December2014 -December 2015

Objectives and activities in 2015, according to the project working plan:

OBIECTIVE 1: *Enzymatic hydrolysis of the synthesized polymers*
> *Activity 1.1. Polyesters hydrolysis*
> *Activity 1.2. Hydrolysis of block copolymers based on polyesters*

Studies on chemical and/or enzymatic hydrolysis of polyesters and block copolymers prepared from polyesters can highlight their biodegradability, a property which could decide the selection of carriers to be used for controlled drug delivery systems (DDS). Susceptibility to chemical degradation (in the absence of enzymes) determines the DDS chemical stability *in vivo*. Degradation in the presence of certain enzymes can be advantageous when these enzymes are over-expressed at the site of DDS action.

Hydrolysis experiments have been performed on polyesters of deoxycholic acid (DCA) and oligoethylene glycols (OEG) with a general code DCA-OEGN, where N is the molar mass of OEG (100, 200, 400, 600 Da), as well as on block-copolymers dextran-polyesters called DexM-(DCA-OEGN), where M is the molar mass of dextran (6 or 10 kDa). Experiments were carried out in phosphate buffered saline (PBS) at pH 7.4 and 37°C, in absence or presence of lipases, which are enzymes well known for their hydrolytic activity against polyesters such as polylactides or polycaprolactones. Lipase from porcine pancreas (PP) and Pseudomonas fluorescence (PF) were selected for our studies. Hydrolysis degree was evaluated by the amount of free DCA and 3-succinoyl-DCA (the DCA derivative used for polyesters synthesis) detected by HPLC in the hydrolysis medium.

1.1. *Polysters hydrolysis*

The rate of polyesters DCA-OEGM hydrolysis is very low in the absence of enzymes (Fig.1A), it increases with increasing OEG'molar mass, but the total amount of free DCA derivatives detected after 5 weeks is as low as 4 wt%. This result indicates a good polyester stability under physiological conditions.

Presence of lipases led to a significant increase of degradation rate (Fig. 1B-F), and hydrolysis degree was influenced by several factors:

- Polyesters hydrophilicity given by OEG' chain length. The increase of OEG length enhances the polyester degradation process (Fig. 1B). The difference between the efficacy of enzymatic and chemical hydrolysis is also increased by increasing OEG length (Fig. 1C-D). For example, the polyester DCA-OEG100 has a similar behavior in the absence and in the presence of PP (Fig.1C), while the polyester DCA-OEG600 releases 20 times more DCA derivatives in the presence of PP than in its absence (Fig. 1D).
- Enzyme type. Variation of the amount of free DCA derivatives released with time in the presence of PP or PF (Fig. 1E-F) indicates a higher hydrolytic activity of PP, but the difference between the two enzymes activity seems to diminish with the increase of polyester hydrophilicity, perhaps due to a higher affinity of PP for more hydrophobic substrates.
- Enzyme concentration. The increase of PP concentration from 1 mg/mL to 2 mg/mL accelerated the hydrolysis rate in the first 2-4 hours of experiment, then the enzyme activity leveled or even decreased. This finding might be the result of enzyme inactivation.

