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Anionic polysaccharide hydrogels with thermosensitive properties

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ABSTRACT

Microparticles of crosslinked carboxymethyl pullulan were chemically modified by introducing hydrophobic thermosensitive Poloxamer groups; these new pullulan derivatives are expected to present, besides their ionic character, both amphiphilic and thermosensitive properties. The obtained microparticles were physico-chemically characterized and their interaction with various proteins, such as enzymes (lysozyme, BSA), vaccines (tetanus anatoxin), was studied. The retention/release behavior of the biomolecules on/from the studied derivatives is influenced by electrostatic and hydrophobic forces, as well as by the presence of thermosensitive Poloxamer groups.

1. Introduction

Study of intelligent (smart) polymers used for controlled drug delivery has been largely extended in recent years; this interest is justified by a multitude of advantages, such as: improved effectiveness, with uniform drug delivery at therapeutical dose, minimal side effects and lower frequency of administration (Brazel & Peppas, 1999).

Hydrogels are three-dimensional chemically or physically crosslinked hydrophilic networks, able to retain large amounts of water or biological fluids (Peppas, Bures, Leobandung, & Ichikawa, 2000), presenting an important potential for drug delivery applications (Hoare & Koane, 2008). As a function of their structure, these hydrogels can have stimuli-sensitive properties, which recommend them for various important applications, such as: controlled and sustained drug delivery, biosensors, bioseparations. The stimuli that can induce responses from hydrogels can be physical (temperature, light, pressure, electric or magnetic field), chemical (pH, ions) or biological ones (antigens or glucose) (Masteikova, Chalupova, & Sklubalova, 2003; Qiu & Park, 2001). The most frequently studied hydrogels are those sensitive to temperature and pH (stimuli present in the body), due to their potential to operate as drug delivery systems; these are specific, controllable and biocompatible drug delivery devices. The drugs widely studied in relation with such hydrogels are: proteins, peptides, anticancerous, antidiabetic (Amin, Rajabnezhad, & Kohli, 2009).

Thermosensitive hydrogels can be: (i) negative temperature sensitive (usually copolymers of N-isopropylacrylamide), in this case, 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; (ii) positive temperature sensitive [copolymers of N-isopropylacrylamide with hydrophilic monomers as hydroxyethyl methacrylate (Ankareddi & Brazel, 2007), interpenetrating network (IPN) of poly[(acrylic acid)-co-(acrylamide)] (Bouillot & Vincent, 2000; Xiao, Zhuo, Xu, & Chen, 2006); in this case, the gels show swelling at high temperature and shrinking at low temperature. The lower critical solution temperature (LCST) of copolymers of N-isopropylacrylamide with various hydrophilic, hydrophobic, ionic co-monomers varies, due to changes in the overall hydrophobicity of the polymer (Feil, Bae, Feijen, & Kim, 1993), the swelling/shrinking behaviors of the hydrogels being strongly influenced by the hydrophobic or hydrophilic nature of the co-monomers (Gutowska, Bae, & Kim, 1992). In the presence of surfactants (especially sodium dodecyl sulfate), the negative temperature sensitive hydrogels of poly(N-isopropylacrylamide-co-acrylamide) are converted to positive ones, due to binding of the surfactant through hydrophobic interactions; the presence of anionic $-O-SO_3^-$ groups induces changes in the number of water layers in the vicinity of the polymer (Caykara, Kiper, & Demirel, 2006). Covalently crosslinked microgels of poly(acrylic acid)-g-PEO-PPO-PEO exhibit a high capacity for loading with hydrophobic or weakly basic drugs; the increase in drug loading at a higher temperature (37 °C comparatively with 20 °C, above critical aggregation temperatures) is explained

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Table 1
Physico-chemical characteristics of POX derivatives of crosslinked carboxymethyl pullulan microparticles.

Sample	Initial molar ratio POX/GU[a]	IExC[b] meq/g	DS with COOH groups	DS with POX chains	True density, g/mL	Pores volume, mL/g	Porosity (∅)	Water uptake g/g	Rose Bengal retention mg/g
CMP	–	3.2	0.7	–	1.14	0.19	0.217	24.1	0
20.01	0.1/1	2.9	0.63	0.07	1.10	0.14	0.154	11.8	4.4
20.03	0.3/1	2.8	0.61	0.09	1.07	0.135	0.144	8.6	5.6
20.05	0.5/1	2.5	0.53	0.17	1.01	0.13	0.131	5.5	8.8
44.01	0.1/1	2.95	0.64	0.06	1.09	0.14	0.153	14.8	6.5
44.03	0.3/1	2.9	0.63	0.07	1.07	0.139	0.148	11.2	7.4
44.05	0.5/1	2.6	0.55	0.15	0.95	0.13	0.125	7.45	11.5

