PEB-*b*-PTBAEMA) as a biocide

for LDPE [202]. These polymers were extruded at 140 °C and the blends were compression-molded into flasks. The antibacterial activity of 10 wt % of the diblock copolymer in LDPE was tested against *E. coli* and effective antimicrobial activity was observed.

Fuchs and Tiller developed a coating method based on emulsion polymerization using water-insoluble antimicrobial emulsifiers [228]. These emulsifiers consist of a hydrophobic polystyrene block and a hydrophilic block of the antimicrobial polymer poly(4-vinyl-*N*-methylpyridinium iodide). The block copolymer was preswelled in water, then styrene and butylacrylate were added followed by free radical polymerization to form a stable suspension of polymer particles. This suspension was cast onto glass slides and air dried to form a thin film of the polymer blend. After thoroughly washing with water, the coated slides were sprayed with a suspension of *S. aureus* and over 24 h, under growth agar, bacterial colonies grew from the individual cells. The authors found that the coated sample affords a reduction of more than 99.9% in the number of viable *S. aureus* cells on the surface.

Domb and coworkers used quaternary ammonium PEI nanoparticles as an antibacterial additive with clinically used dental composite resins [229]. PEI nanoparticles were embedded at 1.0 wt % with the resins and cured by photo-polymerization. These PEI nanoparticles did not alter the original mechanical properties of the composite resin materials. Antimicrobial tests showed that these PEI nanoparticles incorporated in dental composite resins exhibited a strong antibacterial effect against *S. mutans* which lasted for over one month without leaching of the active polymer. The authors found that for composite resin restorations, incorporation of antibacterial nanoparticles may prevent biofilm formation and secondary caries.

Tew and coworkers incorporated AMMs into polyurethane (PU) coatings which showed excellent inhibition of *E.coli* growth on the surface despite immersion in rich growth media for 72 h [67]. More recently, AMM blended into medical grade catheter tubing prevented *S. aureus* growth completely even after repeated exposure. In addition, the exposure time here was less than 3 min suggesting very rapid killing. Fig. 17 shows that the treated PVC surface (left) was able to completely kill bacteria while the untreated PVC surface (right) allowed extensive bacterial growth.

5.2. Methods for evaluating efficiency of biocidal surfaces

Unlike MIC value determination, evaluating surface activity is less well-defined. There are several recognized protocols including the Kirby-Bauer method, shake–flask test, and procedures outlined by the American Society for Testing and Materials (ASTM E 2149-01), Japanese Industrial Standards (JIS Z 2801:2000), and American Association of Textile Chemists and Colorists (AATCC-100-1999). Other groups have put surfaces on agar plates and quantified the zone of inhibition similar to the Kirby-Bauer method while others have adopted modifications including spraying bacteria. As a result, it seemed reasonable to list the methods used in literature. Again, it is hard to be completely inclusive since the literature is so large these days; however, we have included all of the most common methods.

Klibanov and coworkers sprayed their slides with (10^6 cells/mL) of bacterial suspension (to simulate the deposition of airborne bacteria) [218]. After air drying (2 min), slides were placed in a Petri dish and growth agar was added. The Petri dish was incubated overnight at 37 °C. The number of bacterial colonies reflects how many bacteria survived.

Matyjaszewski and coworkers used modified ASTM standard test (E 2149-01) to study the antimicrobial efficiency of the surfaces [198]. In their procedure, modified paper pieces (2.5 × 2.5 cm) were shaken with bacterial suspension for 1 h at 37 °C. A sample of the bacterial suspension was diluted and plated onto agar. After incubation at 37 °C overnight, the number of viable cells was determined as colony forming units (CFU) on agar plates.

Kern and coworkers performed antibacterial testing according to the Japanese Industrial Standards (JIS Z 2801:2000) [227]. In this test, treated polymer was shaped into plates and polymer surfaces were held in contact with bacterial cell suspension (10^6 CFU/mL) using sterile cover and kept for 24 h in humid conditions at 37 °C. The number of viable cells was counted after plating on agar and expressed in colony forming units per milliliter (CFU/mL)

Tew and coworkers tested the activity of AMMs incorporated in PU coatings by spraying the samples with *E. coli* followed by immersion in bacterial growth media for 72 h [67]. Microscopy showed that the untreated sample (left) is significantly colonized while the treated sample (right) does not support *E. coli* growth (Fig 18).

To evaluate the biocidal efficiency of PU containing *N*-halamine biocides, Wynne and coworkers used a modified version of the American Association of Textile Chemists and Colorists (AATCC-100-1999) test method [35]. According to this method, 1 μL of a 10^7–10^8 CFU/mL bacterial suspension was placed on top of the slide surface. An identical slide was placed on top of that surface to "sandwich" the bacterial suspension. A weight was placed on top of the surfaces and the suspension was incubated at room temperature. After a certain time, slides were placed in aqueous sodium thiosulfate solution (to reduce the *N*-halamine and neutralize its biocidal activity) then were vortexed to detach bacteria. To evaluate the number of viable cells in the suspension, 100 μL of this suspension was plated on agar, incubated at 37 °C for 24 h and CFU counted.

For materials that contain leachable biocides, the disc diffusion test (Kirby-Bauer test) can be used to assess their antimicrobial efficacy. Grunlan and coworkers used this method for polyelectrolyte multilayers that contains silver nitrate and/or quaternary ammonium salts [230]. In this test, agar plates were uniformly inoculated with solution of *S. aureus* or *E. coli* containing approximately 5×10^6 CFU/mL. Disks coated with the antimicrobial films were placed on the agar surface and incubated for 24 h at 35 °C. A circular zone of inhibition is formed around active disks. The diameter of the inhibition zone is a measure of film efficacy.

