Scientific Report

to the project

New amphiphilic cationic oligomers as synthetic mimics of antimicrobial peptides and/or external biocides

code PN-III-P4-ID-PCE-2016-0519

December 2017 – December 2018

The main objective of the project is to design, synthesize and evaluate new more biocompatible materials of high complexity with enhanced antimicrobial activity against a wide number of gram positive and gram negative bacteria, yeasts and fungi, with application both as antibiotics alternatives and external powerful biocides. Several steps are necessary for project achievement:

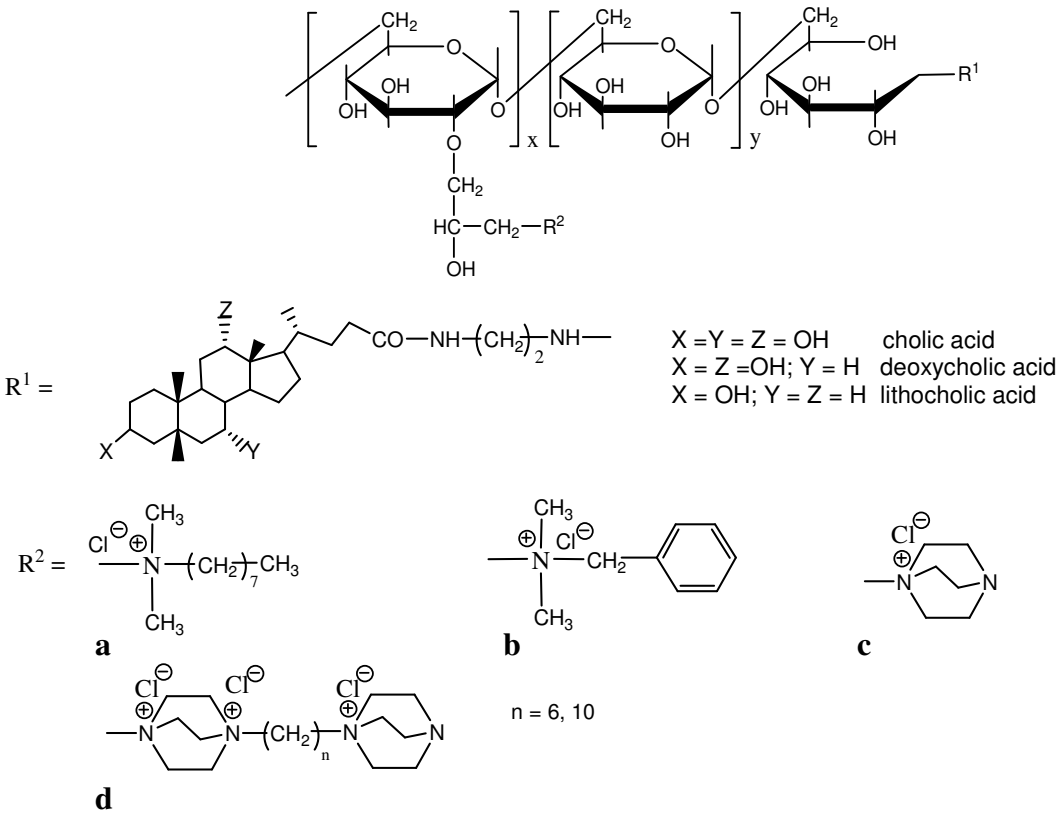
- Synthesis of new polymers based on bile acids, which should have several properties of antimicrobial peptides: facial amphiphilicity, presence of primary amine groups, selective antimicrobial activity and reduced hemolytic activity. These properties are required for their application as alternatives to antibiotics, with a similar or superior activity, and a lower tendency to develop microbial resistance.
- Synthesis of new amphiphilic polymers based on polysaccharides with quaternary ammonium groups and hydrophobic segments located either at the polysaccharide chain end or as pendent groups of polysaccharide backbone. These polymers are designed for application as external biocides (disinfectants) with high antimicrobial activity and enhanced biocompatibility.
- Evaluation of biological activity (antimicrobial, hemolytic) of synthesized polymers.

