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Poly(*N*-isopropylacrylamide-*co*-*N*-vinylpyrrolidone) thermoresponsive microspheres: The low drug loading ensures the pulsatile release mechanism

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Abstract. Poly(*N*-isopropylacrylamide-*co*-*N*-vinylpyrrolidone) (poly(NIPAAm-*co*-NVP)) with a co-monomer molar ratio in copolymer of 91.5/8.5 (NIPAAm/NVP) was synthesized as an interesting thermosensitive material possessing a sharp phase transition at 36 °C under simulated physiological conditions. The effect of the co-monomer molar ratio as well as of the ionic strength and nature of ions on the lower critical solution temperature (LCST) was investigated. Cross-linked poly(NIPAAm-*co*-NVP) thermoresponsive microspheres were synthesized by the suspension polymerization technique respecting the same NIPAAm/NVP molar ratio as for linear polymer. The microspheres were loaded with the model drug diclofenac (DF) by the solvent evaporation method; differential scanning calorimetry (DSC) demonstrates a dispersion of drug crystals within the polymeric matrix. The DF release rate is deeply influenced by the drug loading degree. Only microspheres with low DF loading are able to release the bioactive compound through a pulsatile mechanism.

Keywords: *smart polymers, poly(N-isopropylacrylamide), stimuli-sensitive polymers, drug delivery, microspheres*

1. Introduction

One of the most important achievements in drug therapy is the development of controlled release or retard pharmaceutical formulations [1–3]. These systems have the advantage of maintaining a constant drug concentration in blood and tissues with a single dose, thereby reducing the frequency of administration and increasing the therapeutic efficacy as well as the patient compliance. Although these formulations have brought considerable benefits, their use is not always appropriate since some diseases require the drug administration only when the physiological conditions are altered. Such a goal can be achieved developing self-regulated drug delivery systems, most of them being based on intelligent or stimuli-sensitive polymers [4–6]. These polymers undergo a phase transition when minor changes of the environmental parameters including pH [7], temperature [8], ionic strength [9], light [10], and electric or magnetic field [11, 12] occur. Among these, temperature-sensitive polymers are the most used materials for biomedical applications because they exploit the change of the human body temperature to modify their properties [13, 14]. Poly(*N*-isopropylacrylamide) (poly (NIPAAm)) is the most relevant temperature-sensitive polymer since in aqueous solution it possesses a sharp phase transition (lower critical solution temperature, LCST) at about 32 °C [15]. Under the LCST, the polymer chains are hydrated and solubilize whilst above the LCST, the hydrogen bonds between water molecules and the polymer are disrupted and the polymer becomes hydrophobic and precipitates.

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In turn, the cross-linked hydrogel swells under the LCST and collapses above the LCST, this reversible process being pivotal for the temperature-controlled drug release [16, 17]. However, for many biomedical applications are necessary polymers with a sharp phase transition at a temperature close to that of the human body. In order to increase the LCST, poly (NIPAAm) is usually copolymerized with hydrophilic monomers [18,19]. The percentage of the co-monomer in the copolymer must be low, otherwise the acrylamide sequences of poly(NIPAAm) are highly disturbed and the copolymer may lose its thermosensitive properties [20]. The most important characteristic of a thermoresponsive hydrogel for biomedical applications is to display high rate of swelling and collapsing within a narrow temperature range. The swelling and deswelling rates are governed by the water diffusion throughout the polymer network. The water diffusion depends to the size of the hydrogel: the smaller is the size the quicker is the diffusion and thus the response rate [21, 22]. Therefore, methods for the preparation small-size hydrogels, named microgels, from either preformed polymers [23] or from monomers [24] with relative rapid swelling and deswelling rates have been reported. The use of preformed polymers ensure an advanced purification and characterization in solution, however, they must contain cross-linkable groups that can alter the thermosensitivity of the whole copolymer [25]. The biomedical literature concerning the preparation of poly (*N*-isopropylacrylamide) microspheres from monomers by two phases suspension polymerization is quite insufficient [26] since the process is very sensitive to the presence of the oxygen both in the monomer solution and dispersion medium that causes the inhibition of polymerization. Most of the authors use either microemulsion [27] or precipitation polymerization [28], both processes occurring in a single phase, however the dimensions of the resulted particles is often in the nanometric range. The release rate of physically entrapped drug is usually governed by the average size of microspheres [29], the drug concentration in the polymer matrix [30], porosity [23], and the hydrophobic interactions between drug and the polymeric network [31].

In order to assess the mechanism through which the drug is released from microspheres, appropriate mathematical models were used [32]. Among them, the Korsmeyer-Peppas model is a simple power law model that can explain a mixed release mechanism determined by a range of factors such as swelling of polymers, matrix porosity and drug diffusion [33]. Furthermore, this model is commonly used to examine the pharmaceutical formulations when the release mechanism is not well known or more than one type of release event takes place [34].

Here, poly(*N*-isopropylacrylamide-*co*-*N*-vinylpyrrolidone) with a co-monomer molar ratio (NIPAAm/NVP) of 91.5/8.5 was prepared as an interesting thermosensitive copolymer that possesses a LCST value of 36 °C, under physiological conditions. The effect of the co-monomer molar ratio as well as of the ionic strength and nature of ions on LCST was investigated. Cross-linked microspheres were synthesized by the suspension polymerization technique using a co-monomer molar ratio previously determined for the linear copolymer. The microspheres were loaded with the model drug diclofenac (DF) by the solvent evaporation method and then they were analyzed by differential scanning calorimetry (DSC). Finally, the influence of temperature, the cyclical variation of temperature below and above the volume phase transition temperature (VPTT), and the DF loading on drug release profiles was investigated.

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPAAm) (from Sigma-Aldrich Chemical Co., Milwaukee, WI, USA) was re-crystallized with hexane. *N*-vinylpyrrolidone (NVP), *N*,*N*-methylene bisacrylamide (MBAAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), potassium persulfate (KPS), NaCl, KCl, CH_3COONa, $CaCl_2$, NaH_2PO_4, and diclofenac sodium (DF) were supplied from Fluka AG (Buchs, Switzerland). All the other chemicals were from Fluka AG (Buchs, Switzerland). All chemicals were of the highest analytical grade.

