Scientific Report

Regarding the implementation of the project

New cationic amphiphilic oligomers as synthetic alternatives for antimicrobial peptides and/or external biocides

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

July 2017 – December 2019

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.
- Assessment of antinacterial activity mechanism by the study of the interaction between the synthesized polymers and model membranes (liposomes).

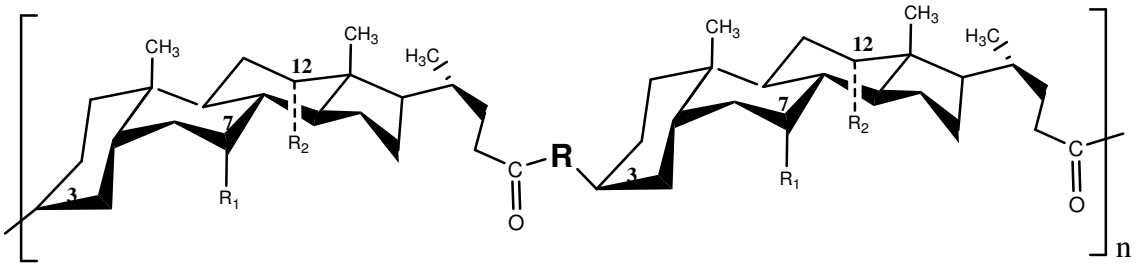
Besides this main objective, the synthesized polymers were tested for their applicability in other domains, especially for purification of industrial waste waters by removal of pollutants (pesticides, clays, dyes) using techniques such as flocculation or adsorption.

Stage 1 (July 2017 – December 2017)

Objective 2017: Synthesis and characterization of bile acid based polymers

Activity 1.1. Synthesis and physico-chemical characterization of polymers

Synthesis of new cationic amphiphilic polymers, which could mimic antimicrobial peptide properties, was based on bile acid intrinsic properties and selective reactivity of functional groups (OH, COOH) bound to steroid nucleus. Bile acids are natural compounds with a rigid structure and facial amphiphilicity due to two faces of the cavity formed by steroid nucleus: one hydrophilic (α) face formed by 2-3 OH groups directed convergentely to the concavity and COOH group at carbon 24; a hydrophobic (β) face where three CH_3 groups are present. Chemically modified bile acid derivatives preserve the facial amphiphilicity, therefore we can expect the same behavior in case of bile acid oligomers and polymers containing bile acid moieties in the main backbone. Antimicrobial activity of bile acid polymers will depend on backbone rigidity and cationic group density. This project takes into account the synthesis of polymers with the general structure depicted in Scheme 1, by binding bile acid molecules at positions 24 and 3 of the steroid nucleus, after the appropriate functionalization of COOH and OH located at these positions. The synthesis of polymers with the structure given in Scheme 1 required two stages: (1) synthesis of bile acid oligomers, and (2) attachment of pendent chains (R_1, R_2 in Scheme 1) with free primary amine groups.



Scheme1.

1.1.1. Synthesis of bile acid oligomers

The building of the oligomer main chain was performed taking into account several key factors for the properties of the final product: **R** group connecting 2 bile acid molecules, which can influence the backbone rigidity; bile acid chemical structure (cholic acid, with $R_1 = R_2 = OH$, or deoxycholic acid, with $R_1 = H$, $R_2 = OH$), which is important for the density of amine groups to be attached to oligomer backbone; oligomer molecular weight, which has to be maintained at a low values ($n \leq 10$). In this stage, two synthetic pathways were experimented for the synthesis of oligomers based

on cholic acid (this bile acid can ensure the highest density of amine groups), which differ by the structure of the group connecting the bile acid molecules in the backbone, namely **R** = triazol amide or phenilen amide.

(a) Polymeric backbone connected *via* triazolic groups (Scheme 2, product **B**) was obtained by 1,3 dipolar cycloaddition of cholic acid modified with propargyl (at position 24) and azide (at position 3) groups (Scheme 2, product **A**)

Scheme 2. Chemical structure of cholic acid with propargyl and azide groups (**A**) and that of the oligomer obtained by Click reaction (**B**). X = NH.

Synthesis of the oligomer **B** required several reaction steps:

i. Propargyl group introduction was carried out be reaction between cholic acid and propargyl amine, in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride as coupling agent and N-hydroxybenzotriazol as catalyst. Reaction progress was confirmed by NMR and FTIR analyses.

ii. Tosylation of OH group in position 3 of propargyl derivative with tosyl chloride. NMR analysis on dried product highlighted the presence of signals specific to the new product, as well the selectivity of tosylation reaction which took place only at OH (3).

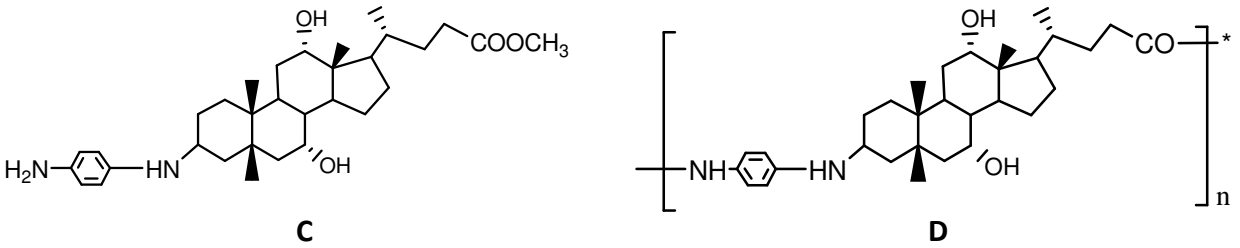
iii. Monomer **A** was prepared by reaction of cholic acid derivative obtained in step (ii) with NaN_3 After purification and drying, FTIR and NMR analyses confirmed the chemical structure of the product.

iv. Oligomer **B** was prepared by Click reaction, and its structure was confirmed by NMR and FTIR. Due to low molecular weight, the signal of monomer functional groups are still present in oligomer NMR spectrum, facilitating the evaluation of number average molecular weight from the ratio between the integral of the signal occurring at 3.06 ppm (end propargyl group) and that at 8.16 ppm corresponding to the triazol cycle in the backbone. The value was $M_n \approx 5000$ g/mol ($n \approx 5$).

(b) Connection of oligomeric backbone by phenylamide bridges (Scheme 3, polymer **D**) was obtained by polycondensation of monomer **C**. Reactions required for preparation of products **C**

and **D** are presented in the following:

i. Synthesis of the cholic acid methyl ester was performed in anhydrous methanol. The success of the reaction was supported by NMR (signal for $COOC\underline{H}_3$ at 3.3 ppm) and FTIR (signal for ester group carbonyl at la 1720 $cm^{-}1$).
ii. Tosylation of OH group in position 3 of the cholic acid methyl ester was performed in a similar way as described for monomer **A**.
iii. Monomer **C** was obtained by reaction of tosylated derivative with 1,4-phenilenediamine and chemical structure of the product was confirmed by NMR (signals of aromatic ring present at 8.1-8.2 ppm).
iv. Monomer **C** polycondensation provided polymer D. FTIR highlighted the occurrence of the signal specific to amide group carbonyl at 1640 cm^{-1}.



Scheme 3. Chemical structure of the cholic acid with methyl ester and phenylenediamine groups (**C**) and of polymer **D** obtained by **C** polycondensation.

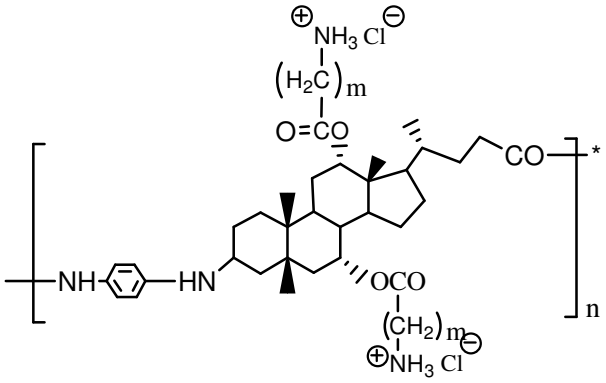
Molecular weight of the oligomer **D** was evaluated with the help of NMR analysis, from the ratio between the integrals of methyl ester end group (3.3 ppm) and aromatic cycle protons, and was $M_n \approx 4200$ ($n \approx 4$).

