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Antimicrobial protonated polydiallylamines: how to retain bactericidal efficiency at minimal toxicity

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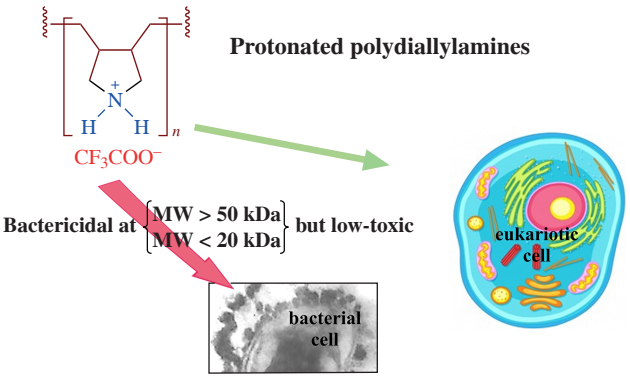
A family of antimicrobial protonated diallylammonium polymers has been synthesized by classical and RAFT polymerization in a wide range of molecular weights (MWs) of about (8–118) × 10^3 g mol^{-1}. Based on the study on toxicity relative to eukaryotic cells (epithelioid line of human lung carcinoma and line of green monkey kidney) and bactericidal activity of polymers (relative to *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *M. smegmatis*), we distinguish two groups of polymers promising as disinfectant and for medical applications. These are polymers with a sufficiently large MW (more than 50 × 10^3 g mol^{-1}) and samples with a low MW (18 × 10^3 g mol^{-1} and lower); their biocidal activity is an order of magnitude higher or slightly higher (selectivity about 1.16) than their cytotoxicity.



Keywords: free radical polymerization, RAFT polymerization, protonated poly(diallylammonium trifluoroacetate), antimicrobial activity, toxicity, eukaryotic cells.

The threat of antibiotic-resistant infections has reached a critical level worldwide today. Therefore, creation of antimicrobial systems both effective and low-toxic, with a non-specific action that does not cause resistance of microorganisms (unlike antibiotics), is the main goal in the field of antimicrobial polymer synthesis.[1–6] Over the past decades, numerous data on synthetic antimicrobial cationic polymers have been accumulated, and it has been shown that achieving a balance between the hydrophobic properties of the (co)polymer and its functional hydrophilic characteristics is an important factor for combining antimicrobial activity with low toxicity.[1–6]

One of the approaches to obtain biocidal but low toxic polymers is the synthesis of degradable macromolecules which are toxic as a whole molecule, while the repeating units of the polymeric backbone itself are not biocidal.[3,7,8] The main idea was combining hydrolytically degradable antimicrobial polymers with the introduction of deactivating satellite end groups.[7,8] Another way to combine antimicrobial and non/low-toxic properties is the creation of polycomplexes formed by polyanion–polycation pairs; these polycomplexes have found application in biomedicine, pharmacology, cosmetology, food, industry, *etc*.[9–11] Polycomplexes of natural compounds are of especial interest due to their low toxicity, high biocompatibility and biodegradability.[10,11] Recently, it was shown that negatively charged polycomplexes formed by cationic poly(diallyldimethylammonium chloride) with excess anionic sodium alginate[12] and sodium polyacrylate[13] may be promising for the development of antibacterial coatings.

Among other polymer systems, water-soluble protonated secondary/tertiary diallylammonium polymers (PDAAs) are of interest. They were first synthesized by radical cyclopolymerization of protonated salts, *i.e*., trifluoroacetates of diallylammonium monomers **M1** [Scheme 1(*a*)], formation of the end vinyl group of the polymer, protonated poly(diallylammonium trifluoroacetate) **P1**, being the result of effective chain transfer to monomer (see Online Supplementary Materials, Figure S1).[14–16] It has been shown that the behavior and many properties of PDAAs are due to the protonated form of amino groups capable of forming hydrogen bonds, which distinguishes secondary and tertiary PDAAs from known quaternized polydiallylamines and other polyamines, and gives the former new properties. In particular, it was found that PDAAs exhibit high nonspecific antimicrobial activity,[17] including rare activity against *Mycobacterium tuberculosis*,[18,19] in contrast to quaternized polymers and low molecular weight biocides.[20]

With the development of the method of controlled radical polymerization with the reversible addition–fragmentation chain transfer (RAFT) mechanism for obtaining polymers with narrow polydispersity,[21–23] it became possible to synthesize polymers with variable functional properties due to the introduction of end groups of the RAFT agent. Thus, it allows one to evaluate the effect of the end groups for polymers with a low molecular

