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New anionic crosslinked multi-responsive pullulan hydrogels

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ABSTRACT

The paper studies the synthesis and characterization of anionic thermoassociative carboxymethylpullulan hydrogels through crosslinking of carboxymethylpullulan with two difunctional Jeffamines: ED-600 and ED-2003; taking into account that the Jeffamines contain polyoxyalkyleneamines (polyethylene oxide, polypropylene oxide) backbone with thermoassociative properties, is expected that the polysaccharide-Jeffamine derivatives also possess amphiphilic and thermosensitive characteristics. The hydrogels were characterized through FTIR spectra, swelling behavior in various media, at various pH or temperatures, retention of hydrophobic molecules, to appreciate their polyelectrolyte and thermoassociative properties. The interaction with biomolecules as proteins: lysozyme and BSA and as antioxidants: lutein was studied to estimate some potential application domains of these newly synthesized hydrogels.

1. Introduction

Hydrogels are tridimensional hydrophilic polymer network, crosslinked through chemical or physical interaction, which can absorb large amounts of water by maintaining their tridimensional structure. A large interest has been devoted to the hydrogels sensitive to external stimuli, such as temperature (Deguchi, Akiyoshi, & Sunamoto, 1994; Fettaka et al., 2011; Singh, Webster, & Singh, 2007), pH (Martínez-Ruvalcaba, Sánchez-Díaz, Becerra, Cruz-Barba, & González-Álvarez, 2009), and electrical field (Kim, Shin, Lee, Kim, & Kim, 2004a, 2004b). This interest is justified through the multitude of application domains of sensitive hydrogels in controlled drug delivery, tissue engineering, biotechnology, etc. Associative properties of the hydrogels are induced by the presence of hydrophobic units on the hydrophilic polymeric backbone (Akiyoshi, Yamaguchi, & Sunamoto, 1991; Henni-Silhadi et al., 2008), while thermoresponsible properties are induced either by the presence of a thermosensitive polymer backbone (PNIPAM, Feil, Bae, Feijen, & Kim, 1993; hydroxypropylcellulose, Fettaka et al., 2011), or by thermosensitive units linked on the polymer chains [poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)] (Deguchi et al., 1994); Jeffamine (Mocanu, Mihai, Dulong, Picton, & LeCerf, 2011). The presence of the ionic groups on the polymeric chains provides them pH-dependent properties (Dulong, LeCerf, Picton, & Muller, 2006); if on the polymer backbone both thermosensitive units and ionic groups are present, the multi-responsive hydrogel will have both temperature and pH sensitive properties (Dumitriu, Mitchell, & Vasile, 2011; Rodriguez-Felix et al., 2011).

The use of difunctional Jeffamines as crosslinking agent has been reported for crosslinking of diglycidyl ether of bisphenol A (Epon 828) (Shan, Verghese, Robertson, & Reifsnider, 1999) or of glycidyl methacrylate copolymer (Luo, Li, & Yang, 2011); the obtained copolymers presented improved properties, which recommend them for special uses, as for example potential candidates for the construction of rechargeable lithium batteries.

The paper presents the synthesis of new thermoassociative, pH-sensitive hydrogels, through crosslinking of carboxymethylpullulan (CMP) with two difunctional Jeffamines: ED-600 and ED-2003; taking into account that the Jeffamines contain polyoxyalkyleneamines (polyethylene oxide [PEO]), polypropylene oxide [PPO]) backbone with thermoassociative properties, is expected that the polysaccharide-Jeffamine derivatives possess amphiphilic and thermosensitive characteristics, too; also, the ionic groups of CMP will induce pH-dependent properties. No study has been reported in literature to the use of difunctional Jeffamines as crosslinking agents of the polysaccharides. Moreover, Jeffamines were presented in the literature as biocompatible products (Marie, Landfester, & Antonietti, 2002). Physico-chemical characterization of the obtained hydrogels evidenced the supposed multi-responsive properties; the study of the interaction with biomolecules as proteins (lysozyme, BSA) and antioxidants (lutein) revealed their potential for use in controlled drug delivery.

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2. Experimental

2.1. Materials

- Carboxymethylpullulan (CMP) synthesized in laboratory, as described (Mocanu, Mihai, Picton, LeCerf, & Muller, 2002).
- Jeffamines (Jeff) ED-600 and ED-2003 (Aldrich); paraffin oil (Iassypharm, Romania); Span 85 (Fluka); N,N′-dicyclohexyl carbodiimide (DCCI) (Fluka), dimethylaminopyridine (DMAPy) (Fluka), DMSO; Rose Bengal sodium salt (Sigma Aldrich); Brilliant Blue (Fluka); acid orange (Fluka), lysozyme (Lys), (Sigma); albumine from bovine serum (BSA, Fluka); lutein (ethanolic extract of lutein capsules, Medica Lab., Romania).

2.2. Methods

Synthesis of Jeffamine-carboxymethylpullulan crosslinked hydrogels. 0.5 g CMP H^+, swollen in 15 mL DMSO was dispersed under stirring in 50 mL paraffin oil containing 2.5 mL Span 85 as stabilizer; after 30 min 0.27 g (1.3 meq) DCCI was added in 2.5 mL DMSO and the reaction was continued for 2 h at room temperature; then, 1.3 g Jeff ED(2000) and 0.03 g DMAPy were added and the reaction was continued for 48 h at room temperature. Then, the hydrogel microparticles were filtered, washed on the filter with acetone (to remove the unreacted Jeff and the dicyclohexylurea formed), then with water and dried from methanol. Yield: 0.59 g. The other syntheses were performed in the same conditions, but using various molar ratios Jeff/CMP, or other Jeff. The diameter of the hydrogel microparticles in dry state, measured with an optical microscope, ranged between 20 and 100 μm.