Polymers **B** and **D** were soluble in DMSO, DMF, THF and insoluble in water, methanol and ethyl acetate.

1.1.2. Attachment of amine groups to bile acid oligomers

In order to obtain polymers with chemical structure **E** (Scheme 4, exemplified for oligomer **D** backbone), OH groups in position 7 and 12 of cholic acid were reacted with carboxylic groups of several amino acids (glycine, β-alanine, aminocaproic acid). For this goal, a sequence of reactions was necessary, including protection and deprotection of amine groups. After final purification, the obtained polymers E were water soluble due to the presence of amine groups as hydrochloride. The

content in amine groups was determined by potentiometric titration (with NaOH 0.1N), and correspond to 70% of OH groups modification.



Scheme 4. Chemical structure of cationic amphiphilic oligomers with cholic acid as part of the backbone and amino acid side chains (**E**). m = 1, 2, 5.

Activity 1.2. Study of bile acid based polymer self-assembling

Amphiphilic polymers can associate (self-assemble) in aqueous medium forming ordered aggregates (micelles, vesicles) as a result of hydrophobic interactions. Bile acids and their derivatives aggregate by interactions between hydrophobic segments located on molecule (β) faces, and usually the formed aggregates contain a few bile acid molecules. Aggregation degree can influence the biological activity of a polymer, as the conformation and compactness of the polymer chains can favor or impair their interaction with cell membranes.

Study of polymers **E** self-assembling was focused on the choice of the most appropriate procedure for aggregate preparation, as well as on the determination of aggregate properties such critical aggregation concentration (*CCA*), size, shape and compactness.

(a) Procedure of aggregate preparation in aqueous medium can be decisive for aggregate properties. 2 methods were used: (i) direct dissolution in water and (ii) solvent exchange method, according to which the polymer was first dissolved in DMSO, then water was added drop-wise (1/1 v/v water/DMSO), the obtained mixture was stirred for 1 h, and finally dialyzed against water for 2-3 days. The final polymer concentration in the resulted colloidal solutions was determined by evaporation to dryness of a known solution volume.

(b) Polymer critical aggregation concentration was determined by fluorescence measurements in the presence of pyrene as fluorophore, as the break-point in the plot I_1/I_3 versus concentration (I_1 and I_3 are the intensities of the peaks I and III, respectively, in the pyrene emission spectrum).

CCA values were not influenced by the polymer backbone (corresponding to **B** or **D** structure), but depended on the amino acid alkyl chain length and were: 0.05 mg/ml (glycine), 0.03 mg/ml (β-alanine) si 0.005 mg/ml (aminocaproic acid), showing a decrease of *CCA* with increasing side chain hydrophobicity. Aggregate compactness and hydrophobicity (evaluated with the minimum value of the ratio I_1/I_3, also called polarity parameter) increase in the same order found for the decrease of *CCA*s.

(c) Aggregate size was measured by DLS and was in the range 50-150 nm, and was higher and with a wider distribution in case of the aggregates prepared by direct dissolution in water. No significant influence of the amino acid alkyl chain length was observed, nevertheless a small decrease of size was found for the polymers carrying aminocaproic acid side chains.

(d) Aggregate shape observed in TEM images taken on sample solutions dried on carbon grids was predominantly spherical, with few rods. Samples prepared by direct dissolution gave aggregates with more irregular and disperse size and shape.

Conclusion

Oligomers having bile acids in the backbone and primary amine groups on side chains were prepared in this stage. Oligomer backbone was obtained by connecting bile acid molecules at their C3 and C24 positions, through triazol cycles or phenilenamide bridges. The synthesized polymers can associate in aqueous medium with formation of spherical aggregates (micelles) of nanometric size, more or less compact as a function of the side chain length. Antimicrobial activity as a function of polymer chemical structure will be evaluated during the next stages of the project.

References

Small, D. M.; The physical chemistry of cholanic acids. In *The Bile Acids. Chemistry, Physiology and Metabolism*, *Vol. 1*, Nair, P. P and Kritchevsky, D., Eds.; Plenum Press: New York, 1971, pp. 249-354.

X. X. Zhu, M. Nichifor. *Acc. Chem. Res*. 2003, 35, 539-546

A. Som, S. Vemparala, I. Ivanov, G.N. Tew. *Biopolymers* 2008, 90,83-93

A. Kumar, R. K. Chhatra, P. S. Pandey, *Org. Lett*. 2012, 12, 24-27

Z. Zhong, Y. Zhao. *J. Org. Chem.* 2008, 73, 5498–5505

W. Li, T. Tian, W. Zhu, J. Cui, Y. Ju, G. Li. *Polym. Chem*. 2013, 4, 3057-3068

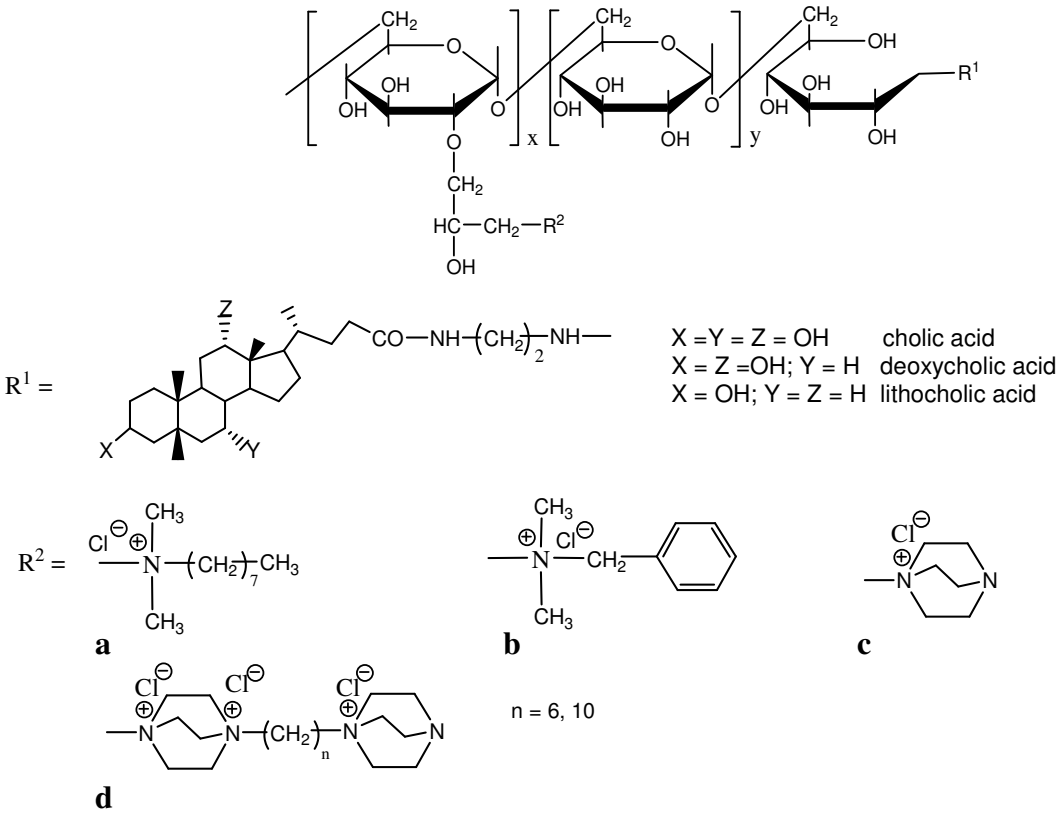
J. Sun, W. Li, L. Xiao, G. Yub, J. Shi. *RSC Adv*. 2016, 6, 62200–62207

Stage 2 (December 2017 – December 2018)

Objective 2018): 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:

v. 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$\underline{H}_3$ at 3.3 ppm) and FTIR (occurrence of ester band at 1720 cm^{-1} and disappearance of carboxyl band at 1700 cm^{-1}).
vi. 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 (–$\underline{CH}_2$-NH_2) and 3.2 ppm (–C$\underline{H}_2$-NH-CO-)) and FTIR (presence of the band assigned to amide carbonyl at 1680 cm^{-1} and disappearance of the carboxyl band).
vii. 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 reagent 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

Table1. 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

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 ≤ 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.

