Scientific report

regarding the implementation of the project

Stimuli-sensitive micro-/ nano-particles bsed on maleic copolymers with biomedical applications

from June 2013 to November 2015

The aim of the present project is to obtain micro/nano-particles based on maleic copolymers that are sensitive to the temperature and pH and that have application in controlled drug release. In order to attain the objective **O1** "**Synthesis and characterization of stimuli-sensitive micro-/nano- particles by covalent cross-linking of maleic copolymers",** in the first stage (June-Dec 2013) copolymers of maleic acid with N-vinylcaprolactam were synthesized and their behavior in aqueous solution was studied (Activity A.1.0). In the second stage (Jan-Dec 2014) microparticles were obtained by suspension cross-linking of the previous synthesized copolymers. The morphology of the microparticles was study by scanning electron microscopy. The stimuli-sensitivity was estimated by measuring their swelling in aqueous solvent at different temperatures and pH values (Activity A1.1).

Another direction that was follow in order to achieve the objective O1 was to synthesized ionic microparticles by crosslinking the alternant copolymer of maleic acid and styrene, followed by the covalent attachment of thermo-sensitive oligomers (Activities A1.2 and A1.3). Due to the fact that potential applications of these microparticles are related to controlled drug delivery, the microparticles were loaded with model drugs: diphenhydramine and metoclopramide. The release of the dugs was studied in buffer solutions that simulated the pH from the gastro-intestinal tract.

In order to attain the objective O2 "**Obtaining and characterization of micro-/nano-particles based on interpolymeric complexes**", the interaction between poly(maleic acid-alt-styrene) and poly(N-vinylcaprolactam) was studied (Activity A.2.1), then spheric particles were obtained based on these interpolymer complexes (Activity A.2.2). The entrapment and the release of the drug from the obtained microparticles were then studied (Activity A.2.3)**.**

O1 "Synthesis and characterization of stimuli-sensitive micro-/nano- particles by cova s-linking of maleic copolymers"

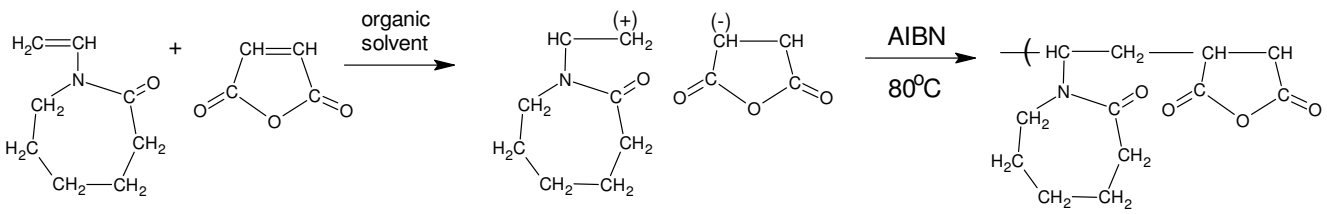
A 1.0 . Synthesis and characterization of poly(N-vinylcaprolactam – maleic acid) copolymer (VCL-MAc)

Thermosensitive polymers dehydrate with the increase of the temperature, forming aggregates above the low critical solution temperature (LCST). Poly(N-izopropylacrilamide), the most studied polymer, have problems of biocompatibility. Poly(N-vinylcaprolactam) was also thermosensitive, but have a better biocompatibility because it does not decompose at hydrolysis. The LCST of this polymer is between 31 and 38^{o}C, depending of the molar mass [1] and concentration [2, 3].

Maleic acid copolymers are pH-sensitive, the dissociation of the carboxylic groups and the conformation of the polymeric chain being determined by the pH [4]. Maleic anhydride/acid copolymers with divinylether, N-vinylpirrolidone, styrene, methyl vinyl ether proved to have important pharmaceutical and medical applications [5]. The two adjacent carboxylic groups determine the dissociation in two steeps, that confere an enlarged pH-sensitivity to that copolymers. The biocompatible copolymer of maleic acid with N-vinylpirrolidone was used as support for covalent attachment of drugs in polymer-drug conjugates [6]. Because N-vinylcaprolactam is the homologue of N-vinylpirrolidone, both being cycling amines, it is expected that the N-vinylcaprolactam – maleic acid copolymer (VCL-MAc) to be also biocompatible.

One of the aim of this project was to obtain VCL-MAc copolymers sensitive both to pH and temperature. This copolymers were synthesis by radical copolymerization of N-vinylcaprolcatam (VCL) with maleic anhydride (MAn) in organic solvent followed by the hydrolysis of the obtained copolymer, but also by radical copolymerization of VCL with maleic acid (MAc) in aqueous solvent, when VCL-MAc copolymer is directly obtained. Better yields were obtained in aqueous solution. The copolymers were characterized by the determination of their composition and by the study of their behavior in aqueous solution. The LCST of the copolymer was between 31 and 35^{o}C, depending on the copolymers composition, concentration, but also on the solution pH. Another interesting behavior of this copolymers is their lack of solubility at acidic pH.

Radical copolymerization of maleic anhydride (MAn) with N-vinylcaprolactam (VCL) in organic solvents was realized using azobisisobutyronitrile (AIBN) or benzoyl peroxide (POB) as initiators. The reaction conditions were presented in Table 1, the monomers concentration being 5 moles/L. The reactions were conducted for 8 hours at 80^{o}C, then the reaction mixture was precipitated into diethyl ether, and the obtained VCL-MAn copolymer was dried at reduced pressure for 48 h. A charge transfer complex was formed between MAn (electron acceptor) and VCL (electron donor): a yellow color appear when the two monomers are put in contact in organic solvents. That charge transfer complex participate into copolymerization process, leading to the formation of alternant sequences of VCL-MAn into the copolymer (Scheme 1). That explain the high content of MAn in the copolymers (Table 1) regardless of the ratio between the monomers in the reaction feed. The VCL-MAn copolymer is soluble in dimethyl sulfoxide, dimethylformamide, acetone, aqueous NaOH solution, but it is insoluble in water, chloroform, ethylic ether, hexane, etc.



Scheme1. Copolymerization reaction of VCL with MAn in organic solvent

Table 1. Reaction conditions and characteristics of VCL-Man copolymers

Probe code	Reaction conditions			Reaction yield	% MAn in the copolymer	
	Solvent	VCL: Man ratio in the reaction feed	Initiator		Determined by conductometry	Determined by potentiometry
O1	Benzene	90:10	AIBN, 0.3%	5.2%	39%	40%
O2	Benzene	97:3	AIBN, 0.3%	6%	24%	25%
O3	DMF	95:10	POB, 0.3%	6.5%	37%	38%
O4	Dioxane	95:5	AIBN, 0.3%	7.7%	37%	37%

The copolymers composition was determine by conductometric titration in acetone: water mixture and by potentiometric titration in aqueous solution. The conductometric titration curve of VCL-MAn copolymer (sample synthesized in DMF) with 0.1 M NaOH is presented in Figure 1.The potentiometric titration curve in the presence of $CaCl_2$ 0.002 M together with the first derivative is presented in Figure 2. The maleic acid copolymers dissociate in two steeps because the two adjacent carboxylic groups have different strength, but in order to reveal both carboxylic groups in potentiometric titration, the addition of a uni-uni-valent or even di-uni-valent electrolyte is necessary. [7]. Thus, a low concentration of $CaCl_2$ lead to the obtaining of two equivalent points (inflection point in the potentiometric curve or maximum in the first derivative curve), the volume of NaOH for the second equivalent point being double the volume for the first equivalence point (Figure 2). The content of MAc in the copolymers, determined from these volumes is presented in Table 1. A good accordance was observed between the two electrochemical methods: potentiometry and conductometry

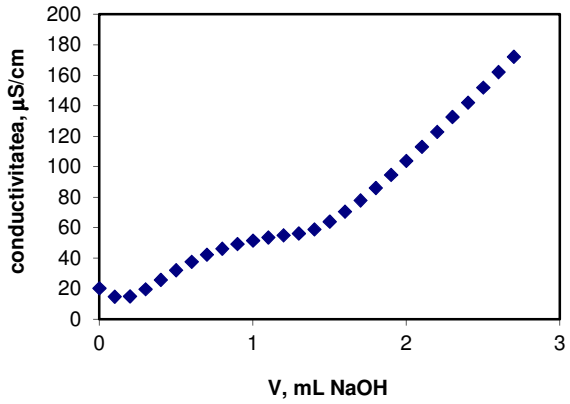


Figure 1. Conductometric titration curve of VCL-MAn copolymer (O3 sample)

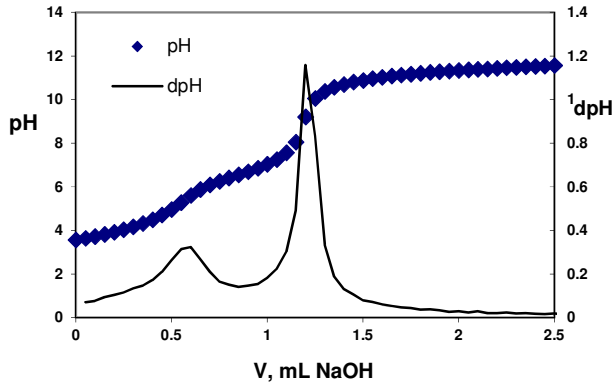


Figure 2. Potentiometric titration curve of VCL-MAn copolymer in the presence of $CaCl_2$ (O3 sample)

The structure of the obtained copolymers was confirmed by FTIR spectroscopy. Figure 3 presents the spectrum of a VCl-MAn copolymer compared with the spectrum of the homopolymer poly(N-vinylcaprolactam). The copolymer presents the characteristic picks for caprolactam: 1635 cm^{-1} (C=O from amidic group), 1482 and 1444 cm^{-1} (methylene groups from the caprolactam ring). In addition to this peaks, in the copolymer spectrum the appearance of the characteristic peaks for maleic anhydride at 1855 and 1780 cm^{-1}, and for carboxylic groups from the hydrolyzed maleic groups at 1728 cm^{-1}.

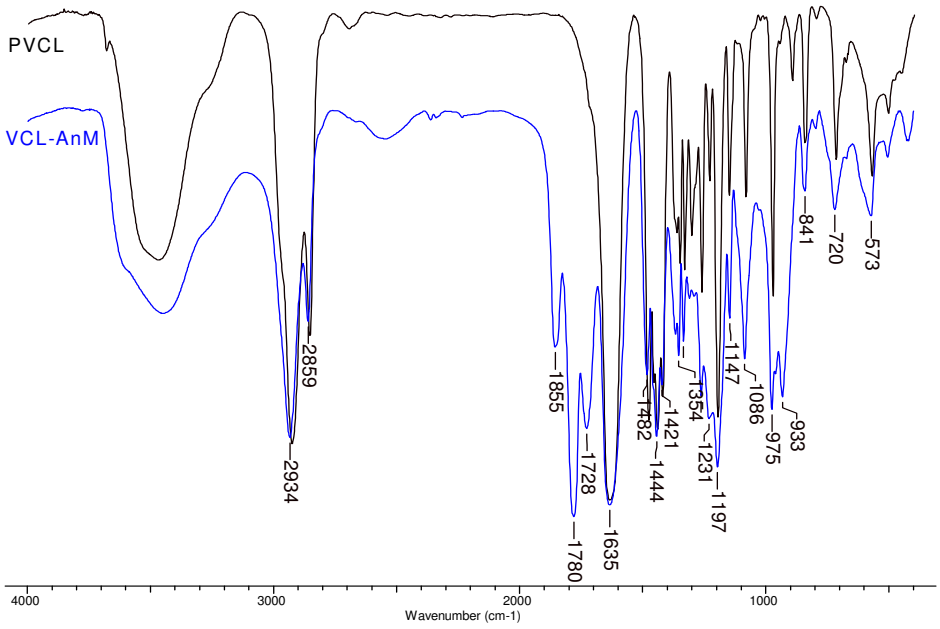


Figure 3. FTIR spectrum of VCL-MAn copolymer compared with the spectrum of poly(N-vinylcaprolactam)

The ^{1}H NMR spectrum of the VNC-MAn copolymer is presented in Figure 4, together with the attribution of the signals. The composition of the copolymer can be calculated taking into account that protons from maleic anhydride appears at 3-4 ppm together with the methinic proton from VCL, and the signal from 1.5-2 ppm is attributed to the 8 protons from VCL ring. A 40 % MAn composition was calculated for the copolymer synthesized in DMF, this composition being in accordance with the other methods.

