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pH- and temperature-sensitive polymeric microspheres for drug delivery: the dissolution of copolymers modulates drug release

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Abstract Most pH-/temperature-responsive polymers for controlled release of drugs are used as cross-linked hydrogels. However, the solubility properties of the linear polymers below and above the lower critical solution temperature (LCST) are not exploited. Here, the preparation and characterization of poly (*N*-isopropylacrylamide-co-methacrylic acid-co-methyl methacrylate) (poly (NIPAAm-co-MA-co-MM)) and poly (*N*-isopropylacrylamide-co-acrylamide) (poly (NIPAAm-co-AAm)), known as "smart" polymers (SP), is reported. Both poly (NIPAAm-co-MA-co-MM) and poly (NIPAAm-co-AAm) display pH- and temperature-responsive properties. Poly (NIPAAm-co-MA-co-MM) was designed to be insoluble in the gastric fluid (pH = 1.2), but soluble in the intestinal fluid (pH = 6.8 and 7.4), at the body temperature (37°C). Poly (NIPAAm-co-AAm) was designed to have a lower critical solution temperature (LCST) corresponding to 37°C at pH = 7.4, therefore it is not soluble above the LCST. The solubility characteristics of these copolymers were exploited to modulate the rate of release of drugs by changing pH and/or temperature. These copolymers were solubilized with hydrophobic cellulose acetate butyrate (CAB) and vitamin B_{12} (taken as a water soluble drug model system) in an acetone/methanol mixture and dispersed in mineral oil. By a progressive evaporation of the solvent, the liquid droplets were transformed into loaded CAB/SP microspheres. Differential scanning calorimetric studies and scanning electron microscopy analysis demonstrated that the polymeric components of the microspheres precipitated separately during solvent evaporation forming small microdomains. Moreover, vitamin B_{12} was found to be molecularly dispersed in both microdomains with no specific affinity for any polymeric component of microspheres. The release of vitamin B_{12} was investigated as a function of temperature, pH, and the CAB/SP ratio.

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1 Introduction

Among the stimuli-sensitive polymers, poly(*N*-isopropylacrylamide) (poly(NIPAAm)) has attracted the most attention due to its sharp transition behaviour in aqueous solution [1–3]. Indeed, poly(NIPAAm) shows a lower critical solution temperature (LCST) at ~33°C [4, 5]. Furthermore, in aqueous solution poly(NIPAAm) exhibits a reversible solubility and a remarkable hydration–dehydration change in response to temperature. Below the LCST, poly(NIPAAm) is in a hydrated state. However, it becomes hydrophobic and insoluble when the environmental temperature is higher than the LCST.

The biomedical and biotechnological applications of poly(NIPAAm) requires the chemical modifications of the polymer. By copolymerization with small amounts of hydrophilic monomers, the LCST value could be increased reaching that of the body temperature [6–8]. Also, the incorporation of small amounts of weakly acidic or basic monomers makes poly(NIPAAm) both pH- and temperature-responsive [9, 10].

Most studies on intelligent polymers deal with their potential role in drug delivery systems. To date, most investigation took into account the hydrogel form of these polymers, obtained by cross-linking the linear form [11–13].

However, few researchers investigated the polymer solubility properties under and above LCST for the controlled drug delivery. Thus, the kinetics of insulin release from beads prepared from pH-/temperature-sensitive polymers were controlled by the molecular weights of polymers. At pH = 7.4 and body temperature (37°C), the low molecular weight polymeric beads dissolved within 2 h and released insulin totally, whereas insulin release from high molecular weight polymeric beads is controlled by the matrix swelling [14]. Moreover, the escape of DNA loaded poly(vinyl alcohol) microspheres from multinucleated microcapsules took place through the holes induced by dissolution of the polymer at colonic pH and temperature [15].

Beside poly(NIPAAm), cellulose acetate butyrate (CAB) is a hydrophobic polysaccharide obtained by esterification of hydroxyl groups of cellulose. The hydrophobic characteristics as well as its biodegradability render it as a support for long-term delivery of drugs [16, 17].

Here, poly(*N*-isopropylacrylamide-co-methacrylic acid-co-methyl methacrylate) (poly(NIPAAm-co-MA-co-MM)) was prepared as a new pH-/temperature-responsive polymer. Poly(NIPAAm-co-MA-co-MM) is not soluble in the gastric juice (pH = 1.2), but solubilizes in the intestinal fluids (i.e., at pH = 6.8 and 7.4), at 37°C. Poly(*N*-isopropylacrylamide-co-acrylamide) (poly(NIPAAm-co-AAm)) was designed to have a LCST of ~37°C at pH = 7.4. Both copolymers were mixed with the hydrophobic cellulose acetate butyrate (CAB), solubilized in an acetone/methanol mixture and transformed into microspheres by the oil/oil solvent evaporation method. The role of poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm) as a drug delivery system was investigated using vitamin B_{12} as a drug model system.

