Electronic supplementary materials *Mendeleev Commun*., 2023, **33**, 255–258

Doxorubicin compositions with biocompatible terpolymer of *N*-vinylpyrrolidone, methacrylic acid and triethylene glycol dimethacrylate

Svetlana V. Kurmaz, Irina I. Ivanova, Nina S. Emelyanova, Dmitry V. Konev, Vladimir A. Kurmaz, Natalia V. Filatova, Anastasia A. Balakina and Alexei A. Terentiev

Table of Contents

Experimental

Materials

N-vinylpyrrolidone (99%, VP, Alfa Aesar) was distilled in vacuum to remove the inhibitor (NaOH). Triethylene glycol dimethacrylate (95%, TEGDM, Aldrich) and MAA (99.5%, Acros Organics) and lactose monohydrate were used without additional purification. 2,2'-azo-bis-isobutyronitrile (AIBN) purified by recrystallization from ethanol was used as the initiator of radical polymerization.

Synthesis of the terpolymer

The synthesis of the terpolymer was carried out in a three-necked flask equipped with the reflux condenser and the thermometer under continuous bubbling with argon for 2 h at 80 °C in the thermostat. All components of the reaction mixture were introduced simultaneously. The content of reagents in the ethanol was ~20 wt %. The concentration of the initiator in the solution was 0.02 mol L^{-1}. After completion of the reaction, an ethanol-soluble product was obtained. The isolation of the terpolymer from solution was carried out by precipitation with the tenfold excess of the precipitant, *n*-hexane. The precipitate was filtered on the Buchner funnel. The terpolymer was dried from solvent and precipitant residues to constant weight in air and in vacuum at 60°C. The copolymer yield was ~94%.

The methods

Electronic absorption spectroscopy

The absorption spectra of aqueous solutions of DOX, terpolymer and DOX-terpolymer compositions were recorded on a SPECS instrument (Russia) in quartz cuvettes of 0.5 or 1 cm thickness. The effective binding constant of DOX and terpolymer in aqueous phosphate buffer solution was estimated from the spectral data. It is assumed that the interaction between the ligand L and the substrate S is 1 : 1; for this reason a single complex SL (1 : 1) is formed. It was also assumed that the sites (and all the binding sites) are independent and all species obeyed the Beer's law. The wavelength is selected at which the molar absorptivities ε_S (molar absorptivity of the substrate) and ε_{11} (molar absorptivity of the complex) are different.

Dynamic Light Scattering

The dynamic light scattering method was used to determine the hydrodynamic radii, R_h, of the terpolymer and its nanostructures with DOX in PBS. To prepare samples for measurements, the solutions were filtered using a filter with a pore diameter of 0.45 μm. Before measuring, the vials with the solution were thermostated at a given temperature for 20 min. DLS measurements were carried out using a Photocor Compact setup (Photocor LTD, Russia) equipped with a diode laser operating at a wavelength of 654 nm. All solutions were analyzed at a detection angle of 90°C. The processing of experimental data was carried out using the DynaLS software, version 2.8.3.

NMR spectroscopy

NMR spectra of the terpolymer and $PC1DOX_{3.1}$ in deuterated chloroform were recorded on a superconducting pulsed broadband two-channel AVANCE III 500 MHz Bruker BioSpin NMR spectrometer. Their concentrations were 7 mg mL^{-1}.

The electrochemical measurements

The electrochemical measurements were carried out via cyclic voltammetry with using of an Autolab/PGSTAT302N universal high-speed potentiostat/galvanostat (ECOCHEMIE, Netherlands) in three-electrode glass cells without separation of the cathodic and anodic area, the working volume was from 5 to 15 cm^3. Before the experiments, the working solutions were deaerated by alternately applying a vacuum and filling the electrochemical cells with argon. When the components of the working solution and electrodes were introduced into the cell and during the electrochemical measurements, a slight excess argon pressure (~20 mbar) was maintained above the solutions. The standard Schlenk procedure[S1-S3] was used for the experiments. All the experiments were performed at a room temperature within the potential scan rate range of 10–2000 mV s^{-1}. The working electrodes were a GC disc electrode HTW Segradur-G (Germany) with a diameter Ø ~3 mm soldered into a glass. The auxiliary electrode was a Pt wire, and the reference electrode was a silver/silver chloride electrode ($Ag/AgCl/KCl_{sat.}$) in aqueous solutions. All the potentials were referred with respect to this electrode. The GC electrode was polished immediately before an experiment by a diamond suspension (diameter of 1 μm, and after ~0.25 μm) then rinsed with ultrasonic machining. A detailed description of the CVA experiments can be found elsewhere.[S1, S2, S4]

