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

period July – December 2017

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:

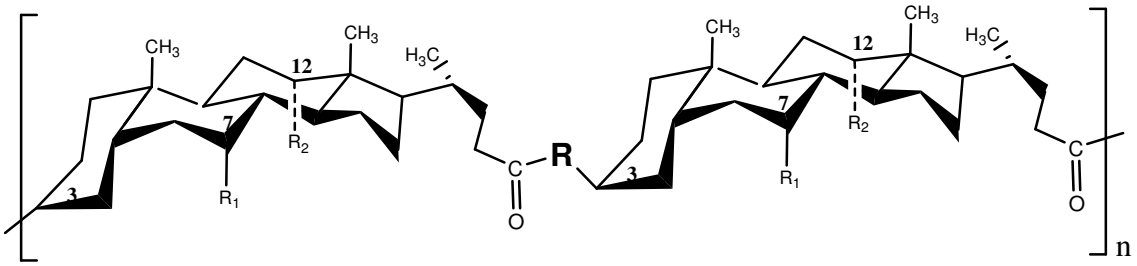
- Synthesis of new polymers based on bile acids, which should have several properties of antimicrobial peptides: facial amphiphilicity, presence of primary amine groups, selective antimicrobial activity and reduced hemolytic activity. These properties are required for their application as alternatives 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 2017 (Stage 1):</u> 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 ($n \approx 5$).

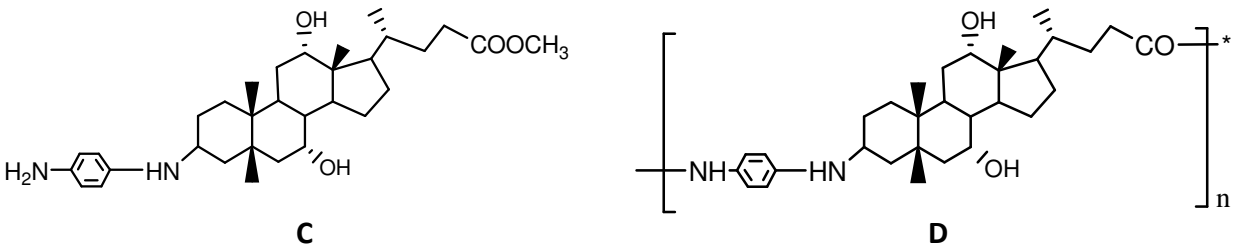
(b) Connection of oligomeric backbone by phenylamide bridges (Scheme 3, polymer **D**) was obtained by polycondensation of monomer **C**. Reactions required for preparation of products **C** and **D** are presented in the following:

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}.



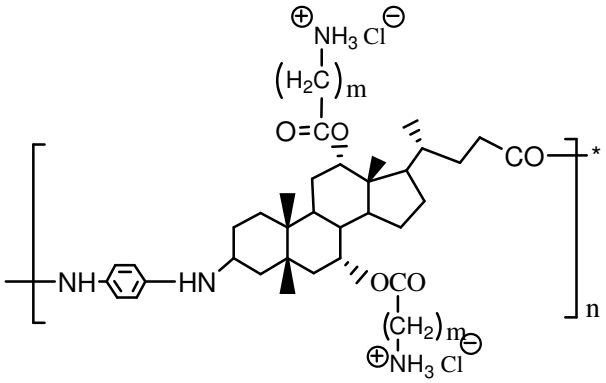
Scheme 3. Chemical structure of the cholic acid with methyl ester and phenilenediamine groups (**C**) and of polymer **D** obtained by **C** polycondensation..

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

Director proiect,