December 2013-Decembrie 2014

Objectives and activities for stage 4 (2014), according to the working plan:

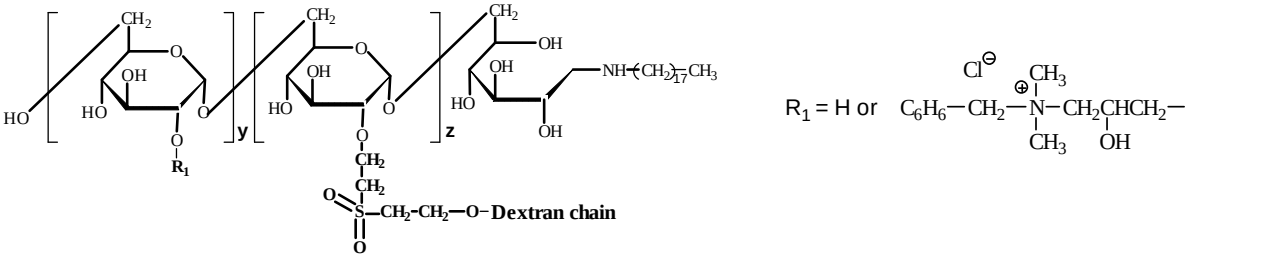
OBJECTIVE 1: <u>Preparation of drug delivery systems based on nanostructured micelles/vesicles</u>

Activity 1.1. Drug encapsulation in nanoparticles

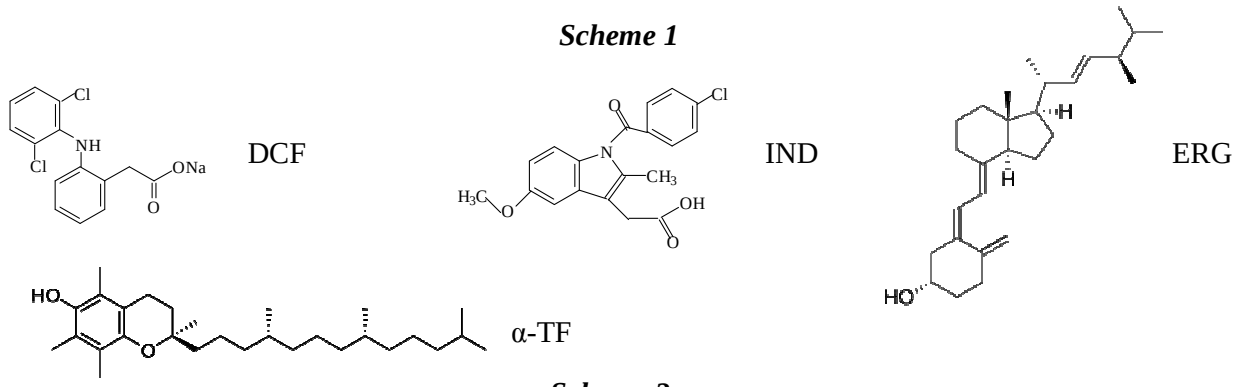
Activity 1.2. In vitro study of drug release rate

1.1. <u>Drug encapsulation in nanoparticles</u>

Drug retention capacity of nanoparticles obtained from modified dextran micelles was investigated by using dextran end-modified with octadecyl amine. This polymer self-assembles in aqueous solution leading to micelles with a hydrophobic core formed by associated alkyl chains and a hydrophilic shell of dextran. In order to increase micelle stability to dilution, the dextran shell was crosslinked with divinylsulfone, and the ability to interact with different bioactive compounds was enhanced by attachment of quaternary ammonium groups along the dextran main chain (Scheme 1). Retention/release studies were performed on a neutral polymer, CM (R_1 = H), and two cationic polymers (CMQ10 and CMQ17, where 10 and 17 indicate the content of cationic pendent groups, in mol/100 glucosidic unities). Two non-steroidal antiimflamatory drugs, diclofenac (DCF) and indometacin (IND), one lipophilic antioxidant, α-tocopherol (α-TF), as well as ergocalciferol (ERG), a provitamin form of vitamin D (Scheme 2), were used as model drugs.



