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Poly(*N*-isopropylacrylamide-co-methacrylic acid) pH/thermo-responsive porous hydrogels as self-regulated drug delivery system

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ABSTRACT

A dual drug delivery system based on a "biosensor" (pH-sensitive unit) and a delivery component (thermosensitive hydrogel) was developed. The pH/thermosensitive hydrogel is able to restore the thermosensitive characteristics after electrostatic interaction of the pH-sensitive units with selected biologically active compounds that act as triggering agents.

The poly(*N*-isopropylacrylamide-co-methacrylic acid) (poly(NIPAAm-co-MA)) was synthesized as an interesting pH/thermo-responsive copolymer by free radical polymerization method. Due to the presence of carboxylic groups in MA units, the copolymer loses its thermosensitivity at physiological pH and temperature. However, when the negatively-charged carboxylic groups of the pH-sensitive units interact electrostatically with the positively-charged drugs with hydrophobic character propranolol, lidocaine or metoclopramide, taken as model biologically active compounds, the copolymer restores the thermosensitive properties around the physiological pH and temperature. The poly(NIPAAm-co-MA) linear copolymer was converted into pH/thermo-responsive porous microgels using oligomers of NIPAAm above their LCST, as porogens. Accordingly, the swelling/collapsing processes of the microgels occur only after the interaction with the positively-charged hydrophobic drugs. The hydrophobic drug acts as a triggering agent and the pH/temperature sensitive hydrogel turns as a biosensor (pH-sensitive units) and a delivery component (thermosensitive hydrogel).

1. Introduction

Controlled drug delivery systems have been a major interest of researchers in the last thirty years (Kawashima et al., 2011; Wu et al., 2009; Yuan et al., 2010; Ying et al., 2009). However, although these systems were in vogue for a long period of time, they have not reached the high level of expectations and as a result a new generation of drug formulation called self-regulated drug delivery systems have been proposed (He et al., 2004; Vihola et al., 2002; Young et al., 1992). These systems are able to recognize changes in the parameters of normal physiological functions and act to address them. Most of self-regulated drug delivery systems are based on smart polymers. They are a group of materials that suffer a phase transition to small changes in external factors such as temperature (Choi et al., 2006), pH (Tan et al., 2007), light (Lin et al., 2010), and ionic strength (Park et al., 2007). Among them, polymers sensitive to temperature and pH are the most used because they exploit changes in temperature and pH as triggering agents for controlled release of drug (Fundueanu et al., 2005; Islam and Yasin, 2012). Most popular thermosensitive polymer is poly (*N*-isopropylacrylamide) (poly(NIPAAm)) because in water possess a sharp phase transition (lower critical solution temperature (LCST)) at a temperature close to body temperature (32 °C) Heskins and Guillet, 1968. Under the LCST, the polymer is hydrated

Abbreviations: AIBN, *N,N′*-azobisisobutyronitrile; APS, ammonium persulfate; CP, cloud point; ESEM, environmental scanning electron microscope; HEAAm, hydroxyethylacrylamide; KS, kanamycin sulfate; LCST, lower critical solution temperature; Lid, lidocaine; MA, methacrylic acid; MBAAm, *N,N′*-methylenebisacrylamide; Met, metoclopramide HCl; MPA, 3-mercaptopropionic acid; NaB, sodium benzoate; NIPAAm, *N*-isopropylacrylamide; ONIPAAm, oligomers of poly(*N*-isopropylacrylamide-co-hydroxyethylacrylamide); PBS, phosphate buffer solution at pH = 7.4; PRP, propranolol HCl; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; VPTT, volume phase transition temperature.

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and soluble while above LCST, the polymer loses water of hydration, becomes insoluble and precipitates. Accordingly, the cross-linked hydrogel swells under the LCST and collapses above the LCST. The swelling/collapsing process is used for pulsed release of drugs (Coughlan et al., 2004; Yan and Hoffman, 1995). However, these hydrogels under physiological conditions show a phase transition far less than human body temperature due to the high ionic strength of these physiological fluids (Fundueanu et al., 2009). Moreover, the hydrogel is characterized by the lack of recognition elements embedded in self-regulating systems. To increase the transition temperature of the hydrogel towards the human body temperature, NIPAAm is copolymerized with hydrophilic monomers (Geever et al., 2006). If the hydrophilic comonomer possesses charged groups (–COOH, $–NH_2$), a pH/thermosensitive copolymer is obtained (Lue et al., 2011; Mai-ngam et al., 2009). However, the charged comonomers due to their high hydrophilicity diminish dramatically or even annihilate the thermosensitivity of the copolymer (Yoo et al., 2000). It was previously reported that electrostatic interactions of the pH sensitive units with opposite charged compounds results in modulation of thermosensitivity of the copolymer (Yoo et al., 1997). In analogy, it can be assumed that the hydrogel obtained from a pH/temperature sensitive copolymer could collapse only after interaction of the charged groups with the opposite charged compound. Another important characteristic of the cross-linked hydrogel is represented by the response rate to temperature changes. Principally, the rates of swelling and shrinking processes of thermosensitive hydrogels depend on water diffusion in/out of the hydrogel (Abd El-Mohdy and Safrany, 2008; Xue et al., 2004). The solvent diffusion depends to the gel porosity, therefore the higher is the porosity of the hydrogel, the faster is the response rate (Kim et al., 2002). The generation of a porous structure is known to be a fundamental factor to avoid the "skin effect" in hydrogels and to promote their fast volume changes in response to temperature modifications. Porous structures allow the water to be expelled or absorbed by convection, a much faster process than by diffusion.

Here, poly(NIPAAm-co-MA) with different comonomer composition was synthesized as a dual pH/thermosensitive copolymer. Due to the high hydrophilicity of carboxylic groups in simulated physiological conditions (phosphate buffer at pH = 7.4), the copolymer loses the thermosensitivity. However, when the carboxylic groups of the pH-sensitive units interact electrostatically with hydrophobic opposite charged bioactive compounds (propranolol, lidocaine, and metoclopramide) the copolymers restore the thermosensitivity. The copolymer was transformed in porous microgels by chemical cross-linking with *N,N′*-methylenebisacrylamide (MBAAm) in the presence of oligomers of NIPAAm at a temperature situated below the LCST of the copolymer and above the LCST of the oligomer. Swelling/collapsing kinetics of microgels were studied in the presence of propranolol, taken as a model triggering agent. The carboxylic group in microgel acts as a biosensor, the thermosensitive microgel represents the delivery component.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm), supplied from Aldrich Chemical Corp. (Milwaukee, WI, USA), was recrystallized from hexane. Methacrylic acid (MA), hydroxyethylacrylamide (HEAAm), 3-mercaptopropionic acid (MPA), *N,N′*-methylenebisacrylamide (MBAAm), ammonium persulfate (APS), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), *N,N′*-azobisisobutyronitrile (AIBN), and 1,4-dioxane were supplied from Fluka AG (Buchs, Switzerland). AIBN was recrystallized from methanol before use, and 1,4-dioxane was purified by refluxing. Propranolol HCl (PRP), lidocaine HCl (Lid), kanamycin sulfate (KS), metoclopramide HCl (Met), and sodium benzoate (NaB) (see their chemical structures in Scheme 1) were provided from different suppliers. The main physico-chemical properties of drugs are presented in Table 1. All chemicals were of analytical or reagent grade and were used without purification unless stated.