The degree of substitution (DS) with Jeffamine units was established through conductimetric titrations (through the difference towards the initial ion exchange capacity with COOH groups).

Determination of uncrosslinked NH_2 groups; during crosslinking reaction it is possibly that only one NH_2 groups of the difunctional Jeff to be involved in the amidation reaction; the content of NH_2 uncrosslinked groups was appreciated by acid orange retention, as used in chitosan NH_2 group determinations (Fras Zemljic, Strnad, Sauperl, & Stana-Kleinschek, 2009).

The water or various solvent regain was determined through centrifugation, for 10 min at 2000 rpm of the previously swollen microparticles for 24 h, by Pepper's method (Pepper, Reichenberg, & Hale, 1952). This was calculated as follows:

$$W_R = \frac{W - W_0}{W_0}$$

where W_R, water (solvent regain); W, weight of the sample swollen at equilibrium; and W_0, weight of the dry sample.

Rose Bengal retention, which is a measure of support hydrophobicity, was determined by the method described by Gigimol and Mathew (2003). A 50 mg support was equilibrated with a 125×10^{-6} M aqueous solution of Rose Bengal; the amount of dye bound by the polymer was determined in UV at 548 nm, from the difference in the concentrations of the dye solution, before and after binding. In the same manner the retention of *Brilliant blue* was determined, through UV spectroscopy, at 586 nm wavelength.

The retention of proteins was performed under "batch" conditions, in glass-stoppered flasks; solutions with known concentration were added to 50 mg dry support, in the presence of sodium azide as a preservative; aliquots were withdrawn and the protein concentration in the supernatant was determined according to the modified Folin method (Lowry, Rosebrough, Lewis Farr, & Randall, 1951). The amount of retained protein is calculated as the difference from the initial protein content of the solution used. After equilibration of the solution concentration, the microparticles were filtered, washed with water to remove the physically entrapped protein, dried from ethyl alcohol, then in vacuum.

The enzymatic activity was determined with a *Micrococcus Lysodeikticus* (Sigma) substrate, at 450 nm (Prasad & Litwack, 1963), on a 3 mg sample mixed directly in the spectrophotometric cell with 3 mL solution of substrate (0.3 mg/mL); a decrease in absorbance was recorded every 15 s. The enzymatic activity was expressed as 1/A.

Lutein retention was also performed under "batch" conditions, in glass-stoppered flasks; solution of lutein with known concentration was added to 50 mg dry support previously swollen in water; the amount of retained lutein is calculated as the difference from the initial lutein content of the solution used.

The antioxidant activity of the lutein-containing hydrogels was determined through DPPH method (Ara & Nur, 2009) and compared with those of ascorbic acid and of the initial lutein. Briefly, various amounts of samples containing the antioxidant product were immersed in 2 mL solution NaCl 1 g/L and 1 mL methanolic DPPH solution (0.128 g/L methanol); after 30 min the absorbance at 517 nm was measured. Decreased absorbance of DPPH indicates increased antioxidant effect. The radical scavenging activity was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \frac{A_0 - A_1}{A_0} \times \quad 100$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any sample, and A_1 is the absorbance of the samples.

3. Results and discussion

3.1. Synthesis and characterization of the supports

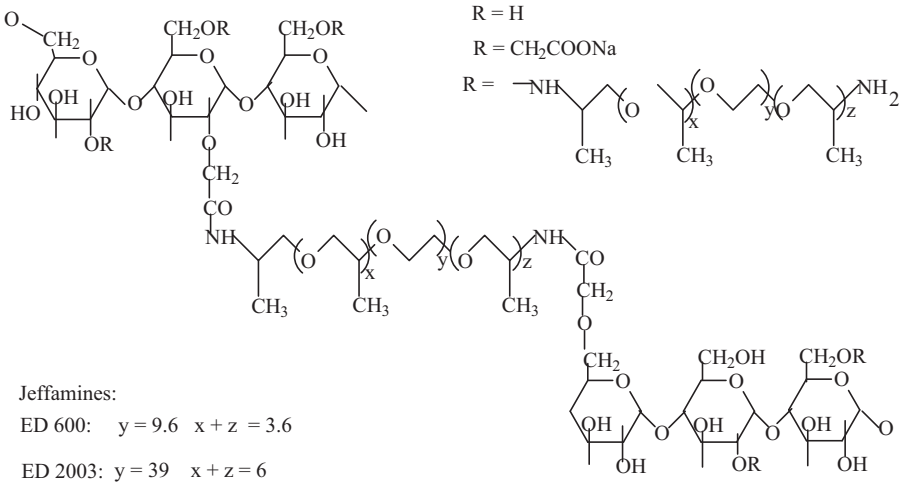
Reaction of carboxymethylpullulan with difunctional Jeff occurs with crosslinking through amide bonds; the macromolecular tridimensional network has the structure presented in Scheme 1.

The FTIR spectra of Jeff-CMP hydrogels confirmed their structure through the presence of the characteristic band of amide I (C=O) at 1652 cm^{-1}, amide II (C–N–H) group at 1550 cm^{-1} and of carboxylic group at 1717 cm^{-1}.