[a] Glucopyranosic unit.
[b] Ion exchange capacity.

by a mechanism of hydrophobic solubilization into micelle–like aggregates inside the microgels (Bromberg & Hatton, 2003); (iii) thermoreversible gels are mainly based on copolymers of Pluronics (Poloxamer) (PEO-PPO-PEO block copolymers) (Bromberg, 1998; Jeong, Kim, & Bae, 2002; Wang, Zhu, Zhang, Yang, & Ding, 2006).

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 pH (unionized anionic group) and swollen in basic pH, where the ionized acidic groups determine electrostatic repulsions. This behavior is important in controlled drug release for oral administration. By using these hydrogels, the drug release will be minimal in the stomach, where swelling is lower, and will be increased in the intestinal tract, due to the increase of pH, leading to the ionization of the carboxylic groups. Anionic hydrogels are recommended for use as intelligent controlled release supports for site-specific drug delivery of therapeutic proteins to the large intestine, where their biological activity is prolonged (Satish, Satish, & Shivakumar, 2006), while cationic hydrogels can be used for localized drug delivery in the stomach for the treatment of *Helicobacter pylori* (Patel & Amiji, 1996).

The paper studies the synthesis, characterization and interaction with drug proteins of crosslinked microparticles of carboxymethyl pullulan, chemically modified with various amounts of Poloxamer chains. In relation to their structure, these microparticles can be considered as potential carriers for controlled release of cationic drugs (which are retained through electrostatic forces) or hydrophobic drugs (retained through hydrophobic forces, due to the Poloxamer units); the electrostatic and hydrophobic forces can also act cooperatively in binding drug capacity. The carboxymethyl pullulan anionic microparticles used as supports for drug delivery are pH sensitive and can be recommended for oral drug delivery; the presence of Poloxamer units on the polysaccharide network can induce temperature sensitive properties in the drug release, as well as improved adherence onto the mucosal tissues, thus providing potential for sustained release of the loaded drugs through diffusion (Bromberg & Hatton, 2003). Moreover, Poloxamer block copolymers are reported to present improved delivery of a variety of antigens in vaccination schemes, the IgG antibody responses being significantly greater than those elicited by aluminium salts (Coeshott et al., 2004).

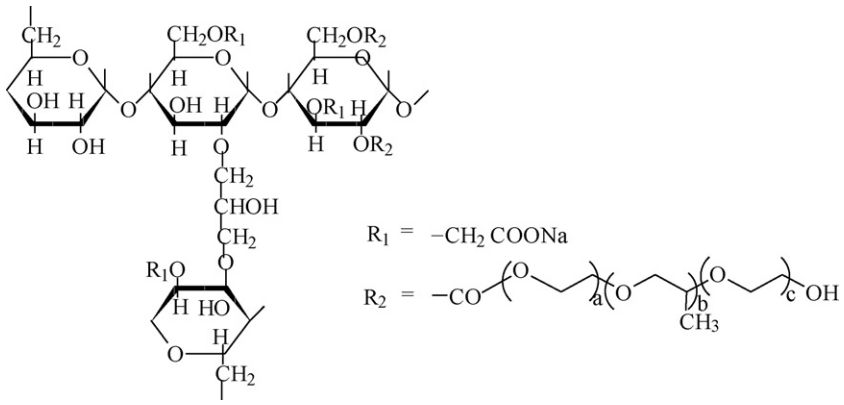
2. Experimental

2.1. Materials

- Crosslinked carboxymethyl pullulan microspheres synthesized in the laboratory, as described elsewhere (Mocanu, Mihai, Picton, LeCerf, & Muller, 2002).
- Poly(ethylene glycol)-block poly(propylene glycol)-block poly(ethylene glycol): Poloxamer (Aldrich): Mn ~ 4400: POX 44; Mn ~ 2000: POX 20.
- Rose Bengal sodium salt (Sigma Aldrich).
- Lysozyme (Lys, Sigma); tetanus anatoxin (AT, kindly supplied by the "I. Cantacuzino" National Institute of Research and Development for Microbiology and Immunology, Iasi, Romania); albumine from bovine serum (BSA, Fluka).
- Dimethyl sulfoxide (DMSO), dicyclohexyl carbodiimide (DCCI), dimethylamino pyridine (DMPy), acetone.

