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Fast-responsive porous thermoresponsive microspheres for controlled delivery of macromolecules

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ABSTRACT

Porous thermoresponsive microspheres with a homogeneous dimension and distribution of the pores are synthesized by an original method. Poly(N-isopropylacrylamide-co-acrylamide) (poly(NIPAAm-co-AAm)) copolymer was obtained as a thermoresponsive material with a lower critical solution temperature (LCST) under physiologic-like conditions (*i.e.*, at 37 °C and pH 7.4, 50 mM phosphate buffer). Semitelechelic oligomers of NIPAAm (ONIPAAm) were also synthesized in the presence of 3-mercaptopropionic acid (MPA) (chain transfer molecule) which acts as a pore-forming agent. Poly(NIPAAm-co-AAm) and ONIPAAm were solubilized in acidified aqueous solution, dispersed in a mineral oil, and transformed in stable microspheres by crosslinking the amide group with glutaraldehyde at temperatures below and above the LCST of the oligomers, and always below the LCST of the polymer. Microspheres obtained at temperatures below the LCST of ONIPAAm are characterized by a homogeneous porous structure with a narrow distribution of the pore size. These microspheres are characterized by a very rapid response rate when the temperature changes below and above the body temperature. The higher is the amount of the porogen in the polymer solution, the larger is the pore size and faster is the response rate. The porous microspheres with suitable pore size are a conveyable matrix for loading and temperature-controlled release of the high molecular weight model drug blue dextran (BD).

1. Introduction

Poly(N-isopropylacrylamide) (poly(NIPAAm)) is the most popular thermoresponsive polymer since it exhibits a sharp phase transition around 32 °C (Heskins and Guillet, 1968; Priest et al., 1986). The temperature at which this transition occurs is called the lower critical solution temperature (LCST). Below the LCST the polymer chain is hydrated and adopts an extended coil conformation, while above the LCST the polymer is dehydrated and adopts a globular conformation. Correspondingly, the crosslinked hydrogels obtained from these polymers swell under the LCST and shrink above the LCST (Zhang and Zhuo, 2000; Yildiz et al., 2002a). The biomedical and biological applications of the gels usually involve the chemical modifications of the poly(NIPAAm) gels. These modifications are usually performed with the aim: (i) to introduce some functional groups able to increase the LCST towards body temperature and (ii) to produce networks with rapid swelling/deswelling kinetics.

Usually, the LCST is increased towards the body temperature by copolymerization of NIPAAm with small amounts of hydrophilic monomers (Liu et al., 2004; Khan, 2007). However, conventional thermoresponsive hydrogels are limited for practical applications because of their slow swelling and deswelling rates (Park and Hoffman, 1992; Park, 1999). Basically, the swelling and deswelling of conventional hydrogels is the results of solvent diffusion and is a slow process depending on the gel size (Tanaka and Fillmore, 1979; Sato-Matsuo and Tanaka, 1988). The smaller is the size of the hydrogel, the faster is the response rate to the input signal. In addition, the so-called "skin effect" significantly decreases the deswelling of large hydrogels because a surface layer of the sample deswells, becomes hydrophobic and less permeable ("skin") for solvent transport, and thus additionally hinders further deswelling (Strachotová et al., 2007).

The generation of a porous structure is known to be a key solution to avoid the "skin effect" in hydrogels and to promote their fast volume changes in response to temperature modifications.

Abbreviations: AAm, acrylamide; AIBN, N,N′-azobisisobutyronitrile; BD, blue dextran; CP, cloud point; D.C., degree of crosslinking; GA, glutaraldehyde; LCST, lower critical solution temperature; MPA, 3-mercaptopropionic acid; NIPAAm, N-isopropylacrylamide; ONIPAAm, oligomers of NIPAAm; PB, phosphate buffer at pH 7.4; poly(NIPAAm-co-AAm), poly(N-isopropylacrylamide-co-acrylamide); SEM, scanning electron microscopy; VPTT, volume phase transition temperature.

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Porous structures allow the water to be expelled or absorbed by convection, a much faster process than by diffusion. Several reports in the literature describe the preparation of porous hydrogels. Recently, porous PNIPAAm hydrogels were prepared by an emulsion templating model. Accordingly, hydrogels are synthesized in an oil-in-water emulsion by free radical copolymerization with a crosslinker, followed by removal of the dispersed oil used as the pore template (Tokuyama and Kanehara, 2007). Macroporous PNIPAAm hydrogels were also synthesized in aqueous solution at temperatures above the LCST, under these conditions the polymer phase separates as it is formed. Then, crosslinking between the aggregated polymer chains produced an heterogeneous macroporous structure in the thermally reversible hydrogel (Yan and Hoffman, 1995). Macroporous hydrogels were also obtained by using hydroxypropyl cellulose as a pore-forming agent. Such a hydrogel responds to temperature changes much faster than hydrogels prepared without using pore-forming agents. However, the polymerization temperature was above LCST and the macroporous hydrogel had a heterogeneous structure (Wu et al., 1992).

The most part of porous thermoresponsive hydrogels reported in literature are prepared as large devices such as discs or cylinders with a diameter of several millimeters (*e.g.*, 10 mm) (Xue et al., 2002; Asoh et al., 2006). The preparation of small microspheres with a relative fast swelling/deswelling rate is also reported but they do not display a porous structure (D'Emanuele and Dinarvand, 1995; Fundueanu et al., 2005).

