New pH and temperature sensitive delivery systems based on renewable resources

Project PN-RU-TE-2014-4-0437

Contract No.: **93** from **01/10/2015**

Acronym: **SensCurdSyst**

Scientific Report 2017

O.4. *Obtaining of microcapsules based on ionic polysaccharides*

The layer-by-layer assembly by alternating adsorption of complementary charge polyelectrolytes was developed for the first time by Decher [1] when he obtained film onto macroscopically flat substrates (e.g. silicon, glass, gold wafers). Donath et al. [2] applied this technique to colloidal particles, transferring the planar deposition to tri-dimensional deposition. The LbL technique consists in the construction of thin polymeric films with controlled architecture by auto-assembling (**Figure 1**). The auto-assembling process of the polymers is attributed to the electrostatic interactions, hydrogen bonding, hydrophobic interactions, and van der Waals forces. This method allows the assembling on any ionic surface. The thickness of the films depends on the intrinsic properties of the polyelectrolytes and on the experimental conditions (pH, ionic strength, etc.). The thickness of two deposit layers can usually vary between 3 and 7 nm [3].

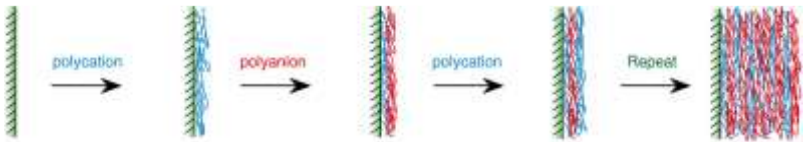


Figure 1. Preparation of thin films on a plane surface using the LbL technique [4]

Even if most of the studies regarding multilayers use synthetic polyelectrolytes, in the last years the multilayers using biopolymers were studied more. Thus, an increased interest was observed for the obtaining of microcapsules based on ionic polysaccharides using the LbL technique, because polysaccharides are not toxic, biocompatible, biodegradable and are obtained from re-generable resources. Negatively charged ionic polysaccharides used in LbL deposition are: chondroitin sulphate, heparin sulphate, hyaluronic acid, alginic acid, etc. or

1. G. Decher, *Science* 277 (1997) 1232–1237
2. E. Donath, G. B. Sukhorukov, F. Caruso, S. A. Davis, H. Möhwald, *Angew Chem Int Ed* 37 (1998) 2202–2205
3. L.L. del Mercato, M.M. Ferraro, F. Baldassarre, S. Mancarella, V. Greco, R. Rinaldi, S. Leporatti, *Adv Coll Interface Sci* 207 (2014) 139–154
4. K. Sato, K. Yoshida, S. Takahashi, J-ichi Anzai et al. / *Advanced Drug Delivery Reviews* 63 (2011) 809–821

can be obtained by chemical modification of non-ionic polysaccharides with sulphate, phosphate or carboxylic groups [5, 6]. In the case of cationic polysaccharides, the choice is limited to chitosan or polysaccharides modified with amine groups [7].

Möhwald [2] and the co-workers obtained hollow microcapsules by deposition of polyelectrolytes on the surface of the colloidal particles, followed by the dissolution of the support (**Figure 2**). This method was used for drug encapsulation with applications in pharmaceutical and medical field. Inorganic particles like $CaCO_3$ or $MnCO_3$ were used because they can be easily dissolved in aqueous solutions with HCl or sodium ethylenediaminetetraacetate (EDTA). When organic particles are used as support, complex conditions are required for their dissolution.

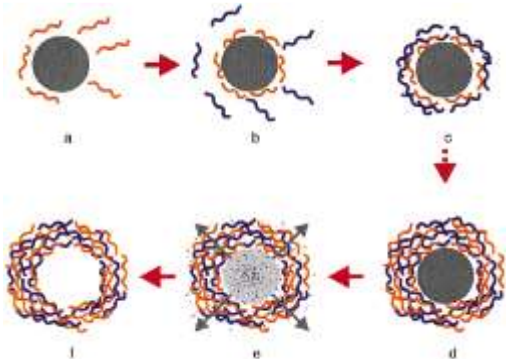


Figure 2. Preparation of microcapsules using LbL technique [2]

A.4.1. Obtaining of micro/nano-capsules from the new ionic derivatives using layer by layer deposition.