By using various amounts of Jeff ED-600 or ED-2003 different hydrogels were obtained whose characteristics are presented in Table 1. By increasing the Jeff ratio, the ion exchange capacity of CMP decreases; the amount of uncrosslinked NH_2 groups, determined from acid orange retention (expressed as meq acid orange/g support) is relatively low and decreases as molar ratio of Jeff increases.

Thermogravimetric analyses of the synthesized supports showed a higher thermal stability of the samples more crosslinked (either with Jeff ED-600 or ED-2003), than that of the samples less crosslinked (Fig. 1). The TG curves of the gels are wide and show that the weight loss occurs in more than one step. Water loss occurs up to 100 °C; an important weight loss begins after 200 °C, with a sharper slope for less crosslinked gels. At about 400 °C, around 90% from less crosslinked gels are degraded, while for the more crosslinked ones the same degradation level occurs at about 500 °C.

The swelling of the hydrogels in various media and different temperatures was studied, to appreciate their thermoresponsive character. As can be seen from the data presented in Fig. 2a, the hydrophilicity of the hydrogels decreases with increasing of the ratio of crosslinking agent used; the hydrogels obtained using Jeff ED-600 as crosslinking agent are more hydrophilic as those using Jeff ED-2003 as crosslinking agent. In NaCl 0.1 M solution, all hydrogels are in collapsed state, due to the screening effect of the salts on the ionic charges of CMP conjugates. With temperature increase, the hydrogels behave differently: those crosslinked



Scheme 1. Chemical structure of Jeffamine-crosslinked hydrogel microparticles.

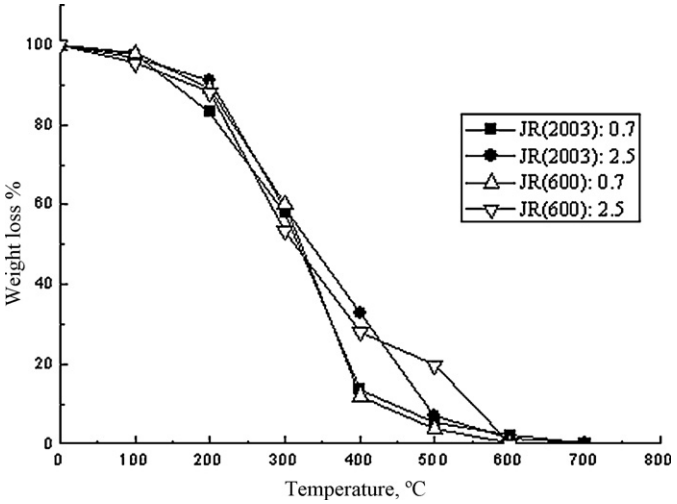


Fig. 1. TG curves of the carboxymethylpullulan-Jeff hydrogels.

with Jeff ED-600 in molar ratio ≤1.3/1 and those crosslinked with Jeff ED-2003 in molar ratio ≥1.3/1 swell to a greater extent. This behavior is specific for positive temperature sensitive hydrogels (that show swelling at high temperature and shrinking at low temperature), mentioned in literature for copolymers having both hydrophilic, ionic and hydrophobic units (Ankareddi & Brazel, 2007); the swelling/shrinking behaviors of the hydrogels are strongly influenced by the hydrophobic or hydrophilic nature of the comonomers (Gutowska, Bae, & Kim, 1992). Probably, the number of layers of water molecules around the acidic groups increases, as temperature rises, as mentioned in literature for NIPAM copolymers in the presence of surfactants containing –O–SO_3^- groups (Caykara, Kiper, & Demirel, 2006). The samples JR(600):2.5 and JR(2003):0.7 behave as negative temperature sensitive hydrogels; the temperature increase is accompanied by the reinforcement of the hydrophobic interactions between hydrophobic segments and by the weakening of hydrogen bonding with water molecules, which results in the shrinkage of the hydrogel, due to the hydrophobic intermolecular interactions. One can suppose that, for Jeff(600) the hydrophobic interactions between Jeff units are reinforced as temperature rise preponderantly at higher amounts of Jeff(600) (namely 2.5), while for Jeff(2003), the hydrophobic interactions manifest preponderantly at low Jeff(2003) content (namely 0.7); higher Jeff(2003) amounts favors probably the increase in the number of layers of water molecules, around POE units, as temperature rise. Hence, the anionic hydrogels obtained using difunctional Jeff as crosslinking agents present thermosensitive properties; the negative or positive character of the thermoresponsivity is influenced by the nature and ratio of Jeff used, by the balance between hydrophobic or hydrophilic units of the macromolecular network.

Thermosensitive character of the crosslinked hydrogels was evidenced also through absorbance measurements (at 400 nm wavelength) as temperature variation (Fig. 3). The samples JR(600):2.5 and JR(2003):0.7 presented an absorbance increase with temperature (in the domain 20–40 °C), while, for the other samples the absorbance increased after 50 °C. These results confirm those concerning the temperature influence on the swelling of the hydrogels, presented above.

pH-sensitive hydrogels contain pendant anionic (carboxylic acid, sulfonic acid) or cationic (ammonium salts) groups that change protons as a function of the environmental pH. The presence of ionizable groups on the polymer chain determines swelling/shrinkage of the hydrogels, due to the electrostatic interactions, as a function of pH, ionic strength and type of counterions. Thus, the hydrogels containing weakly acidic carboxylic groups are shrunk in acidic

Table 1
Reaction conditions and physico-chemical characteristics of obtained hydrogels.