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.

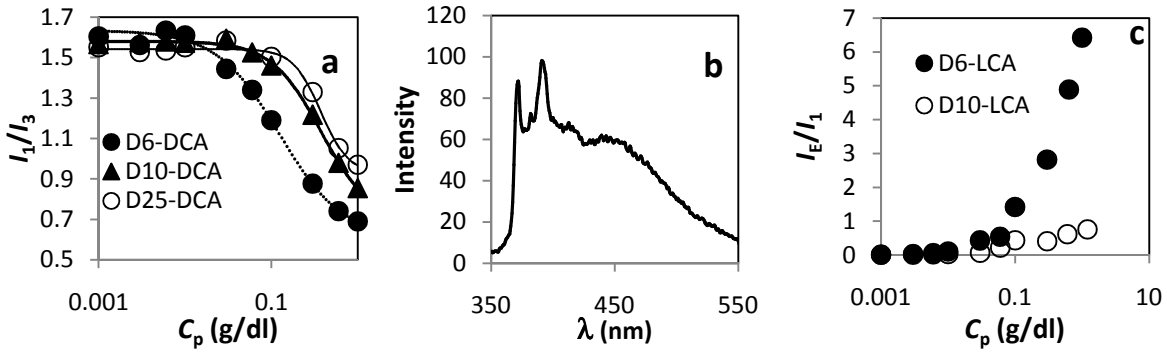


Fig.1. Fluorescence data obtained for aqueous solutions of polymers with bile acid end groups. (a) Variation of I_1/I_3 with concentration of polymers having DCA end groups; (b) Pyrene fluorescence emission spectrum in D5 (D6-LCA) solutions, at C_p = 0.06 g/dl; (c) Ratio I_E/I_1 variation with concentration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

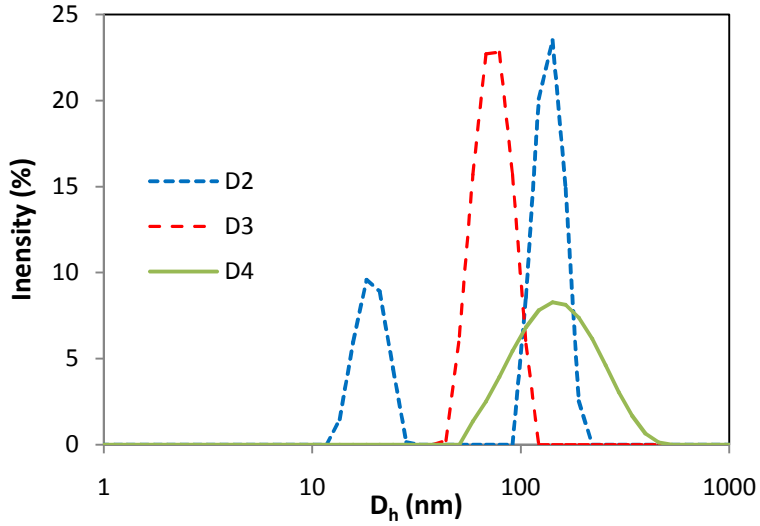


Fig.2. Size distribution of the aggregates formed by neutral polymers with DCA end groups

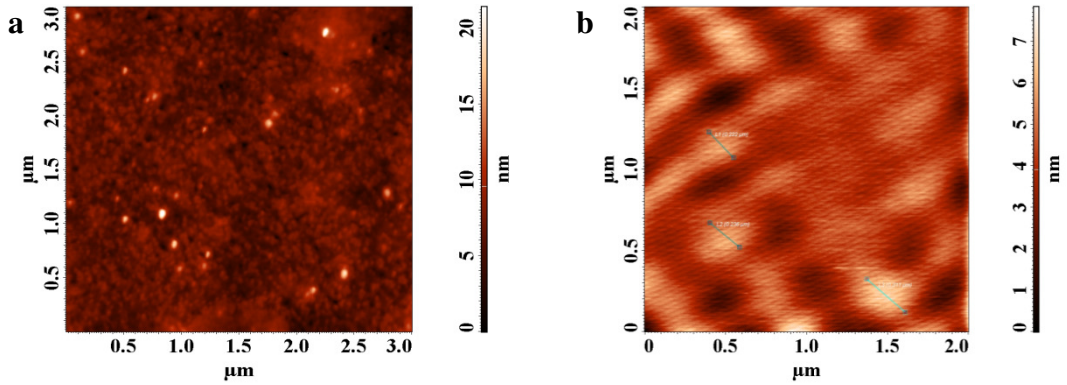


Fig. 3. AFM images taken for aggregates formed by polymers **D2** (a) and **D11**(b).

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

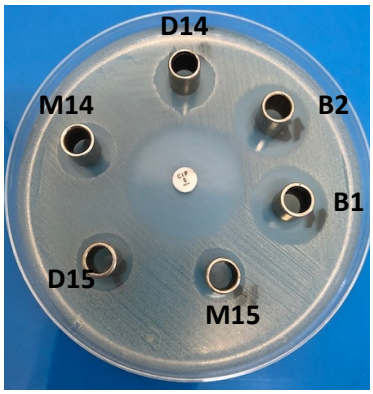
method, which consists in the measurements of the cell growth inhibition zone diameter. Commercially available disks containing Ciprofloxacin (5 µg/disc), Voriconazole (1 µg/disc) or

Table 2. Antibacterial and antifungal activity of tested polymers

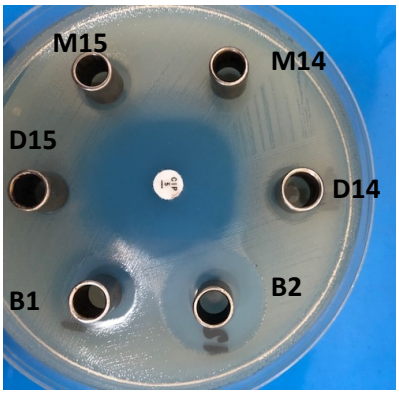
Sample	Inhibition zone diameter (mm)						
	S. aureus ATCC 29213	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*C. albicans* ATCC 90029	*C. albicans* ATCC 90028	*Candida glabrata* ATCC MYA 2950
D2	18.1±0.05	13.3±0.57	0	0	15.3±0.57	13.1±0.05	9.1±0.05
D5	12.0±0.00	13.1±0.17	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

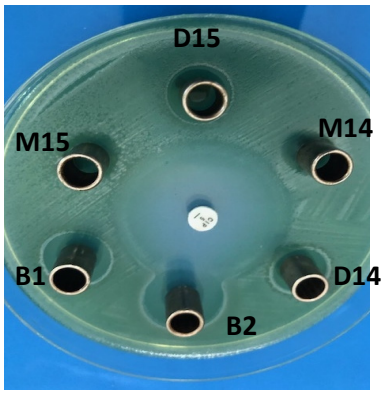
Nystatin (100 µg/disc) were used as antimicrobial agents specific to each cell strain. The cells were seeded on Petri dishes, and stainless steel cylinders (5 mm internal diameter; 10 mm height) were applied on the agar medium surface. Samples (0.1 ml aqueous polymer solutions having 1 g /dl concentration)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



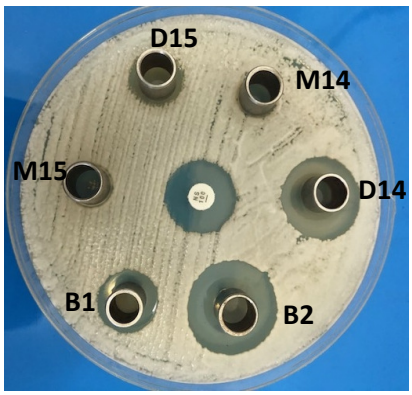
S. aureus ATCC 25923



E. coli ATCC 25922



P. aeruginosa ATCC 27853

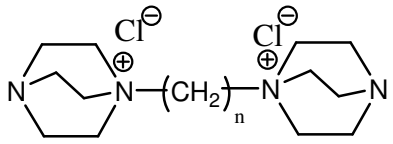


C. albicans ATCC 90028

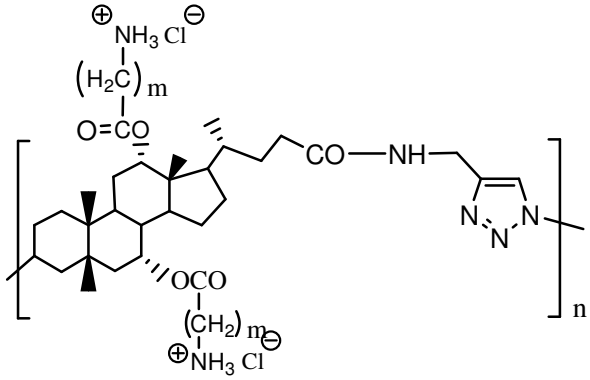
Fig. 4. Images of the Petri dishes with cells incubated in the presence of various polymers.