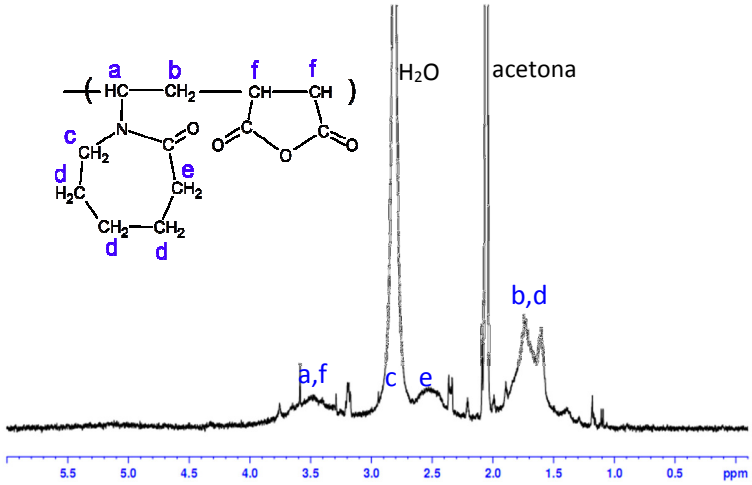


Figure 4. ^{1}H NMR spectrum for the VCL-MAn copolymer (sample O4) in d6-acetone.

The thermo-sensitivity of the obtained copolymers was studied by measuring the optical density of the copolymer solution in water with the increase of the temperature. In this madia the anhydride cycles were hydrolyzed with the formation of maleic acid units on the polymeris chains. The solution was placed into a thermostated cell that was connected to an external thermostatic bath. In Figure 5 are presented the turbidity profiles for two samples of VCL-MAc copolymer. The increase of the turbidity (100-T%, where T represents the transmittance) was due to the insolubility of the copolymer is solution. Due to the fact that the solution opalescence is different at different samples, LCST was taken as the temperature where the first signs of turbidity appear. The LCST values (at pH=8, Cp=3 g/L) was 33^{o}C for the sample O4 (with 25 %

MAc) and 34°C for the sample O2 (with 37% MAc), proving that the LCST increase with the MAc content in the copolymer.

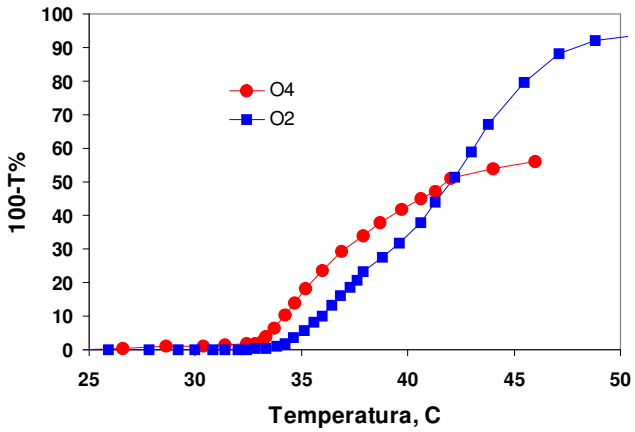
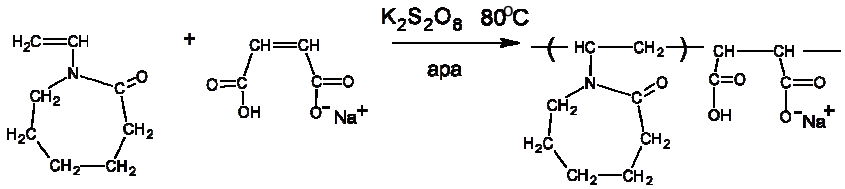


Figure 5. Turbidity profiles with the temperature variation for VCl-MAc copolymers solution (samples O2 and O4, cp=3 g/L, pH=8)

The reaction yield in organic solvents was very low (between 5 and 8%), as it can be seen from Table1, that led us find another method to synthesize this copolymers: in aqueous solution. The maleic anhydride monomer hydrolyzed with the formation of two carboxylic acids that does not formed a charged transfer complex with VCL, leading to the possibility to obtain copolymers with a lower maleic acid content

Copolymerization reaction of VCL with MAc in water. Maleic anhydride was dissolved in water, then after complete hydrolysis and transformation into MAc, the solution pH was adjusted to 5.5 using 1M NaOH. VCL was added under vigorous stirring, when a suspension was formed. Dried nitrogen was bubbled for 60 min, then KPS was added and the temperature was raised to 80°C, when a clear solution was formed. After 8 hours, the reaction mixture containing a white precipitate was cooled down and the resulting viscous solution was diluted with water. The copolymers were purified by dialysis (MWCO cut off 12 – 14,000 g mol-1) and recovered by freeze-drying. The ratio between the monomers in the reaction feed were given in the Table 2, and the reaction is presented in Scheme 2. The reaction yield was between 50 and 80 %.



Scheme 2.Radical copolymerization reaction of VCL with MAc in aqueous media

Table 2. Reaction conditions and characteristics of VCL-MAc copolymersobtained in aqueous solution

Sample code	VCL:Mac ratio in the reaction feed	% AcM in the copolymer		Molar mass Mw, determined by GPC
		Determined by NMR	Determined by potentiometry	
VCL-AcM6	95 : 5	-	6 %	33500
VCL-AcM10	92 : 8	12 %	9 %	-
VCL-AcM16	88 :12	18 %	16 %	-
VCL-AcM18	82 : 16	21 %	22 %	64000
VCL-AcM40	75 : 25	41 %	40 %	-

The copolymer structure was confirmed by FTIR spectroscopy. In the copolymers spectrum, presented in Figure 6, it can be observed the characteristic peaks for VCL units (1635 cm^{-1} –C=O from amide, 1482 și 1444 cm^{-1} -CH_2- stretching), but also the characteristic peaks of the carboxylic groups from MAc at 1725 cm^{-1}. The ratio between the intensity of the carboxylic peaks and the intensity of the amidic peaks increase with the increase of the MAc content in the copolymer from sample VCL-AcM16 to sample VCL-AcM40.

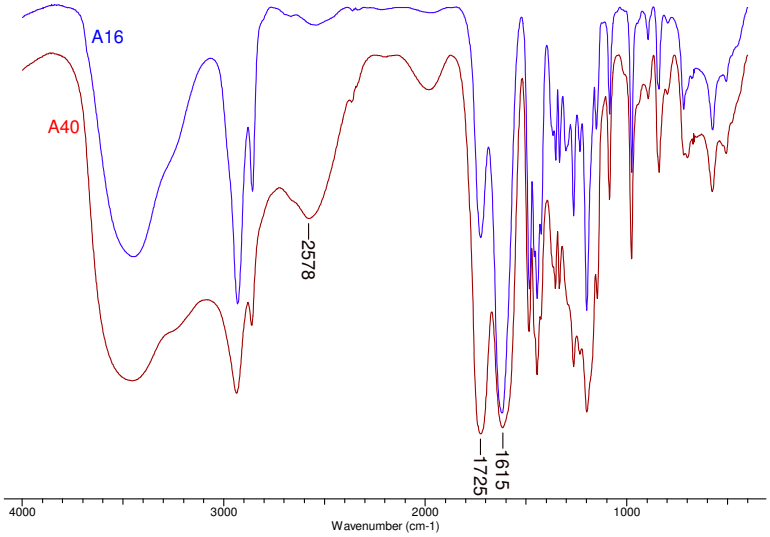


Figure 6. FTIR spectra of VCL-MAc copolymers, samples A16 and A40

The ^1H NMR spectra of the VCL-MAc copolymers is presented in Figure 7, together with the assignment of the signals. The ratio between the monomers was determined from the integrated area of the signal between 2 and 2.7 ppm (A_{efg}), where methine protons of maleic acid are overlapped with $OCCH_2$-protons from caprolactam cycle, and from the area of the signal between 3-3.5 ppm (A_c) ascribed to -NCH_2- protons of VCL. The molar fraction of MAc units can be calculated as

$$\%MAc = \frac{A_{efg} - A_c}{A_c} * 100$$

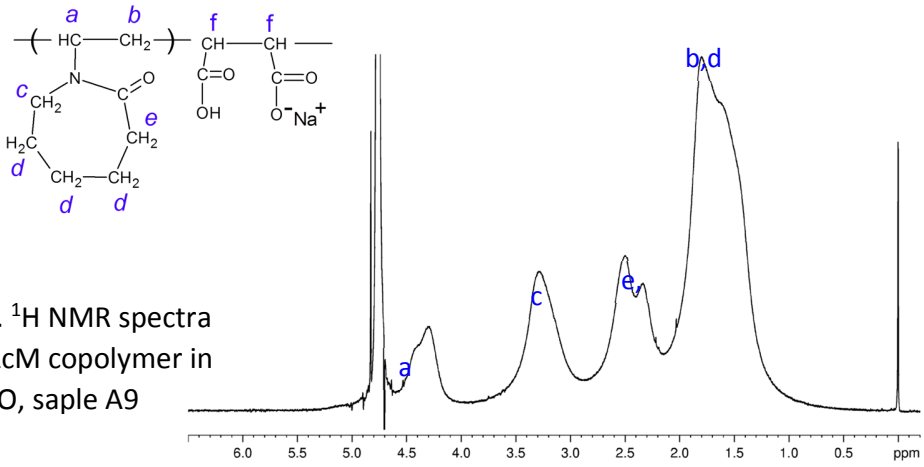


Figure 7. ^{1}H NMR spectra of VCL-AcM copolymer in D_2O, saple A9

Potentiometric titration was also performed in order to confirm the copolymers composition. The purified copolymers presents carboxylic acid groups, but also dissociated carboxylate groups, so they could be titrated with NaOH, but also with HCl, as it can be seen from Figure 8. It also be specified that at the addition of HCL, when the pH decrease, the copolymers become insoluble due to the hydrogen bonds between the amidic C=O groupfrom VCL and the protonated COOH groups from MAc.

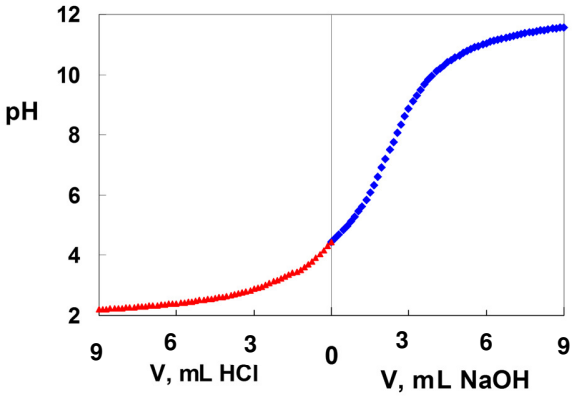


Figure 8. Potentiometric titration of VCL-MAn copolymer in pure water with NaOH and with HCl (sample VCL-MA22)

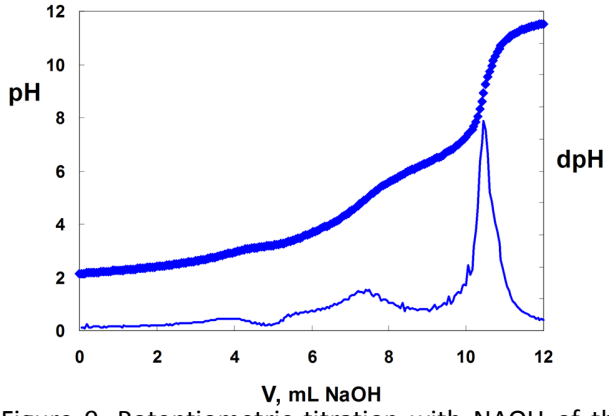


Figure 9. Potentiometric titration with NAOH of the VCL-MAc copolymer in the presence of an excess of HCl and 0.005 M $CaCl_2$ (sample VCL-MAc22)

In pure water, only one of the carboxylic groups can be evidenced by potentiometric titration. In order to evidence both groups and to determine the composition, an excess of HCl and $CaCl_2$ (0.005 M) were added on the copolymer solution. The titration was then performed with NaOH

0.1 N. The potentiometric titration curve and the first derivative were presented in Figure 9. At small volumes of NaOH (4.1 mL), the first derivative present a weak maximum corresponding to the titration of HCl in excess, at 7.3 mL NaOH the inflection point of the potentiometric curve and the maximum of the derivative correspond to the titration of the first carboxylic groups, and at 10.5 mL NaOH the titration of the second carboxylic groups can be observed. The content of maleic acid in the copolymer was calculated using the difference between the base volumes corresponding to the last two equivalence points. The results, in good agreement with those determined by NMR, were presented in Table 2.