1.2. *Hydrolysis of block copolymers based on polyesters*

Block-copolymers DexM-(DCA-OEGN) hydrolysis was performed under the same conditions described for polyesters, in PBS, without or with lipase PP. Fig.2 shows the results obtained with two different forms of a block-copolymer, in comparison with the corresponding polyester. Block-copolymer Dex6-(DCA-OEG100) containing 60 wt % polyester was used either as a powder (bulk) (0.2-0.5 mm in size) suspended in the hydrolysis medium, or as micelles prepared by the solvent exchange method (DMSO-water) followed by dialysis against water (average micelles diameter, determined by DLS, was 500 nm). Polyester hydrolysis rate was low, both in the absence and in the presence of PP. The hydrolysis of polyester block of copolymer as powder was faster than the polyester homopolymer, but the polyester block from micelles has the same hydrolysis rate as the homopolymer. The difference in behavior can be assigned to the different accessibility of lipase to its hydrophobic substrate. In micelle form, hydrophobic polyester block is located in the micelle
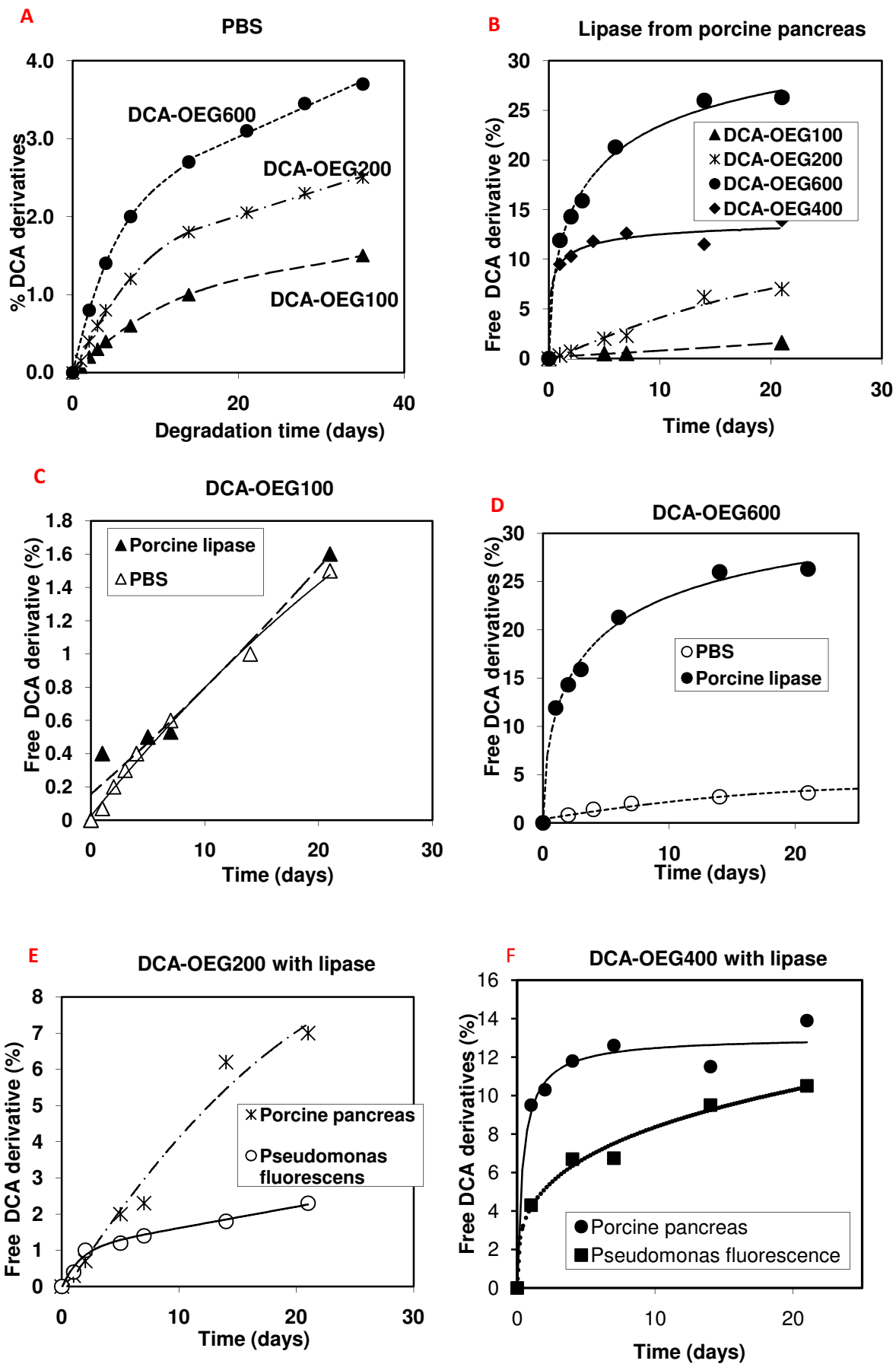
Fig. 1. Comparative data for the chemical and enzymatic hydrolysis of polyesters DCA-OEGN, in PBS (phosphate buffered saline 0.05M), in the absence or in presence of lipase from porcine pancreas or from Pseudomonas fluorescence (1 mg/mL). All hydrolysis were performed at pH 7.4 and 37 $^{\circ}$C.