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** and **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

- All tested polymers have a good anti-staphilococcal 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 biocide.

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.

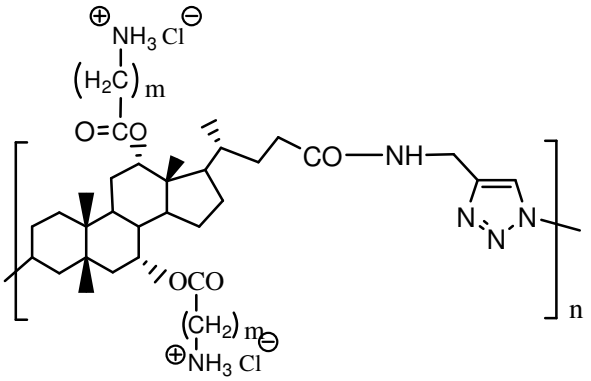
- Dextran based polymers can associate in aqueous media with formation of nanosized spherical aggregates (micelles), the compactness of which depends on dextran molecular weight, hydrophobicity of the end bile acid, structure and content of pendent amino groups.
- All tested polymers display significant 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.

Stage 3 (December 2018 – December 2019)

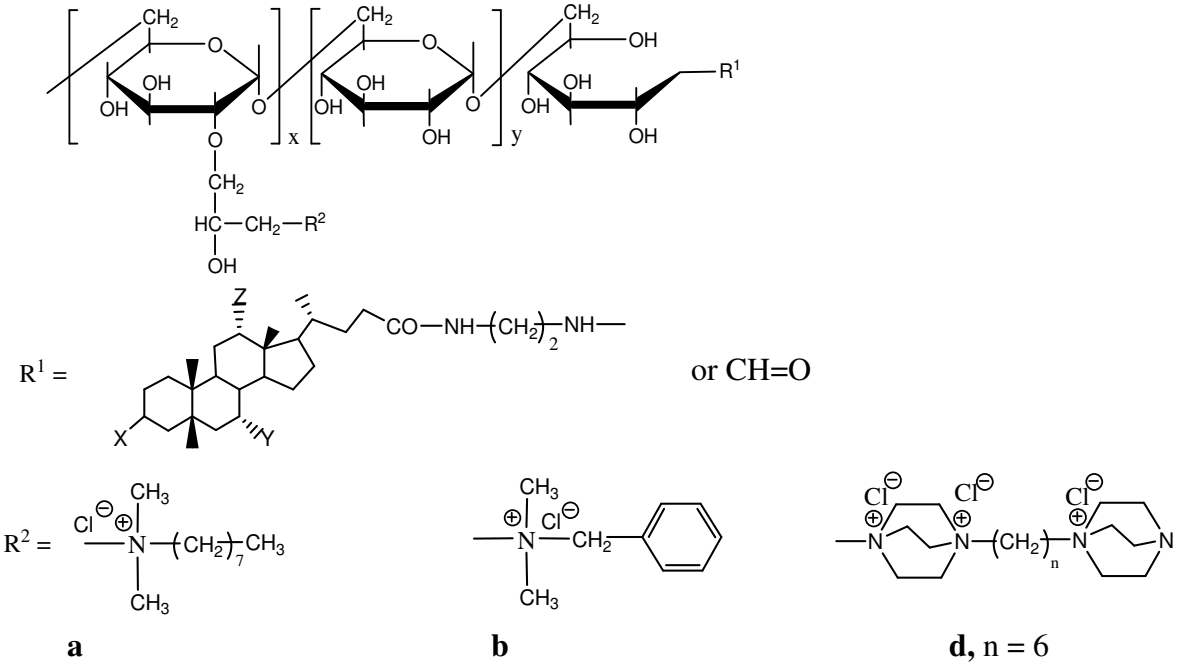
<u>Objective 2019</u>: Selection of polymers with optimal properties and final biological evaluation

Activity 3.1 – ***Experiments for polymer properties improvement***

Antimicrobial tests performed during Stage 2 (2018) showed that polymers with the structure **B** (Scheme 1) and **D** (Scheme 2) have the best antimicrobial activity and a wide activity spectrum against both gram positive or gram negative bacteria, as well as against pathogenic fungi. Therefore, we studied the possibility to improve the activity by variation of these polymers chemical composition and physico-chemical properties. The synthetic method was the same as those described in the reports for Stages 1 and 2.



Scheme 1. Polymers with cholic acid molecules in the backbone linked through triazolic cycles (structure **B**). m = 2; n = 3-7



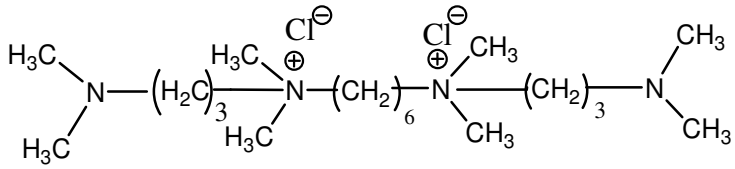
Scheme 2. Chemical structure of polymers **D** prepared by attachment of quaternary ammonium groups to dextran with or without bile acid unities at main chain end.

3.1.1. *Polymers with structure **B***

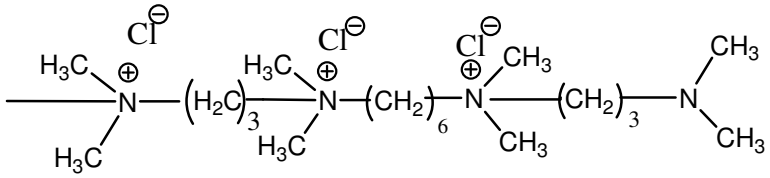
These polymers were synthesized by 1,3 dipolar cycloadition of a cholic acid derivative with propargyl group at position 24 and azide group at position 3. (Stage 1, 2017). In order to study the influence of polymerization degree n on antimicrobial activity, polymers with different degree of polymerization were prepared: n = 3 (**B2**), n = 5 (**B3**) and n = 7 (**B4**). Variation of polymerization degree was achieved by using different amount of catalyst (CuI) and ligand (N,N,N',N'',N''- pentamethyldiethylenetriamine) during cycloaddition reaction. The resulting polymers have low water solubility, therefore, polymer solution in DMSO were used for biological tests.

3.1.2. *Polymers with structure **D***

Biological tests performed in 2018 showed that the polymers with bile acid end groups have a moderate antimicrobial activity, which is not significantly improved by attachment of quaternary ammonium groups to dextran backbone. However, the presence of cationic groups enhances antifungal activity. Polymers with each pendent chain containing several cationic groups (structure **D**, where R^2 = **d**) had better performances, therefore, the influence of sume parameters such as dextram molecular mass and degree of substitution with pendent chain was studied. Moreover, derivative **e** was prepared by reaction between N,N,N',N'-propane diamine with dichlorhexane. Reaction of dextran with an equimolar mixture epichlorhidrin/derivat **e** afforded a polymer with the strucure **D**, where the substituent R^2 = **f** (Scheme 3).



Derivative **e**



Pendent group **f**

Schema 3. Chemical structure of the derivative **e** and pendent group resulted from the reaction of dextran with the mixture **e**/ECH.