Here, we report the preparation and the characterization of porous thermoresponsive microspheres with a very fast response rate to temperature changes. In addition, the microspheres display a very homogeneous porous structure. The preparation of porous thermoresponsive microspheres was performed by chemically crosslinking of preformed poly(N-isopropylacrylamide-co-acrylamide) (poly(NIPAAm-co-AAm)) in the presence of oligomers of NIPAAm acting as the pore-forming agent. The crosslinking was performed in water-in-oil suspension at temperatures below and above the LCST of the oligomers, and below the LCST of the thermoresponsive polymer. The loading and release of blue dextran (BD), taken as a high molecular weight drug model, were investigated.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm), obtained from Aldrich Chemical Corp. (Milwaukee, WI, USA), was recrystallized with hexane. Acrylamide (AAm), 1,4-dioxane, glutaraldehyde (GA) aqueous solution (25%, w/v), 3-mercaptopropionic acid (MPA), and N,N′-azobisisobutyronitrile (AIBN) were supplied from Fluka AG (Buchs, Switzerland). AIBN and 1,4-dioxane were purified in methanol and by refluxing, respectively. Blue dextran (BD) was provided from Pharmacia (Uppsala, Sweden). Light mineral oil (d = 0.84 g/ml) was supplied by Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of the highest analytical grade and used without purification unless stated.

2.2. Synthesis of poly(NIPAAm-co-AAm)

Synthesis of linear poly(NIPAAm-co-AAm) was carried out by free radical copolymerization in 1,4-dioxane using AIBN as the initiator. Typically, 1.13 g NIPAAm (10 mmol), 0.142 g AAm (2 mmol), and 0.010 g AIBN (0.06 mmol) were solubilized in 10 ml 1,4-dioxane. Dried nitrogen was bubbled through the solution for 30 min prior to polymerization. The reaction mixture was allowed to react at 70 °C for 10 h. The polymer was precipitated into diethyl ether, and dried under vacuum. Finally, the copolymer was solubilized in distilled water, dialysed for 5 days at 20 °C, and recovered by freeze-drying.

2.3. Synthesis of semitelechelic NIPAAm oligomers (ONIPAAm)

Semitelechelic NIPAAm oligomers (ONIPAAm) were prepared as follows. Typically, 1.13 g NIPAAm (10 mmol), 43.5 μl MPA (0.5 mmol), and 0.050 g AIBN (0.3 mmol) were solubilized in 6 ml 1,4-dioxane. Dried nitrogen was bubbled through the solution for 30 min prior to polymerization. The reaction mixture was allowed to react at 80 °C for 16 h. The polymer was precipitated into diethyl ether, and dried under vacuum.

2.4. Determination of poly(NIPAAm-co-AAm) and ONIPAAm molecular weight

The number-average (M_n) and weight-average (M_w) molecular weight of poly(NIPAAm-co-AAm) was determined by GPC using the GPC-PL-EMD 950 instrument (Polymer Laboratories, Shropsire, UK) in dimethylformamide at 120 °C and at a flow rate of 0.7 ml/min. Calibration was carried out with monodisperse polystyrene standards.

The molecular weight of oligomers was determined by end-group titration with 0.01 N NaOH in the presence of phenolphthalein, at 22 °C. Accordingly, the carboxyl groups at the end of oligomers were determined. The molecular weight was calculated according to Eq. (1):

$$M_n\,(\mathrm{g/mol}) = \frac{1}{Cs} \times 1000 \qquad (1)$$

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

2.5. Copolymer composition

The copolymer composition was determined by ^{1}H NMR analysis. ^{1}H NMR spectra of poly(NIPAAm-co-AAm) were recorded in deuterated DMSO on a Varian Mercury Plus 400/Varian VXR 200 spectrometer operating at 400 MHz frequency. The molar fraction of AAm in poly(NIPAAm-co-AAm) was calculated according to Eq. (2):

$$3\,\mathrm{AAm} = (3\,\mathrm{AAm} + 3\,\mathrm{NIPAAm} + 6\,\mathrm{NIPAAm}) - Y\,9\,\mathrm{NIPAAm} \qquad (2)$$

where *Y* is the molar fraction of NIPAAm calculated as the area of the methynic proton at 3.88 ppm, and (3 AAm + 3 NIPAAm + 6 NIPAAm) is the total area of the peaks between 0.5 and 2.3 ppm, corresponding to the main backbone protons (3 AAm + 3 NIPAAm) plus the NIPAAm methylenic protons (6 NIPAAm).

2.6. LCST determination

LCST was determined from the dependence of absorbance at 450 nm on temperature using an UV–vis spectrophotometer (Specord 200, Analytic Jena, Jena, Germany) coupled with a temperature controller. The polymer solution was prepared under standard acidic solution (pH 1.2, 64 mM HCl + 50 mM KCl), standard phosphate buffer (pH 7.4, 50 mM Na_2HPO_4 + NaOH) (PB), and in simulated preparation conditions. The heating rate was 2 °C every 10 min and 0.2 °C in the vicinity of cloud point (CP). CP was defined as the temperature at 10% absorbance in the curve of the normalized absorbance versus temperature.

2.7. Microsphere preparation

Microspheres were obtained as follows. Typically, 0.6 g poly(NIPAAm-co-AAm) and 0.1 g ONIPAAm were solubilized in 4 ml

distilled water, at 4 °C. 0.2 ml H_2SO_4 (0.5 M) and 0.5 ml glutaraldehyde (GA) solution (25%, w/v) were added just before dispersion of the aqueous copolymer solution in the dispersion phase. The continuous phase consists in 50 ml light mineral oil in which 0.25 g soybean lecithin, as the dispersing agent, was added. The reactor consists in an open cylindrical vessel (h = 8 cm, i.d. = 8 cm) with a round bottom. The mixture was stirred at 350 rpm by a three-blade turbine impeller. The reaction temperature was fixed below (28 °C) and above (34 °C) the LCST of ONIPAAm, and the reaction time was 48 h. Finally, crosslinked microspheres were washed successively with diethyl ether, methanol, water, and methanol, and dried from diethyl ether. The microspheres were immersed in distilled water for 5 days, at 4 °C, to remove all the un-crosslinked polymers and oligomers.

2.8. Morphological and dimensional analysis

The morphology of the microspheres was evaluated by optical and scanning electron microscopy (SEM). Microsphere size was determined on SEM photomicrographs, considering at least 100 microspheres for each sample.