Phase transition of VCL-MAc copolymers in aqueous solution

The thermosensitivity of poly(N-vinylcaprolactam) is given by balance between the hydrophilic (carbonyl from amidic groups) and hydrophobic groups (methylene groups from caprolactam ring). In the case on VCL-MAc copolymers, besides the hydrogen bonds with water molecules and hydrophobic forces, intra- and inter-molecular hydrogen bonds between COOH groups of MAc and –C=O groups from VCL also interfere.

Temperature-sensitivity and the effect of the pH on the temperature sensitivity of the copolymers in solution was followed by turbidimetry and dynamic light scattering. The solution pH was measured with the pH electrode connected to a potentiometric titration unit, and the pH was adjusted to the desired value with 0.1 NaOH or 0.1 HCl.

Influence of the copolymer concentration

The phase separation of the VCL-AcM copolymer with 22%MAc in the composition was followed at pH=8 at two concentrations: 1g/L and 3 g/L. The absorption at 450 nm (A_{450}) was measured by UV-vis spectroscopy, the temperature being modified with 1^oC every 10 minutes until the phase separation and with 0.2^oC every 10 minutes around the LCST. The LCST was considered the temperature where A_{450} begin to increase. As it can be seen from Figure 10, the LCST decrease with the concentration, being 34.6^oC at 1 g/L and $32.6\ ^oC$ at 3 g/L.

The dynamic light scattering (DLS) studies (Figure 11) show the same behavior for the two concentrations. The conformational changes of the polymeric chains and the formation of the nano-aggregates was evidenced by DLS. At 1 g/L, the hydrodynamic diameter (Dh) decrease between 34 and 35^oC by the collapsing of the polymeric chains, and between 35 and 36^oC, the intermolecular aggregation occurred, 210-270 nm aggregates being formed. At 3 g/L, the chains collapsed until 32^oC, after that some of them formed 230 nm diameter aggregates, some of them remained free in solution. With the increase of the heating, the aggregates and the individual chains continue their collapsing up to 36^oC, but over this temperature all the macromolecular chains were included into larger aggregates (Dh around 250 nm). Due to the fact that at 3 g/L, above 32^oC, the measurement indicate the presence of two populations, we worked hereinafter at 1g/L, when the presence of a single population indicate a uniform aggregation.

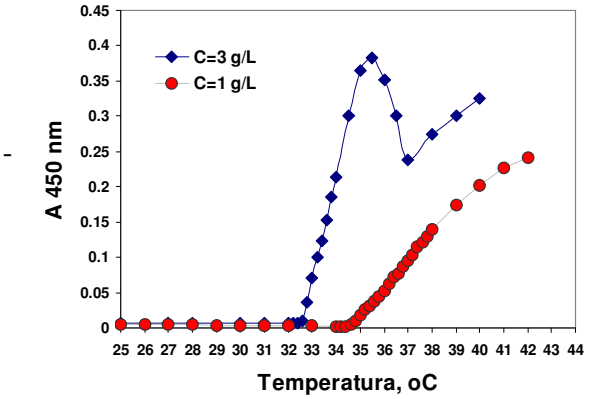


Figure 10. Thephase transition profiles of VCL-MAc 22 copolymer solutions at two concentrations: 1 g/L and 3 g/L.

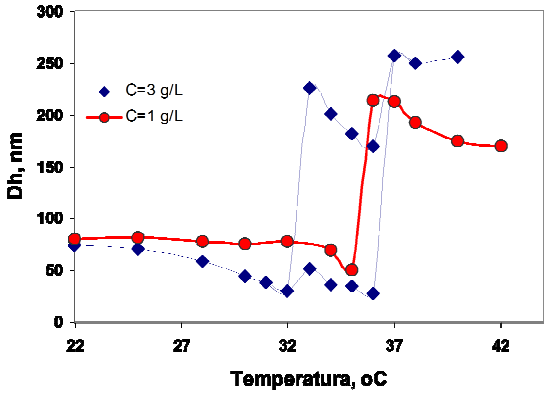


Figure 11. The variation of the hydrodynamic diameter of VCL-MAc copolymer with the temperature at two concentrations: 1 g/L and 3 g/L.

Influence of the copolymer composition

The maleic acid copolymers were proved to formed hydrogen bonds between the adjacent carboxylic groups, the more bonds being at semi-neutralization, when one of the carboxylic groups was dissociated and the other one is in acid form [8].In the case of VCL-MAc copolymer, hydrogen bonds also formed between maleic –COOH groups and C=O groups from caprolactam, that assure the water solubility of the VCL units (by hydrogen bonds with water molecules). At acid pH, when both carboxylic groups became protonated, intermolecular hydrogen bonds and the hydrophobicity of the caprolactam groups lead to the aggregation and to the phase separation.

In Figure 12 is presented the increase of the turbidity with the decrease of the pH for solutions of VCL-MAc copolymers with different composition. The solutions become turbid with the decrease of the pH between pH= 3.5 and 2.5, even at room temperature. With the increase of the MAc content in the copolymer, the transition pH have higher values and the turbidity of the solution at acidic pH increase due to the increase number of hydrogen bonds.

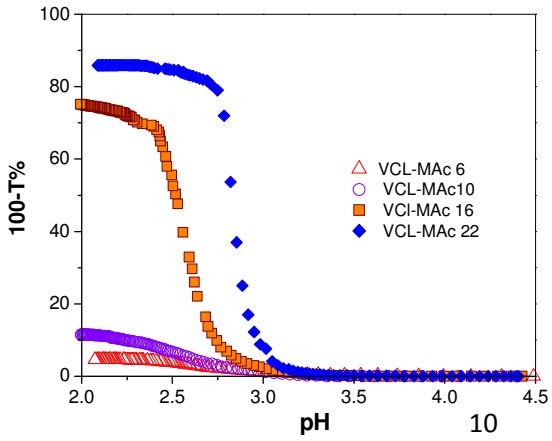


Figure 12. Turbidity increase with the decrease of the solution pH for VCL-MAc copolymers (C_P = 8 g/L, 22 C)

In Figure 13 is presented the modification of the A_{450} with the increase of the temperature for the copolymers solutions (at 1 g/L and pH=8). With the increase of the MAc content in the copolymer, the LCST moved through smaller values, and the turbidity of the system was lower. This was explained by the fact that at pH=8 the carboxylic groups are dissociated and contribute to the increase of the copolymer hydrophobicity, increasing the LCST. In the Figure 14 the increase of the LCST with the increase of the MAc content is presented. At 1g/L, but also at 3 g/L, this increase was linear as far as 22% MAc. It should be mentioned that no phase transition was observed for the copolymer with 40% MAc, meaning that a high MAc content disturb the continuity of VCL units that is responsible for the thermosensibity of the copolymer.

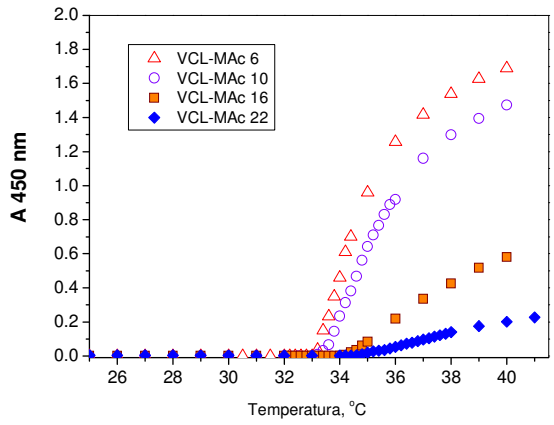


Figure 13. LCST profiles for VCL-MAC copolymers with different compositions (concentration 1 g/L, pH=8)

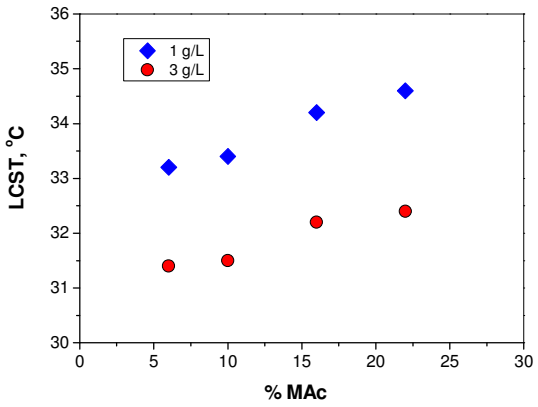


Figure 14. The influence of the MAc content in the copolymer on the LCST, at two concentrations.

The influence of the pH

The influence of the pH on the LCST was studied for the copolymer with the high content of MAc that also present temperature-sensitivity, VCL-MAc22. The pH is known to influence the dissociation of the carboxylic groups and the conformation of the macromolecular chains in solution [4], but in the case of these copolymers, the pH also influence the formation of hydrogen bonds. In Figure 15 is presented the variation of the A450 with the temperature at different pH values. The influence of the pH on the LCST is presented in Figure 16. At basic pH values, the LCST do not vary with the pH. With the decrease of the pH below 5, the LCST increase, being 40°C at pH=3.9, but after that it decrease at room temperature. Such a behavior was found in the

literature for the copolymer of VCL with methacrylic acid, where hydrogen bonds were also formed between the monomeric units [9].

In Figure 17 is presented the variation of the hydrodynamic diameter of VCL-MAc copolymer with the increase of the temperature, at different pH values. A good accordance between DLS and UV measurements was observed. The increase of A_{450} in the order: pH=3.9, pH=5, pH=8, pH=4.2 match with the order of the hydrodynamic diameters of the aggregates formed above the LCST. At pH=5 and pH=8, the collapsing of the chains above the LCST can be seen in UV measurements by the appearance of the opalescence, and in DLS by the increase of the chains hydrodynamic diameter. At pH=4.2, one of the two carboxylic groups are dissociated, together with a part of the adjacent ones, so they begin to form hydrogen bonds with the amidic groups of VCL and those to increase the copolymer hydrophobicity. The macromolecular chains have a collapsed conformation at pH=4.2 compared to pH = 5 or 8, even at the room temperature, at can be seen from the lower hydrodynamic diameter (Figure 17). Due to that fact, the aggregation of the chains was retarded and the phase transition was observed at higher temperatures. The same thing occur at pH=3.9, when the aggregates formed only above 40°C, and their dimensions was very low, so the opalescence of the solution was very weak and cannot be visually detected (A_{450} have small values even above 40°C).

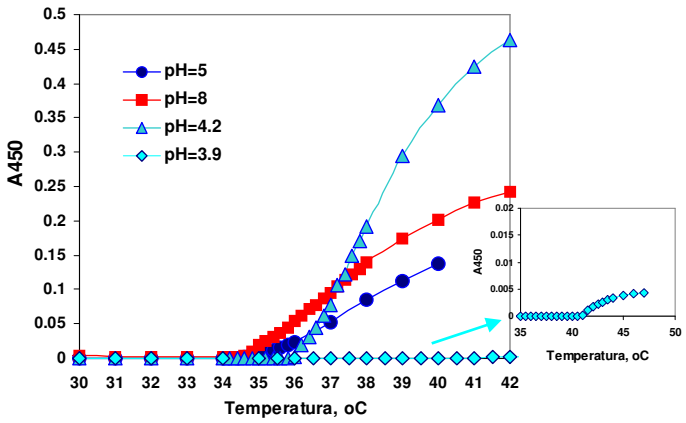


Figure 15. LCST profiles for VCL-AcM22 copolymer, at different pH values (c=1g/L)

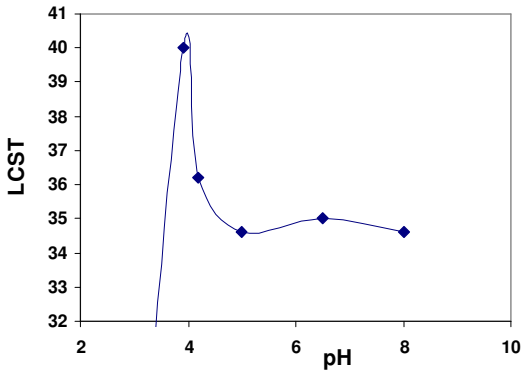


Figure 16. LCST variation with the pH for VCL-AcM22 copolymer

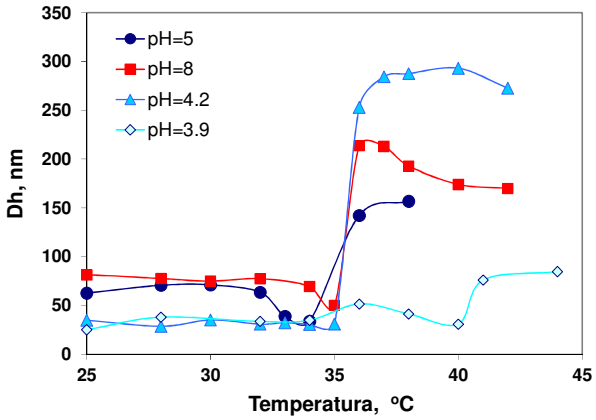


Figure 17. Variation of the hydrodinmic diameter of the VCL-MAc22 chaines/aggregates with the increase of the temperature (c=1g/L).

