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Multi-responsive carboxymethyl polysaccharide crosslinked hydrogels containing Jeffamine side-chains

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ABSTRACT

The paper studies the synthesis and characterization of crosslinked carboxymethylpullulan hydrogels containing Jeffamine (Jef) (M-600 and M-2005) [polyoxyalkyleneamines (polyethylene oxide, polypropylene oxide)] units as side chains, linked through amide bonds. These hydrogels present pH sensitive properties due to the presence of anionic functional groups and thermoassociative properties due to the Jeff units. They were characterized through FTIR spectra, swelling behavior in various media, at various pH or temperatures, retention of hydrophobic molecules, to appreciate their pH-sensitive and thermoassociative (multi-responsive) properties. The interaction with biomolecules as proteins: lysozyme, BSA and antioxidants as: lutein and alpha-tocopherol was studied, to estimate some potential application domains of these new synthesized hydrogels.

1. Introduction

Intelligent (smart) polymers responding to external stimuli have been received great attention over the last years, due to their interesting properties with respect to various applications in biotechnology, pharmacy and medicine. In many cases these polymers are represented by hydrogels, which are three-dimensional chemically or physically crosslinked hydrophilic networks, able to retain large amounts of water or biological fluids (Peppas, Bures, Leobandung & Ichikawa, 2000). Physico-chemical properties of the hydrogels have been determined the particular interest in their use in drug delivery applications (Hoare & Kohane, 2008; Amin, Rajabnezhad & Kohli, 2009). Many kinds of stimuli can induce responses from hydrogels: physical (temperature, light, pressure, electric or magnetic field), chemical (pH, ions) or biological ones (antigens or glucose) (Qiu & Park, 2001; Jeong, Kim & Bae, 2002; Masteikova, Chalupova & Sklubalova, 2003). Between them, the most frequently studied hydrogels are those sensitive to temperature and pH (stimuli acting in the body), due to their potential to be used as drug delivery systems; these are specific, controllable and biocompatible drug delivery devices. Thermoresponsible properties of the hydrogels can be induced by thermosensitive polymer chain [PNIPAM (Feil, Bae, Feijen & Kim, 1993), hydroxypropylcellulose (Fettaka et al., 2011)], or by thermosensitive units linked on the pullulan chains: poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (Deguchi, Akiyoshi & Sunamoto, 1994). pH-sensitive hydrogels contain pendant anionic (weakly, strongly acidic) or cationic (amine) 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 (Dulong, LeCerf, Picton & Muller, 2006). Multiresponsive hydrogels were also reported, containing on the polymer backbone both thermosensitive units and ionic groups, (Bomberg, Temchenko & Hatton, 2002; Rodriguez-Felix et al., 2011; Dumitriu, Mitchell, & Vasile, 2011).

The hydrogels containing both hydrophilic and hydrophobic segments on the macromolecular chains present amphiphilic (associative) interesting properties, determined by their hydrophilic/hydrophobic balance (HLB). Hydrophobized polysaccharides have been intensively studied due to their main property to form hydrogel nanoparticles by self-assembling, being recommended for various applications in biotechnology, pharmacy and medicine. For the most studied cholesteryl pullulan (Akiyoshi, Nagai, Nishikawa & Sunamoto, 1992), which forms nanoparticles through self-assembling, was evidenced the property to include through hydrophobic forces various biologically active substances as: drugs – adriamycin (Akioshi, Taniguchi, Fukui & Sunamoto, 1996), proteins (Ayame, Morimoto & Akiyoshi, 2008), [BSA (Akiyoshi et al., 1996), insulin (Akiyoshi et al., 1998)]; these

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studies revealed the potential use of associative polysaccharides in controlled release drug systems.

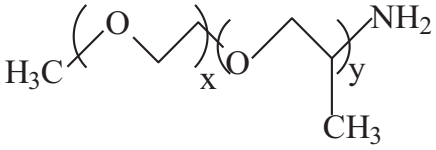
Jeffamines were used to obtain thermosensitive derivatives through their grafting onto the surface of cellulose nanocrystals (Azzam, Heux, Putaux & Jean, 2010), on linear pullulan (Belbekhouche, Ali, Dulong, Picton & LeCerf, 2011), or carboxymethylpullulan (Dulong, Mocanu, Picton, & Le Cerf, 2012; Mocanu, Mihai, Dulong, Picton & Lecerf, 2011); also, thermoresponsive micelles from Jeffamine-*b*-poly(L-glutamic acid) double hydrophilic block copolymers were reported (Agut, Brûlet, Taton & Lecommandoux, 2007).

The present paper studies the synthesis of crosslinked polysaccharide hydrogels containing on the macromolecular network both pH-sensitive–carboxymethyl groups and thermoassociative – Jeffamine [polypropyleneglycol (PPG)-polyethyleneglycol (PEO)] units, linked through amide bonds; physico-chemical characterization of the synthesized hydrogels was performed to verify their multiresponsive properties. The interaction with biomolecules: antioxidants: lutein, alpha-tocoferol; proteins: bovine serum albumine (BSA), lysozyme was also studied to appreciate their potential for use as controlled release drug systems.

2. Experimental

2.1. Materials

Jeffamines (Jeff): Jeffamine M-600 (Fluka) LCST = 59 °C at 10 g L^{-1} in water; M-2005 (Hunstman) LCST = 25 °C at 10 g L^{-1} in water.