2.2. Synthesis of linear poly(NIPAAm-co-MA)

The synthesis of linear poly(NIPAAm-co-MA) was performed by free radical co-polymerization in aqueous solution. In a typical example, 1.13 g of NIPAAm (10 mmol) and 168 μL of MA (2 mmol) were dissolved in 20 mL of PBS. Dry nitrogen was purged through the solution for 60 min prior to polymerization. Then, the initiator (0.020 g of APS) and the accelerator (30 μL of TEMED) were added to the solution and the copolymerization was achieved within 10 h at room temperature (22 ± 2 °C). Thereafter, the polymer solution was dialyzed for 5 days at 22 °C (molecular weight cut off 10,000–12,000 Da; from Medicell International, London, United Kingdom), and recovered by freeze-drying.

2.3. Synthesis of semitelechelic NIPAAm/HEAAm oligomers

The synthesis was carried out following a method previously reported (Fundueanu et al., 2009). Typically, 1.13 g NIPAAm (10 mmol), 0.230 g HEAAm (2 mmol), 52 μL MPA (0.596 mmol) and 0.030 g AIBN (0.18 mmol) were dissolved in 6 ml 1,4-dioxane. Dry nitrogen was purged through the solution for 60 min. before polymerization. The reaction mixture was allowed to react at 80 °C for 20 h. The oligomers were precipitated into diethyl ether, re-dissolved in methanol, precipitated again in diethylether, and dried under vacuum.

2.4. Copolymer composition

The copolymer structure and composition were determined by ^{1}H NMR analysis. ^{1}H NMR spectra of poly(NIPAAm-co-MA) were recorded in deuterated dimethyl sulfoxide on a Varian Mercury Plus 400/Varian VXR 200 spectrometer operating at 400 MHz frequency. The molar fraction of MA in poly(NIPAAm-co-MA) was calculated according to Eq. (1):

$$6MA = (3MA + 3MA + 3NIPAAm + 6NIPAAm) - Y \times 9NIPAAm \quad (1)$$

where *Y* is the molar fraction of NIPAAm calculated as the area of the methynic proton at 3.83 ppm, and (3 *MA* + 3 *MA* + 3 *NIPAAm* + 6 *NIPAAm*) is the total area of the peaks between 0.2 and 2.4 ppm, corresponding to the main backbone protons (3 *MA* + 3 *NIPAAm*) plus the NIPAAm (6 *NIPAAm*) and MA (3 *MA*) methyl protons.

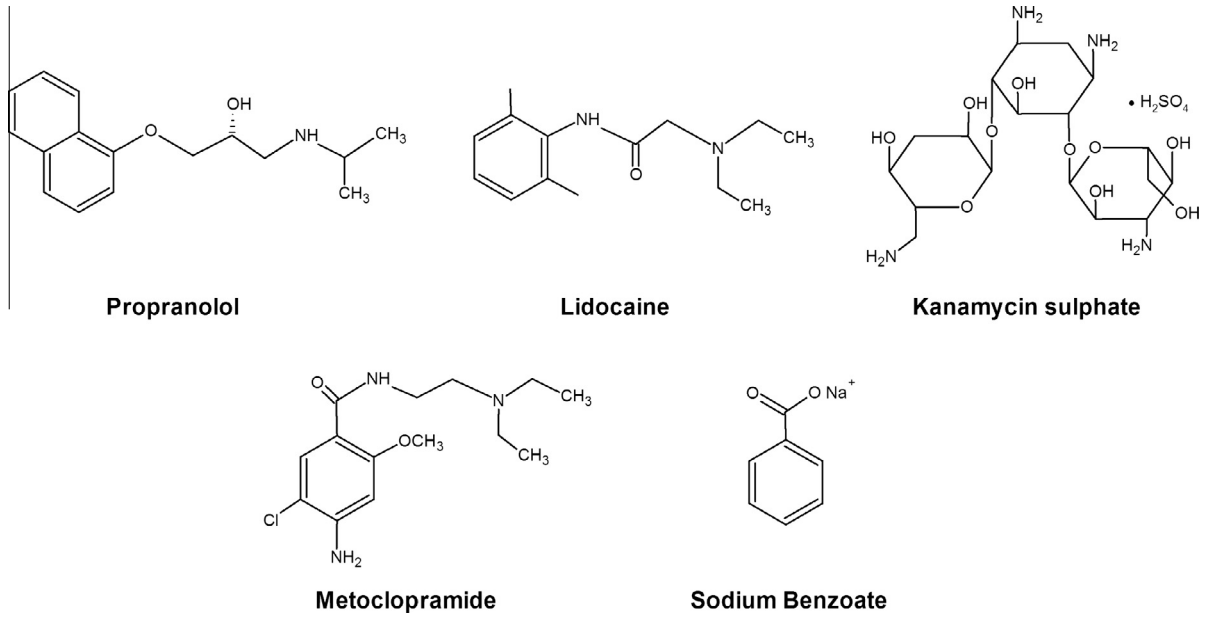
2.5. Determination of molecular weights

The number-average (M_n) and weight-average (M_w) molecular weights of poly(NIPAAm-co-MA) were determined by GPC using the GPC-PL-EMD 950 instrument (Polymer Laboratories, Shropsire, UK) in 0.1 M LiCl in dimethylformamide at 120 °C and a flow rate of 0.7 mL min^{-1}. Calibration was performed with monodisperse polystyrene standards.

The molecular weights of semitelechelic oligomers were determined by end-group titration using 0.01 N NaOH and phenolphthalein, at 22 °C, which detect the carboxyl groups at the end of oligomer chains.

The molecular weight is calculated according to Eq. (2):

$$Mn(g/mol) = \frac{1}{Cs} \times 1000 \quad (2)$$



Scheme 1. Chemical structures of drugs.

Table 1
Main physico-chemical properties of drugs.

Drug	Property		
	pK_a	Log*P*	Log*S*
Propranolol	9.42	3.48	−3.5
Lidocaine	8.01	2.44	−2.6
Metoclopramide	9.27	2.62	−3
Kanamycin	7.2	−6.3	−0.72
Benzoic acid	4.19	1.87	−1.55

where *Cs* is the exchange capacity (meq/g).