6. Conclusions

The need to continually control infectious disease presents challenges on many levels. Here we attempted to summarize the burgeoning area of Facially Amphiphilic AMMs, which are designed to mimic the essential features of HDPs but with simpler structures. These are perhaps the first examples of endowing synthetic polymers with protein-like biochemical activity. There is little question that this area represents an enormous opportunity for macromolecularly- and biologically-oriented scientists to come together. The already designed AMMs have potencies (even *in vivo*) and selectivities rivaling many natural HDPs. Further, in this review, we have attempted to bridge HDPs and biocidal polymers; two fields that rarely reference each other. On first examination these areas may appear quite different; however, a little deeper look shows strong overlap and it is clear to us that researchers in each field have much to gain from interacting with each other.

The importance of molecules that interact with phospholipid membranes in a specific and controlled manner cannot be overstated and is an enormously important area in contemporary science. As protein researchers will attest, the area of membrane proteins is rich with opportunity yet dogged by frustration at the limited number of crystal structures, and therefore the limited availability of high resolution information. Lipid rafts, yet another important but difficult membrane system, are likely to shed new insight on molecular-membrane interactions. Because the action of HDPs/AMMs is intimately related to their interactions with membranes, much is to be learned about how molecules interact with membranes from these interesting molecules. A plethora of biophysical techniques have been used to elucidate the interaction of

these antimicrobial molecules with membranes. Each technique has certain advantages and disadvantages, although it is possible to answer important questions using a variety of techniques. Often several complimentary techniques are used to provide further insight and this approach looks to be very promising. We have not attempted to highlight every technique used in this large field. Instead, we have tried to illustrate those with historical importance in this area (NMR, SANS, IR, dye leakage) and ones that have been used more recently (SAXS, ITC, SFG). More specifically, we discussed the lipid composition and how the differences in bacterial/eukaryotic cells are often not captured in the biophysical experiments. Having worked on this problem for sometime, we certainly appreciate the challenges involved with multi-component lipid mixtures and their properties. In many ways, the lipid complexity makes the systems simultaneously interesting and daunting to work on. The evidence that very specific lipid types greatly influence HDP/AMM interactions is growing and what appears to be an important role played by negative curvature lipids needs to be flushed out in greater detail. Cleverly designed experiments in this area are expected to provide exciting and novel insight. At the same time, the unique membrane activity of these molecules is likely to provide insight into other macromolecular-membrane interactions like those involved in the larger fields of membrane proteins, fusion, endocytosis, translocation, etc.

Although there is no question that HDPs and AMMs represent very interesting membrane active structures, in general, studies focused on membrane interactions are almost always concerned with the mode of antimicrobial activity. The reader should be reminded that HDPs and AMMs may have multiple targets including essential interactions inside the cell. Therefore, mode of action studies should continue to consider intercellular targets in addition to the membrane. Techniques like gene and protein chip analysis appear to be valuable tools but are not covered here due to the limited reports. Knowledge gained from these analytical tools is expected to be of great value. Of course any intercellular targets require the molecule to transverse the membrane, again highlighting the importance of understanding these interactions.

Finally, the materials area is rampant with biocidal polymers. It appears in general that the activities (MICs) of these polymers are no more potent than AMMs, which are selectively toxic, suggesting these mimics are a better approach. Nevertheless, these biocidal polymers represent a significant contribution. Efforts to turn these biocidal polymers into selective AMMs is underway in at least a few laboratories.[49,68,231] Scientifically, the control of physicochemical properties and biological activities is an important challenge. It also appears there is significant practical impact for antimicrobial molecules that limit the spread of infectious disease. As EPA regulations continue to strongly influence this research area, selective agents will become more and more desirable. Learning to make materials with AMMs that remain potently active is no small challenge; however, it will make important contributions to society. Previous studies with biocidal polymeric materials/surfaces will be a rich resource to draw upon for these new selectively toxic materials.

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Appendix

4VP	4-Vinylpyridine
AFM	Atomic Force Microscopy
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
AMP	Antimicrobial Peptide
AMM	Antimicrobial Macromolecule
ASTM	American Society for Testing and Materials
ATRP	Atom Transfer Radical Polymerization
C_0	Intrinsic Curvature
CD	Circular Dichroism
CFU	Colony Forming Units
CH	Cholesterol
CL	Cardiolipin

DABCO	1,4-Diazabicyclo-[2.2.2]-octane
DFT	Density Functional Theory
DHPC	1,2-Diheptanoyl-*sn*-Glycero-3-Phosphocholine
DLS	Dynamic Light Scattering
DMPC	1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine
DOPC	1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine
DOPE	1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine Ethanolamine
DOPG	1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]
DPhPC	Diphytanoyl Phosphatidylcholine
DPPC	1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
DPPG	1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]
d-DPPG	Deuterated-1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]
DSC	Differential Scanning Calorimetry
EPR	Electron Paramagnetic Resonance
FA	Facially Amphiphilic
GIXD	Grazing Incidence X-ray Diffraction
GS	Gramicidin S
GUV	Giant Unilamellar Vesicles
H_{II}	Inverse Hexagonal
HC	Hemolytic Concentration
HDP	Host-Defense Peptide
HDPE	High-Density Polyethylene
IDSA	Infectious Diseases Society of America
ITC	Isothermal Titration Calorimetry
LC	Liquid Crystalline
LCST	Lower Critical Solution Temperature
L_{β}	Lamellar Gel Phase
L_{α}	Lamellar Crystalline Phase
LDPE	Low-Density Polyethylene
LLDPE	Linear Low-Density Polyethylene
Log K_{OW}	Log of octanol/water partition coefficient
LPS	Lipopolysaccharide
LUV	Large Unilamellar Vesicles
MBC	Minimum Bactericidal Concentration
MC	Monte Carlo
MD	Molecular Dynamics
MIC	Minimum Inhibitory Concentration
MW	Molecular Weight
N-alkyl PEI	*N*-alkylated polyethylenimine
NIPAAm	*N*-isopropylacrylamide
NMR	Nuclear Magnetic Resonance
OCD	Oriented Circular Dichroism
PC	Phosphatidyl Choline
PE	Phosphatidyl Ethanolamine
PEB	Poly(ethylene-*co*-butylene)
PEI	Polyethylenimines
PEOMA	Poly(ethyleneoxide) methacrylate
PET	Polyethylene terephthalate
PG	Phosphatidyl Glycerol
PG-1	Protegrin-1
PHMB	Polyhexamethylene biguanide
PS	Phosphatidyl Serine
P/L	Peptide to Lipid Ratio
*P/L**	Threshold Peptide to Lipid Ratio
PLPC	1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine
POPC	1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
POPE	1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine
POPG	1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]
PP	Polypropylene
PU	Polyurethane
PVC	Polyvinychloride
PVP	Poly(4-vinylpyridine)
RBC	Red Blood Cells
SFG	Sum Frequency Generation
SM	Sphingomyelin
SAXS	Small Angle X-ray Scattering
SEM	Scanning electron microscopy
SOPC	1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
SUV	Small Unilamellar Ve sicles
XR	X-ray Reflectivity
TBAEMA	2-(*tert*-butylamino)ethyl methacrylate
TBAM	(2-*tert*-butylaminoethyl)methacrylate
TEM	Transmission Electron Microscopy
TPMP	Thrombin-induced Platelet Microbiocidal Protein
Trp	Tryptophan
TSD	Temperature Scanning Densitometry
TTC	Triphenyl-Tetrazolium Chloride
T_{pre}	Pre Transition Temperature
T_{m}	Gel/liquid-crystalline phase transition temperature

