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Hydrophilic Quaternary Ammonium Ionenes—Is There an Influence of Backbone Flexibility and Topology on Antibacterial Properties?

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The antimicrobial properties of polycations are strongly affected by the structural features such as the backbone flexibility and topology (isomerism) through the polymer ability to attain proper conformation in interaction with the cell membrane. In this paper, a synthesis and biocidal properties evaluation of ionenes characterized by different backbone topology (isomerism) and flexibility are presented. The findings reveal influence of variation in topology on activity against different microorganisms, and general positive effect of improved flexibility. Furthermore, one of the obtained ionenes displays degradable properties in near physiological environment (phosphate-buffered saline pH 7.4, 37 °C). The degradation proceeds via Hofmann elimination reaction and the products are not of acidic character. For the first time a new class of degradable ionenes with a high antimicrobial potential is presented.

1. Introduction

The routine use of broad-spectrum antibiotics significantly reduced mortality rate associated with infectious diseases and postoperative inflammations. Unfortunately, the drug misuse in combination with the short life cycle of bacteria resulted in a steep rise of antibiotic resistance.[1–4] The hospital-acquired infections caused by antibiotic-resistant strains are responsible for 100 000 death annually in the USA alone.[4] Almost 11 000 of these deaths can be attributed to Methicillin-Resistant *Staphylococcus aureus*.[3] Decrease in effectiveness of traditional antibiotics creates intensive pressure to develop new classes of drugs.[5] Polycations mimicking antimicrobial peptides (AMPs) become a promising group of novel antimicrobial agents combining low toxicity of AMPs, especially host defense peptides, with cost-effective synthesis of polymeric disinfectants.[6–8] The polycations disrupt the bacterial cell membrane integrity and their mechanism of action frequently exploits nonspecific interactions not present in traditional antibiotics limiting development of bacterial resistance against polycations.[7,9,10]

During the last two decades many physicochemical properties and structural factors, responsible for biocidal activity of polycations, have been identified.[7] Average molecular weight,[11–16] type,[17–21] density of cationic groups[22–24] responsible for attraction to negatively charged cell membrane, the position of alkyl hydrophobic chains relative to cationic groups (same center vs. different center),[25–27] molecular architectures of polymer (homopolymers, random or block copolymers, telechelic polymers, branched polymers, etc.)[28–31] and amphiphilic balance[26,32–40] were broadly studied.

The amphiphilic balance is an important factor strongly influencing biocidal activity of polycations. Many research groups have shown that excessively hydrophilic polycations are inactive due to problems with incorporation into cell membrane, whereas too hydrophobic polycations are unable to interact with phospholipids bilayer due to aggregation or limited solubility in water.[26,32–40] However, some very hydrophilic polycations containing quaternary ammonium group along the polymer backbone (called *ionenes*), and lacking hydrophobic groups, have shown excellent antimicrobial activity and very low hemolytic properties.[41–46] Moreover, very good skin compatibility and in vivo bactericidal activity of the ionenes were proved.[41]

Polycations are able to attain different conformations in water environment and it could be assumed that some of those conformations stronger interact with a cell surface. According to popular theory amphiphilic polycations have a random coil structure in solution which turn into a globally amphiphilic conformation upon contact with a cell membrane (biomembrane-induced globally amphiphilic conformation).[9,10,47] The polymer conformational changes and possible conformers are dependent on the backbone flexibility and topology, therefore these factors are expected to have an influence on the biocidal activity and selectivity. However, reports on this topic in case of

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polycations are rather limited.[48–50] The influence of molecules conformational flexibility on biocidal activity was broadly investigated in case of AMPs.[51–55] The AMPs flexibility modulation involves macrocycle expansion[53] and incorporation of glycine or proline[51,52,54,55] what additionally can modify amphiphilicity of the molecule. The study on polycations exhibiting difference in backbone flexibility only, and evaluation of their biological properties, can bring us closer to understanding structure-activity correlation not only for polycations themselves, but also for AMPs. The polymeric backbone topology in terms of aryl group isomerism was shown to have a great impact on ionenes behavior in solution. It was manifested as a difference in ability to hydrogel formation[56] and azo dyes aggregation induction.[57] Moreover, the influence of the backbone topology of ionenes on the hydrogels self-healing and self-standing properties[58] as well as on the properties of n-doped ionenes modified electrodes[59] were reported with support of Molecular Dynamic simulations. Published works strongly support the hypothesis that the topology of ionenes backbone should also have a strong impact on their biocidal activity.

In this work we try to answer the question, how the backbone flexibility and topology of hydrophilic ionenes affects their antimicrobial and hemolytic activity? To address this hypothesis, reaction between different isomers of α,α'-dibromoxylenes and different tertiary diamines was used to obtain a series of ionenes. Incorporation of 1,4-diazabicyclo[2.2.2]octane (DABCO) or tetramethylethylenediamine (TMEDA) into the polymer backbone allowed us to tune molecular flexibility without affection of another structural features like the amphiphilic balance, positive charge density, and type of the end groups in order to isolate desired polymer parameters. Additionally, to further study isomeric effect, two polymers possessing higher positive charge density were obtained using a novel cationic linker consisted with two DABCO subunits connected with the ethylene group. The novel linker is degradable in physiological conditions (phosphate-buffered saline (PBS) pH 7.4, 37 °C) via Hofmann elimination reaction and, what is rather unique, degradation products are not of acidic character. Herein a new class of degradable and nonhemolytic ionenes possessing excellent antimicrobial activity is reported.

