Received: 7 April 2020 | Revised: 5 October 2020 | Accepted: 19 October 2020

DOI: 10.1002/app.50222

ARTICLE

Applied Polymer SCIENCE WILEY

Polymerized ionic liquids-based hydrogels with intrinsic antibacterial activity: Modern weapons against antibiotic-resistant infections

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Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: FKZ 03ZZ0910B; Deutsche Forschungsgemeinschaft, Grant/Award Number: KR 2491/12-2

[Correction added on 2 Feb 2021, after first online publication: The copyright line was changed.]

Abstract

In this study, the inherent antibacterial activity of 11 different polymerized ionic liquids (PILs)-based hydrogels as well as their corresponding monomers was examined in an extensive screening. The methicillin-resistant *Staphylococcus aureus Xen 30* (MRSA Xen 30) and *Pseudomonas aeruginosa Xen 5* (*P. aeruginosa* Xen 5) were chosen as test microorganisms. Both are typical representatives of gram-positive and gram-negative bacteria, respectively. Six of the 11 tested monomers were able to eradicate more than 80% of *P. aeruginosa* Xen 5 cells in suspension. Unfortunately, the anionic, neutral and zwitterionic representatives lost their function after polymerization. However, the cationic gels retained their antibacterial activity with nearly 100% eradication of selected microorganisms - even at the smallest amount tested. Bactericidal activity against gram-positive MRSA Xen 30 was high when the bacteria were treated with the imidazolium-based monomers. Five of the tested compounds showed rather limited bactericidal activity <50% killed bacteria. The weak antibacterial activities could be significantly increased by crosslinking them to three-dimensional networks. As a result, all the hydrogels possessed strong killing efficiencies of at least 68% and were able to maintain this activity even at low hydrogel volume fractions. These findings are very promising for the development of new antibacterial materials for medical applications, for example, stent coatings.

1 | INTRODUCTION

Bacteria are one of the oldest forms of life on our planet and can be found almost everywhere, even under inhospitable conditions like extreme pH values or temperatures. For the human health they are both a curse and a blessing while playing an important role in a number of vital processes in the human body. By now, bacteria have also been established in a number of industrial processes.[1] Nevertheless, bacteria that are able to cause infections, can have severe negative consequences in the fields of public health, medical devices and food safety.[2] In the food industry, for example, toxicogenic strains cost millions of dollars annually.[3] More important is the constantly rising number of infectious diseases caused by pathogenic microorganisms despite the conspicuous progress in the standards of health care and medical technology.[4,5] Microbial infections have become once more a

public health concern during the last decades.[6] One of the major reasons is the widespread and rising number of antimicrobial resistances.[7] Several microorganisms have already developed resistance to all known antimicrobial agents resulting in an increased number of infections for which we have no therapeutic options.[8] Among others, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are typical examples of important bacterial strains causing infections.[9] *Staphylococcus aureus* names a gram-positive coccus consisting of yellow-pigmented spherical cells, preferably arranged in grape-like clusters, but more rarely also occurring individually or paired. *Staphylococcus aureus* is facultative anaerobic, non-motile, does not breed spores and can be found nearly everywhere in nature including on the human skin and in upper airways of the human respiratory system.[10,11] Healthy subjects can be colonized by these bacteria without consequences, but having a weakened immune system combined with optimal growing conditions can lead to life treating infections including pneumonia, osteomyelitis, meningitis and arthritis.[10] Unfortunately, some *S. aureus* strains show resistance to common antibiotics such as penicillins and cephalosporins. Additionally, some strains are resistant to vancomycin (VISA/VRSA).[12] Resistance to methicillin (MRSA) indicates insensitivity to β-lactam antibiotics, except for ceftaroline.[13] Very often MRSA strains are also resistant to other antibiotic groups such as macrolides.[14] *Pseudomonas aeruginosa* represents a gram-negative opportunistic bacterium. This species is rod-shaped, asporogenous, monoflagellated and an obligate respire needing oxygen for an optimal metabolism, though an anaerobic respiration is also possible.[15] The hospital strains of *P. aeruginosa* have recently shown an increasing resistance to antibiotics. Since *P. aeruginosa* is a widespread soil and water germ, it also occurs in all other humid environments like sinks, showers, medical devices and medicines making it one of the most frequent hospital

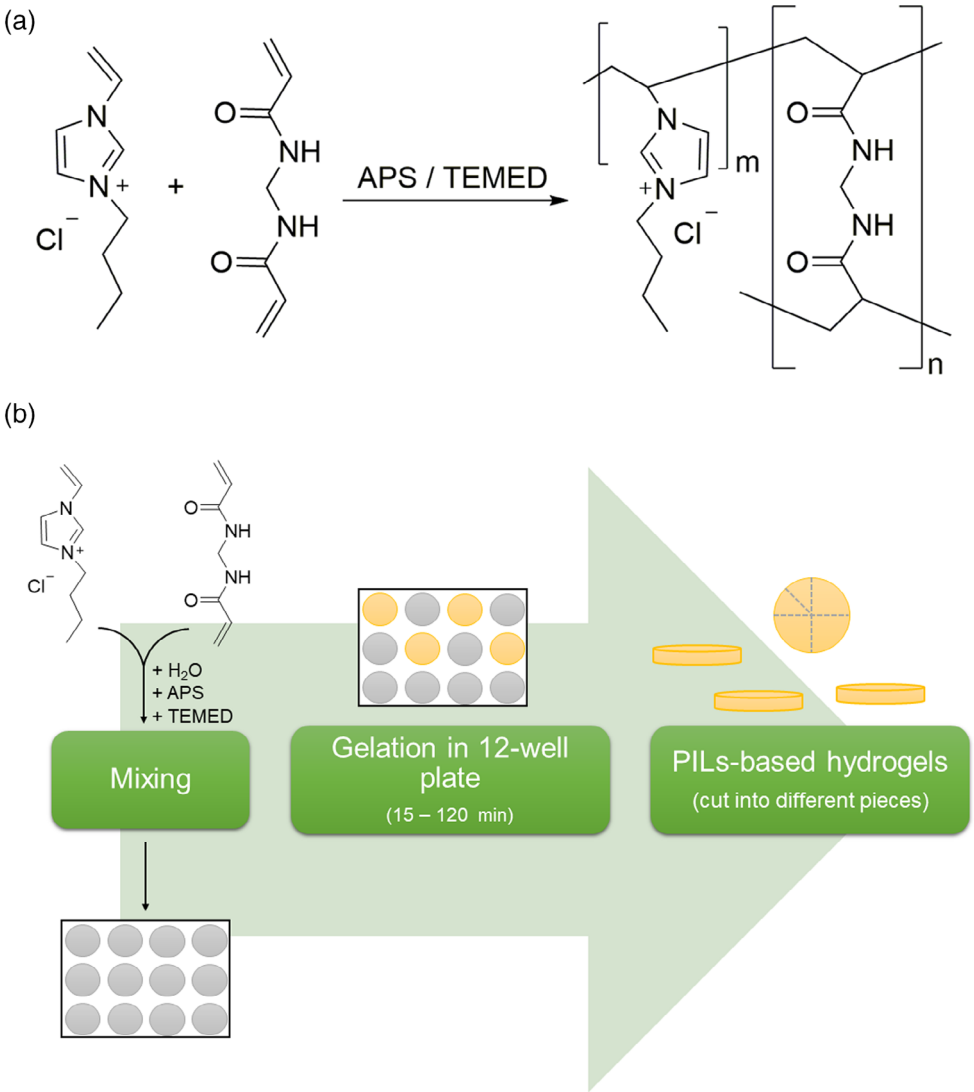

FIGURE 1 (a) Synthesis of poly(VBImCl) via radical polymerization with MBAA as a crosslinker. (b) Schematic illustration of the synthesis of poly(VBImCl) [Color figure can be viewed at wileyonlinelibrary.com]