<u>Objective 2018 (Stage 2):</u> Synthesis and characterization of polymers based on polysaccharides, biological evaluation of synthesized polymers

Activity 2.1. Synthesis and physico-chemical characterization of polymers based on polysacharides

New amphiphilic cationic polymers were synthesized by chemical modification of polysaccharides. The polymers with general chemical structure presented in Scheme 1 (exemplified for dextran) have a block-like structure, with hydrophilic block (the polysaccharide with pendent quaternary ammonium groups) and hydrophobic block (segment R^1) spatially separated, a property supposed to ensure a better antimicrobial activity. The decisive factor for a good antimicrobial activity of this

polymer structure is an appropriate hydrophilic/lipophilic balance, which depends on polysaccharide molecular weight, end group R^1 hydrophobicity, chemical structure and density of cationic groups R^2 attached to the polysaccharide backbone.



Scheme 1. Polymer general chemical structure

Two polymer series based on dextran were synthesized by (1) covalent binding of the hydrophobic group R^1 at the end of polysaccharide chain (Scheme 1), followed by attachment of quaternary ammonium groups along polysaccharide backbone (one cationic center on each side group), or (2) attachment of pendent side groups, each of them bearing several quaternary ammonium groups.

2.1.1 Chemical modification of polysaccharide chain end

All linear polysaccharides have an aldehyde group at one chain end, which allows a selective modification with attachment of a single end segment/polysaccharide chain. The end aldehyde group can react with a primary or secondary amine with intermediate formation of an imine group (Schiff base), followed by imine reduction to amine in the presence of a hydride. Reductive amination of dextran samples with *MW* 6000 (D6), 9000-11000 (D10) or 25000 Da was performed with bile acids (BA) aminated derivatives in order to obtain derivatives **D1**-**D7** with the structure detailed in Scheme 1 and Table 1. Polymers **D1**-**D7** synthesis required three reaction steps:

i. Synthesis of methyl esters of cholic (CA), deoxycholic (DCA) or lithocolic (LCA) acids.

Products were analyzed by ^{1}H-NMR (presence of the signal for COOC<u>H</u>$_3$ at 3.3 ppm) and FTIR (occurrence of ester band at 1720 cm^{-1} and disappearance of carboxyl band at 1700 cm^{-1}).

ii. Reaction of bile acid methyl esters with ethylendiamine (EDA) for preparation of (2'-aminoethylene)-5β-cholanoamides (NH_2-CH_2-CH_2-NH-CO-BA) (BA-EDA). Products structure was confirmed by ^{1}H-NMR (occurrence of signals for methylene protons at 2.8 ppm (–<u>C</u>H$_2$-NH$_2$) and 3.2 ppm (–C<u>H</u>$_2$-NH-CO-)) and FTIR (presence of the band assigned to amide carbonyl at 1680 cm^{-1} and disappearance of the carboxyl band).

iii. Reductive amination of dextran with BA-EDA derivatives. A high conversion degree (95-98 % modified chain ends) was obtained under optimal conditions. Conversion degree was calculated from ^{1}H-NMR spectra, using integrals of the peaks assigned to dextran anomeric protons (4.9 ppm) and protons of methyl group in 19 position of the steroidic cycle (0.89 ppm).

2.1.2 Synthesis of polymers with pendent quaternary ammonium groups

These polymers were prepared with a procedure previously developed (M. Nichifor, M. C. Stanciu, B. C. Simionescu, *Carbohydr. Polym*. 82, 965-975, 2010) using as reagents an equimolar mixture of epichlorohydrin and a tertiary amine. A wide range of polymers with various amphiphilicity can be obtained as a function of hydrophobicity of substituents bound to nitrogen atom of the tertiary amine. Reaction was performed on both unmodified and end chain modified dextran. Chemical structure was confirmed by ^{1}H-NMR spectra where the signals of specific substituents of amine groups (alkyl, benzyl of cyclooctan) were identified, besides signals characteristics to dextran protons. Substitution degree (DS) was established by elemental analysis (nitrogen content) and potentiometric titration with $AgNO_3$ solution (chloride ions content). The procedure was employed for preparation of two cationic polymer series: (i) with a single quaternary ammonium group on a side chain, or (ii) with several quaternary ammonium groups on a side chain.

i. Cationic polymers with a single quaternary ammonium group/side chain were obtained by using the following tertiary amines as reagents: N,N-dimethyl-N-octyl amina (R^2 = **a**), N,N-dimethyl-N-benzyl amina (R^2 = **b**) or 1,4-diaza bicyclo[2.2.2]octane (DABCO, (R^2 = **c**). The cationic group content was limited to values ≤ 30 mol%, as previous studies showed that at higher DS the polymers loose the amphiphilic character (they do not self-assemble anymore).

