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Hydrogels of *N*-isopropylacrylamide copolymers with controlled release of a model protein

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ABSTRACT

Temperature- and pH-sensitive hydrogels, based on *N*-isopropylacrylamide (NiPAAm) and itaconic acid (IA), were synthesized by free radical crosslinking copolymerization in the presence of lipase from *Candida rugosa*. The samples were characterized for their sensitivity to the changes of external conditions and the ability to control the release of a hydrophilic model protein, lipase. These hydrogels were highly responsive to temperature and pH, at constant ionic strength. Parameters, such as the crosslinking degree and non-ionic/ionic (NiPAAm/IA) ratio, were found to impact the hydrogel structure, mechanical properties, morphology and swelling kinetics at different pH and temperatures. The hydrogels demonstrated protein loading efficiency as high as 95 wt%. Release studies of a hydrophilic model protein at a physiological temperature of 37 °C were performed at different pH values. High dependence of lipase release kinetics on hydrogel structure and the environmental pH was found, showing generally low release rates, lower in acidic media (pH 2.20) and higher at higher pHs (6.80). Lipase activity was retained even after treatment conditions that would provoke denaturation of the enzyme if it was not protected in the gel. The obtained hydrogels were found suitable for releasing therapeutic proteins in a controlled manner at specific sites in gastrointestinal tract.

1. Introduction

Due to widely variable and easily adjusted properties, of which permeability is among the most dominant, hydrogels based on synthetic polymers have been studied extensively as vehicles for the controlled release of both, low-molecular mass drugs and macromolecular drugs such as therapeutic proteins, enzymes and DNA (Peppas et al., 2000). The gel structure may be controlled by varying the formulation and preparation conditions. Therefore, gel properties, like porosity, swelling behavior, stability, biodegradability, gel strength and biocompatibility may be tailored for a particular application. A special class of hydrogels, which are called "intelligent" or "stimuli-sensitive" hydrogels, exhibit significant volume change in response to small changes in external stimuli, such as pH, temperature, light, etc. Among the most studied "intelligent" hydrogels are pH- and temperature-sensitive hydrogels for pharmaceutical applications, as well as those which respond to both stimuli, pH and temperature, which have been used extensively in the development of drug delivery systems. These polymers are expected to reside in the body for a desired period of time and respond to local pH and temperature values in order to modulate the drug release profile.

The entrapment of biological materials, e.g. enzymes, in synthetic hydrogels could be of interest for a variety of pharmaceutical and biotechnological applications (Tang and Singh, 2009; Wang et al., 2006). Besides being used as the catalyst in chemical reactions, hydrogels with incorporated enzyme may allow its sustained and localized release enhancing the therapeutic efficacy. The ability of pH-sensitive hydrogels to respond to environment makes them suitable for the site-specific delivery of proteins along the gastrointestinal (GI) tract which has pH variations throughout its length (Qiu and Park, 2001). The use of such hydrogels in drug delivery systems is particularly important since the therapeutic application of hydrophilic macromolecules is associated with some serious problems, especially upon their oral administration. First, high molecular weight results in limited distribution across biological barriers and low bioavailability. Second, peptides, proteins, oligonucleotides, and genes are unstable compounds that need to be protected from degradation in the biological environment (low pH, gastric enzymes). Thus, the design of an efficient oral drug delivery system for the delivery of hydrophilic macromolecules such as proteins, possessing high loading capacity, extended delivery time, ability to accumulate in required pathological sites in the body, is still having many unresolved issues (Schmaljohann, 2006; Zhang and Wu, 2004).

Hydrogels based on *N*-isopropylacrylamide (NiPAAm) have been used for developing reversible temperature-controlled

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release systems. Therefore, a volume phase transition of poly(*N*-isopropylacrylamide) (PNiPAAm) matrix at 32 °C is narrow and is not operational at physiological temperature of 37 °C. As the range of physiological pH values is wider, 2.20 in stomach to 6.80 in intestine (mean values), it is easier to tune the release of drug by temperature- and pH-sensitive systems. It is found that by incorporating small amount of ionizable groups (such as carboxyl) into PNiPAAm chains, the thermosensitivity can be controlled by pH (Erbil et al., 1999). In our earlier investigations, it was confirmed that by introducing small amounts of itaconic acid (IA) the Lower Critical Solution Temperature (LCST) of NiPAAm/IA copolymers is transferred to higher temperature (Kalagasidis Krušić et al., 2009) which is in accordance to literature (Jones, 1999). Therefore, the LCST can be adjusted to be around body temperature by the amount of IA. However, it should be noted that below its LCST, release kinetics of drug (or solute) from swollen PNiPAAm based network has disadvantageous burst effect (Liu et al., 2004a,b, 2006). The addition of hydrophilic ionic comonomers, such as acrylic, methacrylic or itaconic acid, introduces a pH-sensitivity into the non-ionic PNiPAAm hydrogel and makes it useful for the controlled release of proteins to a specific site in the gastrointestinal tract. In pH-sensitive PNiPAAm/IA hydrogels LCST is shifted to higher temperatures, but still lower than physiological temperature of 37 °C, needed for *in vivo* applications (Bae et al., 1987; Zhang and Zhuo, 1993).