Polymers with the structure **D** synthesized in the present stage and used for antimicrobial tests are included in Table 1. These polymers are water soluble and their water solutions were used for biological evaluations.

Table 1. Chemical composition of polymers **D**

Cod polimer	M_r* dextran, Da	R^1	R^{2**}	DS, molar %***
D5	6000	Lithocholic acid	-	-
D14	6000	-	**d**	20
D16	6000	Lithocholic acid	**a**	25
D17	6000	-	**d**	36
D18	25000	-	**d**	20
D19	40000	-	**d**	20
D20	6000	-	**f**	20

* Molecular mass from supplier
** According to Scheme 2 and 3
*** Degree of substitution, expressed as moles pendent group/100 glucopyranosidic unities

Activity 3.2. ***Final biological evaluation (antimicrobial and hemolytic activity). Selection of polymers with optimal properties***

3.2.1. *Antimicrobial activity*

These studies were performed on cells of Gram positive (*Staphylococcus aureus ATCC* 25923) and Gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) bacteria, as well as on pathogenic fungi (*Candida albicans* ATCC 90028 and ATCC 14053). Antimicrobial activity was evaluated qualitatively by diffusion in agar medium method and quantitatively by broth microdilution method.

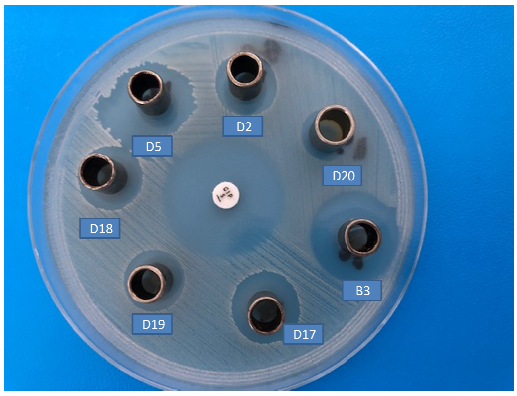
Diffusion method is based on the measurements of the diameter of the microbial growth inhibition zone. Cells were seeded on Petri plates, then, stainless steel cylinders (5 mm inner diameter, 10 mm height) were applied on the medium surface. Polymer samples (0.1 ml solution, 1 g /dl) were added in each cylinder. Plates were kept for 10 min at room temperature to allow a uniform diffusion in the medium, then, they were incubated at 37°C for 24 h (bacteria) and at 24°C for 48 h (fungi). After incubation the diameter of inhibition zone was measured (Fig. 1). Commercially available disks containing Ciprofloxacin (5 µg/disk), Voriconazole (1 µg/disk) and Nystatin (100 µg/disc) were used as antimicrobial agents specific to each cell strain. According to the results are presented in Table2, all polymers have antistaphylococcal activity, most of them have antifungal activity too, but Gram negative bacteria (*E. coli* and *P. aeruginosa*) are sensitive only to polymers with the structure **B.** It is worth mentioning the good activity of polymers **B** against *P. aeruginosa*, one of the most resistant Gram negative bacteria species. Antimicrobial activity of the polymers with the

structure **D** is not significantly influenced by the increase of substitution degree from 20 to 36% (samples **D14** and **D17**) or by the chemical structure of the pendent groups (samples **D14** and **D20**). Dextran molecular mass increase leads to a slight decrease of antistaphylococcal and antifungal activity. Regarding polymers with the structure **B**, a maximum of activity was found for the polymer with an intermediate polymerization degree (n = 5), and this is more evident for *E. coli*, a bacteria very resistant to many polymer types.

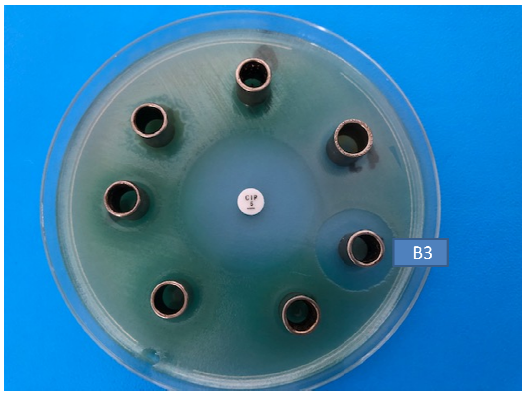
Table 2. Results obtained by diffusion method

Sample	Inhibition zone diameter (mm)				
	S. aureus ATCC 25923	*E. coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. albicans* ATCC 14053
D5	19.0±0.00	0	0	0	0
D16	16.0±0.00	0	0	0	NT*
D14	15.0±0.00	11.1±0.17	12.1±0.17	19.0±0.00	NT*
D17	15.1±0.05	0	0	17.3±0.57	17.0±0.00
D18	14.0±0.00	0	0	15.3±0.57	14.1±0.05
D19	13.1±0.05	0	0	16.0±0.00	15.0±0.00
D20	14.1±0.05	0	0	14.0±0.00	NT*
B2	19.1±0.05	18.1±0.05	19.1±0.05	18.3±0.57	NT*
B3	21.0±0.00	21.1±0.05	18.1±0.05	23.1±0.05	NT*
B4	20.3±0.57	18.1±0.05	18.3±0.57	24.1±0.05	25.1±0.05
Ciprofloxacin (5 µg/disc)	26.7±0.06	30.5±0.50	29.0±0.00	NT*	NT*
Fluconazol (25 µg/disc)	NT*	NT*	NT*	30.0±0.00	28.0±0.00
Voriconazol (1 µg/disc)	NT*	NT*	NT*	31.5±0.50	32.5±0.50

*NT not tested



S. aureus ATCC 25923



P. aeruginosa ATCC 27853

Fig.1. Images of the plates treated with polymers

Broth microdilution method allows the determination of minimum concentrations for growth inhibition (*MIC*), for bactericide (*MBC*) or fungicide (*MFC*) activity. Solutions obtained by successive dilution of polymer stock solution were inoculated with equal volume of microbial cell suspension (10^6 CFU/mL). *MIC* is the lowest concentration for which a complete inhibition of cell growth was observed after an incubation for 24h at 37°C (bacteria bacterii) or at 24°C (fungi). Values of *MBC* / *MFC* were determined by transferring 0.1 mL samples, showing no visible growth of microorganism, on the surface of an agar plates. Subcuktyres were incubated for 24h, and *MBC* / *MFC* were taken as the lowest concentrations required for killing more than 99.9% of tested microorganisms. These tests have been performed for polymer samples with the best activity observed at diffusion tests. The results presented in Table 3 allow the following conclusions:

- Polymer **B3** has an excellent antimicrobial activity for all microorganism strains tested. The lowest *MIC* values (about 10 µg/ml) were obtained for *S. aureus, E.coli,* and *C. albicans*.
- Polymer **B4** has the same wide spectrum of activity as **B3**, against both gram positive and gram negative bacteria, as well as against fungi, but its activity is moderate as the values of minimal concentrations are about 2 orders of magnitude higher than those obtained for **B3**. This test highlights once more the importance of the degree of polymerization for antimicrobial activity of this polymer class.
- The other tested polymers display only a modest antistaphylococcal activity. Among them, the sample **D16** has a good antistaphylococcal activity, the values of MIC being in its case between that of **B3** and those of the other polymers.

- The ratio between *CMI* and *CM*B or *CMF* indicates if the polymer is a bactericidal (the microorganism is completely destroyed) or a bacteriostatic agent (it stops the cells growth). An agent is bactericidal when $MBC/MIC \leq 4$ and bacteriostatic if this ratio is higher than 4. Most of the samples listed in Table 3 can be regarded as bactericidal because $MBC(MFC) \approx 2MIC$. The only bacteriostatic activity was observed for polymer **B3** against *C. albicans.* In this case $CMF \approx 7CMI$.