Quantum-chemical modeling of the structure of DOX-copolymer complexes

Quantum-chemical calculations were carried out within the framework of the density functional theory (DFT) with full optimization of the geometry of the initial molecules and their complexes in the Gaussian 09 program.[S5] The hybrid functional TPSSh[S6] and the basis set 6-31G * // 6-311 ++ G ** were used as a method and basis. The influence of the solvent (water) was also taken into account using the polarizable continuum model (PCM). There are no imaginary vibration frequencies in the calculation results, all optimized structures correspond to the minimum potential energy. Parameters such as the energy of secondary interactions, the Weiberg index and the bond order were found from calculations using natural basic orbitals (NBO).

To analyze the wave functions using the QTAIM method, the AIMALL software package (version 10.05.04)[S7] was used. The wave functions of the structures were calculated in the same approximations as the geometry optimization. In particular, from the analysis of the wave functions, we found the energies of intermolecular bonds (E_{bonds}), the electron density (ρ) and the Laplacian of the electron density (∇2ρ) at the critical points of the bond. The energies of intermolecular bonds were calculated using the formula $E_{a\text{-}b} \approx 1 / 2ve$ (r),[S8] where $E_{a\text{-}b}$ is the A-B bond energy, and v_e (r) is the potential energy density at the critical point of the A-B bond. The illustrations were made using the ChemCraft program (version 1.8).[S9]

Cytotoxicity studies

Experiments were performed on *HeLa* (human cervical carcinoma, subclone M) and *Vero* (African green monkey kidney epithelium) cells. Cells were purchased from the Russian cell culture collection (Institute of Cytology RAS, St. Petersburg, Russia). Cells were grown in EMEM (*HeLa*) and DMEM (*Vero*) media (PanEco, Russia) supplemented with 10% fetal calf serum (BioWest, France), penicillin (50 U mL^{-1}) and streptomycin (50 μg mL^{-1}) at 37 °C in an atmosphere of 5% CO_2.

The cytotoxicity was studied using the MTT test[S10]. Cells were seeded into 96-well plates at a concentration of 5×10^4 cells mL^{-1}. The studied compounds were added to incubation medium 24 h post-seeding. Doxorubicin (Pharmachemie, Netherlands) was added into the incubation medium in the concentration range from 0.54 to 8.15 μg mL^{-1}. Terpolymer was added into the incubation medium at a final concentration from 4.69 to 200 μg mL^{-1}. $PC1DOX_{3.1}$ was added

into the incubation medium at a final concentration from 0.14 to 5.88 μg mL^{-1} (for doxorubicin). After 24-h exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Dia-M, Russia) was added to the incubation medium at a concentration of 0.5 mg mL^{-1} for 4 h. Then the medium was aspirated and MTT formazan was dissolved in 100 μL of 100% DMSO. The optical density was measured at a wavelength of 570 nm and a background wavelength of 620 nm using a Spark 10M multifunctional plate reader (Tecan, Switzerland). IC_{50} values were determined from dose-effect relationships using median effect analysis[S11].

Table S1 Concentrations of reagents and conditions for PC1DOX$_n$ preparation.

[Copolymer] in Pr^{i}OH/mg mL^{-1}	[DOX] in water/mg mL^{-1}	Volume ratios of solutions copolymer and DOX/mL	DOX content per copolymer/ %	[Copolymer] in PBS/mg mL^{-1}	[DOX]×10^5 in PBS/M
2	1	4 : 0	0	2	0
		4 : 0.05	0.625		2.16
		4 : 0.1[a]	1.25		4.3
		4 : 0.15	1.9		6.5
		4 : 0.20[a]	2.5		8.6
		4 : 0.25[a]	3.1		10.8

[a] Volume ratios of Pr^{i}OH and DOX solution in control experiments.

Table S2 Concentrations of reagents and conditions for $PC2DOX_n$ preparation.