core, which is protected by the hydrophilic corona formed by dextran blocks. Under these conditions, the access of the lipase inside the micelle is restricted. This explanation is supported by an increase of the hydrolysis degree of micelles in the presence of more hydrophilic PF.
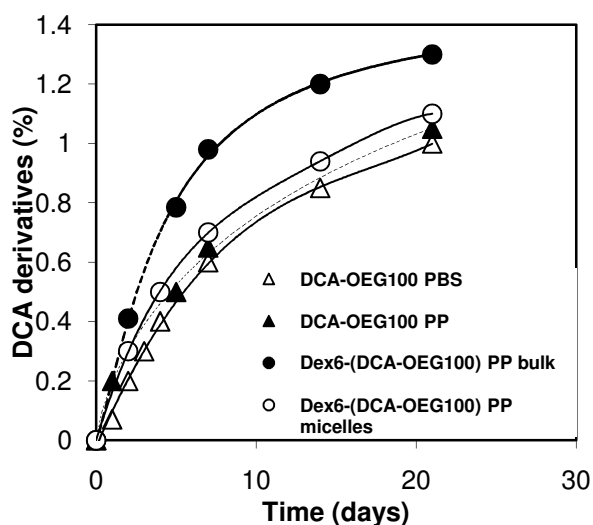

Fig.2. Variation with time of amount of DCA derivatives released during hydrolysis in PBS, in the presence of lipase from porcine pancreas (PP) by the polyester DCA-OEG100 homopolymer and block copolymer Dex6-(DCA-OEG100). PP concentration = 1 mg/mL, pH 7.4, 37°C.

OBJECTIVE 2*: **Prospective study for new applications of synthesized block colpolymers**
 *Activity 2.1.   Pesticide removal from wastewaters*
 Activity 2.2.   Dyes removal from waste waters
 Activity 2.3.   Nanoreactors

2.1. Pesticide removal from wastewaters

Pesticide removal from wastewaters resulted from industrial processes or from agriculture areas is an important issue for environment protection, due to pesticides toxicity and their carcinogenic action against leaving organisms. The use of polymers with cationic groups for pesticide removal can be efficient, as many pesticide particles have a negatively charged surface and electrostatic interaction particle-polymer can accelerate impurities flocculation and sedimentation. Cationic polymers DM-RX (Scheme 1) (M is dextran molar mass, in kDa, R = Ethyl or Octyl and is the variable substituent in pendent cationic groups, and X is the content in cationic groups, in mol/100 glucozidic unities). Fastac 10 EC (a commercial pesticide containing α–Cypermethrin) emulsion in water was used as wastewater experimental model.
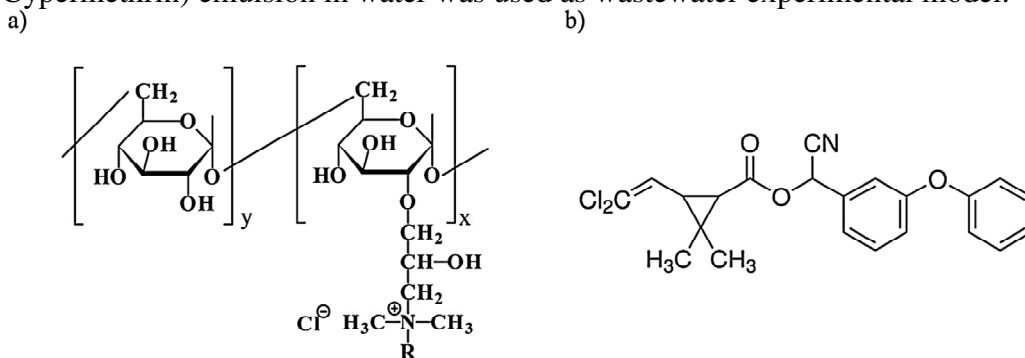