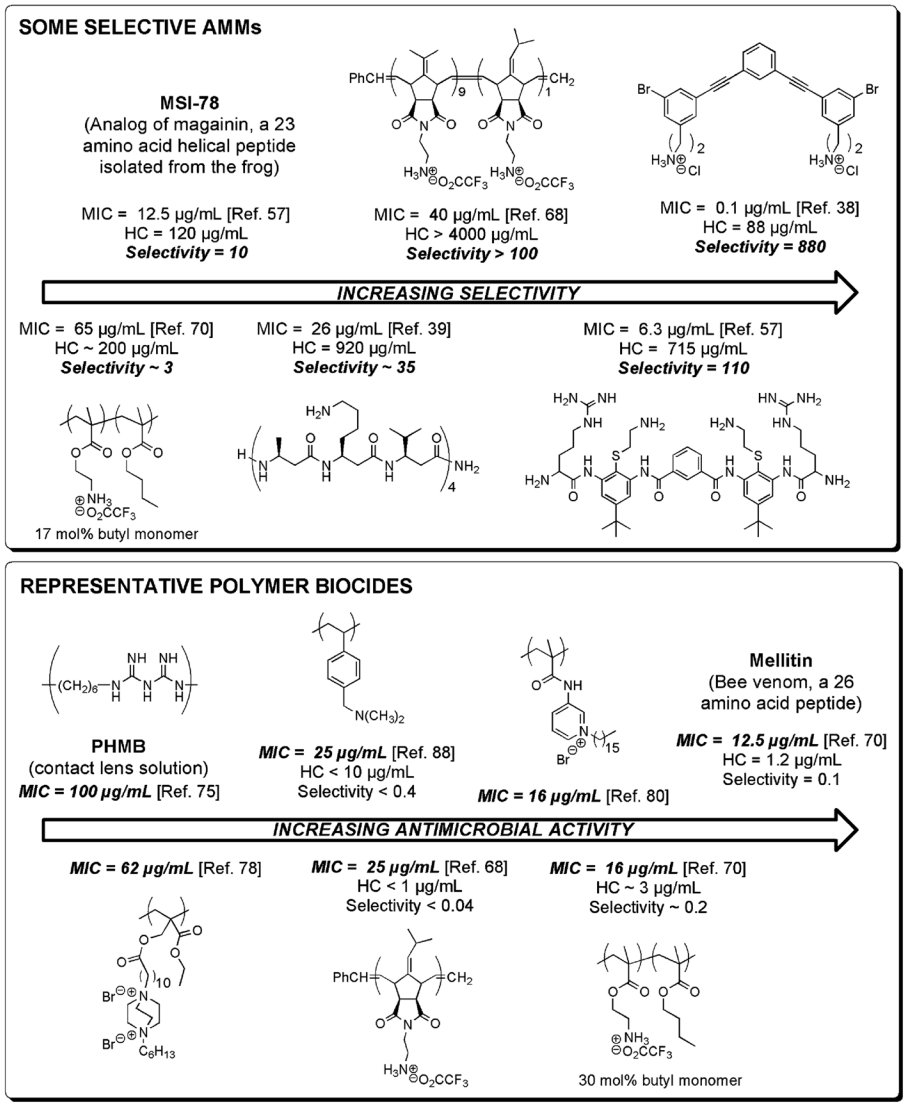


Fig. 1.

Collection of chemical structures to illustrate the terms “selective” AMMs and polymer “biocides” as determined by MIC and HC experiments. For the cited cases, the MIC is the minimum concentration at which *E. coli* growth is inhibited 90 – 100%. HC is the hemolytic concentration to lyse 50%, as convention, of a RBC solution. Polymers that have been traditionally studied for biocidal activity have usually *not* been subjected to HC experiments so that some may in fact be “selective” by other criteria. A classic example is polyhexamethylene biguanides (PHMB), a polymer well-accepted as a “disinfectant” but is non-toxic at the concentration used in contact lens solution (~ 0.0001 wt%).

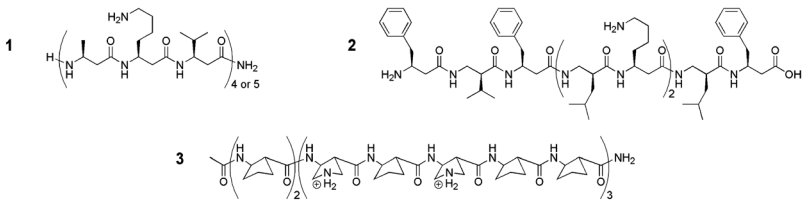


Fig. 2.
1 = Selective antimicrobial β-peptides [39]. **2** = β^2/β^3 peptide [40,41]. **3** = "β-17" β-peptide [42].