2 Experimental section

2.1 Materials

N-isopropylacrylamide (NIPAAm), obtained from Aldrich Chemical Corp. (Milwaukee, WI, USA), was recrystallized from hexane. Cellulose acetate butyrate (CAB; Mw = 40 000 g/mol, degree of substitution (%) with acetyl = 0.2, butyryl = 2.4, hydroxyl = 0.4) was obtained from Eastman Inc. (Kingsport, Tennesse, USA). Acrylamide (AAm), 1,4-dioxane, methacrylic acid (MA), methyl methacrylate (MM), and *N*,*N*′-azobisisobutyronitrile (AIBN) were supplied from Fluka AG (Buchs, Switzerland). 1,4-Dioxane was purified by refluxing. Vitamin B_{12} was kindly supplied from Iassy Pharm SA (Iassy, Romania). All chemicals were of the highest analytical grade.

2.2 Synthesis of poly(NIPAAm-co-MA-co-MM)

Linear poly(NIPAAm-co-MA-co-MM) was synthesized by free radical copolymerization in 1,4-dioxane with AIBN as initiator. Typically, 1.13 g NIPAAm, 0.086 ml MA, 0.15 ml MM, and 0.010 g AIBN were solubilized in 10 ml 1,4-dioxane. Dried nitrogen was bubbled through the solution for 30 min prior to polymerization. After polymerization at 70°C for 20 h, the mixture was precipitated in diethylether, and dried under vacuum. Then, the copolymer was solubilized in distilled water, dialyzed for 5 days at 20°C (molecular weight cut off 10,000–12,000 Da; from Medi Cell International, England), and recovered by freeze drying for 24 h at −57°C and a vacuum of 0.045 mbar (Martin Christ, Freeze Dryer, Alpha 1-2/LD). This polymer has been called "enterosoluble polymer" (EP).

2.3 Synthesis of poly(NIPAAm-co-AAm)

Linear poly(NIPAAm-co-AAm) was synthesized as reported for the preparation of EP with small modifications. Briefly, 1.13 g NIPAAm, 0.142 g AAm, and 0.010 g AIBN 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 70°C until the gel point was reached (about 4 h), then 6 ml of dioxane was added and the reaction continued for an additional 16 h. The recovery of the copolymer was performed as described in section 2.2. This polymer has been called "thermoresponsive polymer" (TP).

2.4 Determination of molecular weight

Values of the number-average (M_n) and the weight-average (M_w) molecular weight of poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm) were determined by gel permeation chromatography 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. The calibration curve was carried out using monodisperse polystyrene standards.

2.5 Copolymer composition

Composition of copolymers was determined by ^{1}H-NMR spectroscopy. ^{1}H-NMR spectra were recorded on a Bruker Avance DRX 400 NMR (Bruker, Rheinstetten, Germany), at 100°C, using deuterated dimethyl sulfoxide as solvent. The molar fractions of NIPAAm and MM in poly(NIPAAm-co-MA-co-MM) were determined from the area of the peak at 3.83 ppm due to the methyne group of NIPAAm and the area of the peak at 3.57 ppm for the methyl protons of MM. The molar fraction of MA was obtained by conductometric titration. The molar fraction of AAm in poly(NIPAAm-co-AAm) was determined from the area of the peak at 6.45 ppm due to the $-NH_2$ protons of AAm. The molar fraction of NIPAAm was obtained from the area of the peak at 3.89 ppm, attributed to the methyne group of NIPAAm.

2.6 LCST determination

LCST was determined from the absorbance of poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm) at 450 nm as a function of temperature using the UV-Vis spectrophotometer coupled to a temperature controller. The polymer solution was investigated under standard acidic (pH = 1.2, 64 mM HCl + 50 mM KCl), and standard neutral (phosphate buffer, PB; pH = 6.8 and 7.4, 50 mM Na_2HPO_4 + NaOH) conditions. The heating rate was 2°C every10 min and 0.2°C in the proximity of the 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 containing vitamin B_{12} were prepared by the oil/oil solvent evaporation method using an open cylindrical reactor (h = 80 mm, i.d. = 80 mm) with a round bottom. Typically, 150 mg CAB, 150 mg SP, and 10 mg of vitamin B_{12} were solubilized in 2 ml of the 1/1 acetone/methanol (v/v) mixture. The polymer solution was poured into 50 ml light mineral oil in which 0.25 g soybean lecithin, as the dispersing agent, was added and stirred at 350 rpm by a three-blade turbine impeller. The reaction temperature was 25°C and the reaction time was 10 h. Finally, the microspheres were recovered by filtration using a sintered glass filter (diameter of pores 25 μm), washed with diethyl ether, and dried under vacuum (48 h at 40°C and 66 mbar). Microspheres without vitamin B_{12}, used for DSC analysis, were prepared as mentioned above.