2. Results and Discussion

2.1. Polymer Synthesis and Characterization

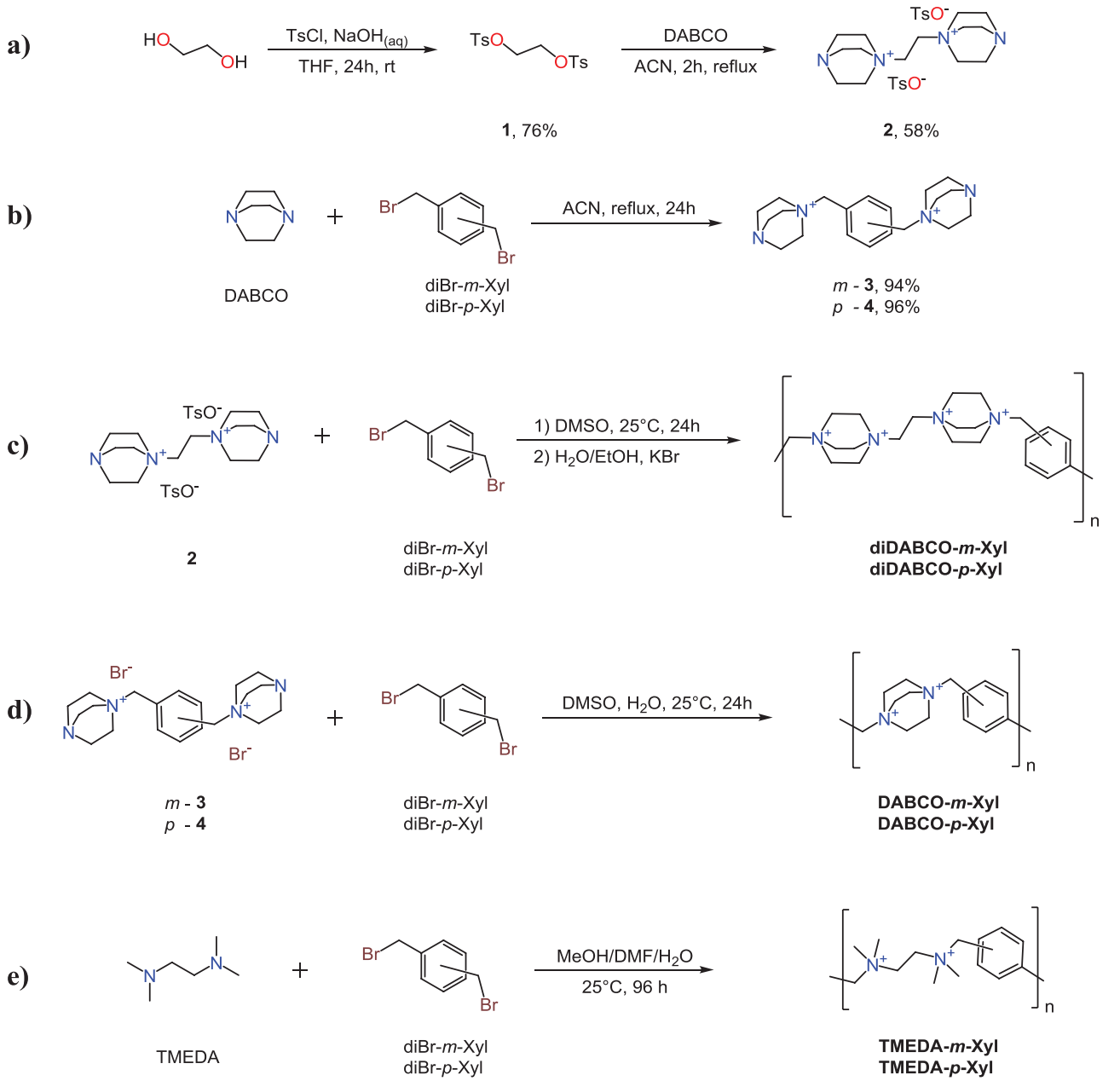
To investigate the influence of polymeric backbone topology and rigidity on bioactivity of ionenes, the series of macromolecules was synthesized using polyaddition of di-tertiary ammines to different isomers of α,α'-dibromoxylenes (**Scheme 1**). A comparative study on topology was performed on the ionenes possessing *meta* or *para* isomers of aromatic subunits along the backbone, whereas the effect of the backbone stiffness was studied by comparison of polymers possessing DABCO and TMEDA subunits. Structures of DABCO-*m*-Xyl, DABCO-*p*-Xyl, TMEDA-*m*-Xyl, and TMEDA-*p*-Xyl polycations were designed to possess the same amphiphilic balance and positive charge density. In each polycation, quaternary ammonium groups are joined alternately using the ethylene group and xylene moiety what enabled to maintain a constant ratio between carbon and nitrogen atoms in different polymers. To further investigate the isomeric effect additional polycations with higher positive charge density along the backbone—diDABCO-*m*-Xyl and diDABCO-*p*-Xyl—possessing linked DABCO moieties were synthesized. Other structural features, such as a type of counter-anion, an average number of quaternary ammonium groups per molecule and a type of terminal groups, which may affect the antimicrobial activity were constant within the series.

To obtain two polycations with the higher positive charge density (diDABCO-*m*-Xyl and diDABCO-*p*-Xyl, Scheme 1c) the monomer 1,2-bis(4-aza-1-azoniabicyclo[2.2.2]oct-1-yl)ethane ditosylate (**2**, diDABCO) was synthesized in reaction between di(*p*-toluenesulfonate) (**1**) and DABCO.[60,61] The polyaddition reaction of **2** to α,α'-dibromo-*m*-xylene (diBr-*m*-Xyl) or α,α'-dibromo-*p*-xylene (diBr-*p*-Xyl) was performed in DMSO at room temperature using 20% molar excess of **2** to control average molar mass of ionenes[62,63] and type of the end groups. The reaction between diBr-*p*-Xyl and **2** gave satisfied results in terms of molar mass distribution (MMD) determined by gel permeation chromatography (GPC). However, the synthesis of diDABCO-*m*-Xyl under the same conditions led to polymeric material with broad and not regular MMD (Figure S2a, Supporting Information). Observed sharp peaks, corresponding to molecules with lower masses, can be assigned to cyclic side products. This was additionally confirmed in polyaddition experiments performed with different monomer concentration and GPC control (Figure S2a, Supporting Information). The polymerization performed under elevated monomer concentration (560 mmol L^{-1}) significantly decreases the amount of low molar weight oligomers (Figure S2b, Supporting Information).

To verify the type of end groups present, the polymers terminated with benzylbromide group were synthesized (Scheme S1a, Supporting Information). The comparison between ^{1}H NMR spectra of ionenes terminated with amine DABCO and benzyl bromide end groups revealed that ^{1}H NMR signals from each group can be easily distinguished (Spectra S1 and S2, Supporting Information), allowing to determine degree of polymerization (DP) (**Table 1**). We can conclude (within the limit of ^{1}H NMR detection) that reaction carried out with 20% molar excess of amine **2** leads to the polymer possessing amine terminal groups only.

The diDABCO containing ionenes were obtained as tosylate salts, whereas other studied ionenes as bromides. Since the type of counter-anion could affect antimicrobial and hemolytic activity of polycations,[64–66] exchange of tosylates for bromides was performed to avoid undesired influence. The tosylates were successfully, and without significant influence on molar mass distribution, exchanged to bromides by precipitation with ethanol (Spectra S7 and S8, Figure S2b, Supporting Information).

Our attempt to obtain rigid ionenes with a single DABCO subunit (DABCO-*m*-Xyl and DABCO-*p*-Xyl, Scheme 1d) using the direct polyaddition between DABCO and α,α'-dibromoxylenes in DMSO resulted with aldehyde side products formation (Scheme S2a, Spectrum S20, Supporting Information). Alkyl bromides undergo Kornblum oxidation reaction to aldehydes in DMSO in presence of a base,[67] but use of different organic solvent was not possible due to poor solubility of DABCO-*p*-Xyl and DABCO-*m*-Xyl. In order to decrease



Scheme 1. Synthesis of monomers and further polyaddition leading to desired ionenes. The polyaddition reaction were performed in diamine excess regime. Bromides were omitted for clarity.

Table 1. Characteristics of obtained ionenes.