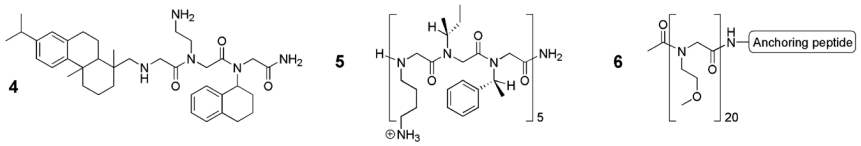


Fig. 3.
4 = Promising hit from peptoid combinatorial library [52]. **5** = Selective peptoid mimic of magainin-II [53]. **6** = Peptidomimetic for Ti surface modification [54].

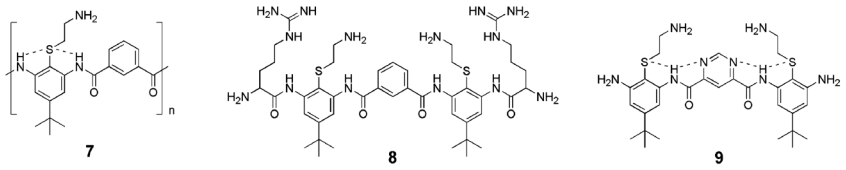


Fig. 4.

7 = General structure of FA arylamide oligomers [11]. **8** = Selective arylamide [57]. **9** = Pyrimidine arylamide oligomer [60].

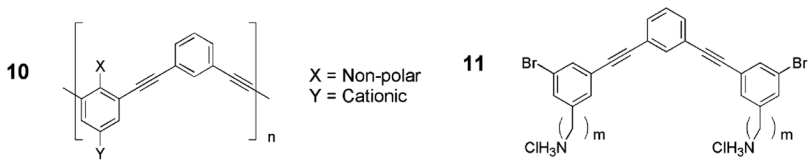


Fig. 5.

10 = General structure of phenylene ethynylenes studied [62,63]. **11** = Trimer derivatives with distinct activities [38].

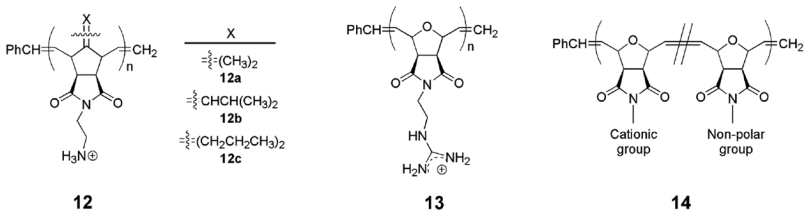


Fig. 6.

12 = Set of polynorbornes whose activities are relatively MW independent [68]. **13** = Guanidinium functionalized polynorbornene [69]. **14** = Design to access copolymer series with a range of hydrophobicities [69].

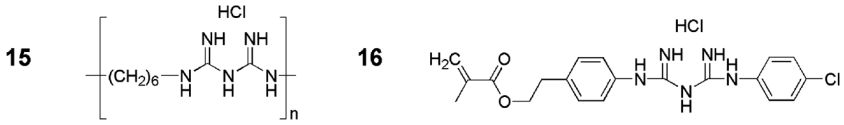


Fig. 7.

15 = Widely used contact lens disinfectant, PHMB [74]. **16** = Methacrylate monomer containing biguanide [7].

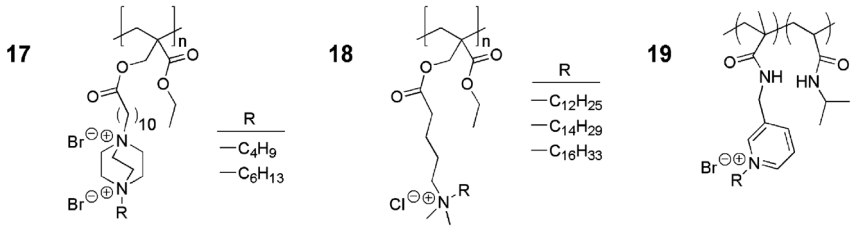


Fig. 8.

17 = DABCO-based quaternary ammonium polymers [78]. **18** = Quaternary ammonium polymers quaternized > 90% after polymerization [79]. **19** = Copolymer of pyridinium containing methacrylamide and NIPAAm [81].

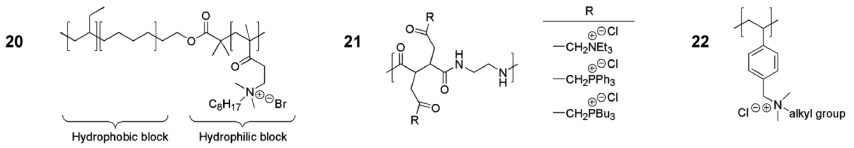


Fig. 9.

20 = Quaternary ammonium containing block copolymer [85]. **21** = Ammonium and phosphonium polymers synthesized from a common reactive backbone [87]. **22** = Polystyrenes with quaternary ammonium groups [8,88].

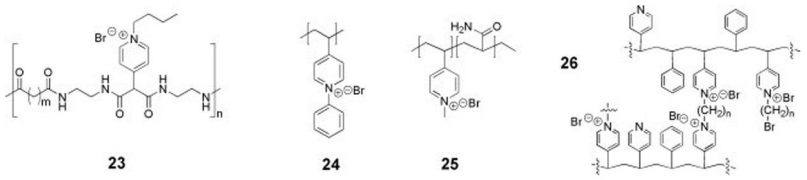


Fig. 10.

23 = Polyamide with pendant quaternary pyridinium groups [89]. **24** = Poly(benzylvinylalkyl pyridinium bromide)s [90]. **25** = Random copolymer of acrylamide and quaternized vinyl pyridine [91]. **26** = Crosslinked polystyrene-*r*-quaternary pyridinium-type polymers [93].