ii. Polymers with several quaternary ammonium groups/side chain (R^2 = **d**) were obtained by using DABCO derivatives in reagents mixture. These derivatives (Scheme 2) have two quaternary ammonium groups bound by an alkylene chain and two tertiary amine groups at molecule opposite ends. Their synthesis involves the reaction of an α, ω-dichloroalkan (1, 6-

dichlorohexan or 1,10-dichlorodecan) with DABCO. Polymers obtained with these derivatives have three quaternary ammonium groups/side chain, and this significant increase of cationic charge density can lead to an enhancement of polymers antimicrobial activity.

Table 1 presents some of the polymers synthesized with the described procedures, together with information about their specific chemical structure.

Activity 2.2. Self-assembling of polysaccharide based polymers (critical micelle concentration, size)

Amphiphilic polymers can associate (self-assemble) in aqueous media due to hydrophobic interactions and form aggregates (micelles, vesicles). Aggregation extent can influence the polymer biological activity, as it can favor or hinder the interaction between the polymer aggregate and cell membrane.

Study of polymer self-assembling aimed at evaluation of aggregates properties: critical aggregation concentration (*CCA*), compactness degree, size, morphology and Zeta potential.

All polymers prepared in this stage are water soluble; consequently aggregate formation depends only on the polymer concentration.

a. *CCA* value (concentration at which the aggregation process starts) of an amphiphilic compound is related to its self-assembling capacity, i.e. a lower *CCA* indicates a higher compound ability to generate stable aggregates. *CCA* was determined by fluorescence measurements in the presence of pyrene as fluorescence probe, as the break point in the graph I_1/I_3 versus concentration (I_1 and I_3 are intensity of peak I and III, respectively, in pyrene emission spectrum) (Fig. 1a). *CCA* values (Table 1) depend on several parameters: (i) polysaccharide chain length (they decrease with decreasing of $MW_{dextran}$); (ii) hydrophobicity of R^1 (bile acid) given by the number of hydroxyl groups in bile acid molecules; consequently, *CCA* decreases in the order R^1 = CA > DCA > LCA; (iii) pendent cationic group presence and density. Attachment of cationic groups leads to the increase of polymer hydrophilicity, which is reflected in the increase of *CCA* values; (iv) cationic group (R^2) chemical structure. Based on the results obtained with polymers carrying various groups R^2, we choose those with R^2 = **a**, **b** and **c** and DS $\leq$ 25-30 mol%, which still have an appropriate hydrophilic/ lipophilic balance for application as antimicrobial agents. Experiments carried out with a different series of similar polymers showed that the presence of more hydrophilic (N,N-dimetil-N-etil amoniu), or more hydrophobic (N,N-dimetil-N-dodecil amoniu) groups vanishes the antimicrobial activity of the corresponding polymers.

Tabelul 1. Detailed chemical structure of cationic amphiphilic dextran derivatives and their aggregate characteristics.

Polymer	Chemical structure				Aggregate characteristics			
	MW dextran	R^1	R^2	moli R^2 /100 UG	*CAC*, g/dl	I_1/I_3*	D_h,* nm	Zeta* potential, mV
D1	6000	CA	-	-	0.010	0.720	85	-
D2	6000	DCA	-	-	0.037	0.689	135	-
D3	10000	DCA	-	-	0.082	0.854	75	-
D4	25000	DCA	-	-	0.170	0.971	156	-
D5	6000	LCA	-	-	0.006	0.606	200	-
D6	10000	LCA	-	-	0.010	0.770	150	-
D7	25000	LCA	-	-	0.100	0.842	110	-
D8	6000	-	a	25	0.300	1.420	450	25
D9	6000	-	b	25	0.255	1.373	320	20
D10	6000	-	c	25	0.450	1.320	400	28
D11	6000	DCA	a	25	0.110	0.953	220	23
D12	6000	DCA	b	25	0.105	1.037	230	24
D13	6000	LCA	b	20	0.085	0.885	175	22
D14	6000	-	d n = 6	23**	-	-	-	40
D15	6000	-	d n = 10	19**	-	-	-	46