Sample	Jeffamine	mol Jeff/G.U.[b]	Ion exchange capacity (meq/g)	DS[a] with ionic groups	meq acid orange/g support	DS_{Jeff}
CMP linear	–	–	3.31	0.72	–	–
JR(600): 0.7	ED-600	0.7/1	3.07	0.67	0.096	0.05
JR(600): 1.3	ED-600	1.3/1	2.57	0.56	0.04	0.16
JR(600): 2.5	ED-600	2.5/1	2.20	0.48	0.03	0.24
JR(2003):0.7	ED-2003	0.7/1	2.20	0.48	0.06	0.24
JR(2003):1.3	ED-2003	1.3/1	1.84	0.40	0.03	0.32
JR(2003):2.5	ED-2003	2.5/1	1.47	0.32	0.014	0.40

[a] Degree of substitution.
[b] Glucopyranosic unit.

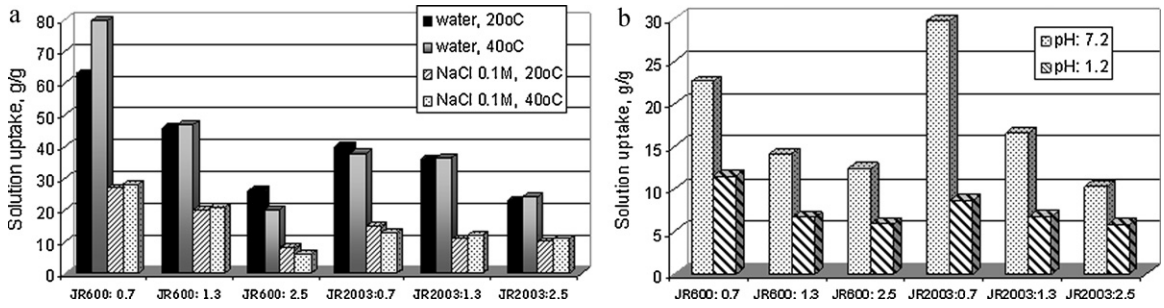


Fig. 2. Swelling behavior of the hydrogels: (a) water and NaCl 0.1 M uptake at 20 °C and 40 °C; (b) swelling in pH: 1.2 and 7.2.

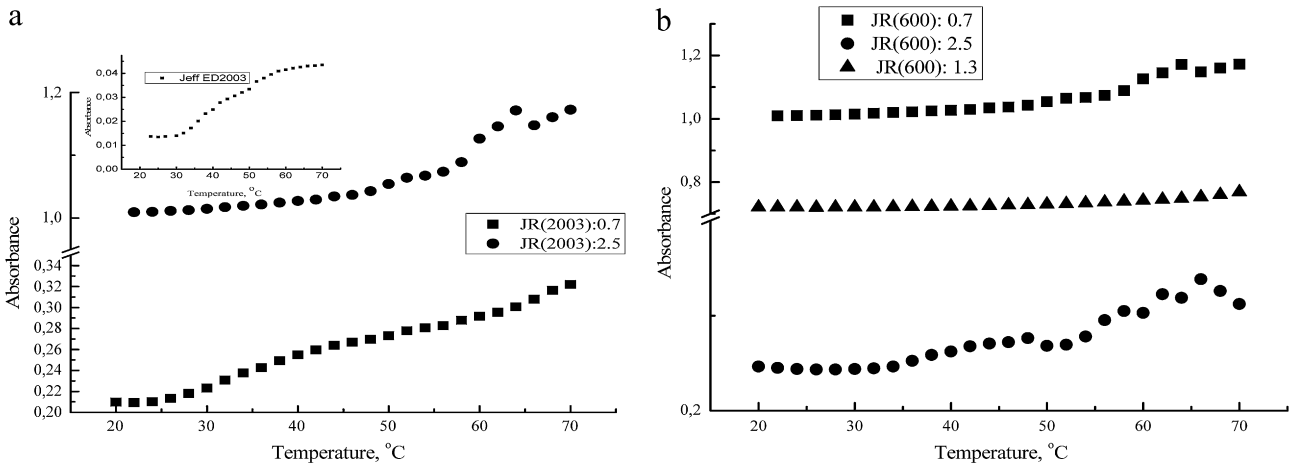


Fig. 3. Absorbance variation as temperature increase; (a) hydrogels of Jeff2003 [in inset is presented Jeff2003 variation as temperature function]; (b) hydrogels of Jeff600.

pH (unionized anionic group) and swollen in basic pH, where the ionized acidic groups determine electrostatic repulsions (Taleb, Samia, Nabil, & hegazy, 2007). This behavior as polyelectrolyte is important in controlled drug release for oral administration. The pH-sensitivity of the hydrogels, assured by the presence of anionic groups, is evidenced through the variation of microparticle volume in acidic, respectively basic media. In acidic pH, the microparticles containing carboxylic group are in a collapsed state, as mentioned in literature (Dong & Hoffman, 1991), while in enteric solutions (pH 6.8–7.2) they are swollen (Fig. 2b).

The amphiphilic character of the hydrogels was evidenced through retention of dyes hydrophobic (Rose Bengal) (Gigimol & Mathew, 2003) and amphiphilic (Brilliant blue) (Dulong et al., 2006). All hydrogels retain the studied dyes. Their retention, presented in Fig. 4 is inversely towards the ratios of Jeff used for crosslinking, but directly proportional with the amount of uncrosslinked Jeff units, expressed as meq. AO retained. Based on these results, one can suppose that the associative character is not only provided both by the crosslinked Jeff units, but also by the uncrosslinked ones.