Tabelul 3. *MIC* and *MBC*/*MFC* for selected polymers

Polymer	*S. aureus* ATCC 25923		*E. coli* ATCC 25922		*P. aeruginosa* ATCC 27853		*C. albicans* ATCC 90028	
	MIC (mg/mL)	*MBC* (mg/mL)	*MIC* (mg/mL)	*MBC* (mg/mL)	*MIC* (mg/mL)	*MBC* (mg/mL)	*MIC* (mg/mL)	*MFC* (mg/mL)
D5	2.5	>5	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
B3	0.01	0.03	0.01	0.03	0.07	0.15	0.01	0.07
D16	0.15	0.31	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
D20	1.25	2.5	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	1.25	5
B4	1.25	2.5	1.25	5	1.25	5	1.25	5

[a] not determined

3.2.2. *Hemolytic activity*

Many antimicrobial agents, especially those with cationic groups, are not selective for microorganisms, i.e. they can have destructive activity against human cells. Evaluation of an antimicrobial agent selectivity is usually made by determination of its hemolytic activity against blood red cells (BRC). This test is based on quantification of the hemoglobin released by RBC in the presence of the agent. Preliminary tests performed in Stage (2018) were made by measuring hemolysis degree after 24 h of contact between RBC and a solution containing 10 mg polymer/mL. In the present stage we performed experiments for determination of HC_{50} (polymer concentration corresponding to 50% hemolysis). The ratio HC_{50}/MIC is a generally accepted indication for the selectivity of an antimicrobial agent for a certain microorganism.. A higher ratio is characteristic for a more selective agent, which might be recommended for systemic administration. An agent with a high antimicrobial activity but a low selectivity can be used only as an external biocide.

Hemolysis experiments were carried out with BRC from human blood (obtained from a volunteer). The fresh blood sample was washed with PBS solution, centrifuged for 3 min at 3000 rpm, followed by supernatant removal. This procedure was repeated 3 times, then the cells were re-suspended in PBS to obtain a stock suspension containing 1% v/v erythrocytes. 0.75 ml of this suspension were mixed in an Ependorf tube with 0.75 ml polymer solution (in PBS or DMSO). The final polymer concentration in the mixture was in the range 0.1-10 mg/ml. The resulting suspension was incubated for 30 min at 37^{o}C, then, the tubes were centrifuged for 10 min at 3000 rpm. The released hemoglobin libera in supernatant was measured by UV – Vis at 414 nm. Control samples for 0% hemolysis (negative control, in the absence of polymer) and 100% hemolysis (positive control, with 0.1% Triton X-100 solution). The results are the average of three independent measurements. Hemolysis extent was quantified with the formula a fost determinat cu formula:

Hemolysis (%) = 100 $(A_t - A_0) / (A_m - A_0)$

where A is the absorbance (at 414 nm) of the sample in the presence of tested polymer (A_t), in in the absence of polymer (A_0) and in the presence of Triton solution (A_m). Polymer concentration corresponding to 50% hemolysis was taken as HC_{50}. The results obtained for the several polymers are presented in the Table 4.

Table 4. Comparison between antimicrobial and hemolytic activity of the polymers Selectivity S = HC_{50}/MIC

Polymer/strain		*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028
B3	*MIC,* mg/ml	0.01	0.01	0.07	0.01
	HC_{50}, mg/ml	4.07			
	S	407	407	58	407
B4	*MIC,* mg/ml	1.25	1.25	1.25	1.25
	HC_{50}, mg/ml	5.50			
	S	4.4	4.4	4.4	4.4
D16	*MIC,* mg/ml	0.15	n.d.[a]	n.d.[a]	n.d.[a]
	HC_{50}, mg/ml	2.56	-	-	-
	S	17	-	-	-

The data included in Table 4 show that oligomer **B3** has a high selectivity for all tested microorganisms, much higher than that of oligomer **B4** with a similar chemical structure but with higher polymerization degree. This difference requires a special attention and supplementary experiments for identification of relationship activity/molecular mass. Polymer **D16** has a rather good selectivity for *S. aureus*. These results recommend the polymer **B3** as potential alternative for antibiotics, with low risk in systemic administration, while the polymer **D16** with good antistaphylococcal activity and selectivity could be used as disinfectant with low toxicity during handling.

Activity 3.3 – ***Studies for application of synthesized polymers in new domains (flocculation, dispersion).***

Cationic polymers synthesized during project period, especially those obtained by dextran chemical modification, can have other applications, for example as biocompatible additives in water purification by removal of different pollutants such as clays, pesticides or dyes.

3.3.1. *Flocculation studies with dextran carrying cationic pendent groups*

Dextran derivatives with pendent quaternary ammonium groups (N-alkyl-N,N-dimethyl-N-(2-hydroxypropyl) ammonium chloride - structure **D**, R^2 = a, b in Scheme 2), have been tested with good results as flocculation agents for suspensions or emulsions containing organic (pesticides: Fastac 10EC, Decis, Dithane M45either) or inorganic (clays, TiO_2, zirconium silicate) contaminants. Influence of polymer properties (cationic group content, pendent group structure, dextran molecular weight) on flocculation efficacy was evaluated by comparison of the minimum doses required for a good separation and the width of flocculation windows (polymer concentration range corresponding to > 90% separated contaminant). Evaluations based on measurements of turbidity/UV-Vis absorbance, zeta potential and flocs size, allowed the appraisal of polymer optimal doses and assessment of the flocculation mechanism.

An example of the influence of polymer dose is presented in Fig. 2. The increase of polymer concentration in the flocculation mixture leads first to a significant decrease of the mixture turbidity, followed by a zone of minimal turbidity (flocculation window), and a final turbidity increase due to suspension re-stabilization. Zeta potential variation with polymer concentration enlightens the occurrence of the three turbidity zones: stepwise neutralization of negatively charged contaminant particles by cationic polymer adsorption on their surface, which leads to particle aggregation and flocculation; complete neutralization (zero potential zeta), corresponding to optimal dose; particle charge reversal from negative to positive due to excess adsorbed polymer, leading to particle re-suspension.

The increase of cationic group (substitution degree, DS) determines a significant decrease of optimal dose, but a decrease of the flocculation windows, the latter aspect being a drawback in practical applications where a larger optimal concentration range avoids the failures caused by dosage errors.

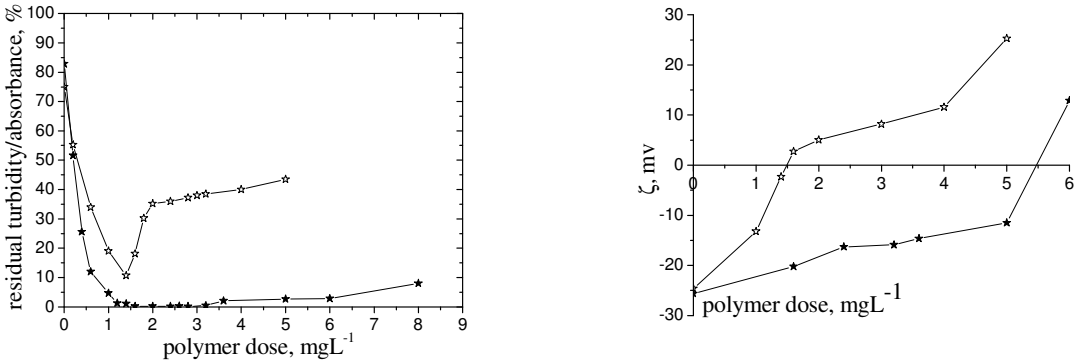


Fig. 2. Influence of the dose of a polymer with DS = 94% on the flocculation of clay suspensions (solid star) and Fastac emulsions (open star), evaluated by absorbance (left) and zeta potential (right) measurements.

3.3.2. *Study of dye adsorption on cationic dextran gels*

A cationic dextran derivative with chemical structure **D** was crosslinked with epichlorhidrin and the resulted gel was used in the study of adsorption of anionic dyes with different chemical structure, number and type of anionic groups: Methyl Orange (MeO) and Orange II (OrII) (azo dyes, with one sulfonic group), Indigo Carmine (IC) (indigoid dye, with two sulfonic groups), and Rose Bengal (RB) (antroquinone dye, with carboxylic groups). The influence of pH, contact time (Fig.3), dye type (Fig. 3a,b), dye initial concentration (Fig.3c) and temperature (Fig.3d) on adsorption efficacy was determined in order to establish the optimal conditions for the dye removal from industrial waste waters.