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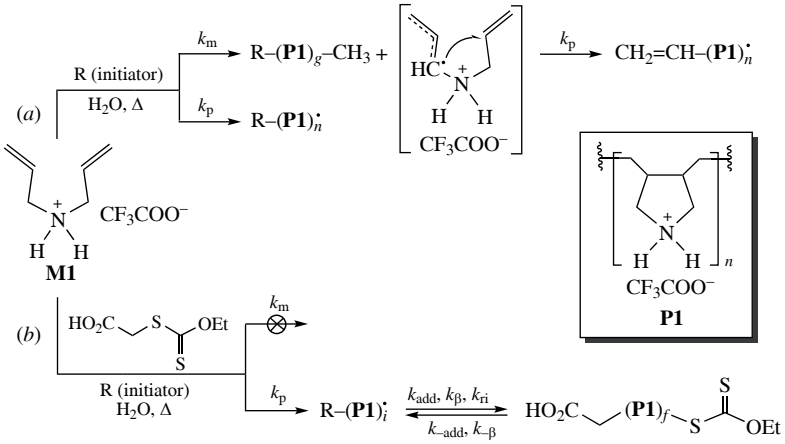

Scheme 1 Synthesis of PDAA polymers. (*a*) Free radical cyclopolymerization of protonated diallylammonium monomer **M1**: k_p is the chain propagation and k_m is the chain transfer to monomer by the α-H atom abstraction from the allyl group with subsequent transformation of the diallyl transfer radical into a chain propagation radical by means of intramolecular cyclization. (*b*) Cyclopolymerization of **M1** that occurs with efficient chain transfer to monomer, in the presence of the RAFT agent, xanthate HOC(O)CH_2SC(=S)OEt: k_p and k_m are the rate constants of propagation and efficient chain transfer to monomer reactions, k_{add}/k_{-add} and $k_\beta/k_{-\beta}$ are the forward/reverse rate constants of addition and fragmentation reactions, and k_i is the rate constant of reinitiation; the efficient chain transfer to monomer is kinetically suppressed by means of a competitive reaction with the RAFT agent to obtain RAFT-**P1**.

weight (MW), in particular, on antimicrobial activity/toxicity.[23,24] Recently, RAFT-**P1** polymers of a low MW of $(8–14)\times10^3$ g mol^{-1} with terminal dithiocarbonyl and acetic groups (originated from xanthate) were synthesized by RAFT polymerization in the presence of the xanthate RAFT agent, namely, 2-[(ethoxycarbonothioyl)sulfanyl]acetic acid, HOC(O)CH_2SC(=S)OEt) [see Scheme 1(*b*)].[25]

To complement the series of classical polymers with samples having a low degree of polymerization, free radical polymerization of **M1** was carried out in excess of ammonium persulfate (APS, 10^{-1} M) as the initiator. The NMR, IR spectroscopy and elemental analysis data indicate that in this case the characteristic reactions of the chain transfer to monomer [reaction (1), see also Scheme 1(*a*)] are largely kinetically suppressed by the interactions of macroradicals with the primary radicals of the initiator R$^\bullet$ [reaction (2)].[26]

$$R–P_n^\bullet + M \longrightarrow R–P_n–CH_3 + CH_2=CH–P^\bullet \quad (1)$$

$$R–P_n^\bullet + R^\bullet \longrightarrow R–P_n–R \quad (2)$$

It was shown that with the excess of APS and, accordingly, a decrease in the molecular weight of polymers, the relative number of characteristic terminal vinyl groups decreases, and the terminal groups formed by the interaction of macroradicals with the primary radicals of the initiator become predominant, sulfate groups in our case[26] (Figure S2).

As a result of classical and RAFT polymerizations, a series of PDAA polymers have been obtained in a fairly wide range of MWs of $(8–118)\times10^3$ g mol^{-1}. Samples with a rather high MW have terminal CH_2=CH-groups, while samples with MW values of $(8–28)\times10^3$ g mol^{-1} have different end groups: RAFT-**P1** with –S–C(=S)–OEt and –CH_2–COOH end groups, the other two samples mostly with –O–S(=O)$_2$O$^-$(NH_4^+) terminal groups (Table 1).

Earlier we have revealed that PDAA biocidal efficiency enhanced with an increase in MWs (*i.e.* polymer length) and depended on the degree of alkyl substitution on the N atom and the alkyl chain length.[17,18] However, it has been shown that increasing the polymer length leads to an increase in such toxicity characteristic as the hemolytic activity.[2,6] Following this and other data, it became necessary to find out whether it is possible to retain the antimicrobial effectiveness of PDAA polymers

Table 1 Cytotoxic concentration (CTD_{50})[a] and minimal bactericidal concentration (MBC) of aqueous solutions of samples at a treatment time of 24 h and $C_{cell} = 10^5$ CFU (of tested eukaryotic and bacterial cells).