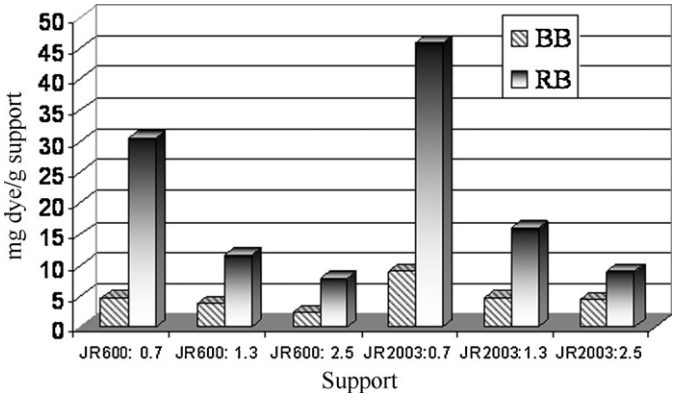


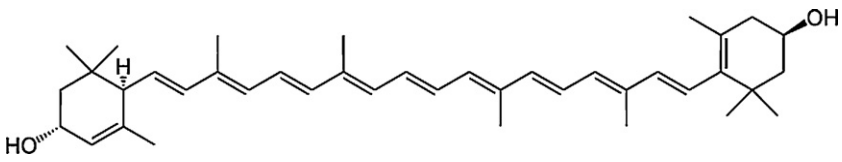
Fig. 4. Dyes retention (hydrophobic – Rose Bengal and amphiphilic – Brilliant Blue) on the Jeff hydrogels.

3.2. Interaction with biomolecules

Lutein (3,3′-dihydroxy-β-ε-carotene) is a carotenoid present in fruits and vegetables with antioxidant function such as quenching of singlet oxygen or other electronically excited molecules and reduces the progress of many degenerative diseases (Di Mascio, Kaiser, & Sies, 1989). Lutein possesses pronounced free radical scavenging activity due to its polarity and number of conjugated double bonds (Scheme 2) (Sindhu, Preethi, & Kuttan, 2010).

The antioxidant activity of carotenoids is conferred by the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize sulphenyl radicals and stabilize peroxyl radicals (Palace, Khaper, Qin, & Singal, 1999).

Commonly, in fruits and vegetables the antioxidants (ascorbic acid, carotenoids) exist in nature in combination, and in combination they certainly cooperate on total antioxidant activity. In the paper the retention of the lutein on the obtained supports was studied; due to its hydrophobic character, lutein will be retained preponderantly through hydrophobic forces. Our studies established that lutein is retained gradually, in time; the samples less crosslinked JR(600): 0.7 and JR(2003): 0.7 retain faster, higher amounts of lutein (Fig. 5). They are more hydrophilic, being less crosslinked, hence, the diffusion process of the biomolecule will be facilitated. They also possess higher amounts of uncrosslinked Jeff units, which probably influence the retention through hydrophobic forces.



Scheme 2. Structure of lutein.

The antioxidant activity of lutein, comparatively with that of ascorbic acid was determined; the scavenging effect of lutein (which is a measure of its antioxidant activity) is smaller (but still important) than that of ascorbic acid (Fig. 6), as was reported otherwise in literature (Kotíková, Lachman, Hejtmánková, & Hejtmánková, 2011). The lutein retained on the studied supports presents antioxidant activity, as can be seen from the data presented in Fig. 6. The scavenging effect of the released lutein is lower than that of lutein substance due to the gradual release of the biomolecule retained on the supports. This behavior can be important in the potential use of the supports for controlled drug delivery.

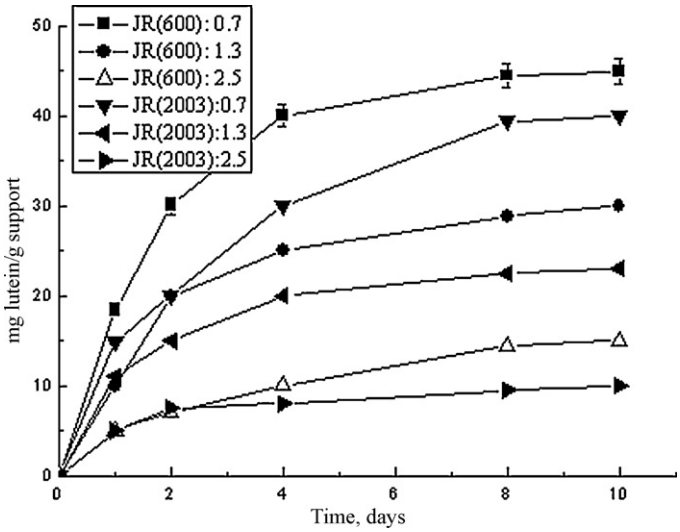


Fig. 5. Lutein retention on the Jeff hydrogels; the values are the mean of three independent measurements that deviated: 2–3%.