* Values determined for aqueous solution with C_p = 1g/dl

** DS = moles side chain/100 GU; each side chain has three quaternary ammonium centers

b. Aggregate compactness and hydrophobicity can be evaluated by the minimum value of the ratio I_1/I_3 (called polarity parameter). These properties increase in the same order found for *CCA* decrease (Table1). The most hydrophobic aggregates are formed by the polymer with $MW_{dextran}$ = 6000 and LCA as end group, polarity parameter of which (≈ 0.60) is similar to that of cycloalkanes. Pyrene excimer formation (indicated by the occurrence, in pyrene fluorescence

emission spectrum, of a specific peak centered at about 450 nm) was observed for polymers **D5** and **D6,** suggesting that these polymers form very large and compact aggregates where several pyrene molecules can be trapped. Excimer formation (evaluated with the ratio I_E/I_1) is enhanced by concentration increase and it is more significant for **D5** (D6-LCA) (Fig. 1c). No excimer formtion was observed in case of polymers with DCA or CA end groups.

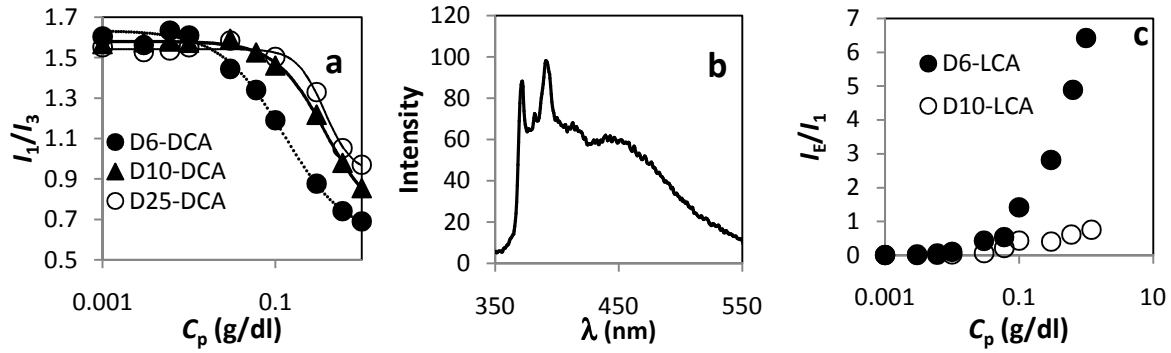


Fig.1. Fluorescence data obtained for aqueous solutions of polymers with bile acid end groups. (a) Variation of I_1/I_3 with concentration of polymers having DCA end groups; (b) Pyrene fluorescence emission spectrum in D5 (D6-LCA) solutions, at C_p = 0.06 g/dl; (c) Ratio I_E/I_1 variation with concentration of polymers carrying LCA end groups.

c. Aggregate size was determined by DLS measurements and was in the range 50-150 nm for neutral polymers (Table 1, Fig. 2) and 200-300 nm cationic polymers. Size distribution was mono-modal, except for polymers obtained from dextran with MW 6000.
d. AFM images of polymers dried on mica plates show that neutral polymers form small spherical aggregates (Fig. 3a). Cationic group attachment leads to a change in aggregate size and shape, which become much larger and ovoid (Fig. 3b).

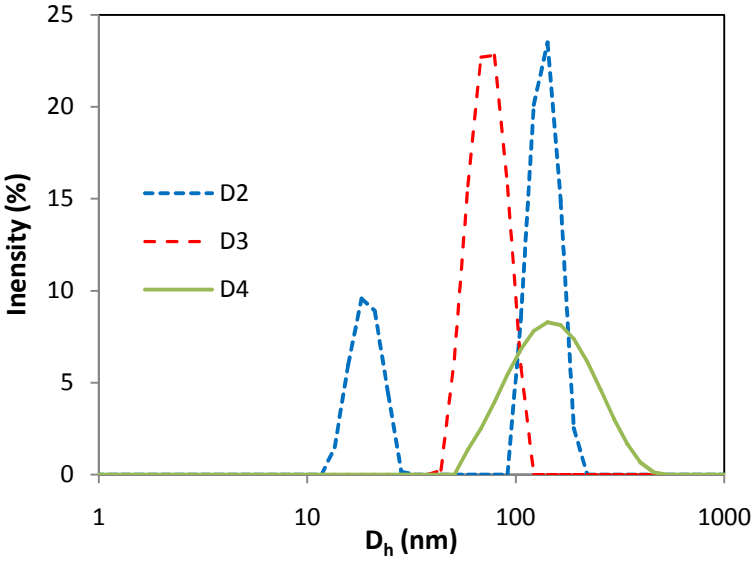


Fig.2. Size distribution of the aggregates formed by neutral polymers with DCA end groups