Preparation of the support $MnCO_3$ microparticles

$MnCO_3$ crystals were formed from NH_4HCO_3 and $MnSO_4$ solutions. The next step was the optimization of the experimental conditions (concentration of the precursors, temperature, pH, stirring speed and reaction time) to obtain micro/nano-capsules. The control of microparticles shape was ensured by adding the nucleation agents. In order to modify the charge and also to influence the crystallization and the shape of the inorganic particles, ionic polysaccharides were added in the synthesis of the $MnCO_3$ microparticles.

5. D.M. Suflet, G.Chitanu, V.I. Popa, *React Funct Polym*, 66(2006), 1240–1249
6. D.M. Suflet, I. Popescu, I.M. Pelin, A. Nicolescu, G. Hitruc, *Carbohyd Polym*, 123(2015) 396–405
7. T. Crouzier, T. Boudou, C. Picart, *Curr Opin Coll Interface Sci*, 15 (2010) 417–426

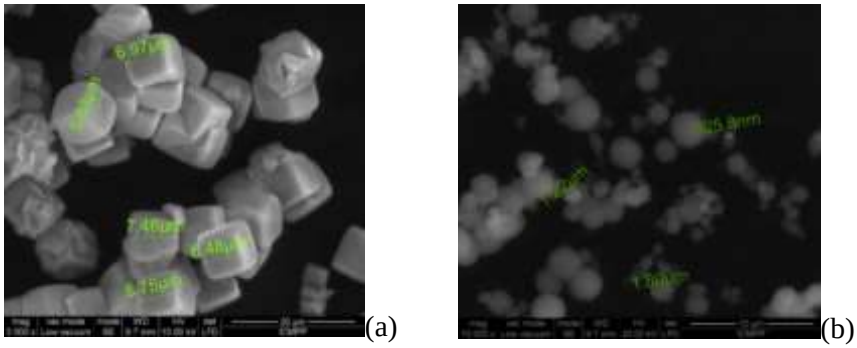
Obtaining of multilayers from PCurd/NCurd (prepared in the previous stages of the project) on the $MnCO_3$ microparticles support

The $MnCO_3$ microparticles were first dispersed in water by vortexing and ultrasonication, then they were alternatively immersed into polyanion and polycation solutions (PCurd and NCurd). Between the immersions, the microparticles were washed three times with distilled water. After each steep, the microparticles were recovered from the solutions by centrifugation. The deposition process was repeated till the obtaining of three double layers. In the end, the inorganic core was dissolved using HCl and EDTA aqueous solutions.

A.4.2. Characterization of microparticles

Characterization of the $MnCO_3$ support microparticles

The morphology of the microparticles was studied by scanning electron microscopy (SEM), when a cubical shape was observed for the $MnCO_3$ microparticles obtained in the absence of the nucleation agents, and a spherical shape was observed for the microparticles obtained in the presence of the nucleation agent (**Figure 3**).



(a) (b)

Figure 3. SEM images of $MnCO_3$ microparticles in the absence (a) and in the presence of nucleation agent (b)

$MnCO_3$ microparticles were also synthesized in the presence of ionic polysaccharides. Microparticles having spherical shape with the diameter between 3 and 4 μm were obtained in the presence of anionic polysaccharide, when cellulose phosphate was used, and between 1 and 8 μm, when phosphorylated curdlan was used. The size of the microparticles was influenced by the polymer concentration. When a cationic polysaccharide (NCurd) was added, the shape of the microparticles was influenced by the concentration of the polymer, so only in the presence of a high amount of NCurd, spherical shapes were obtained.

