December 2012-December 2013

OBJECTIVES and ACTIVITIES for 2016

OBIECTIVE 1*:* <u>Biochemical properties of prepared systems</u>

Activity 1.1. Toxicity, biocompatibility

Activity 1.2. Interaction with different cells

1.1. Toxicity, biocompatibility

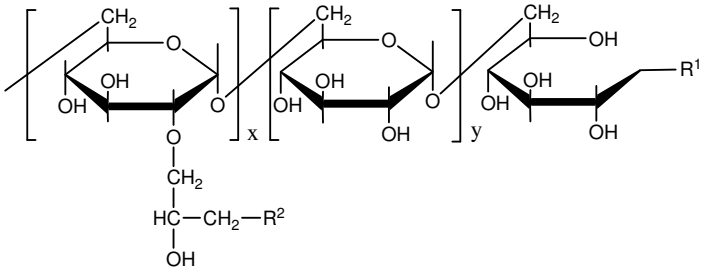
A block-copolymer Dex10-(DCA-OEG100) containing 40 wt% polyester was tested for toxicity by measurement of cell viability in the presence of polymer (citotoxiciy), and biocompatibility was determined by following polymer activity against red blood cells (RBC) (hemolysis test).

<u>*Citotoxicity*</u> was evaluated on osteoblast cells, which were cultured in a medium containing 1 mg/mL polymer. Viability was measured after 24 h at 37 ^{o}C using MTT test, which implies the addition of a MTT solution [(3-(4,5-dimethyl)tiazol-2,5-diphenyl tetrazolium bromide), 5mg/mL in phosphate buffer] in culture medium. The test is based on viable calls ability to metabolize water soluble MTT with formation of insoluble formazan salt. The amount of colored formazan is determined by UV-vis measurements at 550 nm, and cells viability, as % from a control, is calculated with formula: (At/Ac)100, where At si Ac are the absorbance of the cells in the presence and the absence of polymer, respectively. The result of this test indicated a 100% viability after 24 h. Besides, polymer presence did not affect cell proliferation ability.

<u>*Hemolysis test*</u> was realized on RBC cells separated by centrifugation of rabbit blood. 10 mL of RBC suspension were added to 90 mL polymer solution (10 mg/mL) and the mixture was incubated for 24 h at 37 ^{o}C, then centrifuged. The amount of released hemoglobine in supernatant was measured by UV-vis at 540 nm and compared with that obtained for a positive control test (distilled water, zero hemolysis) and for a negative control test (0.2 % Triton X-100, 100% hemolysis). The results proved polymer hemocompatibility, with a hemolysis percent less than 1% after 24 h.

1.2. Interaction with different cells

Interaction of some cationic amphiphilic block-like polymers (having general chemical structure presented in Scheme 1 and detailed chemical composition given in Table 1) with microbial cells (bacteria, fungi) was studied in order to determine polymer antimicrobial activity.



Scheme 1. General chemical structure of cationic amphiphilic polymers used in antimicrobial tests. Dextran has a hydrophobic chain (long alkyl) attached at its reductive end, as well as quaternary ammonium groups attached to the main backbone. Synthesis of these polymers was described in the Report to Stage II (2012).

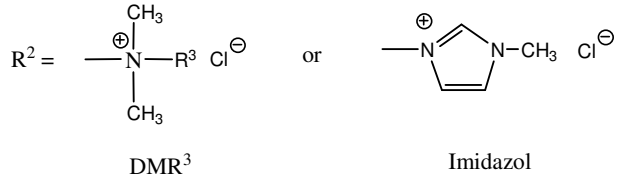


Table 1. Chemical composition and some characteristics of cationic polymers with chemical structure presented in Scheme 1. Degree of substitution with cationic groups was DS = 29 ± 2 mol%

Polymer sample	$M_{n,dex}$	R^1	R^2	R^3	*CAC*,mg/ml	Zeta potential, mV
A1	8000	Alkyl, n = 18	DMR^3	Benzyl	1.06	26
A2	8000	-	DMR^3	Octyl	3.00	40
A3	8000	Alkyl, n = 12	DMR^3	Octyl	1.02	28
A4	8000	Alkyl, n = 12	DMR^3	Benzyl	2.02	26
A5	8000	Dialkyl	DMR^3	Benzyl	1.81	27
A6	4500	Alkyl, n = 18	DMR^3	Benzyl	0.83	30
A7	4500	Alkyl, n = 12	DMR^3	Benzyl	1.02	29
A8	4500	Alkyl, n = 12	Imidazol	-	1.25	30

Antimicrobial activity against several microbe straines. Gram pozitive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea ATCC* 9341), Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and pathogenic fungi (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC MYA 2950, *Candida parapsilosis* ATCC 22019) were used for these tests. Antimicrobial activity was evaluated by: (i) disc diffusion method on agar medium, which provides the diameter of microbial growth inhibition zone, and (ii) broth dilution method, which allows the calculation of minimum inhibition concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC). Antibacterial activity of tested polymers was compared with that of Ciprofloxacin, and positive controls for antifungic activity were Nystatin and Fluconazol. Results presented in Fig. 1 si Table 2 show that the polymers have antimicrobial activity against all used microbe species, except for *P. aeruginosa*, are less efficient than controls, and have better activity against Gram positive bacteria and *C. parapsilosis* than against *E.coli.* Dextan molecular weight had the highest influence on activity, as it determines the hydrophilic/hydrophobic balance of the amphiphilic polymer. Presence of dimethylbenzylammonium groups seems to favor activity, but polymers with dimethyloctylamonium groups are more efficient against *E.coli.* (samples **A2** si **A3**). MIC and MBC/MFC values included in Table 3 confirm the results of microbial susceptibility tests, and the lowest values obtained against *S. aureus* (MIC = 60 µg/mL) are of a similar order of magnitude as those reported for other cationic polymers with different chemical structures.