M- 600 PPO/PEO ratio: 9/1

M-2005 PPO/PEO ratio:29/6

N,N′-dicyclohexyl carbodiimide (DCCI) (Fluka), dimethylaminopyridine (DMAPy), DMSO, Rose Bengal (RB) (Sigma Aldrich), Brilliant Blue (BB) (Fluka), Vitamin B12 (B12), lysozyme (Sigma), BSA (Fluka) lutein (ethanolic extract of lutein capsules, Medica Lab., Romania), alpha-tocopherol (Fluka).

2.2. Methods

Crosslinked carboxymethylpullulan microspheres (CMP) synthesized in the laboratory, as described elsewhere (Mocanu, Mihai, Picton, LeCerf & Muller, 2002).

The Scheme 1 presents the synthesis of the Jeffamine-substituted crosslinked CMP; it comprises many steps, as follows: (1) crosslinking with epichlorohydride, in basic media; (2) carboxymethylation with sodium chloroacetate in basic media; (3) amidation reaction with Jeffamines. After each step, the microparticles were filtered, washed with appropriate solvents and dried.

Synthesis of Jeffamine-carboxymethyl pullulan derivatives (CMP-Jeff). 1.5 g (7.5 mmol) CMP microparticles in H^+ form was swollen in 15 mL DMSO freshly distilled for 24 h at 20 °C; then, 0.46 g (2.25 mmol) DCCI in 10 mL DMSO was added and the reaction mixture was stirred for 2 h; 4.5 g (2.25 mmol) of Jeff M-2005 and 0.01 g (0.08 mmol) DMAPy were added and the reaction was continued for 48 h at 20 °C. After that, the microparticles were filtered, washed on the filter with dichloromethane (to remove the unreacted DCCI), acetone and methyl alcohol [to remove dicyclohexylurea (Leung, Lai, Lau, Yu, & Hsiao, 1996)] then with water, and dried from ethyl alcohol. Yield: 2.64 g. The same procedure was carried out for obtaining various DS with Jef units, using different molar ratios of Jef-M600 or Jef-M2005, and corresponding amounts of DCCI, for activating the COOH groups.

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).

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

$$W_R = \frac{W - W0}{W0}$$

where W_R – water (solvent regain); W – weight of the sample swollen at equilibrium; 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 from the difference in the concentrations of the dye solution, before and after binding (at 548 nm wavelength).

Brilliant blue and Vitamin B 12 retention experiments were also performed upon the same procedure described above (at 586 nm and 550 nm wavelength, respectively).

The retained dyes are not released from the supports during washing with water; that was checked through UV measurements of the effluents.

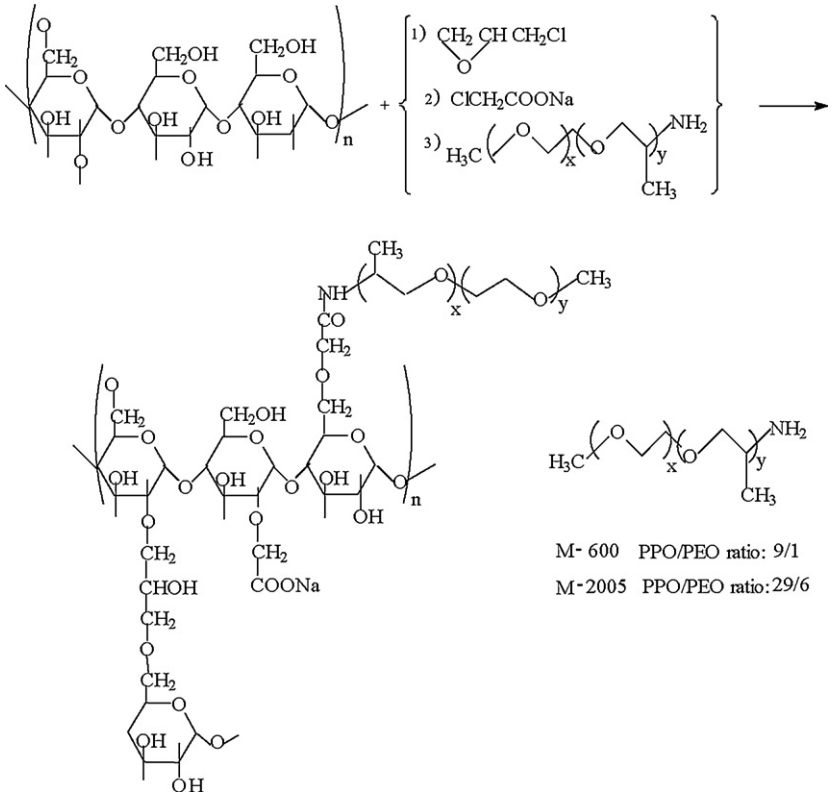
Lutein and alpha-tocopherol retention experiments were performed in similar manner, in solutions containing 1 g drug/L (at 444 nm and 290 nm wavelength, respectively).

Retention of PEG with various Mw values was determined by an adapted method, as described for fluorescein-labeled dextran (F*-dextran) (Lynch & Dawson, 2003). Microparticles previously dried in vacuum and weighed, were equilibrated in a 0.02% sodium azide solution, for 24 h; then, they were placed in solutions of 200 μg/mL concentration of various molecular weight PEG, which was monitored daily. After equilibration, the microparticles were filtered, and the amount of remaining PEG in the solutions has been determined by measurements of total carbon content, with a Shimadzu TOC-V CSN total organic carbon analyzer apparatus. The amount of retained PEG was determined through difference from its initial content.

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.

Release of the proteins retained on the supports was also performed under "batch" conditions, on a 50 mg support–protein complex, in phosphate buffered saline (PBS) solution with pH: 7.4; the protein content was determined also by the Folin method, on a Specord 200 Analytic Yena UV–vis spectrophotometer.