2.2. Synthesis of poly(NIPAAm-*co*-NVP)

The synthesis of the linear poly(NIPAAm-*co*-NVP) was performed by free radical co-polymerization in aqueous solution. In a typical example, 1.13 g of NIPAAm (10 mmol) and 107 µl of NVP (1 mmol) were dissolved in 10 ml distilled water; then, nitrogen was bubbled through the solution for 30 min to remove the solubilized oxygen. The polymerization was initialized with 0.020 g KPS and 40 µl TEMED; the reaction lasted for 24 h at room temperature (22±2 ºC). Finally, the copolymer was diluted with

distilled water, dialyzed for one week at room temperature (dialysis bag from Medicell International, England; molecular weight cut off 10 000–12 000 Da), and recovered by freeze-drying.

2.3. Determination of the poly(NIPAAm-*co*-NVP) molecular weight

The values of the number-average (M_n) and weight-average (M_w) molecular weight of poly(NIPAAm-*co*-NVP) were determined by GPC in chloroform at 30 °C and at the flow rate of 1 ml·min^{-1} by using the GPC-PL-EMD 950 instrument from Polymer Laboratories (Shropshire, UK). Calibration was carried out with monodisperse polystyrene standards.

2.4. Determination of the copolymer composition

The composition of the poly(NIPAAm-*co*-NVP) was determined by proton nuclear magnetic resonance (^{1}H-NMR) analysis. ^{1}H-NMR spectra of poly (NIPAAm-*co*-NVP) were recorded in deuterated water on a Varian Mercury Plus 400/Varian VXR 200 spectrometer (Rheinstetten, Germany) operating at 400 MHz frequency.

The co-monomer molar ratio in the poly(NIPAAm-*co*-NVP) copolymer was calculated according to Equations (1) and (2):

$$2X = 1 \tag{1}$$

$$X + Y = A \tag{2}$$

where X and Y are the molar fractions of NVP and NIPAAm, respectively. Equation (1) represents the area of the methylene protons of NVP (6) at 3.28 ppm and Equation (2) describes the peak area (A) at 3.91 ppm of the methynic protons of NVP and NIPAAm (3 and 4).

2.5. Determination of the lower critical solution temperature

The LCST was determined from the dependence of the absorbance at 450 nm on temperature by using an UV-Vis Specord 200 spectrophotometer (Analytic Jena, Jena, Germany) coupled with a temperature controller. The copolymer solution (1%, w/v) was prepared in distilled water and in simulated physiological fluids: 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 °C every 10 min. The LCST was taken as the temperature at which the first signs of turbidity occurred (onset temperature).

2.6. Microsphere preparation

Poly(NIPAAm-*co*-NVP) microspheres were prepared by the w/o suspension polymerization. Typically, 2.26 g of NIPAAm (20 mmol), 214 µl of NVP (2 mmol), and 0.031 g of MBAAm (0.2 mmol) were solubilized in 9 ml of distilled water. The solution was purged with nitrogen for 30 min. The continuous phase was prepared by solubilizing 0.5 g of Span 80 in 250 ml of sunflower oil. Then, the initiator (0.040 g of KPS) and the accelerator (250 µl of TEMED) were added to the monomer solution and immediately dispersed in the continuous phase using a three-blade turbine impeller. The stirring rate and the polymerization temperature were fixed at 450 rpm and 22±2 °C, respectively; the polymerization reaction lasted 10 hours. Lastly, the cross-linked microspheres were washed consecutively with cyclohexane, methanol, water, and acetone; then microspheres were dried from diethyl-ether.

2.7. Morphological and dimensional analysis of microspheres

The size and the morphology of microspheres were assessed by environmental scanning electron microscopy (ESEM, type Quanta 200, Netherlands). The morphological modifications of the microspheres under and above the VPTT were recorded by an optical microscope provided with a digital camera. Before measurements, the microspheres were swollen in PBS and stained with 0.1% (w/v) Congo Red.

2.8. Swelling degree of microspheres

The effect of temperature on the volume of microspheres was determined at equilibrium by setting the microspheres in a graduated cylinder (*i.d.* = 12 mm) filled with PBS. The swelling degree (*SD*) was calculated according to Equation (3):

$$SD = \frac{V_s}{V_d} \tag{3}$$

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

2.9. DSC analysis

Differential scanning calorimetric investigations (DSC Pyris Diamond, Perkin Elmer, Japan) were

carried out on small samples placed in sealed non-hermetic aluminum pans. All measurements were achieved at the heating rate of 10 °C·min^{-1} in an inert atmosphere of nitrogen gas.

2.10. Volume phase transition temperature of microspheres

The VPTT of microspheres corresponds to the inflection point of the *swelling degree-temperature* curve and was determined by Boltzmann fitting of the experimental data [35].

2.11. Swelling and deswelling kinetics of microspheres

The swelling kinetics was investigated by placing the microspheres in a graduated glass cylinder (*i.d.* = 12 mm) containing PBS at the desired temperature (45 °C). Then, the glass cylinder was moved into a water bath at 4 °C and the increase of the microsphere volume was measured at fixed time intervals. The dynamic swelling ratio (q_{ds}) was calculated according to Equation (4) [36]:

$$q_{ds} = \frac{V_t - V_d}{V_{0(45°C)} - V_d} \tag{4}$$

where $V_{0(45\,°C)}$ is the microsphere volume at equilibrium at 45 °C, V_t is the microsphere volume at a given time, and V_d is the microsphere volume in the dried state.

For deswelling kinetics measurements, the microspheres suspension, previously equilibrated at 4 °C, was moved into a water bath at 45 °C. The dynamic deswelling ratio (q_{dd}) was calculated according to Equation (5) [36]:

$$q_{dd} = \frac{V_t - V_d}{V_{0(4°C)} - V_d} \tag{5}$$

where $V_{0(4\,°C)}$ is the volume of microspheres at 4 °C. Swelling kinetics of poly(NIPAAm-*co*-NVP) microgels was analyzed according to the known as Gompertz model ('one phase association'; Equation (6)) [37]:

$$Y = Y_0 + (plateau - Y_0) \cdot (1 - \exp(-k \cdot t)) \tag{6}$$

where Y_0 is the dynamic swelling ratio at 45 °C, t is the time [min] and *plateau* is the asymptote of the dynamic swelling ratio at 4 °C.

Collapsing kinetics of poly(NIPAAm-*co*-NVP) microgels where analyzed according to the the known as Gompertz model ('one phase dissociation'; Equation (7)) [37]:

$$Y = (Y_0 - plateau) \cdot \exp(-k \cdot t) + plateau \tag{7}$$

where Y_0 is the dynamic collapsing ratio at 4 °C, t is the time [min] and *plateau* is the asymptote of the dynamic swelling ratio at 45 °C.