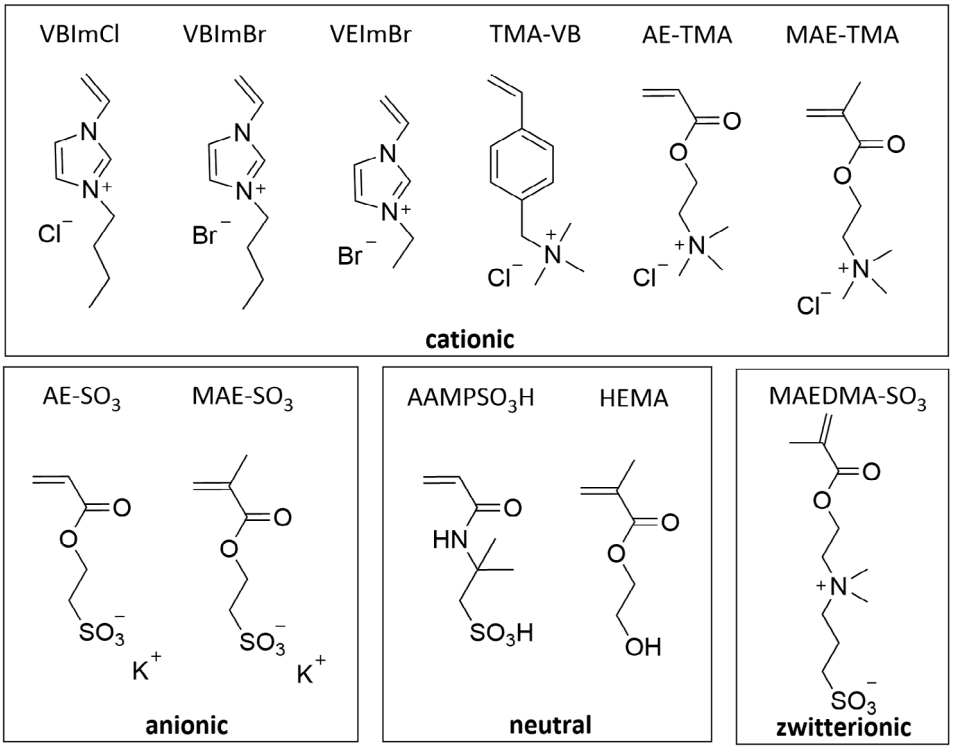

FIGURE 2 Structures of tested monomers

germs. Unfortunately, most hospital strains show resistance to β-lactams, fluoroquinolones and aminoglycosides.[16] *Pseudomonas aeruginosa* causes a wide variety of infections including lung, urinary tract, skin, eyes and ears.[10,17] Facing the problem of drug-resistance, there is a need to design and synthesize novel materials showing antibacterial activity, especially against resistant bacterial strains.[10] This resulted in a large number of new materials over the last years that can be divided into different groups based on the mechanism of their antibacterial effect. Either the substances can be used as a supporting material keeping organic or inorganic substances with antibacterial activity, or their surfaces can be chemically modified to immobilize antibacterial substances. In this context, the functionalized materials inspired by mussel adhesiveness should be mentioned. Generally, this recent approach results in a universal coating procedure for several materials like metals, polymers or ceramics that can be used for several applications.[18–20] In addition, a few substances, such as polymers, show an inherent antibacterial effect, gaining a lot of interest and making them the focus of current research. Having inherent antibacterial effects without the need of any additives results in advantages like longer activity and a minimized risk of toxicity to human cells due to lack of low molecular weight biocides.[21] Polymerized ionic liquids (PILs) are a relatively new and very promising class of functional polymers formed from linked ionic liquids (ILs). Just like ILs, PILs gained special interest based on their effect against gram-positive and gram-negative bacteria as well as fungi and algae.[6,22,23] Especially, imidazolium, pyridinium and quaternary ammonium-based ILs achieved excellent results.[24,25] Furthermore, ILs can also be used for the synthesis of physically or chemically crosslinked polymer networks like hydrogels. These gels are able to absorb more than 90% of water without dissolving or even losing their three-dimensional shape.[26] Combined with their designable mechanical properties and their often good biocompatibility, they were already used for the immobilization of enzymes[27–31] as well as a multitude of other medical applications like drug delivery systems, materials for contact lenses or in tissue engineering.[32–34] Thus, hydrogels provide a suitable approach to develop inherent antimicrobial, especially inherent antibacterial materials.[35,36] The smart combination of the advantages of ILs and hydrogels makes PILs-based hydrogels very attractive for a number of new applications in different fields. Our research group has been dealing with the synthesis and application of these kinds of materials for quite a long time. In our previous work, we reported about mechanical and thermal properties of imidazolium-based PIL-hydrogels, which were also used for the immobilization of enzymes and organocatalysts.[28,37–39] Most recently, the swelling behavior and the rheological properties of different PILs-based hydrogels as well as their biocompatibility have also been investigated. We found that all of these PILs-based hydrogels possessed a good or even a very good compatibility with mouse fibroblasts.[40] In order to complete the overall

picture of these promising materials, we tested the different PILs-based hydrogels as well as their corresponding monomers (Figure 2) with regard to their inherent antibacterial activities against MRSA Xen 30 and *P. aeruginosa* Xen 5. For the first time, an extensive test series of 11 different PILs-based hydrogels having oppositely charged backbones was performed. This broad screening facilitates the identification of correlations between structure and observed effect. Furthermore, possible trends between the different hydrogel groups should be identified.

2 | MATERIALS AND METHODS

2.1 | Chemicals

N,*N*′-methylenebis(acrylamide) (MBAA) (99%; Sigma Aldrich), *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED) (≥99.5%; Sigma Aldrich), ammonium persulfate (APS) (98%; Acros Organics), 3-sulfopropylmethacrylate potassium (MAE-SO_3) (98%; Sigma Aldrich), 3-sulfopropylacrylate potassium (AE-SO_3) (Sigma Aldrich), (vinylbenzyl) trimethylammonium chloride (TMA-VB) (99%; ACROS Organics), [2-(acryloyloxy)ethyl]trimethylammonium chloride (AE-TMA) (80 wt% in H_2O; Aldrich), [2-(methacryloyloxy)ethyl]trimethylammonium chloride (MAE-TMA) (75 wt% in H_2O; Aldrich), 2-hydroxyethylmethacrylate (HEMA) (97%; Alfa Aesar), [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl) ammoniumhydroxide (MAEDMA-SO_3) (95%; Sigma Aldrich), 2-acrylamido-2-methyl-1-propanesulfonicacid ($AAMPSO_3H$) (99%; Sigma-Aldrich), 1-vinylimidazole (≥99%; Alfa Aesar), bromoethane (98%; Alfa Aesar), 1-chlorobutane (≥99%; Acros Organics), 1-bromobutane (99%; Sigma Aldrich), phosphate buffered saline (PBS) (1X solution, pH 7.4; Corning) were used as received. Double distilled water was used throughout this study.