2.6. Determination of the lower critical solution temperature

The LCST was determined from the curve representing dependence of the absorbance change at 450 nm on temperature. The UV–Vis Specord 200 spectrophotometer (Analytic Jena, Jena, Germany) equipped with a temperature controller, was used. The copolymer solution (1%, w/v) was prepared in distilled water, standard acidic solution (pH = 1.2; 64 mM HCl + 50 mM KCl), and standard phosphate buffer solution (PBS) (pH = 7.4; 50 mM Na_2HPO_4 + NaOH). The heating rate was fixed at 0.2 or 1 °C every 10 min. The cloud point (CP) was defined as the temperature at which the absorbance riches a value of 0.5.

2.7. Turbidimetric titration

Twelve mL of PRP solution in PBS (10 mg mL^{-1}) were added in small doses (0.2 mL), to a solution of thermosensitive copolymer in PBS (25 mL, 10 mg mL^{-1}). The temperature of the cell was fixed at 42 °C. After each addition, the solution was left to react for five minutes, and then the transmittance was measured with a Brinkmann PC 900 turbidimeter (SUA) equipped with a 20-23-634-5 type fiber optic cell.

2.8. Synthesis of the microgels

Porous poly(NIPAAm-co-MA) hydrogel was synthesized as follows: 1.13 g of NIPAAm (10 mmol), 168 μL of MA (2 mmol), 0.016 g of MBAAm (0.1 mmol), and 0.2 g of oligomer (porogen) were dissolved in 10 mL of PBS. Dry nitrogen was purged through the solution for 60 min before polymerization. Then, the initiator (0.020 g of APS) and the accelerator (30 μL of TEMED) were added, and the solution was immediately transferred into a syringe. The polymerization was conducted for 12 h at a temperature situated below the LCST of the copolymer but above the LCST of the semitelechelic oligomers (24 ± 2 °C). After polymerization, the resulting hydrogel was washed widely with water to remove the un-reacted species, then with acetone and diethyl ether and dried under vacuum. Finally, the dried hydrogel was crushed in small particles (between 10 and 200 μm). The non-porous hydrogel was prepared under the same conditions without porogen. The carboxyl groups in hydrogel (exchange capacity) were determined by titration method. Firstly, the hydrogel, added in protonated form with 0.1M HCl, was left in an excess of 0.1M NaOH for 24 h. Then, it was washed and the collected solution was titrated with a 0.1M HCl solution.

2.9. Morphological analysis

The hydrogels swollen in PBS at room temperature were quickly frozen in liquid nitrogen and then freeze-dried (−57 °C, 5.5×10^{-4} mbar) for 24 h until all the solvent was sublimed. The freeze-dried hydrogels were fractured carefully and the interior morphology was observed by use of an Environmental Scanning Electron Microscope (ESEM, type Quanta 200, Netherlands).

2.10. Swelling degree

The volume increase of the microparticles (in the absence or in the presence of stoichiometric amount of PRP) at different temperatures was determined at equilibrium in PBS by placing the microparticles in a graduated cylinder (i.d. = 12 mm). The swelling degree (q) was calculated according to Eq. (3):

$$q = \frac{V_s}{V_d} \quad (3)$$

where V_s is the volume of the swollen microparticles and V_d is the dried volume.

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2.11. Determination of the volume phase transition

The volume phase transition temperature (VPTT) of microgels was determined as the midpoint of the section of the *swelling degree-temperature* curve with the maximum slope.

2.12. Swelling/collapsing kinetics

Swelling kinetic of the pH/thermosensitive microgels was determined in PBS in the presence of stoichiometric amount of PRP. The microgels were equilibrated at the required temperature (45 °C) in a graduated glass cylinder (i.d. = 12 mm), then were transferred into a water bath at 4 °C. The volume change was monitored regularly. The dynamic swelling ratio (q_{ds}) was calculated according to Eq. (4) Yıldız et al., 2002:

$$q_{ds} = (V_t - V_d)/(V_{0(45\,^\circ C)} - V_d) \quad (4)$$

where $V_{0(45°C)}$ is the microgel volume at equilibrium at 45 °C, V_t is the microgel volume at a given time, and V_d is the volume of dried microgels.

To evaluate the collapsing kinetic, the microgels equilibrated in PBS at 4 °C were transferred into water bath at 45 °C. The dynamic collapsing ratio (q_{dc}) was calculated according to Eq. (5) Yıldız et al., 2002:

$$q_{dc} = (V_t - V_d)/(V_{0(4\,^\circ C)} - V_d) \quad (5)$$

where $V_{0(4°C)}$ is the microgel volume at equilibrium at 4 °C, V_t is the microgel volume at a given time, and V_d is the volume of dried microgels.

2.13. Binding/collapsing experiments

In a typical example, 0.1 g of microgels were introduced in a glass cylinder and conditioned in 6 mL of PBS at 36 °C for 24 h. Then 4 mL of PRP solution in PBS (22 mg mL^{-1}) were added over the swollen microgels (PRP: MA molar ratio being 2:1). The solution was shaken at a constant temperature. At regular time intervals, the suspension was filtered, sample was withdrawn from filtrate and analysed spectrophotometrically for PRP content. Simultaneously, the swelling degree was measured. The same experiments were performed for non-porous microgels. The amount of bound drug can be determined from the difference in concentration before and after the adsorption. The adsorption capacity of the microgels (A, mg g^{-1}) was calculated according to Eq. (6):

$$A = (C_0 - C_e)V/m \quad (6)$$

where C_0 and C_e were the initial concentration and the equilibrium concentration of the drug (mg mL^{-1}) respectively, V is the volume (mL) of the initial solution, and m (g) is the mass of the dried microgels.

2.14. Statistical analysis

The values are presented as mean ± standard deviations (Graph Pad 5.0 Software). Where appropriate, the deviations were calculated as percentage from the mean values and are given the minimum and the maximum values. The difference of parameters was statistically tested for significance with one-way with ANOVA followed by Turkey–Kramer post test using GraphPad Instant 3 (GraphPad software, Inc., La Jolla, CA) software for Windows. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of the pH/thermosensitive linear copolymer

One of the most important demands of a thermosensitive polymer proposed to be used in a biomedical application is to have a sharp phase transition around the physiological pH and temperature. As it is well known, poly(NIPAAm) is one of the most used thermosensitive polymer because displays a sharp phase transition around 32 °C in aqueous solution (Heskins and Guillet, 1968). However, in simulated physiological fluids (PBS) possessing a large amount of salts, the transition temperature of poly(NIPAAm) may decrease even down to 28 °C (Fundueanu et al., 2009), making it unsuitable for biomedical applications. With the aim to increase the LCST towards that of the human body, NIPAAm is copolymerized with hydrophilic monomers containing neutral functional groups such as –OH and –CO–NH$_2$ (Geever et al., 2006). The copolymerization of NIPAAm with charged hydrophilic monomers possessing carboxyl functional groups gives pH/thermosensitive copolymers, however the amount of carboxylic comonomer must be low to preserve the thermosensitivity of copolymer (Makino et al., 2000a). Here, copolymers of NIPAAm with two different amounts of MA were prepared. As reported in Table 2 and proven by ^{1}H NMR spectra (Fig. 1), the co-polymer formation and the percentage of the co-monomers in the copolymer correspond approximately to those observed in the feed.