2.9. Wrack density

The wrack density was determined by weighting the volume of 1 ml of microspheres measured with a graduated cylinder (i.d. = 12 mm).

2.10. Phosphate buffer retention

The amount of phosphate buffer pH 7.4 (PB) retained in the microsphere network was determined by the centrifuge method (Pepper et al., 1952). Briefly, 100 mg microspheres were introduced into a glass filter-tube fitted with a sintered glass disc. The tube and microspheres were immersed in PB at 22 °C for 24 h, and then centrifuged at 2000 rpm for 30 min. The amount of PB retained by microspheres was calculated by weighting the swollen microspheres after centrifugation.

2.11. Swelling degree

The volume expansion of microspheres at different temperatures was determined at equilibrium, after having placed the microspheres in PB. The volume of the swollen beads (V_s), reported to the dried volume (V_d) and measured by placing the microspheres in a graduated cylinder (i.d. = 12 mm), was defined as the swelling factor (q).

2.12. Swelling/deswelling kinetics

Swelling kinetics of the thermoresponsive microspheres were studied in PB in a graduated glass cylinder (d = 12 mm) placed in a thermostatic transparent water bath. The microspheres were equilibrated at the desired temperature (*i.e.*, 50 °C), thereafter the glass cylinder was transferred into a water bath at 4 °C. The volume increase was monitored periodically. The dynamic swelling ratio is defined by Eq. (3):

$$Q_{ds} = \frac{V_t - V_d}{V_{0\,(50\,^\circ C)} - V_d} \quad (3)$$

where Q_{ds} is the dynamic swelling ratio, $V_{0\,(50\,^\circ C)}$ is the microsphere volume at equilibrium at 50 °C, V_t is the microsphere volume at a given time, and V_d is the microsphere volume in the dried state.

The deswelling kinetics was followed by applying a temperature change in the opposite direction of the swelling behaviour. Microspheres equilibrated in PB at 4 °C were transferred into water bath at 50 °C.

The dynamic deswelling ratio is defined by Eq. (4):

$$Q_{dd} = \frac{V_t - V_d}{V_{0\,(4\,^\circ C)} - V_d} \quad (4)$$

where Q_{dd} is the dynamic deswelling ratio, $V_{0\,(4\,^\circ C)}$ is the volume of microspheres at 4 °C, V_t is the volume of microspheres at a given time, and V_d is the volume of dried microspheres.

2.13. Drug loading

Inclusion of BD in the microspheres was carried out by the evaporation method as follows. 5 ml of an aqueous solution of BD (5 mg/ml) were poured over 100 mg microspheres in a porcelain crucible and kept at 22 °C (±2 °C) until the water was completely evaporated. Then, the microspheres were washed two times with 10 ml distilled water, and the effluents were collected and assayed spectrophotometrically to determine the BD content. Finally, the loaded microspheres were dried under vacuum.

The efficiency of inclusion was calculated according to Eq. (5):

$$\text{Efficiency}\,(\%) = \frac{BD_a}{BD_t} \quad (5)$$

where BD_a is the actual amount of BD in the microspheres and BD_t is the theoretical amount of BD in the microspheres.

2.14. In vitro BD release kinetics

In vitro BD release kinetic studies were performed, at different temperatures, by soaking 200 mg of microspheres in 50–100 ml of PB solution under gentle stirring. At regular time intervals aliquots of the release medium were withdrawn and the drug content was determined spectrophotometrically. The same volume of the release medium was added to replace the volume of the extracted samples.

3. Results and discussions

3.1. Preparation and characterization of poly(NIPAAm-co-AAm) copolymer

Usually, small amounts of hydrophilic monomers are copolymerized with NIPAAm to increase the LCST towards the body temperature (Liu et al., 2004; Khan, 2007). Acrylamide (AAm) is the monomer here used to copolymerize with NIPAAm, because AAm (i) increases the LCST due to its hydrophilicity and (ii) possesses the functional amide group capable of crosslinking (Fundueanu et al., 2009). As reported in Table 1 and proven by ^{1}H NMR spectra (data not shown), the copolymer formation and the percentage of the co-monomers in the copolymer correspond approximately to those observed in the feed. It should be noted that increasing the AAm content of the copolymer the LCST increases, however it shows a sharp phase transition (Fig. 1).

The copolymer containing 79.4% mol NIPAAm and 20.6% mol AAm (sample P_2 in Table 1) was chosen for the preparation of thermoresponsive porous microspheres. Indeed, it displays (i) a sharp phase transition at ~37 °C in physiological fluids (PB) and (ii) possesses enough functional groups capable to react with the crosslinking agent, and to form a stable tridimensional network. Moreover, in the recent studies poly(NIPAAm-co-AAm) was not shown to pose biologically significant risk at relevant human dosages (Ankareddi et al., 2008). Also, poly(NIPAAm) may provide a convenient and useful technology for cell culture (Takezawa et al., 1990).

Table 1
Influence of co-monomer ratio in the feed and in the copolymer on the LCST[a].

Sample	Co-monomer composition				LCST (°C)	
	In the feed (% mol ratio)		In the co-polymer (% mol ratio)		pH = 7.4	pH = 1.2
	NIPAAm	AAm	NIPAAm	AAm		
P_0	100	0	100	0	28.2	31.4
P_1	90.91	9.09	88.06	11.94	34.1	35.2
P_2[b]	83.34	16.66	79.4	20.6	36.8	40.1
P_3	76.93	23.07	66.6	33.3	42.1	46.6

[a] Data are the results of two independent experiments. LCST values were determined by the cloud point technique using a polymer solution of 1% (w/v).
[b] M_n = 37,928 g/mol, M_w = 100,130 g/mol, and IP = 2.64. IP, index of polydispersity.