Scheme 1. (a) Chemical structure of dextran with cationic groups; R is ethyl (Et) or octyl (Oct)(DM-RX), and (b) α–Cypermethrin

Different polymer doses (from 1g/L stock solution) were added to Fastac 10EC emulsions, the mixtures were stirred and then let without stirring for 1200 min for sedimentation. The supernatant was analyzed by UV-vis spectrometry at 276 nm (absorbance maximum of the UV spectrum of pesticide emulsion), zeta potential (Nano-ZS, model ZEN-3500) and floc size measurements (Mastersizer 2000). Separation efficiency was estimated from residual absorbance (%) = $100\ A_f/A_i$ (A_i and A_f are supernatant absorbance of

Fastac emulsion in the absence and in the presence of polymer, respectively). The obtained results are summarized in the following.

- Residual absorbance did not decrease in the absence of polymer, indicating a stable system.
- Supernatant absorbance significantly decreased in the presence of polymers, and the decrease was dependent on the polymer chemical structure. The minimum residual absorbance observed for different polymers was: D40- 46%, D40-Et18 -9.88%, D40-Et94-10.71% si D40-Oct30-30%   (Fig. 3a). This behavior can be explained by preponderance of electrostatic interactions between negatively charged pesticide particles (zeta potential of pesticide aqueous solution was -24.6 mV) and positively charged polymer. Small but significant decrease of absorbance in the presence of the neutral polymer (unmodified dextran D40) can be due to a partial adsorption of this polymer on the surface of pesticide particles, resulting in a screening of the negative charges and partial separation. Lower efficacy of D40-Oct30, which carries a hydrophobic substituent (R = octyl), could be explained by a less efficient coverage of pesticide particle by more compact amphiphilic polymer coils.
- Increase of polymer concentration highlighted three domains for the residual absorbance variation: (i) absorbance decreases until a minimum value; (ii) absorbance increases; (iii) absorbance remains constant. Polymer concentration corresponding to minimum residual absorbance (optimal dose) depends on polymer chemical structure and decreases with increasing cationic group content. It should be mentioned the very narrow flocculation window and the absence of a complete emulsion redispersion, as the residual absorbance is kept at a value lower than 50% on a large range of polymer concentration, for all polymers used in this study.
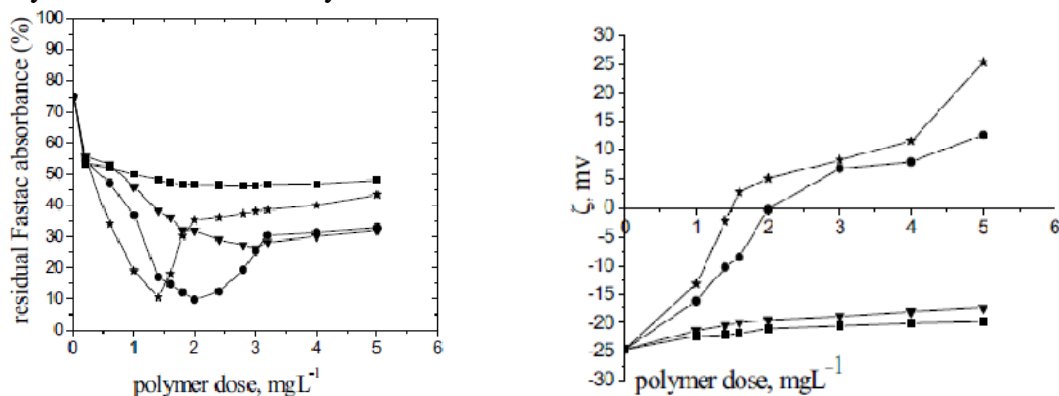

Fig. 3. Variation of residual absorbance (%) (a) and of zeta potential (ζ) (b) with polymer dose, as a function of polymer chemical structur : dextran (square); D40-Et18 (circle); D40-Et94 (star); D40-Oct30 (inverted triangle). pH 4.4, Fastac concentration 0.02%.