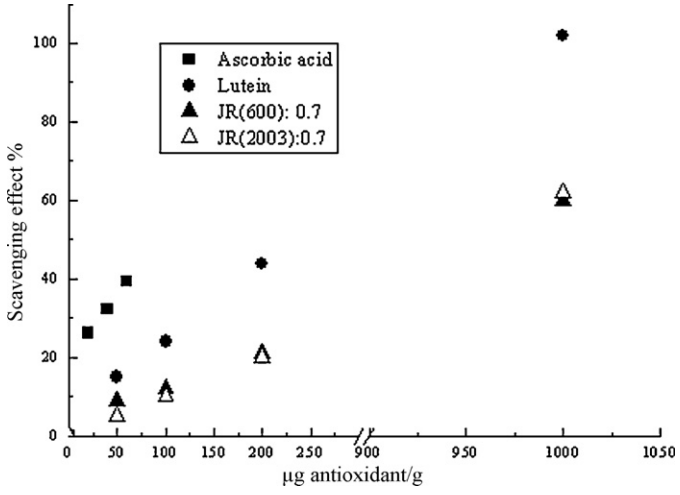


Fig. 6. Scavenging effect of ascorbic acid, lutein and Jeff hydrogels containing lutein; the values are the mean of three independent measurements that deviated: 2–4%.

Lysozyme is a small, globular, basic protein with an isoelectric point of ~11 and Mw = 14,600. It evidences antimicrobial activity and can be used in the treatment of ulcer, viral infections and skin diseases. Due to its basic character, it is retained on the supports containing anionic groups through electrostatic interactions, by inter-polyelectrolyte complex formation; the presence of hydrophobic groups on the same support may improve the interaction with proteins through a cooperative effect.

Lysozyme is retained on the supports in amounts that depend on the nature and amount of Jeff used for crosslinking. The less crosslinked hydrogels retain higher amounts of lysozyme; to this behavior contribute many factors: (i) the higher water swelling, which facilitates the access of the protein inside hydrogel; (ii) the higher ion exchange capacity, which determines a higher protein retention through electrostatic forces; (iii) the higher amount of hydrophobic uncrosslinked Jeff units, which favors protein retention through hydrophobic forces (Fig. 7a).

The enzymatic activity of the lysozyme immobilized on synthesized supports was also investigated. Decrease in the absorbance (increase of the 1/A ratio) of the substrate solution as time function (which is proportional with the enzymatic activity) proves that the lysozyme immobilized on supports manifests lytic activity towards *M. Lysodeikticus* (Fig. 7b). The rate of the enzymatic process depends on the access of the substrate to the immobilized lysozyme. The enzymatic process occurs with the enzyme in immobilized state, an assertion based on the fact that, in a 0.067 M phosphate solution (used to determine the enzymatic activity), no release of lysozyme was obtained. Interpolyelectrolyte complex formation due to the lysozyme binding occurs with shrinkage of the hydrogel volume; as a result, the access of the substrate to the immobilized lysozyme will depend rather on the degree of the collapsing of the complex, than on the hydrophilicity of the initial support. The enzymatic process of the lysozyme itself on *M. Lysodeikticus* occurs with a higher rate in the first time and then is slowed as enzyme consumption. The use of the enzyme in immobilized state provides significant advantages; besides improvement of chemical and storage stability, a controlled release of the enzymatic activity by an appropriate choice of the support can be achieved.

The lysozyme retained on the hydrogels preserves its enzymatic activity; this behavior has been established through determinations of specific activity of the lysozyme released from the hydrogels in NaCl 0.1 N solutions. The specific activity was about 92,700 units/mg ± 10% (1 unit corresponds to the amount of enzyme which decreases the absorbance at 450 nm by 0.001/min, at pH 7.0, 25 °C, using a suspension of *M. Lysodeikticus* as substrate), similar to that of the lysozyme taken into study.

Bovin serum albumin (BSA) is a protein with Mw = 60,000 and isoelectric point of 4.7; studies on the retention of BSA on PNIPAM-carboxymethyl interpenetrating cellulose polymeric network revealed that its maximum absorption capacity was obtained at pH 4.7 (Ekici, 2011). At its isoelectric point the protein solubility in the aqueous media decreases; an acidic or basic medium causes the protein to become positively or negatively charged, increasing the solubility of the protein in the aqueous media. Therefore, lower or higher pH values than isoelectric point resulted in decreased BSA absorption onto the absorbent. BSA absorption can be influenced by several factors: hydrophobic binding arises from hydrophobic

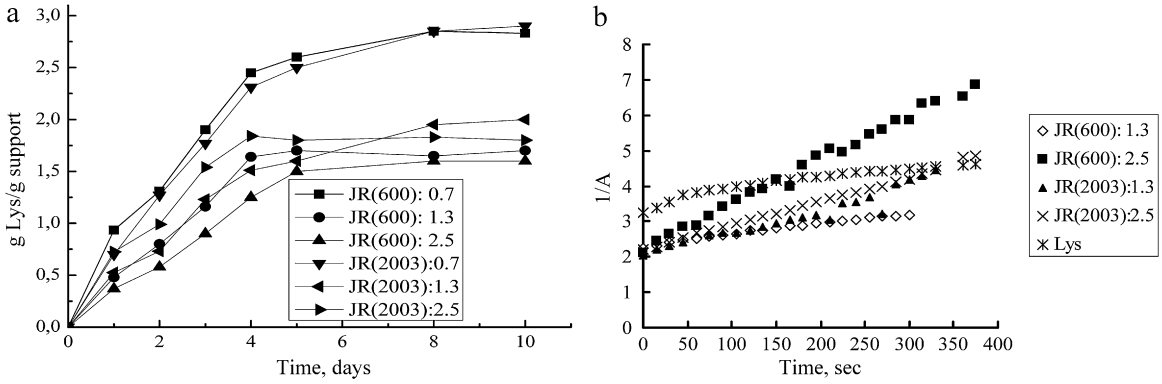


Fig. 7. Lysozyme retention on the Jeff hydrogels (a); enzymatic activity of the lysozyme itself and immobilized on the Jeff hydrogels; the values are the mean of three independent measurements that deviated: 2–5%.