All the experiments were performed in triplicate under simulated physiological conditions (PBS at pH = 7.4).

2.12. DF loading

DF-loaded microspheres were prepared in a vial (20 ml) by immersing 200 mg of dried microspheres in 16 ml solution of DF dissolved in ethanol (1.25 and 2.5 mg·ml^{-1}). After complete evaporation of the solvent at 60±2 °C, the microspheres were washed with diethyl ether and dried under vacuum at 40 °C. The washing solvent was evaporated and the residual drug was solubilized in ethanol; this solution contained also the drug that was not incorporated in the microspheres and sticked on the vial walls. The amount of the retained drug per mg of dried microspheres was determined by difference between the initial amount of the drug and the residual drug. The residual drug, dissolved in ethanol, was quantified by UV–Vis spectroscopy, using a previously made calibration curve. The entrapment efficiency (E) was calculated according to Equation (8):

$$E\,[\%] = \frac{Q_r}{Q_t} \tag{8}$$

where Q_r is the effective amount of the drug entrapped in the microspheres, and Q_t is the theoretical amount of the drug present in the microspheres.

2.13. *In vitro* DF release kinetics

In vitro kinetic studies of DF release were performed below and above the VPTT, by immersing 50 mg of loaded microspheres in 100 ml of PBS, under gentle stirring. At fixed time intervals, 1 ml of the release medium was taken and the DF content was determined spectrophotometrically. When the temperature was cyclically changed, samples were taken every 2 for 10 minutes at a given temperature after which the temperature changed and the operation was repeated in a similar manner. After collecting 5 samples, 5 ml of the fresh buffer were added to replace the volume of the removed samples.

DF release from poly(NIPAAm-*co*-NVP) microgels was analyzed according to the Korsmeyer-Peppas model (Equation (9)) [33]:

$$\log\left(\frac{M_t}{M_\infty}\right) = \log k + n \cdot \log t \quad (9)$$

where M_t is the amount of drug released in time t, M_∞ is the amount of drug released after time ∞, n is the diffusional exponent or drug release exponent, and k is the Korsmeyer release rate constant.

The n value is used to characterize the following different release mechanisms:

$n = 0.43$	Fickian transport
$0.43 < n < 0.85$	Non-Fickian transport
$n = 0.85$	Case II transport
$n > 0.85$	Super case II transport

2.14. Statistical analysis

All 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.

3. Results and discussions

3.1. Preparation and characterization of the thermo-responsive linear copolymer

In most cases, the biomedical applications of thermosensitive polymers imply those polymers that exhibit a fast phase transition at a temperature close to that of the human body. Poly(NIPAAm) is the most used thermoresponsive polymer because it possesses a sharp phase transition (LCST) at 32 ºC [15]. To increase the transition temperature to a value close to that of the human body, NIPAAm is usually copolymerized with hydrophilic monomers [18]. Here, NVP was chosen as a hydrophilic monomer to be copolymerized with NIPAAm. The ^{1}H-NMR analysis (Figure 1) settles the copolymer formation and indicates that the percentage of co-monomers present in the copolymer corresponds approximately to that in the feed (Table 1).

As shown in Table 1, upon increasing the amount of NVP in the initial mixture and therefore in the copolymer, the LCST value increases both in simulated physiological conditions (at pH = 7.4 and 1.2) and in distilled water. The LCST is lower at pH = 7.4 than at pH = 1.2, and both values are lower than that in distilled water. These differences reflect the co-influence of the ionic strength of the solutions as well as the pH since the co-monomers possess weak ionisable groups. Therefore, the LCST is higher at pH = 1.2 than at pH = 7.4, even though the ionic strength of the simulated gastric fluid is greater ($I_{1.2}$ = 114 mM and $I_{7.4}$ = 89 mM). In order to elucidate the influence of ionic strength as well as of the anion or cation species of the salts, different salts at different ionic strengths were investigated. As shown in Table 2, LCST increases on decreasing the ionic strength, the highest values have been obtained in distilled water (I = 0).

In general, the addition of an electrolyte induce flocculation of the aqueous colloidal dispersions; this phenomenon is called the salting out effect. A comparable effect occurs in the case of thermosensitive polymers. Dissolution of poly(NIPAAm) supposes the presence of water molecules around the NIPAAm sequences in copolymer, generating a hydration layer. In the presence of salts, the linkages between highly oriented water molecules and NIPAAm are disrupted and the polymer becomes hydrophobic and precipitates. According to the type and the concentration of

Table 1. Effect of the co-monomer ratio in the feed and in the copolymer on the LCST (the copolymer concentration was 1 %, w/v).

Sample code	Co-monomer composition				LCST [°C]		
	In the feed ·10^{-3} M [% mol ratio]		In copolymer [% mol ratio]		pH = 7.4	pH = 1.2	pH = 5.5 (H_2O)
	NIPAAm	NVP	NIPAAm	NVP			
S_0[a]	10	0	100	0	28.4±0.1	31.5±0.2	32.5±0.2
S_1[b]	10 (90.9)	1 (9.1)	91.5	8.5	36.0±0.2	37.3±0.3	38.4±0.1
S_2[c]	10 (87.7)	1.4 (12.3)	87.5	12.5	37.5±0.2	39.5±0.2	40.5±0.1
S_3[d]	10 (83.3)	2 (16.7)	84.0	16.0	39.4±0.3	41.6±0.3	42.4±0.2
S_4[e]	10 (80.0)	2.5 (20.0)	79.0	21.0	41.2±0.4	43.1±0.4	44.0±0.3

Data are the results of two independent experiments.

[a]LCST values for S_0 are from Ref [29] [b]M_n = 31 740, M_w = 55 460, IP =1.75; [c]M_n = 33 600, M_w = 89 260, IP = 2.66; [d]M_n = 46 450, M_w = 81 170, IP = 1.75; [e]M_n = 37 760, M_w = 83 960, IP = 2.22; IP, index of polydispersity.