3.2. Phase transition characterization

Because poly(NIPAAm-co-MA) possesses both thermo- and pH-sensitive units, it is essential to determine the LCST at different pH values simulating different physiological fluids. As shown in Table 2, in PBS at pH = 7.4, a dramatically shift of the LCST value to higher temperature was observed by increasing the MA content of the poly(NIPAAm-co-MA). In fact, at pH = 7.4, the carboxylic groups of MA are fully ionized (pK_a = 5.4) and therefore possess a significant hydrophilicity. As follows, the phase transition is very slow (Fig. 2A and B), and both copolymers lose in a great extent the thermosensitivity, this renders unsuitable the copolymers for biomedical applications. On the contrary, at acidic conditions (pH = 1.2),

Table 2
Influence of comonomer ratio in the feed and in the copolymer on the LCST (concentration of copolymer solution was always 1%, w/v).

Sample	Comonomer composition				LCST (°C)			Yield (%)
	In the feed × 10^{-3} M (% mol ratio)$^-$		In copolymer (% mol ratio)		pH = 7.4	pH = 1.2	H_2O	
	NIPAAm	MA	NIPAAm	MA				
P_0	10	0	100	0	28.4 ± 0.2	31.5 ± 0.2	32.5 ± 0.2	93.7 ± 4.9
P_1	10 (90.9)	1 (9.1)	n.d.[a]	n.d.	38.3 ± 0.1	28.8 ± 0.3	31.6 ± 0.2	89.2 ± 5.2
P_2[b]	10 (83.3)	2 (16.7)	83.7	16.3	48 ± 0.3	26.8 ± 0.3	32.6 ± 0.3	88.7 ± 4.6

Data are the results of two independent experiments.
[a] n.d. = not done.
[b] M_n = 20088 g/mol, M_w = 26421 g/mol, IP = 1.315, IP = polydispersity index.

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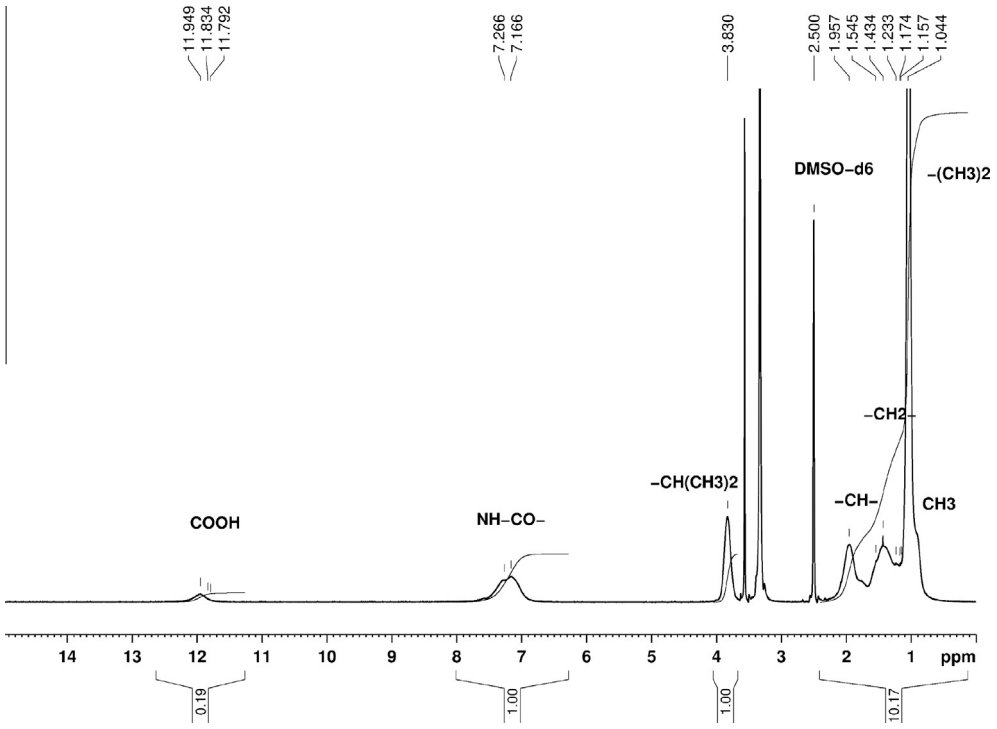

Fig. 1. ^{1}H NMR spectrum of poly(NIPAAm-co-MA) (sample P_2 in Table 1).

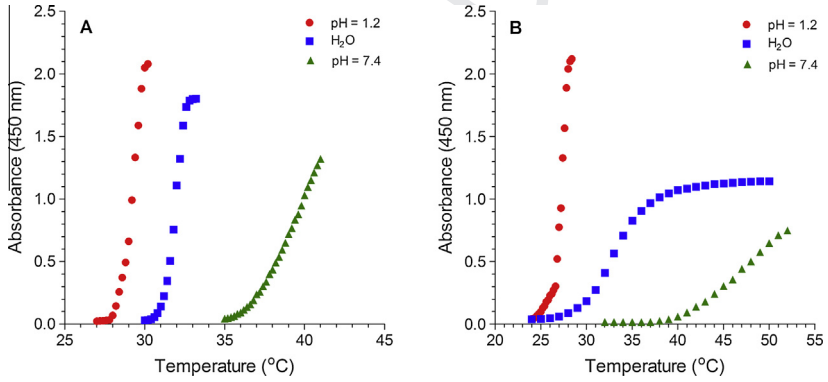

Fig. 2. LCST profile of poly(NIPAAm-co-MA) (P_1, panel (A); P_2, panel (B) in pseudo-physiological fluids: acidic solution at pH = 1.2 (filled circles), distilled water (filled squares) and phosphate buffer at pH = 7.4 (filled triangles). The polymer concentration was 1% (w/v). The absorbance values are the mean of three independent experiments that deviated 2–7%.