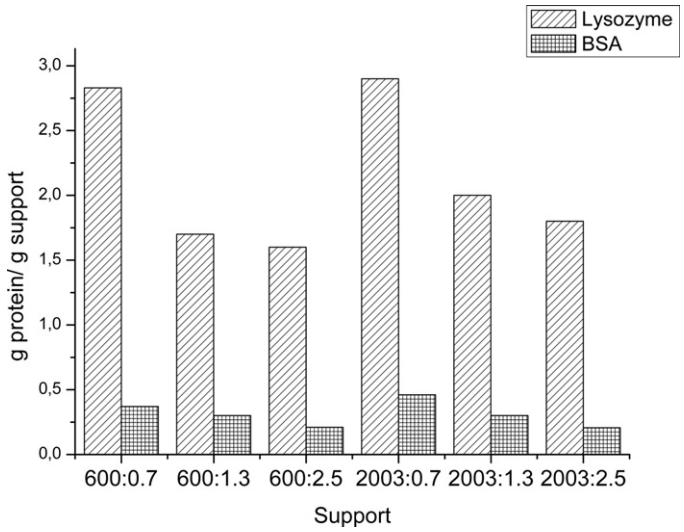


Fig. 8. Comparative retention of lysozyme and BSA on the Jeff hydrogels.

groups of absorbent and protein molecules, hydrophilic interactions between absorbent and protein molecules. This implies that binding of BSA to the hydrogel absorbent was dependent upon the cooperative effects of hydrophobic and hydrophilic interactions. The data presented in Fig. 8 show that BSA is retained on the hydrogels in smaller amounts than lysozyme (its molecular weight is higher); the less crosslinked hydrogels, more hydrophilic, with more uncrosslinked Jeff units retain higher BSA amounts. Its retention can be the result of many accumulative effects, for example, hydrophobic interactions, hydrogen bonds, electrostatic forces (Hou, Liu, Deng, Zhang, & Yan, 2007).

4. Conclusions

The paper presents the synthesis of new microparticles obtained through crosslinking of carboxymethylpullulan with two difunctional Jeffamines; physico-chemical characterization of these hydrogels revealed their thermosensitive, associative, and polyelectrolyte behavior. Their interaction with some biomolecules (antioxidant: lutein; proteins: lysozyme, BSA) was studied with the aim to appreciate potential application domains. Further studies, devoted to the *in vivo* behavior and efficiency of these new supports are under development for providing additional data on the conditions and performance in the retention/release of biologically active substances.

References

Akiyoshi, K., Yamaguchi, S. & Sunamoto, J. (1991). Self-aggregates of hydrophobic polysaccharide derivatives. *Chemistry Letters*, *20*, 1263–1266.

Ankareddi, I. & Brazel, C. S. (2007). Synthesis and characterization of grafted thermosensitive hydrogels for heating activated controlled release. *International Journal of Pharmaceutics*, *336*(2), 241–247.

Ara, N. & Nur, H. (2009). In vitro antioxidant activity of methanolic leaves and flowers extracts of *Lippia Alba*. *Research Journal of Medicine and Medical Sciences*, *4*(1), 107–110.

Caykara, T., Kiper, S. & Demirel, G. (2006). Thermosensitive poly(N-isopropylacrylamide-co-acrylamide) hydrogels: Synthesis, swelling and interaction with ionic surfactants. *European Polymer Journal*, *42*, 348–355.

Deguchi, S., Akiyoshi, K. & Sunamoto, J. (1994). Solution property of hydrophobized pullulan conjugated with poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer. Formation of nanoparticles and their thermosensitivity. *Makromolecular Rapid Communications*, *15*(9), 705–711.

Di Mascio, P., Kaiser, S. & Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quench. *Archives of Biochemistry and Biophysics*, *274*, 532–538.

Dong, L. & Hoffman, A. S. (1991). A novel approach for preparation of pH-sensitive hydrogels for enteric drug delivery. *Journal of Controlled Release*, *15*(2), 141–152.

Dulong, V., LeCerf, D., Picton, L. & Muller, G. (2006). Carboxymethylpullulan hydrogels with an ionic and/or amphiphilic behavior: Swelling properties and entrapment of cationic and/or hydrophobic molecules. *Colloid and Surfaces A: Physicochemical and Engineering Aspects*, *274*, 163–169.

Dumitriu, R. P., Mitchell, G. R. & Vasile, C. (2011). Multi-responsive hydrogels based on N-isopropylacrylamide and sodium alginate. *Polymer*, *60*, 222–233.

Ekici, S. (2011). Intelligent poly(N-isopropylacrylamide)-carboxymethyl cellulose full interpenetrating polymeric networks for protein adsorption studies. *Journal of Material Sciences*, *46*, 2843–2850.

Feil, H., Bae, Y. H., Feijen, J. & Kim, S. W. (1993). Effect of comonomer hydrophilicity and ionization on the lower critical temperature of N-isopropylacrylamide copolymers. *Macromolecules*, *26*, 2496–2500.

Fettaka, M., Issaadi, R., Moulai-Mostefa, M., Dez, I., Lecerf, D. & Picton, L. (2011). Thermo sensitive behavior of cellulose derivatives in dilute aqueous solutions: From macroscopic to mesoscopic scale. *Journal of Colloid Interface Sciences*, *357*, 372–379.