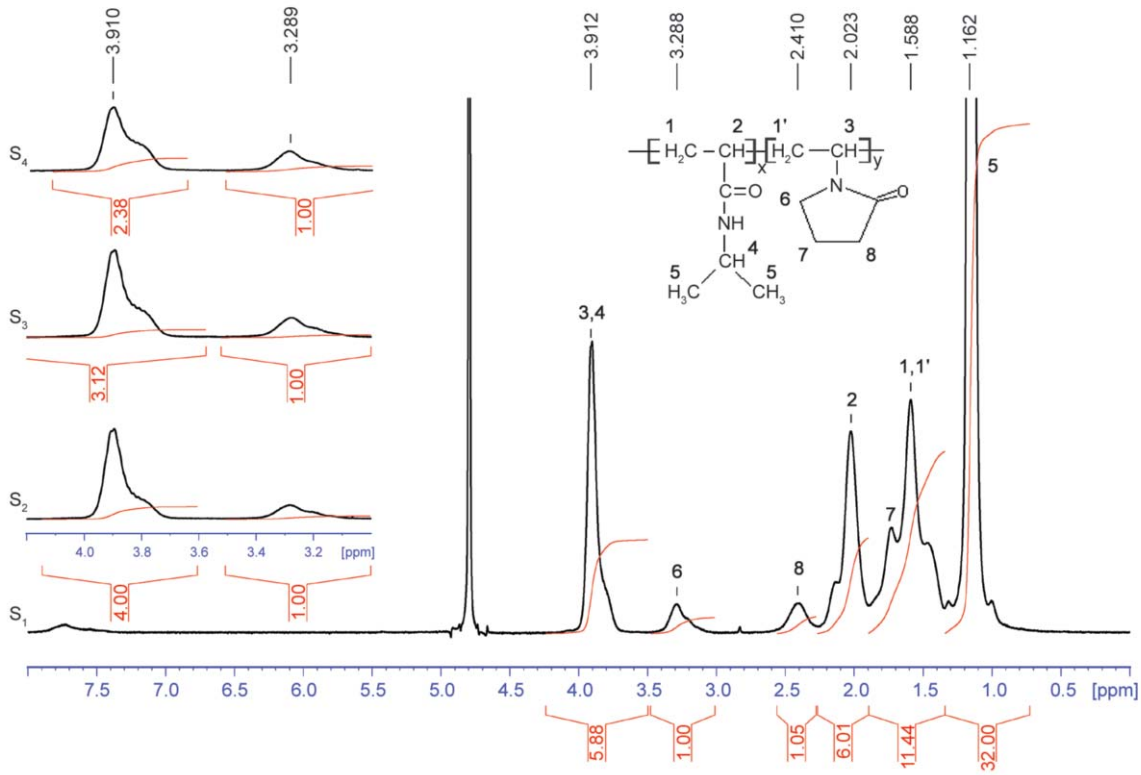

Figure 1. ^{1}H-NMR spectrum of poly(NIPAAm-*co*-NVP) (sample S_1 in Table 1). For samples S_2, S_3, and S_4, only the portions of the graph used to calculate the composition are shown.

Table 2. Influence of the ionic strength and of ion species on LCST (determinations were performed on sample S_1 in Table 1)

Ionic strength / Salt	LCST [°C]					
	1 M	**0.5 M**	**0.2 M**	**0.1 M**	**0.05 M**	**0.0 M (H_2O)**
NaCl	26±0.3	32±0.3	35±0.2	37±0.2	37.5±0.2	38.4±0.2
KCl	26±0.2	32±0.2	35±0.3	37±0.2	37.3±0.1	–
CH_3COONa	20±0.3	29±0.2	34±0.3	35.5±0.3	37±0.3	–
$CaCl_2$	25±0.2	31±0.1	35±0.2	36±0.3	37±0.4	–
NaH_2PO_4	Not soluble	19±0.1	31±0.2	35±0.2	35.8±0.2	–

ions, the hydration layer will be more or less destructured [38, 39].

Changing monovalent cation, i.e. Na^+ (ionic radius = 1.02 Å) with K^+ (ionic radius = 1.38 Å), does not alter the value of LCST as long as the volume of ions are not very different. When the role of the bivalent cation Ca^{2+} (ionic radius = 1.0 Å) was considered, LCST slightly decreased. Dramatic changes of LCST were observed when the monovalent anion Cl^- was replaced with complex species such as $H_2PO_4^-$ or even CH_3COO^-. Thus, LCST decreases from 32 °C in the presence of 0.5 M NaCl to 19 °C in the presence of 0.5 M NaH_2PO_4. Moreover, in 1 M NaH_2PO_4 the copolymer is no longer soluble.

Therefore, while the valence of ion has little effect, the size of the ion seems to influence dramatically the LCST.

Lastly, data shown in Table 1 indicate that the copolymer with a co-monomer molar ratio of 91.5:8.5 (NIPAAm:NVP) displays LCST values of 36.0 °C and 37.3 °C at pH = 7.4 and 1.2, respectively (i.e., under simulated physiological conditions).

3.2. Phase transition characterization of the linear copolymer

One of the most important characteristics of a thermosensitive polymer planned for biomedical applications is to display a phase transition around the human body temperature in a narrow temperature range. The cloud point is the method used in this study to determine the LCST which is taken as the temperature when first opalescence appears in the polymer aqueous solution. In fact, the values given in Table 1 do not indicate the sharpness of the phase

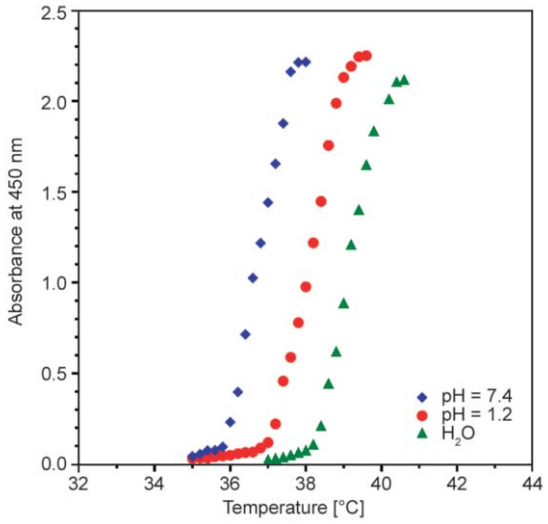

Figure 2. LCST profiles of poly(NIPAAm-*co*-NVP) (sample S_1 in Table 1) under simulated physiological conditions (i.e., at pH = 7.4 and 1.2) and in distilled water.

transition and therefore the allure of the curves are represented in Figure 2.

As shown in Figure 2, the phase transitions occur within an interval of two Celsius degrees both under physiological conditions at pH = 7.4 and 1.2; therefore this copolymer appears to be suitable for biomedical applications.