2.2 | Bacterial growth

Staphylococcus aureus Xen 30 (MRSA Xen 30) strains and *Pseudomonas aeruginosa Xen 5* (*P. aeruginosa* Xen 5) strains were cultivated on LB media. Medium for MRSA Xen 30 was prepared by dissolving 20 g LB-Miller Broth (10 g/L NaCl; Fisher) and 15 g Agar (BD Difco™ Dehydrated Culture Media: Granulated Agar; Fisher) in 1 L double distilled water. For *P. aeruginosa* Xen 5, a Cetrimide Agar (Fisher) was used to ensure preferential growing conditions. Media were sterilized for 1 h by autoclaving and agar plates were casted subsequently. Then a pure culture of the respective bacteria was inoculated on the agar plate and incubated overnight at 37°C.

2.3 | Synthesis of polymerizable monomers

1-vinyl-3-ethyl-imidazoliumbromide (VEImBr), 1-vinyl-3-butyl-imidazoliumchloride (VBImCl) and 1-vinyl-3-butyl-imidazoliumbromide (VBImBr) were prepared according to the published procedures.[41–44]

VEImBr: ^{1}H NMR (300 MHz, DMSO-d_6, δ/ppm relative to tetramethylsilane [TMS]): 9.64 (s, 1H, N═C$\underline{H}$—N), 8.24 (s, 1H, N—C$\underline{H}$═CH—N), 7.98 (s, 1H, N—CH═C$\underline{H}$—N), 7.32 (dd, J = 15.77 Hz, J = 8.82 Hz, 1H, N—C$\underline{H}$═CH_2), 5.99 (dd, J = 15.73 Hz, J = 2.37 Hz, 1H, N—CH═C$\underline{H_2}$), 5.41 (dd, J = 8.78 Hz, J = 2.29 Hz, 1H, N—CH═C$\underline{H_2}$), 4.24 (q, J = 7.41 Hz, 2H, *ethyl-α*-C$\underline{H_2}$), 1.45 (t, J = 14.64 Hz, 3H, *ethyl-β*-C$\underline{H_3}$).

VBImCl: ^{1}H NMR (250 MHz, DMSO-d_6, δ/ppm relative to tetramethylsilane [TMS]): 9.84 (s, 1H, N═C$\underline{H}$—N), 8.27 (s, 1H, N—C$\underline{H}$═CH—N), 7.98 (s, 1H, N—CH═C$\underline{H}$—N), 7.34 (dd, J = 15.75 Hz, J = 8.80 Hz, 1H, N—C$\underline{H}$═CH_2), 6.02 (dd, J = 15.63 Hz, J = 2.37 Hz, 1H, N—CH═C$\underline{H_2}$), 5.41 (dd, J = 8.78 Hz, J = 2.29 Hz, 1H, N—CH═C$\underline{H_2}$), 4.23 (t, J = 7.18 Hz, 2H, *butyl-α*-C$\underline{H_2}$), 1.81 (q, 2H, *butyl-β*-C$\underline{H_2}$), 1.28 (sxt, 2H, *butyl-γ*-C$\underline{H_2}$), 0.89 (t, 3H, *butyl-δ*-C$\underline{H_3}$).

VBImBr: ^{1}H NMR (300 MHz, DMSO-d_6, δ/ppm relative to tetramethylsilane [TMS]): 9.64 (s, 1H, N═C$\underline{H}$—N), 8.24 (s, 1H, N—C$\underline{H}$═CH—N), 7.98 (s, 1H, N—CH═C$\underline{H}$—N), 7.32 (dd, J = 15.58 Hz, J = 8.78 Hz, 1H, N—C$\underline{H}$═CH_2), 5.99 (dd, J = 15.71 Hz, J = 2.39 Hz, 1H, N—CH═C$\underline{H_2}$), 5.42 (dd, J = 8.76 Hz, J = 2.29 Hz, 1H, N—CH═C$\underline{H_2}$), 4.23 (t, J = 7.27 Hz, 2H, *butyl-α*-C$\underline{H_2}$), 1.82 (q, 2H, *butyl-β*-C$\underline{H_2}$), 1.28 (sxt, 2H, *butyl-γ*-C$\underline{H_2}$), 0.88 (t, 3H, *butyl-δ*-C$\underline{H_3}$).

2.4 | General procedure for hydrogel syntheses

All hydrogels were synthesized in a standardized process by radical polymerization (Figure 1(a)) at room temperature (20 ± 2°C). This has already been described in prior works of our group.[37,40] The monomer and the according amount of crosslinker (MBAA, 2 mol%) were dissolved in deionized water to adjust the total monomer concentration to the required value (2 mol/L). Afterwards, the according amount of APS-solution was added (0.1 mol% of total monomer amount). TEMED (1.9 mol% of total monomer amount) was added immediately before the

reaction solution was mixed and added into cylindrically shaped molds (22.4 mm diameter).

2.5 | Bacterial killing assay using different polymerizable monomers

For testing the polymerizable monomers, a 50% (w/w) stock solution of monomer 1–4, 7, 8, 10 and 11 (Table 1) dissolved in PBS1X was prepared. Monomers 5, 6 and 9 were used as provided by the respective supplier (Table 1). All samples were sterilized under UV for 30 min. Tests were performed using 96-well plates and a total sample volume of 100 µl consisting of 50 µl bacteria stock solution (ca. $2X10^5$ CFU/ml, OD_{600} 0.3–0.8), x µl monomer solution (x = 50 µl/20 µl/5 µl/1 µl) and 50 µl–x µl PBS1X. After an incubation for 1 h at 37°C, a progressive dilution from 10^5 CFU/ml to 10^2 CFU/ml was prepared. For the first dilution, 10 µl sample solution were mixed with 90 µl PBS1X. For the subsequent dilutions, 10 µl of the solution of a higher concentration were added to 90 µl PBS1X. Finally, two spots (V = 10 µl) of each dilution were plated on LB agar. The plates were left to dry upright and then placed upside down in the incubator for overnight grow at 37°C. To determine the colony forming units (CFU/ml) the number of all bacteria colonies of each spot was counted, averaged for each dilution and multiplied by the respective dilution factor. The percentage of the colony forming units was determined by dividing the obtained value by the reference value of a blank measurement and then multiplied by 100. Subtracting this value from 100, resulted in the percentage of killed bacteria per mL sample solution. The described experiment was performed three times for each monomer and the respective volumes. Additionally, the killing assay was performed over 1 week to determine the duration of the antibacterial effect. The tests were performed by incubating 50 µl of monomer and 10^5 CFU/ml of bacteria at the start of the experiment. The bacteria were maintained in PBS1X supplemented with 20% LB. Samples were taken at 1, 3, 5, 8, 24, 48, 72 h as well as 168 h (1 week) and treated according to the procedure described above.