Scheme 1



Scheme 2

Encapsulation of biologically active compounds was performed by using different retention media, as a function of each drug solubility. DCF was dissolved in water, IND in a mixture ethanol/water (8/2, v/v), while α-TF and ERG solutions were prepared in pure ethanol. The drug solution with a known concentration (20 ml) was added to 20 mg dried particles (in case of DCF and IND) or particles pre-swollen in 2 mL water (for α-TF and ERG). The mixtures were left under stirring for 48 h at ambient temperature, then they were transferred in dialyzing tubes and dialyzed against water (for DCF experiments) or against water /methanol (1/1 v/v) (for IND, α-TF, ERG), until UV absorbance of external solution, measured at 276 nm (DCF), 319 nm (IND), 292nm (α-TF), and 265 nm (ERG) indicated the absence of the drug. Finally, particles were recovered by freeze-drying, and the amount of drug retained by particles (expressed as mg/g support) was determined by UV measurements carried out on ethanol/water (8/2 v/v) suspensions.

DCF and IND, which have carboxylic groups, were retained in higher amounts by cationic polymers particles, in the following retention order: CM (50 mg DCF/g; 75 mg IND/g) < CMQ10 (300 mg DCF/g; 100 mg IND/g) < CMQ17 (375 mg DCF/g; 120 mg IND/g), indicating the predominant role of electrostatic interaction in retention process. Contribution of other interaction types, for example hydrophobic associations, is suggested by the low but significant retention of these drugs on neutral particles. In

comparison to DCF, IND showed a lower affinity for cationic particles, which can be the result of IND higher molecular mass and hydrophobicity.

Retention of hydrophobic drugs, α-TF and ERG, is not significantly influenced by support chemical composition (with or without cationic groups), showing that preferential partition of these compounds inside the micelle hydrophobic core is the preponderant retention mechanism. The big difference between the α-TF (~ 200 mg/g) and ERG (~ 100 mg/g) retention can be assigned to the different chemical structure of the two drugs. ERG has a bulky cyclic molecule, while α-TF has a linear alkyl chain which can favor both the diffusion inside particles and interaction with similar alkyl chains forming nanoparticle hydrophobic core.

1.2. In vitro study of drug release rate

In vitro release of encapsulated drugs was studied by dialysis method. A mixture of particles loaded with drug (30 mg) and phosphate buffered saline (PBS, 0.1N, pH 7.4) (3 ml) was introduced into a dialysis tube (cellulose acetate membrane, cut-off 10000), and PBS (30 mL) was used as external solution. In case of more hydrophobic drugs (IND, α-TF and ERG) Tween 80 (0,1 g%) was added to PBS external solution. This emulsifying agent is frequently used in evaluation of water insoluble drug release. At established time intervals, external solution was removed and replaced with an equal volume of fresh PBS. Each withdrawn solution was analyzed by UV for quantification of the released drug. The cumulative amount of drug released was calculated for each time interval, and expressed as % of initially loaded drug. Dialysis of the free drug (in the absence of a polymeric support) was carried out as a control experiment.

In all studied cases, the free drug was faster recovered by dialysis than the drug loaded on polymer, and the release from nanoparticles showed a “burst effect” in the first 1-4 h, perhaps due to the drug bound at the nanoparticle surface. Thus, 90% of free DCF was recovered after 24 h, while loaded polymeric nanoparticles released 60-70 % bound drug after 48 h. Un-bound IND was almost completely recovered after 48 h, but its release from polymeric supports was slow and continuous over one week interval. The release profile and the influence of the polymeric support chemical structure were similar for these two drugs, what is an indication that the release from support is mainly controlled by the same factors, namely the electrostatic interactions between cationic groups located in the micelle shell and carboxylic groups of the drugs . The release rate of both compounds was lower in acidic medium (pH 1,2), due to the decrease of drug solubility in water with decreasing pH (Fig. 1).

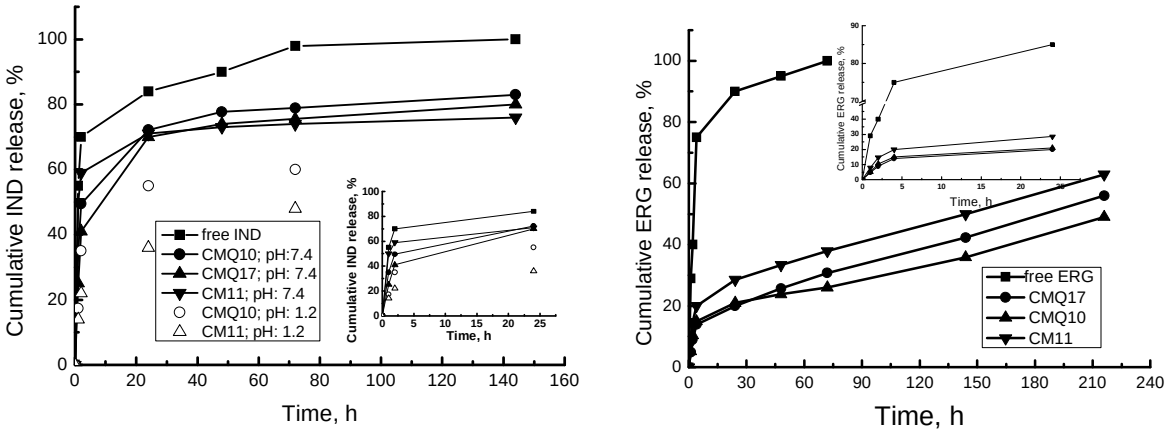


Fig. 1.