3.3. Microsphere preparation and characterization

For most of the biomedical and biotechnological applications, the thermoresponsive hydrogels should possess high rates of swelling and deswelling at the body temperature. The rate of volume change depends to the hydrogel size: the smaller is the diameter, the higher are the swelling and collapsing rates. Only few data are available for the preparation of poly(*N*-isopropylacrylamide)-based microspheres by the two phases suspension cross-linking polymerization [26] since this polymerization reaction is inhibited by oxygen dissolved both in the monomer solution and in the dispersion medium. Suspension polymerization allows the microsphere size control by varying the rate of dispersion and the viscosity of both phases. To optimize the microsphere synthesis, both aqueous and oil phases were long-time purged with nitrogen and the initiator and accelerator were added in the polymerization solution immediately before dispersion. Thus, small and discrete microspheres were synthesized as shown in Figure 3a.

To analyze the morphology of the microspheres in their swollen state, they were frozen with liquid nitrogen and then lyophilized. As shown in Figure 3b, microspheres display a porous structure in their swollen state. To demonstrate that the cross-linked microspheres preserve the thermosensitive properties of the linear copolymer, the effect of temperature on swelling degree was investigated under simulated physiological conditions (PBS at pH = 7.4). As shown in Figure 4, the swelling degree slowly decreases with temperature. Nevertheless, near the LCST the swelling degree decreases dramatically and the swelling ratio between the swollen and dried microspheres reaches

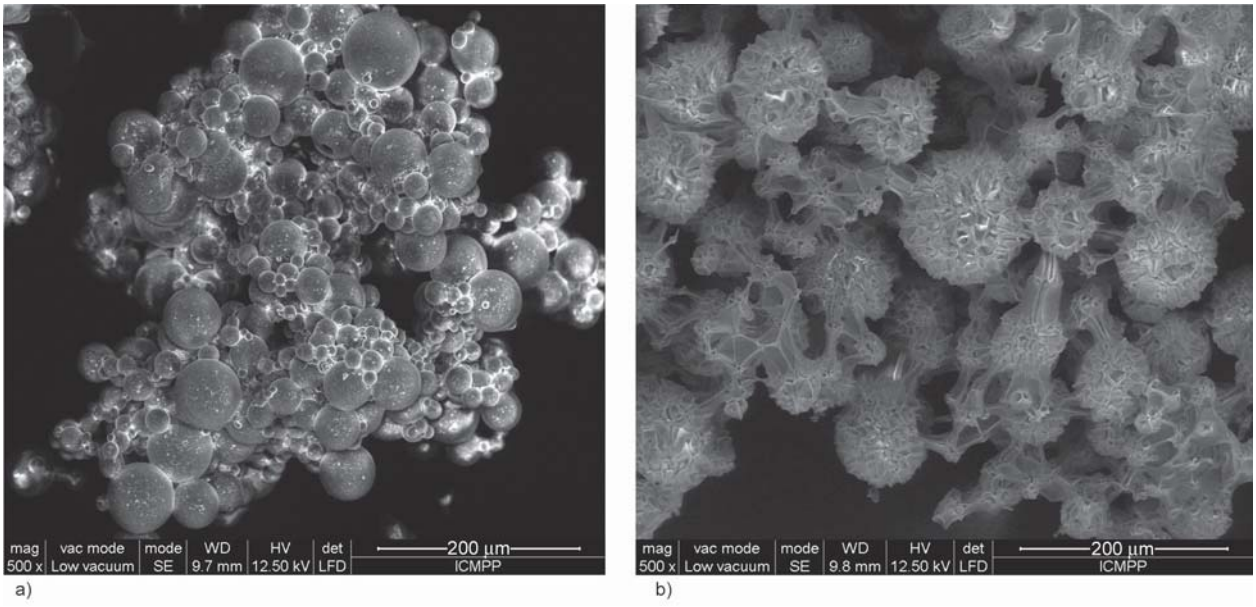

Figure 3. Environmental scanning electron micrographs of poly(NIPAAm-*co*-NVP) microspheres dried by progressive removal of water with organic solvents (a) and by lyophilization (b).

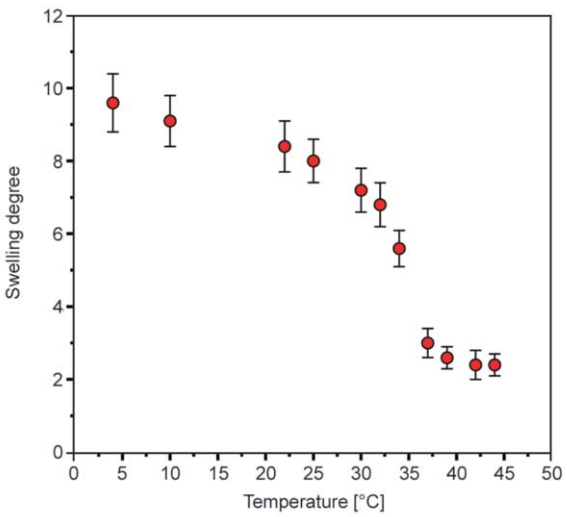

Figure 4. Effect of temperature on the swelling degree of poly(NIPAAm-*co*-NVP) microspheres. Data were obtained in PBS at pH = 7.4 and are the results of three independent experiments.

a very low value (= 2.6) at 39 °C. The VPTT value (= 33.6 °C) of cross-linked microspheres determined by the Boltzmann fitting of the experimental data is slightly lower than LCST of the linear copolymer. This discrepancy may be the result of the different approach to determine the transition temperature. In fact, LCST is taken as the onset temperature while VPTT is taken as the inflection point in the swelling degree-temperature curve.

Moreover, pictures of the microspheres in the swollen state below and above the LCST were taken (Figure 5), stressing the thermosensitive properties of cross-linked particles.