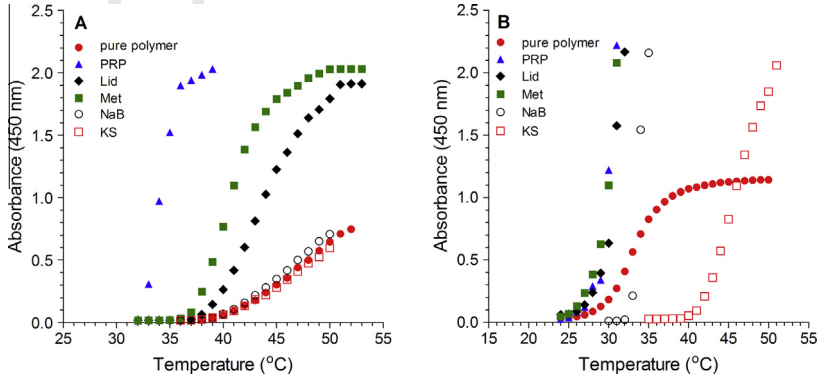

Fig. 3. LCST profiles of poly(NIPAAm-co-MA) (sample P_2 in Table 1), in the absence of any drug (filled circles), "complexed" with a stoichiometric amount of the positively charged drugs: PRP (hydrophobic drug; filled triangles), Lid (hydrophobic drug; filled diamonds), Met (hydrophobic drug; filled squares) or KS (hydrophilic drug; empty squares), and in the presence of a stoichiometric amount of the "un-complexed" drug NaB (empty circles). The absorbance values are mean of three independent measurements that deviated 2–8%. The experiments were performed in phosphate buffer at pH = 7.4 (panel A) and in pure distilled water (panel B). The polymer concentration was 1% (w/v).

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the MA are fully protonated, and the LCST decreases to temperatures much lower than that of the human body (Fig. 2A and B). The higher is the percentage of MA in copolymer, the lower is the LCST (Table 2). Moreover, at pH = 1.2, the phase transitions are very sharp for both kind of copolymers (Fig. 2A and B).

Remarkably, in physiological conditions at pH = 7.4, when the hydrophilic carboxylate groups of MA, responsible for the low thermosensitivity of poly(NIPAAm-co-MA), interact electrostatically with the positively charged amino groups of the hydrophobic drugs (see Table 1) (the ionically linked compound is called also "complex") such as propranolol (PRP), metoclopramide (Met) and lidocaine (Lid) (1:1 molar ratio drug/MA), the copolymer restores the thermosensitive properties, the phase transition becomes more abrupt and the LCST is adjusted towards the body temperature (Fig. 3A). The more hydrophobic is the drug the lower is the LCST. Therefore, PRP possessing two benzene rings seems to be the most hydrophobic drug since its "complex" with the copolymer has the lowest LCST (33.4 °C, Fig. 3A). Indeed, PRP possesses the highest absolute values of hydrophobicity index ($\log P = 3.48$; $\log S = -3.5$) (see Table 1). Metoclopramide ($\log P = 2.62$; $\log S = -3$) displays a higher LCST (39 °C) than PRP, but LCST is lower than that of lidocaine (41.5 °C) which in turn possesses the lowest absolute values of hydrophobicity index ($\log P = 2.44$; $\log S = -2.6$). These results confirm our hypothesis that only drugs able to interact electrostatically with the carboxylic groups of MA, generating a new comonomer, change the hydrophilic/hydrophobic balance of the copolymer. It should be noticed that the interactions between drugs and carboxylate groups occurred even in the presence of competitive ions from PBS, because the drugs possess relatively strong amino groups (see their chemical structure in Scheme 1 and the pK_a values in Table 1). If the drug (kanamycin) interacting electrostatically with poly(NIPAAm-co-MA) has a hydrophilic character ($\log P = -6.3$; $\log S = -0.72$), LCST is expected to increase to higher temperature. In fact, LCST is almost the same as for pure polymer (Fig. 3A). The same behaviour was observed in the presence of sodium benzoate. Even though this drug has a hydrophobic character ($\log P = 1.87$; $\log S = -1.55$), it is negatively charged and does not interact electrostatically with carboxylic groups of MA from the copolymer. However, it should be taken into account that kanamycin possesses relatively weak primary amino groups ($pK_a = 7.2$) interacting with the carboxylic groups of MA. In the presence of competitive ions from PBS, these weak interactions may be suppressed, and the ionic "complex" can be broken down.

In order to prove these suppositions, the same experiments were performed in pure water in the absence of competitive ions (Fig. 3B). In these conditions, the ionic "complex" between kanamycin and MA is stable and therefore the LCST is much higher than that of pure copolymer. Moreover, the "complexes" between the strong amino groups and MA are more stable in water, and therefore the LCSTs are lower in water than in PBS.

3.3. Preparation and characterization of hydrogel

Poly(NIPAAm-co-MA) hydrogel was prepared by free radical cross-linking copolymerization of NIPAAm and MA with small amount of MBAAm, as the cross-linker, and APS and TEMED were used as the redox initiator system. The amount of MBAAm was in such a manner adjusted, thus to obtain a mechanically stable