A 1.1. Obtaining and characterization of microparticles by the cross-linking of N-vinylcaprolactam - maleic acid copolymers

In order to cross-linked the thermo- and pH-sensitive copolymers base on N-vinylcaprolactam and sodium maleate, it was necessary to transformed the copolymers into acid form when they were soluble in organic solvents such as DMSO or dioxane. HCl was added into the aqueous solution of VCL-MAc copolymer until the pH was 2.5, and the precipitated copolymer was heated and then filtrated. The obtained copolymer in acid form was dried at reduce pressure and 80°C for 48 hours. The transformation of the carboxylate groups into carboxylic acid groups could be evidenced by FTIR spectroscopy (Figure 18) by the disappearance of the shoulder from 1580 cm^{-1} characteristic for the $-COO^-$ groups and by the increase of the peak from 1725 cm^{-1} characteristic for the COOH groups.

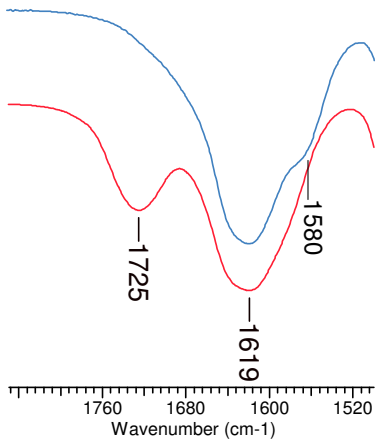
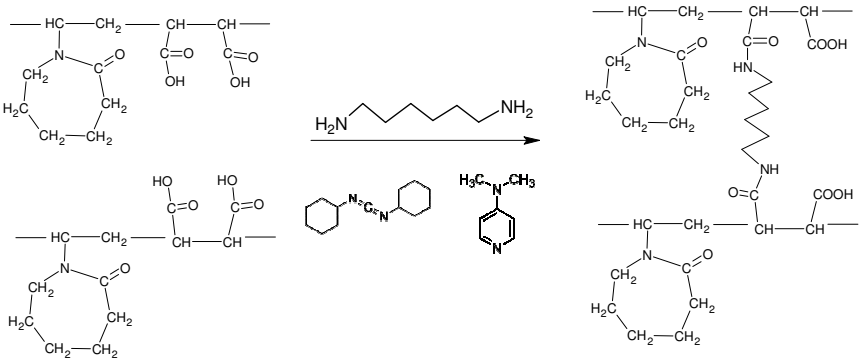


Figure 18. A detail of the FTIR spectra of the VCL-MAC copolymer in the form of sodium salt (up) and in the acid form (down)

The VCL-MAc copolymer was then cross-linked with hexamethylendiamine. The amidation reaction took place in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine, according to Scheme 3.



Scheme 3. Cross-linking reaction of VCL-MAC copolymer used for the obtaining of MCL microparticles

In order to obtained microparticles (MCL), the cross-linking was made in suspension: 1.5 g VCL-MAc copolymer in acid form was solubilized in 12.5 mL DMSO by heating the solution at 70^{o}C, and after cooling, 0.81 g dicyclohexylcarbodiimide was added and the DMSO solution was dispersed under stirring (450 rpm) into 50 mL paraffin oil that contained 3.3 g Spam 80 (as dispersing agent). After 30 min 0.38 g hexamethylendiamine and 0.096 g 4-dimethylaminopyridine dissolved into 2 mL DMSO were added, and the stirring was mentained for 6 hours at room temperature. The obtained microparticles were washed with diethyl ether, methanol, water, acetone, in this order. The reaction yield was around 60 %.

The structure of the MCL microparticles was confirmed by the FTIR spectrum (Figure 19) by the presence of the carboxylic acid groups at 1724 cm^{-1}, of the caprolactam ring at 2935, 2860 cm^{-1} , of the amidic C=O groups at 1622 cm^{-1}, C-N at 1480 cm^{-1}. The new amidic bonds and the amidic groups from VCL units are overlapped at 1622 cm^{-1}

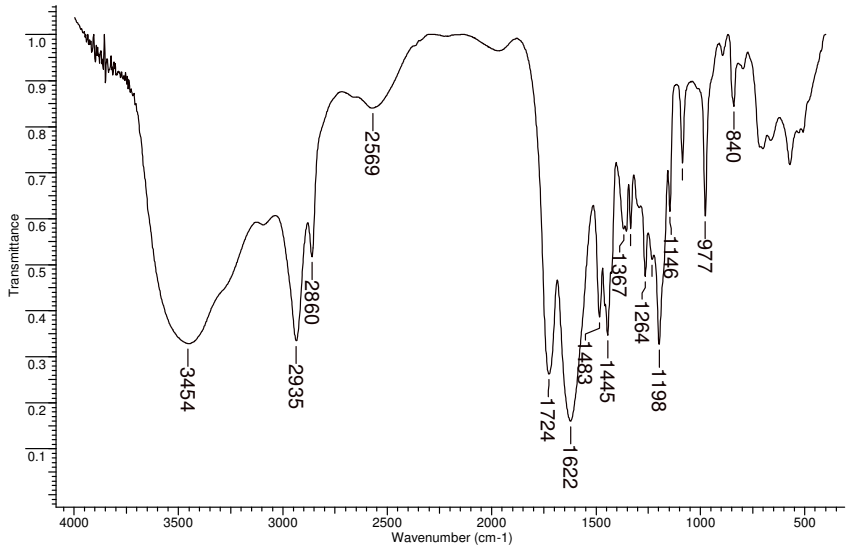


Figure 19. FTIR spectrum of MCL microparticles

Scanning electron microscopy (SEM) shows that the obtained microparticles have dimensions between 100 and 200 μm, but are formed from united 2-4 μm microspheres (Figure 20). Thus, the microparticles have a high specific surface.

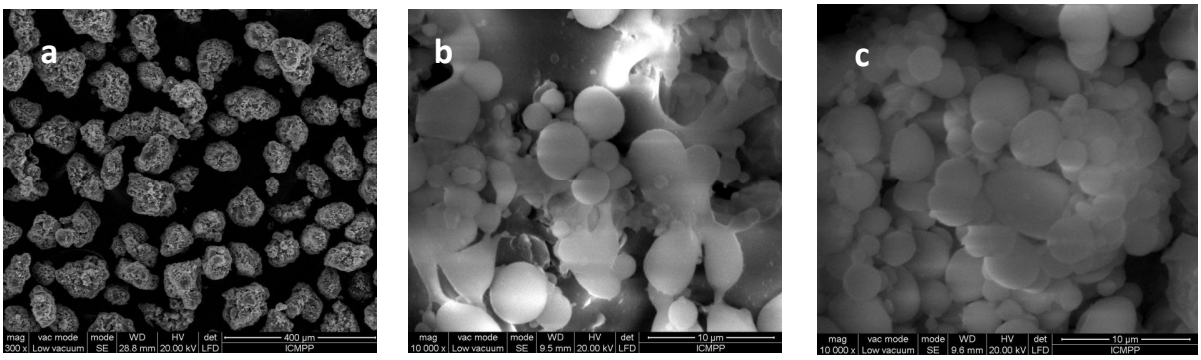


Figure 20. SEM pictures of MCL microparticles (a) and surface detailes (b and c)

The exchange capacity of MCL particles was determined by indirect titration of the carboxylic groups: 0.1 g microparticles were swollen for the night in 10 ml 0.1 M NaOH solution. The concentration of the NaOH solution after the neutralization of the carboxylic groups was determined by potentiometric titration of 5 mL supernatant solution with 0.1 M HCl. The exchange capacity of MCL particles was $2,8*10^{-3}$ eq/g.

In order to highlight the influence of the pH on the swelling of the microparticles in aqueous solution, the water/buffer solution retention was measured using the centrifugation method [10]. The dry microparticles (0.1 g) were placed into stainless steel baskets that have a sieve and a filter paper at the bottom, and the baskets were immersed into water with a certain pH (adjusted with NaOH or HCl), or into buffer solutions. After the swelling of the microparticle (24 hours), the baskets were centrifuged at 1000 rpm for 15 minutes, when only the swell microparticles remained, without the solvent in excess. The solvent retained into the microparticles, measured by weighing, was reported to the amount of dry microparticles.

In the Figure 21 is presented the water retention into the microparticles with the variation of the pH (the pH was adjusted with NaOH or HCl). The water retention was constant between pH=2 and pH=6, but it increase with the increase of the pH above pH=6, with the dissociation of the second carboxylic groups from the maleic units. Above pH=9, when both carboxylic groups were dissociated, the water retention remained constant (13 g water/g miclopartilces) with further increase of the pH.

The solvent retention of buffer solutions: pH=7.4, pH=4 and pH=1.2, into microparticles are presented in Figure 22. The solvent retention also increase with the pH. In phosphate buffer solution pH=7.4 water retention was 9 g solution/g microparticles. With the increase of the temperature from the room temperature (22^{o}C) to 40^{o}C, the solvent retention decrease, but the modification was not significant.

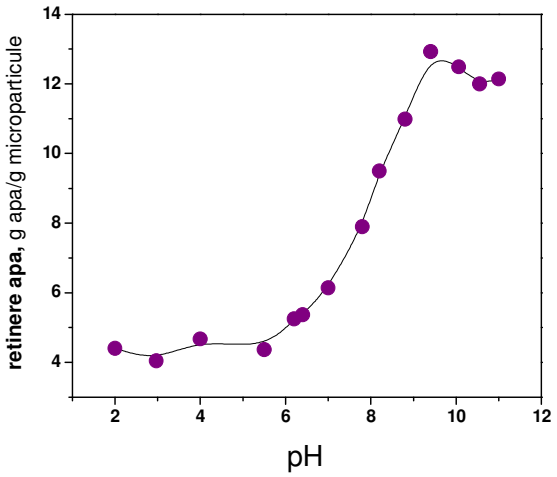


Figure 21. Influence of the pH on the water retention in the MCL microprticles

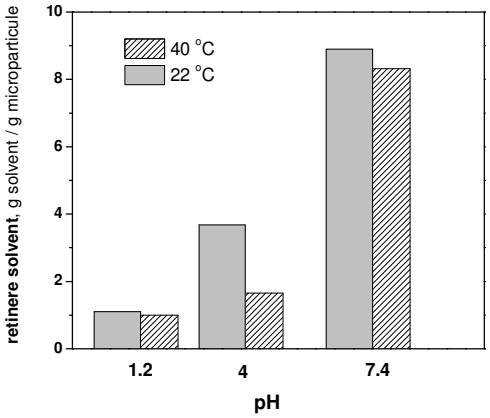
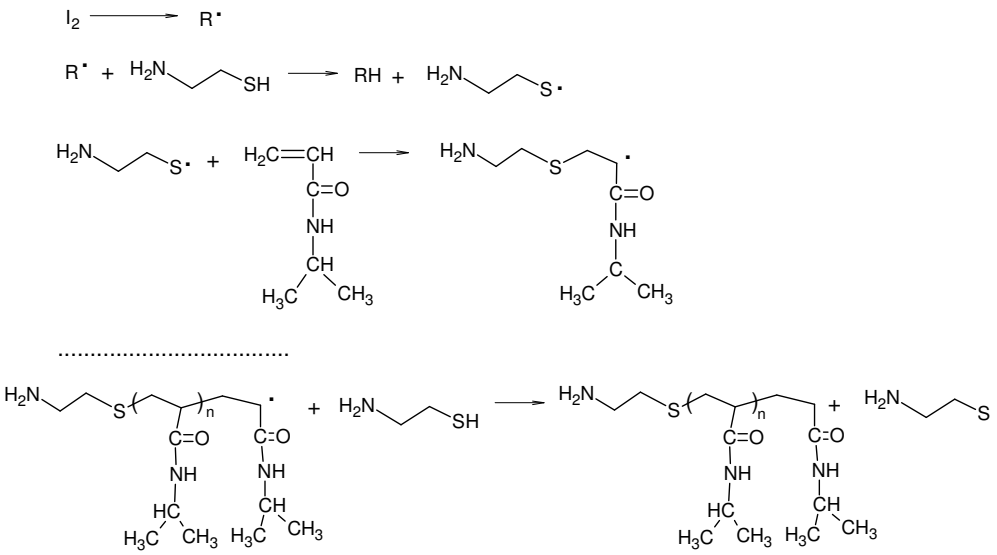


Figure 22. Influence of the temperature on the buffer solution retention into the MCL microparticles

A 1.2. Synthesis and characterization of thermosensitive oligomers

The semitelechelic oligomers were synthesized by radical copolymerization of N-isopropylacrylamide in the presence of cysteamine hydrochloride as chain transfer agent, according to Scheme 4. Briefly, 1.3013 g N-isopropylacrylamide (NIPAM), 0.065 g cysteamine hydrochloride and 0.025 g azoizobutironytrile were solubilized in 6 mL dimethylformamide. Dry nitrogen was bobbled for one hour, then the reaction was heated at 80^{o}C and maintained at this temperature for 16 hours. The oligomer was precipitated into diethyl ether and hen dried.