PNiPAAm hydrogels and interpenetrating networks, biomaterials containing two polymers, each in network form, as well as NiPAAm copolymers with acrylic, methacrylic and itaconic acid (Xue and Hamley, 2002; Zhang and Zhuo, 2002; Zhang et al., 2004; Kalagasidis Krušić and Filipović, 2006), as well as itaconic acid esters have been investigated as drug release systems (Vallés et al., 2000). However, the pH- and temperature-sensitive hydrogels of NiPAAm and itaconic acid have not been studied as matrices for the release of proteins. Due to the fact that IA is obtained from the non-petrochemical resources it is of a great interest for polymeric biomaterials. IA is obtained by fermentation from renewable resources such as carboxydrate materials containing sucrose and glucose (molasses and hydrolyzed starch) (Kirimura et al., 1997; Petruccioli et al., 1999; Willke and Vorlop, 2001).

The aim of this work was the synthesis of the novel temperature- and pH-sensitive NiPAAm/IA hydrogels, having incorporated model protein drug lipase from *Candida rugosa*, with improved mechanical and swelling properties which could be used as site specific protein drug delivery system. A series of hydrogel formulations, with varying NiPAAm/IA weight ratios, were synthesized and evaluated regarding morphology, mechanical properties, swelling degree, protein loading efficiency and release profiles. The main goal was to elucidate the effect of the hydrogel structure and environmental conditions on the *in vitro* release behavior of a therapeutic protein, as a function of hydrogel composition and pH, at the physiological temperature of 37 °C. Since a therapeutic protein must be biologically active when released, the biological activity of the released lipase was also examined by the specific enzyme activity assay.

2. Materials and methods

2.1. Materials

The monomers used in this study, itaconic acid (IA) and *N*-isopropylacrylamide (NiPAAm), were obtained from Acros Organics (Belgium). The crosslinking agent *N*,*N*′-methylenebisacrylamide (MBA) was obtained from Serva Feinbiochemica (Germany). Potassium Persulphate (PPS) and Potassium Pyrosulphate (PPyroS), the initiator and accelerator, were purchased from Merck & Co Inc. (Germany) and Acros, respectively. Lipase from *C. rugosa* (CRL), was obtained from Sigma–Aldrich Chemie Gmbh (Germany). NiPAAm was recrystallized from benzene/*n*-hexane mixture (35/75) before use. Other materials were used as received, without purification.

Aqueous media with different pH values were prepared using hydrochloric acid (La Chema, Czech Republic), potassium chloride (Zorka Šabac, Serbia), sodium dihydrogen phosphate dihydrate and di-sodium hydrogen phosphate dodecahydrate (Lach–Ner, s.r.o., Czech Republic). Distilled water was used for all copolymerizations and the preparation of the buffer solutions.

2.2. Synthesis of the hydrogels

Hydrogel synthesis was performed at 25 °C in the nitrogen atmosphere by free radical crosslinking copolymerization. The reaction time depended on the concentration of the crosslinking agent, as well as on the NiPAAm/IA comonomer weight ratio in the initial mixture and was in the range of 12 and 48 h (Table 1).

The monomers were separately dissolved in water. Redox couple of PPS and PPyroS, in an amount of 1.0 wt% with respect to monomers was added to the IA solution, prior to polymerization. In order to immobilize a model protein into the system, the hydrogel synthesis was performed in the presence of the enzyme. This is a common method for drug entrapment in hydrogels (Wang et al., 2006). In this method, the lipase is added in the monomers solution (*in situ* polymerization) at a concentration of 20.0 wt% with respect to monomers. The concentration of the crosslinking agent, MBA, was 2.0 and 4.0 wt% with respect to monomers. After being purged by the nitrogen, the reaction mixture was poured between two glass plates (21 cm × 6 cm × 0.4 cm), sealed with a PVC spacer (0.3 cm thick).

After the reaction was completed, the gels were cut into discs and immersed in water for a week to remove unreacted reactants. The water was changed daily. The discs were dried at room temperature for a day and then at the temperature of 37 °C to xerogels (0.10 ± 0.01 cm thick and 0.70 ± 0.10 cm in diameter). The PNiPAAm hydrogel has been synthesized under the same conditions as were used for the copolymers with 2.0 and 4.0 wt% of MBA. The samples were labeled as NiPAAm/IA/MBA 85/15/2, 90/10/2, 95/5/2, 100/0/2 and 85/15/4, 90/10/4, 95/5/4, 100/0/4. The first two numbers correspond to the comonomer NiPAAm/IA weight ratio, and the third one corresponds to the concentration of the crosslinking agent, MBA.

2.3. The network parameters

The basic parameters of the hydrogel network, the molecular weight between the crosslinks (M_c) and the mesh size (ξ), have been calculated by applying the equilibrium swelling theory. The molecular weight between the crosslinks, for non-ionic hydrogels, is calculated by the following equation (Peppas et al., 2000):

$$\bar{M}_c = -\frac{(1 - 2/\phi)V_1 \nu_{2r}^{2/3} \nu_{2m}^{1/3}}{\bar{\nu}[\ln(1 - \nu_{2m}) + \nu_{2m} + \chi \nu_{2m}^2]} \quad (1)$$

where ν_{2r} represents the polymer fraction within the gel during relaxation period (defined as the state of the polymer just after synthesis, but before swelling), ν_{2m} is the polymer fraction within the gel in its swelled state, $\bar{\nu}$ is the specific polymer density, V_1 is the molar volume of water or the swelling media, ϕ is the crosslinking agent functionality and χ represents the interaction parameter between the polymer and the solvent.

Table 1
The amount of lipase entrapped and the entrapment efficiency for different hydrogel formulations.

Sample	2 wt% of the crosslinking agent			4 wt% of the crosslinking agent		
	t, h	P_g, mg/$g_{dry\ hydrogel}$	η, %	t, h	P_g, mg/$g_{dry\ hydrogel}$	η, %
85/15	48	188.6	94.3	24	174.5	87.2
90/10	48	188.1	94.1	24	155.3	77.6
95/5	48	189.3	94.6	24	157.0	78.5
100/0	24	190.4	95.2	12	178.7	89.3

t, reaction time for the sample polymerizations; P_g, the amount of lipase entrapped; η, the entrapment efficiency.