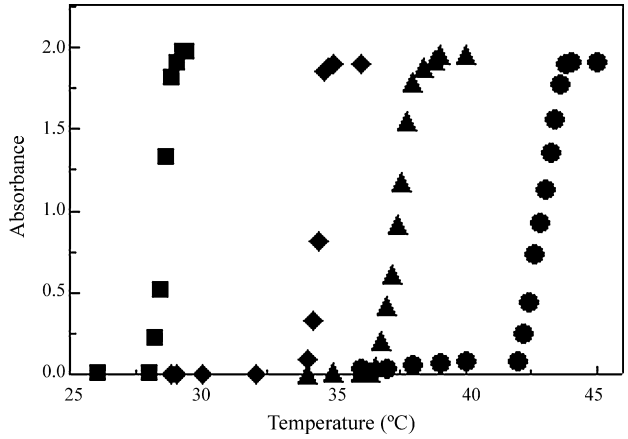

Fig. 1. LCST profiles of poly(NIPAAm-co-AAm) containing different amounts of AAm in the copolymer (expressed as % mol): 0 (■; sample P_0), 11.94 (♦; sample P_1), 20.6 (▲; sample P_2), and 33.3 (●; sample P_3). The values are means of two independent measurements. Data were obtained in PB.

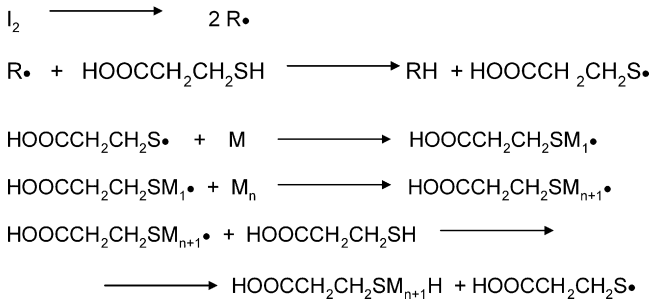

Fig. 2. Radical oligomerization reaction of NIPAAm.

3.2. Synthesis of semitelechelic NIPAAm oligomers

Semitelechelic NIPAAm oligomers having carboxyl end group were synthesized by free radical polymerization using MPA as the chain transfer agent as shown in Fig. 2. The results are summarized in Table 2.

As expected, the molecular weight of oligomers decreases as the amount of the chain transfer agent increases. However, the molecular weight is low in both cases and therefore oligomers are appropriate candidates to be used as pore-forming agents because of their low viscosity. In addition, the –COOH end group and the entire oligomer are chemically inert; indeed, they do not interact with the crosslinker (GA) during microsphere preparation. The oligomer with molecular weight of 2000 g/mol was chosen to be used in this study because of its low molecular weight and very low viscosity. This oligomer can be easily removed from the microsphere network by washing.

Table 2
Preparation conditions and characterization of semitelechelic NIPAAm oligomers.

Sample	[T]/[M][a]	Yield (%)	M_n (g/mol)
S_1	0.05	62.2	2000
S_2	0.025	74.6	4200

[a] [T]/[M] represents the molar ratio between the chain transfer agent and the monomer.

3.3. Phase transition temperature of the reaction mixture

In order to produce porous microspheres at temperatures below and above the LCST of the pore-forming agent (ONIPAAm) and below the LCST of poly(NIPAAm-co-AAm), the phase transition temperature of the oligomer under simulated preparation conditions (acidified solution containing GA and the polymer) was determined (Fig. 3). Also, the LCST of the pure oligomer and of the pure poly(NIPAAM-co-AAm) was determined under the same conditions.

As shown in Fig. 3, the LCST of pure oligomer is 31.1 °C under simulated preparation conditions in the absence of poly(NIPAAM-co-AAm). In the presence of poly(NIPAAm-co-AAm), the LCST slightly decreases to 30.1 °C. The LCST of poly(NIPAAm-co-AAm) under simulated preparation conditions is 41.2 °C in the absence of the oligomer. Since the LCST of the polymer solution cannot be determined in the presence of the oligomer, we estimated a slightly decrease of the LCST, no more than 1 °C, as previously stated. Accordingly, we fixed the experimental temperatures at 28 °C (*i.e.*, below the LCST of the oligomer), and at 34 °C (*i.e.*, above the LCST of the oligomer) for the preparation of porous microspheres. However, porous microsphere preparation was carried out below the LCST of poly(NIPAAm-co-AAm).

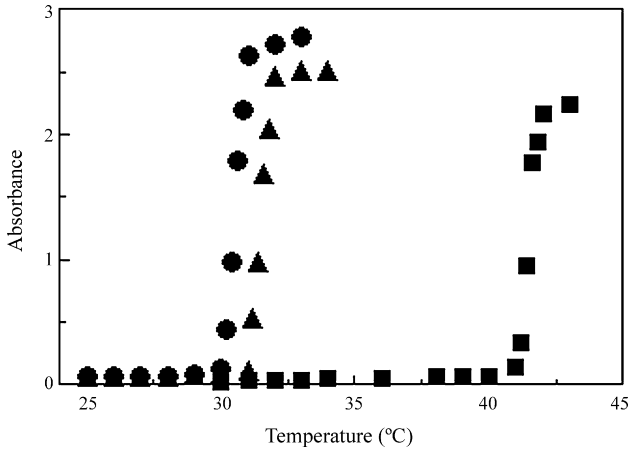

Fig. 3. LCST profile of ONIPAAm in the absence (▲) and in the presence of poly(NIPAAm-co-AAm) (●). For comparison, the LCST profile of poly(NIPAAm-co-AAm) in the absence of ONIPAAm (■) is shown. Data were obtained under simulated preparation conditions (presence of GA and H_2SO_4).