2.6 | Killing assay using different hydrogels

All hydrogels were synthesized according to the procedure mentioned before. After polymerization, the gels were cut into wedge-shaped pieces of 100, 50 and 25 µl and washed in 4 ml PBS1X overnight to remove the unpolymerized monomer. When the PBS1X was removed, the gels were dried for approximately 4 h at room temperature and sterilized for 30 min under UV light. Tests were performed using 48-well plates and a total sample volume of 1000 µl consisting of 500 µl bacteria stock solution (ca. 2×10^5 CFU/ml, OD_{600} 0.3–0.8), x µl polymerized hydrogel (x = 100 µl/50 µl/25 µl) and 500 µl–x µl PBS1X. After an incubation for 1 h at 37°C, a sample of 100 µl was taken. Again, starting from this sample, a progressive dilution from 10^5 CFU/ml to 10^2 CFU/ml was made by pipetting the 10 µl sample solution and adding 90 µl PBS1X as already described in the

TABLE 1 Stock solutions of the tested monomers

No.	Monomer	Physical state	Concentration of stock solution	Concentration of added monomer in			
				50 µl	20 µl	5 µl	1 µl
1	VBImCl	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
2	VBImBr	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
3	VEImBr	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
4	TMA-VB	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
5	AE-TMA	Liquid[a]	80% (w/w) in H2O[b]	400 mg/ml	160 mg/ml	40 mg/ml	8 mg/ml
6	MAE-TMA	Liquid[a]	75% (w/w) in H_2O[b]	375 mg/ml	150 mg/ml	37.5 mg/ml	7.5 mg/ml
7	MAE-SO_3	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
8	AE-SO_3	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
9	AAMPSO_3H	Liquid[a]	50% (w/w) in H_2O[b]	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
10	HEMA	Liquid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml
11	MAEDMA-SO_3	Solid	50% (w/w) in PBS1X	250 mg/ml	100 mg/ml	25 mg/ml	5 mg/ml

[a]Delivered as solution by the supplier.

[b]As delivered by the supplier.

previous section. The described experiment was performed in triplicate for each kind of hydrogel and respective hydrogel volumes. For determining the duration of the antibacterial effect, the killing assay was performed over 1 week by incubating 100 µl of hydrogel and 10^5 CFU/ml of bacteria at the start of the experiment. The bacteria were maintained in PBS1X supplemented with 20% LB. Samples were taken at 1, 3, 5, 8, 24, 48, 72 h as well as 168 h (1 week) and treated according to the procedure described above.

3 | RESULTS AND DISCUSSION

3.1 | Antibacterial effect against *P. aeruginosa* Xen 5

The monomers that were tested in this study are summarized in Figure 2. They can be divided into cationic, anionic, neutral and zwitterionic species according to their structure. Using the highest concentration of 50 µl monomer solution mixed with 50 µl bacteria stock solution, all substances showed an antibacterial activity against *P. aeruginosa* Xen 5 and killed at least 30% of those gram-negative bacteria (Figure 3(a)). As expected, the dilution of the test solutions with PBS is associated with a reduced killing efficiency of ≥38% in case of HEMA, so that bacterial growth increases comparatively. In two cases, the effect even disappears almost completely at a maximum dilution: the AE-TMA allows bacterial growth of approximately 84% and AE-SO_3 of 94% (Table 2).

It has already been described in the literature that cationic substances have a stronger effect on microorganisms as they favor the interaction with the negatively charged bacterial cell membrane compared to anionic or neutral substances.[6] This trend is also reflected in the results. Using the highest amount of 50 µl, four of the six cationic monomers showed a 100% killing efficiency: VBImCl, VBImBr, TMA-VB and AE-TMA. In contrast to AE-TMA, VBImCl and TMA-VB also showed a very strong effect at 20 µl, which also decreased at lower monomer contents. After adding 1 µl monomer solution both killed ≥60% of the bacterial cells. VBImBr consistently showed the best effect against *P. aeruginosa* Xen 5. Even at the two highest dilution levels, VBImBr was able to eliminate 99.1 ± 0.6% (5 µl) and 68.4 ± 9.9% (1 µl) of the bacteria (Figure 4).

The explanation for this marked antibacterial activity is given in the mechanism of action. Conventional antibiotics exert their antibacterial effect in different ways, for example by blocking DNA replication and RNA synthesis, disturbance of the synthesis of essential metabolites or inhibiting bacterial protein synthesis.[45,46] Instead, the studied monomers probably interact with certain components of the cell membrane. This has been shown for a

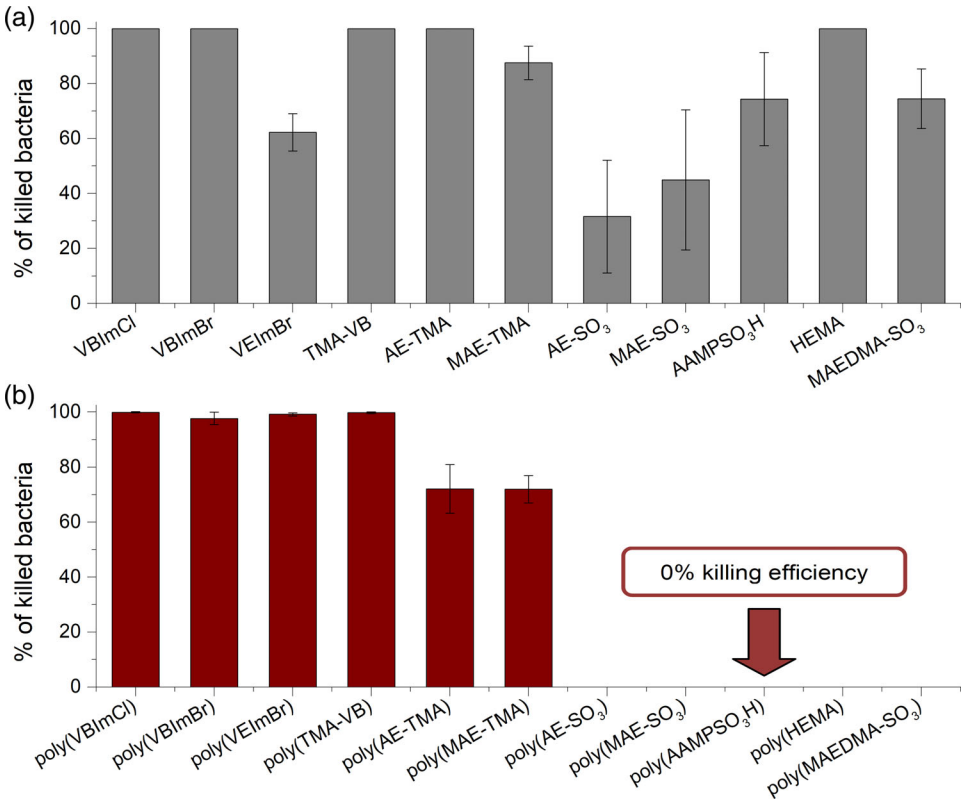

FIGURE 3 Killing efficiency against *P. aeruginosa* Xen 5 of (a) monomers. *Cond.*: 50 µl monomer solution (concentrations in Table 1), 50 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$. (b) hydrogels. *Cond.*: 100 µl hydrogel (c = 2 mol/L), 400 µl PBS1X, 500 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Antibacterial effect at highest dilution of AE-TMA and $AESO_3$ against *P. aeruginosa* Xen 5 ($n = 3$)

	Killed bacteria	
Monomer	**50 µl monomer solution**	**1 µl monomer solution**
AE-TMA	100.0% ± 0.0%	15.7% ± 9.0%
AE-SO_3	31.6% ± 20.6%	6.0% ± 7.0%