The molecular weight between the crosslinks, for ionic hydrogels, is calculated as follows:

$$\left[\frac{2K_{a1}K_{a2}+10^{-\mathrm{pH}}K_{a1}}{2(10^{-\mathrm{pH}})^2+10^{-\mathrm{pH}}K_{a1}+K_{a1}K_{a2}}\right]^2\left(\frac{V_1\nu_{2m}^2X^2}{4I\bar{\nu}^2}\right)=\ln(1-\nu_{2m})+\nu_{2m}+\chi\nu_{2m}^2+\frac{(1-2/\phi)V_1\nu_{2r}^{2/3}\nu_{2m}^{1/3}}{\bar{\nu}\bar{M}_c} \quad (2)$$

where K_{a1} and K_{a2} represent the first and the second dissociation constants of the itaconic acid, X gives the mass fraction of the itaconic acid and I represents the ionic strength of the medium.

The hydrogel mesh size was calculated with the following equation:

$$\xi=\alpha\cdot(\bar{r}_0^2)^{1/2} \quad (3)$$

where α represents the fraction of the polymer chain stretching in any direction, while $(\bar{r}_0^2)^{1/2}$ is the average distance between two adjacent crosslinks in the solvent-free state. The case of isotropic swelling of the hydrogel gives the following equation:

$$\alpha=\nu_{2m}^{-1/3} \quad (4)$$

$(\bar{r}_0^2)^{1/2}$ depends on the molecular weight between the crosslinks by the expression:

$$(\bar{r}_0^2)^{1/2}=l\cdot\left(\frac{2C_n\bar{M}_c}{M_r}\right)^{1/2} \quad (5)$$

where C_n represents the characteristic Flory ratio ($C_{n,\mathrm{IA}}=4.63$ (Veličković et al., 1994) and $C_{n,\mathrm{NiPAAm}}=11.7$ (Kalagasidis Krušić, 2006)), l is the length of the C—C bond (1.54×10^{-10} m for the vinyl polymers) and M_r molecular weight of the basic structural unit within the polymer chain.

2.4. Swelling degree studies

To determine the swelling degree of the hydrogels, the xerogel discs were immersed in buffer solutions with pH values of 2.20 ± 0.01 and 6.80 ± 0.01 to simulate the gastric and intestinal pH at 37 °C. The swelling process was monitored gravimetrically.

The swelling degree was calculated using the equation:

$$q=\frac{W_t}{W_0} \quad (6)$$

where W_0 is the weight of the xerogel and W_t is the weight of the swollen hydrogel at time t.

2.5. Mechanical properties

Strain-frequency sweeps were performed on hydrogel discs swollen to equilibrium, using a Rheometrics 605 Mechanical Spectrometer, with parallel plate geometry, 25 mm in diameter. The complex shear moduli were measured as a function of angular velocity (ω), from 0.1 to 100 rad s^{-1}, at 37 °C. The strain applied was 20%. The sizes of the discs were 25 mm in diameter to fit the parallel plate diameter; the gap for the samples was about 2.55 mm.

2.6. Morphology of the hydrogels

The morphology of the hydrogel samples was performed by the JEOL JSM-5800 Scanning Electron Microscope. Before the SEM morphological experiments took place the hydrogel samples were liophylized. To avoid the deformations caused by the fracture of the samples, the swollen liophylized samples were frozen in liquid nitrogen, and then broken. After on, the samples were coated with platinum under vacuum using Polaron SC502 sputter coater.

2.7. Protein release study from the hydrogels

The release experiments were carried out at 37 °C to investigate the effect of the hydrogel composition and crosslinking agent concentration on protein release profiles. The *in vitro* release experiments were conducted by immersing the hydrogel samples (the average volume of the xerogels prior to the release kinetic studies was 0.0387 ± 0.0037 mL) in a beaker filled with 10 mL buffer solution of a targeted pH value simulating biological fluids at 37 °C. The loaded hydrogels were stored in the dry state before the release experiments. The simulation of the GI tract was performed by immersing the hydrogel samples in the buffer solution of pH 2.20 ± 0.01 for 2 h and then all the samples were transferred to the beaker filled with buffer solution of the pH value 6.80 ± 0.01 to completion of one week. At proper intervals, 2 mL of solution were taken for released protein measurement and returned back to the beaker to maintain the same condition throughout the experiment. The sterile, as well as sink conditions, have been maintained throughout the experiments. To simulate the gut movement the samples were placed in a shaking incubator with a mild shaking motion (50 rpm) during the studies. The samples were analyzed spectrophotometrically at 225 nm by using bovine serum albumin as a standard (Ultrospec 3300 *pro* UV/Visible Spectrophotometer, Biochrom Ltd.). The enzyme concentration was determined from a standard curve which was constructed for each measurement. Mean and standard deviation of the results from three independent experiments were calculated using Microsoft Excel (Redmond, WA, USA) software. All data were reproducible within ±5% accuracy. The results of release studies were presented in term of cumulative release as a function of time. The enzymatic activity of samples was also analyzed using the Sigma lipase activity method as previously described (Knežević et al., 2006). For comparison purposes, the stability of native lipase was also studied at pH 2.20 and 6.80. Aliquots of lipase (10 mg, corresponded to the amount of the immobilized lipase) were incubated at 37 °C in the buffer solution of pH 2.20 or pH 6.80. At predetermined time intervals, samples were withdrawn and enzyme activity was assayed by Sigma method.