[Copolymer] in Pr^iOH/mg mL^{-1}	[DOX] in water/mg mL^{-1}	Volume ratios of solutions copolymer and DOX/mL	DOX content per copolymer/ %	[Copolymer] in PBS/mg mL^{-1}	[DOX]×10^5 in PBS/M
0	1	4 : 0.1	-	-	4.3
0.5		4 : 0.1	5.0	0.5	4.3
1.0		4 : 0.1	2.5	1.0	4.3
2.0		4 : 0.1	1.25	2.0	4.3
5.0		4 : 0.1	0.5	5.0	4.3

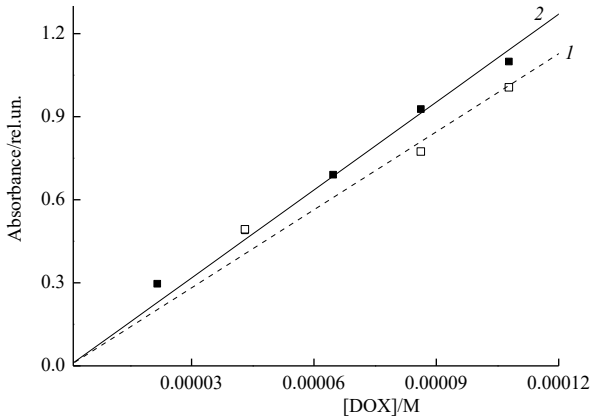
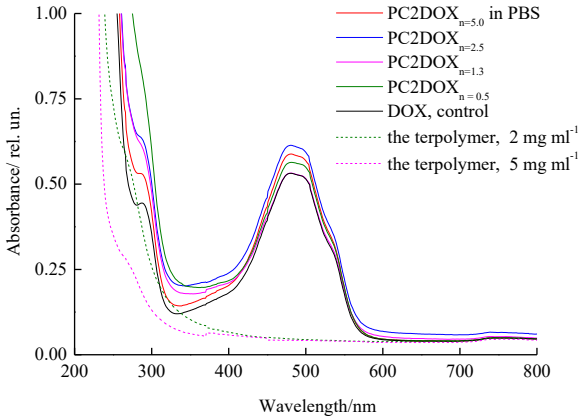
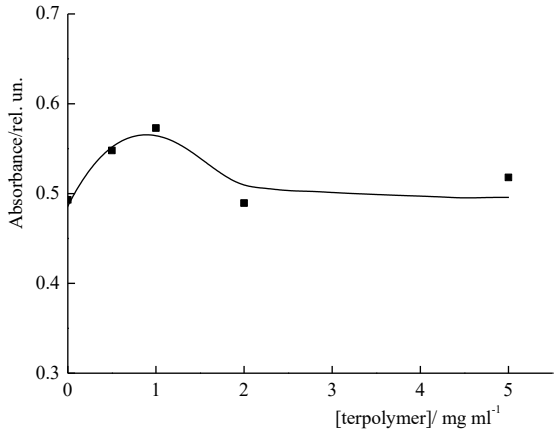


Figure S1 The dependencies of the optical density *A* of the absorption band at 490 nm of DOX (*1*) and PC1DOX$_n$ (*2*) on concentration in aqueous phosphate buffer solution.



(*a*)



(*b*)

Figure S2 Absorption spectra: (*a*) of DOX and PC2DOX$_n$ aqueous phosphate buffer solutions; (*b*) dependencies of the optical density *A* of the absorption band of DOX at 490 nm on copolymer concentration. Cuvette is 1 cm.

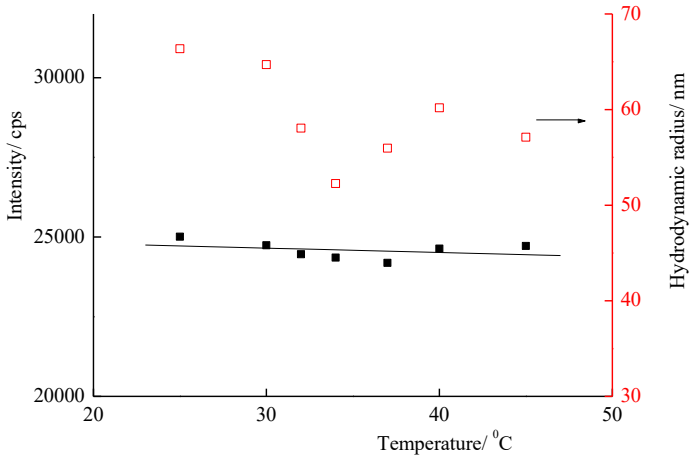


Figure S3 The dependencies of light scattering intensity *I* and hydrodynamic radii R_h of scattering centers of $PC1DOX_{3.1}$ in PBS on temperature.