3.4. Preparation of porous microspheres

Stable thermoresponsive microspheres with a porous structure were prepared with an original procedure based on the water-in-oil suspension crosslinking of preformed polymers. The thermoresponsive poly(NIPAAm-co-AAm) copolymer and ONIPAAm oligomer were solubilized in an acidified distilled water and dispersed in mineral oil at a temperature below the LCST of the polymer and below (28 °C) or above (34 °C) the LCST of ONIPAAm. The droplets suspension was then hardened by crosslinking

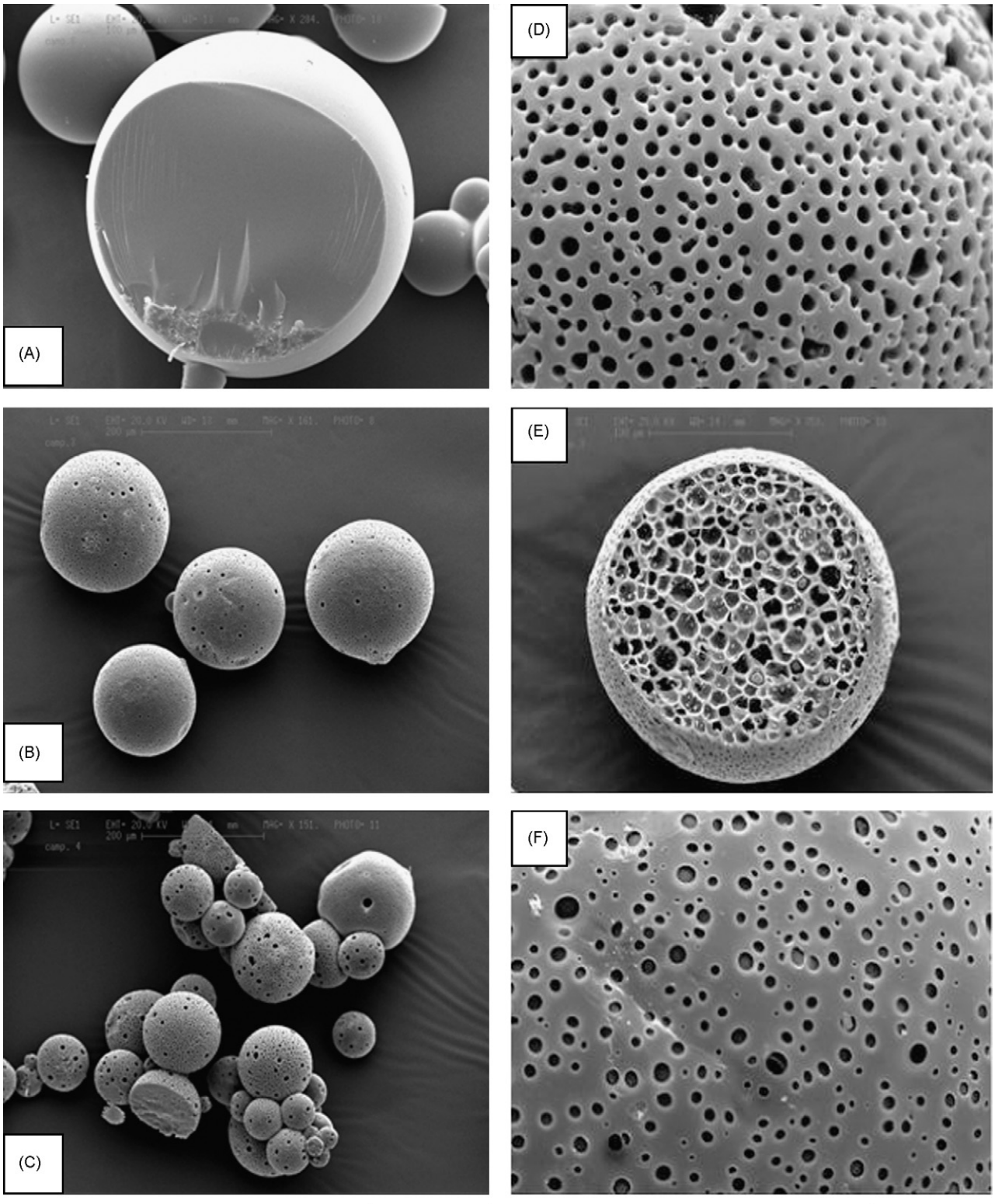

Fig. 4. Scanning electron micrographs of poly(NIPAAm-co-AAm) microspheres synthesized in the absence of ONIPAAm (POR_0) (panel A), and in the presence of ONIPAAm: 14.3% (w/w) (POR_2) (panel B) and 25% (w/w) (POR_3) (panel C–E). For comparison, the scanning electron micrograph (surface detail) of the microspheres obtained at a temperature above the LCST of ONIPAAm (sample POR_{1B}) is shown in panel F. The bar shown in panels A, B, and C corresponds to 100, 175, and 190 μm, respectively, and the bar shown in panels D, E, and F corresponds to 20, 85, and 25 μm, respectively.

Table 3
Preparation conditions and main characteristics of porous poly(NIPAAm-co-AAm) microspheres[a].

Sample	Amount of poly(NIPAAm-co-AAm) (g)	Amount of ONIPAAm (g) (%, w/w)	Recovery (%)	Mean diameter (μm)	PB regain at 22 °C (ml/g)	Swelling degree in PB at 22 °C	Wrack density (g/ml)
POR_0	0.6	0 (0.0)	89.5 ± 6	160 ± 18	5.84 ± 0.4	10.2 ± 0.3	0.56 ± 0.1
POR_1	0.6	0.05 (7.7)	82.2 ± 7	168 ± 15	6.54 ± 0.2	11.4 ± 0.6	0.50 ± 0.1
POR_2	0.6	0.1 (14.3)	77.8 ± 6	182 ± 20	7.50 ± 0.1	12.1 ± 0.2	0.44 ± 0.05
POR_3	0.6	0.2 (25.0)	68.9 ± 5	201 ± 22	8.62 ± 0.2	13.9 ± 0.5	0.40 ± 0.0
POR_{1A}	0.6	0.05 (7.7)	80.4 ± 6	158 ± 18	7.41 ± 0.18	12.6 ± 0.8	0.48 ± 0.05
POR_{1B}	0.6	0.1 (14.3)	73.1 ± 7	171 ± 21	8.22 ± 0.23	13.1 ± 0.3	0.41 ± 0.02
POR_{1C}	0.6	0.2 (25.0)	aggregates	–	–	–	–