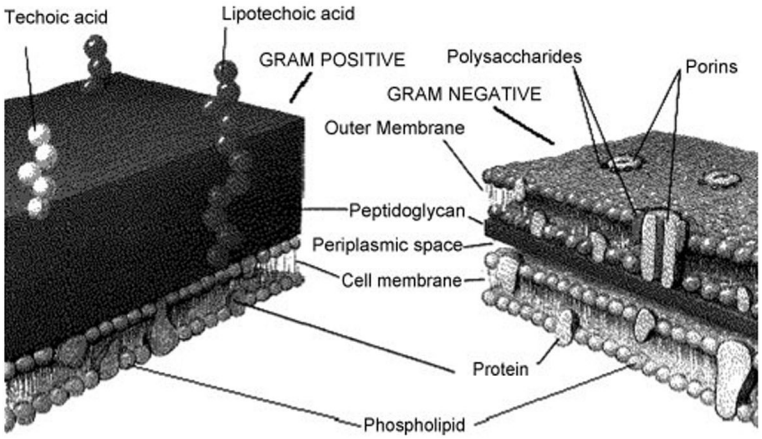


Fig. 11.
Cell wall components of Gram-positive and Gram-negative bacteria, taken from http://filebox.vt.edu/users/chagedor/biol_4684/Methods/cellwalls.html.

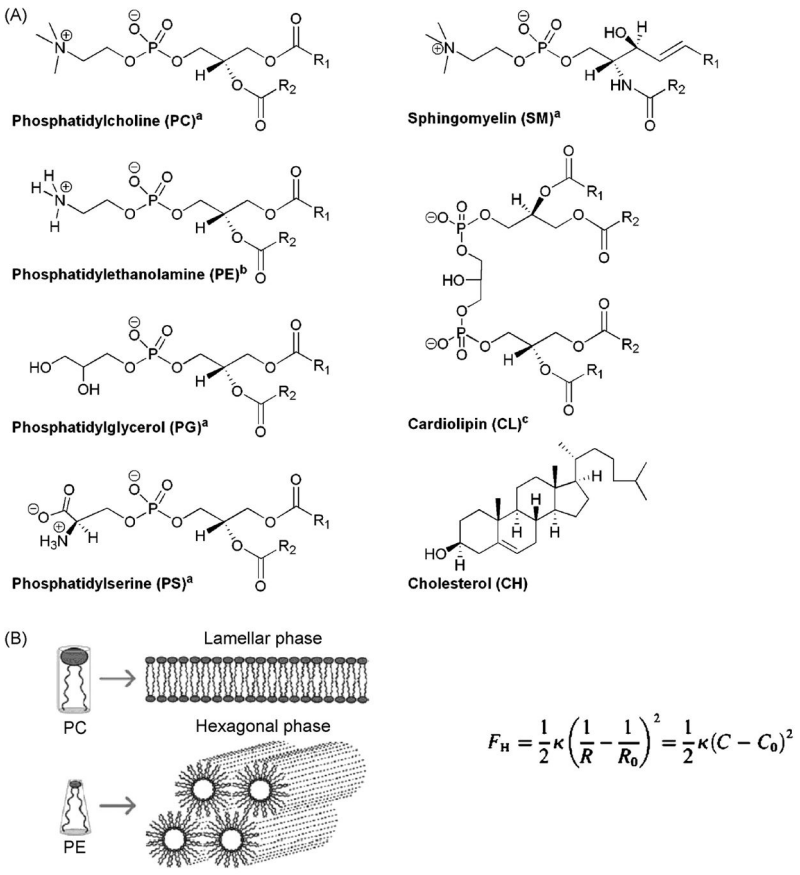


Fig. 12.

(A) General structure of the common phospholipids and cholesterol. All the lipids have a polar phosphate head group and hydrophobic fatty acyl tails R_1, R_2 ($R_1 = R_2$ for symmetric lipid or R_1 ? R_2 for asymmetric lipid). [a] $C_0 > 0$ or $C_0 \sim 0$, [b] $C_0 < 0$, [c] $C_0 < 0$ when bound to Ca^{2+}. (B) Lamellar (top) and hexagonal (bottom) phases promoted by intrinsic curvature of the lipid, $C_0 \sim 0$ (e.g. PC) and $C_0 < 0$ (e.g. PE), respectively. The free energy (F_H) per unit area in the lipid monolayer of the hexagonal phase is approximated by the above equation, where k is the bending modulus for the monolayer, R is the radius of a pivotal plane, and R_0 is the radius of intrinsic curvature describing the lipid assembly in a stress-free state with the minimum energy. Fig. 12(B) reproduced with permission from *Biophysical Journal* [106].