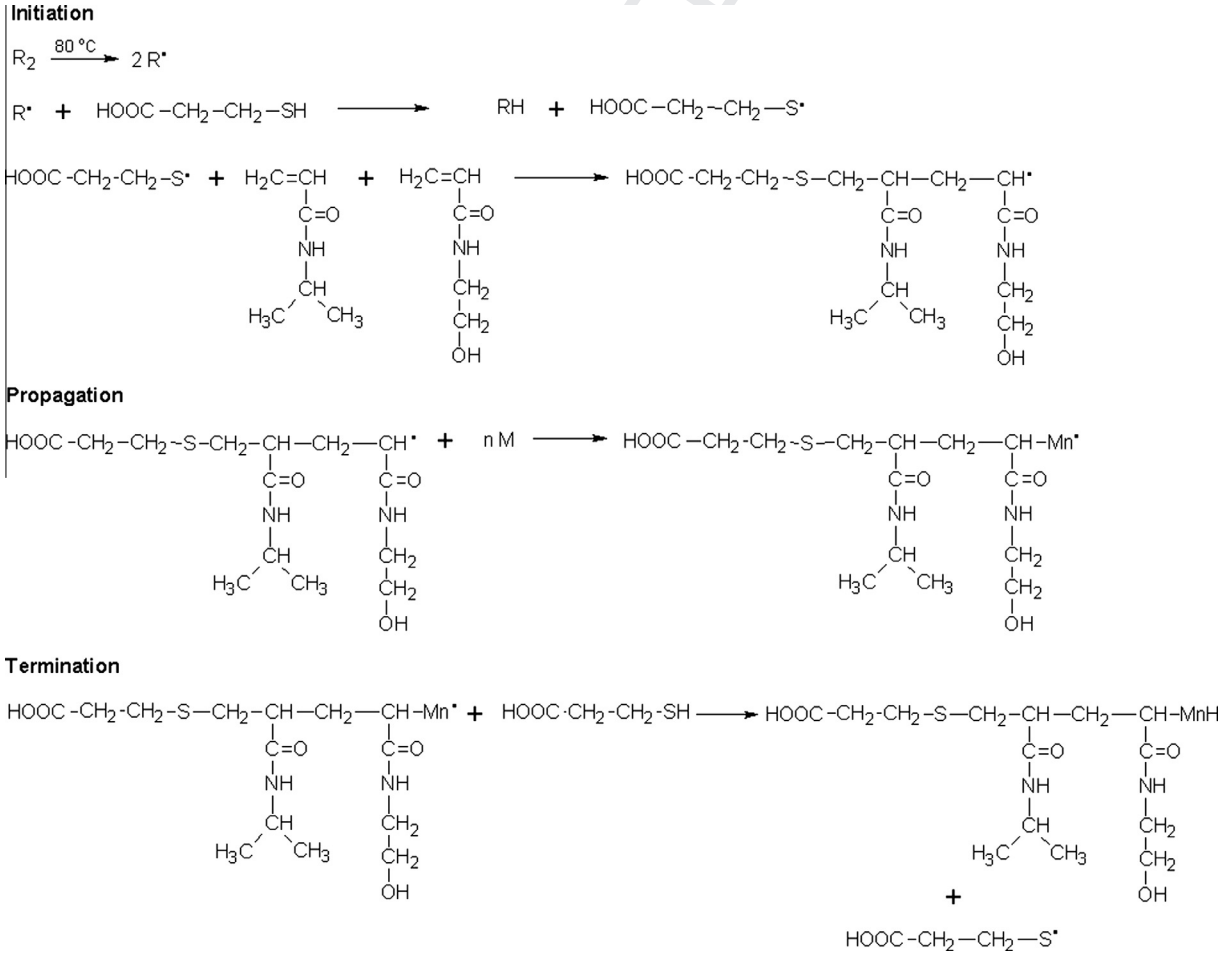

Fig. 4. Synthesis of semitelechelic poly(*N*-isopropylacrylamide-co-hydroxyethylacrylamide) oligomers.

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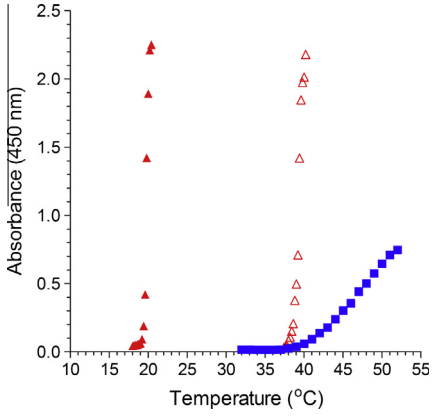

Fig. 5. LCST profiles of the semitelechelic oligomer in simulated preparation conditions (phosphate buffer at pH = 7.4, in the absence (empty triangles) and in the presence of the monomers and crosslinker (filled triangles)). For comparison, the LCST profile of poly(NIPAAm-co-MA) (sample P_2 in Table 1) in PBS is represented (filled squares). The absorbance values are mean of three independent measurements that deviated 3–8%.

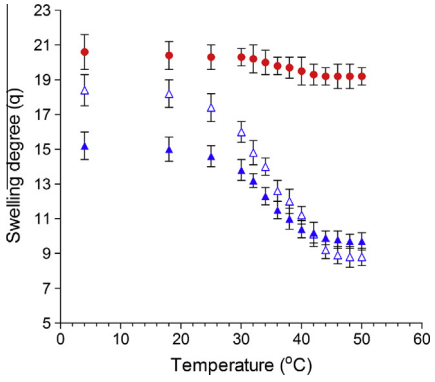

Fig. 7. Effect of temperature on the swelling degree of porous poly(NIPAAm-co-MA) microgels in the absence (filled circles) and "complexed" with PRP (empty triangles), under simulated physiological conditions (PBS at pH = 7.4); (n = 3). For comparison the phase transition of non-porous microgels "complexed" with PRP is represented (filled triangles).

hydrogel with high capacity of swelling. Moreover, with the aim to obtain a high porous structure, usually responsible for a fast swelling/collapsing rate, oligomers of poly(*N*-isopropylacrylamide-co-hydroxyethylacrylamide) (ONIPAAm), were used as porogens. It is expected that at a temperature above their LCST, the oligomers form globular structures within the hydrogel matrix and large halls remain after their removal. Semitelechelic ONIPAAm oligomers possessing carboxyl end groups were synthesized by free radical polymerization using 3-mercaptopropionic acid as chain transfer agent as shown in Fig. 4. The molecular weight was estimated at 2380 g/mol by end group titration. The LCST of the ONIPAAm in simulated preparation conditions of hydrogel ("in situ", PBS at pH = 7.4, presence of the monomers and cross-linker) was determined (Fig. 5). It can be observed that the phase transition temperature of the oligomer decreased with around 20 °C in the presence of monomers and cross-linker.

Following these preliminary experiments, the synthesis of the hydrogel was performed in PBS at pH = 7.4 and 22 °C, below the LCST of the poly(NIPAAm-co-MA) but above the LCST of the oligomers. As can be seen in Fig. 6, the hydrogel obtained in the presence of the porogen displays a more porous and regular structure than that obtained in the absence of the porogen. The exchange capacity of porous and non-porous were determined, being 1.46 ± 0.05 meq/g and 1.38 ± 0.04 meq/g, respectively.

In order to ensure that the poly(NIPAAm-co-MA) microgels maintain the pH/thermosensitive properties of the linear polymer, the volume phase transition temperature (VPTT) was determined under physiological conditions (PBS, pH = 7.4). The method is based on the measurement of the swelling degree at equilibrium, at different temperatures below and above the LCST (Fundueanu et al., 2009). This method is simple and accurate and could be applied to hydrogels which are in the form of small particles. It was previously established that poly(NIPAAm-co-MA) itself does not display temperature sensitivity around the human body temperature. Therefore, the microgels were first equilibrated into PBS (pH = 7.4) containing stoichiometric amount of PRP, then the swelling degree was measured at different temperatures. As shown in Fig. 7, the phase transition occurs when the temperature changes around the LCST of the complex formed by the linear polymer and the drug. The porous microgels display a sharper transition than those non-porous. In the absence of drug, the microgels do not show any transition temperature following the behaviour of the linear polymer. However, a slightly decrease of the swelling degree with temperature was noticed, both below and above LCST, this behaviour was found also in other thermosensitive cross-linked networks (Makino et al., 2000).