Fig.1. Results obtained by disc diffusion method with samples **A2**-**A7** and *S. aureus* ATCC 25923 (left) and *Candida parapsilosis* ATCC 22019 (right)

Table 2. Antibacterial and antifungal activities of the studied polymers ale polimerilor studiati

Polymer	Diameter of inhibition zone (mm)						
	S. aureus ATCC 25923	*S. lutea* ATCC 9341	*E. coli* ATCC 25922	*Pseudom. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. glabrata* ATCC MYA 2950	*C. parapsilosis* ATCC 22019
A1	13.6±0.57	14.0±0.00	0	0	11.0±0.00	12.5±0.50	13.0±0.00
A2	13.6±0.57	15.0±0.57	11.0±0.00	0	12.0±0.00	13.0±0.00	20.3±0.57
A3	13.0±0.00	13.7±0.06	10.6±0.57	0	12.0±0.00	12.0±0.00	20.3±0.57
A4	13.3±0.57	13.0±0.00	0	0	12.3±0.57	12.3±0.57	20.0±0.00
A5	10.0±0.00	14.0±0.00	0	0	13.0±0.00	12.3±0.57	20.3±0.57
A6	15.3±0.57	17.8±0.28	0	0	14.7±0.06	13.0±0.00	22.3±0.57
A7	15.0±0.00	17.8±0.28	10.6±0.57	0	14.3±0.57	15.0±0.00	23.3±0.63
A8	0	0	0	0	10.3±0.57	10.7±0.06	15.0±0.00
Ciprofloxacin (5 µg/disc)	24.7±0.06	25.0±0.00	30.5±0.50	30.0±0.00	*NT	*NT	*NT
Fluconazol (25 µg/disc)	NT*	NT*	NT*	NT*	30.5±0.50	23.5±0.50	22.0±0.00
Nystatin (100 µg/disc)	NT*	NT*	NT*	NT*	23.5±0.50	22.0±0.00	23.0±0.00

*NT-not tested

Table 2. MIC si MBC/MFC values for tested polymers

Polymer	*S. aureus* ATCC 25923		*C. albicans* ATCC 90028	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
A1	1.25	2.5	2.5	5
A2	1.25	2.5	2.5	5
A3	1.25	2.5	2.5	5
A4	1.25	2.5	2.5	5
A5	1.25	2.5	2.5	5
A6	0.06	1.25	1.25	2.5
A7	0.06	1.25	1.25	2.5
Ciprofloxacin	1[a]	2[a]	n.d.[b]	n.d.[b]
Fluconazol	n.d.[b]	n.d.[b]	8[a]	16[a]

[a] values expressed in µg/mL; [b] not determined

OBIECTIVE 2*:* <u>Prospective study for the new applications of block copolymers</u>

Activity 1.1. Nanoreactors (additional studies)

During the present stage we resumed the studies initiated in 2015 using a block-copolymer Dex10-(DCA-OEG100) containing 69 % g/g polyester and forming vesicles in aqueous media. The vesicles were loaded with lipase from porcine pancreas, which was previously labeled with fluorescein izothiocianat (LP-FITC). Two polymer samples containing 0. 1 and 0.5 g LP-FITC /g were prepared and tested as potential nanoreactors. Retention of enzyme inside vesicles for a longer period of time was checked by UV and/or fluorescence measurements performed at different time intervals on supernatant obtained after centrifugation of loaded vesicles suspension. The results showed that LP-FITC did not diffuse through vesicle outer wall, as the labeled enzyme was not detected in supernatant after 3 month storage. In order to test the possibility

to carry out enzymatic reactions inside the inner aqueous compartment of vesicle, 4-nitrophenyl benzoate (NPB) was used as substrate for lipase. NPB is a colorless compound, which releases, after hydrolysis, 4-nitrophenol, a yellow product with UV absorption (λ_{max} = 402 nm, ε_M = 18400 L mol^{-1} cm^{-1}, in 0.1N NaOH). Experiments were based on the assumption that enzyme is located only in the vesicle inner compartment, but NPB and its hydrolysis product can permeate through vesicle outer wall, and were performed as follows: 500 µl loaded vesicle aqueous suspension (5 mg/mL) were mixed with a NPB aqueous solution (containing 1% DMSO), in the amount required to reach a ratio NPB/LP of 100/1,g/g, and the mixture was introduced in a 10 mL centrifuge tube. After 60 sec at ambient temperature the mixture was centrifuged (1200 rpm), an aliquot from supernatant was diluted with 0.1 N NaOH, and the value of absorbance at 402 nm, was used for calculation of 4- nitrophenol released after enzymatic hydrolysis of NPB. Conversion rate after 60 sec was 4.5 10^{-2} h^{-1}, showing that the enzymatic reaction can take place inside the aqueous compartment of vesicles, consequently these vesicle forming block-copolymers can act as nanoreactors.

Results obtained up to now were included in 2016 in 2 accepted articles and 1 article under review

- G. Mocanu, M. Nichifor, L. Sacarascu. Dextran based polymeric micelles as carriers for delivery of hydrophobic drugs. *Current Drug Delivery*, accepted, DOI: 10.2174/1567201813666160513132 45
- L. Ghimici, C. E. Brunchi, A. Deleanu. Removal of some commercial pesticides containing a-Cypermethrin, Deltamethrin and Mancozeb as active ingredients by chitosan solution. *Cellulose*, accepted, DOI: 10.1007/s10570-016-1056-1
- C.G. Tuchilus, M. Nichifor, G.Mocanu, M. C. Stanciu. Antimicrobial activity of chemically modified dextran derivatives. *Carbohydrate Polymers*, under review

Project director