The antioxidant activity of the lutein or alpha-tocopherol containing hydrogels was determined through DPPH method (Ara &



Scheme 1. Synthesis of the Jeffamine-substituted crosslinked CMP.

Nur, 2009) and compared with those of ascorbic acid, initial lutein or alpha-tocopherol. Briefly, various amounts of samples containing the antioxidant product were immersed in 2 mL solution NaCl 1 g L^{-1} and 1 mL methanolic DPPH solution (0.128 g L^{-1} 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:

$$\text{Scavenging effect } \% = \frac{A_0 - A_1}{A_0} \times 100$$

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 physico-chemical characterization of microparticles

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}.

Reaction conditions and physico-chemical characteristics of Jeff-substituted carboxymethylpullulan microparticles are presented in Table 1.

The obtained microparticles present ion exchange capacities and degrees of substitution with Jeff units depending on the initial Jeff molar ratio; for both Jeff used, the efficiency of the amidation reaction is about 50%.

The swelling of the hydrogels in various media and different temperatures was studied, to appreciate their hydrophilicity as well as their polyelectrolyte and thermoresponsive character. As can be seen from the data presented in Fig. 1, the swelling of the microparticles diminishes with the increase of degree of substitution with M-2005 Jeff hydrophobic units. Surprisingly, the microparticles substituted with M-600 Jeff units are more hydrophilic than the initial CMP. The thermosensitive character of the hydrogels is evidenced through their swelling behavior at various temperatures; thus, the swelling of M-2005 Jeff hydrogels diminishes with temperature increase (negative temperature sensitive hydrogels), while the swelling of M-600 Jeff hydrogels rises at 40 °C and diminishes around 60 °C (cloud point of Jeff M-600). The

Table 1
Reaction conditions and physico-chemical characteristics of Jeff-substituted CMP microparticles.

Sample	Jeff	Molar ratio Jeff/GU[a]	Ion exchange capacity, meq/g	$DS^{b}_{COO^-}$	DS_{Jeff}
CMP	–		2.97	0.63	–
J-1	M-2005	0.3/1	0.935	0.475	0.15
J-2	M-2005	0.1/1	2.12	0.595	0.035
J-3	M-600	0.3/1	1.96	0.52	0.11
J-4	M-600	0.1/1	2.55	0.59	0.04

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

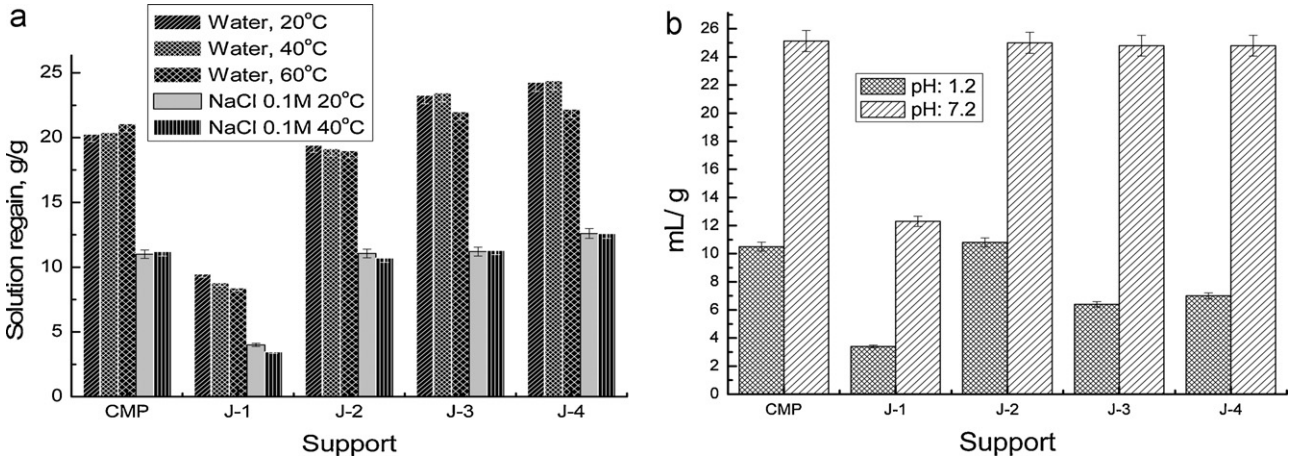


Fig. 1. Swelling of hydrogels in water or NaCl 0.1 M solution at various temperatures (a) and in solutions of various pH (b).

swelling/shrinking behavior of the hydrogels is strongly influenced by the hydrophobic or hydrophilic nature of the comonomers (Gutowska, Bae & Kim, 1992). For 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. The swelling increase as temperature function may be attributed to the increase of the number of layers of water molecules, around POE units, as temperature rise. 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. (Fig. 1a)

In NaCl 0.1 M solutions all hydrogels collapse, due to the screening effect of the salts on the ionic charges of CMP conjugates through the polyelectrolyte effect. The temperature influence on the swelling behavior in saline solutions is the same as in pure water.

The pH-sensitivity of the hydrogels is assured by the presence of anionic groups, the pK_a values of CMP and its Jeffamines-substituted derivatives ranging between 4.1 and 4.8. This is evidenced through the variation of the microparticles' volume in acidic, respectively basic media. In acidic pH 1.2 (below pK_a), the microparticles containing unionized anionic carboxylic groups are in a collapsed state, as mentioned in literature (Dong & Hoffman, 1991), while in enteric solutions (pH 6.8–7.2) (above pK_a) they are swollen (Fig. 1b), due to the ionized acidic groups which determine electrostatic repulsions (Taleb, Abdel-Aal, El-Kelesh & Hegazy, 2007). This behavior as polyelectrolyte is important in controlled drug release for oral administration.