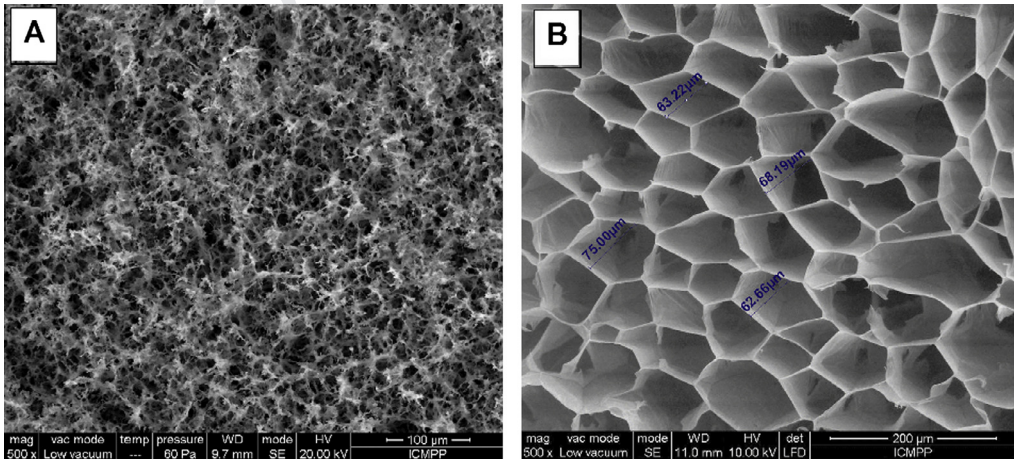

Fig. 6. Scanning electron micrograph of lyophilized poly(NIPAAm-co-MA) hydrogel (cross-section) obtained in the absence (panel A) and in the presence of the porogen (panel B).

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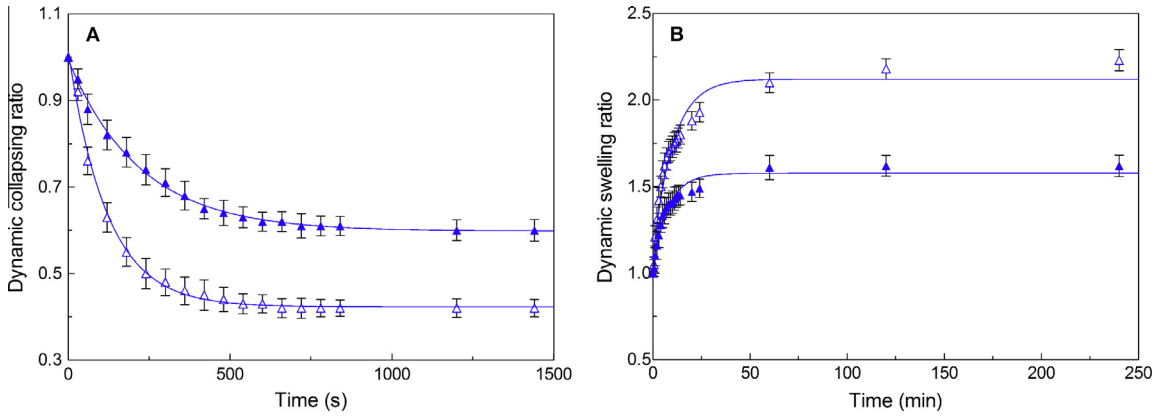

Fig. 8. Collapsing (panel A) and swelling (panel B) kinetics of porous (empty triangles) and non-porous (filled triangles) poly(NIPAAm-co-MA) microgels in the presence of PRP, under physiologically-like conditions (PBS, pH = 7.4); (n = 3). Data reported in panel A were analysed according to Eq. (7):

$$Y = \text{Span} \times e^{-kt} + \text{Plateau} \quad (7)$$

with values of Span = 0.59 ± 0.01, k = 0.0084 ± 0.0004 min^{-1} ($t_{1/2}$ = 82.5 min), and Plateau = 0.42 ± 0.004 (empty triangles), and Span = 0.39 ± 0.006, k = 0.0045 ± 0.0002 min^{-1} ($t_{1/2}$ = 154 min), and Plateau = 0.60 ± 0.004 (filled triangles). Data reported in panel B were analysed according to Eq. (8):

$$Y = Y^{\max} \times (1 - e^{-kt}) + Y^0 \quad (8)$$

with values of $Y^{\max}$ = 1.02 ± 0.05, k = 0.10 ± 0.01 min^{-1} ($t_{1/2}$ = 9.3 min), and Y^0 = 1.1 ± 0.04 (empty triangles), and $Y^{\max}$ = 0.55 ± 0.02, k = 0.13 ± 0.01 min^{-1} ($t_{1/2}$ = 7.1 min), and Y^0 = 1.0 ± 0.02 (filled triangles). Data analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

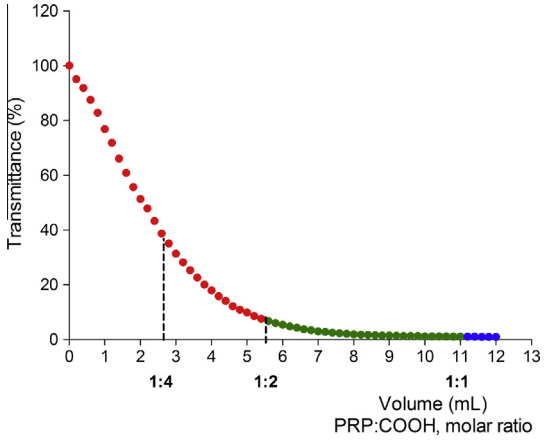

Fig. 9. Turbidimetric titration of linear poly(NIPAAm-co-MA) (sample P_2 in Table 1) with propranolol in PBS at 42 °C.

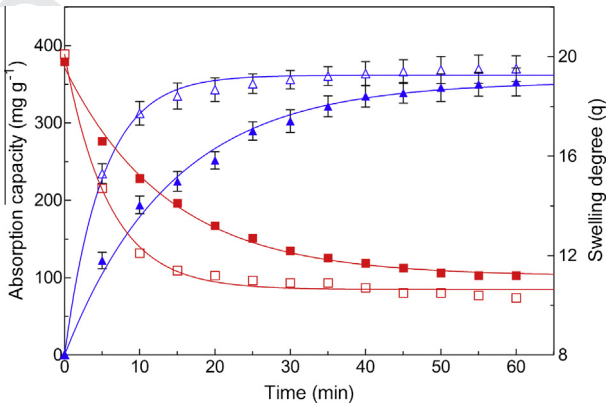

Fig. 10. Propranolol absorption (filled and empty triangles) correlated with collapsing (filled and empty squares) kinetics of poly(NIPAAm-co-MA) microgels determined under physiologically-like conditions (PBS at pH = 7.4, T = 36 °C). Filled and empty symbols represent the non-porous and porous microgels, respectively. Data are the results of three independent experiments. Data representing propranolol absorption were analysed according to Eq. (9):

$$Y = Y^{\max} \times (1 - e^{-kt}) \quad (9)$$

with values of $Y^{\max}$ = 362 ± 7 mg g^{-1} and k = 0.20 ± 0.01 min^{-1} ($t_{1/2}$ = 3.5 min) (empty triangles), and $Y^{\max}$ = 353 ± 12 mg g^{-1} and k = 0.071 ± 0.004 min^{-1} ($t_{1/2}$ = 9.8 min) (filled triangles). Data representing collapsing kinetics were analysed according to Eq. (10):

$$Y = \text{Span} \times e^{-kt} + \text{Plateau} \quad (10)$$

with values of Span = 0.95 ± 0.2, k = 0.17 ± 0.01 min^{-1} ($t_{1/2}$ = 4.1 min), and Plateau = 10.6 ± 0.08 (empty squares), and Span = 8.4 ± 0.2, k = 0.073 ± 0.004 min^{-1} ($t_{1/2}$ = 9.5 min), and Plateau = 11.2 ± 0.2 (filled squares). Data analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3.4. Swelling/shrinking experiments

One of the most important requirements of thermosensitive hydrogels for use in biomedical applications is to assure a high swelling/collapsing rate to small changes in external temperature. As it was previously stated, the microgels become thermosensitive only after electrostatic interactions between the carboxylic groups of MA in copolymer and the amine groups of PRP. As a result, the microgels were first equilibrated in PBS in the presence of a stoichiometric amount of PRP, and then the swelling/collapsing tests were performed by changing the temperature around the VPTT.