2.2. Methods

- *Synthesis of POX-substituted carboxymethyl pullulan microparticles* (CMP), with various degrees of substitution (DS), with POX units was performed as follows: 1 g CMP (4.4 mmoles) in H^+ form was swollen in 10 mL dry DMSO for 2 h, at room temperature; then 0.09 g (0.44 mmoles) of DCCI in 2 mL DMSO was added and the reaction mixture was stirred for 2 h at 20 °C; further, 1.94 g (0.44 mmoles) of POX 44 in 10 mL DMSO and 0.006 g (0.05 mmoles) DMPy (as catalyst) were added and the reaction was continued for 48 h at 20 °C. Finally, the microparticles were filtered, washed on the filter with dichloromethane (to remove the unreacted DCCI), acetone and methyl alcohol (to remove dicyclohexylurea (Beilstein; Leung, Lai, Lau, Yu, & Hsiao, 1996)) then with water, and dried from ethyl alcohol. Yield: 1.76 g. The same procedure was carried out for obtaining various DS with POX units, using various molar ratios of POX 44 or POX 20, and corresponding amounts of DCCI, for activating the COOH groups.
- *Degree of substitution with POX groups* (DS) was determined through conductimetric titrations (Eyler, Klug, & Diephuis, 1947), as a difference from the initial carboxymethyl group content (Table 1).
- FTIR spectra confirmed the formation of ester linkages between POX and the carboxylic groups (the band at 1734 cm^{-1}).
- The water 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).
- *The true density* of the microspheres (d) was determined in volumetric flasks, using cyclohexane as a suspension medium; the pore volume (Vp) of the microparticles was determined from cyclohexane retention; the porosity of microparticles was expressed as: $\varnothing = Vp/V_0$, where V_0 is the true volume of beads, calculated from: $V_0 = W_0/d$ (where W_0 is the weight of the dry beads and d is the true density), as described in literature (Bai & Li, 2006).
- *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.



Scheme 1.

- *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 solutions simulating gastric juice (pH 1.2), intestinal fluid (pH 7.2) or isotonic NaCl 1 g/L solution (which mimics blood osmolality); the protein content was again determined by the Folin method, on a Specord 200 Analytic Yena UV–vis spectrophotometer.

3. Results and discussion

3.1. Synthesis and characterization of polymeric supports

The chemical structure of pullulan microparticles and their synthesized functional derivatives is presented in Scheme 1.

Several samples were synthesized at various molar ratios of POX 20 and POX 44, for obtaining derivatives with different hydrophobic/hydrophilic ratios. Their physico-chemical characteristics are presented in Table 1.

As one can see, with the introduction of POX chains, the amount of ionic groups was reduced proportionally with the increase in the degree of substitution with POX units. Also, with the introduction of POX chains, pore volume and microparticle porosity decrease. As known, pore volume and porosity of hydrogels in a dry state are insignificant, while, in a swollen state, their porosity increases important. Water regain was diminished through the introduction of hydrophobic POX chains, proportionally with the degree of hydrophobic substitution of the CMP derivatives.

Rose Bengal is an anionic dye that can be absorbed through electrostatic and/or hydrophobic interactions on the surface of/inside microparticles. As shown in Table 1, no anionic dye was retained on the CMP support while, on the POX modified microparticles, the amount of retained Rose Bengal increases with the increase of DS and/or molecular weight of the hydrophobic groups. The anionic carboxymethylic groups of the CMP microparticles could give rise to the electrostatic repulsion of the anionic dye Rose Bengal sodium salt, which determined no dye absorption, while the presence of hydrophobic groups on the macromolecular backbone favored dye retention; similar results were obtained in the reten-