The release of hydrophobic drugs α-TF and ERG in PBS medium containing 0.1% Tween 80 is presented in Fig. 2. 80% of both free drugs were recovered after 24 h. The burst phenomenon was also observed in the first 4 h of the bound drug release, when 7-15% α-TF and 14-20% ERG were recovered. This first interval was followed by a slow and controlled release of 50-60% drugs over one month interval. Both neutral and cationic crosslinked micelles released much slower the hydrophobic drugs than the more hydrophilic DCF or IND, showing that these nanoparticles are more efficient supports for the controlled release of hydrophobic drugs.

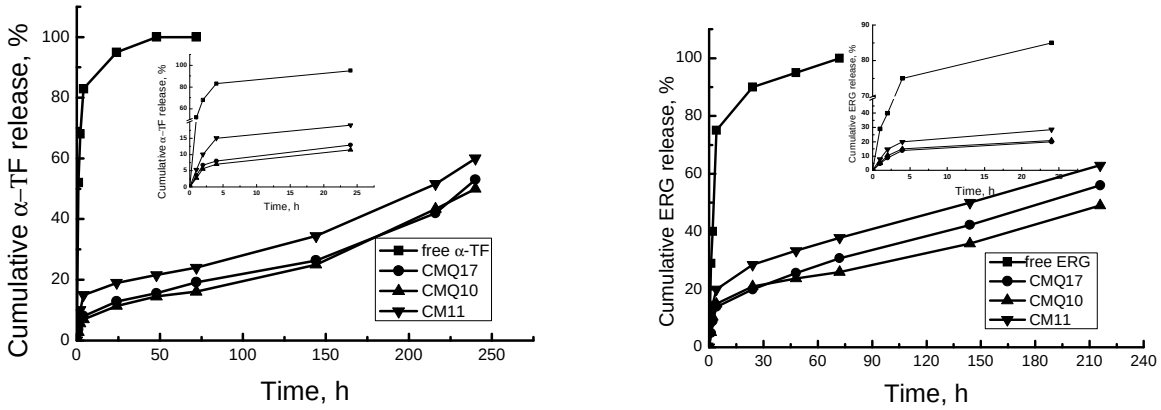


Fig. 2.

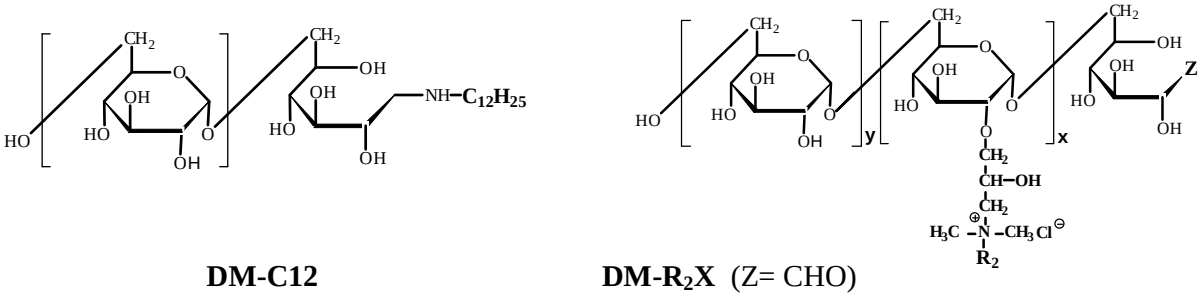
OBJECTIVE 2: ***<u>Prospective study for new applications of synthesized block colpolymers</u>***

Activity 2.1. Flocculants for inorganic particles

Activity 2.2. Dispersants for emulsions/suspensions

2.1. <u>Flocculants for inorganic particles</u>

Inorganic particles present in surface waters or domestic and industrial waste waters are undesired impurities which can be removed by flocculation/sedimentation processes in the presence of natural or synthetic polymers. We studied the applicability of our polymers in separation of zirconium silicate (kreuzonit) from its aqueous suspensions, as kreuzonit is an impurity often present in the waste water delivered by ceramic industry, where it is used as opacifying agent. At this end, block-like cationic amphiphilic polymers, obtained from dextran (MW 10 kDa) with hydrophobic end groups (dodecyl) and pendent cationic groups (DM-C12-R_2X) were used as flocculating agents. A neutral amphiphilic polymer and several cationic hydrophilic polymers (without hydrophobic end group) of DM-R_2X type, were used for comparison. The polymers are defined by M – dextran molar mass, in kDa, the variable substituent in pendent cationic group, R = Etyl or Octyl , and the content in cationic groups, X, in mol/100 glucosidic unities) (Scheme 3).