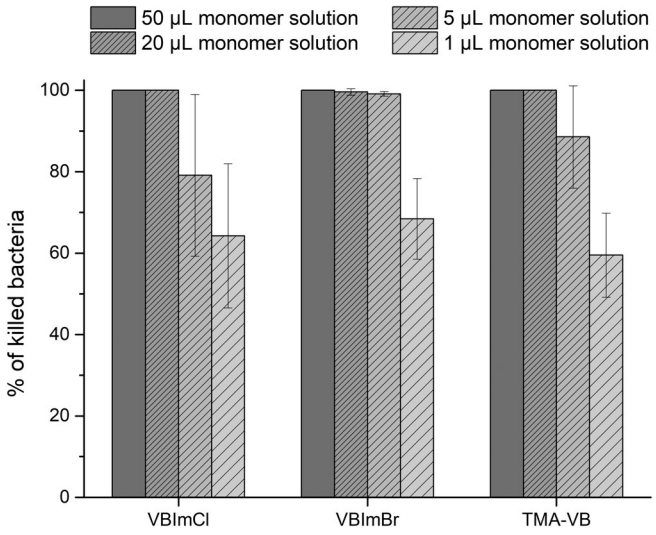

FIGURE 4 Antibacterial effect of different diluted monomer solutions against *P. aeruginosa* Xen 5. *Cond.*: X µl monomer solution (concentrations in Table 1), 50 µl–X µl PBS 1X, 50 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$

number of non-polymeric and polymeric bactericides with cationic components, but also with neutral ones.[47–50] These substances can lead to death without even penetrating the bacterium. Superficially described, first of all, the formation of pores is caused by the incorporation of the positive charged units of the compounds in the bacterial membrane. The more components are laid in, the more permeable the membrane becomes. Finally, the cell leaks out and the associated cell death occurs. This process takes place very quickly and is based on physicochemical alteration of the bacterial membrane, which makes the development of bacterial resistance very difficult.[51,52] For a more detailed explanation of the mechanism of action, the proceeding itself as well as individual substance groups (cationic/anionic) have to be examined more closely. Cationic substances are assumed to come into contact with the negatively charged phosphate and carboxylate groups of the cell wall via electrostatic interactions. This is followed by the described storage of the hydrophobic parts of the compounds, resulting in cell death. So, the antibacterial effect of the cationic species increased with the intensity of the interactions.[53] Based on this knowledge, an attractive interaction between the negatively charged cell membrane of the bacteria and the anionic compound seems rather unlikely. However, the results show a bactericidal effect that is possibly caused by positive counterions. In general, these ions have a certain influence on the bactericidal activity - also in cationic substances, where the influence of the negative counterions is overcompensated by the positively charged framework of the substances. Nevertheless, in anionic compounds the positive counterions are the central point of interaction resulting in antibacterial activity, which is, however, smaller than that of the cationic substances. These results are in strong contrast to the antibacterial effects of the resulting hydrogels, which are referred to by using the prefix "poly" and summarized in Figure 3(b). It is noticeable that only hydrogels based on cationic monomers kill the gram-negative bacteria cells, for example poly(TMA-VB) and imidazolium-based hydrogels with outstanding killing efficiencies of at least 97.7%. Poly(AE-TMA) and poly(MAE-TMA) also had a good effect, killing approximately 72% of bacteria. Also, the reduction of gel volumes by half resulted in >60% of bacteria being killed in these cases (Figure 5).

Although the mechanism of the inherent antibacterial activity of hydrogels is not yet fully understood, it has been hypothesized that it is similar to the mechanism by which these synthetic cationic molecules function. In general, the attraction of the negatively charged parts of the bacterial plasma membrane (inner phospholipid

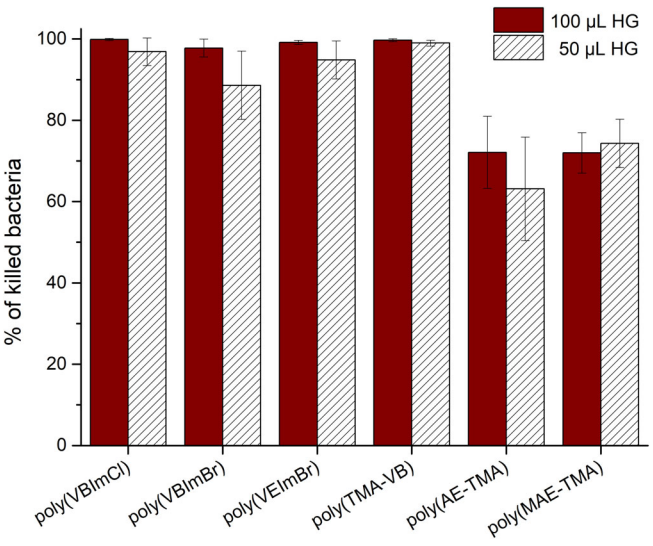

FIGURE 5 Antibacterial effect of different hydrogel amounts against *P. aeruginosa* Xen 5. *Cond.*: X µl hydrogel (c = 2 mol/L), 500 µl–X µl PBS1X, 500 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

membrane) by the cationic nanoporous hydrogels is based on electrostatic interactions. As a consequence, some parts of the inner bacterial bilayer might be pulled into the hydrogel pores as shown schematically in Figure 6 in a highly simplified form. In this process, some of the inner anionic lipid molecules also move to the outside for further interaction. This results in cell wall disruption, membrane lysis and subsequent cell death.[5,50,54] Unfortunately, in these tests neither anionic nor neutral or zwitterionic monomer-based gels showed an inherent antibacterial effect on *P. aeruginosa* Xen 5. This could be explained by the structural differences of the cell envelope between gram-negative bacteria like *P. aeruginosa* Xen 5 and gram-positive bacteria, including the lack of teichoic acid in gram-negative bacteria as a part of the peptidoglycan layer. Teichoic acids are placed on the plasma membrane of gram-positive bacteria, penetrate the peptidoglycan layer and protrude into the extracellular space to interact with other substances, facilitating the penetration of external substances. In case of gram-negative bacteria, the peptidoglycan layer is covered by an additional phospholipid bilayer (outer membrane) and not connected with the plasma membrane. This outer membrane is a natural barrier that makes it difficult for substances to enter the cells. It possesses negatively charged lipopolysaccharides, which have a positive influence on the interaction with cationic substances. However, the additional (outer) membrane, combined with the reduced mobility of the cationic sections in the three-dimensional cross-linked hydrogels, hinders the efficient destruction of the inner cell membrane of gram-negative bacteria when testing the polymerized substances.[55–57] For an effective interaction resulting in cell death, the anion is also crucial.[58] Potassium (K^+) acts as counterion for the two tested anionic representatives poly(MAE-SO_3) and poly(AE-SO_3). Previous studies have shown that polyanionic membranes containing K^+ as a counterion showed no antibacterial activity.[59] In addition to the influence of the already described cationic component of the hydrogel and the permeability of the microbial membrane, the antibacterial activity also depends on the porosity of the hydrogel and the amphiphilicity of the polymer.[5] It appears that an interaction of all these components had a negative effect on the antibacterial activity.

3.2 | Antibacterial effects against MRSA Xen 30

Compared to the effects of the tested components on *P. aeruginosa* Xen 5, the response was very different for some monomers when using MRSA Xen30. It is difficult to see a trend between the different groups when looking at the results of the experiment with the highest amount of monomer of 50 µl (Figure 7(a)). MAEDMA-SO_3, a zwitterionic monomer, killed approx. 67% of the bacterial cells. In comparison, the representatives of the anionic group showed a smaller impact with 43.2 ± 18.3% efficiency for AE-SO_3 and 30.4 ± 14.3% for MAE-SO_3. In the group of cationic and neutral monomers there are great differences regarding their influence on MRSA Xen 30. The imidazolium-based monomers again achieved very good results by killing 98.5 ± 2.5% (in the case of VEImBr) to 100% (for VBImBr) of the bacteria. HEMA also eliminated approx. 99% of bacteria after the prescribed incubation period.