The amphiphilic character of the hydrogels was evidenced through retention of the dyes: hydrophobic (Rose Bengal – RB) (Gigimol & Mathew, 2003), amphiphilic (Brilliant blue – BB) (Dulong et al., 2006) and hydrophilic vitamin B12 (Fig. 2). RB and BB are retained through hydrophobic forces on the hydrogels, in higher amounts on Jeff-substituted derivatives. Vitamin B12 is retained through formation of interpolymer complexes, as in cases of complexing with a weak acid cation exchange resin (Mahore, Wadher, Umekar & Bhoyar, 2010) or with poly(methacrylic acid-g-ethylene glycol) hydrogels (Fogueri & Singh, 2009). The fact that it is retained in higher amounts on Jeff-substituted derivatives can be explained through an additional influence of the hydrophobic interactions between the support and the solute.

Attempts to determine the porosity of the hydrogels in swollen state were made (in addition to that determined through water

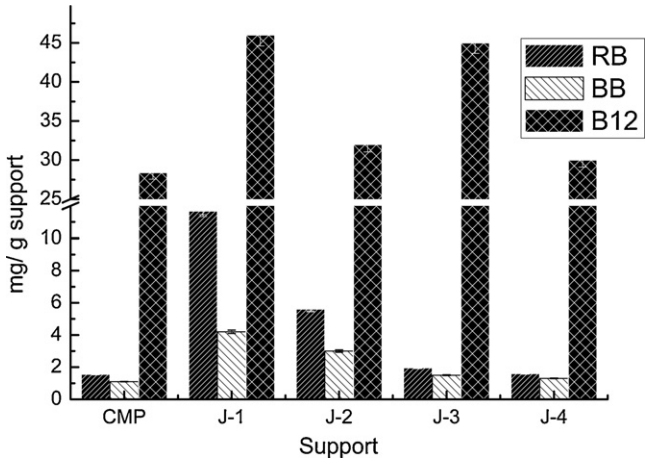


Fig. 2. Retention of Rose Bengal (RB), Brilliant Blue (BB) and Vitamin B12 (B12) on the hydrogels.

regain). Generally, in swollen state, a hydrogel has a certain porosity which can be defined as average "pore radius" of the network and can be determined from permeability data, by using probes of known molecular dimensions (Garret, Chantelier, Griesser & Milthorpe, 1998). PEG samples of various molecular weights were used: 600; 2000; 6000; 10,000; 13,500, with gyration radius of: 1; 2; 3.5; 5 and 6 nm, respectively (Mocanu, Mihai, LeCerf, Picton & Dulong, 2007). The data presented in Fig. 3 show that the supports

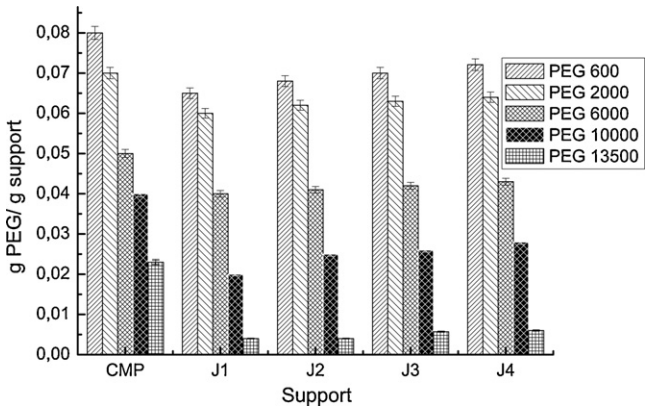
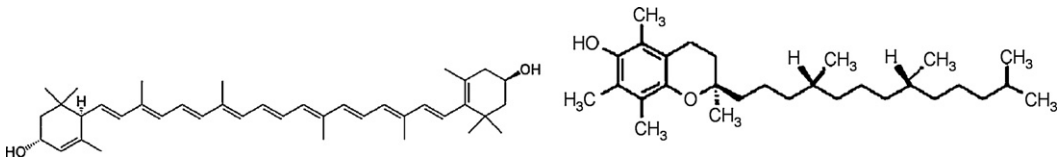


Fig. 3. Retention of PEG having different Mw on the hydrogels.



Scheme 2. Structure of lutein (a) and alpha tocopherol (b).