[a] Data are the results of two independent experiments ± SD. Microspheres were prepared at 28 °C (sample POR_0, POR_1, POR_2, and POR_3) and at 34 °C (sample POR_{1A}, POR_{1B}, and POR_{1C}). The stirring speed and temperature were 350 rpm and 48 h, respectively.

the amide group of acrylamide with GA (Fundueanu et al., 2009). The results given in Table 3 demonstrate that porous microspheres were obtained both below and above the LCST of the poly(NIPAAm) oligomer. However, aggregates occurred at high porogen concentration, above the LCST of the oligomer (sample POR_{1C}). The precipitation of the oligomer inside of the polymer droplets during the crosslinking process leads to a destabilization of the suspension and to aggregation. As shown in Table 3, high amounts of the porogen caused low percentage of microsphere recovery because the porogen hinders the crosslinking process; a large amount of the polymer is not linked to the microsphere network and is removed from the system during the washing step. Moreover, high amounts of the porogen induce the increase of the microsphere size because of the increased viscosity of the polymer solution.

The dimension of the pores is obviously affected by the amount of the porogen in the reaction mixture. In the absence of the porogen no pores are formed and the microspheres display a compact inner structure and a smooth surface (Fig. 4, panel A). By increasing the amount of the oligomer, the size of the pore increases obtaining a stable porous structure (Fig. 4, panels B and C).

The size distribution and homogeneity of the pores seems to be narrower and more uniform, respectively, in microspheres obtained under LCST than in microspheres obtained above LCST. Under LCST, at 28 °C, the crosslinking process took place in a homogeneous solution of the polymer and of the oligomer. Therefore, uniform porosity was obtained (Fig. 4, panels D and E). On the opposite, above LCST, at 34 °C, the oligomers collapsed inside of the droplets, and the dimension and shape of the pores depend on the dimension and shape of the small aggregates (Fig. 4, panel F). Both types of microspheres possess relatively high values of water regain and swelling degree which is a general characteristic of hydrophilic and porous structures (Table 3).

Water regain and swelling degree increase as the amount of the porogen increase; indeed, networks with more porous structure and weaker crosslinking degrees were obtained at high amounts of the porogen. Large amount of the porogen caused also a decrease of the wrack density.

3.5. Determination of the transition temperature of the porous hydrogel

With the aim to verify if the porous microspheres preserve the thermoresponsive properties of the linear polymer, values of the volume phase transition temperature (VPTT) of the hydrogel microspheres were determined under physiological conditions. The method is based on the measurement of the swelling degree in PB at different temperatures below and above the LCST. This method is simpler than the classical method that involves the determination of water regain at different temperatures (Wang et al., 2003; Yildiz et al., 2002b). Moreover, the classical method could apply to large hydrogels showing the shape of discs or slabs (Xue et al., 2002; Asoh et al., 2006). For microspheres, this method is not adequate since it does not discriminate between the water present in the microspheres and the water present between microspheres. As shown in Fig. 5, the microspheres preserve the thermoresponsive properties of the linear polymer.

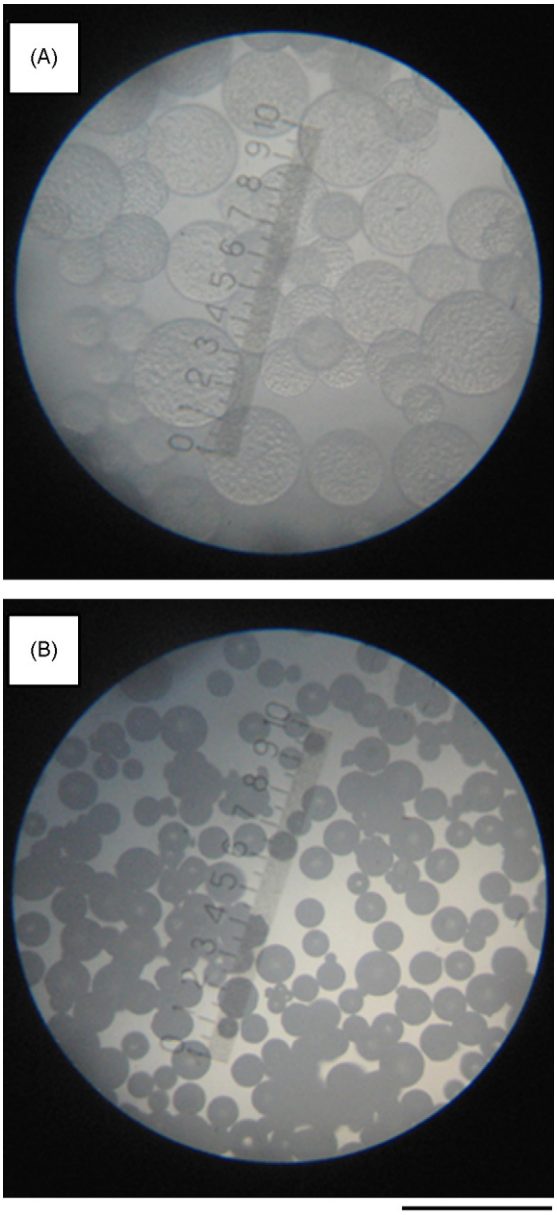

Fig. 5. Optical photomicrographs of porous thermoresponsive microspheres (POR_3) in the swollen state, at pH 7.4 (PB), below the LCST at 22 °C (panel A), and above the LCST at 45 °C (panel B). The bar shown in panels A and B corresponds to 1000 μm.