At lower monomer contents of 5 and 1 µl, the percentage of bacteria killed by the imidazolium-based VEImBr decreased from barely 99% to just under 87% and approx. 38% respectively. VBImCl and VBImBr were able to maintain their pronounced effect even at the highest dilution level (1 µl monomer solution) and eliminate at least 85% of the bacteria cells (Figure 8). AE-TMA and MAE-TMA are also cationic in nature, but have only low or negligible activity in the highest concentration.

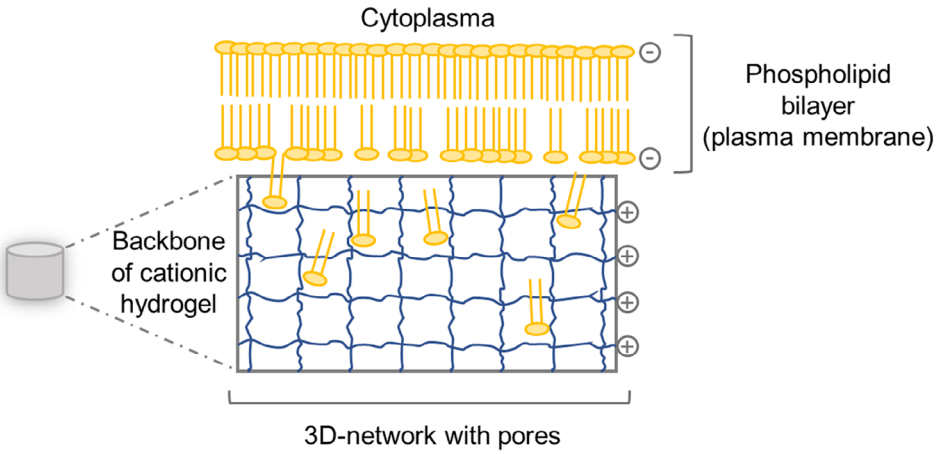

FIGURE 6 Schematic diagram showing the interactions between negatively charged parts of the bacterial plasma membrane of gram-positive and gram-negative bacteria and hydrogels having a cationic framework [Color figure can be viewed at wileyonlinelibrary.com]

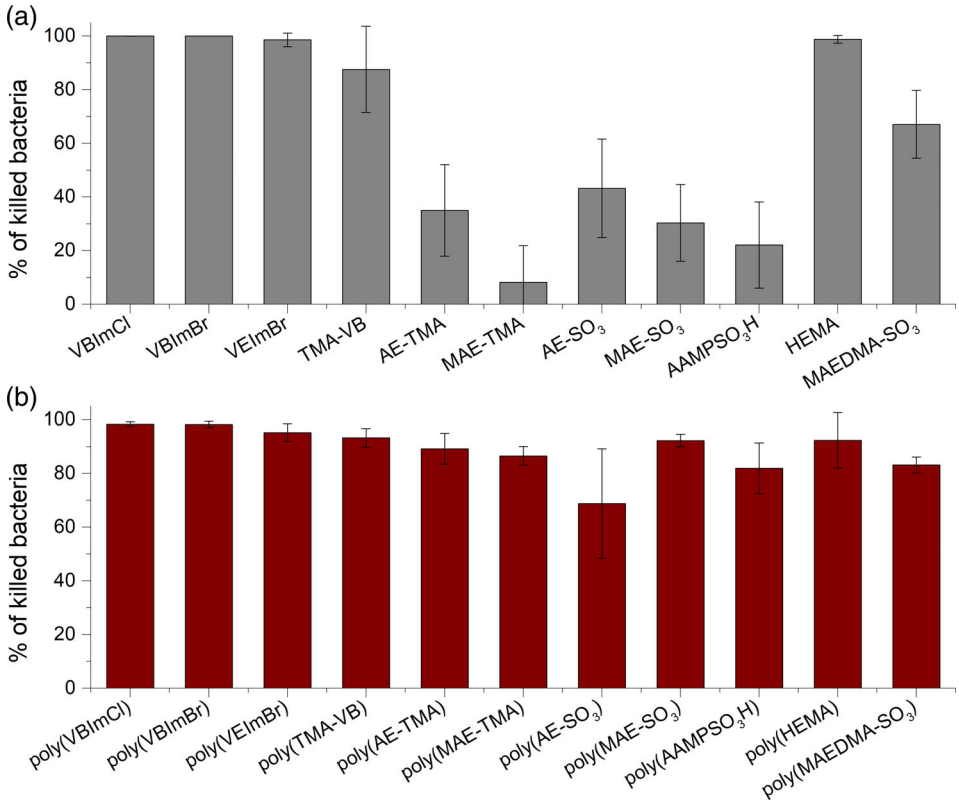

FIGURE 7 Killing efficiency against MRSA Xen 30 of (a) monomers. *Cond*.: 50 µl monomer solution (concentrations in Table 1), 50 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$. (b) hydrogels. *Cond*.: 100 µl hydrogel (c = 2 mol/L), 400 µl PBS1X, 500 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

The results contrast strongly with these generally good results of monomers from the same group. $AAMPSO_3H$, also a formal neutral monomer, had a low effect of <25% killed bacteria cells using the highest concentration of 50 µl.

As can be seen in Figure 7(b), the polymerization of monomers into hydrogels was in most cases accompanied by an increase in antibacterial activity. Since the absorption of large water amounts is a main characteristic of hydrogels and their application as draw agents for osmotic processes has already been reported,[60–62] this phenomenon might be explained by the osmotic pressure gradient between the hydrogel and the bacterial cell. Generally, hydrogels can be classified as strongly swelling and slightly swelling with regard to their swelling degree. According to previous results, all PILs-based hydrogels proved to be swelling agents in distilled water at 37°C. In case of poly(MAE-TMA) and poly(TMA-VB) very strong swelling could be observed resulting in degrees of 13.7 and 20.8, respectively. In contrast to that, poly(HEMA) achieved rather poor swelling degrees of 0.3.[40] This is also reflected in the almost constant antibacterial activity of the monomeric and polymeric form. Consequently, it seems that the ability to absorb water is enhancing the antibacterial effect by an additional cell dehydration. Unlike the small monomers, the polymerized species are much bigger. They are not able to enter the bacterial membrane and probably stick onto the bacterial surface, forming a hydrogel-bacterium-interface that facilitates the diffusion of water out of the bacterium into the hydrogel. As mentioned, this process is driven by the different osmotic states between the bacterial cell and the hydrogel. The latter was swollen in PBS and dried for 4 h before the experiments, resulting in a reduced water and an increased ion content that is probably reinforcing this effect.