Scheme 4. Obtaining of N-isopropylacrylamide oligomer by radical polymerization in the presence of chain transfer agent

The oligomer structure was confirmed by ^{1}H NMR and FTIR spectroscopy. In the Figure 23 is presented the NMR spectra together with the assignment of the signals.

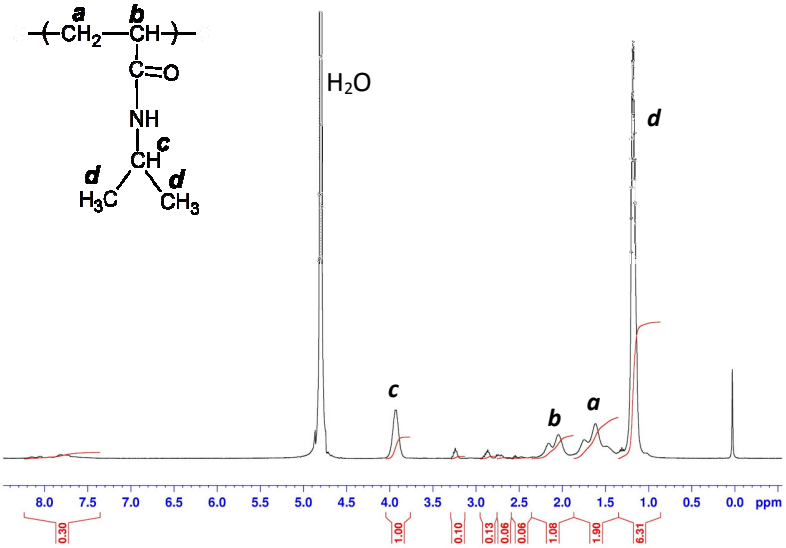


Figure 23. ^{1}H NMR spectrum of NIPAM oligomer

In the FTIR spectrum of the oligomer (Figure 24) asymmetric stretching vibration of $–CH_2$ groups was observed at 270 cm^{-1}, stretching vibration of amidic N-H at 3296 cm^{-1}, amide I at 1649 cm^{-1}, and amide II at 154 cm^{-1} was observed. If the band from 2935 cm^{-1} can be attributed to terminal ammonium groups ($-NH_3^+$), the band from 354 cm^{-1} can be also attributed to the terminal primary amine ($-NH_2$).

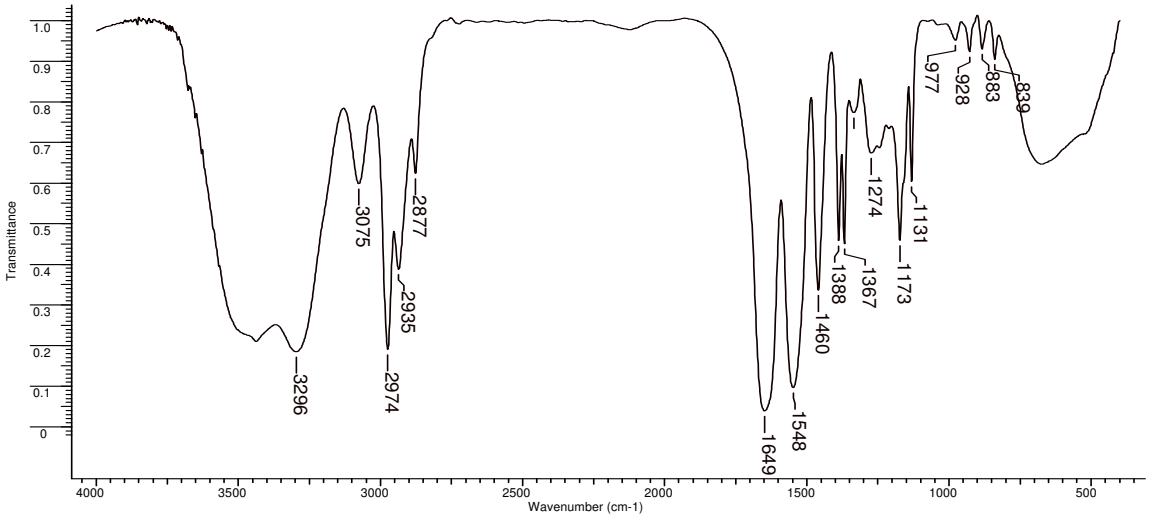


Figure 25. FTIR spectrum of the NIPAM oligomer

In order to transform the terminal ammonium groups into reactive amino groups ($-NH_2$), the oligomer was solubilized into water, passed through an ionic exchange column with a cationic exchange resin Dowex 21 K, then the copolymer solution was lyophilized. The average molar

mass of the oligomer, determined by potentiometric titration of the terminal -NH_2 groups with HCl 0,1 M, was 3600 Da.

It is known that poy(N-isopropylacrylamide) have an LCST around 32°C. In order to determine the LCST of the oligomer the variation of the A_{450} was measured with the increase of the temperature. From Figure 26 it can be seen that cloud point temperature was 31°C.

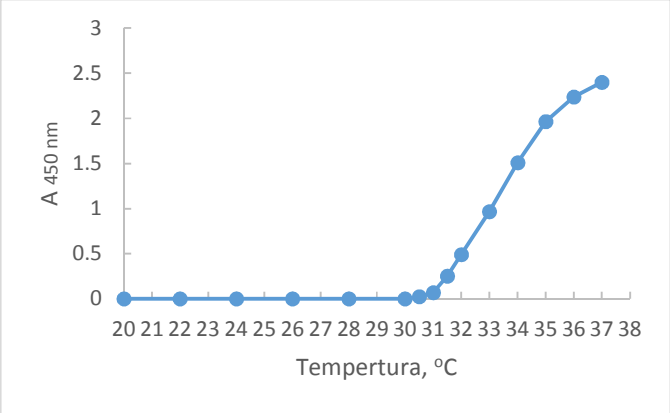
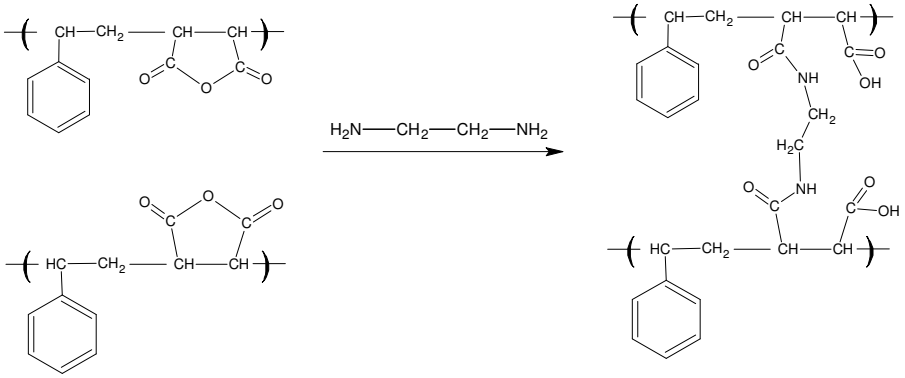


Figure 26. LCST profile of of NIPAM oligomer in aqueous solution (Cp= 1 g/L)

A 1.3. Obtaining and characterization of micro/nano-particles with stimuli-sensitive pendant groups

pH-sensitive microparticles (MPSAM) were obtained by cross-linking of the alternant maleic anhydride-styrene copolymer with ethylene diamine, according to Scheme 5. Briefly, 2 g poly(maleic anhydride-styrene) were dissolved in 17 ml DMSO, and the obtained solution was dispersed in 60 ml mineral oil with 3 g Spam 80. The dispersion was maintained under stirring for 30 min using an anchor-stirrer shaft and a mechanic stirrer, then 0.1485 g ethylenediamine dissolved in 3 mL DMSO as added. The dispersion was maintained under stirring for 12 hours at 60°C. The obtained MPSAM microparticles were washed with diethyl ether (in order to remove the mineral oil and Spam 80), acetone (in order to remove the unreacted copolymer), again with diethyl ether and acetone, then they were dry at reduced pressure.



Scheme 5. Cross-linking reaction of the MAn-styrene copolymer with ethylenediamine

From the FTIR spectrum of MPSAM microparticles (Figure 27) the characteristic bands for the maleic C=O groups can be observed at 1859 and 1774 cm^{-1}, the characteristic bands for C-O-C from the anhydride cycle at 1226, 1079 și 954 cm^{-1}, and the aromatic phenyl ring can be observed at 703 cm^{-1}. The copolymer crosslinking was proved by the appearance of the amide peaks at 1649, 1558 and 1541 cm^{-1}, and by the 1717 cm^{-1} peak corresponding to carboxylic acid groups obtained by the opening of the anhydride cycle.

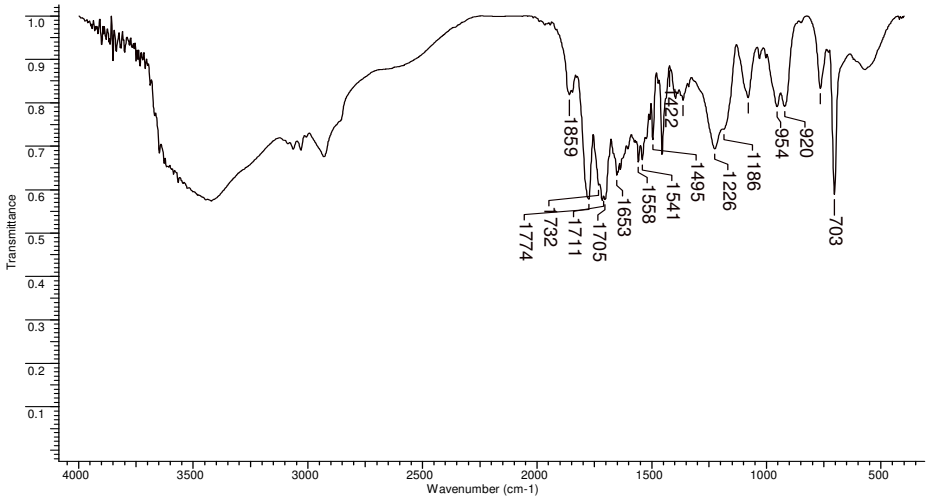


Figure 27. Spectrul FTIR spectrum of MPSAM microparticles obtained by cross-linking of mleic anhydride-styrene copolymer

The morphology of the obtained microparticles was studied by SEM. As it can be seen in the Figure 28, the microparticles dimensions were between 100 and 400 µm, and their surface presents spherical structures of 8-17 µm.