Polymer	DP (NMR)	n $N^{+d)}$ (NMR)	M_n [kDa] (NMR)	M_n [kDa] (GPC)	$Đ_M$ (GPC)
diDABCO-*p*-Xyl[a)]	8.2	34.8	5.9	3.9	1.68
diDABCO-*m*-Xyl[c)]	12.2	50.8	8.7	6.9	1.58
DABCO-*p*-Xyl[b)]	26.3	63.4	10.4	4.9	2.11
DABCO-*m*-Xyl[a)]	21.2	44.4	8.5	4.8	1.73
TMEDA-*p*-Xyl[b)]	21.6	45.2	8.6	8.2	3.70
TMEDA-*m*-Xyl[c)]	14.5	31.0	5.9	4.0	1.48

[a)]Polymer obtained as hemihydrate: $N^+ \cdot 1/2H_2O$; [b)]polymer obtained as monohydrate: $N^+ \cdot H_2O$; [c)]polymer obtained as sesquihydrate: $N^+ \cdot 3/2\ H_2O$; [d)]average number of cationic group per molecule.

nucleophilicity and increase pK_a of DABCO tertiary amine group, the monoalkylated DABCO monomers **3** and **4** were synthesized using known procedure[68] (Scheme 1b). The polyaddition reactions of **3** and **4** with diBr-*m*-Xyl and diBr-*p*-Xyl led to desired DABCO-*m*-Xyl and DABCO-*p*-Xyl polymers respectively. Similarly to diDABCO containing polymers, the product of polyaddition with 40 mmol mL^{-1} concentration of *para* monomers (diBr-*p*-Xyl and **4**) was not contaminated with cyclic side products while the product of *meta* polyaddition (diBr-*m*-Xyl and **3**) in the same condition contained cyclic oligomers mainly. The reaction performed at high concentration of the monomers (up to 380 mmol L^{-1}) successfully reduced amount of the cyclic oligomers (Figure S3, Supporting Information). DP was determined using ^{1}H NMR spectra and the absence of bromide end groups was confirmed by comparison with spectra of polymers obtained in polyaddition reactions with excess of α,α'-dibromoxylenes (Scheme S1b, Spectra S3 and S4, Supporting Information).

The ionenes possessing the flexible TMEDA derivative linker, TMEDA-*m*-Xyl and TMEDA-*p*-Xyl (Scheme 1e), were synthesized in polyaddition reactions of TMEDA with diBr-*m*-Xyl and diBr-*p*-Xyl in mixture of MeOH/DMF/H_2O. Other synthetic attempts to obtain this polymer, like the direct polyaddition of α,α'-dibromoxylenes and TMEDA in DMSO as well as the reaction between monoalkylated TMEDA derivative **S4** and α,α'-dibromo-*p*-xylene (Scheme S2c,d, Supporting Information) led to polymers with substantial number of aldehyde end groups (Scheme S2c, Spectrum S15, Supporting Information). This difference in reactivity between **S4** and **4** is likely an effect of a fixed monoalkylated DABCO conformation in which quaternary ammonium center reduces availability of free electron pair through the field effect.[60] As a result, TMEDA analogues produce aldehyde side products in Kornblum reaction much easier than DABCO derivatives **2**, **3**, and **4**. DP of flexible polycations was determined using ^{1}H NMR spectroscopy as described for previously presented polymers (Scheme S1c, Spectra S5 and S6, Supporting Information).

Elemental analysis confirmed high the purity of all obtained polycations and revealed that the isolated products are hydrated salts. ^{1}H NMR spectra confirmed structures and GPC analysis revealed comparable molar mass distribution of obtained ionenes (**Figure 1**). The average amount of positively charged group in tested polycations, estimated by ^{1}H NMR spectroscopy, is in the range of 31.0–63.4 per polymeric chain and M_n determined by GPC is in the range of 3.9–8.2 kDa (Table 1). Polymer dispersity $Đ_M$ remains in the range between 1.58 to 3.7, typical values for the step-growth polymerization reaction.

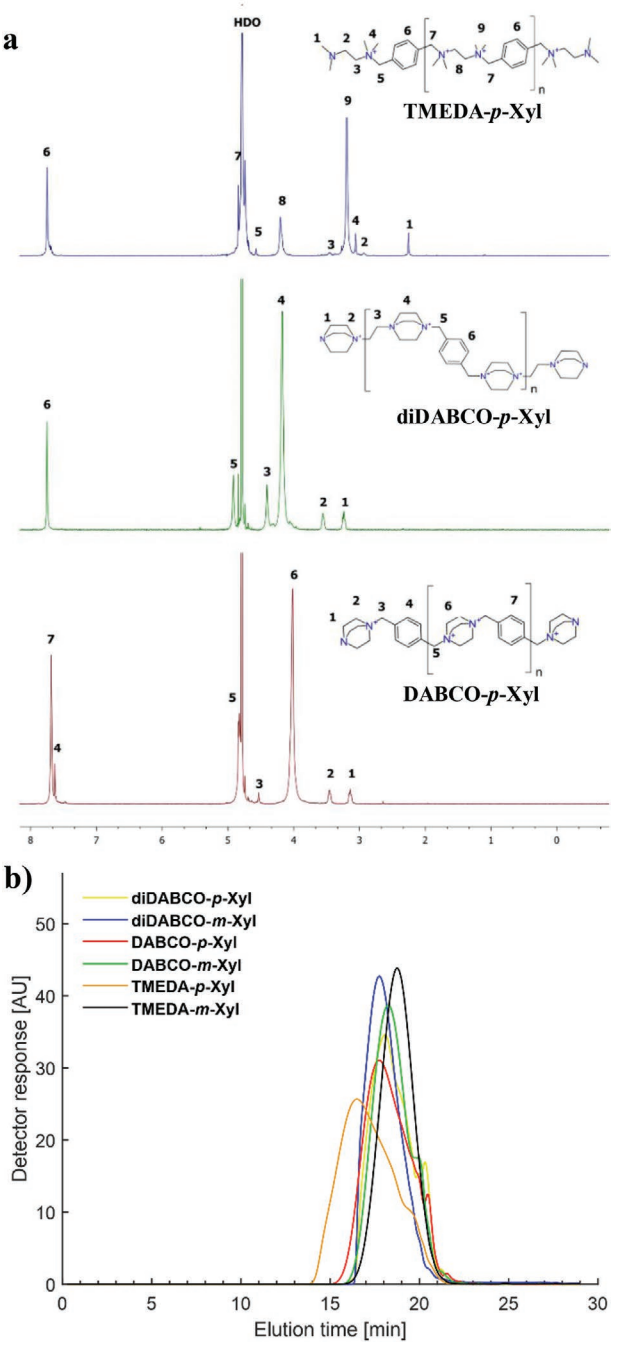


Figure 1. a) 1H NMR spectra of para isomer of studied polymers (for other spectra see Supporting information); b) stack of GPC traces of studied polycations.

2.2. Antimicrobial and Hemolytic Properties of Ionenes

The minimum inhibitory concentration (MIC) (**Table 2** and **Figure 2**) was determined against model microorganisms using the broth microdilution method, the phrase "antimicrobial activity" used in this paper refers to an inhibitory activity only, if not otherwise stated. The obtained results indicate high activity of all investigated polymers with MIC values falling between 4 to 64 μg mL^{-1}. The inhibitory activity is comparable with linear polyethyleneimine (L-PEI),[69] however in contrast to L-PEI our polymers display negligible hemolytic activity even in concentration of 5 mg mL^{-1}. Combination of low MIC values and low hemolytic activities of hydrophilic quaternary ammonium ionenes were also reported by others.[41,43,46]

Discussed in this work polymers display comparable or higher inhibitory activity against gram-positive *S. aureus* than against gram-negative *Escherichia coli*. This trend is reported for many antibacterial ionenes[39,41,42,70–72] and likely can be

Table 2. Antimicrobial and hemolytic properties of tested ionenes.