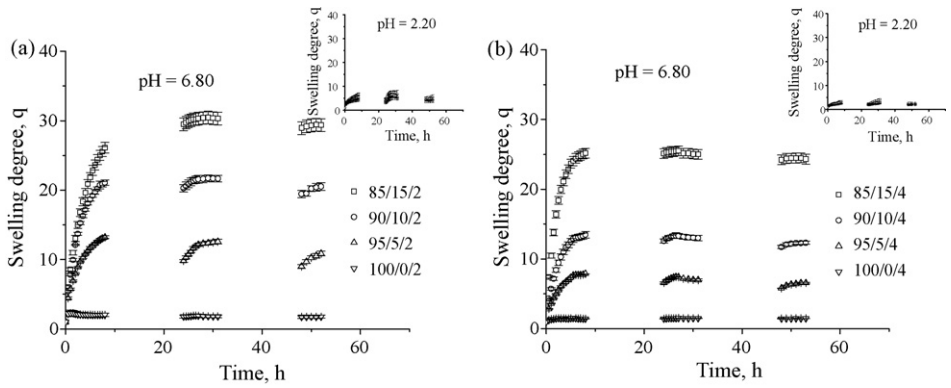

Fig. 1. The swelling behavior of the investigated hydrogels with (a) 2.0 wt% of crosslinking agent at 37 °C: pH 6.80, (insert) pH 2.20 and (b) 4.0 wt% of crosslinking agent at 37 °C: pH 6.80, (insert) pH 2.20. Values are reported as mean ± standard deviation.

3. Results and discussion

3.1. Swelling kinetics

Experiments at two pH values of 2.20 ± 0.01 and 6.80 ± 0.01 to simulate the gastric and intestinal pH at 37 °C were carried out to analyze its influence on the swelling kinetics. All the experiments have been done in triplicate and the mean values for the replicated data with the standard deviations are presented in Fig. 1. The standard deviations were < ±10% of the mean values, suggesting a rather high reproducibility. The results in Fig. 1 show that NiPAAm/IA hydrogels are highly pH-sensitive, since the difference in swelling is evident when the pH value changes from 2.20 to 6.80. At low pH value (2.20) the swelling degrees are low and slightly depend on the IA content in the samples. The low q_e values are primary due to the intermolecular physical crosslinking, via hydrogen bond formation, between carboxylic and amide groups in IA and NiPAAm residues, respectively.

For pH of 6.80, which is above the nominal pK_a values of IA (3.85 and 5.45 (Weast, 1974)), the ionization of IA occurs resulting in the increased hydrophilicity and electrostatic repulsion of carboxyl groups. As a result the swelling degrees were higher. The most pronounced pH-sensitivity was observed for the samples with highest acid content (Fig. 1a and b).

The results presented in Fig. 1 clearly show that the IA carboxyl group gives the hydrogel anionic character and affects the hydrogel swelling behavior. When the pH value of the medium is 2.20, lower than both pK_{a1} and pK_{a2} values, the swelling degree is low and almost similar for all the samples. At this pH, carboxyl groups are not ionized, so hydrogen bonds are formed between carboxyl and amide groups, acting as the additional physical crosslinking. This difference in swelling is a little more pronounced for the samples with 2.0 wt% of the crosslinking agent, due to the influence of the crosslinking agent concentration. As it was expected, higher crosslinking degrees led to the lower swelling degree owing to the higher network density which makes the network chains inflexi-

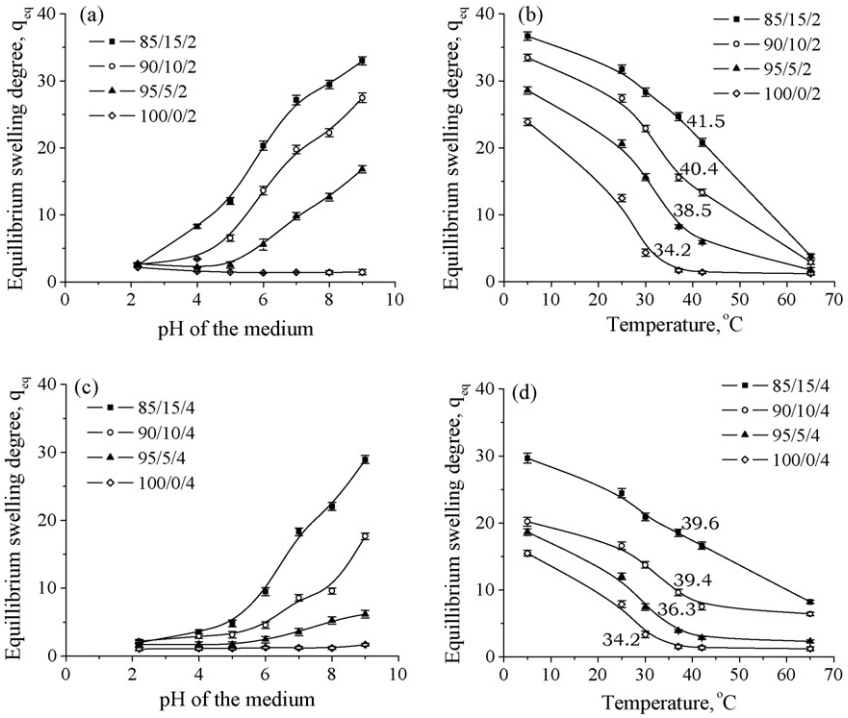

Fig. 2. The equilibrium swelling degree of the investigated hydrogels: with 2.0 wt% of crosslinking agent at 37 °C in different pH media (a), at different temperatures in pH 6.80 (b), with 4.0 wt% of crosslinking agent at 37 °C in different pH media (c), at different temperatures in pH 6.80 (d).