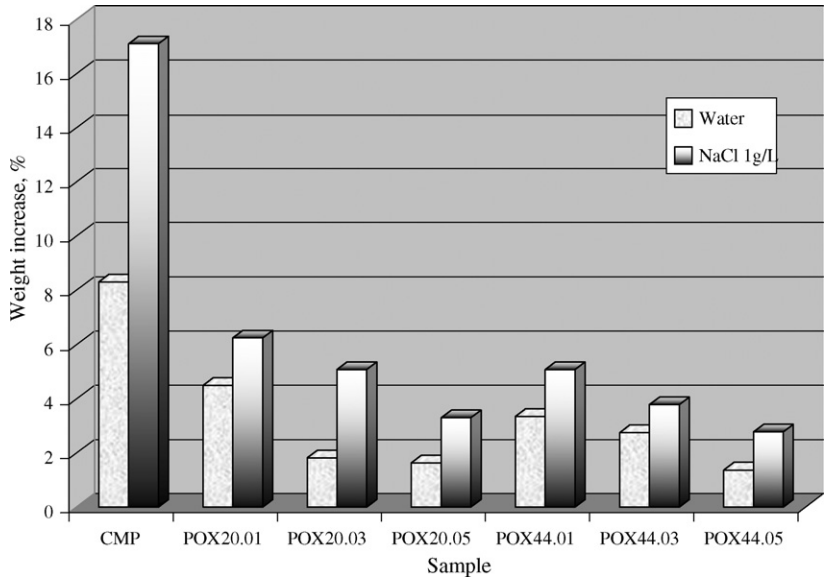


Fig. 1. Weight increase of the supports as a function of temperature (R—regain of water or NaCl 1 g/L solution).

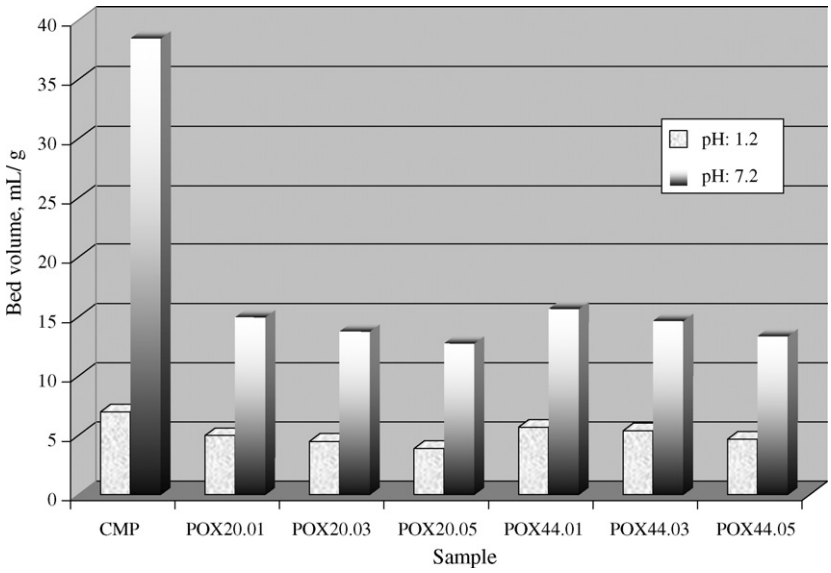


Fig. 2. Microparticles variation volume as a pH function.

tion of Rose Bengal on polystyrene or polystyrene substituted with anionic SO_3^- groups beads (Hou, Liu, Deng, Zhang, & Yan, 2007).

When increasing temperature, the water regain of microparticles increases; however, the increase for CMP microparticles is much higher than that for the POX-substituted ones. 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 et al., 2006); such an increase is less pronounced in the case of POX-substituted derivatives, but their thermosensitivity is still positive (Fig. 1). Temperature variation also influences the retention of 1 g/L NaCl solution to a higher extent, even higher than the water regain; one can therefore assume that, with temperature increase, the screening of the ionic charges due to electrostatic forces is less pronounced.

The pH-sensitivity of the supports, 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. 2). This behavior can be used in oral drug delivery.

SEM photos (Fig. 3) showed an irregular fine porous structure; this structure changed from the CMP microparticles to the POX-substituted ones, a pore size decrease being observed. The change of surface morphology is induced by introduction of POX groups and varied with their degree of substitution.

3.2. Interaction of polymeric supports with biomolecules

In order to find an application domain of these supports, their interaction with various biological active proteins, such as: lysozyme, tetanus anatoxine, BSA, was studied. The interaction of the ionic supports with proteins occurs principally through electrostatic interactions, by inter-polyelectrolyte complex formation, in an aqueous medium. The hydrophobic groups which are also linked on the supports can act cooperatively in binding biomolecules through hydrophobic forces.