One of the most important characteristics of thermoresponsive hydrogels planned for biomedical applications is to possess high swelling/collapsing rates in biological fluids. The swelling and collapsing rates depend to the water diffusion in and out of the hydrogel. Water diffusion rates depend on the size and porosity of the hydrogel: the smaller is the size and the higher is the porosity the faster is the response rate. Taking into account the micrometric size and porous structure (Figure 3b) of poly(NIPAAm-*co*-NVP) microspheres, the swelling process takes place very rapidly (Figure 6a) reaching equilibrium within 100 seconds. The collapsing process is faster and the equilibrium is reached in almost 50 seconds (Figure 6b). The collapsing rate is higher than the swelling rate (values of k being 0.058 and 0.027 sec^{-1}, respectively), because during shrinkage the water is expelled mechanically from the microspheres, a process that is faster than the solvent diffusion within the microspheres. In fact, the collapsing and swelling are almost instantaneous processes, the lag time (= 15 sec; arrows) corresponds to the time interval needed to change the sample temperature from 45 to 4 °C (Figure 6a) and from 4 to 45 °C (Figure 6b). Data shown in Figure 6 indicate that the swelling and collapsing kinetics of poly(NIPAAm-*co*-NVP) microgels are mono-exponential processes that can be ascribed to the simplest mechanism known as Gompertz model

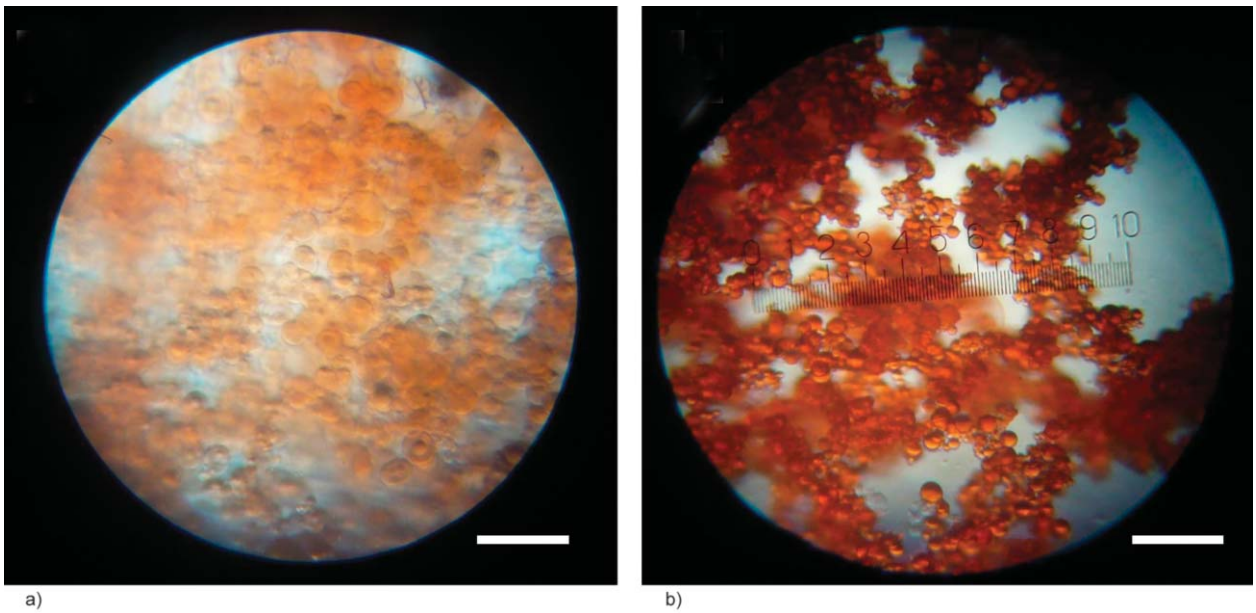

Figure 5. Optical photomicrographs of stained poly(NIPAAm-*co*-NVP) microspheres in the swollen state in PBS at pH = 7.4 below (a) and above (b) the LCST. The bars correspond to 250 μm.

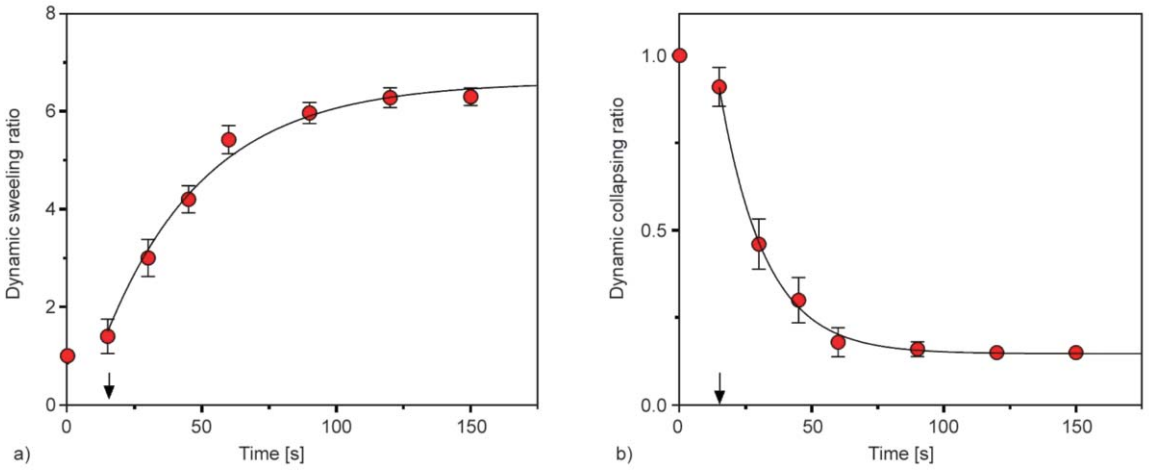

Figure 6. Swelling (a) and collapsing (b) kinetics of poly(NIPAAm-*co*-NVP) microgels (PBS at pH = 7.4). The continuous lines were calculated according to Equations (6) (a) and (7) (b) with the following parameters: (a) Y_0 = 1.4, *Plateau* = 5.2 and k = 0.027 sec^{-1}, and (b) Y_0 = 0.76, *Plateau* = 0.15 and k = 0.058 sec^{-1}.v

[37]. Moreover, the relative high value of *Plateau* for swelling (= 5.2) as well as the low value of *Plateau* for collapsing (= 0.15) indicate that microspheres are very swellable and compressible, their volume in the collapsed state being close to that in the dried state.

3.4. Loading and release studies

The loading process was performed in ethanol since DF is soluble in this solvent and the microspheres display a high degree of swelling ($SD_{ethanol} = V_s/V_d$ = 17.4). Moreover, ethanol is a volatile solvent that can be removed easily from the microspheres. By a progressive evaporation of the solvent, the drug is forced to diffuse within the microspheres. The percentage of the entrapped drug increases from 7.62% (w/w) (E = 83.8%), when the initial drug concentration is 1.25 mg·ml^{-1}, to 13.08% (w/w) (E = 78.5%) when the initial drug concentration is 2.5 mg·ml^{-1}. The high degree of entrapment could also reflect the ability of vinylpyrrolidone to form complexes with H-donor compounds such as the carboxylic groups of DF [40]. DSC measurements give important information on drug dispersion in the polymer matrix (Figure 7). The thermogram of pure DF (Figure 7 curve a) shows a sharp peak at 290 °C which corresponds to the melting point of the drug in the crystalline form. The empty microspheres do not show any peak in the region of the DF melting point (Figure 7 curve b). For drug loaded microspheres, the thermograms demonstrate that DF forms small microdomains within the polymeric network. Broad peaks at around 260 °C, resulting from the melting of DF crystals, appear for either the low (7.62%, w/w) (Figure 7 curve c) or high (13.08%, w/w) loading degree microspheres (Figure 7 curve d).