Polymer	MIC [μg mL^{-1}]			HC_{50} [μg mL^{-1}][a)]	Selectivity		
	E. coli	*S. aureus*	*C. albicans*		*E. coli*	*S. aureus*	*C. albicans*
diDABCO-*p*-Xyl	8	4	16	>5000 (3%)	>625	>1250	>313
diDABCO-*m*-Xyl	16	4	16	>5000 (1%)	>313	>1250	>313
DABCO-*p*-Xyl	8	8	64	>5000 (25%)	>625	>625	>78
DABCO-*m*-Xyl	16	4	32	>5000 (1%)	>313	>1250	>156
TMEDA-*p*-Xyl	8	8	32	>5000 (1%)	>625	>625	>156
TMEDA-*m*-Xyl	8	4	8	>5000 (2%)	>625	>1250	>625
Polymyxin B	≤0.5	32	>64	>5000 (1%)	>10^4	>156	n.d.

[a)]Hemolytic yield for the polymer concentration of 5 mg mL^{-1} is given in parentheses.

derived from presence of the additional outer cell membrane in gram-negative bacterium cell which prevents ionenes from reaching deeper into the cell wall and the inner membrane.[10] Herein presented polycations are characterized by relatively low molar mass what allows for easy diffusion through the thick cell wall (sieving effect) of gram-positive strains.[12] Inhibitory activity against fungi *Candida albicans* is generally lower than the antibacterial activity, what probably is a result of a different cell membrane composition and a presence of thick cell wall composed of chitin.[10]

Difference in polymer activity between gram-positive and gram-negative bacteria and yeast is also extending into effect of the backbone isomerism and flexibility. When the activity against gram-negative *E. coli* is considered, two of *meta* rigid ionenes—diDABCO-*m*-Xyl and DABCO-*m*-Xyl—are slightly less active then their *meta* counterparts, whereas both isomers of flexible polycations—TMEDA-*p*-Xyl and TMEDA-*m*-Xyl—showed the same activity (Figure 2). The activity against gram-positive *S. aureus* displays rather different trend, two of *para* ionenes—DABCO-*p*-Xyl and TMEDA-*p*-Xyl—showed higher MIC values in comparison with their meta analogues and the isomeric effect was not observed for diDABCO-*p*-Xyl and diDABCO-*m*-Xyl. There is no difference between the activity of flexible and rigid ionenes (DABCO versus TMEDA ionenes) against *S. aureus*. The *meta* ionenes containing DABCO and TMEDA were also more active against *C. albicans* than their *para* analogues and the clear effect of backbone flexibility is present—flexible TMEDA

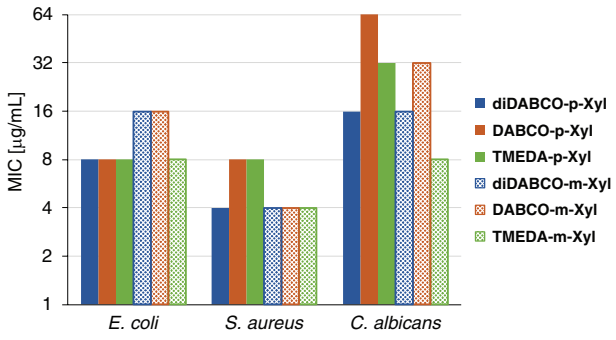


Figure 2. MIC values of studied ionenes—a graphical representation of Table 2 to facilitate data analysis and discussion.

containing polymers are more active against *C. albicans* than DABCO containing polymers.

Recently, Palermo et al. published a viewpoint article in which the nature of amphiphilic balance of antibacterial polymers is discussed.[73] Following presented concept our ionenes could also be considered as amphiphilic, thanks to presence of distinguishable hydrophilic ammonium and hydrophobic aryl repeating units (**Figure 3**). The amphiphilic balance resulting from the ratio between hydrophobic and hydrophilic groups is the same among DABCO and TMEDA containing ionenes (DABCO-*p*-Xyl, DABCO-*m*-Xyl, TMEDA-*p*-Xyl, and TMDA-*m*-Xyl). However, a conformation of *meta*-structure with clear hydrophilic and hydrophobic domain segregation can by proposed (Figure 3), what seems to be unlikely for *para*- structure. Therefore, despite the same amphiphilic balance *meta* ionenes can achieve an suitable amphiphilic conformation at the water-lipid interface easier in comparison with their *para* analogues. Additionally rigid *para* ionenes seem to be more susceptible to attain straight, extended conformation at the interface than their *meta* analogues. Simplified model depicted at the Figure 3 will be further investigated using Molecular dynamic simulation.

We observed a slightly higher activity against *E. coli* for *para* DABCO-*p*-Xyl and diDABCO-*p*-Xyl compare to their *meta* analogues and slightly higher activity of DABCO-*m*-Xyl and TMEDA-*m*-Xyl then *para* analogues against *S. aureus* and *C. albicans*. We speculate that the difference in the polymer topology displayed as segregated or mixed polymeric domains in various conformations of *para* and *meta* molecules are responsible for an interaction with bacterial cell envelop. This interaction is manifested differently depending on composition of outer cell structure in gram-negative and gram-positive bacteria and yeasts. Results obtained by Mayr et al. for ionenes possessing quaternary ammonium groups separated alternately by ethylene, propylene or butylene and different isomers of bis-benzamide moieties showed also higher activity of *para* isomers against *E. coli*.[48]

Separately, the elevated charge density (the number of quaternary ammonium groups within the repeating unit) influence can be discussed for diDABCO and DABCO analogues. This effect is significant only for activity against *C. albicans* showing lower MICs for diDABCO structures with higher charge. The charge density effect was discussed previously showing positive influence of the parameter on antimicrobial activity.[22,74,75]

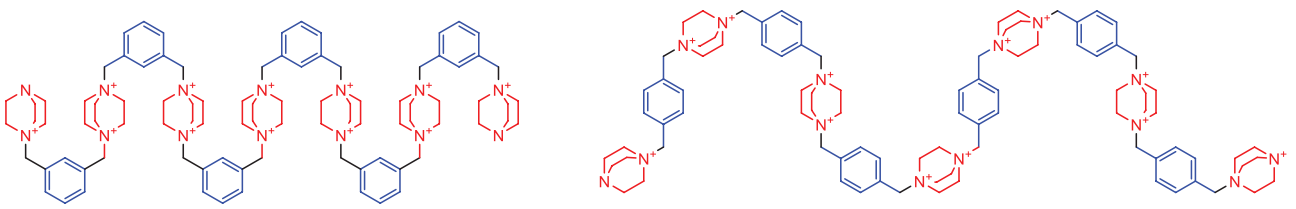


Figure 3. Illustrative structures of DABCO-*m*-Xyl and DABCO-*p*-Xyl in their simplified amphiphilic conformations. Positively charged hydrophilic fragments are marked in red and hydrophobic aromatic fragments are marked in blue. Further explanation in the text.