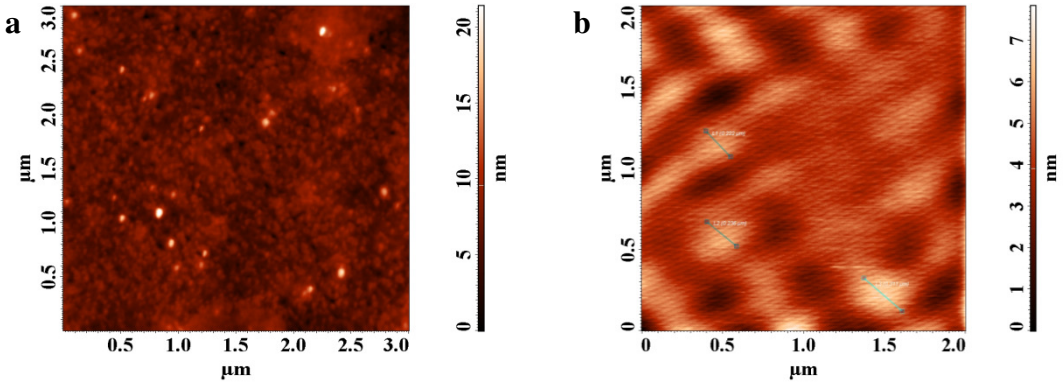


Fig. 3. AFM images taken for aggregates formed by polymers **D2** (a) and **D11**(b).

Activity 2.3. Biological evaluation of synthesized polymers (antimicrobial and hemolytic activity)

Antimicrobial activity was tested on many cell strains, and hemolytic activity was evaluated on red blood cells (RBC). These tests can provide information on the polymers' selectivity for microbial cells (high antimicrobial activity, low hemolytic effect) and allow a selection of the most appropriate chemical structures for use as antibiotic replacements or external biocides.

2.3.1. Antimicrobial activity

Studies were performed using different cells: Gram positive bacteria (*Staphylococcus aureus* ATCC 25913 and *ATCC* 25923), Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and pathogen fungi (*Candida albicans* ATCC 90028 and ATCC 90028, *Candida glabrata* ATCC MYA 2950). Antimicrobial activity was evaluated by agar disk diffusion method, which consists in the measurements of the cell growth inhibition zone diameter. Commercially available disks containing Ciprofloxacin (5 µg/disc), Voriconazole (1 µg/disc) or

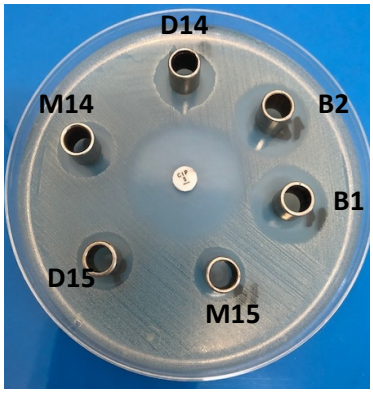
Table 2. Antibacterial and antifungal activity of tested polymers