3.2.1. Interaction with lysozyme

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

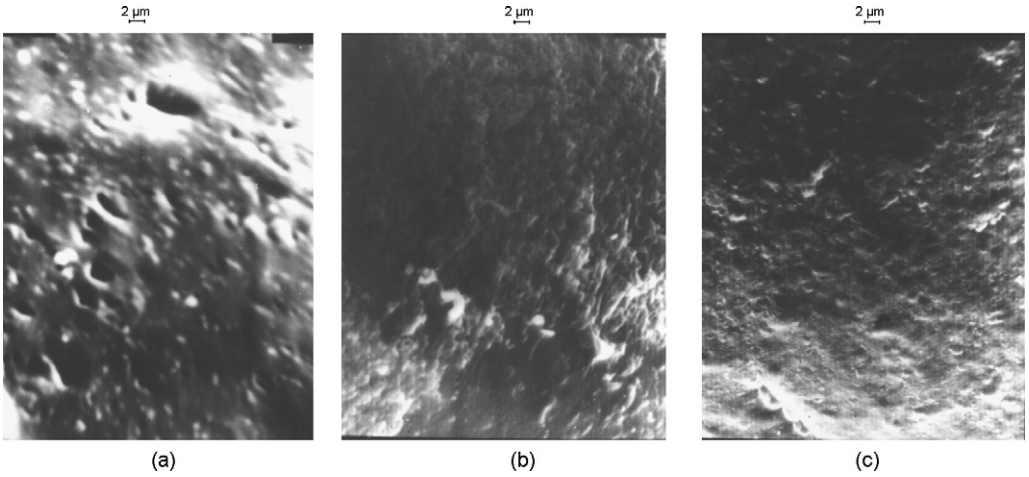


Fig. 3. SEM photos of CMP (a); POX 44.01 (b); POX 44.05 (c) microparticles.

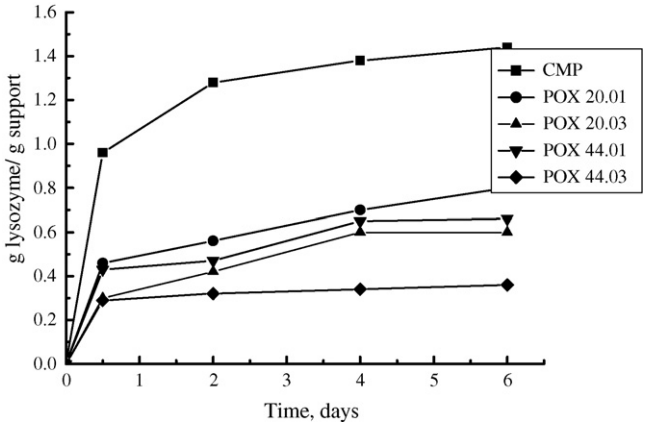


Fig. 4. Retention of the lysozyme on the studied supports.

supports containing anionic groups; the presence of hydrophobic groups on the same support may improve the interaction with proteins.

The data presented in Fig. 4 show that lysozyme is retained in higher amounts on CMP supports without hydrophobic groups; hence, the main force of its retention is represented by electrostatic interactions. Taking into account the low dimension of the enzyme, as well as the higher hydrophilicity of the CMP support, one can assume that lysozyme is retained both inside and at the surface of the microparticles. In the case of hydrophobized microparticles, the lower amount of immobilized lysozyme can be explained both by lower hydrophilicity and lower ionic charges of the support.

The release of lysozyme occurs gradually, as a function of time and pH (Fig. 5a, b). A burst release was observed during the initial stage, and was attributed to the release of the drug immobilized at the surface of the microparticles; the same burst release was reported in the case of lysozyme retained on poly(glutamic acid)–poly(NIPAM-co-2-hydroxyyethyl methacrylate) hydrogels (Zhao et al., 2009). After the initial burst, the release of the lysozyme is controlled mainly by the diffusion mechanism. In acidic pH 1.2 solutions, lysozyme release occurred more slowly than in buffered solution with pH 7.2, due to the more collapsed polysaccharide network. This behavior can be useful in oral administration of therapeutically active protein, the polysaccharide microparticles assuring protection against acidic stomachal medium. Diffusion-controlled lysozyme release was more evident in the case of POX-substituted derivatives, whose swelling degrees in both acidic and basic media were lower (Fig. 2). With increasing temperature, the rate of lysozyme release increased; this behavior agrees with the higher swelling of microparticles at elevated temperature (Fig. 5c). A faster release of drugs at higher temperatures was also reported for PNIPAM-based hydrogels (at some functional monomer/crosslinker ratios), although they presented negative thermosensitivity (Iemma et al., 2009).