- Variation of zeta potential with polymer dose (Fig. 3b) has a similar profile for all hydrophilic cationic polymers. Zeta potential increases monotonously over the entire polymer dose range and changes from negative to positive. Polymer concentrations necessary for pesticide particle neutralization (zeta potential zero) were very close to those corresponding to the minimum residual absorbance, showing again that the flocculation in mainly the result of system charge neutralization. Small variation of zeta potential for dextran and D40-Oct30 suggests a reduced interaction of these polymers with pesticide particles and explains their reduced efficacy in separation process.

- Increase of Fastac emulsion pH from 4.4 to 7.4 leads to an increase of flocculation efficacy, highlighted by the optimal dose decrease and occurrence of flocculation window. Further increase of pH to 10 do not enhance the separation process.

- Increase of initial Fastac emulsion concentration improved the efficacy of separation process, expressed by a significant decrease of residual absorbance and the presence of a wider flocculation window

- The values obtained for the pesticide floc size as a function of cationic polymer dose confirmed the preponderance of electrostatic interactions in separation process.

2.2. *Dyes removal from waste waters*

Dyes occur in waste waters emerging from many industrial processes, and their removal is very important for environment protection. Experiments carried out in the project framework aimed at finding a polymeric support able to absorb a large amount of an anaionic dye, methylene blue (MB), which is widely used in textile industry. For this purpose, amphiphilic block copolymers were synthesized by attachment of a hydrophobic compound (alkyl, bile acid) at the dextran reductive end, followed by the chemical modification of the dextran OH groups in order to introduce cationic side groups and/or crosslinking divinyl sulfone (DVS) bridges (Scheme 2).

Scheme 2.
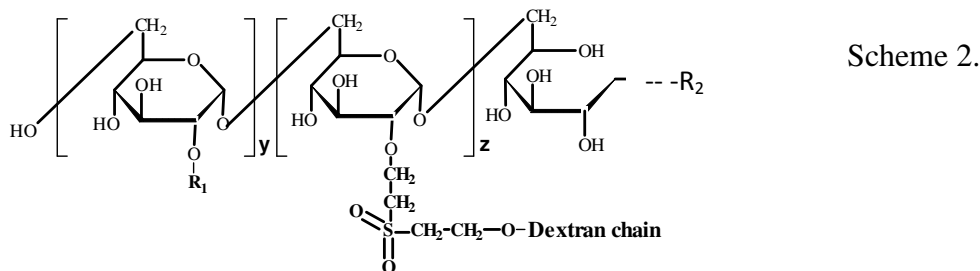
According to the results presented in Fig. 4, crosslinking and presence of cationic groups increase the amount of anionic dye retained by polymeric supports. The nature of hydrophobic moiety attached at the dextran chain end (alkyl or bile acid) does not influence support affinity for MB.
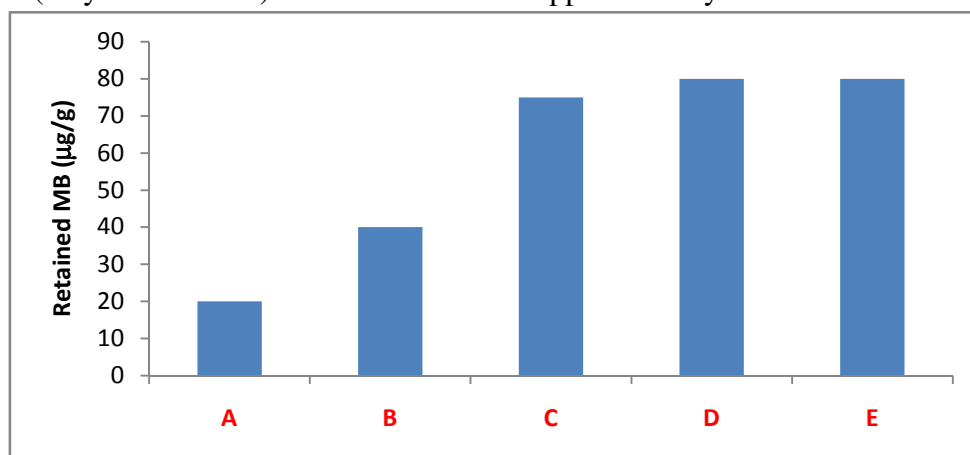
Fig.4. MB amount retained on different supports. **A** –R_1 = H, R_2 = $C_{18}H_{37}$, Z=0; B- R_1 = H, R_2 = $C_{18}H_{37}$, Z=20 mol%; **C**- R_1 = $(C_6H_5CH_2)(CH_3)_2$-N-$(CH_2CHOHCH_2)$Cl; Y=20 mol%, R_2 = $C_{18}H_{37}$, Z =0; **D -** R_1 = $(C_6H_5CH_2)(CH_3)_2$-N-$(CH_2CHOHCH_2)$Cl; Y=20 mol%, R_2 = $C_{18}H_{37}$, Z =20 mol% ; **E** - R_1 = $(C_6H_5CH_2)(CH_3)_2$-N-$(CH_2CHOHCH_2)$Cl; Y=20 mol%, R_2 = DCA, Z =20 mol%;

