Electronic supplementary materials *Mendeleev Commun.*, 2025, **35**, 450–453

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

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Materials and Methods

Materials. Trifluoroacetic acid (TFA, "for synthesis", ≥99.0%; Merck, Germany), and radical initiators ammonium persulfate (APS, 99+%, for molecular biology, DNAse, RNAse and protease free, Acros, Belgium) and 4,4′-azobis(4-cyanovaleric acid) (ACVA, 98.0%; Aldrich) were used without additional purification. Diallylamine (DAA) reagents (for synthesis, 97%; Acros; Belgium), and solvents hexane and diethyl ether ("analytically pure", Khimmed; Russia) were distilled before use. Chromatographically pure DAA: T_b = 111–112°C. ^{1}H NMR (Me_2CO-d_6): = 3.20 (d, 4 H, 2 α-CH_2, J = 5.89 Hz), 5.12 (m, 4H, 2α-CH_2), 5.87 (m, 2H, 2β-CH).

Synthesis. The procedures for obtaining trifluoroacetic salts from DAA monomers were described previously.[S1-S3] The structures were confirmed by ^{1}H NMR spectra (characteristic spectrum is given in[S2]). ^{1}H NMR for DAATFA: (Me_2CO-d_6) - 3.71 (d, 4 H, 2 α -CH_2, J = 6.43 Hz), 5.47 (m, 4H, 2γ-CH_2), 6.00 (m, 2H, 2 β-CH).

DAATFA polymerization. Polymerization of the DAATFA and DAMATFA was carried out according to the elaborated method[S1-S3]. Aqueous solutions of DAATFA, [M] = 2 mol/L, at several concentrations of the APS initiator, [APS] = 2×10^{-2}, 4×10^{-2} and 10^{-1} mol/L, and T = 40 and 50ºC were prepared. Example 1: DAATFA (10.575 g, 2 mol/L) was dissolved in a small amount of double distilled water in a pycnometer, then APS (0.57 g, 10^{-1} mol/L) was added and the volume was adjusted to 25 ml with double distilled water (pH 2.5 solution). The ampoule with the solution was degassed by freezing with liquid nitrogen 9-10 times under vacuum down to 5×10^{-3} Torr, sealed and thermostated at 40 or 50°C. The polymer was taken into Et_2O, then purified three times by reprecipitation from a solution in MeOH into Et_2O, and dried under vacuum over P_2O_5. The samples 7 and 10 were prepared with [APS] = 2×10^{-2} and 10^{-1} mol L^{-1} correspondingly at 50ºC, the samples 8 and 9 were prepared with [APS] = 4×10^{-2} and 10^{-1} mol L^{-1} correspondingly at 40ºC.

DAATFA RAFT polymerization. Sample P11 (RAFT-**P1**) was synthesized in the presence of the RAFT agent xanthate as follows. Radical polymerization of DAATFA was carried out in aqueous solution with initiator ACVA, [M] = 2 mol L^{-1}, [ACVA] = 5×10^{-3} mol l^{-1}, at the ratio of concentrations [xanthate]/[ACVA] = 3, T = 70°C for 20 h. Example: DAATFA (10.575 g,

2 mol L^{-1}) and xanthate (0.068 g, 1.5×10^{-2} mol L^{-1}, corresponding to the [xanthate]/[ACVA] = 3) was dissolved in a small amount of bidistilled water; next, initiator ACVA (0.035 g, 5×10^{-3} mol L^{-1}) and bidistillate were added until the entire volume was 25 ml (see also date[S4]).