3.2.2. Interaction with tetanus anatoxin

Tetanus is characterized by prolonged contraction of the skeletal muscle fibers; the mortality rates reported vary from 40% to 78%. In recent years, approximately 11% of reported tetanus cases have been fatal. The highest mortality rates occur with unvaccinated persons and persons over 60 years of age. Tetanus can be prevented by vaccination with tetanus toxoid (anatoxin). An important aspect in the use of adjuvants for human vaccines is the toxicity and adverse side effects of most adjuvant formulations (Gupta & Siber, 1995). The most common adjuvants for human use are aluminium hydroxide and aluminium phosphate. The study tries to estimate whether

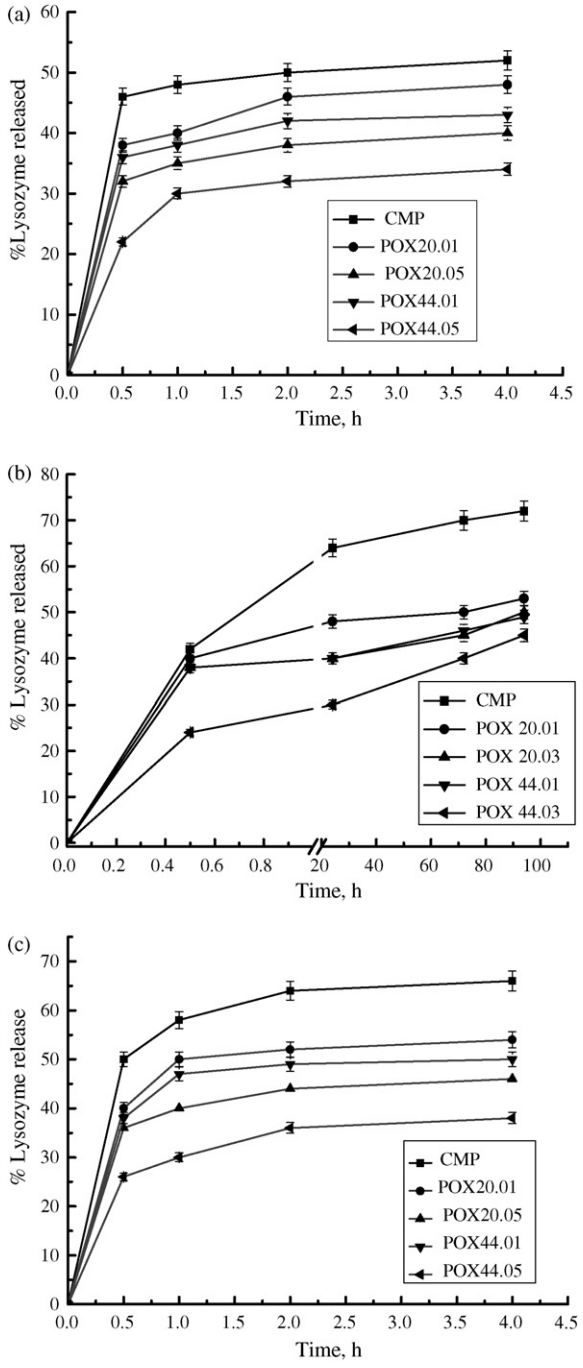


Fig. 5. Release of the lysozyme (a) pH 1.2, 20 °C; (b) pH 7.2, 20 °C; (c) pH 1.2, 37 °C; the values are the mean of three-independent measurements that deviated 2–4%.

the thermoassociative hydrogels based on anionic pullulan can be employed as tetanus anatoxin adjuvants, assuring its controlled release.

Tetanus anatoxin has higher molecular weight (150,000 g/mol) than lysozyme. Under such conditions, one may suppose that retention of AT occurs preponderently on the surface of microparticles. As shown in Fig. 6, the CMP microparticles retain a lower amount of AT than the derivatives that contain hydrophobic POX substituents, despite their high hydrophilicity, which would allow protein access inside the polysaccharide network. Hence, one can suppose that the hydrophobic interactions occur preponderently as compared to the electrostatic ones in the retention of antigen on the studied supports. Based on these data, one may assume that the POX-

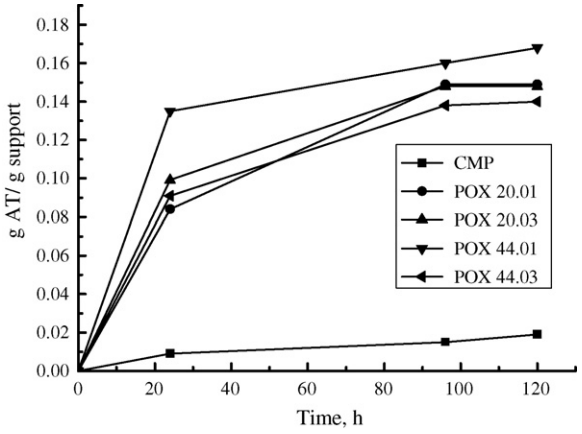


Fig. 6. Tetanus anatoxin retention on the studied supports.

substituted polysaccharides can be viewed as promising vaccine adjuvants.