Table 2
The mesh sizes for different hydrogel formulations at different temperatures.

Sample	ξ, nm (2 wt% of the crosslinking agent)				ξ, nm (4 wt% of the crosslinking agent)			
	85/15	90/10	95/5	100/0	85/15	90/10	95/5	100/0
5 °C	2.838	3.114	2.686	2.153	2.614	2.010	1.913	1.786
25 °C	2.678	2.606	1.953	1.405	2.138	1.465	1.233	0.925
30 °C	2.378	2.263	1.485	0.514	1.968	1.445	0.798	0.440
42 °C	1.986	1.375	0.688	0.140	1.586	0.640	0.360	0.160
65 °C	0.450	0.365	0.204	0.120	0.948	0.634	0.270	0.138

ble and the free volume between the chains less available. As the pH value of the surrounding medium raises to 6.80, which is above pK_a values of both carboxyl groups, those groups are transformed to carboxylate anions, the hydrogen bond are broken and the swelling is much higher.

Fig. 2 shows the variation of the swelling degree with pH at 37 °C (a and c) and the variation of the swelling degree with temperature at pH 6.80 (b and d), for the lipase loaded samples with 2.0 and 4.0 wt% of the crosslinking agent. It is clearly seen that the variation of pH and temperature significantly influences the swelling degree of hydrogels, as expected. It is also evident that the addition of itaconic acid shifts the LCST to higher temperatures and the transition range is broader (Fig. 2b and d).

The pH dependent thermosensitivity of NiPAAm/IA is attributed to the influence that the ionization of carboxyl group has on the LCST transition of PNiPAAm (Fig. 2b and d). Although PNiPAAm exhibits a LCST around 32 °C in water, incorporation of a small amount of IA into PNiPAAm chain can offer pH dependent LCST of PNiPAAm due to the ionization of carboxyl groups. At pH value of 2.20 carboxyl groups are not ionized and the temperature sensitive phase transition is very sharp. With the increase in pH value, the electrostatic repulsion of ionized carboxyl group causes the copolymer LCST to increase. Therefore, the LCST below body temperature can rise above the body temperature by ionization of pH responsive moieties.

The mesh size is one of the most important parameters determining the velocity of the protein release from the hydrogel matrices. According to the mesh size, all hydrogels have been divided into few categories: microporous, macroporous, nonporous and superporous (Peppas et al., 2000). The mesh size was calculated applying the equilibrium swelling theory, and the results are presented in Tables 2 and 3. The obtained results revealed that hydrogel composition, temperature and pH of the external media significantly influence the mesh size. By increasing the crosslinking agent concentration, available free volume between the chains and chain flexibility decreases, causing lower values of the mesh size. Further, the mesh size for PNiPAAm hydrogel decreases with increasing temperature, as shown in Table 2. PNiPAAm network tends to collapse as the temperature increases above the LCST, due to the scission of the hydrogen bonds between the hydrophilic groups in the side chains of PNiPAAm and water. Hence, the hydrophobic interactions prevail and the mesh size decreases by raising the temperature from 5 to 65 °C (Table 2). On the other hand, increasing the pH values of the external media and IA content in hydrogels, the mesh size of the samples increases (Table 3) owing to the increase of the gel hydrophilicity.

3.2. Mechanical properties

The mechanical properties of hydrogels are of a great importance for their use as carriers for the controlled protein release. The comonomer ratio and the crosslinking agent concentration substantially affect the mechanical properties of hydrogels.

The mechanical properties of the hydrogel samples are presented as a shear storage moduli versus angular velocity at 37 °C in Fig. 3. The shear storage modulus, G', was practically angular velocity independent for all samples. Mechanical properties of hydrogels depend on crosslinking agent concentration. The greater the crosslinking density, the stronger, less swellable and less diffusable hydrogel is obtained. The optimal content of the crosslinking agent is desirable to achieve. By increasing the crosslinking concentration from 2.0 to 4.0 wt%, the mechanical properties of the hydrogel samples strengthen 2–3 times. The hydrogel comonomer ratio prior to polymerization reaction affects the final product properties. The shear storage moduli decrease with an increase of the itaconic acid content, giving the hydrogels weaker mechanical properties, which is in accordance with the equilibrium swelling results. Greater values of the shear storage moduli were obtained by the less swellable hydrogels.

G' dependence on the angular velocity matches with the results of the swelling behavior of the hydrogel samples. From Fig. 3 it can be concluded that the influence of the crosslinking agent concentration on mechanical properties is more pronounced than that of the IA content.

3.3. SEM analysis

Figs. 4 and 5 show the morphology of the NiPAAm/IA copolymer hydrogels with lipase. It can be seen that the difference between the samples with 15.0 wt% of IA and the samples with 0.0 wt% of IA, with the same crosslinking agent concentration exists (Fig. 4a and b). Larger mesh sizes are also visible for the samples with the same IA content and higher crosslinking agent concentration (Fig. 5a and b). It is resulted from the differences in composi-

Table 3
The lipase velocity release constants and mesh sizes for the hydrogels with 20.0 wt% of lipase where lipase has been immobilized by *in situ* polymerization at 25 °C.