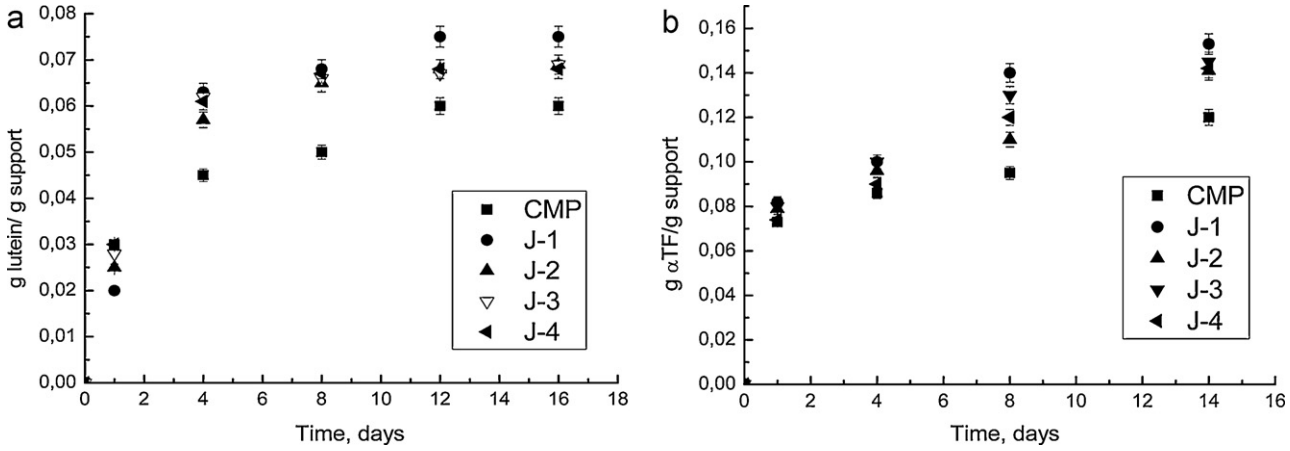


Fig. 4. Retention of lutein (a) and of alpha-tocopherol (b) on the hydrogels; the values are the mean of three independent measurements that deviated: 2–3%.

retain various amounts of PEG 600-10.000; the Jeff-substituted hydrogels retain lower PEG amounts than parent CMP; this fact corelates with their smaller water regain (Fig. 1a). PEG 13500, retained in very small amounts on Jeff-substituted hydrogels can be considered as their average exclusion limit (hydrodynamic radius: 6 nm).

3.2. Interaction with biomolecules

3.2.1. Interaction with antioxidants

Lutein (3,3′-dihydroxy-β-ε-carotene) (Scheme 2a) 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 (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).

Tocopherols (Scheme 2b) are a class of chemical compounds of which many have vitamin E activity. It is a series of organic compounds consisting of various methylated phenols. The tocopherols occur in alpha, beta, gamma and delta forms, determined by the number and position of methyl groups on the chromanol ring. Alpha-tocopherol is the most biologically active form of vitamin E that is preferentially absorbed and accumulated in humans; tocopherols are fat-soluble antioxidants. As it is fat-soluble, it is incorporated into cell membranes, which protects them from oxidative damage. However, there are other functions that have also been recognized to be of importance; α-tocopherol has a regulatory effect on enzymatic activities, on gene expression, also plays a role in neurological functions.

In the paper was studied the retention of the lutein and alpha tocopherol (α-TF) on the obtained supports; due to their hydrophobic character, one can suppose that they will be retained preponderantly through hydrophobic forces. The data presented in Fig. 4 show that both lutein (Fig. 4a) and (α-TF) (Fig. 4b) were retained gradually in time, in higher amounts on J-1 substituted, most hydrophobic hydrogel; (α-TF) is retained in higher amounts than lutein, probably due to its lower molecular weight, which determines an easier access into macromolecular hydrogel network.

With the aim to appreciate possible application domains of hydrogels containing the mentioned biomolecules, their antioxidant activity (expressed as Scavenging effect %) was studied, comparatively with that of the ascorbic acid (AA) and of the drugs used. The data presented in Fig. 5 show that the antioxidant effect of the parent drugs follows the order: AA > α-TF > lutein, as already mentioned in literature (Kotíková, Lachman, Hejtmánková & Hejtmánková, 2011); α-TF and lutein retained on the studied supports present also antioxidant activity. The scavenging effects of the released α-TF and lutein are lower than those of the parent substances due to the gradually release of the biomolecules retained

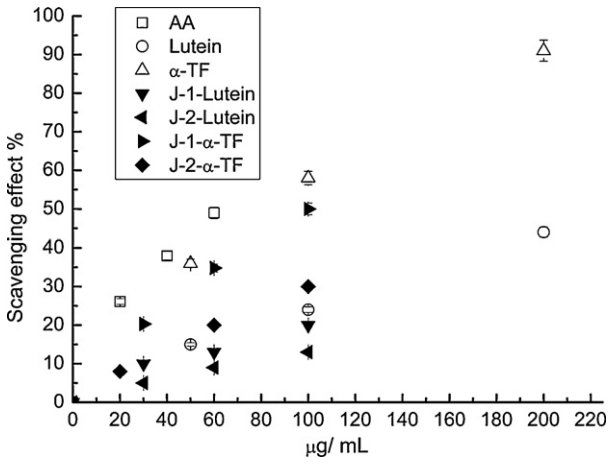


Fig. 5. Scavenging effect of alpha-tocopherol and lutein retained on the hydrogels, comparatively with that of the drugs as such and of the citric acid; the values are the mean of three independent measurements that deviated: 2–3%.