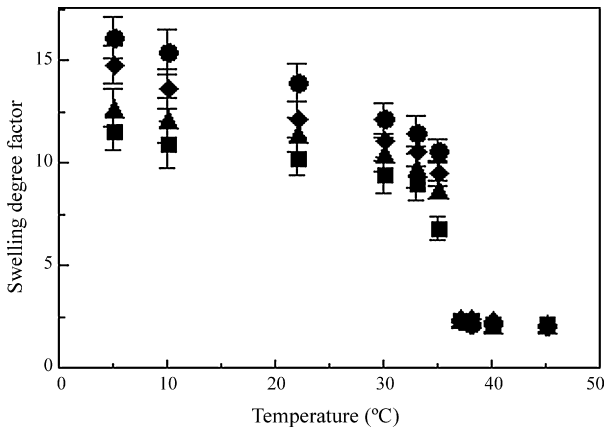

Fig. 6. Effect of temperature on the swelling degree of poly(NIPAAm-co-AAm) microspheres with different porosity: POR_0 (■), POR_1 (▲), POR_2 (♦), and POR_3 (●). Where statistical error bars are not shown, they are smaller than the symbols. Data were obtained in PB.

A sharp phase transition occurs when the temperature changes around the LCST (Fig. 6). Assuming that the crosslinking degree of microspheres is almost the same, it can be noticed that microspheres with the highest porosity display the sharpest phase transition around the LCST. However, the most important characteristic of a thermoresponsive hydrogel is the rapidity of the volume change to the input signal. Tanaka and co-workers showed that the promptness of the swelling/deswelling response to the temperature change is inversely related to the size of the hydrogel (Tanaka and Fillmore, 1979). Also, a porous structure facilitates the migration of water through the pores ("convective" transport) of the hydrogels, increasing the response rate (Strachotová et al., 2007). The microspheres synthesized in this study cumulate both the small size and porous structure. As follows, the porous microspheres display a very rapid volume change when the temperature increases or decreases around VPTT (Fig. 7). In particular, after 1 min the swelling as well deswelling process of microspheres with the highest porosity reach the equilibrium. In fact, the swelling/deswelling processes take place more rapidly than reported, almost instantaneously, since the rate limiting step is represented by heat transfer from the external water bath to microspheres. Obviously, less porous microspheres swell/shrink slower, but still very rapid for practical applications (Fig. 7).

3.6. Loading and release studies

Blue dextran (BD) was used as a high molecular weight drug model for loading and release studies, after testing several compounds displaying different molecular weights (data not shown). The inclusion of BD into porous microspheres was performed in cold water at 22 °C, indeed the swelling degree of microspheres is very high at this temperature ($q_0 = 10.2$, $q_1 = 11.4$, $q_2 = 12.1$, and $q_3 = 13.9$ in PB, for samples POR_0, POR_1, POR_2, and POR_3, respectively). By progressive water evaporation, BD is practically forced to penetrate into the pores of the microspheres (Table 4).

In the absence of the porogen, BD was not entrapped into the microspheres because of the lack of porosity (Fig. 4, panel A). The size of the pores of these microspheres even in the swollen state is smaller than the size of BD (the gyration radius of BD is about 490 Å (Fundueanu et al., 1999)). The highest amount of entrapped BD was found in the microspheres with the lowest porosity. The microspheres possessing the largest pores display the lowest amount of entrapped BD because the most part of BD is easily removed from the microspheres during the washing step.

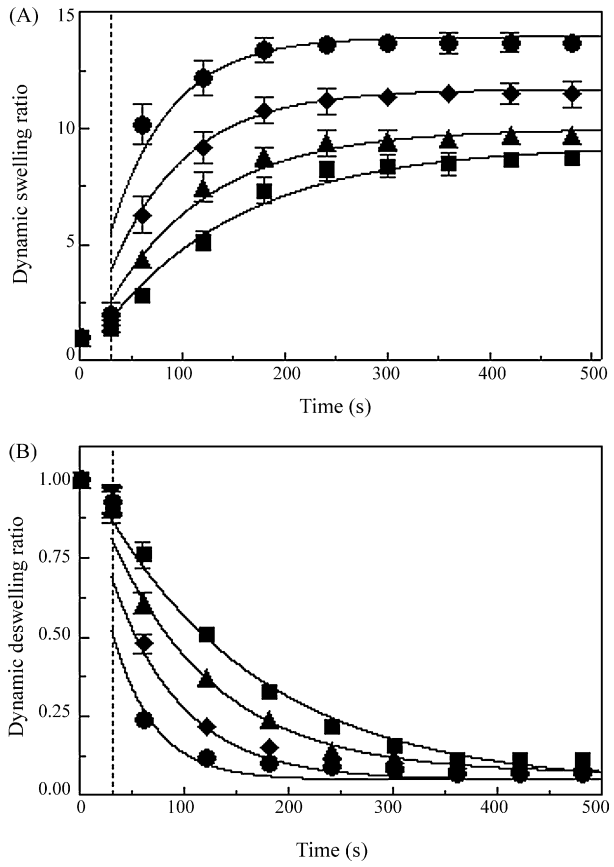

Fig. 7. Dynamic swelling kinetics of poly(NIPAAm-co-AAm) microspheres with different porosity: POR_0 (■; $t_{1/2} = 94.8$ s), POR_1 (▲; $t_{1/2} = 67.9$ s), POR_2 (♦; $t_{1/2} = 51.3$ s), and POR_3 (●; $t_{1/2} = 40.7$ s) (panel A). Dynamic deswelling kinetics of poly(NIPAAm-co-AAm) microspheres with different porosity: POR_0 (■; $t_{1/2} = 106$ s), POR_1 (▲; $t_{1/2} = 67.3$ s), POR_2 (♦; $t_{1/2} = 46.2$ s), and POR_3 (●; $t_{1/2} = 27.7$ s) (panel B). Vertical dashed lines indicate the dead-time (30 s) required for heat transfer. Where statistical error bars are not shown, they are smaller than the symbols. Data were obtained in PB. Data reported in panels A and B have been fitted according to equation: $Y = Y_{max} \times (1 - e^{-kt})$ and $Y = \text{Span} \times e^{-kt}$, respectively. K was transformed to $t_{1/2}$ according equation: $t_{1/2} = 0.693/k$.