Sample	pH 2.20 ± 0.01 (2 h) → pH 6.80 ± 0.01 (24 h) at 37 °C					SA, IU/mg × 10^2
	k, h^{-1}	n	$t_{1/2}$, h	M_c	ξ, nm	
85/15/2	–	–	13.23	178.43	2.686	48.4
90/10/2	–	–	15.15	105.27	1.876	43.2
95/5/2	0.019	1.02	19.51	54.48	1.095	41.0
100/0/2	0.015	1.09	20.75	5.97	0.205	36.5
85/15/4	–	–	13.75	142.06	2.268	43.9
90/10/4	–	–	17.22	62.45	1.223	42.5
95/5/4	0.018	1.05	19.44	32.71	0.726	40.0
100/0/4	0.011	1.12	20.29	4.27	0.164	35.4

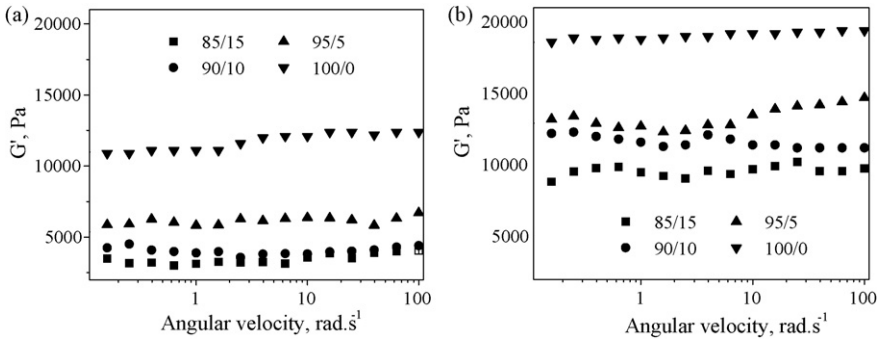

Fig. 3. The G' versus angular velocity for hydrogels with (a) 2.0 wt% and (b) 4.0 wt% of the crosslinking agent swollen to equilibrium in pH 6.80 at 37 °C.

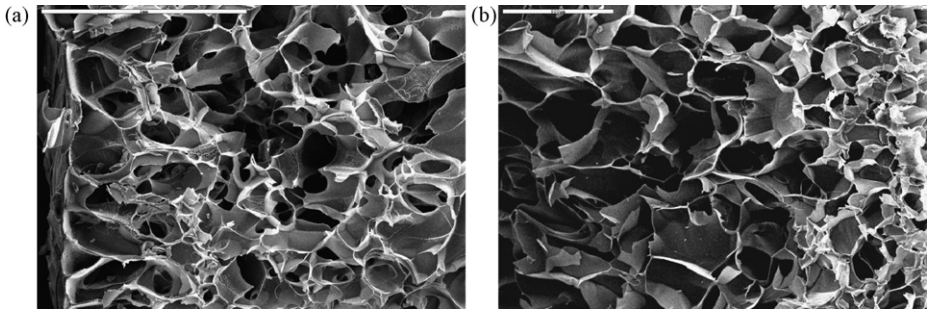

Fig. 4. The SEM micrographs of the investigated hydrogels with 4.0 wt% of crosslinking agent concentration and (a) 15.0 wt% of IA ('bar' 1 mm, 70×) and (b) 0.0 wt% of IA ('bar' 500 μm, 70×).

tion, including comonomer content and crosslinking degree of the specimens. According to these micrographs, the copolymerization/crosslinking reaction did not occur uniformly throughout the hydrogel, as expected. Obviously, the crosslinking agent concentration and the IA content affect the morphology of the hydrogels, as well as the swelling and the mechanical properties.

3.4. Lipase loading in hydrogels

The amount of lipase loaded in hydrogels (Table 1) varied from 188.1 to 190.4 mg/g of the dry hydrogel for 2.0 wt%, and from 155.3 to 178.7 mg/g for 4.0 wt% crosslinking agent concentration. The average standard deviation of the loaded lipase was 0.5 and 7.2% for 2.0 and 4.0 wt% of the crosslinking agent concentration, respectively, revealing that the hydrogel composition did not have a marked effect on the loading efficiency. However, the results also indicate that higher degree of the crosslinking resulted in somewhat lower loadings and entrapment efficiencies.

Since the candidates for controlled release systems often include enzymes, peptides, and proteins, which can be very expensive, preparation of temperature- and pH-sensitive hydrogels with maximal protein content and activity is of high importance. The corresponding encapsulation efficiencies of lipase determined in this work were in the range of 77.6–95.2%, revealing that the entrapment technique provided rather high protein loadings with some loss of enzyme for any given hydrogel formulation. These values are comparable to the results obtained in some related studies concerning protein entrapment in hydrogels (Chen et al., 2005). On the other hand, the encapsulation efficiency achieved in the present work are higher than those previously reported in the literature for protein incorporated in various hydrogels based on natural polymers such as alginate (Nochos et al., 2008; Simi and Abraham, 2007) or chitosan (Alsarra et al., 2004).

However, some protein loss has occurred during the manufacturing process (22.4–4.8%) including both polymerization process and washing the hydrogel to remove unreacted reactants. The explanation could be found in irregular or incomplete polymer network formation resulting in non-uniform lipase distribution. It is possible that some loss of lipase was during the actual polymerization time when the polymer network has not yet fully formed to form hydrogel. In addition, the other possible explanation could be diffusion of the entrapped lipase out of the systems during the

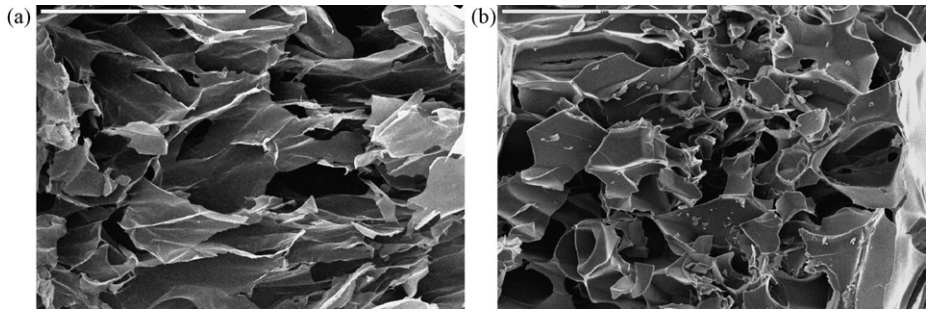

Fig. 5. The SEM micrographs of the investigated hydrogels with 10.0 wt% of IA concentration and (a) 2.0 wt% and (b) 4.0 wt% of crosslinking agent concentration ('bars' 1 mm, 70×).