The structural flexibility can be discussed by comparison of DABCO and TEMDA containing polymers. Obtained results indicates that more flexible structure is beneficial in case of activity against *E. coli* and *C. albicans* and has no effect for *S. aureus*. We associate the effect with conformational freedom of polymeric chain which can adapt easier to appropriate interacting form and such effect was discussed before.[7] It should be noted that flexibility influence is strongly linked to the molecular structure and opposite observations were also reported.[49] Synergistic effect of flexibility and topology influence is well visible in TMEDA-*m*-Xyl—the most active ionene against *C. albicans*. It can be concluded that combination of the elastic linker and *meta* isomer allow for effective segregation of the polymeric domains and formation of the active amphiphilic structure

Two of presented ionenes, TMEDA-*p*-Xyl and DABCO-*p*-Xyl, were recently reported in literature by other research teams. S. Liu et al. studied ionenes containing hydrophobic group along polymer backbone and reported MIC values of TMEDA-*p*-Xyl comparable to our results (3.9, 7.8, and 7.8 for *E. coli*, *S. aureus*, and *C. albicans* respectively).[41] Recently, Y. Yuan et al. studied antibacterial and fungicidal activity of ionenes composed of DABCO and imidazolium moieties.[46] The authors found MIC value for DABCO-*p*-Xyl higher than MIC determined by us (*E. coli* >125 μg mL^{-1}; *S. aureus* 62 μg mL^{-1}; *C. albicans* 125 μg mL^{-1}), what can be related to significant difference in average molar mass.

To investigate the bactericidal activity the time-kill kinetic studies against *S. aureus* were performed (Figure S4, Supporting Information). Bacteria were treated with polymers at 2 × MIC and 4 × MIC concentrations since antimicrobial polycations are typically characterized with minimum bactericidal concentrations (MBCs) close to their MIC values.[7,14,76]. All of the presented ionenes displayed very high bactericidal efficiency completely eradicating bacteria (no colonies observed) after the shortest incubation time applied (15 min) under polymers concentration of 2 × MIC (Figure S4, Supporting Information). Such rapid bactericidal effect strongly indicates that the mechanism of action does not require diffusion through the cell envelope and it is rather based on interaction with the cell membrane.[13,41,40] The MBC values against *S. aureus* of the presented ionenes is equal or smaller than respective 2 × MIC values.

The selectivity indexes, defined as a ratio of HC_{50} to MIC, of our polymers display values as high as 625 against *E. coli*, 1250 against *S. aureus* and 625 against *C. albicans*. Almost all polymers showed insignificant hemolytic activity in concentration of 5 mg mL^{-1}, only DABCO-*p*-Xyl caused hemolysis of 25% of RBCs. Interestingly, Y. Yuan et al. have also observed extended hemolytic activity for ionenes containing DABCO-*p*-Xyl as a repeating unit.[46] The most active TMEDA-*m*-Xyl can be considered as a broad spectrum non-hemolytic antimicrobial hydrophilic polycation.

2.3. Degradation Studies of diDABCO Containing Ionenes

Most of reported antibacterial polycations do not degrade easily, what usually is considered as an advantage, for example, it elongates time of activity and enables lower dosage usage.[77] Nevertheless, biodegradability of antibacterial agents is important to control over toxicity toward human cells and bacterial resistance development.[7] The most common way to construct antibacterial polycations susceptible to degradation is to implement polycarbonate[19,34,78] or polyamide[36] functional groups. Mizutani et al. showed other approach obtaining polymers that undergo "self-degradation" via intermolecular ester aminolysis[79] and very recently Krumm et al. presented biodegradable ionenes with ester groups along the polymers backbone.[80] Herein, we propose an alternative approach for degradable polycations through Hofmann elimination reaction under mild conditions, following our previous work on degradation of methylated **2** derivative under near physiological conditions (PBS pH 7.4, 37 °C).[60] Since similar property was expected for diDABCO containing polycations, the stability studies of diDABCO-*p*-Xyl were conducted.

The polymer degradation study was additionally performed at pH 6.5 PBS buffer to reflect pH conditions of bacterial infection.[81] The reaction progress was followed by the ^{1}H NMR (**Figure 4**, Spectrum S16, Supporting Information) thanks to full identification of degradation products (Spectrum S23, Supporting Information). The process was completed in 8% after 17 days (pH 7.4), the degradation rate, just like the Hofmann elimination reaction is about four times slower at pH 6.5 and as such one can expect that process will proceed faster in healthy tissue compare to the infected one.

The diDABCO-*p*-Xyl and diDABCO-*m*-Xyl polymers can be considered as a novel class of biodegradable ionenes with strong antimicrobial properties. The degradation is enzyme independent, its rate depends mainly on pH conditions and importantly pH decreasing carboxylic groups are not formed in the process.

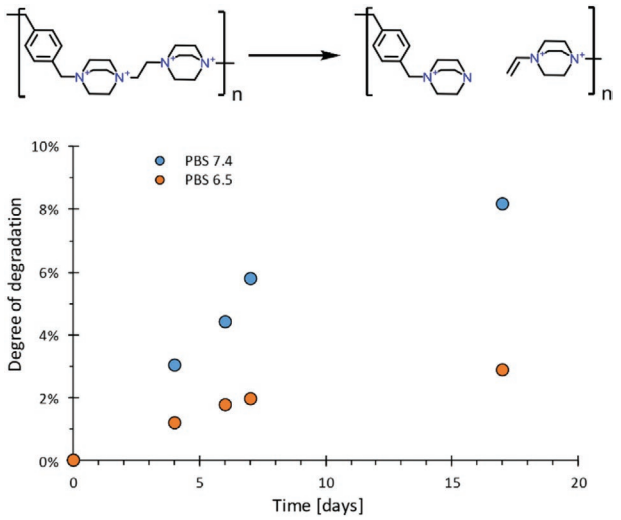


Figure 4. Hofmann elimination reaction of diDABCO-*p*-Xyl and the time course of its degradation in PBS pH 7.4 and PBS pH 6.5 at 37 °C.

3. Conclusion

The influence of the backbone flexibility and topology (in terms of *meta, para* isomerism of aromatic subunit) on antimicrobial and hemolytic activity was investigated in the series of ionenes. The employed polyaddition reaction (step-growth polymerization) between different isomers of α,α'-dibromoxylenes and tertiary diamines required different synthetic approach for each diamine due to side reactions (mainly Kornblum oxidation) and solubility issues. Furthermore, the increase of monomer concentration allowed to overcome the problem of side cyclic product formation during the synthesis of *meta*-polymers.

The investigated polymers showed high antimicrobial activity against *E. coli, S. aureus,* and *C. albicans* (MIC within range of 4–64 μg mL^{-1}) and negligible hemolytic activity (in 5 mg mL^{-1} hemolysis mainly within range of 1–3%). *Para* isomers of rigid ionenes were slightly more active against gram-negative *E. coli* then *meta,* while flexible ionenes did not display differences between isomers. *Meta* isomers of DABCO and TMEDA containing polymers were a little more active against gram-positive *S. aureus* and yeast *C. albicans* than *para* isomers, and the most active were molecules possessing diDABCO moiety what can be affiliated with the higher positive charge density (the number of quaternary ammonium groups within repeating unit) along the backbone. We can conclude that straight and extended conformation of ionenes is responsible for interaction with cell envelope of gram-negative *E. coli* bacteria, whereas interaction with gram-positive *S. aureus* and yeast *C. albicans* cells is improved by higher positive charge density as well as possibility to attain amphiphilic conformation with segregated hydrophobic and hydrophilic domains. Higher activity of flexible ionenes against *E. coli* and *C. albicans* can be a result of higher conformational freedom which facilitate better interaction with the cell envelope. Moreover, all presented ionenes showed strong bactericidal activity against *S. aureus* completely killing bacterial cells within 15 min at concentration of two times MIC values.