Entry	Sample	MW/ $\times10^{-3}$ g mol^{-1}	CTD_{50}/μg ml^{-1}		MBC/μg ml^{-1}		
			A-549	MA-104	*S. aureus*	*P. aeruginosa*	*M. smegmatis*/ *M. tuberculosis*
1	Pyr[b]	0.071	1050±250 (without neutr.) – 1570±346[c]		Not active[b]	Not active[b]	
2	PyrTFA[b]	0.185	3840±1070	–	Not active[b]	Not active[b]	
3	**P1**	118[d]	14.63±0.88	26.03±1.15			
4	**P1**	85[d]	19.87±0.99	32.67±1.11			
5	**P1**	62[d]	15.13±0.35	25.74±1.12	1.5±0.3 (**10^7 CFU**)[e]	125±7.5 (**10^7 CFU**)[e]	7.0±0.7/ 62±6.0(Tb) (**10^6 CFU**)[f]
6	**P2**[g]	60[d]	19.17±0.96	31.67±1.10	7.0±1.0 (**10^7 CFU**)[e]	31±3.5 (**10^7 CFU**)[e]	15±1.5/250±25(Tb) (**10^6 CFU**)[f]
7	**P1**	43[h]	14.93±0.36	25.44±1.14			
8	**P1**	40[h]	16,46±0.92	24.87±0.10			
9	**P1**	28[h]	22.02±2.28	31.14±1.81	37.5±7.5	62±6.2	
10	**P1**	17.9[h]	34.53±1.75	43.65±1.17	37.5±7.5	62±6.2	
11	RAFT-**P1**	8[h]	16.68±0.42	25.80±1.08	31±3.1	31±3.1	31±3.1

[a] CTD_{50} is the concentration which causes the death of 50% (one half) of test cells *in vitro*. [b] 500 μg ml^{-1} was the highest studied concentration of samples. [c] Toxicity of both the initial solution and the solution previously neutralized with 0.1 M HCl was tested. [d] MW was determined by the static light scattering method. [e] Ref. 17, $C_{cell} = 10^7$ CFU, exposure 1.5 h. [f] Ref. 18, $C_{cell} = 10^6$ CFU. [g] **P2** is tertiary poly(diallylmethylammonium trifluoroacetate). [h] Hydrodynamic molecular weight $M_{D\eta}$ was determined using data on intrinsic viscosity [η] of samples in 1 M NaCl, and translational diffusion coefficients D_0 of samples and the value of the hydrodynamic invariant A_0 were determined experimentally earlier.[27]

without causing significant cytotoxic effects on human cells. To this end, the influence of MWs in a wide range, including the monomeric chain, on the toxic action of PDAA relative to eukaryotic cells, and in parallel on the antimicrobial activity of these polymers relative to a number of bacteria, has been studied.

Investigations of cytotoxicity, using the *in vitro* method on cell cultures, are being increasingly used in biochemical and toxicological studies and are alternative to classical tests on experimental animals. All data obtained during 30 years in the Program MEIC (the Fund for the Replacement of Animals in Medical Experiments) indicated that the parameters of basal toxicity *in vitro* were very similar irrespective of the species and tissue origin of the mammalian and human established cell lines, and are universal for all types of cells.[28–30] It should be noted that research on cell cultures is used for the long-term testing (up to three days), opposite to testing of hemolytic activity, which can be used only for the rough estimation of cellular membrane damage for about 1–2 h exposure.

In the work, permanent (established) cell lines A-549 (epithelioid line of human lung carcinoma) and MA-104 (epithelioid line of green monkey kidney) were used for the study (see Table 1). The dose of the substance in the well was determined, at which 50% destruction of the cellular monolayer, CTD_{50}, was observed (methodology of toxicity research[31] is presented in Online Supplementary Materials). Since the antimicrobial activity of PDAAs with sufficiently large MWs was studied earlier,[17–19] the *in vitro* antibacterial activity of PDAA samples with a low MW relative to antibiotic-resistant bacteria was investigated from the collection of FRC 'Fundamentals of Biotechnology': *Staphylococcus aureus* 209P, *Pseudomonas aeruginosa* PAO1 and *M. smegmatis* mc2155 (ATCC 700084) (the closest fast-growing relative of *M. tuberculosis* with a similar cell wall structure). The spread plate method was used and minimal bactericidal concentration (MBC) values were estimated, *i.e.*, concentration that required to eliminate detectable growth of cells. (The detection limit of the spread plate method, using a 100 μl plating volume, was estimated between 10 and 30 CFU ml^{-1} compared to initial 10^5 CFU ml^{-1}, methods of antimicrobial studies are given in Online Supplementary Materials.)