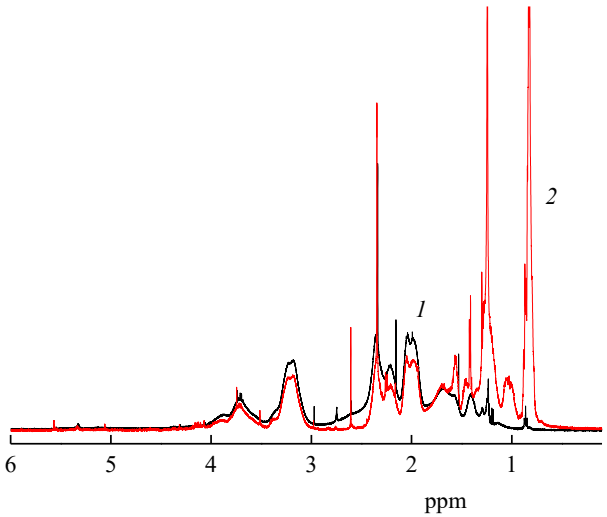
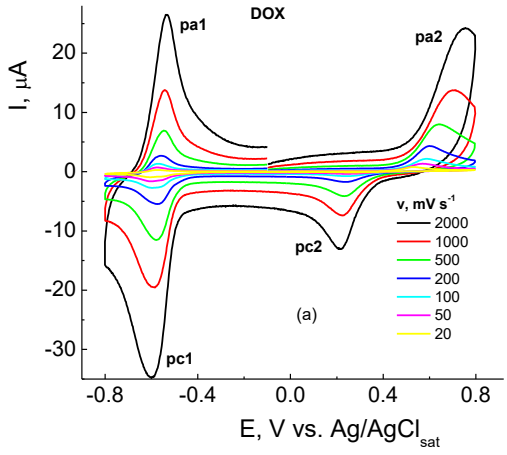
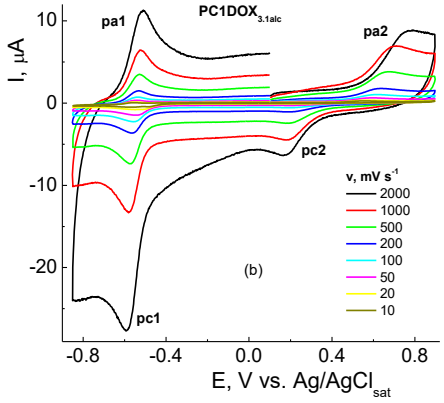


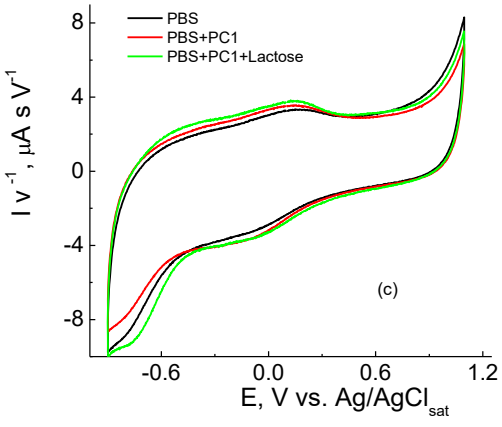
Figure S4 ^{1}H NMR spectra of the terpolymer (*1*) and PC1DOX$_{3.1}$ (*2*) in deuterated chloroform. The spectra were normalized to the solvent signal.



(*a*)



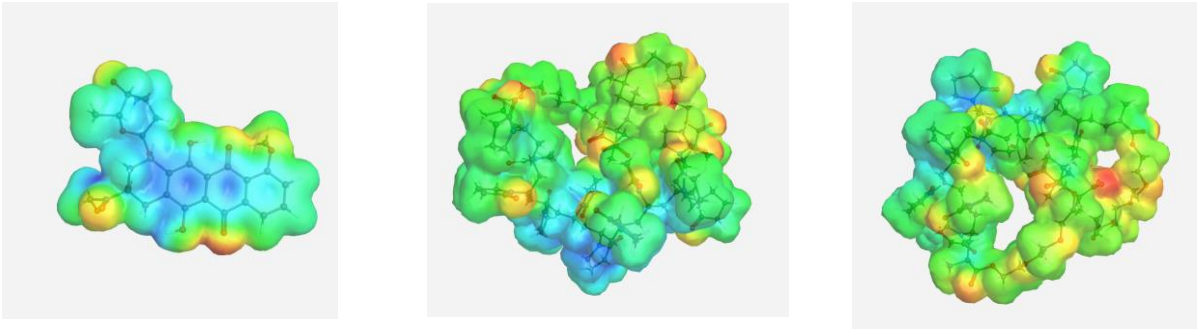
(*b*)