The release of the AT retained on the supports has been studied as a function of temperature and pH. It is interesting to note that, despite the remarkably different degree of swelling of hydrogels at different pH values, the release rate does not differ very much in these media, probably because the hydrophobic interactions control the release of the protein, independently from the volume phase transition of the hydrogels (Fig. 7a, b). The release rate is slower for the hydrogels substituted with more POX hydrophobic

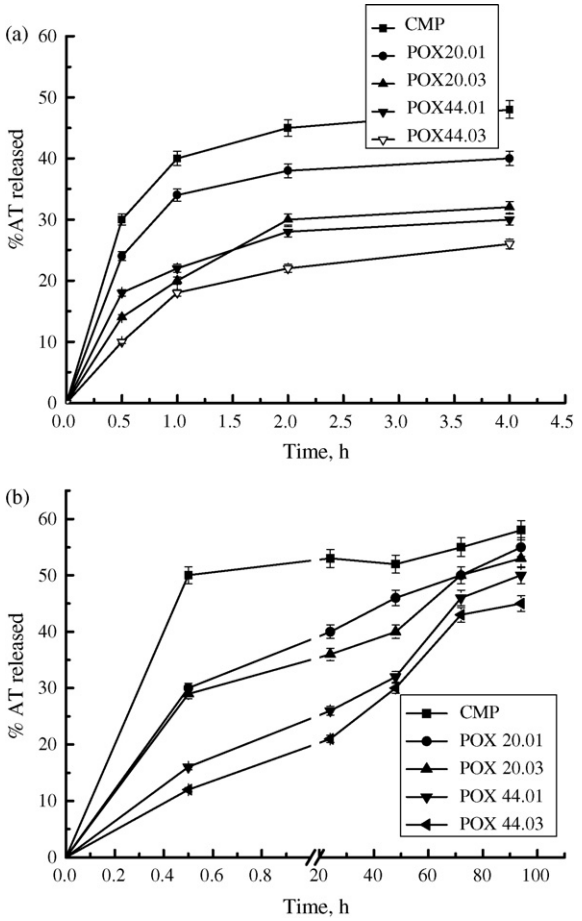


Fig. 7. Release of tetanus anatoxin (a) pH 1.2, 20 °C; (b) pH 7.2, 20 °C; the values are the mean of three-independent measurements that deviated 2–4%.

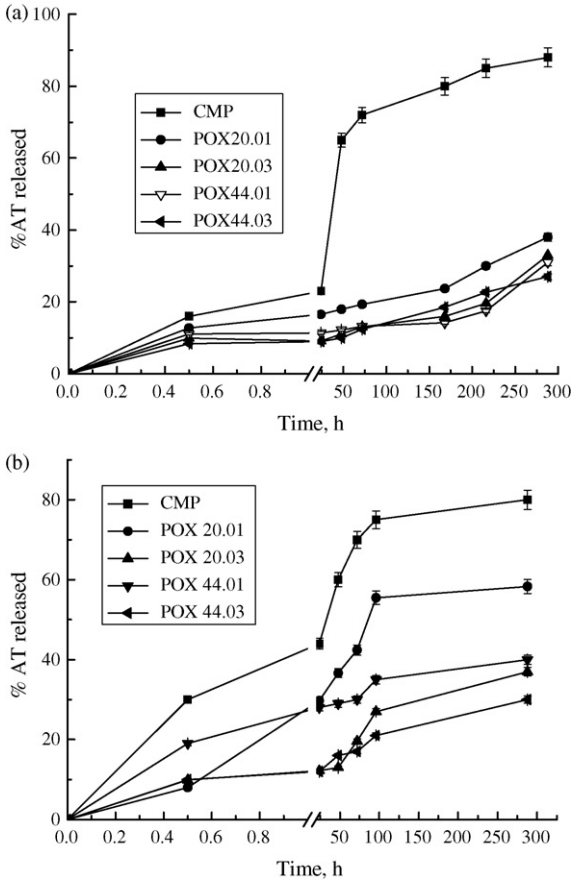


Fig. 8. Release of tetanus anatoxin (a): isotonic NaCl solution, 20 °C, (b): isotonic NaCl solution, 37 °C; the values are the mean of three-independent measurements that deviated 2–4%.

units, which also confirms the role of the hydrophobic forces in the AT release mechanism. An important observation is that the presence of POX units on the hydrogel backbone assures a controlled AT release, both in acidic and basic media; hence, these hydrogels can be useful for obtaining drug delivery systems as well as vaccine adjuvants.