The tested hydrogels achieved a killing efficiency of at least 68%. Again, the best results were achieved by 100 µl of the imidazolium-based hydrogels. Even in the polymerized form, these tested substances showed an excellent antibacterial activity of at least 95%. The vinylbutylimidazolium-based species were able to maintain this high elimination rate even at a hydrogel volume of 25 µl (Figure 9). In the case of poly(VEImBr), the activity decreased to barely 58%. When considering the imidazolium-based representatives, the results of the monomer tests showed that VBImCl and VBImBr performed better than VEImBr. While this effect only became apparent with low hydrogel volumes when PILs-based hydrogels were used, the trend in monomers could already be seen at the lowest dilution level. Thus, the influence of the chain length in this case seems to be stronger than that of the counterion. With regard to the antibacterial mechanism of these substances, this fully coincides with the reported effects. Since a C4-chain is more hydrophobic than the C2-chain of VEImBr, the

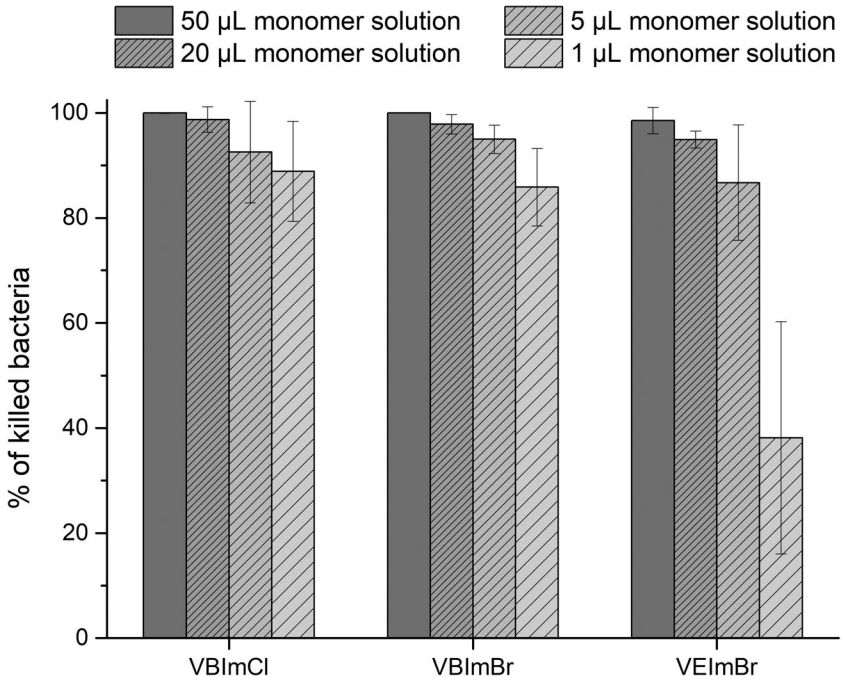

FIGURE 8 Antibacterial effect of different diluted imidazolium-based monomer solutions against MRSA Xen 30. *Cond.*: X µl monomer solution (concentrations in Table 1), 50 µl–X µl PBS 1X, 50 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$

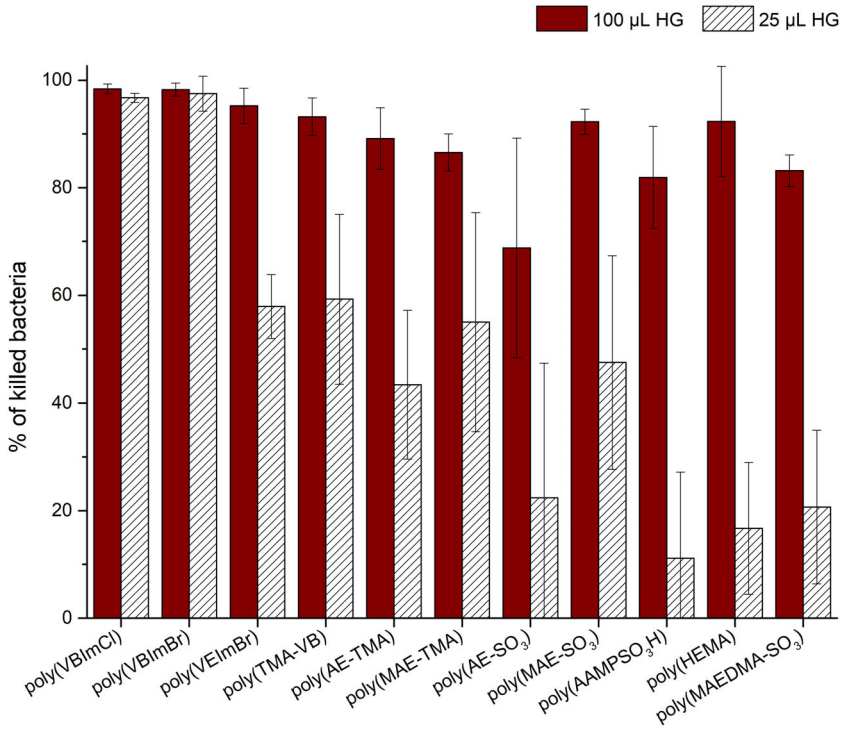

FIGURE 9 Antibacterial effect of different hydrogel amounts against MRSA Xen 30. *Cond.*: Different hydrogel volume (c = 2 mol/L), 400 µl PBS1X, 500 µl bacteria stock solution, 1 h incubation at 37°C, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

former can penetrate more strongly into the phospholipid bilayer to perforate the membrane more and promote cell death. The results are also in line with previous investigations performed using ILs of this type.[8] In general, it should be emphasized that the other hydrogels, which are based on cationic monomers, produced good results with a reduced hydrogel volume of 25 µl. Poly(AE-TMA) also killed nearly half (43.4%) of the bacteria cells. Only poly(MAE-TMA) and poly(TMA-VB) had killing efficiencies of <50%. Compared to the results with gram-negative *P. aeruginosa* Xen 5, it is noticeable that the hydrogels from the anionic, neutral and zwitterionic group also achieved very good results at higher volumes when using gram-positive MRSA Xen 30. The reason for this very different influence on MRSA Xen 30 and *P. aeruginosa* Xen 5 might probably be the differences in the cell envelope

again. Gram-positive bacteria contain the anionic teichoic acid which is embedded in the peptidoglycan layer. It is attached to the phospholipid bilayer (inner membrane) and is able to introduce an additional negative charge resulting in a stronger interaction between the cations (or counter-cations) and the membrane. In this way, the influence of the anion on the killing efficiency becomes marginal.[58] Therefore, gram-positive bacteria generally show a higher susceptibility towards bactericidal substances compared to gram-negative ones.[63–65] Unfortunately, this marked antibacterial effect decreased strongly at lower volumes or disappeared completely. For example, poly(AE-SO_3) has very little antibacterial effect (22.4% killed bacteria cells). The methylated analogue also lost nearly 45% of its original killing efficiency. A drastic reduction of the effect can also be observed with poly(AAMPSO_3H) and poly(MAEDMA-SO_3). Here the effect is just ≤20% when using 25 µl hydrogel.