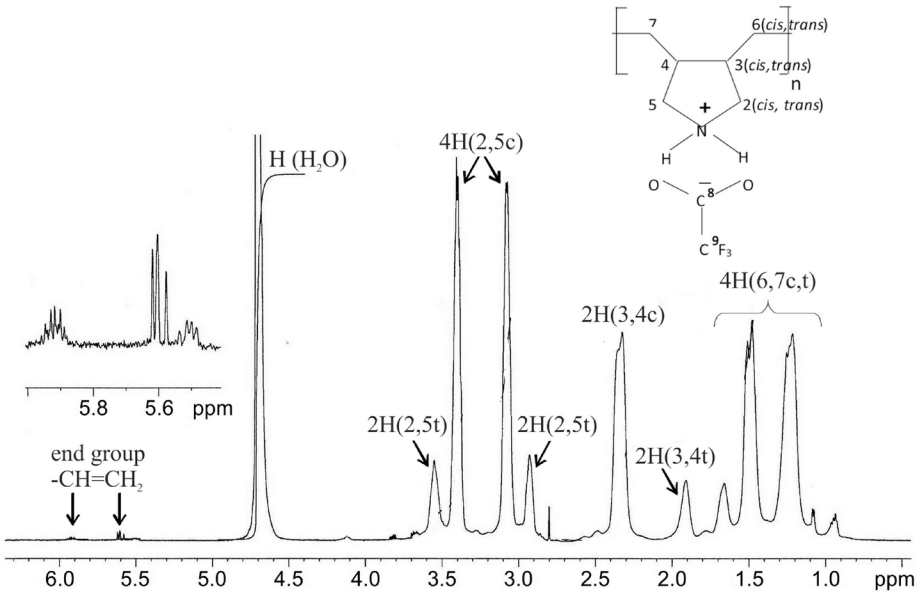

Figure S1 ^{1}H NMR spectrum of polymer PDAATFA (samples 3 and 5, Table 1), Bruker AVANCE III HD (400 MHz ^{1}H), D_2O, signals of H atoms of macrochain are assigned based on the two-dimensional HSQC spectrum, data[S3]; signals in the region of 5.5 – 6.0 ppm are due to H atoms of the end vinyl group (see text, Scheme 1(a))[S1-S3].

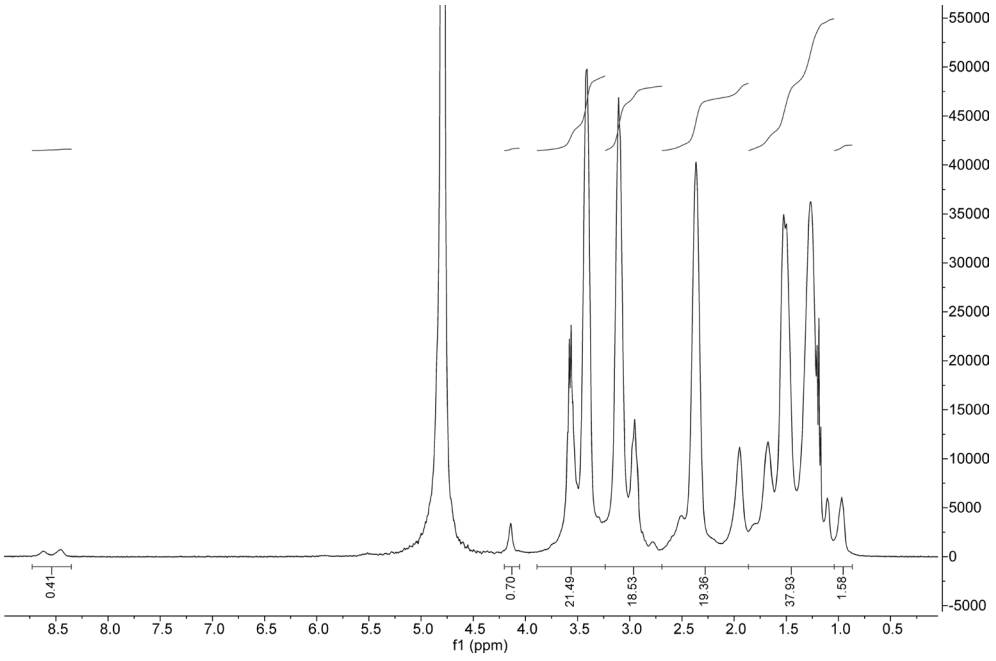

Figure S2 ^{1}H NMR spectrum of polymer PDAATFA, sample 10, Bruker AVANCE III HD (400 MHz ^{1}H), D_2O, the numeration of atoms is similar to the one in Figure 1; signal in the region of 4.15 ppm should be assigned due to H atoms of the $-CH_2-O-S(=O_2)-O^{-}(NH_4^{+})$ end

group, while signals due to the end vinyl groups (region 5.5 – 6.0 ppm) are poorly detected (compare Figure S1, see text).

Measurements. ^{1}H and ^{13}C NMR spectra of the synthesized samples were obtained on a Bruker AVANCE III HD spectrometer (400 MHz ^{1}H).

Determination of molecular characteristics of polymers. The molecular characteristics of the synthesized polymers were determined by hydrodynamic and dynamic light scattering (DLS) methods. The values of the intrinsic viscosity [η] of the samples in 1 M NaCl (Ostwald viscometer, solvent flow time 70.5 sec) and the translational diffusion coefficients D_0 were determined according to DLS data (Photokor complex, Russia). The experimental values of [η] and D_0 of the synthesized samples were used to calculate their hydrodynamic molecular weight $M_{D\eta}$ according to the equation (1)[S5]:

$$M_{D\eta} = (A_0 T/\eta_0 D_0)^3 \, (100/[\eta]) \qquad (1)$$

Here A_0 is the hydrodynamic invariant, T is the absolute temperature, η_0 is the viscosity of the solvent. The value of the hydrodynamic invariant $A_0 = 3.0\times10^{-10}$ erg $K^{-1}\cdot mol^{-1/3}$, which is included in equation (*1*), for the homologous series of PDAATFA was determined experimentally in[S6]. The methodology of all measurements and formalism were described in detail earlier[S4,S6].