Sample	Inhibition zone diameter (mm)						
	S. aureus ATCC 29213	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*C. albicans* ATCC 90029	*C. albicans* ATCC 90028	*Candida glabrata* ATCC MYA 2950
D2	18.1±0.05	13.3±0.57	0	0	15.3±0.57	13.1±0.05	9.1±0.05
D5	12.0±0.00	13.1±0.17	0	0	13.3±0.57	9.3±0.57	0
D8	NT	13.6±0.57	11.0±0.00	NT	NT	12.0±0.00	13.0±0.00
D9	12.3±0.57	14.3±0.57	0	0	10.1±0.17	10.3±0.57	0
D10	10.5±0.50	11.5±0.50	0	0	20.1±0.17	18.3±0.57	18.3±0.57
D12	10.1±0.17	11.5±0.50	0	0	15.0±0.00	12.3±0.57	9.1±0.05
D13	10.1±0.17	9.1±0.05	0	0	17.1±0.05	10.3±0.57	0
D14	NT	15.0±0.00	11.1±0.17	12.1±0.17	NT	19.0±0.00	NT
M14	NT	13.1±0.17	0	0	NT	0	NT
D15	NT	13.5±0.50	10.1±0.17	11.1±0.17	NT	11.3±0.57	NT
M15	NT	11.1±0.17	0	0	NT	0	NT
B1	NT	17.5±0.50	13.5±0.50	14.0±0.00	NT	14.0±0.00	NT
B2	NT	19.1±0.17	20.1±0.17	19.1±0.17	NT	20.1±0.05	NT
Ciprofloxacin (5 µg/disc)	26.0±0.00	25.0±0.00	30.5±0.50	30.5±0.50	*NT	*NT	*NT
Nystatin (100 µg/disc)	NT*	NT*	NT*	NT*	22.0±0.07	22.1±0.05	13.1±0.17
Voriconazole (1 µg/disc)	NT*	NT*	NT*	NT*	36.5±0.50	35.5±0.50	31.1±0.17

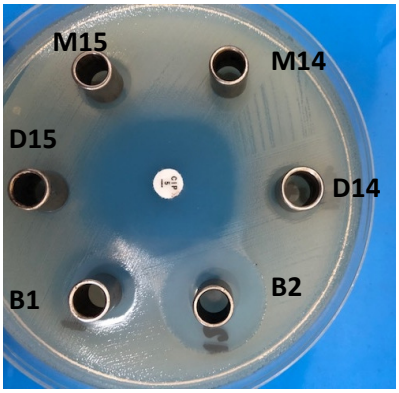
*NT-not tested

Nystatin (100 µg/disc) were used as antimicrobial agents specific to each cell strain. The cells were seeded on Petri dishes, and stainless steel cylinders (5 mm internal diameter; 10 mm height) were applied on the agar medium surface. Samples (0.1 ml aqueous polymer solutions having 1 g /dl

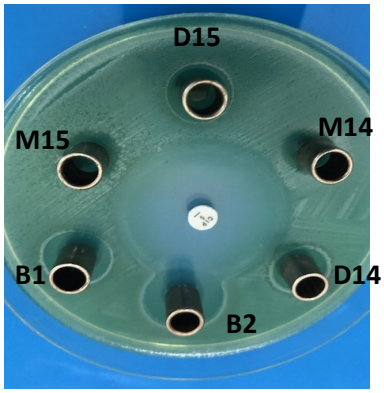
concentration) were added in cylinders and the dishes were incubated at 37°C for 24 h (bacteria) or at 24°C for 48 h (fungi). After incubation, the diameters of inhibition zones around the stainless steel cylinders were measured (Fig. 4, Table 2).



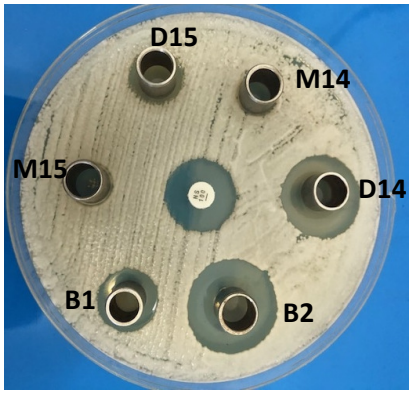
S. aureus ATCC 25923



E. coli ATCC 25922



P. aeruginosa ATCC 27853

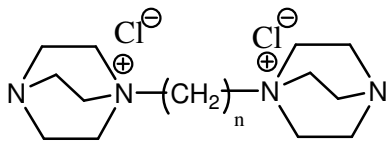


C. albicans ATCC 90028

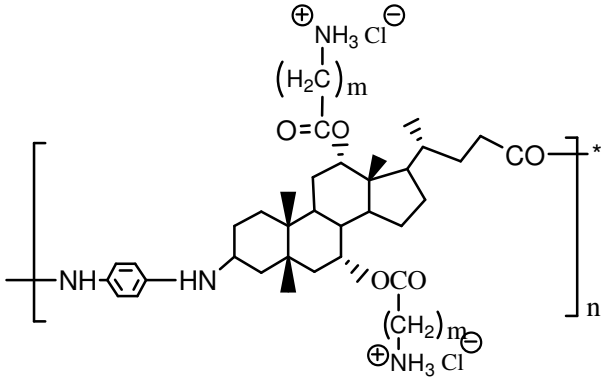
Fig. 4. Images of the Petri dishes with cells incubated in the presence of various polymers.