The stability studies of diDABCO containing diDABCO-*p*-Xyl polymer in conditions simulating physiological (PBS 7.4, 37 °C) revealed its degradation via Hofmann elimination reaction. This process was slower in simulated conditions of infected wound (pH 6.5). Importantly, carboxylic groups responsible for decrease of pH and additional inflammation were not generated in this reaction. For the first time a new class of degradable polycations with high antimicrobial properties is reported.

4. Experimental Section

Materials and Methods: All chemical reagents were purchased from Sigma-Aldrich, TCI Chemicals, Fluorochem and were used without further purification. The AR grade solvents were purchased from Chempur and used without further purification. Regenerated-cellulose dialysis tubing (Spectra/Por 7, MWCO 1 kDa) were pre-treated according to the instruction provided by the manufacturer before use. Water for dialysis, antimicrobial assays and GPC analysis was purified with a Milli-Q system (Millipore). Freeze-drying was performed using Labconco FreeZone 2.5 Liter Benchtop Freeze Dry Systems. The ^{1}H and ^{13}C NMR spectra were recorded using Varian 400 MHz spectrometer using D_2O and $CDCl_3$ as solvents. ^{1}H NMR chemical shifts were referenced to the resonance for residual protonated solvent (δ 7.26 for $CDCl_3$ and 4.79 for D_2O). ^{13}C NMR chemical shifts were referenced to the solvent (δ 77.16 for $CDCl_3$). Elemental analyses were performed using Vario EL III CHNS Elemental.

GPC analyze was performed using Agilent 1260 Infinity liquid chromatograph equipped with RID and UV DAD detector (detection at 268 nm). UV spectra (Figure S1, Supporting Information) were recorder using Cary 3 spectrophotometer to preselect proper detection wavelength. The PSS NOVEMA Max 5 μm analytical 300 × 8 mm column with precolumn (PSS GmbH) was applied using mobile phase containing: 54/23/23 (v/v/v%) water/methanol/acetic acid and 0.5 M sodium acetate, at a flow rate of 0.4 mL min^{-1}. All chemicals were of HPLC grade. Column was thermostated at 50 °C. Calibration was performed using series of Poly(2-vinylpyridine) standards (PSS GmbH) in the range of molar masses: 620 Da–539 kDa. Samples, dissolved in eluent at 5 mg mL^{-1} concentrations, were injected in 20 μL volumes. GPC results (average molar masse and dispersity $Đ_M$) were calculated using Agilent GPC Addon Rev. B.01.02 software.

Broths for antimicrobial assays were prepared using commercially available Mueller-Hinton Broth powder (Biocorp), Mueller-Hinton Agar powder (Biocorp) and Sabouraud broth powder (Merck). American Type Culture Collection (ATCC) bacterial and yeast strains were used in this work: *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 10231) were used.

Synthesis of Ethylene di(p-toluenesulfonate) (**1**): Ethylene glycol (10.00 g, 161.1 mmol) in THF (100 mL) was added to solution of NaOH (14.80 g, 370 mmol) in water (19 mL), and the mixture was stirred at room temperature for 2 h. Upon cooling to 0 °C, tosyl chloride (61.44 g, 322.2 mol) was slowly added over a period of 2 h. Low temperature (0 °C) was maintained for additional 2 h, cooling bath was removed and stirring was continued overnight in room temperature. Reaction mixture was poured into $HCl_{(aq)}$ (700 mL, 2 mol L^{-1}) and the precipitate was isolated by filtration. The solid was washed with water, until neutral pH of the filtrate was reached, and subsequently washed with a small amount of methanol. The product was dried under vacuum overnight yielding 47 g of a white solid (76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.45 (s, 6H, $PhCH_3$), 4.17 (s, 4H, $-CH_2-$), 7.33 (d, J = 8.4Hz, 4H; ArH), 7.72 (d, J = 8.4 Hz, 4H; ArH); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 21.6, 66.7, 127.9, 129.9, 132,1, 145,2.

Synthesis of 1,2-Bis(4-aza-1-azoniabicyclo[2.2.2]oct-1-yl)ethane ditosylate (**2**): A solution of 1,4-diazabicyclo[2.2.2]octane (DABCO) (22.7 g, 200 mmol) in MeCN (60 mL) was added dropwise, at 40 °C, to a stirred solution of **1** (25.0 g, 68 mmol) in MeCN (220 mL) over a period of

20 min. The reaction mixture was refluxed for 3 h and subsequently cooled down to room temperature forcing precipitation of the product. The precipitate was filtered off, washed twice with MeCN, and dried under vacuum overnight yielding 22.5 g of a brown crystals (58%).

^{1}H NMR (400 MHz, D_2O) δ: 2.31 (s, 6H, $PhCH_3$), 3.13 (t, J = 7.2 Hz, 12H; $-CH_2-$), 3.43 (t, J = 7.2 Hz, 12H; $-CH_2-$), 3.85 (s, 4H, $-CH_2-$), 7.30 (d, J = 8 Hz, 4H; ArH), 7.62 (d, J = 8 Hz, 4H; ArH); ^{13}C NMR (400 MHz, D_2O) δ: 20.6, 44.1, 53.0, 54.9, 125.4, 129.6.

Synthesis of α,α′-(1-Azonia-4-azabicyclo[2.2.2]octyl)-para-xylene dibromide (**3**): A solution of α,α′-dibromo-*p*-xylene (6.60 g, 25 mmol) in MeCN (180 mL) was added dropwise at room temperature to a stirred solution of DABCO (14.00 g, 125 mmol) in MeCN (80 mL) over a period of 30 min and the reaction mixture was refluxed overnight. After cooling down to room temperature the solid was filtered off, washed with MeCN (20 mL) and diethyl ether (150 mL), and dried under vacuum overnight yielding 11.42 g of a white solid (94%).

^{1}H NMR (400 MHz, D_2O) δ: 3.14 (t, J = 7.6 Hz, 12H; $-CH_2-$), 3.45 (t, J = 7.6 Hz, 12H; $-CH_2-$), 4.53 (s, 4H, $-CH_2-$), 7.61 (s, 4H, ArH); ^{13}C NMR (400 MHz, D_2O) δ: 44.3, 52.2, 67.6, 128.7, 133.9. Analytics. calculated for $C_{20}H_{32}Br_2N_4{\cdot}H_2O$: C, 47.44; H, 6.77; N, 11.07; found: C, 47.44; H, 6.60; N, 11.05.

Synthesis of α,α′-(1-Azonia-4-azabicyclo[2.2.2]octyl)-meta-xylene dibromide (**4**): A solution of α,α′-dibromo-*m*-xylene (6.60 g, 25 mmol) in MeCN (180 mL) was added dropwise at room temperature to a stirred solution of 1,4-diazabicyclo[2.2.2]octane (DABCO) (14 g, 125 mmol) in MeCN (80 mL) over a period of 30 min and the reaction mixture was refluxed overnight. After cooling down to room temperature the precipitated product was filtered off, washed with MeCN (20 mL) and diethyl ether (150 mL), and dried under vacuum overnight yielding 11.66 g of a white solid (96%).

^{1}H NMR (400 MHz, D_2O) δ: 3.12 (t, J = 7.2 Hz, 12H; $-CH_2-$), 3.44 (t, J = 7.2 Hz, 12H; $-CH_2-$), 4.52 (s, 4H, $-CH_2-$), 7.59 (s, 1H, ArH), 7.63 (s, 3H, ArH); ^{13}C NMR (400 MHz, D_2O) δ: 44.3, 52.1, 67.7, 127.3, 130.3, 135.5, 137.4. Analytics. calculated for $C_{20}H_{32}Br_2N_4{\cdot}3H_2O$: C, 44.29; H, 7.06; N, 10.33; found: C, 43.76; H, 7.01; N, 10.23.