The influence of temperature release was studied in an isotonic 1 g/L NaCl solution (which mimics the osmolality of blood). The CMP support releases the AT at rates which do not depend very much on the temperature used (Fig. 8a, b). The AT release from POX-substituted hydrogels is influenced by the following factors: (i) the DS with hydrophobic groups (the higher the DS, the lower the release rate), which also confirms the influence of hydrophobic interactions in the release mechanism of AT; (ii) the temperature (a higher release rate at higher temperatures, corresponding to volume variation as a function of temperature).

3.2.3. Interaction with BSA

BSA was used as a model protein for retention experiments on the studied supports; taking into account that its molecular weight is approximately 60,000, should be expected for it to be retained in amounts varying between those of lysozyme and AT. Indeed, as evidenced in Fig. 9, the values of BSA retention range between those of lysozyme and AT. Sephadex crosslinked dextran-based gels have various fractionation ranges for globular proteins, as a function of their swelling porosity; Sephadex G-75, with a water regain of 7.5 g/g, has a fractionation range for globular proteins up to 70,000 (Kremmer & Boross, 1979). Our pullulan-based hydrogels have water regains between 5.5 and 12 g/g for POX-

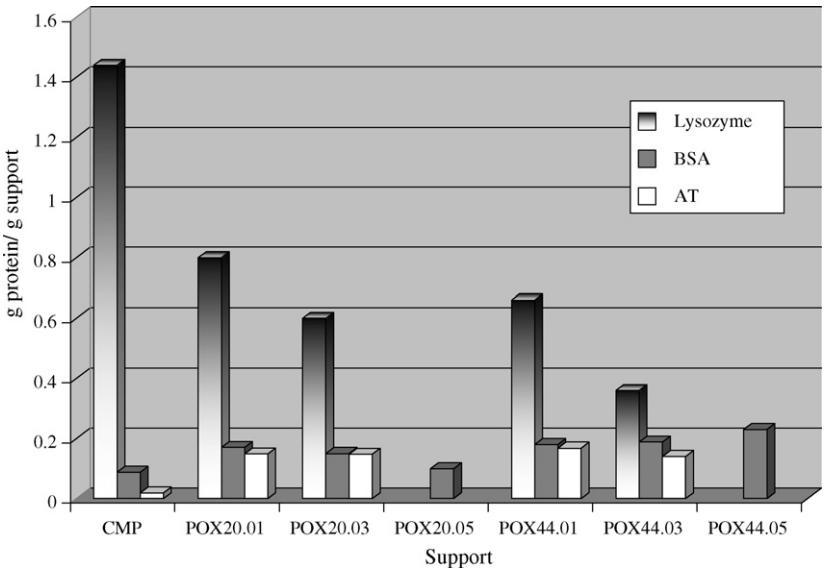


Fig. 9. Comparative retention of the proteins: Lys/BSA/AT on the studied supports.

substituted derivatives, and 24 g/g for CMP microparticles. Hence, one can assume that BSA will be retained on the supports both on the surface of/and inside microparticles. Its retention can be the result of many accumulative effects, for example, the interactions between protein molecules and microspheres, including hydrophobic interactions, hydrogen bonds, electrostatic forces (Hou et al., 2007).

BSA release profiles are intermediates between those of Lys and AT, at all pH and/or temperatures applied; the release rate decreased with increasing the protein size, the release mechanism being governed by diffusion, as also reported (Censi et al., 2009) for lysozyme/BSA/IgG release from thermosensitive methacrylated p(HPMAm-lac)-PEG-p(HPMAm-lac) hydrogels.

4. Conclusions

New thermosensitive microparticles based on crosslinked anionic pullulan have been synthesized and characterized; they present positive thermosensitivity, as already evidenced for other hydrophilic thermosensitive hydrogels. These microparticles retain various amounts of biologically active proteins, as a function of their molecular weight; the smaller lysozyme is retained preponderently through ionic forces both inside and on the surface of the microparticle; AT is retained, due to its high molecular weight, on the microparticle surface, while BSA, which has intermediate molecular weight, is retained, as a function of the support used, both inside and on the microparticle surface. The proteins are released *in vitro* as a function of pH, temperature and duration. In acidic pH, the release rate is lower than in a basic one; this behavior can be promising for controlled release in oral administration.

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.

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