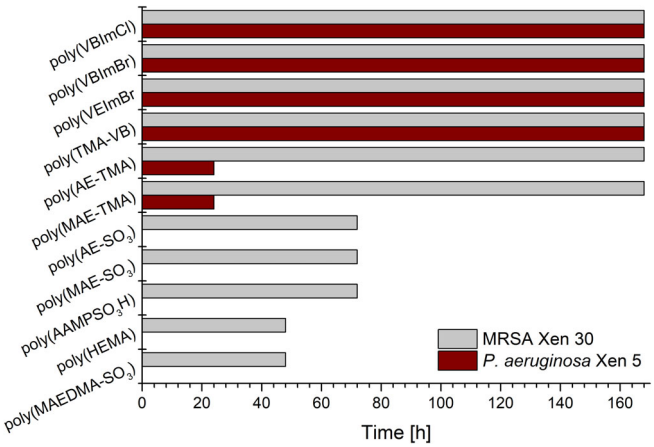

FIGURE 10 Time span of the antibacterial effect (≥50% killing efficiency) of the tested hydrogels against MRSA Xen 30 (dark red) and *P. aeruginosa* Xen 5 (gray). *Cond.*: 100 µl hydrogel volume (c = 2 mol/L), 400 µl PBS1X, 500 µl bacteria stock solution, 168 h (1 week) incubation at 37°C, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Time span of the antibacterial activity

The fields of application of hydrogels with inherent antibacterial properties seem to be broadly diversified. However, for an adequate application it is important to know the length of time of action. In this work the time span of the inherent antibacterial effect was tested concerning two different aspects. First, the influence of the storage time has been tested with 14 days old hydrogels. In these measurements neither a significant increase nor a significant decrease of antimicrobial activity could be detected. Generally, the imidazolium-based hydrogels as well as poly(TMA-VB) were able to maintain their very high killing efficiencies for both types of bacteria. The other hydrogels also exhibited approximately the same efficiencies compared to the results of the freshly synthesized representatives. Therefore, the storage in an airtight container for 2 weeks has no influence on the antibacterial activity of the gels.

Furthermore, the antibacterial effect was monitored over a period of 1 week by determining the killing efficiency after 1, 3, 5, 8, 24, 48, 72 h and 168 h (1 week) of incubation at 37°C. It was observed that all substances with a 100% killing efficiency were able to maintain this effect even after 1 week. In the case of incomplete killing, the big majority of the hydrogels retained an efficiency of ≥50% for at least 72 h. Only poly(AE-TMA) and poly (MAE-TMA) showed shorter durations of action of at least 24 h when working with gram-negative *P. aeruginosa* Xen 5. Unfortunately, the effect vanished after 48 h. In contrast to that, the imidazolium-based hydrogels as well as poly(TMA-VB) retained their killing efficiencies of 100% for at least 1 week. In case of MRSA Xen 30, all hydrogels having positively charged backbones retained their effect over the period of investigation. Poly(AE-SO_3) and poly(MAE-SO_3), both hydrogels having a negatively charged backbone, as well as poly(AAMPSO_3H) showed an efficiency of >50% for at least 72 h. After 1 week, their efficiency dropped under 50%. The lowest activities after 3 days were seen in poly(HEMA) and poly(MAEDMA-SO_3) killing 45.6% and 31.1% of the gram-positive bacteria, respectively. After 1 week of incubation, poly(HEMA) was still able to kill 11.9% of the bacteria but poly(MAEDMA-SO_3) lost its antibacterial properties (Figure 10).

A similar trend could be observed for the time span experiments of the monomers. As expected, storage under airtight and cool conditions was no problem for the monomers. The time killing assay showed that for both bacteria species, the imidazolium-based monomers VBImCl and VBImBr as well as TMA-VB and HEMA were able to maintain their high killing activities of at least 87% over the whole testing period of 1 week. VEImBr also retained its antibacterial effect for at least that time. Testing the influence on gram-negative *P. aeruginosa* Xen 5 showed that the good killing efficiencies of AAMPSO_3H (74.4%) and MAEDMA-SO_3 (74.5%) did not decrease to <50% before 48 h. In case of MRSA Xen 30, MAEDMA-SO_3 showed the same development. In contrast to that AAMPSO_3H showed an initial activity of <50% anyway.

4 | SUMMARY AND CONCLUSION

For the first time, a broad screening of 11 different PILs-based hydrogels and their corresponding monomers with regard to their inherent antibacterial effects against *P. aeruginosa* Xen 5 and MRSA Xen 30 was performed ($n = 3$) to examine structural trends. The tested substances can be divided into four different groups based on their structural differences: six cationic, two anionic, two neutral and one zwitterionic representative. The monomers also serve as starting materials for the production of hydrogels. Using a crosslinker, they are linked by radical polymerization to form three-dimensional networks. After gelation and washing, the gels were tested without adding any antibiotics. The killing efficiency of the resulting hydrogels was of particular interest due to the variety of possible applications of hydrogels in the medical field, for example, as implants, drug delivery systems, contact lens material, bone substitutes and stent coatings.[26,66,67] The results showed that all hydrogels with a cationic framework have a good to very good inherent antibacterial effect (>70%) against both, gram-negative and gram-positive bacteria. Outstanding results were achieved by using imidazolium-based hydrogels having a killing efficiency of at least 95%. It was possible to demonstrate and confirm the excellent antibacterial effect of ILs and PILs in the form of PILs-based hydrogels as well. Especially, poly(VBImCl) and poly(VBImBr) showed very high killing effects even at the lowest hydrogel content of 25 µl. In the case of gram-positive MRSA Xen 30, there was also a strong effect (≥68.8% killed bacterial cells) observed for the anionic, neutral and zwitterionic species. In contrast, these polymerized species did not show any effect on the bacterial growth of *P. aeruginosa* Xen 5 due to the differences in the cell envelope structure between gram-positive and gram-negative bacteria. Furthermore, it could be shown that storing the hydrogels in an airtight container for 14 days does not have a significant effect on the antibacterial effect. Therefore, freshly synthesized hydrogels as well as stored gels can be used. Incubation tests also showed that the inherent antibacterial activity of monomers and hydrogels lasts for several days. When testing hydrogels and MRSA Xen 30, a decrease <50% killing efficiency could be observed not earlier than after 72 h. In the case of gram-negative *P. aeruginosa* Xen 5, four of the six effective hydrogels maintained their 100% activity over the test period of 1 week.

For the investigations in this study, different volumes of wedge-shaped gels were used. Since a dependence on the killing efficiency on the surface is very likely, further experiments with varying surface-to-volume ratios are planned. Additionally, the toxicity aspect needs to be further investigated. One of our previous works has already shown good to very good cell compatibility in direct contact with mouse fibroblasts. The best result was achieved by poly(VEImBr) that shows a relative cell viability of 98.4%. With a reduction of cell vitality by not more than 20%, poly(MAE-TMA), poly(AE-SO_3), poly(MAE-SO_3), poly(MAEDMA-SO_3) and poly(HEMA) possess just a low cytotoxicity. These results are in the same range of hydrogels based on hyaluronic acid or alginate that are commonly used in the medical field.[40] Nevertheless, additional biocompatibility tests with macrophages and a haemolysis test could be implemented in the future.

ACKNOWLEDGEMENTS

We acknowledge the financial support by the Deutsche Forschungsgemeinschaft and the University of Rostock within the funding programme Open Access Publishing. Funding by the Federal Ministry of Education and Research within RESPONSE "Partnership for Innovation in Implant Technology" (FKZ 03ZZ0910B) and the Deutsche Forschungsgemeinschaft (DFG; grant KR 2491/12-2) as well as the HERMES research funding of the University of Rostock is gratefully acknowledged. We also thank Katrin Feest for proof reading and Fitzroy Byfield for the technical support.

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How to cite this article: Claus J, Jastram A, Piktel E, Bucki R, Janmey PA, Kragl U. Polymerized ionic liquids-based hydrogels with intrinsic antibacterial activity: Modern weapons against antibiotic-resistant infections. *J Appl Polym Sci*. 2021;138:e50222. https://doi.org/10.1002/app.50222