Adsorption data were evaluated by equilibrium, kinetic and thermodynamic analysis. Equilibrium adsorption data fit well to Langmuir model, and the kinetic data correspond to pseudo-second order kinetic model. The calculated thermodynamic parameters and activation energies have shown that adsorption mechanism depends on the dye type and is governed by diffusion (RB), chemi-sorption (IC) or a combination of physical and chemical interactions

(MeO si Or II). The gel highest adsorption capacity was about 650-730 mg/g and depended on dye chemical structure and its molecular weight. The cationic gel could be regenerated by desorption of adsorbed dye, using a dynamic procedure (in a column) with sequential elution

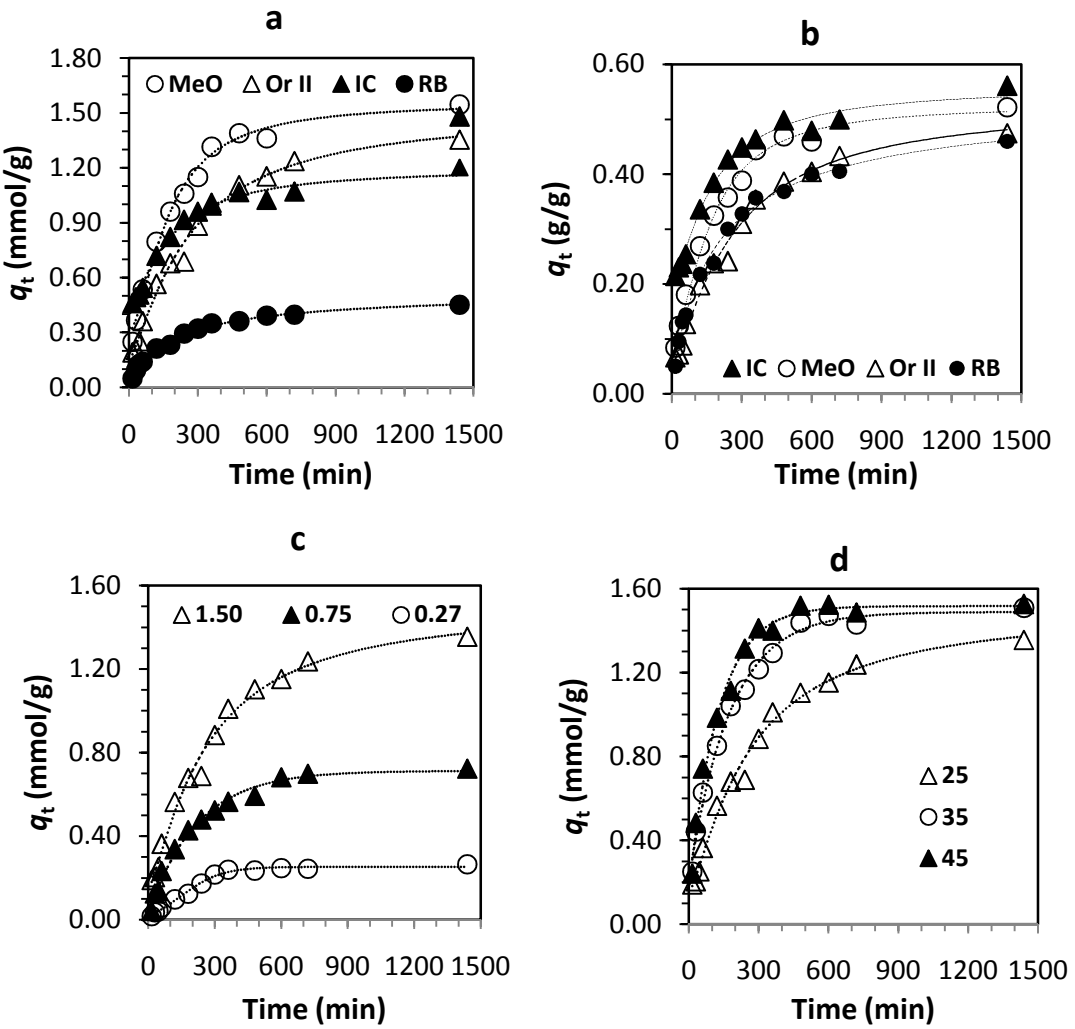


Fig. 3. Variation of dye adsorption with contact time as a function of dye typei (**a**, **b**), Or II initial concentration (mM) (**c**), and temperature (^{o}C) (for Or II) (**d**). Initial concentration was 1.5 mM (**a, b,d**), and temperature 25^{o}C (**a,b,c**).

with water, 0.5 N NaCl, and methanol.

Activity 3.4 – ***Study of polymer interaction with model membranes (liposomes)***

Activity mechanism of an antimicrobial agent can be evaluated by the study of its interaction with model membranes obtained by preparation of liposomes from lipid mixtures characteristic to each microorganism and blood red cells. This study based on the encapsulation inside liposomes of a fluorescent dye (calcein) with self-quenching properties and measurement of fluorescence of calcein released due to the interaction between liposome membrane and tested

polymer. Several lipid mixtures similar to those found in the membrane of cells were used for liposome preparation, according to the following table.

Nr.	Cell type	Lipide (%)					Calcein
		Phosphatidyl coline	Phosphatidyl ethanolamine	Phosphatidyl glycerol	Cardiolipin	Cholesterol	
1	E.coli	-	78	17	5		0.1
2	S. aureus	-	-	57	43		0.1
3	Globule rosii	33	9	-	-	25	0.1

Preparation of large unilamelar vesicles (LUV) was performed using a simple procedure with solvent dispersion which avoid very long mechanical methods (with repeated extrusions). Lipid mixture and calcein were dissolved in ethylic ether and chloroform, and the resulted solution was slowly injected with a syringe in a PBS aqueous solution heated at 60^{o}C. Vesicles settled on the flask bottom during gradual solvent evaporation. Afer cooling, the suspension is washed with PBS and centrifuged several times for removal of free calcein. DLS and TEM measurements showed that the obtained particles have a diameter of 150-200 nm and a morphology corresponding to unilamelar vesicles (Fig. 4)

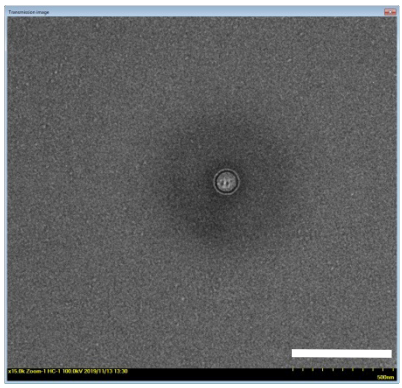


Fig. 4. TEM image of vesicles obtained from the lipid mixture 1 (*E. coli* membrane) The bar corresponds to 500 nm.

Vesicles were diluted with PBS solution to a content of 1/20 v/v fluorescence emission intensity (excitation at 470 nm, emission at 503 nm) of the mixture was checked and the obtained value was taken as negative standard. Fluorescence intensity value measured after the vesicles interaction with a Triton X100 solution (0.1%) was considered as maximum value due to the release of the whole encapsulated calcein release (positive standard). Tests for interaction liposome-polymer were carried out using polymer aqueous solution/ suspensions with concentrations equal to *MIC* value determined for each pair polymer- microorganism.

Interaction was quantified by calculation of the calcein amount release from vesicles as a function of time, using formula:

Free calcein (%) = $100(F_t - F_0)/(F_m - F_0)$

where F is fluorescence emission intensity determined in the presence of polymer (F_t), PBS solution (F_0), and Triton X solution (F_m).

The results of experiments performed with polymers and vesicles prepared from the lipid mixture 1 (*E. coli* membrane model) and 2 (*S. aureus* membrane model) are presented in Fig. 5 and they support the antimicrobial test results, suggesting that antimicrobial activity is due to the polymer interaction with cell membrane, followed by the membrane disruption and eventual cell death.