3.7. Release studies

In vitro release studies give important information on the efficiency of a delivery system. Release studies were performed at pH 7.4 (in PB) below and above the VPTT. The effect of temperature on drug release profiles is shown in Fig. 8. In a porous thermoresponsive drug delivery system, the main factors that control the drug release from microspheres are the swelling/deswelling process, the porosity of the hydrogel, and the hydrophilic/hydrophobic balance of the polymeric network. Therefore, the diffusion of BD from microspheres to the release fluid will be controlled by steric and hydrophobic interactions. Below the VPTT, the microspheres are in the swollen state and almost no steric and hydrophobic interactions occur, consequently BD was released quickly. The larger is

Table 4
Incorporation efficiency of BD in thermoresponsive microspheres[a].

Sample	Encapsulated BD (%, w/w)		Efficiency (%)
	BD_a	BD_t	
POR_0	0	20	0
POR_1	7.5 ± 0.4	20	37.5 ± 2
POR_2	2.1 ± 0.2	20	10.5 ± 1
POR_3	1.2 ± 0.2	20	6.0 ± 1

[a] Data are the results of three independent experiments ± SD.

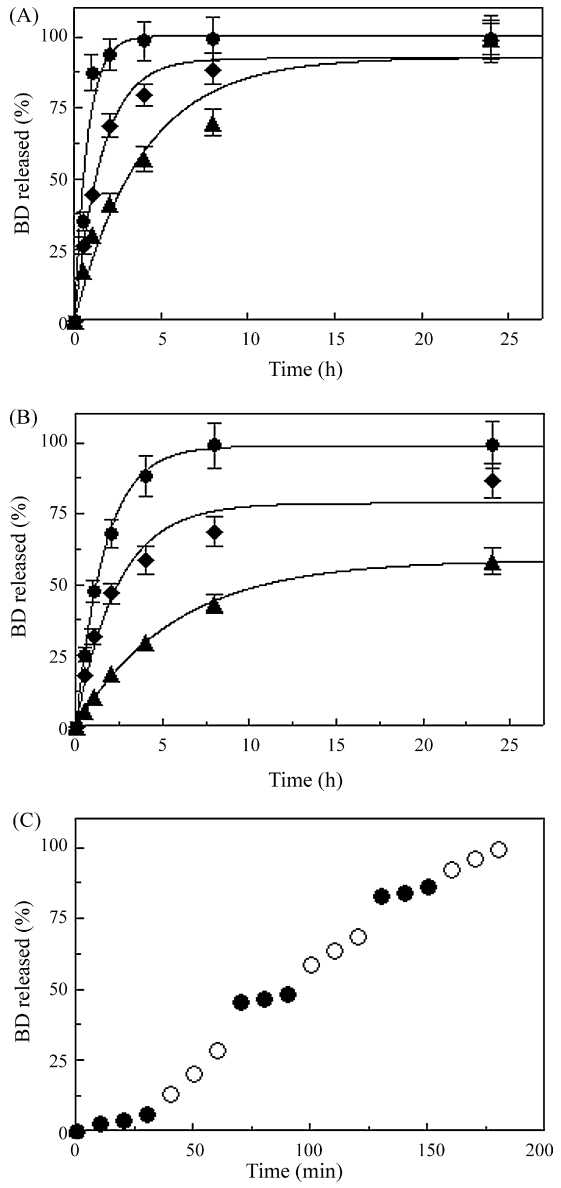

Fig. 8. Kinetics of BD release from poly(NIPAAm-co-AAm) microspheres, below the VPTT (33 °C) POR_1 (▲; $t_{1/2}$ = 2.7 h), POR_2 (♦; $t_{1/2}$ = 1.1 h), and POR_3 (●; $t_{1/2}$ = 0.52 h) (panel A). Kinetics of BD release from poly(NIPAAm-co-AAm) microspheres, above the VPTT (38 °C): POR_1 (▲; $t_{1/2}$ = 3.9 h), POR_2 (♦; $t_{1/2}$ = 1.7 h), and POR_3 (●; $t_{1/2}$ = 1.2 h) (panel B). Effect of temperature cycling (33 °C (○) and 38 °C (●)) on BD release from poly(NIPAAm-co-AAm) microspheres (POR_1) (panel C). Data were obtained in PB. Data reported in panels A and B have been fitted according to equation: $Y = Y_{max} \times (1 - e^{-kt})$. K was transformed to $t_{1/2}$ according equation: $t_{1/2} = 0.693/k$.

the dimension of the pores the higher is the release rate of BD (Fig. 8, panel A).

When the temperature was raised above the VPTT, the microspheres collapsed and become hydrophobic, and steric and hydrophobic interactions reduced the release rate of BD (Fig. 8, panel B). This difference could be higher since the increase in temperature has two opposite effects: it decreases the dimensions of the pores and increases the solubility and the diffusion rate of the drug. However, the contribution of each factor cannot be determined. The difference between the release rate of BD below and above the VPTT was exploited for delivery of BD under cycling changes of temperature. This difference is highest for microspheres with lowest porosity (POR_1). Fig. 8 (panel C) shows kinetics of BD release from porous thermoresponsive microspheres (POR_1) under thermal cycling operation between 33 and 38 °C in PB. A pulsatile release of BD was observed, the first pulse occurring in the swollen state of the microspheres. However, the most part of BD was expelled during the shrinkage of the matrix when BD is mechanically squeezed out from the system.

4. Conclusions

Thermoresponsive hydrogels displaying both a small size (microspheres) and an homogeneous porous structure were obtained by an original procedure. The poly(NIPAAm-co-AAm) preformed polymer, with the LCST corresponding to that of the body temperature (37 °C), was used for the preparation of the microspheres. Oligomers of NIPAAm were used as pore-forming agents below and above their LCST. The microspheres obtained at temperatures below the LCST of oligomers display an homogeneous porous structure and a very fast swelling/deswelling rate around the VPTT. The porous microspheres are suitable matrix for the temperature-controlled release of macromolecular drugs.

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