Generally, the swelling/collapsing rate of common hydrogels is the result of water diffusion in/out and depends mainly on the hydrogel porosity (Kim et al., 2002). The higher is the porosity of the hydrogel, the faster is the water diffusion and therefore the response rate to the change of temperature.

The microgels possess a porous structure, therefore, the swelling/shrinking kinetics occur relatively rapidly when the temperature changes around the VPTT (Fig. 8). As expected, the microgels obtained in the presence of porogen display a more rapid swelling/shrinking rate.

It must be noticed that the collapsing rate are higher than the swelling rate because the water is expelled off mechanically

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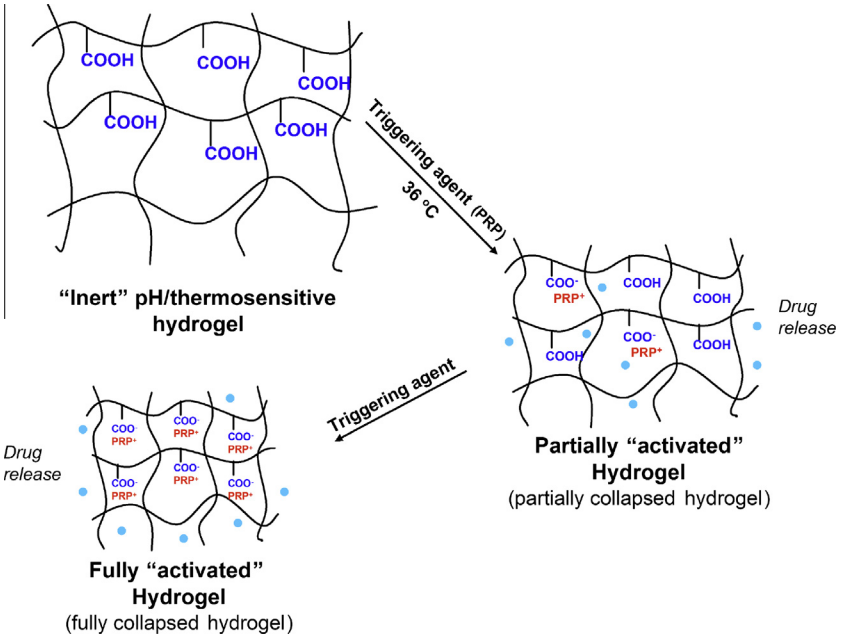

Fig. 11. Schematic representation of the principle of operation of pH/thermosensitive poly(NIPAAm-co-MA) microgels in the presence of the triggering agent, under simulated physiological conditions.

throughout the collapse of the hydrogel, a process much faster than its diffusion into the hydrogel.

As it was previously stated, the collapsing/swelling experiments were carried out only after the complete "complexation" of the microgels with PRP (PRP:COOH molar ratio = 1:1). However, the behaviour of the microgels at lower degree of "complexation" should be investigated. To an accurate evaluation of this hypothesis, a turbidimetric titration of the linear copolymer with PRP was performed at a temperature above the LCST, at which the polymer-drug "complex" (1:1, molar ratio) is completely precipitated (42 °C) (Fig. 9).

As it is shown in Fig. 9, the progressive addition of PRP induces a linear decrease of the transmittance of the copolymer solution. When half of the carboxylic groups were "complexed" with PRP, the transmittance decreases by about 92.4%. The further "complexation" of the remaining carboxylic groups determines a decrease of transmittance of about 7%. In analogy, it can be supposed that the decrease of the transmittance of the linear copolymer solution with 92.4% could be hypothetically correlated with a dramatic and almost complete shrinkage of the hydrogel. It must be underlined that in solution, the linear copolymer (MA) interacts almost instantaneously with PRP. On the contrary, in a hydrogel possessing a tridimensional network, the interaction between MA and PRP is controlled by diffusion. As follows, the shrinkage of the microgels was investigated taking into account the absorption kinetic of PRP and absorption capacity of the support (Fig. 10).

The progressive absorption of the PRP is accompanied by the gradual shrinkage of the microgels.

The propranolol absorption by porous microgels is faster than drug absorption by non-porous microgels by about 3 folds. Moreover, the rate of porous microgel collapsing is faster than that of non-porous microgels by about 2 folds.

It should be noted that as the degree of absorption of the drug increases, the tridimensional network collapse progressively and the access to the free carboxyl groups becomes increasingly more difficult or even impossible. As a result, at the end of the absorption process, the amount of linked PRP is 85.6 ± 3.84% and 86.4 ± 4.43% from theoretical, for porous and non-porous microgels, respectively. However, this difference is not significant if p value is considered ($p > 0.05$).

From the biomedical point of view, this progressive shrinkage of the microgels could determine, for example, the release of the drug, previously loaded within the hydrogel (Fig. 11). As previously stated, under the physiological conditions (PBS, $T = 36$ °C) the hydrogel is "inert" but restores the thermosensitive properties (becomes "active") and shrinks only after electrostatic interactions of carboxyl groups of MA with PRP (Fig. 11). In these conditions, the self-regulated drug delivery device contains a "biosensor" (MA), a delivery component (thermosensitive microgels), and PRP acts as a triggering agent.

4. Conclusions

The common pH/thermosensitive poly(NIPAAm-co-MA) copolymer acquires new valences after electrostatic interaction with biologically active substances with different hydrophilic/hydrophobic balance. The copolymer possesses both pH- and thermo-sensitive units, however, in physiological conditions (PBS at pH = 7.4) the carboxylic groups are ionized, and due to their high hydrophilicity it loses the themosensitivity. However, when the carboxylic groups interact electrostatically with positively charged molecules with hydrophobic character, the copolymer restores the thermosensitivity. This dual system could represent the basement of the next generation of drug delivery systems capable to detect some biochemical substances (triggering agents) released by the organism in pathological conditions. After electrostatic interactions with the triggering agents, the delivery component (thermosensitive hydrogel) collapses and releases the drug.

Acknowledgements

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