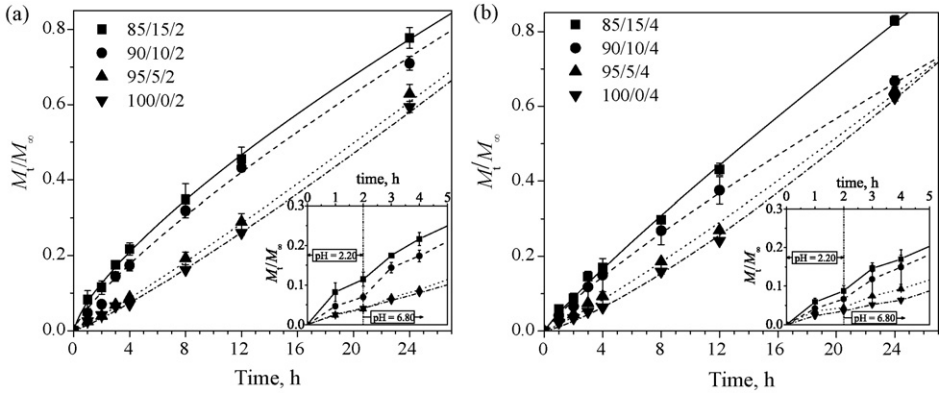

Fig. 6. Normalized average cumulative values of released protein (M_t/M_∞) versus time for hydrogels with 20.0 wt% of lipase and with (a) 2.0 wt% and (b) 4.0 wt% of the crosslinking agent concentration in simulated conditions at 37 °C, given for the first 60% of a time (85/15 straight solid (—); 90/10 straight dash (– –); 95/5 straight dot (···); 100/0 straight dash dot (– · –)). Each point represents the mean value ($n = 2$).

washing process which occurs when the hydrogel is placed in an environment that provokes the hydrogel to swell and the mesh size to increase.

3.5. Lipase release profiles and mathematical analysis

The various hydrogel formulations were subjected to lipase release studies at 37 °C in media simulating biological fluids. Thus, the percent of released lipase was measured first in pH 2.20 buffer in duration of 2 h, simulating pH conditions in stomach, followed by release measurements at pH 6.80, simulating the pH of intestinal media, to completion of a week. The lipase release was expressed as normalized average cumulative values of released protein (M_t/M_∞). The mean values for the replicated data with the standard deviations are presented in Fig. 6. Each data point is calculated based on at least two measurements. The standard deviation values were $< \pm 5\%$ of the mean values in all cases indicating a high technical reproducibility.

The results showed that the pH value of the medium has a strong effect on the protein release kinetics. Low amounts (<3.5%) of lipase were released from hydrogels at pH 2.20 and higher levels of protein release were observed at pH 6.80 (up to 26% in 24 h). This result agrees well with the swelling study, revealing that the lower hydrogel swelling ability at pH 2.20 reduces the release of lipase from the samples.

There is an obvious dependence of the kinetics of the protein release at pHs 2.20 and 6.80 on IA concentration. The hydrogels with lower concentration of IA were generally associated with slower release rate. Namely, around 16.7% of the entrapped lipase was released after 24 h from the hydrogel 100/0/4 while about 25.7% was released from the hydrogel 90/10/4, indicating that the presence of IA increases the release rate. The overall release studies showed that the incorporation of IA in hydrogel, even at small ratio, increased the release of the model protein at pH 6.80, probably due to high swelling of hydrogels with IA.

First, the duration of protein release and the total protein mass that is released in this time period was determined. The percent of lipase mass that was released from the hydrogel formulations for up to one week was in the range between 21.3 and 34.1% depending on hydrogel content and did not correlate with the concentration of lipase entrapped in the hydrogels. This could be a result of the formation of protein aggregates or collapsed state of the hydrogel at 37 °C whereby the protein is largely trapped within the polymer network, therefore limiting drug release at this temperature (Chen et al., 2005). From Fig. 6a and b it can be seen that copolymers with 2.0 and 4.0 wt% of the crosslinking agent and lowest IA content, 95/5/2 and 95/5/4, show slower release than the copolymers with higher IA contents for both crosslinking agent concentrations.

The temperature- and pH-sensitive NiPAAm/IA hydrogels have a very complex release behavior, which is strongly dependent on copolymer composition and pH at a given temperature of 37 °C, and can also be influenced by the swelling behavior of the samples, polymer–protein interactions, and the solvent–protein interactions. The PNiPAAm homopolymer has a sharp phase volume transition of 34.2 °C (Fig. 2b) in pH 6.80 buffer. That phase transition is still sharp for the copolymers with lowest IA content, 95/5/2 and 95/5/4, which have the LCST value nearest to 37 °C, the temperature at which release measurements were performed. The LCST transition becomes less pronounced for copolymers 90/10/2 and 90/10/4 and it practically does not exist for copolymers with the highest IA content, 85/15/2 and 85/15/4.