DM-C12 **DM-R_2X** (Z= CHO)

DM-C12-R_2X (Z = NH-$C_{12}H_{25}$)

Scheme 3. $R_2$is ethyl (Et) or octyl (Oct)

Kreuzonit suspension (5g/L) were mixed with different polymer doses (1 g/L stock solution in water), stirred and left 1200 min for sedimentation, then the supernatant was analyzed by UV-Vis spectrometry at 400 nm (residual turbidity, % of initial turbidity) and zeta potential measurements (Nano-ZS, model ZEN-3500). The obtained results can be summarized as follows:

- In the polymer absence, residual turbidity was as high as 90%, what indicates a relatively stable system.
- Presence of all polymers determines a significant decrease of suspension turbidity, but separation efficiency was influenced by polymer dose and its chemical structure. Hence, at the same polymer dose (0.2 mg/L), residual turbidity decreases in the order : no polymer (90 %) > hydrophilic neutral polymer (dextran) (50%) > amphiphilic neutral polymer (D10-C12) (30 %) > amphiphilic cationic polymer (D10-C12-Oct30) (4 %) (Fig. 3). This behavior can be explained by preponderance of electrostatic interactions between negatively charged kreuzonit particles (zeta potential in aqueous suspension is

– 27.9 mV) and positively charged groups on polymer. Low but significant turbidity decrease in the presence of neutral polymers is a proof for partial adsorption of these polymers on the kreuzonit particle surface, leading to a partial separation due to either negative charge screening (dextran) or particle aggregation as a result of hydrophobic association between polymers adsorbed on different particles (D10-C12).

- With increasing polymer concentration, three domains for turbidity variation were highlighted: (i) turbidity decreases until a minimum value; (ii) turbidity is maintained at a value close to the minimum; (iii) turbidity increases until a constant value. Polymer concentration at which the minimum turbidity is observed (optimal dose) and the width of concentration range corresponding to the minimum turbidity domain (flocculation window) are very important parameters for flocculation process and depend on polymer chemical structure. All cationic polymers gave an optimal dose lower than 1 mg/L, and its values slightly increases in the order D10-Oct30 (0.1 mg/L) < D10-C12-Oct30 (0.2 mg/L) < D10-C12-Et30 (0.4 mg/L). Polymer chemical structure had a more significant influence on the flocculation window width. D10-C12-Et30 gave the widest flocculation window (0.4 -1.4 mg/L), while D10-C12-Oct30 kept the minimum turbidity over a very narrow concentration range (0.2-0.4 mg/L). The different behavior can be assigned to the presence of a more hydrophobic substituent (octyl instead of ethyl), which favor a hydrophobic interaction between the polymer adsorbed on particle surface and the free polymer in solution, leading to the increase of cationic charges at the particle surface and, consequently, to particle redispersion.

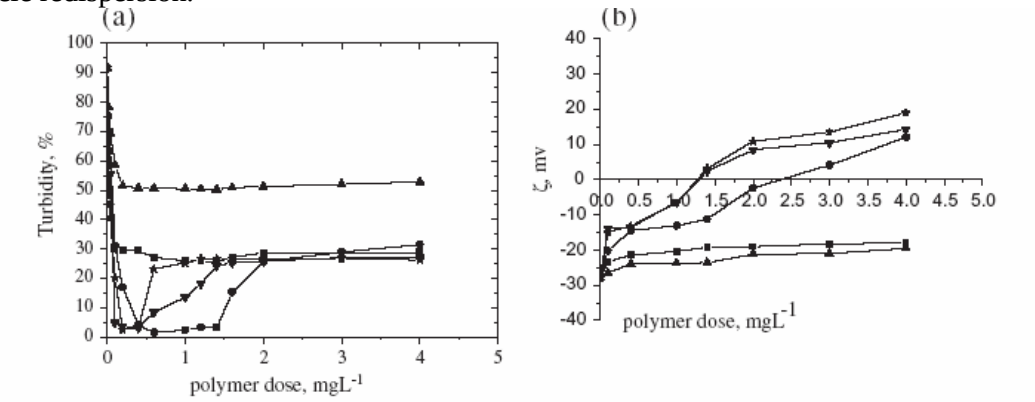


Fig. 3. Variation of residual turbidity (T%) (a) and zeta potential (f) (b)with polymer dose, as a function of polymer chemical structure : dextran (triangle); D10-C12 (square); D10-C12-Oct30 (star); D10-Oct30 (inverted triangle); D10-C12-Et30 (circle).