Synthesis of Poly([1,2-bis(1,4-diazoniabicyclo[2.2.2]oct-1-yl)ethane]-alt-[1,4-phenylenebis(methylene)]) (diDABCO-p-Xyl): A solution of α,α′-dibromo-*p*-xylene (2.244 g, 8.50 mmol) in DMSO (38 mL) was added dropwise at room temperature to a stirred solution of **2** (6.067 g, 10.2 mmol), DMSO (66 mL) and water (6 mL). The reaction mixture was stirred at room temperature for 24 h and poured into acetone (0.9 L). The obtained suspension was centrifuged, supernatant was removed and a solid was washed with acetone, diethyl ether and dried under vacuum. In order to exchange tosylates for bromides, the solid (10.20 g) was dissolved in water (25 mL), a solution of KBr 5% w/v (38 mL) was added and subsequently the mixture was poured into ethanol (410 mL). The suspension was centrifuged to isolate solid polymer and the exchange procedure was repeated two more times. Then, in order to remove remains of DMSO, wet solid was stirred with methanol (170 mL) for 40 min, centrifuged, washed with fresh methanol and dried under vacuum for 2 days. Quantitative removal of methanol required freeze-drying of water polymer solution resulting with 4.49 g of a light brown solid (70%).

^{1}H NMR (400 MHz, D_2O) δ: 3.24 (t, 6H, terminal $N-CH_2$-), 3.56 (t, 6H, terminal $N^{\oplus}-CH_2-$), 4.17 (s, 24H, $N^{\oplus}-CH_2-$), 4.41 (s, 4H, $N^{\oplus}-CH_2-$), 4.92 (s, 4H, $Ar-CH_2-$), 7.75 (m, 4H, ArH). Analytics calculated for $(C_{22}H_{36}Br_4N_4{\cdot}2H_2O)_n$: C, 37.10; H, 5.66; N, 7.87; found: C, 36.83; H, 5.72; N, 8.12.

Synthesis of Poly([1,2-bis(1,4-diazoniabicyclo[2.2.2]oct-1-yl)ethane]-alt-[1,3-phenylenebis(methylene)]) (diDABCO-m-Xyl): To a stirred solution of **2** (4.8165 g, 8.1 mmol) in mixture of DMSO (7.5 mL) and water (4.5 mL) a crushed α,α′-dibromo-*m*-xylene (1.7820 g, 6.75 mmol) was added at room temperature. The reaction mixture was further stirred for 24 h and poured into acetone (500 mL). The obtained suspension was centrifuged, supernatant was removed and solid was dried under vacuum. In order to remove cyclic side products the crude product was stirred with methanol (13 mL) for 3 h, then the suspension was centrifuged and the solid was washed two times with fresh methanol (15 mL) and dried under vacuum yielding 2.49 g of product in the tosylate salt form. To exchange tosylates the polymer was dissolved in 7.5 mL of KBr 5% w/v aqua solution at 50 °C, obtained clear solution was poured into ethanol (125 mL). The suspension was centrifuged to isolate the polymer and the exchange procedure was repeated four times more. The wet polymer was washed with ethanol, diethyl ether and dried under vacuum overnight. Quantitative removal of ethanol required freeze-drying of water polymer solution and led to 1.43 g of a light brown solid (28%).

^{1}H NMR (400 MHz, D_2O) δ: 3.24 (t, 6H, terminal $N-CH_2-$), 3.55 (t, 6H, terminal $N^{\oplus}-CH_2-$), 4.18 (s, 24H, $N^{\oplus}-CH_2-$), 4.42 (s, 4H, $N^{\oplus}-CH_2-$), 4.94 (s, 4H, $Ar-CH_2-$), 7.80 (m, 4H, ArH). Analytics calculated for $(C_{22}H_{36}Br_4N_4{\cdot}4H_2O)_n$: C, 35.31; H, 5.93; N, 7.49; found: C, 35.82; H, 6.10; N, 7.83%.

Synthesis of Poly([1,4-diazoniobicyclo[2.2.2]octane]-alt-[1,4-phenylenebis(methylene)]) (DABCO-p-Xyl): A solution of α,α′-dibromo-*p*-xylene (0.983 g, 3.73 mmol) in DMSO (37 mL) was added dropwise, at room temperature, to a stirred solution of **4** (2.000 g, 4.10 mmol), DMSO (46 mL), and water (5 mL). The reaction mixture was stirred at room temperature for 24 h and poured into acetone (0.8 L). In order to agglomerate fine solid *n*-hexane was added (0.4 L) and suspension was centrifuged. Subsequently, the supernatant was removed, the solid was washed with acetone and diethyl ether and dried under vacuum. To remove remains of DMSO, the polymer was stirred with methanol (120 mL) for 2 h, centrifuged, washed with fresh methanol and dried under vacuum for 2 days. Quantitative removal of methanol required freeze-drying of water polymer solution leading to 1.59 g of a white solid (53%).

^{1}H NMR (400 MHz, D_2O) δ: 3.20 (t, 6H, terminal $N-CH_2-$), 3.51 (t, 6H, terminal $N^{\oplus}-CH_2-$), 4.06 (s, 12H, $N^{\oplus}-CH_2-$), 4.58 (s, 2H, terminal $Ar-CH_2-$), 4.86 (s, 4H, $Ar-CH_2-$), 7.73 (m, 4H, ArH). Analytics calculated for $(C_{14}H_{20}Br_2N_2{\cdot}2H_2O)_n$: C, 40.80; H, 5.87; N, 6.80; found: C, 41.34; H, 5.89; N, 7.11.

Synthesis of Poly([1,4-diazoniobicyclo[2.2.2]octane]-alt-[1,3-phenylenebis(methylene)]) (DABCO-m-Xyl): A solution of α,α′-dibromo-*m*-xylene (0.594 g, 2.25 mmol) in DMSO (2.5 mL) was added, at room temperature, to a stirred solution of **3** (1.319 g, 2.70 mmol) in mixture of DMSO (3.5 mL) and water (300 µL). The reaction mixture was stirred at room temperature for 24 h and poured into acetone (250 mL). In order to agglomerate fine solid diethyl ether was added (100 mL) and suspension was centrifuged. The supernatant was removed and the solid was dried under vacuum. To remove cyclic side products, the polymer was stirred with methanol (20 mL) for 3 h, then the suspension was centrifuged and the solid was washed twice with fresh methanol (20 mL), and dried under vacuum. Quantitative removal of methanol required freeze-drying of water polymer solution and yielded 1.2 g of a white solid (63%).

^{1}H NMR (400 MHz, D_2O) δ: 3.14 (t, 6H, terminal $N-CH_2-$), 3.47 (t, 6H, terminal $N^{\oplus}-CH_2-$), 4.06 (s, 12H, $N^{\oplus}-CH_2-$), 4.57 (s, 2H, terminal $Ar-CH_2-$), 4.86 (s, 4H, $Ar-CH_2-$), 7.77 (m, 4H, ArH). Analytics calculated for $(C_{14}H_{20}Br_2N_2{\cdot}3H_2O)_n$: C, 39.09; H, 6.09; N, 6.51; found: C, 39.21; H, 6.25; N, 6.80.