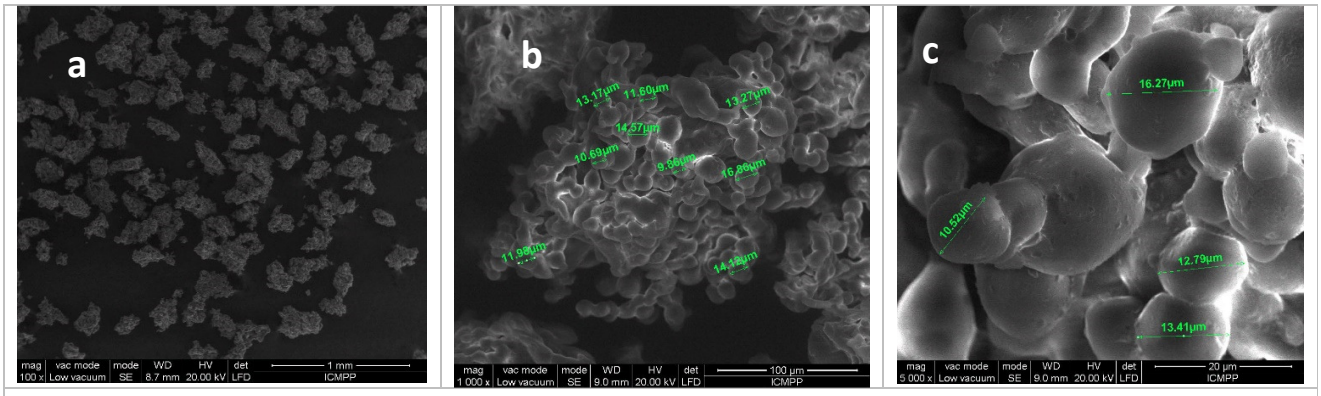
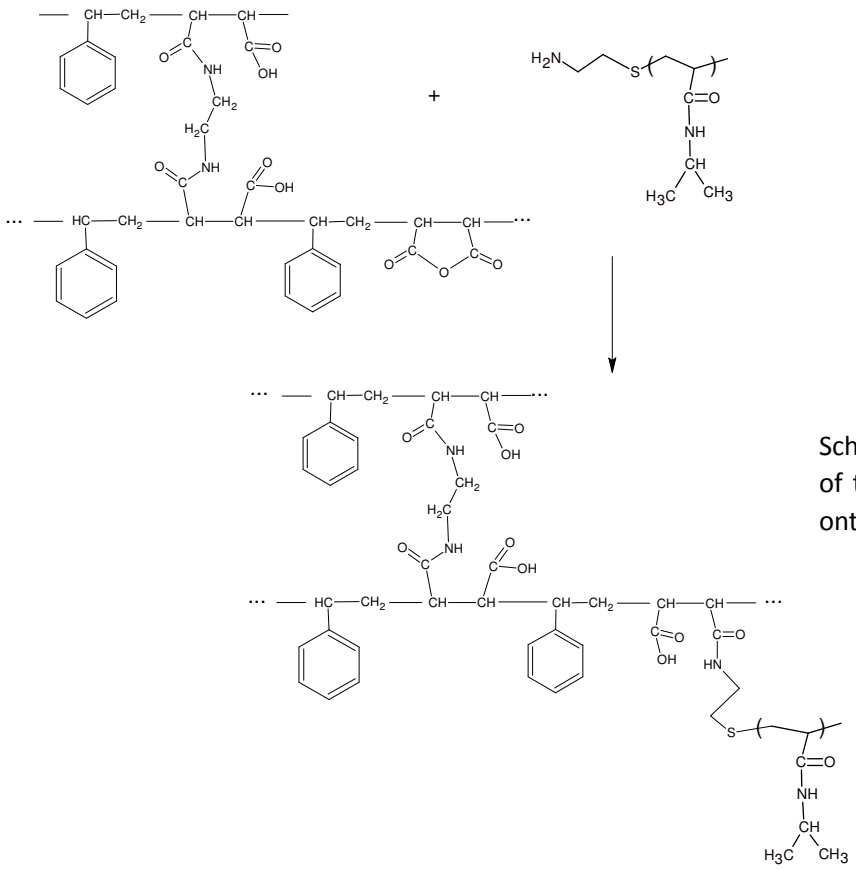


Figure 28. SEM images of the MPSAM microparticles (a) and surface detailes (b și c)

In order to add thermosensitivity to the obtained pH-sesitive maleic anhydride-styrene microparticles, the thermosensitive oligomers with terminal amine groups were added at the remained anhydride cycles. The MPSAM microparticles (0.2g) were swollen in 10 mL DMSO, then semitelechelic oligomers (0.32 g) dissolved into DMSO (5 ml) were added and the reaction was mentained at 80°C for 24 hours. The coupling reaction, with the obtaining of MPSAM-NIPAM microparticles was presented in the Scheme 6.



Scheme 6. The coupling reaction of the semitelechelic oligomers onto MPSAM microparticles

The FTIR spectrum of MPSAM-NIPAM microparticles is presented in Figure 29. Compared with the spectrum of MPSAM microparticles (Figure 27), in the spectrum of the grafted microparticles the characteristics peaks of methylene groups from NIPAM were observed at 2974 and 2877 cm^{-1}, and the peaks for the new amide groups were presented at 1653 and 1541 cm^{-1}.

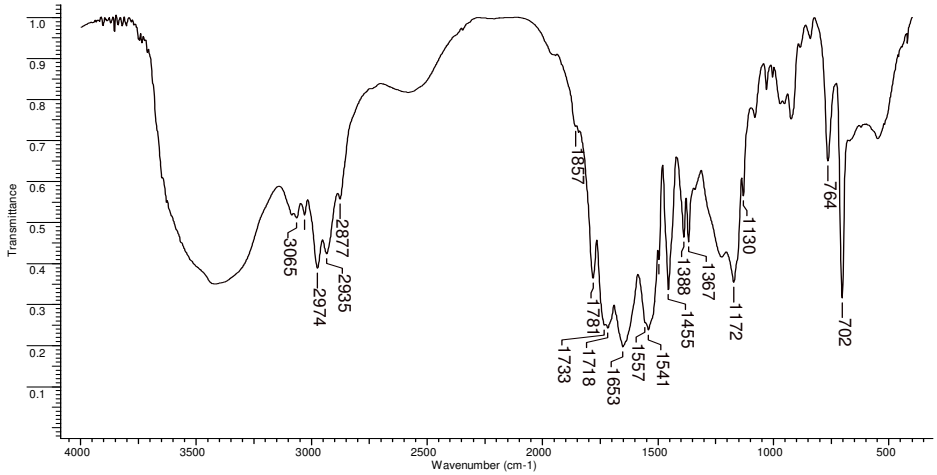


Figure 29. FTIR spectrum of MPSAM-NIPAM

The exchange capacity of the MPSAM was 4,96*10^{-3} echiv./g, and of the MPSAM-NIPAM microparticles was 2,92*10^{-3} echiv./g. Using that values, the substitution degree of the carboxylic groups for the derivative micropaticles was calculated as being 41%.

Temperature sensitivity of the grafted microparticles was verified by measuring the water retention capacity at different temperatures. The blue dextran method [11] was used in order to have a good sensibility of the measurements. The method consist in the swelling of the microparticles in an aqueous solution with a known concentration of blue dextran with a high molar mass (Mw=2.000.000), that do not entered in the hydrogel pores. The determination of the water retention was based on the measurement of the increase of the blue dextran concentration in the supernatant by UV-vis spectroscopy. 0.05 g MPSAM-NIPAM microparticles were immersed into 3 ml blue dextran solution 1 mg/ml and maintained at the room temperature for 24 hours in order to reach the equilibrium. A 0.5 mL sample was then taken from the solution and the blue dextran concentration was measured by measuring the absorbance at 618 nm, based on a previously made calibration curve. In Figure 30 are presented the water retention values at different temperatures. Below the critical temperature of the oligomer (31^{o}C) the microparticles retain around 29 ml water/g microparticles, but above the LCST the water retained decrease due to the collapse of the thermosensitive grafted oligomers.

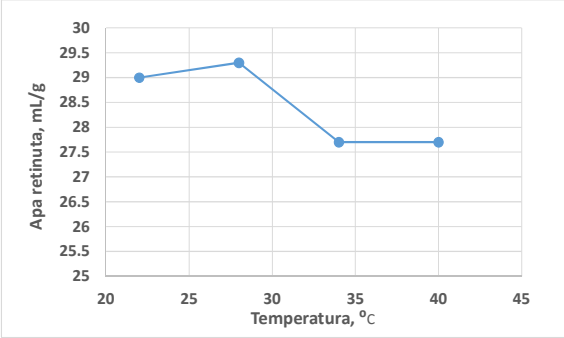


Figure 30. Influence of the temperature on the water retention of MPSAM-NIPAM microparticles

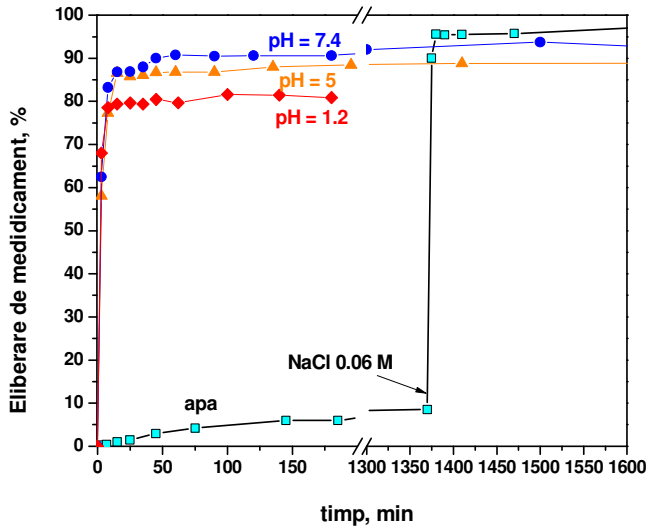
A 1.4. Loading and release of active principles in/from the obtained micro-/nano- particles

The MCL microparticles were loaded with model cationic drugs: diphenhydramine and metoclopramide. 0.1 g microparticles were immersed into 20 ml drug solution. The drug amount was calculated so that the ratio between the cationic drug and the anionic groups from the microparticles to be 2: 1. The drug loaded after 48 hours was determined by UV spectroscopy using the previously made calibration curve (at 218 nm for diphenhydramine and at 272 nm for metoclopramide). Thus, the drug loaded was 0.2505 g diphenhydramine /g MCL and 0.712 g metoclopramide/ g MCL. The microparticles were then filtrated, washed with distilled water and dried using water:acetone mixtures in different ratios.

The studies of the drug release from the loaded microparticles were performed into buffer solutions that simulate the body fluids, but also in water. Briefly, 50 mg MCL microparticles were immersed in the release medium (50 ml) under gently stirring. At regular time intervals,

samples were taken from the release medium and the drug amount was determined by UV spectroscopy. The same volume of buffer solution were then put back into the release medium.

In the Figure 31 are presented the release profiles of diphenhydramine from the MCL microparticles at 22°C. A burst release of the drug was observed in the buffer solutions, no matter of the pH. Still, the decrease of the pH from 7.4 to 1.2 led to the decrease of the drug release amount. This is due to the fact that the swelling of the microparticles in buffer solutions decrease with the decrease of the pH (Figure 22). When water was used as release medium, only 9% from the drug was released after one day, but when salt was added (0.06 M NaCl), almost all the diphenhydramine was released very fast. Thus, the burst release of the drug in the buffer solution is explained to the salt that shield the electrostatic attraction between the drug and the polymeric matrix.



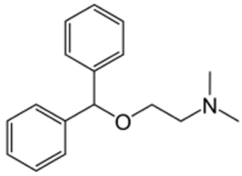
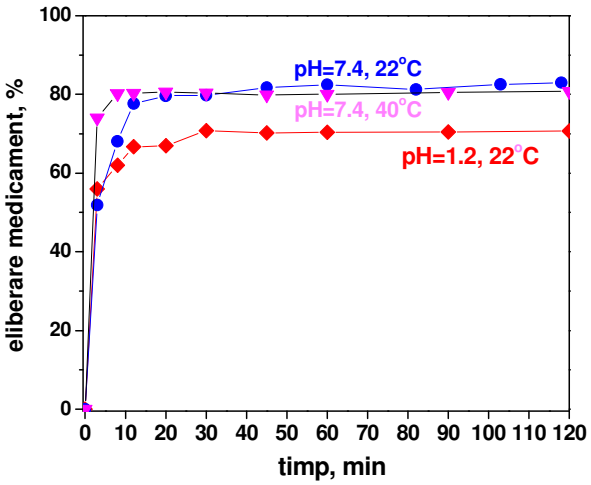


Figure 31. Drug release profiles of the diphenhydramine from the MCL microparticles into buffer solution and water

In the Figure 32 are presented the release profiles of metoclopramide from MCL microparticles in the buffer solutions pH=7.4 and pH=1.2. It can be seen that the drug is also sharply release, and the amount released at acidic pH is smaller than the amount released at basic pH. In order to study the effect of the temperature on the release profiles, the release in phosphate buffer pH=7 was performed at room temperature, but also at 40°C. The two release profiles were not different, because the burst release due the ionic strength do not led the weak influence of the temperature to manifest.