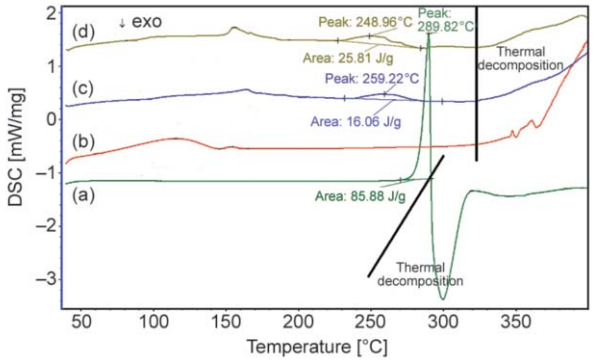

Figure 7. DSC thermograms of pure DF (a), empty poly (NIPAAm-*co*-NVP) microspheres (b), and loaded microspheres with DF: 7.62% (c) and 13.08% (w/w) (d). Scanning rate: 10 °C·min^{-1}.

Broad temperature ranges of melting accompanied by quite low phase transition thermal effects frequently indicate that the polymer crystallites are different in size and perfectness, thus resulting different but close melting temperatures. In fact, the small and irregular crystallites with different degree of perfectness induce broad melting DSC peaks. It has been also suggested that the miscibility of the drug with a polymer could lead to the melting point depression of drug crystals embedded in the polymer matrix [41]. The temperature as well as the dispersion of molecular DF or drug crystallites within the polymer matrix influences the release profiles of DF from polymeric microspheres. Therefore, release studies were performed under simulated physiological conditions (PBS at pH = 7.4) and temperatures situated below (32 °C) and above (38 °C) the VPTT (Figure 8).

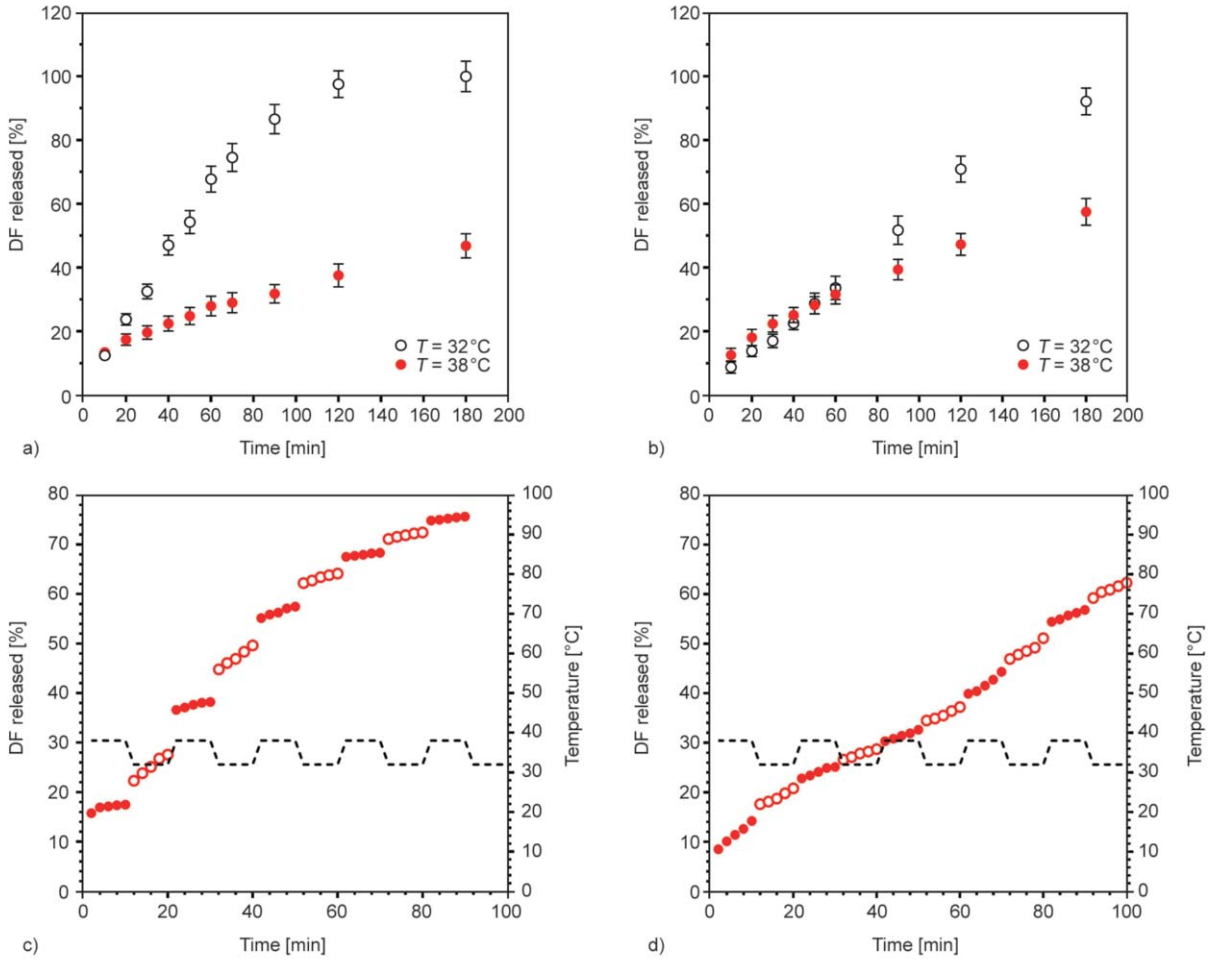

Figure 8. Effect of temperature (a and b) and temperature cycling (c and d) on DF release profiles from poly(NIPAAm-*co*-NVP) microspheres. The release studies were performed in PBS at pH = 7.4, using microspheres with 7.62% (w/w) DF (a and c) and with 13.08% (w/w) DF (b and d).