Synthesis of Poly([tetramethylethylenediammonium]-alt-[1,4-phenylenebis (methylene)]) (TMEDA-p-Xyl): A solution of α,α′-dibromo-*p*-xylene (1.663 g, 6.30 mmol) in DMF (14 mL) was added dropwise, at room temperature, to a stirred solution of TMEDA (0.805 g, 1.040 mL, 6.93 mmol) in MeOH (13 mL). The reaction mixture was stirred at room temperature for 96 h. In order to maintain homogeneity of the mixture after 8 and 24 h, 14 and 35 mL of water was added respectively. Then, the reaction mixture was diluted with water (80 mL), transferred to dialysis tubing (MWC 1 kDa) and dialyzed against Milli-Q water for 3 days. The solution was freeze-dried yielding 0.477 g of a white solid (20%).

^{1}H NMR (400 MHz, D_2O) δ: 2.25 (s, 6H, terminal $N-CH_3$), 2.93 (t, 2H, terminal $N-CH_2-$), 3.06 (s, 6H, terminal $N^{\oplus}-CH_3$), 3.20 (s, 12H, $N^{\oplus}-CH_3$), 3.46 (t, 2H, terminal $N^{\oplus}-CH_2-$), 4.20 (t, 4H, $N^{\oplus}-CH_2-$), 4.57 (s, 2H, terminal $Ar-CH_2-$), 4.74 (s, 4H, $Ar-CH_2-$), 7.70 (m, 4H, ArH). Analytics calculated for $(C_{14}H_{24}Br_2N_2{\cdot}2H_2O)_n$: C, 40.40; H, 6.78; N, 6.73; found: C, 39.95; H, 7.08; N, 6.73.

Synthesis of Poly([tetramethylethylenediammonium]-alt-[1,3-phenylenebis (methylene)]) (TMEDA-m-Xyl): A solution of α,α'-dibromo-*m*-xylene (1.663 g, 6.30 mmol) in DMF (14 mL) was added dropwise, at room temperature, to a stirred solution of TMEDA (0.805 g, 1.040 mL, 6.93 mmol) in MeOH (13 mL). The reaction mixture was stirred at room temperature for 96 h. In order to maintain homogenous mixture 14 mL of water was added 8 h after start of the reaction. Subsequently, the reaction mixture was diluted with water (110 mL), transferred to dialysis tubing (MWC 1 kDa) and dialyzed against Milli-Q water for 3 days. The solution was freeze-dried yielding 0.356 g of a white solid (14%).

^{1}H NMR (400 MHz, D_2O) δ: 2.27 (s, 6H, terminal N—CH_3), 2.94 (t, 2H, terminal N—CH_2—), 3.06 (s, 6H, terminal $N^{\oplus}$—CH_3), 3.21 (s, 12H, $N^{\oplus}$—CH_3), 3.49 (t, 2H, terminal $N^{\oplus}$—CH_2—), 4.21 (t, 4H, $N^{\oplus}$—CH_2—), 4.61 (s, 2H, terminal Ar—CH_2—), 4.79 (s, 4H, Ar—CH_2—), 7.67 (m, 1H, ArH), 7.80 (m, 2H, ArH), 7.91 (m, 1H, ArH). Analytics calculated for $(C_{14}H_{24}Br_2N_2{\cdot}3H_2O)_n$: C, 38.73; H, 6.96; N, 6.45; found: C, 38.94; H, 6.99; N, 6.53.

Antimicrobial Assay: Antimicrobial activity was evaluated by determination of the MIC against *E. coli*, *S. aureus*, and *C. albicans*. The broth microdilution method was applied according to CLSI M07-A9 Vol. 32 No. 2 (for bacteria) and CLSI M27-A2 Vol. 22 No. 15 (for yeast).

Single colonies of bacteria or yeast were used to inoculate 5 mL of Mueller-Hinton Broth (MHB) or Sabouraud broth (SAB), respectively, and the cultures were grown overnight at 37 °C with shaking (240 rpm) in Lab Companion SI-600R bench top shaker (Ramsey, MN, USA).

The polymer stock solutions (5120 μg mL^{-1}) prepared in Milli-Q water were diluted with the appropriate broth (MHB or SAB) to concentration of 1024 μg mL^{-1} and used to prepare series of different polymer concentrations in broth (from 512 to 2 μg mL^{-1}; 100 μL each) in 96-well plates by twofold dilution method. Subsequently, 100 μL of microbial suspension (2×10^5 CFU mL^{-1} for bacteria and 2×10^3 CFU mL^{-1} for yeast) was added to each well. Uninoculated broth, uninoculated broth with polymer solutions, inoculated broth with polymyxin B and inoculated broth without any antimicrobial agent were used as controls. Four replicates were performed for each concentration of polymer and the control. The plates were incubated for 20 h at 37 °C (Thermo Scientific Heratherm Compact Microbiological Incubator (Waltham, MA, USA)). The optical density at 600 nm (OD_{600}) were measured using Synergy H4 Hybrid Microplate Reader, Biotech (Winooski, VT, USA). The recorded MIC value was the lowest concentration of the polymer at which no microbial growth was observed with the microplate reader.

Time-Kill Kinetics Assay: The *S. aureus* cells were inoculated and prepared according to the same procedure in the MIC measurement described above. The samples were treated with the polymer concentration of 2 × MIC and 4 × MIC at 37 °C and at proper time intervals (15 min, 30 min, 45, 60, and 120 min) microbial samples were taken out (20 μL) from each well and series of tenfold dilutions in phosphate buffer solution were made immediately. The final dilutions were spread on agar plates (MHA) and left for incubation for 18 h at 37 °C. After incubation the colony-forming units were counted.

Hemolysis Assay: Fresh blood was obtained from the Regional Center for Blood Donation and Blood Treatment in Warsaw. Samples were centrifuged (700 *g*, 10 min), supernatant plasma was rejected, and erythrocytes were washed with ice cold PBS three times (by centrifuging, 700 *g*, 10 min). After final centrifugation, erythrocytes were diluted ten times with PBS. Polymer solutions were prepared in PBS buffer. Erythrocyte suspensions (500 μL) were added to polymer solutions (500 μL) at investigated concentrations. Samples were incubated for 1 h at 37 °C, then samples were centrifuged (10 min, 700 *g*), and hemoglobin release was measured in supernatants using spectrophotometric method (absorption at λ = 540 nm). PBS buffer and 0.2% Triton X-100 served as negative and as positive controls, respectively. Experiments were performed at the Faculty of Chemical and Process Engineering WUT with OSH approval for research with human blood.

Degradation Study: Deuterated phosphorus buffered saline solution pH 7.4 was prepared by freeze-drying of 10 × PBS 7.4 (500 μL), triple dissolution of solid residue in D_2O (5 mL) and subsequent freeze-drying. Deuterated buffer salts were dissolved in D_2O (5 mL) and diDABCO-*p*-Xyl (20 mg) was dissolved in 1 mL of such solution. Then, the mixture was transferred to NMR tube under inert gas atmosphere. The NMR tube was incubated at 37 °C except periods of ^{1}H NMR spectrum recording (about 15 min each).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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antimicrobial activity, ionene, polyaddition, polycation, quaternary ammonium compounds

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