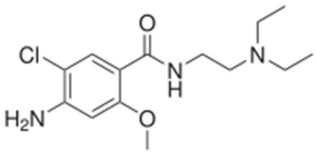


Figure 32. pH and temperature influence on the metoclopramide release from the microparticles

The problem of burst release can be solved by the entrapment of the loaded microparticles into neutral polymeric matrices. Thus, the pH and thermo-sensitive microparticles were entrapped into cellulose acetate butyrate (CAB) microcapsules using the solvent evaporation method [12]. 0.4 g CAB were dissolved in 2 ml chloroform, then 0.02 g Tween 80 were added as surfactant and 0.6 ml cyclohexane were added as inert solvent for the formation of pores. 0.08 g MCL microparticles loaded with metoclopramide were swollen in 0.4 ml chloroform, then suspended into the initial chloroform solution. The homogenous suspension was dispersed at 450 rpm into an external aqueous phase, 100 ml polyvinyl alcohol 1%. The temperature of the fromed emulsion was then raised to 52°C for the evaporation of the chloroform and formation of CAB microcapsules. After 3 hours, the formed microcapsules were separated by filtration, washed with distilled water, and then dried.

SEM pictures, presented in Figure 33, show that the obtained microcapsules are almost spheres with 100-300 μm diameter. From the surface detail (Figure 33 c) the presence of the pores can be observed. The porosity, obtained using cyclohexane in the obtaining process, is necessary for the release of the drug from the inner microparticles.

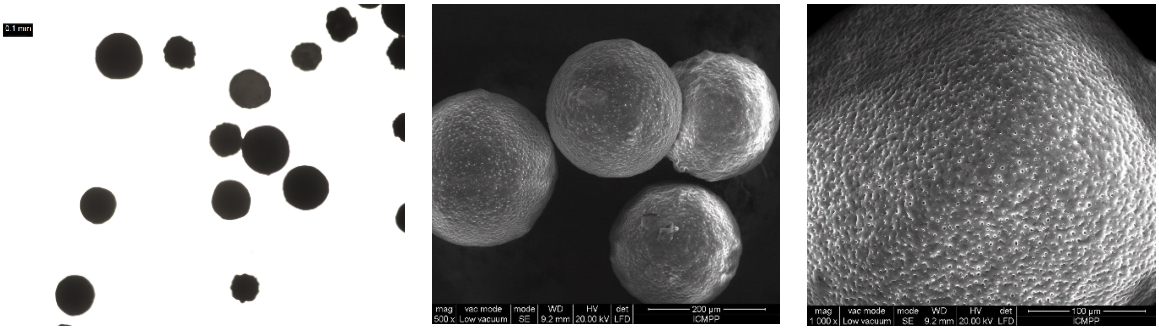


Figure 33. Optical microscopy (a) and SEM images (b,c) of the CAB microcapsules with incorporated MCL

The drug release from the CAB microcapsules was studied in phosphate buffer pH=7.4 (Figure 34). The drug is released progressively: only 25% of the drug is released in the first 2 hours. Thus, the problem of burst release was solved by incorporation into CAB microcapsules.

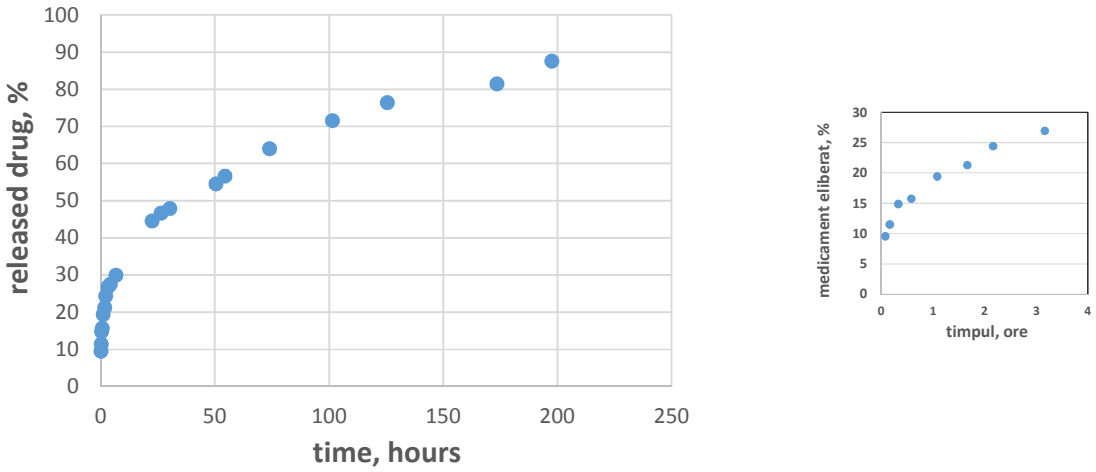


Figure 34. Metoclopramid release profile from CAB microcapsules with incorporated loaded MCL (buffer pH=7.4). In the figure from the right is presented the release from the first hours

O2. Obtaining and characterization of micro-/nano-particles based on interpolymeric complexes

A2.1. Study of the interaction between maleic copolymers with proton-accepting polymers

The interactions through hydrogen bounds between proton-donors polymers (polycarboxylic acids) and proton-acceptor polymers (that contain amide, lactam, ether or hydroxyl groups) can lead to the formation of interpolymeric complexes. Because the pH influence the protonation of the polyacid, this is the main factor in hydrogen bonding: in acidic media the complexes can precipitate, but with the increase of the pH the interaction between the polymers decrease or evan disappear and the complex can dissociate.

In this study we followed the interaction between poly(vinylcaprolactam) (PVCL) and poly(maleic acid–*alt*-styrene) (AcMSt). The interactions were studied in aqueous solution using a concentration of 10^{-2} N for each copolymer. The mixture composition was expressed using the polyacid molar fraction defined as:

$$N_{PA} = \frac{[COOH]}{[COOH]+[PVCL]} \qquad (1)$$

where [COOH] is the equivalent concentration of the maleic copolymer, and [PVCL] is the concentration of the PVCL polymer in the final mixture solution.

In the Figure 35 is presented the influence of the pH on the optical density of the PVCL-MAcSt mixture. At different polyacid ratios (NPA=0.5, 0.3 and 0.1) the mixture precipitate at around pH=2.9 (critical pH).

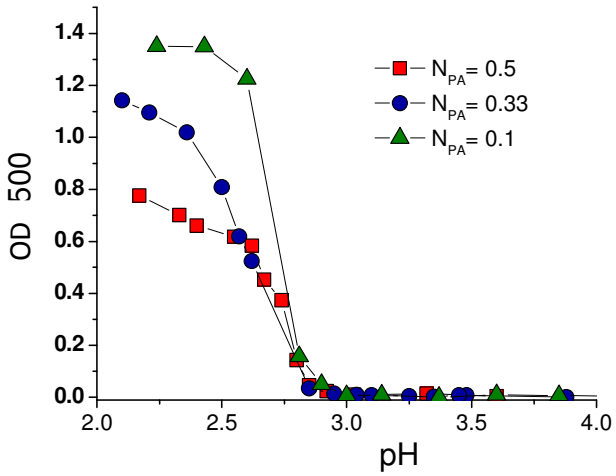


Figure 35. Influence of the pH on the optical density of different mixtures of MACSt and PVCL

In the Figure 36 is presented the variation of the optical density of the mixture with its composition at two pH values. At pH=2.5, but also at pH = 2.7, the optical density presented two maxima: at N_{PA}=0.25 and also at N_{PA}=0.5. From our knowledge, such a behavior was not observed until now in the literature.

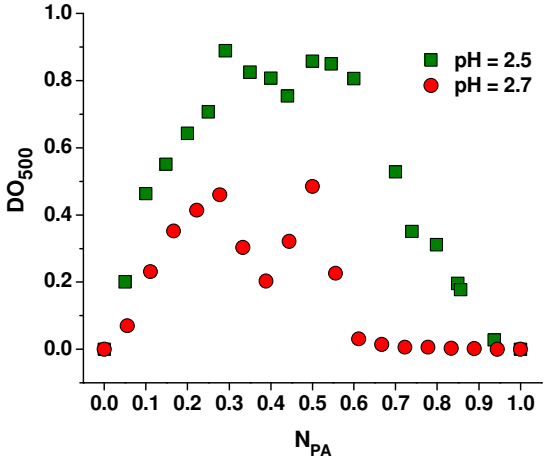


Figure 36. Variation of the optical density of the solution function of the polyacid content at pH=2.5 and at pH=2.7

The interaction between the polymers was also studied at pH values higher than the critical pH, when the hydrogen-bonded interpolymer complex remain in solution but the pH is small enough so the carboxylic groups of MAcSt are in protonated form. The interaction between

the two polymers was evidenced by potentiometry, viscometry, and also fluorescence of pyrene used as probe.

When the two solutions brought to the same pH (pH=3.5) were mixed, an increase of the pH could be observed as shown in Figure 37. The increase of H+ ions concentration was due to the formation of hydrogen bonded between –COOH groups of MAcSt and C=O groups of PVCL. The maximum of the potentiometric curve at around N_{PA}=0.3 means that the hydrogen interactions are maximum at this composition of the mixture.

The deviation of the reduced viscosity of the mixture from the ideal value also reflects the interaction between the polymers. In the Figure 38 is presented the ratio between the experimental reduced viscosity and the ideal reduced viscosity of the mixture, function of the composition. The deviations from ideality were not very high, but the ratio was lower than 1, showing that the polymers in the mixture are more compact that when they are separately. The more compact structure of the complex is observed at N_{PA}=0.5, but a change of slope can be seen at around N_{PA}=0.3

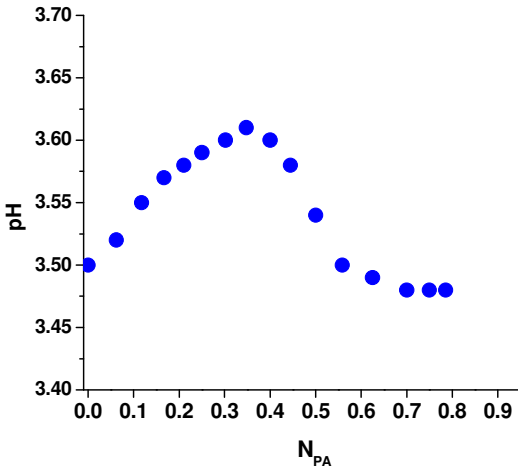


Figure 37. Variation of the pH with the polyacid mole fraction when MAcSt was added on the PVCL solution

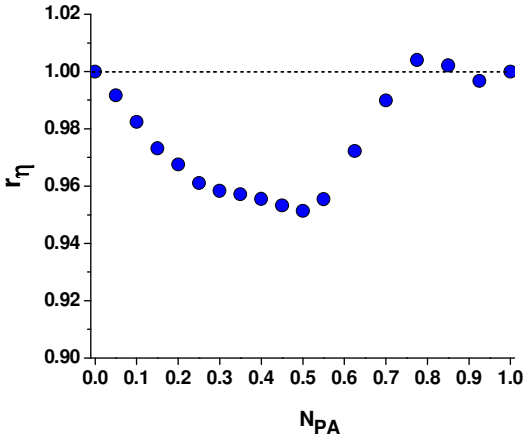


Figure 38. Figure 4. Variation of the reduced viscosity ratio with the composition of the mixture (pH = 3.5)

The influence of the MAcSt copolymer on the LCST of PVCL was studied (Figure 39). At pH=4.5, addition of small amounts of MAcSt lead to the increase of the cloud point temperature, probably because at this pH some of the carboxylic groups are in pronated form and interact with the PVCL chains, but some of them are in dissociated form and lead to the increase of PVCL solubility. At this pH and at N_{PA} over 0.16 the phase transition of PVCL was not longer observed. At pH=3.4, the temperature were the turbidity start to increase remain 33.5°C irrespective of MAc-St content, but the phase transition profiles are influenced by the amount of added polyacid.