Characterization of microparticles/microcapsules

In order to establish the order of layer deposition on the support microparticles, zeta potential measurements were first performed onto $MnCO_3$ microparticles, using a Zetasizer Nano ZS from Malvern (equipment purchased in the frame of this project). The variation of the zeta potential of the microparticles during the deposition of the polyelectrolyte layers was also pursued. The zeta potential always increased after the deposition of cationic polysaccharide and decreased after the deposition of anionic polysaccharide, giving an indication about the polymeric film growth on the surface of the $MnCO_3$ microparticles.

The inorganic core of the microparticles was removed using EDTA aqueous solution. The obtaining of hallow microcapsules was evidenced by SEM microscopy.

O5. Retention/delivery studies of therapeutic agents into/from the micro-/nanoparticles/capsules. Biological tests.

The retention studies were performed using model molecules as methylene blue (MB) and rhodamine 6G (R6G) in order to simulate the retention of low-molecular drugs, but also lysozyme in order to simulate the retention/delivery of high-molecular drugs like proteins. The aim of the studies was to establish the mechanisms and the kinetics of the retention/delivery process. The studies were performed using the batch technique. The concentration of the model substances was determined by spectrophotometric measurements, using the adsorption at 663 nm for MB, at 526 nm for R6G, and at 280 nm for lysozyme.

The dye/protein amount adsorbed at equilibrium (q_e) was calculated with equation (1):

$$q_e = \frac{V(C_0 - C_f)}{m} \quad (1)$$

where C_0 and C_f are the initial and the final concentrations of the dye/protein, V is the volume of the solution, and m is the adsorbent amount. The efficiency of the dye/protein adsorption was calculated with equation (2):

$$R(\%) = \frac{100(C_0 - C_f)}{C_0} \quad (2)$$

The equilibrium adsorption experiments were fitted with different theoretical models.

The constants from Langmuir isotherm (K_L and a_L), from Freundlich isotherm (K_F and $1/n_f$), from Dubinin-Radushkevich isotherm (k_{DR}, q_{DR}) and from Temkin isotherm (a_T, b_T) could be calculated from the representation of the equations (4), (6), (8) and (10), respectively. Each of these constants give important information about the adsorption process, the distribution of the adsorption sites, and about the adsorption energy.

Table 1. Theoretical models of the adsorption isotherms

Model	Ecuation		Liniarized equation		Ref.
Langmuir isotherm	$q_e = \frac{K_L C_e}{1+a_L C_e}$	(3)	$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e$	(4)	[8]
Freundlich isotherm	$q_e = K_F C_e^{1/n_F}$	(5)	$lnq_e = lnK_F + \frac{1}{n_F} lnC_e$	(6)	[9]
Dubinin-Radushkevich izotherm	$q_e = q_s e^{-k_{DR}\varepsilon^2}$	(7)	$lnq_e = lnq_s - k_{DR}\varepsilon^2$	(8)	[10]
Temkin isotherm	$q_e = \frac{RT}{b_T} ln(a_T C_e)$	(9)	$q_e = \frac{RT}{b_T} lna_T + \frac{RT}{b_T} lnC_e$	(10)	[11]

The sorption kinetics offers information about the adsorption mechanisms. Four models were applied to the experimental data: pseudo-first-order model, pseudo-second-order model, intra-particle diffusion model, and Boyd model [12, 13].

A.5.1. Studies regarding the retention of active principles in/on microparticles

The porosity of the microparticles was taken into consideration in order to select the model compound used for the study of the retention/delivery mechanism and kinetics. Thus,

- for PCellMS microparticles, with small pores, cationic dyes (rhodamine 6G - R6G and methylene blue -MB), which are used as disinfectant in the medical field, were chosen.
- for Curd-MA_NIPAM, with large pores, lysozyme was chosen as model high-molecular molecule. Lysozyme (molar mass of 14.4 kDa) is an antimicrobial enzyme against gram-positive bacteria that can be found in secretions (saliva, tears, milk or mucus) or in cytoplasmic granules of macrophages.