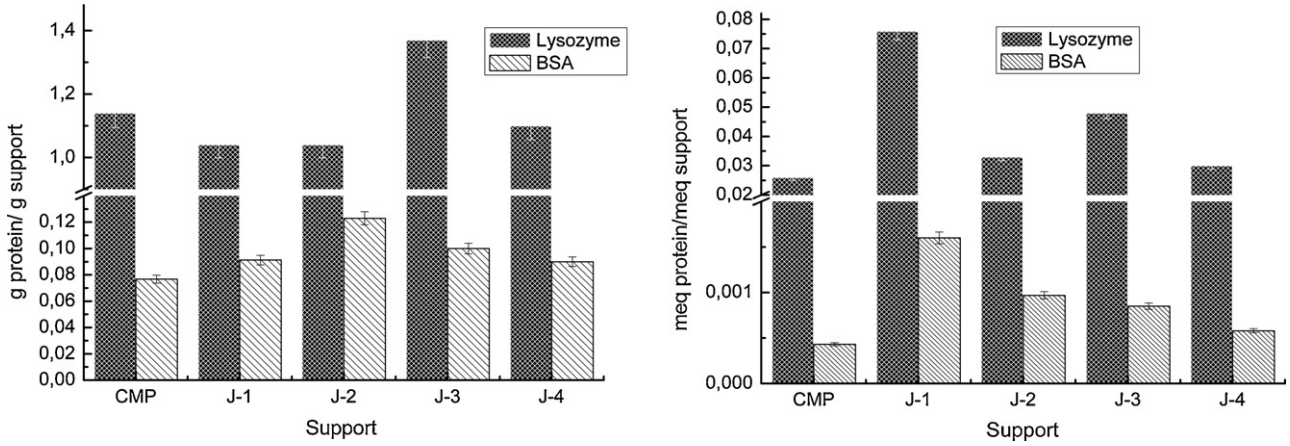


Fig. 6. Retention of the proteins (BSA, lysozyme) on the hydrogels, expressed as g protein/g support (a) and meq protein/meq support (b); the values are the mean of three independent measurements that deviated: 3–5%.

on the supports. This behavior can be important in the potential use of the supports for controlled antioxidant substances delivery.

3.2.2. Interaction with proteins

Lysozyme is a small (Mw = 14,600), globular, basic protein with an isoelectric point of ~11; it presents antimicrobial activity and can be used in the treatment of ulcer, viral infections or 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.

Bovin serum albumin (*BSA*) is a protein with Mw = 60,000 and isoelectric point of 4.7; the maximum absorption capacity was obtained at pH 4.7 on the retention of BSA on PNIPAM-carboxymethyl interpenetrating cellulose polymeric network (Ekici, 2011). BSA absorption on the supports can be influenced by several factors: hydrophobic or hydrophilic interactions between the corresponding groups of the absorbent and of protein molecules. Hence, the BSA retention on the hydrogel support will be dependent upon the cooperative effects of hydrophobic and hydrophilic interactions. Its retention can be the result of many accumulative effects, for example, hydrophobic interactions, hydrogen bonds, electrostatic forces (Hou, Liu, Deng, Zhang & Yan, 2007). The data presented in Fig. 6a show that lysozyme is retained in higher amounts than BSA on the studied supports; that can be explained through the smaller dimensions of the lysozyme which allow its free diffusion inside hydrogel pore network. In this context, many indications about molecular dimensions of lysozyme were found: (a slightly ellipsoidal shape and dimensions: 30 Å × 30 Å × 45 Å (Imoto, Johnson, North, Phillips & Rupley, 1972); d_h: 1.8 nm (Colvin, 1952); 1.8 nm (Hiinenberger, Mark & van Gunsteren, 1995); 4.1 nm (Merril, Dennison & Sung, 1993); 2.8 nm × 3.2 nm × 3.0 nm (Yaminsky, Gvozdev, Sil'nikova & Rashkovich, 2002). Even these reported dimensions are fairly different, one can suppose that lysozyme can penetrate into hydrogel pores (determined average exclusion limit: 6 nm). But, bigger BSA molecule (3.5 nm (Champagne, Luzzati & Nicolaieff, 1958); 7.2 nm (Merril et al., 1993); ellipsoidal shape and dimensions: 40 Å × 140 Å (Bloomfield, 1966) is retained in smaller amounts, the uptake being primarily by surface adsorption; some diffusive penetration into the matrix can be also possible. By representing the protein retention expressed as: meq protein/meq ion exchange capacity of the support (Fig. 6b) is evidenced better the influence of hydrophobic interactions on the protein binding: the Jeff-substituted hydrogels retain higher protein amounts. Even the Jeff-substituted derivatives have a smaller ion exchange capacity than parent CMP, the amount of retained protein is higher; this means that other interactions (hydrophobic) cooperate in an important extent to the protein binding.

The in vitro release of the proteins in PHB was also studied, as time and temperature function. Lysozyme is faster released from the more hydrophilic supports (CMP, J-4); possible included in the hydrogel pores, the lysozyme is released upon a diffusion

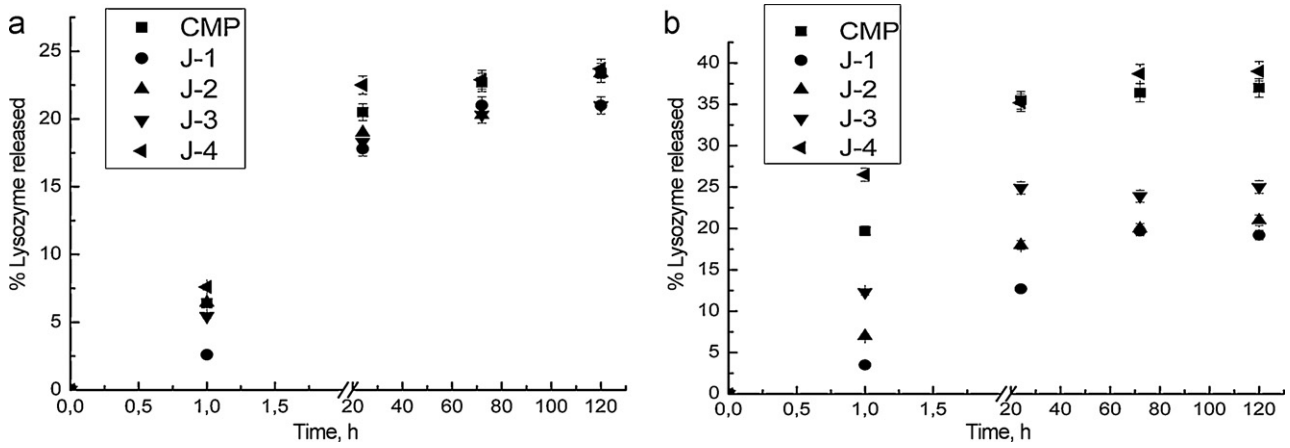


Fig. 7. *In vitro* release of the lysozyme retained on the hydrogels at 20 °C (a) and 40 °C (b); the values are the mean of three independent measurements that deviated: 3–4%.