- The profile of zeta potential variation with polymer dose (Fig. 3b) is similar for all cationic polymers. Zeta potential increases monotonously over entire concentration range corresponding to flocculation window, then, just at the end of flocculation window, a sudden increase take place, eventually leading to charge reversal from negative to positive. Concentrations at which neutralization of kreuzonit particles was observed (zeta potential zero) were higher than those corresponding to the end of flocculation windows. This finding, together with negative zeta potential observed over the entire flocculation window, are good arguments for a flocculation process governed by a „patch" mechanism, according to which particle aggregation is the result of the electrostatic interaction between oppositely charged areas on the surface of particles partially covered with polymer.

- In case of neutral polymers, dextran and D10-C12, zeta potential increases slightly over the entire studied concentration range from -27.9 mV (for kreuzonit particle in the absence of polymer) to about -18-19 mV. These data agree with the turbidity results, which point for a partial adsorption of neutral polymers on particle surface, having as result a moderate aggregation due to negative charge screening (dextran) or to the hydrophobic interaction between polymer chains adsorbed on different particles. (D10-C12).

- Dextran molar mass (10, 200, 400 kDa) used for cationic polymer DM-Oct30 synthesis, did not influence the flocculation process efficacy, a finding reported for other polymers with medium or high charge density. Nevertheless, zeta potential measurements highlighted a decrease of polymer

concentration required for particle charge neutralization with increasing dextran molar mass (1.3 mg/L for D10-Oct30, 1.15 mg/L for D200-Oct30, 1 mg/L for D400-Oct30), indicating a moderate improvement of flocculation efficacy with increasing molar mass.

2.2. Dispersants for emulsion/suspension

It was shown in the previous section that, above a certain polymer concentration, kreuzonit suspension undergoes redispersion and becomes stable. The concentration required for redispersion and dispersion stability depends on polymer chemical structure. For this purpose, the most efficient polymers are amphiphilic cationic polymers with hydrophobic substituents at the cationic groups (D10-C12-Ict30).

Conclusions

In this stage we synthesized and characterized block-like amphiphilic polymers obtained from a polysaccharide, dextran, as hydrophilic block, and long alkyl chains (dodecyl, octadecyl) attached at the dextran reductive end, as hydrophobic block. These polymers can form micelles in aqueous solutions. In order to improve polymer performances and micelle stability, the main dextran backbone was chemically modified by attachment of pendent cationic groups and/or crosslinking bridges. Studies aiming at evaluation of potential application of these polymers as carriers for drug controlled release or as flocculants/dispersants led to several conclusions:

- Nanoparticles obtained by crosslinking of the micelle outer shell (dextran) are able to retain high amounts of biologically active compounds through hydrophobic and /or electrostatic interactions and release them slowly, in a controlled manner. The synthesized nanosized micelles are mainly recommended as carriers for hydrophobic drug controlled delivery.

- Cationic amphiphilic polymers can be used for separation/dispersion of inorganic particle suspensions (kreuzonit). Polymer D10-C12-Oct30 is more efficient in the flocculation processes requiring small doses of polymeric additive, while D10-C12-Et30 would be preferred if the flocculation window width is an important parameter. Polymer D10-C12-Oct30 is also recommended as dispersion agent for inorganic particle suspensions.

The results obtained in this stage were published in 4 articles

1. M. Nichifor, G. Mocanu, M.C. Stanciu " Micelle-like association of polysaccharides with hydrophobic end groups" *Carbohydrate Polymers* **110**, 209-218, **2014**.
2. G. Mocanu, M. Nichifor, L. Picton, E. About-Jaudet, D. Le Cerf. "Preparation and characterization of anionic pullulan thermoassociative nanoparticles for drug delivery" *Carbohydrate Polymers* **111**, 892–900, **2014.**
3. L. Ghimici, M. Nichifor, Flocculation performance of different cationic amphiphilic dextran derivatives in zirconium silicate suspension, *Separation and Purification Technology*, **133**, 254-259, **2014.**
4. G. Mocanu, M. Nichifor, M. C. Stanciu, New shell crosslinked micelles from dextran with hydrophobic end groups and their interaction with bioactive molecules, Carbohydrate Polymers, accepted, DOI 10.1016/j.carbpol.2014.11.047

Project Director

Marieta Nichifor, PhD