I. Studies regarding the adsorption on PCellMS microspheres

pH was shown to influence the adsorption of dyes on PCellMS: at low pH values the monobasic phosphate groups from the polysaccharide were in protonated form and the dye adsorption was almost zero; with the increase of the pH the phosphate groups begin to

8. I. Langmuir, The constitution and fundamental properties of solids and liquids, *J. Am. Chem. Soc*. 38 (1916) 2221–2295.
9. X. Song, Y. Pan, Q.Wu, Z. Cheng,W. Ma, Phosphate removal from aqueous solutions by adsorption using ferric sludge, *Desalination* 280 (2011) 384–390.
10. M. Dubinin, L. Radushkevich, Equation of the characteristic curve of activated charcoal, *Proc. Acad. Sci. USSR* 55 (1947) 331–333
11. M. Tempkin, V. Pyzhev, Kinetics of ammonia synthesis on promoted iron catalysts, *Acta Phys. Chim. USSR* 12 (1940) 327–356
12. G.E. Boyd, A.W. Adamson, L.S. Myers Jr., *J. Am. Chem. Soc*., 69, 2836 (1947)
13. Y.S. Ho, G. McKay, *Chem. Eng. J*., 70, 115 (1998)

dissociate and electrostatically attract the dye molecules leading to the increase of the dye adsorption amount (q_e).

The influence of the microparticles dose on the dye adsorption was also studied maintaining constant the dye concentration and the volume solution. As expected, the increase of the microparticles dose led to the increase of the q_e and of the adsorption efficiency (R%) to 90%.

The equilibrium adsorption isotherms experiments were also performed in order to establish the adoption mechanisms. The experimental data were fitted with the theoretical models (**Figure 4**) and the adsorption parameters were calculated. The data are better fitted with Langmuir model compared with other models (Freundlich, Tempkin, and Dubinin-Radushkevich), which shows a uniform distribution of the adsorption sites, a uniform distribution of the adsorption energy, and that each adsorption site adsorbs only one dye molecule (monolayer adsorption). The theoretical limit of adsorption, q_m from Langmuir model, was found to be 58.8 mg MB/g microparticles and 78.13 mg R6G/g microparticles.

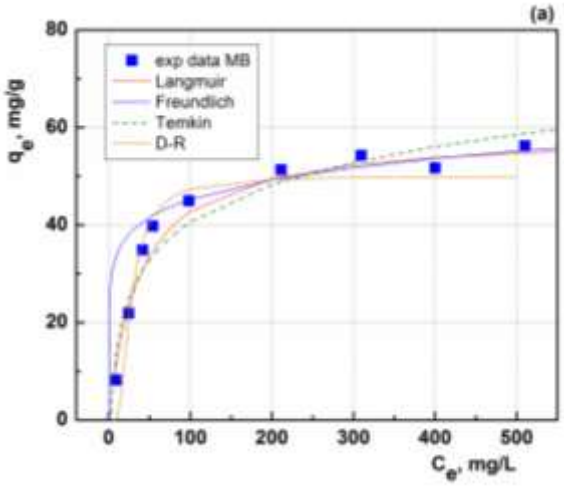


Figure 4. Adsorption isotherms of MB on PCellMS: experimental data and the Fitting of the theoretical models.

The adsorption kinetics is very important in order to evaluate an adsorption process. The kinetics depends on the sorbet-adsorbent interaction and on the conditions of the system. **Figure 5** present the influence of time and of the concentration of the dye solution on the adsorption. As expected, the general tendency was the increase of dye adsorption with time, until 24 hours, after with the dye adsorption is constant. The dye adsorption also increased with the dye concentration in solution.

In order to evaluate the adsorption mechanism, the experimental data were fitted with different theoretical models: pseudo-first-order, pseudo-second-order, intra-particle diffusion, and Boyd model. The data were better fitted with the pseudo-second-order model ($R^2 > 0.98$), meaning that the dye was retained on the surface of the PCellMS by physical forces.