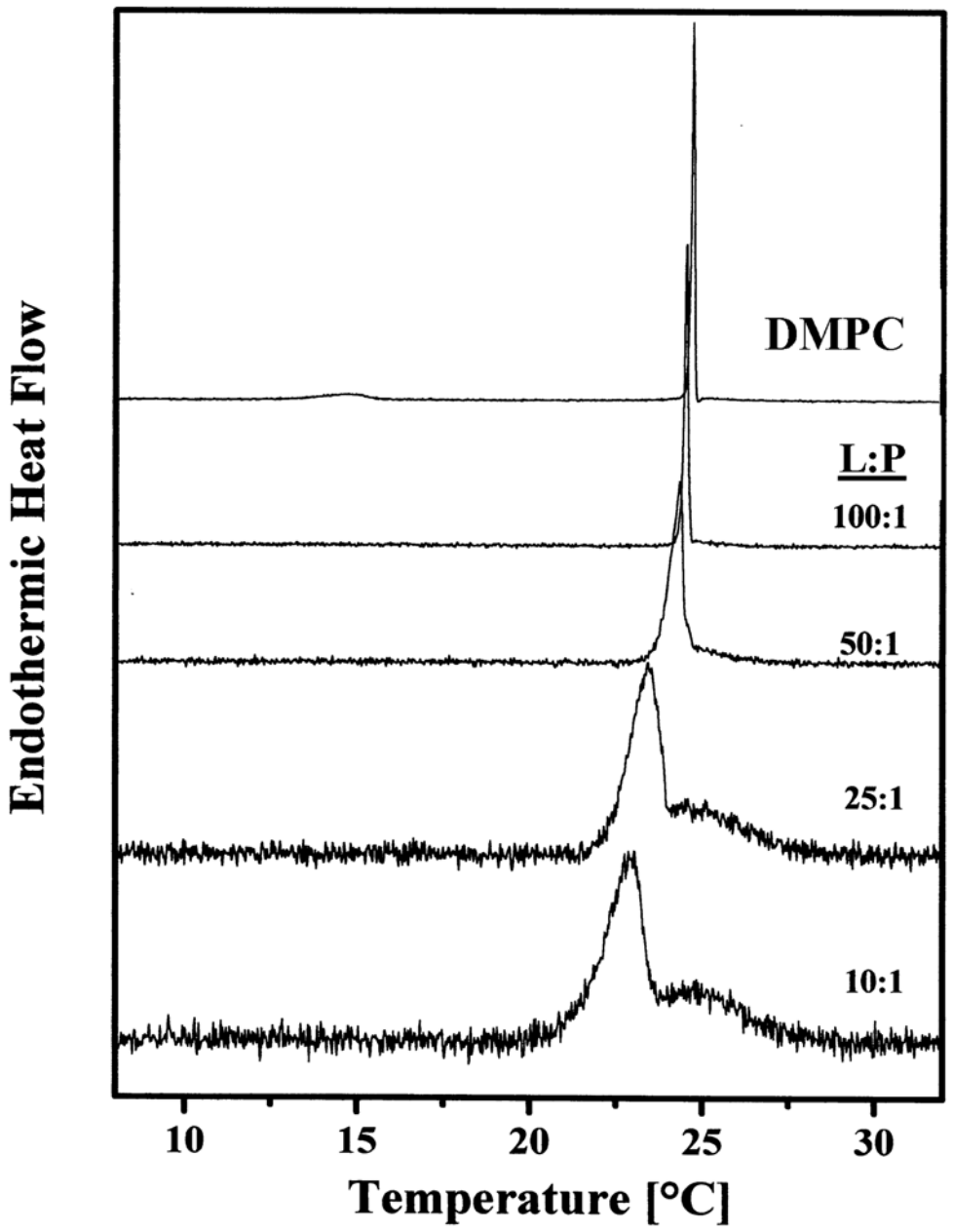


Fig. 13.

High-sensitivity DSC heating scans illustrating the effect of the presence of increasing quantities of gramicidin S (GS) on the thermotropic phase behavior of DMPC MLVs. The top scan is of DMPC alone and the DMPC/GS molar ratios of the lower scans are indicated on the figure itself. Reproduced with permission from *Biochimica et Biophysica Acta, Biomembranes* [129].

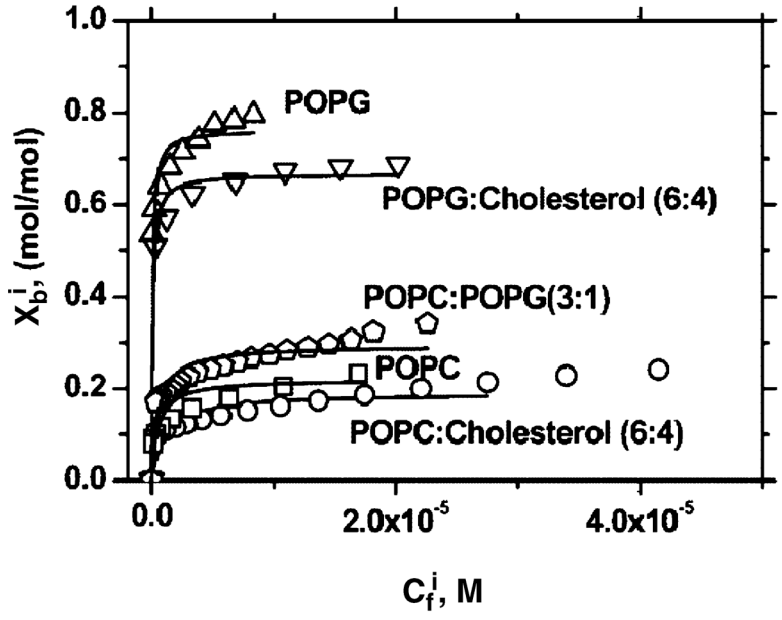


Fig. 14.

Binding isotherms for binding of GS to various LUVs derived from the ITC measurements at 25 °C. The degree of binding (X_b^i) is plotted as a function of free peptide concentration (c_f^i). Each data point represents an individual titration step. The solid lines represent theoretical fits according to the one-site binding mo del. Reproduced with permission from *Biochemistry* [186].

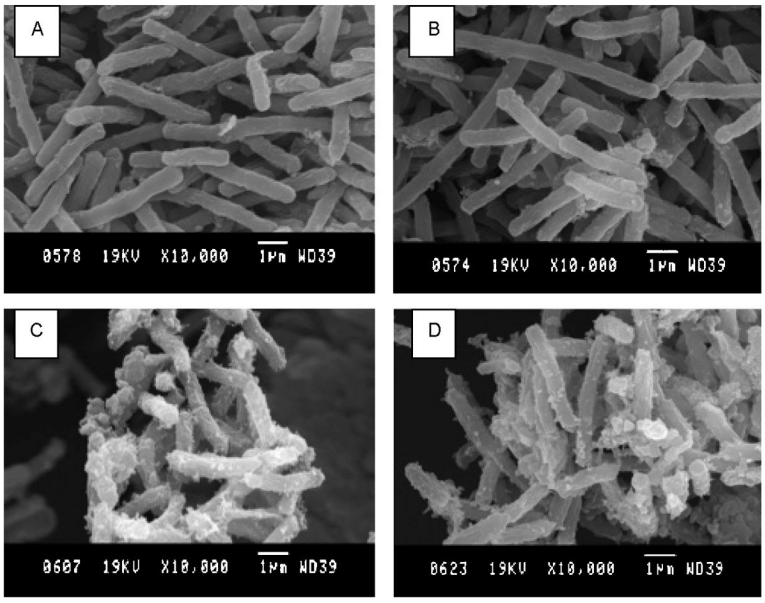


Fig. 15.
SEM micrgraphs of *E. coli* in contact with neat LDPE (A) and modified LDPE (B–D) after 15 (B), 30 (C), and 60 min (A, D) of contact time. Reproduced with permission from *Biomacromolecules* [202].