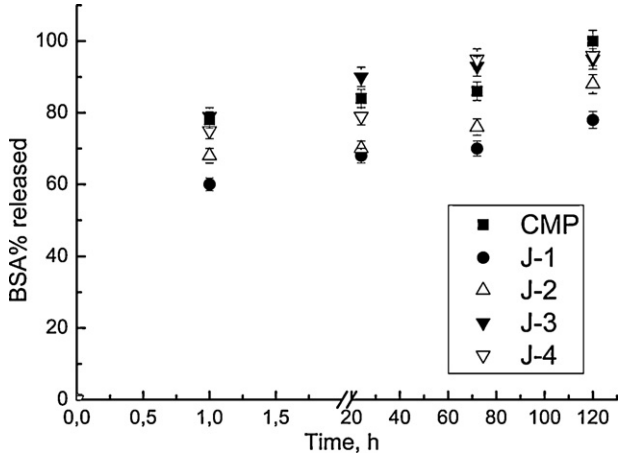


Fig. 8. *In vitro* release of the BSA retained on the hydrogels at 20 °C; the values are the mean of three independent measurements that deviated: 3–4%.

governed mechanism (Censi et al., 2009). (Fig. 7a) At 40 °C the release behavior is slightly different towards 20 °C: the samples J-1 and J-2, with negative thermosensitivity (shrunken at higher temperatures) release the lysozyme slower than the samples CMP, J-3, J-4, with positive thermosensitivity; this fact is also due to the influence of the diffusion on the release process (Fig. 7b).

Comparatively, BSA is released with higher rate (Fig. 8), in amounts which are very few dependent on the temperature used; this behavior can be explained through the fact that BSA is retained mainly on the hydrogel surface, hence the diffusion and the temperature have a smaller influence on BSA release, unlike the thermosensitive methacrylated p(HPMAm-lac)-PEG-p(HPMAm-lac) hydrogels which present a diffusion governed mechanism release for some protein, BSA including (Censi et al., 2009). More hydrophobic J-1 and J-2 hydrogels release slower BSA, due to its hydrophobic interactions with the mentioned supports.

It is important to know if the proteins retained on the hydrogels preserve their enzymatic activity; this aspect was verified for lysozyme. The specific activity of the lysozyme released from the hydrogels in NaCl 0.1 N solutions 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 *Micrococcus lysodeikticus* as substrate), similar with that of the lysozyme taken into study.

4. Conclusions

Crosslinked polysaccharide multi-responsive (thermo-, pH-sensitive) associative hydrogels having Jeffamine side chains, linked through amide bonds were synthesized and physico-chemically characterized. The thermo- and pH-sensitive characters were evidenced through appropriate swelling measurements, while the associative character was evidenced through hydrophobic/amphiphilic/hydrophilic dyes retention. The retention of antioxidants: lutein, alpha-tocopherol on the hydrogels was studied; their scavenging effect was evidenced. The proteins: lysozyme, BSA were also retained on the hydrogels in various amounts through electrostatic and/or hydrophobic forces; they were released in PHB as time function. Lysozyme, which can penetrate inside the hydrogel pore network, presented a temperature-controlled release. Hence, the study of the interaction of the hydrogels with biomolecules demonstrates their potential use as biomaterials for controlled drug or protein delivery.

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References

Agut, W., Brûlet, A., Taton, D., & Lecommandoux, S. (2007). Thermoresponsive micelles from Jeffamine-*b*-poly(L-glutamic acid) double hydrophilic block copolymers. *Langmuir*, *23*, 11526–11533.

Akiyoshi, K., Nagai, K., Nishikawa, T., & Sunamoto, J. (1992). Self-aggregates of hydrophobized polysaccharide as a host for macromolecular guests. *Journal of Chemistry Letters*, *21*, 1727–1730.

Akioshi, K., Taniguchi, I., Fukui, H., & Sunamoto, J. (1996). Hydrogel nanoparticles formed by self-assembly of hydrophobized polysaccharide. Stabilization of adriamicin by complexation. *European Journal of Pharmaceutics and Biopharmaceutics*, *42*(4), 286–290.

Akiyoshi, K., Nishikawa, T., Mitsui, Y., Miyata, T., Kodama, M., & Sunamoto, J. (1996). Self-assembly of polymer amphiphiles: thermodynamics of complexation between bovine serum albumin and self-aggregate of cholesterol-bearing pullulan. *Journal of Colloid Surfaces. A: Physicochemical Engineering Aspects*, *112*(2–3), 91–95.

Akiyoshi, K., Kobayashi, S., Shichibe, S., Mix, D., Baudys, M., Kim, S. W., et al. (1998). Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: complexation and stabilization of insulin. *Journal of Controlled Release*, *54*, 313–320.

Amin, S., Rajabnezhad, S., & Kohli, K. (2009). Hydrogels as potential drug delivery systems. *Scientific Research Essay*, *3*(11), 1175–1183.

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.