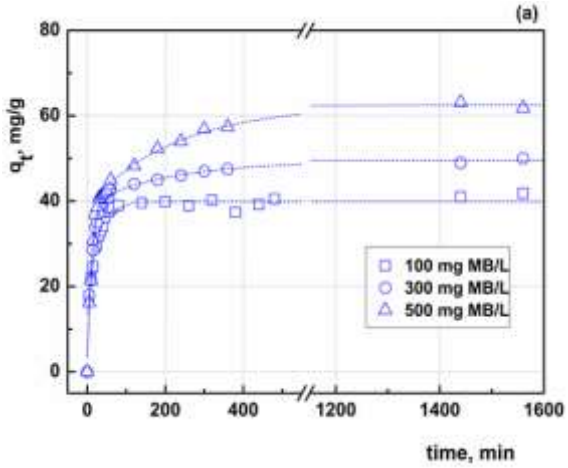


Figure 5. Adsorption kinetics of MB onto PCellMS microspheres at different dye concentrations.

Plotting the experimental data according to the intra-particle model the multi-linearity of curves was observed. The first region was attributed to the diffusion of dyes through the solution to the external surface of microspheres or to the boundary layers diffusion of solute molecules (film diffusion or external mass transfer) with an instantaneous blocking of the available sites on the external surface of microspheres. The second region represents the intra-particle diffusion process, when the dye molecules enter the porous structure of microparticles. The last region, which was the slowest stage, was attributed to the equilibrium phase where the adsorption site on the microspheres and the concentration of the dye solution are limited.

II. Studies regarding the retention of lysozyme onto Curd-MA_NIPAM microparticles

As an active principle, lysozyme was chosen as model in the retention/delivery studies onto/from the Curd-MA_NIPAM microparticles. This enzyme has antimicrobial activity against gram-positive bacteria. This globular protein has an isoelectric point around 11, meaning that at normal pH values it has positive charges and can electrostatically interact with anionic charges from the microparticles. The protein concentration in the solution was monitored by UV spectroscopy using the adsorption at 280 nm. The protein retention studies performed under batch conditions showed that the equilibrium is attained after two days.

A.5.2. "In vitro" studies of the release of active principles from micro/nano particles/capsules

In vitro" studies of lysozyme release from NCurd-MA_NIPAM microparticles

The release of the protein from the microparticles was performed in phosphate buffer solutions at pH=7.4 and at pH=5.4 to simulate the physiological conditions. In order to study the influence of the temperature on the protein release, the studies were performed at 20°C and at 40°C. NCurd-MA_NIPAM microparticles loaded with protein were immersed in the release medium under gentle stirring. At predetermined time intervals, small solution volumes were withdrawn and the same volume of fresh buffer was added to maintain the total volume of the release medium. The lysozyme concentration was determined by UV spectroscopy, and the released lysozyme amount was reported on the total amount of lysozyme from the microparticles. Due to the fact that the pH influences the dissociation of the carboxylic groups from the polymer network and the swelling of the microparticles, the pH was also shown to influence the protein release. Thus, at pH=7.4 lysozyme was released faster compared with the release at pH=5.4, both experiences performed at 20°C. When the pH was maintained at 5.4, but the temperature was increased to 40°C, the release was even slower. Due to the collapsing of the polymer network over the volume phase transition temperature (VPTT) at 40°C, the release of the entrapped protein was hindered. This behavior was different compared to the release of low-molecular drugs from microparticles based on NIPAM, but was also observed at the release of proteins from other thermosensitive hydrogels with high porosity [14, 15].

The difference between the release rates at 20 and at 40°C allows the pulsatile release of lysozyme from NCurd-MA_NIPAM microparticles. In order to study this possibility, the release was performed at pH=5.4 and the temperature was cyclically modified: 15 minutes at 40°C and 15 minutes at 20°C. When the temperature was lower than VPTT, the swollen microparticles enabled the release of the lysozyme by diffusion, but when the temperature was raised over the VPTT, the collapsing of the polymeric network hindered the diffusion of the high-molecular protein.