2.3. *Nanoreactors*

Nanoreactors are nano-sized particles allowing the control of the site and microenvironment for chemical or enzymatic reactions. They found applications in very specific domains such as (bio)sensors production, controlled drug delivery, nanometric metal particle preparation. Micelles obtained from amphiphilic block-copolymers can retain in their hydrophobic core a catalyst, which can act with high specificity for selected substrates. Enzymes encapsulated in aqueous core of block-copolymer vesicles are protected against proteolitic degradation and preserve their *in situ* activity.

Preliminary studies were performed for the immobilization of an enzyme (lipase) in vesicles (Fig. 5) obtained from a block-copolymer Dex10-(DCA-OEG100) containing 69 wt% polyester DCA-OEG100. The final purpose of these studies is the preparation of nanoreactors designed for the controlled enzymatic hydrolysis of specific substrates. Several steps were carried out: (a) fluorescent labeling of lipase with fluorescein isothiocyanate (LP-FITC); (b) Vesicle preparation with simultaneous LP-FITC encapsulation inside vesicles by solvent exchange method (DMSO/water, with block- copolymer dissolved in DMSO,

lipase in water); (c) Quantification of immobilized lipase (fluorescence measurement of supernatant after particle centrifugation); (d) evaluation of vesicle stability (DLS, TEM, HPLC)
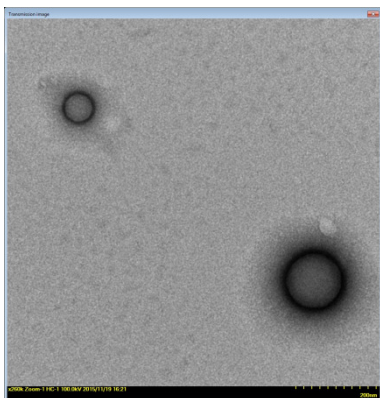
Fig. 5. TEM image of vesicles prepared from block-copolymer Dex10-(DCA-OEG100**).** Vesicle average size was 288 nm (DLS measurements) and the width of vesicle wall was 10 nm.

Results obtained in this stage were dissaminated in 4 published articles in 2015 and an article submitted to publication

1. 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* 119, 228-235, **2015**.
2. M.C. Stanciu, M. Nichifor " New biocompatible amphiphilic diblock copolymer based on dextran, *European Polymer Journal* 71, 352-363, **2015**.
3. L. Ghimici, M. Nichifor" Ionic dextran derivatives for removal of Fastac 10 EC from itaqueous emulsions, *Carbohydrate Polymers* 134, 46-51, **2015.**
4. L. Ghimici, M. Constantin "The separation of the pyrethroid insecticide Fastac 10 EC by cationic pullulan derivatives" Reacive and Functional Polymers 95, 12-18, **2015**.
5. G. Mocanu, M. Nichifor " Dextran based polymeric micelles as carriers for delivery of hydrophobic drugs" *International Journal of Biological Macromolecules,*under review.

Project Director,

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