(*c*)

Figure S5 CVA-curves of DOX (*a*) and PC1DOX$_{3.1}$ (*b*) in aqueous buffer solution (pH 7.24) and (*c*) background electrolytes in coordinates *I*, *E* (a, b) and *I*/*v* (*c*) coordinates at *v* = 10-2000 mV s^{-1}. DOX concentrations in (*a*, *b*) are 4.0 × 10^{-5} and 1.4 × 10^{-5} M, resp.

We model the cavity of the branched copolymer consisting of the maximum accessible amounts of VP, MAA and TEGDM units in the same monitoring in which they are present in experimental samples using the semi-empirical method of AM1 in the Gaussian 09 program. On the basis of an electrostatic surface [Figure S6 (*a*)], DOX guest molecule concludes that the negative charge is concentrated on carbonyl groups, on it and methoxy groups, and due to partially positive hydrogen atoms it will form connections with the atoms of the oxygen of the terpolymer. For terpolymer, the most negative atoms of oxygen of carboxyl groups of the MAA units, the carbonyl group of the lactate ring of the VP, as well as C=O group of TEGDM units and a slightly weaker negative charge are concentrated on the oxygen group atoms TEGDM units [Figure S6 (*b*, *c*)]. At the same time, the rest part of terpolymer is charged to one degree or another positively, therefore, almost any hydrogen atom of the copolymer can participate in hydrogen ties with the donor atoms of the guest molecule.



(*a*) (*b*) (*c*)

Figure S6 Electrostatic potential of DOX molecule (*a*) and the terpolymer moiety (*b*, *c*).

Table S3 IC_{50} values for test compounds after 24 h exposure on *HeLa* and *Vero* cells.

Compound	IC_{50}, $\mu g \cdot mL^{-1}$	
	HeLa	*Vero*
DOX	1.35	3.26
$PC1DOX_{3.1}$	3.02	>6.00
terpolymer	–	–

References

S1. S. V. Kurmaz, V. D. Sen', A. V. Kulikov, D. V. Konev, V. A. Kurmaz, A. A. Balakina, A. A. Terent'ev, *Russ. Chem. Bull.*, 2019, **68**, 1769.

S2. D. V. Konev, K. V. Lizgina, D. K. Khairullina, M. A. Shamraeva, C. H. Devillers, M. A. Vorotyntsev, *Russ. J. Electrochem*., 2016, **52**, 778.

S3. Z. N. Gafurov, I. F. Sakhapov, V. M. Babaev, A.B. Dobrynin, V.A. Kurmaz, K.E. Metlushka, I. Kh. Rizvanov, G. R. Shaikhutdinova, O. G. Sinyashin, D. G. Yakhvarov, *Russ. Chem. Bull.*, 2017, **66**, 254.

S4. S. V. Kurmaz, N. V. Fadeeva, B. S. Fedorov, G. I. Kozub, N. S. Emel'yanova, V. A. Kurmaz, R. A. Manzhos, A. A. Balakina, A. A. Terentyev, *Mendeleev Commun.*, 2020, **30**, 22.

S5. M. J. Frisch, G. W. Trucks, H. B. Schlegel et al (Gaussian, Inc., Wallingford CT, 2009).

S6. J. M. Tao, J. P. Perdew, V. N. Staroverov, G. E. Scuseria, *Phys Rev Lett.*, 2003, **91**, 146401.

S7. AIMAll (Version 10.05.04), Todd A. Keith, T. K Gristmill Software, Overland Park KS, USA, 2010 (aim.tkgristmill.com).

S8. E. Espinosa, E. Molins, C. Lecomte, *Chem Phys Lett.*, 1998, **285**, 170.

S9. Chemcraft - graphical software for visualization of quantum chemistry computations. https://www.chemcraftprog.com

S10. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.

S11. T.-C. Chou, *Pharmacol. Rev.*, 2006, **58**, 621.