A.5.3. Studies regarding the biological activity of new micro particles/capsules synthesized

The biological (cytotoxicity and biocompatibility) and antibacterial activity of synthesized polysaccharide derivatives and micro particles/capsules were studied together with researchers from the Faculty of Medical Bioengineering of Gr.T. Popa University of

14. Hu, J., Zheng, S. and Xu, X., *J. Polym. Res*. 19 (2012) 9988
15. Zheng, S., Hu, J., Xu, X., Chen, X. Shen, D., *Monatsh. Chem*. 145 (2014) 39

Medicine and Pharmacy and Al.I. Cuza University, Iasi. In this stage, four samples were chosen to be tested: P1 (NCurd_DMOctA), P2 (PCurd), P3 (microspheres based on Curd) and P4 (microspheres based on PCurd).

Viability test

The MTT assay was chosen to study the viability cells. The results obtained for cell viability in the presence of polymeric samples after 24 and 48 hours concluded:

- only P1 sample presented the cytotoxicity even at a lower concentration than 2.5 mg/mL;
- the P2, P3 and P4 samples do not present cytotoxicity;
- the results obtained with MTT assay were confirmed by microscopy images performed in phase contrast and in fluorescence.

Anti-tumoral test

The aim of this test was to observe the selectivity of cellular destruction by a maximum efficiency in destroying tumor cells and to keep the viability of normal cells. The experiments were carried on two tumoral cell lines (MCF-7 and MG 63) and one normal cell line (PF - primary fibroblasts). Based on the vitality tests, when only P1 sample presented cytotoxicity, the tumoral test was performed on the P1 sample. In this test the solutions of polymer with concentrations between 0.05 and 1 mg/ml were used.

The P1 cytotoxicity begins to manifest itself starting from the concentration of 0.25 mg/ml only for the tumoral cell lines but not for the PF line, when a cell viability of about 85% was found. The decrease of viability continues with the increase of the polymer concentration, up to 1 mg/ml concentration of P1, when 0% viability was achieved for MCF-7, close to 0% for MG-63, and about 15 % viability of primary fibroblasts was found.

Anti-microbial test

The test was performed in liquid medium in triplicate using *E.coli DH5α.* The medium was sterilized (30 min at 120 °C) and the kanamycin was introduced in liquid medium and kept overnight at 37°C, under gentle stirring (100 rpm). Using the UV-vis method to determine the anti-bacterial activity, the samples were incubated at 37 °C for 5h, and then the UV adsorption at λ = 600 nm was measured. **Figure 5** presents the anti-bacterial activity of the tested samples.

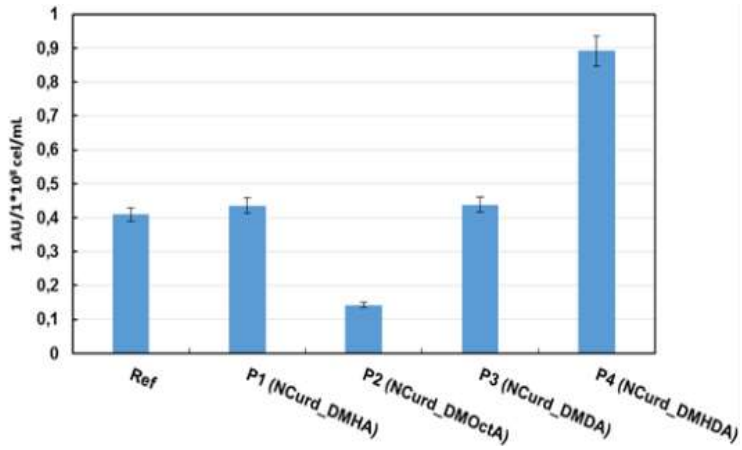


Figure 5. The anti-bacterial activity of samples

The objectives and the activities proposed on 2017 were accomplished and the results will be published in journals with impact factor.