Besides polymers **D** listed in Table 1, several other samples were tested: **M14** and **M15** (Scheme 2), which are the amine derivatives used for polymers **D14** and **D15** synthesis, as well as oligomers containing bile acids in backbone (**B1** si **B2**, Scheme 3) synthesized in the previous stage (Stage 2017).

According to the results presented in Table 2, all tested samples have a significant antimicrobial activity, but the activity efficacy and spectrum depend on samples' chemical structure, as follows:



Scheme 2. Chemical structure of compounds **M14** (n = 6) si **M15** (n = 10).



Scheme 3. Chemical structure of compounds **B1** (m = 5) si **B2** (m = 2). n = 3

- Al tested polymers have a good antistaphilococic activity and a very good antifungal activity. The best results were obtained with polymers **B2**, **B1** and **D14**. Polymers **D14** and **D15** are more efficient then their precursors **M14** and **M15**, suggesting that attachment of these derivatives as pendant groups along dextran backbone enhances antimicrobial activity. Among the tested samples, only derivatives **M14** and **M15** have no antifungal activity.
- Most of the tested polymers have no activity against Gram negative bacteria (*E.coli* si *P. aeruginosa*), except for polymers **B1**, **B2**, **D14** and **D15**. These polymers activity against *E. coli* increases in the order **D14** ≈ **D15 < B1 << B2.**
- Comparison of the results with polymers with similar structure highlights several aspects:
  - (i) Polymers with DCA end groups seem more efficient than those with LCA end groups. Attachment of cationic pendent groups to polymers with BA end groups does not result in a significant improvement of antibacterial activity, but increases antifungal activity.
  - (ii) The structure of amine group R^2 influences the polymers **D2-D13** selectivity against certain cell strains. Only polymers containing R^2 = **a** have a measurable activity against *E. coli*, and polymers with R^2 = **c** have the most efficient antifungal activity.
  - (iii) Activity of polymers **D14** and **D15** decreases with increasing alkyl chain length between positively charged centers, perhaps due to the lower charge along the side chains.
  - (iv) Polymer **B2** cu with β-alanine side chains is more efficient than polymer **B1** with aminocaproic acid side chains.

2.3.2. Hemolytic activity was studied using cells separated by centrifugation from rabbit blood. RBC suspension was incubated with sample aqueous solution (10 mg/ml) for 24 h la 37 ^{o}C, then centrifuged. The amount of hemoglobin released in supernatant was measure by UV-vis at 540 nm and compared with that found in a positive test (distilled water, zero hemolysis) and in a negative test (0.2 % Triton X-100, 100% hemolysis). The results showed that the polymer **B2** is hemocompatible, with a hemolysis effect lower than 1% after 24 h. Hemocompatibility is lower for polymers **B1** (20% hemolysis) and **D14** (40% hemolysis). These results recommend polymer **B2** as antibiotic replacement, and polymer **D14** as external biocid.

Conclusion

Cationic amphiphilic polymers with bile acid end groups and/or cationic pendent groups were synthesized by dextran chemical modification. Results of the studies concerning the self-assembling properties of these newly synthesized polymers and biological activity of all polymers synthesized in the project framework are summarized in the following.

- Dextran based polymers can associate in aqueous media with formation of nanosized spherical aggregates (micelles), the compactness of which depends on dextran molecular weight, hydrophobicity of the end bile acid, structure and content of pendent amino groups.
- All tested polymers display significant antimicrobial activity, but selectivity against different cell strains depends on polymer chemical structure.
- Hemolytic activity also depends on polymer chemical structure and hemolysis degree varies between 1 and 40%.
- Polymer **B2** (an oligomer based on cholic acid, with pendent β-alanine moieties) with the most efficient antimicrobial activity, the widest activity spectrum and the best hemocompatibility, has encouraging potential for application as antibiotic replacement.
- Polymer **D14** (dextran having pendent groups with several cationic centers separated by a C6 alkyl chain), which showed a good antifungal activity and a moderate antibacterial activity, can be recommended only as external biocide due to its lower hemocompatibility.
- The studies to be performed in the next stage (2019) will be focused on the improvement of polymers **B1** and **D14** performances.