Ayame, H., Morimoto, N., & Akiyoshi, K. (2008). Self-assembled cationic nanogels for intracellular protein delivery. *Bioconjugate Chemistry*, *19*(4), 882–898.

Azzam, F., Heux, L., Putaux, J. L., & Jean, B. (2010). Preparation by grafting onto, characterization, and properties of thermally responsive polymer-decorated cellulose nanocrystals. *Biomacromolecules*, *11*, 3652–3659.

Belbekhouche, S., Ali, G., Dulong, V., Picton, L., & LeCerf, D. (2011). Synthesis and characterization of thermosensitive and pH-sensitive block copolymers based on polyetheramine and pullulan with different length. *Carbohydrate Polymers*, *86*(1), 304–312.

Bloomfield, V. (1966). The structure of bovine serum albumin at low pH. *Biochemistry*, *5*, 684–689.

Bomberg, L., Temchenko, M., & Hatton, T. A. (2002). Dually responsive microgels from polyether-modified poly(acrylic acid); swelling and drug loading. *Langmuir*, *18*(12), 4944–4952.

Censi, R., Vermonden, T., van Steenbergen, M. J., Deschout, H., Braeckmans, K., De Smedt, S. C., et al. (2009). Photopolymerized thermosensitive hydrogels for tailorable diffusion-controlled protein delivery. *Journal of Controlled Release*, *140*(3), 230–236.

Champagne, M., Luzzati, V., & Nicolaieff, A. (1958). The size and shape of bovine serum albumin as a function of pH, determined by small-angle scattering of X-rays. *Journal of American Chemical Society*, *80*(4), 1002–1003.

Colvin, J. R. (1952). The size and shape of lysozyme. *Canadian Journal of Chemistry*, *30*, 831–834.

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 quencher. *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.

Dulong, V., Mocanu, G., Picton, L., & Le Cerf, D. (2012). Amphiphilic and thermosensitive copolymers based on pullulan and Jeffamine®: synthesis, characterization and physicochemical properties. *Carbohydrate Polymers*, *87*(2), 1522–1531.

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.

Fogueri, L. R., & Singh, S. (2009). Smart polymers for controlled delivery of proteins and peptides: a review of patents. *Recent Patents on Drug Delivery & Formulation*, *3*, 40–48.

Garret, Q., Chatelier, R. C., Griesser, H. J., & Milthorpe, B. K. (1998). Effect of charged groups on the adsorption and penetration of proteins onto and into carboxymethylated poly(HEMA) hydrogels. *Biomaterials*, *19*, 2175–2186.

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.

Hiinenberger, P. H., Mark, A. E., & van Gunsteren, W. F. (1995). Computational approaches to study protein unfolding: hen egg white lysozyme as a case study. *Proteins: Structure, Function, and Genetics*, *21*, 196–213.

Hoare, T. R., & Kohane, D. S. (2008). Hydrogels in drug delivery: progress and challenges. *Polymer*, *49*, 1993–2007.

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.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972). In P. D. Boyer (Ed.), *The enzymes* (3rd ed., pp. 665–868). New York: Academic Press.

Jeong, B., Kim, S. W., & Bae, Y. H. (2002). Thermosensitive sol–gel reversible hydrogels. *Advanced Drug Delivery Reviews*, *54*, 37–51.

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 antioxidant. *LWT – Food Science and Technology*, *44*, 1703–1710.

Leung, M., Lai, J. L., Lau, K. H., Yu, H., & Hsiao, H. J. (1996). S,S-Dimethyl dithiocarbonate: a convenient reagent for the synthesis of symmetrical and unsymmetrical ureas. *Journal of Organic Chemistry*, *61*, 4175–4179.

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.

Lynch, I., & Dawson, K. A. (2003). Effect of a polymeric additive on the pore-size distribution and shrinking process of a hydrogel network. *Macromolecular Chemistry Physics*, *204*, 443–450.

Mahore, J. G., Wadher, K. J., Umekar, M. J., & Bhoyar, P. K. (2010). Ion exchange resins: pharmaceutical applications and recent advancement. *International Journal of Pharmaceutical Sciences Review and Research*, *1*(2), 8–13.

Masteikova, R., Chalupova, Z., & Sklubalova, Z. (2003). Stimuli-sensitive hydrogels in controlled and sustained drug release. *Medicina*, *39*(2), 19–24.

Merril, E. W., Dennison, K. A., & Sung, C. (1993). Partitioning and diffusion of solutes in hydrogels of poly(ethylene oxide). *Biomaterials*, *15*(3), 1117–1126.

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., LeCerf, D., Picton, L., & Dulong, V. (2007). New polysaccharide-based microparticles crosslinked with siloxanic units. I. Synthesis and characterization. *Reactive and Functional Polymers*, *67*(1), 60–66.

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

Peppas, N. A., Bures, P., Leobandung, W., & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, *50*, 27–46.

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.

Qiu, Y., & Park, K. (2001). Environment-sensitive hydrogels for drug delivery. *Advanced Drug Delivery Reviews*, *53*(3), 321–339.

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.

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, A. M. F., Abdel-Aal, S. E., El-Kelesh, N. A., & Hegazy, A. (2007). Adsorption and controlled release of chlortetracycline HCl by using multifunctional polymeric hydrogels. *European Polymer Journal*, *43*, 468–477.

Yaminsky, I. V., Gvozdev, N. V., Sil'nikova, M. I., & Rashkovich, L. N. (2002). Atomic force microscopy study of lysozyme crystallization. *Crystallography Reports*, *47*(Suppl. 1), S149–S158.