Fras Zemljic, L., Strnad, S., Sauperl, O. & Stana-Kleinschek, K. (2009). Characterization of amino groups for cotton fibers coated with chitosan. *Textile Research Journal*, *79*(3), 219–226.

Gigimol, M. G. & Mathew, B. (2003). Effect of the nature and degree of crosslinking on Rose Bengal binding by DVB-, NNMBA-, HDODA- and TTEGDA-crosslinked poly(*N*-vinylpyrrolidone)s. *Polymer International*, *52*, 973–980.

Gutowska, A., Bae, Y. H. & Kim, S. W. (1992). Heparin release from thermosensitive hydrogels. *Journal of Controlled Release*, *22*(2), 95–104.

Henni-Silhadi, W., Deyme, M., Ruiz de Hoyos, M., LeCerf, D., Picton, L. & Rosilio, V. (2008). Influence of alkyl chains length on the conformation and solubilization properties of amphiphilic carboxymethylpullulans. *Colloid Polymer Sciences*, *286*, 1299–1305.

Hou, X., Liu, B., Deng, X., Zhang, B. & Yan, J. (2007). Monodisperse polystyrene microspheres by dispersion copolymerization of styrene and other vinyl comonomers: Characterization and protein adsorption properties. *Journal of Biomedical Materials Research A*, *83*(2), 280–289.

Kim, S., Shin, S., Lee, S., Kim, L. & Kim, S. I. (2004). Electromechanical properties of hydrogels based on chitosan and poly(hydroxyethyl methacrylate) in NaCl solution. *Smart Materials and Structures*, *13*, 1036–1039.

Kim, S. J., Shin, S. R., Lee, S. M., Kim, I. Y. & Kim, S. I. (2004). Electromechanical properties of hydrogels based on chitosan and poly(hydroxyethyl methacrylate) in NaCl solution. *Smart Materials and Structures*, *13*, 1036–1040.

Kotíková, Z., Lachman, J., Hejtmánková, A. & Hejtmánková, K. (2011). Determination of antioxidant activity and antioxidant content in tomato varieties and

evaluation of mutual interactions between antioxidants. *LWT – Food Science and Technology*, *44*, 1703–1710.

Lowry, O. H., Rosebrough, N. J., Lewis Farr, A. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Luo, D., Li, Y. & Yang, M. (2011). Preparation and characterization of novel crosslinked poly[glycidyl methacrylate–poly(ethylene glycol) methyl ether methacrylate] as gel polymer electrolytes. *Journal of Applied Polymer Science*, *120*, 2979–2984.

Marie, E., Landfester, K. & Antonietti, M. (2002). Synthesis of chitosan-stabilized polymer dispersions, capsules, and chitosan grafting products via miniemulsion. *Biomacromolecules*, *3*(3), 475–481.

Martínez-Ruvalcaba, A., Sánchez-Díaz, J. C., Becerra, F., Cruz-Barba, L. E. & González-Álvarez, A. (2009). Swelling characterization and drug delivery kinetics of polyacrylamide-co-itaconic acid/chitosan hydrogels. *Express Polymer Letters*, *3*(1), 25–32.

Mocanu, G., Mihai, D., Picton, L., LeCerf, D. & Muller, G. (2002). Associative pullulan gels and their interaction with biological active substances. *Journal of Controlled Release*, *83*, 41–52.

Mocanu, G., Mihai, D., Dulong, V., Picton, L. & LeCerf, D. (2011). New anionic amphiphilic thermosensitive pullulan derivatives. *Carbohydrate Polymers*, *84*(1), 276–281.

Palace, V. P., Khaper, N., Qin, Q. & Singal, P. K. (1999). Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. *Free Radical Biology and Medicine*, *26*, 746–761.

Pepper, K., Reichenberg, D. & Hale, D. K. (1952). Properties of ion-exchange in relation to their structure. IV. Swelling and shrinkage of sulfonated polystyrenes of different crosslinking. *Journal of Chemical Society*, 3129–3136.

Prasad, A. L. & Litwack, G. (1963). Measurement of the lytic activity of lysozymes (muramidases). *Analytical Biochemistry*, *6*, 328–334.

Rodriguez-Felix, D. E., Castillo-Ortega, M. M., Real-Felix, D., Romero-Garcia, J., Ledezma-Perez, A. S. & Rodriguez-Felix, F. (2011). Synthesis and swelling properties of pH and temperature-sensitive interpenetrating polymer network composed of polyacrylamide and poly(γ-glutamic acid). *Journal of Applied Polymer Sciences*, *119*, 3531–3537.

Singh, S., Webster, D. C. & Singh, J. (2007). Thermosensitive polymers: Synthesis, characterization and delivery of proteins. *International Journal of Pharmaceutics*, *341*, 68–77.

Shan, L., Verghese, K. N. E., Robertson, C. G. & Reifsnider, K. L. (1999). Effect of network structure of epoxy DGEBA-poly(oxypropylene)diamines on tensile behavior. *Journal of Polymer Science: Part B: Polymer Physics*, *37*, 2815–2819.

Sindhu, E. R., Preethi, K. C. & Kuttan, R. (2010). Antioxidant activity of carotenoid lutein in vitro and in vivo. *Indian Journal of Experimental Biology*, *48*, 843–848.

Taleb, M. F., Samia, A. E., Nabil, A. & Hegazy, A. (2007). Adsorption and controlled release of chlortetracycline HCl by using multifunctional polymeric hydrogels. *European Polymer Journal*, *43*, 468–477.