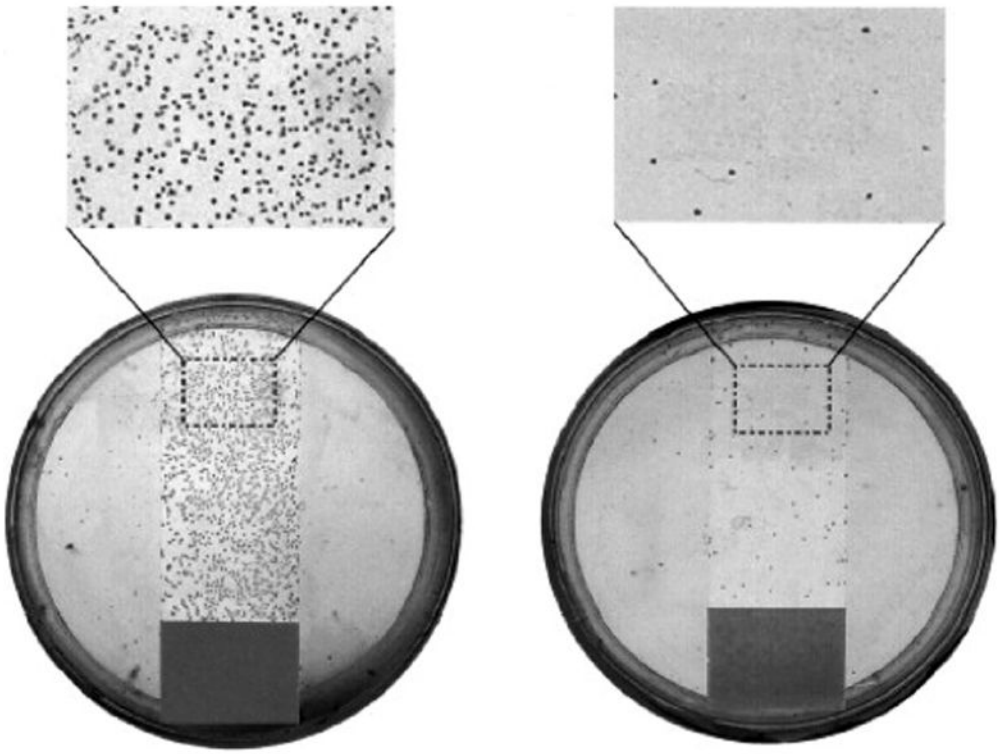


Fig. 16.
Photographs of amino glass slide (*Left*) and a hexyl-PVP-modified slide (*Right*) onto which aqueous suspensions (10^6 cells/mL of distilled water) of *S. aureus* cells were sprayed, air dried for 2 min, and incubated under 0.7% agar in a bacterial growth medium at 37 °C overnight. Reproduced with permission from *Proceedings of the National Academy of Sciences* [218].

Fig. 17.
Photographs of modified medical grade PVC from catheter tubing (blended with AMM), *(Left)*, and unmodified PVC, *(Right)*, after spraying with aqueous suspensions of *S. aureus* cells (10^5 cells/mL), air drying for 3 min, and incubating under rich growth media at 37 °C for 24 h.

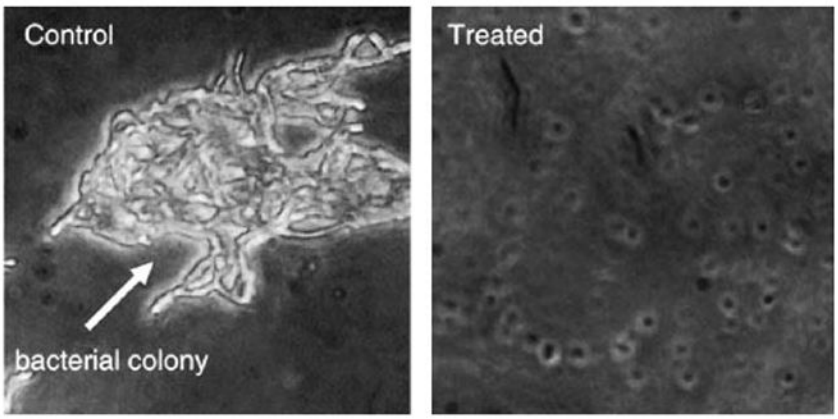
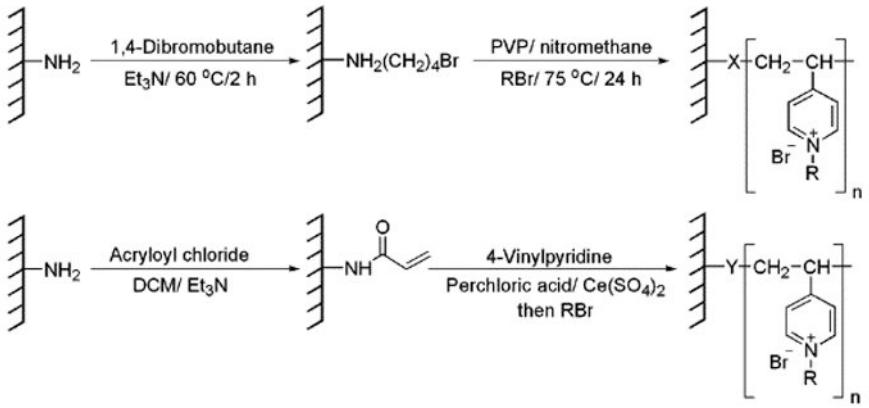
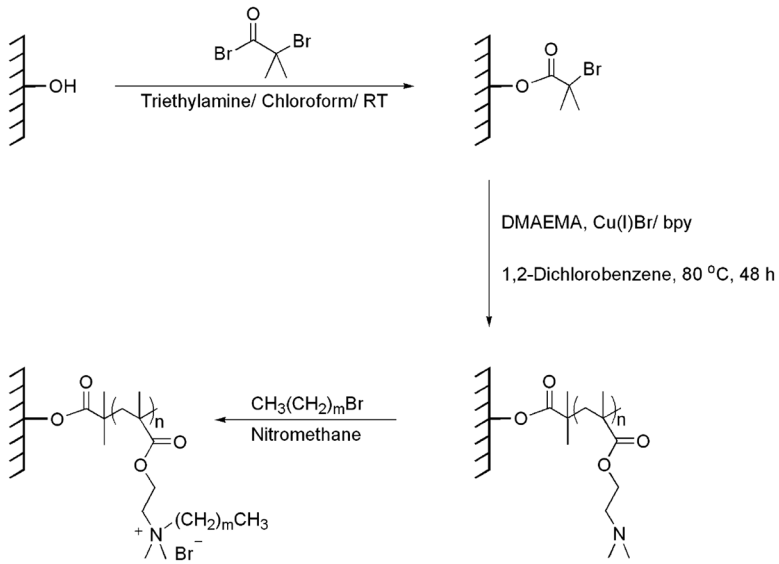