As seen from Table 1, pyrrolidine (Pyr) and pyrrolidinium trifluoroacetate (PyrTFA), which model the monomer unit of PDAA polymers, exhibit a weak cytotoxic effect and their CTD_{50} is more than two orders of magnitude higher than that of all polymers studied (entries 1, 2; note that PyrTFA does not exhibit antimicrobial activity within the studied maximum cells concentration of 500 μg mol^{-1}).[17,18] Low toxicity of pyrrolidine is not surprising: it is the starting scaffold for multiple biologically active compounds.[32,33] Even lower toxicity of PyrTFA salt was unexpected. This allows us to conclude that the monomer unit of PDAA polymers is low-toxic, which, apparently, can be also expected for oligomers of small length. However, in the MW range of $(28–118)\times10^3$ g mol^{-1}, PDAA polymers are very toxic due to the cooperative action of the hydrophobic content and the total charge of the polycation, and the CTD_{50} values vary little in this MW range (see Table 1).

On the contrary, the antibacterial activity of these samples increases by more than an order of magnitude with increasing MW. Importantly, the MBC against *S. aureus* values (determined relative to *S. aureus* at a high concentration of 10^7 cells) for entries 5 and 6 are an order of magnitude lower than the CTD_{50} values of these samples (see Table 1). This indicates the action selectivity of polymers with a rather high MW (about 60×10^3 g mol^{-1} and more) over 10. The sample of entry 10 with a low MW value (18×10^3 g mol^{-1}) obtained by free radical polymerization turned out to be the least toxic in the PDAA series in spite of a decrease in its biocidal efficiency with decreasing MW. Comparison of its CTD_{50} with MBC (against *S. aureus*) rates a selectivity of biological action of about 1.16.

At the same time, the high toxicity of RAFT-**P1** [entry 11, –SC(=S)OEt end group] was unexpected because it is the sample with the lowest molecular weight from the tested polymers (MW of 8×10^3 g mol^{-1}). Not only did it not detect a further decrease in toxicity, oppositely, it has shown toxicity comparable to that of samples with MWs five times higher. The effect of the dithiocarbonyl group on PDAA toxicity turned out to be more significant than that on antimicrobial activity (see Table 1). When comparing the CTD_{50} values for two samples of entries 10 and 11 with a rather close MW, it could be concluded that in the case of a polymer with a small MW, the polar lipophilic group has a strong cytotoxic effect on eukaryotic cells (it should be added that the negatively charged end sulfate groups could slightly reduce both activity and toxicity for these samples). The conclusion on strong impact of the end –SC(=S)OEt group on cytotoxicity relative to eukaryotic cells does not coincide with the data[24] on the weak influence of $SC(=S)S(CH_2)_{11}CH_3$ or –SC(=S)SEt groups on hemolytic activity (as a measure of toxicity) of the polymethacrylates. Although, those polymers which contain the long alkyl $S(CH_2)_{11}CH_3$ group had the highest antimicrobial activity.[24] Note that it is not fully correct to compare the result on toxicity obtained in previously published work[24] and in this paper because very different cells were investigated and for different exposures.

Another type of action of the satellite end groups was demonstrated for the above mentioned biodegradable antibacterial polymers.[7,8] In particular, in the case of polymers based on polyionenes with inserted ester functions, OH and COOH end groups both deactivated the polymer to some extent by partial hydrolysis (under physiological conditions) to form a nontoxic fragment.[8] Higher toxicity to human malignant cells than to non-transformed kidney cells was expected (see Table 1), as the activity of polycations relative to cancer cells is well known. Thus, PDAA toxicity values obtained for the MA-104 cell line seem to be more adequate.

Summarizing, a family of antimicrobial polymers PDAA has been obtained in a wide range of MW values of about $(8–118)\times10^3$ g mol^{-1}, exhibiting biocidal activity relative to a range of hospital pathogens. Using the study on toxicity relative to eukaryotic cells and bactericidal activity of polymers, two areas of MW values can be distinguished such that biocidal activity of the polymers with given MWs is slightly higher or much higher than their toxicity. The polymers with a sufficiently large MW (more than 50×10^3 g mol^{-1}) have strong biocidal activity; their MBC (against *S. aureus*) values are an order of magnitude lower than CTD_{50} concentrations, which leads to toxic effects on mammalian cells. These polymers can be used today in medical institutions for the purpose of disinfection of premises and medical equipment. The MBC value for the sample with a small MW of 18×10^3 g mol^{-1} is slightly lower than its toxic CTD_{50} concentration. However, to reduce its (and polymers with a lower MW) cytotoxic effect, a low inhibitory concentration should be used in order to achieve the effect of total death of bacterial cells in a longer period of time (two to three days). Such PDAA polymers seem to be promising for the creation of new antimicrobial transdermal agents.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7621.

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