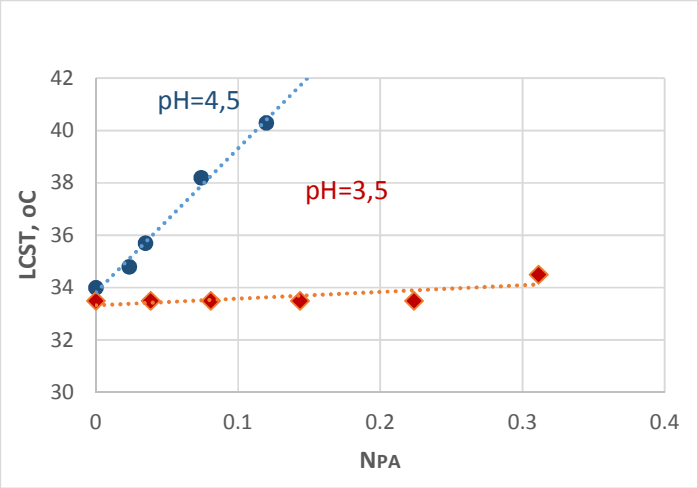


Figure 39. Influence of the MAcSt addition on the cloud point of PVCL aqueous solution at pH=4.5 and 3.5

A2.2. Obtaining and characterization of micro/nano-particles using interpolymeric complexation

A mixture of MAcSt and PVCL at a basic pH was added using a syringe in a precipitation bath of 0.1N HCl. The drops precipitated when they got in the acid bath. The microsphere were washed with distilled water and dried. In Figure 40 are presented pictures of the particles during their obtaining and SEM images of the dried particles. The microparticles have a spherical shape with diameters about 2 mm in swollen state and 0,8 mm in dried state.

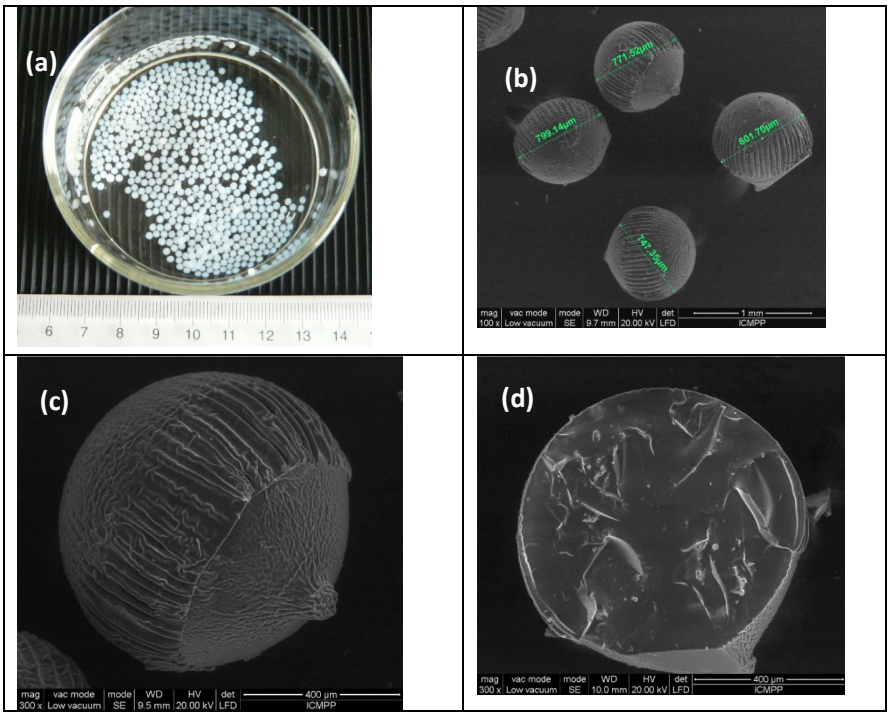


Figure 40. The microparticles in the precipiatation bath (a). SEM images of micropaticles (b,c) and of a cross-section (d)

A2.3. Release studies of the active principles from the obtained micro-nano-particles

The adsorption of a cationic drug, metaclopramid, in the microspheres was studied. In the Figure 41 is presented the adsorption isotherm. The data were fitted with the Langmuir model with a good correlation coefficient (R2=0,995). The maximum monolayer adsorption capacity in pure water is q_m= 25mg metaclopramid/g microparticles.

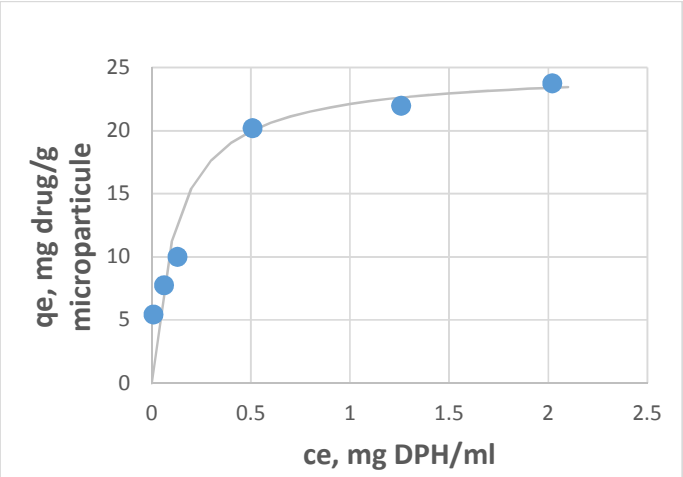


Figure 41. The isotherm of metaclopramid adsorption into the microspheres based on MAcSt-PVCL polymer complex (points-experimental values and line – calculated values using the Langmuir equation)

The drug release was studied in phosphate buffers at pH=7.4 and pH=5. As it can be seen from Figure 42, the released is influenced by the pH. At basic pH (7,4) the drug was almost completely released after 3 hours because at this pH the polymeric complex is soluble and the drug is release by the dissolution of the matrix. In the weak basic media (pH=5) the release is retarded, the diffusion of the drug being the determined factor in the release kinetic.

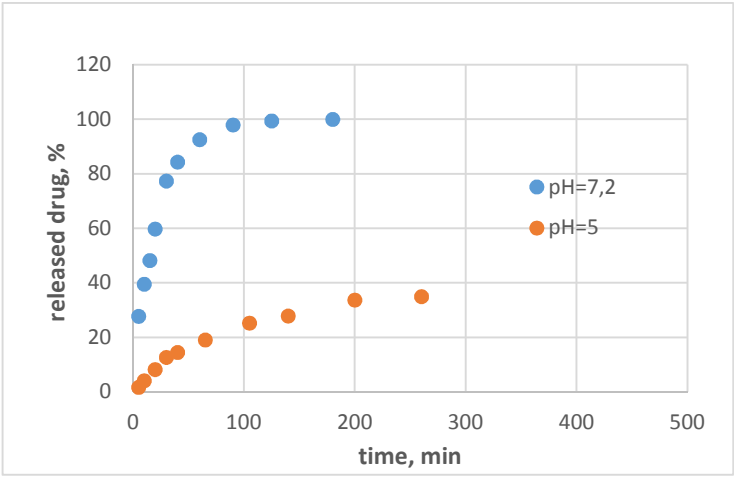


Figure 42. Influence of the pH on the drug release kinetic

Dissemination of the results

Five paper were published in ISI journals in the frame of this project:

1. I. Popescu, D.M. Suflet, "Poly(N-vinyl caprolactam-co-maleic acid) microparticles for cationic dye removal", *Polym. Bull.*, **2015**.
2. A. Filippov, E. Tarabukina, M. Simonova, T. Kirila, G. Fundueanu, V. Harabagiu, M. Constantin, I. Popescu, "Synthesis and investigation of double stimuli-responsive behavior of N-isopropylacrylamide and maleic acid copolymer in solutions", *J. Macromol. Sci. Part B: Physics*, **2015**, 54 (9), 1105-1121.
3. I. Popescu, A. I. Prisacaru, D. M. Suflet, G. Fundueanu, "Thermo- and pH-sensitivity of poly (N-vinylcaprolactam-co-maleic acid) in aqueous solution**",** *Polym. Bull.*, **2014**, 71 (11), 2863-2880.
4. M. Constantin, S. Bucatariu, V. Harabagiu, I. Popescu, P. Ascenzi, G. Fundueanu, "Poly(*N*-isopropylacrylamide-co-methacrylic acid) pH/thermo-responsive porous hydrogels as self-regulated drug delivery system", *Eur J Pharm Sci.* **2014**, 62, 86-95.
5. D.M. Suflet, I.M. Pelin, D. Timpu, I. Popescu, "pH-sensitive multilayers based on maleic acid terpolymers with weak and strong acid moieties", *Colloid Surface A*, **2013**, 436, 113–122.

Five comunications were presented at scientific meetings:

1. I. Popescu, D. M. Suflet, "Microparticles obtained by the cross-linking of maleic acid copolymers and their applications in dye removal", The 3rd CEEPN workshop on Polymer Science, 23-26 Sept. **2015**, Iasi
2. I Popescu, "Removal of cationic dyed from aqueous solutions by adsorption using poly(N-vinyl caprolactam-co-maleic acid) microparticles", *8th Edition of symposium with international participation - New trends and strategies in the chemistry of advanced materials with relevance in biological systems, technique and environmental protection*", June 4-5 **2015**, Timișoara
3. I. Popescu, G. Fundueanu, "Separation de phases en solution aqueuse de poly(N-vinylcaprolactame-*co*-acide maleique)", *XI^{ème} Colloque Franco-Roumain sur les Polymères*, 27-29 août **2014**, Pitești
4. I. Popescu, D. M. Suflet, G. Fundueanu, "Stimuli-sensitive microparticles based on poly(n-vinyl caprolactam-co-maleic acid)", *A XXXIII-a Conferinţă Naţională de Chimie*, 01-03 octombrie **2014**, Călimăneşti-Căciulata
5. I. Popescu, D. Timpu, „Multilayer films based on maleic acid terpolymers and weak polycations with pH-dependent loading and release behaviour of small molecules", *European Workshop Polymer Science at Nanoscale*, 22-23 oct **2013,** Iasi

Three posters were presented at the internation symposium *Polymers for Advanced Technologies*, Berlin, Germany, 29 sep 29 – 2 oct **2013:**

1. I. Popescu, G. Fundueanu, "Synthesis and solution properties of N-vinylcaprolactam/maleic acid copolymers".
2. I. Popescu**,** D.M. Suflet, I.M. Pelin, G. Fundueanu, "New pH-sensitive microparticles. Synthesis, characterization, and *in vitro* drug release studies".

3. I. Popescu, D. M. Suflet, G. Fundueanu, “pH-sensitive multilayers based on maleic acid copolymers”.

Conclusions:

In that project, new copolymers of N-vinylcaprolactam and maleic acid with different ratios between the monomers were synthesized. The behavior of this copolymers in aqueous solution is the result of electrostatic, hydrophobic and hydrogen bonding interactions. Thus, the copolymers in aqueous solution present a phase transition with the increase of the temperature, but also with the decrease of the pH or at complexation with hydrophobic drugs. The LCST of the copolymer increase with the increase of the content of maleic acid, with the decrease of the concentration, but was also influenced by the pH.

By suspension cross-linking of the N-vinylcaprolactam – maleic acid copolymers, 100-200 μm microparticles were obtained. The swelling of this microparticles in aqueous media increase with the increase of the pH and decrease with the increase of the temperature from 22 to 40^{o}C. A burst drug release from the microparticles was observed in the buffer solutions or in the presence of salt due to the electrostatic interaction beween the drug and the polymer matrix. A progressively drug release was obtained only when the loaded microparticles were incorporated into cellulose acetate butyrate microcapsules.

pH-sensitive microparticles were also obtained by suspension cross-linking of maleic anhydride-styrene copolymer. This microparticles were then grafted with N-isopropylacrylamide semitelechelic oligomers for the addition of thermo-sensitivity, fact proved by the measurement of water retention at different temperatures.

The hydrogen-bonded interaction between poly(N-vinylcaprolactam) and poly(maleic acid-alt-styrene) was studied in aqueous solution by turbidimetry, viscometry, potentiometry. The mixing ratio, but especially the pH influence the interpolymer interaction: under pH=2,8 the polymer complex precipitate even at low ratios of polyacid, at pH between 2.8 and 5 the complex is formed but it is soluble, and at basic pH the interaction do not occurred/the complex is dissociated.

Spherical particles were obtained based on hydrogen-bonded complexation in acidic pH. They can adsorbed cationic drugs and the adsorption isotherm fits well the Langmuir model. The drug was released only in small extend in acidic pHs, but totally and rapid released in basic pHs, that is an advantage when the release of the drug in the lower parts of the gastrointestinal tract is envisaged.

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