Fig. 18.
Untreated (*left*) and treated (*right*) PU film. The treated sample was able to completely kill *E. coli* whereas the untreated surface allowed bacterial colonization. Reproduced with permission *Journal of Industrial Microbiology & Biotechnology* [67].



Scheme 1.
Synthetic pathways for the formation of quaternized PVP on glass surfaces.



Scheme 2.
Synthetic route for the ATRP and quaternization of DMAEMA on solid surfaces.

Table 1

Classes of non-natural polymers/oligomers studied for their antimicrobial activity.

Chemical structure	Selected groups[a]	Data available[b]	Selected techniques of interest
Antimicrobial peptidomimetics			
β-peptides	WF DeGrado	A, B	Conformation in micelles, vesicle leakage studies, determination of kinetics of vesicle lysis
	SH Gellman and RM Epand	A, B	Protease stability, enzyme-based leakage assay, DSC, ITC, FRET, lipid dependence studies
	D Seebach	A	Determination of broad spectrum activity
Peptoids	J Winter	B	Deconvolution of libraries strategies, flow cytometry of stained cells, mice studies
	AE Barron and PB Messersmith	A, B, C	Helicity assessment in vesicles, protein absorption using OWLS
Cyclic peptides	MR Ghadiri	A, B	ATR/FTIR, depolarization assays, mice studies, drug resistance studies
Facially amphiphilic antimicrobial polymers and oligomers			
Arylamide oligomers and analogues	WF DeGrado, GN Tew, and ML Klein	A, B	DFT computational methods, MD at octane/water interface, logK_{OW} measurements, SFG vibrational spectroscopy
Phenylene ethynylenes	GN Tew	A, B, C	SAXS, lipid movement assays, fluorescence microscopy, MTD assays, toxicity trials on liver cells, resistance assays
Polynorbornenes *Polymethacylates*	GN Tew and EB Coughlin WF DeGrado, I Ivanov, and GN Tew	A, B A,B,C	Designed copolymerization to rationally improve selectivities, lipid studies MD in solution and water-lipid interfaces, incorporation into plastics and onto surfaces
Biocidal cationic polymers			
Polymers with biguanides	S Tazuke, Y. Zhang independently	B	Fluorescence depolarization, DSC, fractionation studies
Oligoguanidines	M Albert and H Hönig	D	Structural characterization of product mixtures with ^{13}C-labelling and MALDI-TOF MS
Polymers containing quaternary ammoniums	LJ Mathias	D	Bactericidal determination, broth-dilution and spread-plate methods, LCST measurements
	R Jérôme	C	Evaluation of block copolymers and materials
	ER Kenawy	D	Novel synthesis and zone of inhibition studies
	S Tazuke, SH Gellman, independently	A	pKa determination
Polymers containing quaternary pyridiniums	S Tazuke, N Kawabata, independently	D	Early report of counting colonies by spread plate method
	B Gao	B	pH dependence studies, galactosidase and TTC-dehydrogenase assays
	G Li	B	Activity against fungi and yeasts, allergy and acute toxicity in animals, SEM
	JS Yoon	D	Comparison of block and random copolymers
Cationic polymers used in materials (found in Section 5)			

[a] Other noteworthy groups are cited within the text.

[b] Data of particular interest to this review includes, A = Selectivity over mammalian cells tested, B = Biophysical conformational or mode of action studied, C = Materials studies reported, D = Mainly the synthesis, characterization, and antimicrobial activity of polymers in solution have been studied thus far by the indicated groups.

Table 2

Lipid distribution in different cell types.[a]

Cell type	PC	PE	PG	PS	SM	CL	CH
E. coli (Gram-negative)	-	80%	20%	-	-	5%	-
S. typhimurium (Gram-negative)	-	60%	33%	-	-	7%	-
P. cepacia (Gram-negative)	-	82%	18%	-	-	-	-
B. subtilis (Gram-positive)	-	12%	70%	-	-	4%	-
S. aureus (Gram-positive)	-	-	57%	-	-	43%	-
RBC (outer leaflet)	33%	9%	-	-	18%	-	25%
RBC (inner leaflet)	10%	25%	-	10%	5%	-	-

[a]There are major differences in the phospholipid composition (by weight percent) of the exposed membrane surfaces among Gram-negative bacteria, Gram-positive bacteria, and erythrocytes (red blood cells) [50,102–104].