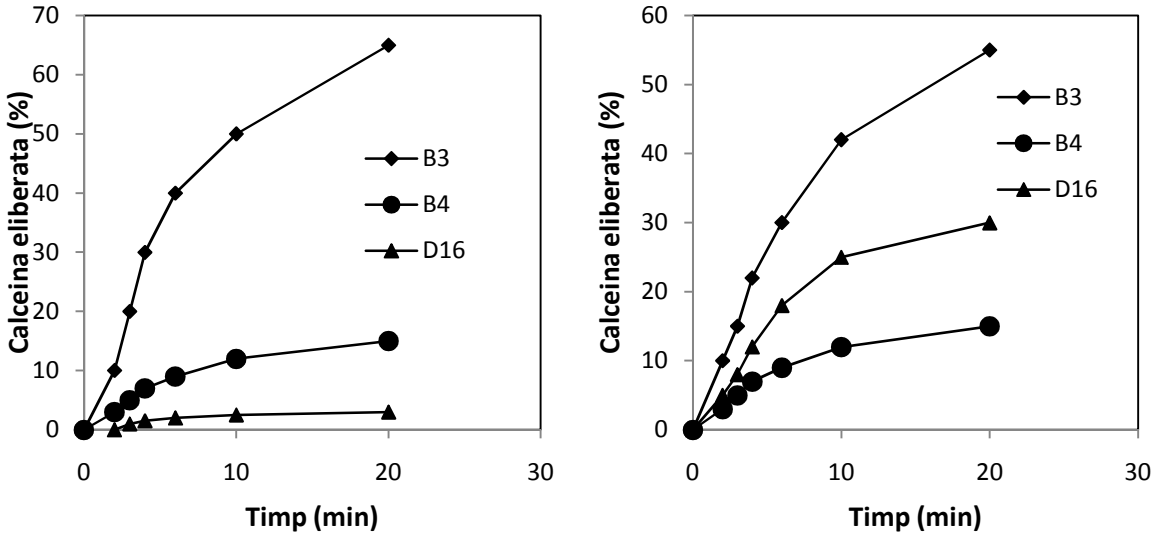


Fig.5. Evolution with time of the calcein amount released from the liposomes type 1 (left) and 2 (right) in the presence of selected polymers

CONCLUSIONS AND GENERAL EVALUATION OF THE OBTAINED RESULTS

Studies performed during the entire project period lead to the synthesis of two new polymer/oligomer based on natural compounds – bile acids and polysaccharides – with self-assembling properties and antimicrobial activity.

- New oligomers with a bile acid, cholic acid, in the backbone and primary amino groups in the side chains were prepared. Synthesis of these polymers started with the hypothesis that the bile acid facial amphiphilicity, as well as amino groups from amino acids, might lead to polymers with similar structure and activity displayed by antimicrobial peptides. The new polymers can self-associate in aqueous and form spherical particles (micelles) with nanometric size (50-150 nm in diameter), more or less compact as a function of the amino

acid forming the side chains. Antimicrobial tests against gram-positive or gram-negative bacteria and pathogenic fungi lead to the selection of an oligomer with a polymerization degree ≈ 5 and β-alanine in the side chains having a very good antimicrobial activity (*MIC* ≈ 10 μg/ml) and an excellent selectivity (HC_{50}/MIC ≈ 400) for the most of the tested microorganisms.

It should be stressed that the exclusive use of bile acids for the preparation of antimicrobial polymers able to simulate the facial amphiphilicity of antimicrobial peptide has not been reported yet. Facial amphiphilicity was obtained mainly with synthetic polymers [1] such as poly(norbornene) [2,3] ,poly(phenylene ethynylene) [4] poly(arylamide) [5,6]. Most of these polymers have a very good antimicrobial activity but a low selectivity, therefore they have been recommended for preparation of materials with antimicrobial surface[6,7].

Better performances were reported for a new poly(arylamide) class [6], which combine antimicrobial activity with good selectivity. These performances are similar or even superior to those of the polymer synthesized and selected in the present project. Additionally, the procedure used for the synthesis of the oligomer based on cholic acid is simpler and its biocompatibility is superior to that of polymers obtained only from synthetic raw materials.

In order to have a more complete evaluation of applicability of cholic acid oligomer as antibiotic alternative, the future research will be focused on determination of polymer cytotoxicity (*in vitro*) for different human cells, *in vivo* cytotoxicity (on animals) and antimicrobial activity against microorganisms resistant to antibiotics.

- A new class of amphiphilic polymers based on dextran was synthesized by attachment bile acid molecules at the end of the dextran backbone and or side chain containing one or more cationic groups. These polymers self-associate in spherical micelles having diameters of 50-150 nm (neutral polymers) or 200-300 nm (cationic polymers). Their antistaphylococcal and antifungal activity is good and hemocompatibility is moderate, being appropriate for external use as disinfectants. The major novelty of this polymer class is the preparation of dextan with side chains containing each three quaternary ammonium groups linked by long alkyl chains. Such a chemical structure leads to a significant increase in cationic group density, which could enhance biocidal activity. Up to now only the synthesis of polymers with many cationic groups in the main chain (ionenes) obtained by polycondensation of tertiary diamines with dihalogenoalkanes was reported, and there antimicrobial activity is moderate [8]. The new polymer class developed during the project will be further studied

for the improvement of their antimicrobial performances. For this purpose, some structural parameters will be taken into account such as charge density, the length of the alkylic chain between cationic centers, or usage of other tertiary amine, for example imidazol. Among polymers obtained at date from dextran, the best results were found for a dextran derivative with lithocholic acid end groups and dimethyloctylammoniu chloride pendent groups (**D16**) with good antistaphylococcal activity (MIC 0.15 mg/g) and a selectivity (S = 17) much greater than that of well known antimicrobial polymers based on biguanidine and poly(vinylpiridine) (S < 1) or that of peptide like melitin (S = 0.1) [7].

- Cationic polymers based on dextran were tested for application in different domains. They were used as such, or after some chemical modification (crosslinking), as biocompatible additives for removal of some pollutants such as clays, pesticides or dyes, from surface or waste waters. Good results were obtained at flocculation of suspensions or emulsions containing organic (pesticides: Fastac 10EC, Decis, Dithane M45either) or inorganic (clays, TiO2, zirconium silicate) contaminants, as well as at adsorption of anionic dyes. Consequently, these polymers with reduced toxicity can be applied purification processes or surface waters or residual waters disposed by pesticides or dye industrial producers.

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1 . M. Hartlieb, E. G. L. Williams, A. Kuroki, S. Perrier, K. E.S. Locock. Antimicrobial Polymers: Mimicking Amino Acid Functionality, Sequence Control and Three-dimensional Structure of Host-defense Peptides. *Current Medicinal Chemistry* 2017, *24,* 1-26.

2. K. Lienkamp, G. N. Tew. Synthetic Mimics of Antimicrobial Peptides—A Versatile Ring-Opening Metathesis Polymerization Based Platform for the Synthesis of Selective Antibacterial and Cell Penetrating Polymers. *Chemistry – A European Journal* 2009, 15, 11784-11800.

3. A. Al-Ahmad, D. Laird, P. Zou, P. Tomakidi, T. Steinberg, K. Lienkamp. Nature-Inspired Antimicrobial Polymers – Assessment of Their Potential for Biomedical Applications. *PLOS ONE* 2013, 8, e73812.

4. R. B. Breitenkamp, L. Arnt, G. N. Tew. Facially amphiphilic phenylene ethynylenes. *Polymers for Advanced. Technologies* 2005; 16, 189–194.

5. R. W. Scott, W. F. DeGrado, G. N. Tew. De novo designed synthetic mimics of antimicrobial peptides. *Current Opinion in Biotechnology* 2008, 19, 620–627.

6. H. D. Thaker, A. Som, F. Ayaz, D. Lui, W. Pan, R. W. Scott, J. Anguita, G. N. Tew. Synthetic Mimics of Antimicrobial Peptides with Immunomodulatory Responses. *Journal of American Chemical Society* 2012, 134, 11088−11091.

7. G. J. Gabriel, A. Som, A. E. Madkour, T. Eren, Gregory N. Tew. Infectious disease: Connecting innate immunity to biocidal polymers. *Materials Science and Engineering* R 2007, 57, 28–64.

8 . J. Guo, J. Qin, Y. Ren, B. Wang, H. Cui, Y. Ding, H. Mao, F. Yan. Antibacterial Activity of Cationic Polymers: Side-chain or Mainchain Type? *Polymer Chemistry*, 2018, 9, 4611-4616.