Microspheres with a low loading degree (7.62%), display a substantially difference between the DF release rate at temperatures situated below and above the VPTT (Figure 8a). In fact, below the VPTT, the microspheres are in the swollen state and are highly hydrophilic, thus facilitating the DF release. Above the VPTT, the microspheres are in the collapsed state and are more hydrophobic, impairing the drug diffusion. As shown in Figure 8b, the release profiles of DF from microspheres with high loading degree (13.08%) at temperatures lower and higher the VPTT are almost superimposable for the first 90 min; then, they diverge, the higher release rate being that observed at the lower temperature. For microspheres with a high loading degree, a larger fraction of the drug is on the surface of the microspheres, therefore the release rate in the first stage is not influenced by the degree of either swelling or collapse of the polymeric network. In addition, a high drug loading degree (more crystals) induces a reduced flexibility of the macromolecules and therefore the collapsing process is hampered, the polymeric network is permissive and the drug is easily released. After dissolution and diffusion of the drug from the surface, the influence of the temperature on the DF release is more pronounced. The increase of temperature has two opposite effects. In fact, the temperature: (i) increases the dissolution rate of the crystallized DF and diffusion rate of each drug molecules, and (ii) decreases the diffusion rate of free drug because induces the collapse of the polymeric network. Thus, the release of the drug is influenced by the balance between these parameters. The microspheres characterized by a large difference between release rates at temperatures situated below and above the VPTT are appropriate supports for achieving pulsatile drug delivery. Therefore, the microspheres with low loading degree (7.62%) have been incubated in PBS at pH =7.4, and the temperature was cyclically changed from below to above the VPTT and vice versa. Notably, the DF is

released in a pulsatile manner: above the VPTT almost no drug is released because of the collapsed network while below the VPTT the drug is released in a substantially amount (Figure 8c). However, it must be underlined that a large amount of the drug is released during the network collapsing when the temperature changes from below to above the VPTT. Actually, the drug is expelled mechanically from the hydrogel. The release profiles of DF from microspheres with high drug loading, during cyclical modification of temperature (below and above the VPTT), are presented in Figure 8d. In the first 10 min. a large amount of drug is released even though the temperature is situated above the VPTT (collapsed microspheres). This drug is supposed to be that located on the surface of microspheres. Then, the release rates are almost the same at temperatures both below and above the VPTT until 50 min. The lack of difference between the release rates is due to a cumulus of factors with opposite effects previously mentioned. Particularly, the presence of high amount of drugs within microspheres as small crystals hampers a complete collapse of polymeric network and therefore the drug diffusion rate is not enough reduced. Moreover, after 60 min., when a fraction of crystals dissolved, there is a minor difference between the release rates at the two temperatures and the appearance of a slight pulsating effect when the temperature changes from 32 to 38 °C (network collapse and mechanical expulsion of the drug). In conclusion, the smaller the amount of drug in the microspheres, the greater the difference between the release rates of drug at the two temperatures.

In order to understand the preponderance of different factors on release mechanism, experimental drug release data was fitted with Korsmeyer-Peppas model (Table 3).

The Korsmeyer-Peppas model is a simple power law model that can explain mixed release mechanisms associated to a variety of factors such as swelling of polymers, matrix porosity and drug diffusion rates in swelling systems. All profiles exhibited good fitting with this model as long as they are characterized by high values of the correlation coefficients (R^2), which were determined from the linear regression analysis of the release data of samples.

Table 3. Release parameters obtained using Korsmeyer-Peppas model.

DF [%]	Temperature [°C]	Korsmeyer-Peppas: $Q = kt^n$		
		K	n	R^2
7.62	32	1.45	0.93	0.997
	38	4.76	0.43	0.991
13.08	32	3.64	0.53	0.998
	38	0.74	0.94	0.988

For low loading degree microspheres (7.62%) the release exponent (n) from Korsmeyer-Peppas equation is 0.93 and 0.43 for release studies performed at 32 and 38 °C, respectively. As follows, the release mechanism corresponds to the Super case II transport at a temperature situated below the VPTT when DF release is controlled by diffusion and swelling process. Above the VPTT, the polymeric microspheres are in collapsed state and therefore the release of DF is controlled only by diffusion (n = 0.43). For high loading degree microspheres (13.08%), the release exponent is 0.53 and 0.94 for release studies performed at 32 and 38 °C, respectively, leading to the conclusion that the release mechanism corresponds to the non-Fickian transport and Super case II transport. Indeed, for high loading degree microspheres, the swelling and deswelling processes of polymeric network are obstructed, moreover the presence and distribution of drug crystals as well as their dissolution deeply influenced the release profiles.

4. Conclusions

Poly(NIPAAm-*co*-NVP) was synthesized as an innovative copolymer that possesses, under simulated physiological conditions (PBS at pH = 7.4), a sharp phase transition at the human body temperature. The decrease of LCST with the increase of ionic strength of the copolymer solution was proven for all studied salts. However, if the change of monovalent cation Na^+ with K^+ does not alter the value of LCST, when the monovalent anion Cl^- was replaced with $H_2PO_4^-$ or even CH_3COO^-, substantial changes of the LCST values are observed.

Discrete poly(NIPAAm-*co*-NVP) microspheres with thermoresponsive properties were synthesized by suspension polymerization of the monomers upon extensive purging of nitrogen both in the dispersed and continuous phase. DF was incorporated in the microspheres as small crystalline microdomains since drug inclusion by the solvent evaporation method induces a progressive uptake of the drug within polymer network until drying. Pronounced differences of the release rates at temperatures below and above the VPTT were obtained for low loading microspheres (7.62%). On the opposite, this difference

is substantially reduced for high loading microspheres because: (i) the presence of the drug on the microsphere surface could annul the effect of temperature, and (ii) a high drug loading degree (i.e., the abundance of drug crystals) reduces the flexibility of the polymer chains and therefore the microsphere collapse, which is responsible for slowing down the rate of drug release. Therefore, only low loading microspheres are able to ensure a pulsatile release mechanism when the temperature is cyclically modified and appear to be suitable for biomedical applications.

Abbreviations

DF	diclofenac
DSC	differential scanning calorimetry
ESEM	environmental scanning electron microscopy
IP	index of polydispersity
KPS	potassium persulfate
LCST	lower critical solution temperature
MBAAm	*N*,*N*-methylene bisacrylamide
M_n	number-average molecular weight
M_w	weight-average molecular weight
NIPAAm	*N*-isopropylacrylamide
NVP	*N*-vinylpyrrolidone
PBS	phosphate buffer solution
TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
VPTT	volume phase transition temperature

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