Methodology of toxicity investigations

Investigations of cytotoxicity were performed using *in vitro* method on cell cultures. The dose of the substance in the well was determined, at which 50% destruction of the cellular monolayer, CTD_{50}, was observed. The CTD_{50} is the standard and omnipresent index of toxicity estimation *in vitro*. It is used as the main parameter in comparative studies of toxicity in all International Programs of toxicity testing *in vitro* like ECVAM (EU), CAAT (the USA), ZEBET (Germany), in the databases, like TOXLINE or INVITTOX, as well as in the National Guidelines of Toxicity testing *in vitro*[S7].

In this work, permanent (established) cell lines of eukaryotic cells A-549 (epithelioid line of human lung carcinoma) and MA-104 (epithelioid line of green monkey kidney cells) were used. Cells were grown in the α-MEM cell culture medium (Biolot, St.-Petersburg, Russia) supplemented with 10% calf serum, seeded in 96-well tissue culture plates (Nunc, Denmark) and allowed to grow in the CO_2-incubator at 5% CO_2 until the formation of confluent cellular monolayer (usually 24 h). The medium was discarded and replaced with a solution of tested compounds in serial dilutions in the serum-free α-MEM medium. Cells were further incubated for 24 h and their viability was assessed by the MTT (Thiazolyl blue, Sigma, USA) test[S8]. The

OD of colored product was measured in ThermoFisher Varioscan Plate Analyzer (Waltham, MA, USA) at 570 nm.

Mathematical/statistical analysis of the results: Each concentration of a compound under study was tested at least in 4 wells of a culture plate (n = 4). Control (intact) cells were represented at n≥4 wells. Each experiment was tripled. CTD_{50} (50% cytotoxic concentration) – the concentration which provoked 50% destruction of cellular monolayer, was calculated with the software package GraphPadPrism (GraphPadSoftware, SanDiego, California) in the non-linear regression fit: log(inhibitor) vs. response – Variable slope (four parameters).

Procedure for antibacterial activity research

Standard reference strains were used for polymer activity testing, namely *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *M. smegmatis* strain mc^2155 (ATCC 700084) obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures of State Research Center for Applied Microbiology and Biotechnology, Russia. Bacteria *P. aeruginosa* and *S. aureus* were grown in NB medium (Himedia, India) for 20 h. Bacterial inoculums were adjusted with sterile NB medium to a 1 McFarland standard with an organism density of approximately 3×10^8 colony forming units (CFU)/mL, then the suspension was diluted with NB broth to make a 1:3000 bacterial dilution (1×10^5 CFU/mL).

Estimation of bacterial viability. Bacteria *P. aeruginosa* and *S. aureus* were then inoculated at a concentration of 10^5 CFU per 1mL into 15 mL test tubes containing 2 mL of NB medium (Himedia, India) and polymer aqueous solutions of different concentration prepared by serial dilutions. After 24 hours of incubation at 37°C and 120 rpm., the culture from each tube was spread on agar-solidified NB medium by streak seeding method and incubated at 37 °C. The viability of bacteria was determined after 2 days (presence or absence of bacterial growth all along the streak), the minimal bactericidal concentrations corresponding to each treatment time (MBC_{100}, or MBC) were determined, 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 $CFU{\cdot}mL^{-1}$ compared to initial 10^5 $CFU{\cdot}mL^{-1}$. All experiments were carried out at least 4 times, and the data are reported as the mean values ± ER (experimental errors, which were calculated according to the recommended procedures).

M. smegmatis was pre-cultured for 24 h at 37°C in an orbital shaker (200 rpm.) in 20 mL of the rich Broth E LabM (NBE) medium containing (per litre) 5 g peptone, 5 g NaCl, 1.5 g beef extract, and 1.5 g yeast extract supplemented with 0.05% (v/v) Tween 80 in 50 mL flasks. Bacteria were then inoculated at a concentration of 10^5-10^7 CFU/mL into 15 mL test tubes containing 2 mL of Sauton's medium according to standard procedure (0.5 g of KH_2PO_4 L^{-1},

1.4 g of $MgSO_4$ L^{-1}, 4 g of l-asparagine L^{-1}, 60 mL of glycerol, 0.05 g of ferric ammonium citrate L^{-1}, 2 g of citric acid L^{-1}, 0.1 mL of 1% $ZnSO_4$ L^{-1}, pH 7 adjusted with 1 M NaOH)[S9] and polymer aqueous solutions of different concentration. After 24 hours of incubation at 37°C and 120 r.p.m., the culture from each tube was spread on agar-solidified NBE by streak seeding method and incubated at 37°C. The viability of bacteria was determined after 5 days (presence or absence of bacterial growth along the entire streak), i.e. MBC concentrations corresponding to each treatment time were determined (about the detection limit of the spread plate method see previous paragraph). All experiments were repeated no less than 4 times, the data are reported as the mean values ± ER (experimental errors, which were calculated according to the recommended procedures).

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