Copolymer hydrogel formulations 90/10/2, 85/15/2, 90/10/4 and 85/15/4 appear to show a biphasic release profile of lipase, with slower release at pH 2.20 for the first 2 h, followed by an accelerated release at pH 6.80, due to pH sensitive nature of the samples (Fig. 6, insert). On the other hand, for the homopolymers 100/0/2 and 100/0/4 and the samples with lowest IA content, 95/5/2 and 95/5/4, such a difference in release rates at different pH values of 2.20 and 6.80 is not present. The *in vitro* lipase release studies also show that no initial burst release is present for the all hydrogel formulations, suggesting that the lipase molecules are all entrapped inside the hydrogel. It appears that all hydrogel formulations possess prolonged release characteristics where the released percent range in 24 h was between 54.2 and 82.9%, which agreed with some previously reported results with other protein drug delivery systems which show a slow protein release during the time (Chen et al., 2005; Hiemstra et al., 2007).

Lipase release kinetics was analyzed by Peppas mathematical models, which were applied considering the amounts of protein released from 0 to 24 h for the first 60% of the drug release. The experimental points were fitted with the following Ritger–Peppas equation (Ritger and Peppas, 1987a):

$$\frac{M_t}{M_\infty} = k \cdot t^n \tag{7}$$

where M_t is the total cumulative mass of drug released at time t, M_∞ is the total cumulative mass of drug released at infinite time (at equilibrium), M_t/M_∞ represents the fractional release of the drug with respect to the value at infinite time, and k and n are constants related to diffusion coefficient and the specific transport mechanism (Liu et al., 2004a). Eq. (7) is termed the power law model, with n equivalent to the diffusional exponent. It can be easily linearized by plotting the log of fractional release ver-

sus the log of time, where the slope corresponds to the order of release and the *y*-intercept relates to the structural and diffusional characteristics. It is evident that when *n* has a value of one, the drug release is independent of concentration and time, corresponding to zero-order release. The kinetic parameters were calculated for the first 60% of the drug release, but also the cumulative lipase release half time ($M_t/M_\infty = 0.5$) was calculated and the obtained values presented in Table 3 (the kinetic parameters for the samples with 10 and 15 wt% of the IA are missing, due to the non-linear kinetics for the first 60% of a release time). For disc geometries the *n* values of below and 0.5 points to Fickian diffusion transport through hydrogel network (Case I). The range above 0.5 denotes non-Fickian diffusion transport mechanism. When *n* is equal to 1, or very close under that value, the so-called Case II appears, leading to zero-order release. Case III appears when *n* is greater than 1 (Ritger and Peppas, 1987a,b).

Notably, hydrogels with higher IA content or lower crosslinking density were associated with higher release rates consistent with the pattern of swelling and suggesting less resistance to diffusion with increased swelling. Thus, for the crosslinking ratios and initial IA concentrations involved in this study, $t_{1/2}$ varied from 13.2 to 20.8 h. It is also important that samples 95/5/2 and 95/5/4 exhibit almost zero-order release kinetics. For hydrogels with very high loading of active ingredient, as it is the case of NiPAAm/IA hydrogels, the release of contained protein follows near zero-order kinetics (Landgraf et al., 2005).

Overall, the results reveal that NiPAAm/IA hydrogels may be used for controlled delivery of proteins in a biologically active form for an extended period of time by adjusting the copolymer compositions and crosslinking agent concentration.

Since a therapeutic enzyme must be biologically active when released, the samples of released lipase from all hydrogel formulations withdrawn after 96 h were tested for specific enzyme activity using the Sigma method. The results are also presented in Table 3. It appeared that all hydrogel formulations provided the immobilized lipase to keep high activity in media simulating GI fluids, even at pH 2.20, a pH level at which native enzyme underwent deactivation completely after 15 min (data not shown). A higher specific activity was observed for hydrogels produced with high concentration of IA. The highest enzyme activity of released lipase was about 0.5 IU/mg for the hydrogel formulation 85/15/2. Released lipase from other formulations showed a slightly but insignificantly lower specific activity (STD = 10.1%).

4. Conclusion

This paper reports on the NiPAAm/IA copolymer hydrogel formulations, synthesized in the presence of the model protein, lipase from *C. rugosa*, by *in situ* polymerization. The samples were characterized regarding morphology, mechanical properties, swelling degree, protein loading efficiency and release profiles. It was found that all hydrogels showed the temperature and pH sensitivity with variations of hydrogel composition. Further, with increasing the IA content in the samples, the LCST of NiPAAm/IA hydrogels are shifted to higher temperatures, along with the broadening of the phase transition region and higher swelling ratios. The SEM analysis confirmed a significant increase in porosity with the increase of IA content. The hydrogel mechanical properties were dependent on the IA content and the crosslinking agent concentration. Therefore, these parameters can be tuned to produce hydrogels suitable for the controlled drug delivery with high preservation of the enzymatic activity of the released protein.

An appealing advantage of the NiPAAm/IA hydrogels and immobilization technique used for preparation of the lipase loaded hydrogels is that the protein loading efficiency can be as high as 95%. The release profiles of the model protein were found to be dependant on pH and hydrogel composition with slow release rates. The composition of hydrogels could be adjusted to follow near zero-order kinetics, as in the case of 95/5/2 and 95/5/4 samples. Such a matrix system is expected to protect drugs during its residence in highly acidic conditions of the stomach and release them in the lower parts of the GI tract. A direct correlation between the extent of swelling of the hydrogels and the protein release properties is apparent. The hydrogel demonstrated capacity to incorporate model protein therapeutics and are promising systems to be employed as pH responsive drug delivery systems.

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