2.8 Efficiency of microsphere recovery

After washing and drying, the microspheres were weighed and the weight compared to the initial mass of the polymers plus the drug. The recovery yield was calculated by the following equation:

$$Recovery\,(\%) = \frac{W_{microsph}}{W_{polym} + W_{vit}} \times 100 \quad (1)$$

where $W_{microsph}$, W_{polym}, and W_{vit}, represent the weight of isolated microspheres, of polymers (CAB + SP), and of vitamin B_{12}, respectively.

2.9 Determination of vitamin B_{12} loading

The amount of vitamin B_{12} entrapped in microspheres was determined after dissolution of 25 mg microspheres in 50 ml warm methanol. The amount of vitamin was determined by UV-Vis spectrophotometry (λ = 362 nm), using a previously made calibration curve in methanol (range of linearity: 5–25 μg/ml). Loading was calculated by the following equation:

$$Loading\,(\%) = \frac{W_{vit}}{W_{microsph}} \times 100 \quad (2)$$

where W_{vit} and $W_{microsph}$ represent the weight of vitamin B_{12} and of isolated microspheres, respectively.

2.10 Encapsulation efficiency

The encapsulation efficiency of the vitamin B_{12} was calculated as the ratio between the actual vitamin content and the theoretical content, and expressed as percentage (Eq. 3):

$$Efficiency\,(\%) = \frac{Vit_a}{Vit_t} \times 100 \quad (3)$$

where Vit_a is the actual amount of the vitamin in microspheres, and Vit_t is the theoretical amount of the vitamin in microspheres.

The theoretical content of the vitamin in microspheres was calculated by the following equation:

$$Vit_t\,(\%) = \frac{W_{vit}}{W_{polym} + W_{vit}} \times 100 \quad (4)$$

where W_{vit} and W_{polym} represent the weight of vitamin and of polymers (CAB + SP) in the initial reaction mixture, respectively.

2.11 Morphological and dimensional analysis

Microcapsule morphology was evaluated by optical and electron microscopy. Dried microcapsules were analyzed at 15–20 kV by scanning electron microscopy (SEM) (Cambridge S 360) after metallization by gold coating (Edwards Sputter coating S 150). Size and size distribution were evaluated by optical microscopy using an inverted

microscope (Nikon Diaphot, Tokyo, Japan) equipped with a digital camera. Microcapsule size and size distribution were determined by examining the microcapsule diameter on digital photomicrographs, considering at least 200 microspheres for each analysis. Samples were counted, and each fraction was compared with the total number of microspheres.

2.12 DSC analysis

Differential scanning calorimetric measurements (DSC-60, Shimadzu, Japan) were carried out on 2–10 mg samples, placed in sealed non-hermetic aluminum pans and using an increasing temperature rate of 10°C/min. Prior to this, for glass transition temperature (Tg) determination, samples were heated from 20 to 150°C at 10°C/min to remove all the residual moisture and erase the effect of previous thermal history. Tg was considered at the mid-point temperature of the endothermic drift in the heating curves.

2.13 In vitro vitamin B_{12} release studies

In vitro vitamin B_{12} release studies were performed by the bath method [18], using different solutions simulating the gastric juice (pH = 1.2, KCl + HCl) and the intestinal fluid (phosphate buffer at pH = 6.8 and 7.4, 50 mM Na_2HPO_4 + NaOH). Samples were dispersed in flasks containing buffered solutions at different temperatures and gently stirred (50 rpm). Samples of the receiving buffer were withdrawn at different time intervals and the content was determined by spectrophotometric analysis (λ = 360 nm). The same volume of the fresh receiving buffer was added to replace the volume of the withdrawn samples. After vitamin B_{12} release, the morphological changes in the structure of CAB microcapsules were investigated by SEM.

3 Results and discussion

3.1 Preparation and characterization of "smart" polymers

Poly(NIPAAm) is one of the widely used temperature-responsive polymers for delivery of drugs [19] due to its sharp phase transition temperature (~33°C), in aqueous solution [4, 5]. However, in the gastric juice (pH = 1.2) and in the intestinal fluid (pH = 7.4) at physiological temperature (37°C), this polymer is completely insoluble and is not suitable to be used for biomedical applications (Fig. 1). Therefore, poly(NIPAAm) was copolymerized with small amounts of hydrophilic/hydrophobic monomers in order to increase the LCST from 33°C to 37°C. Firstly,

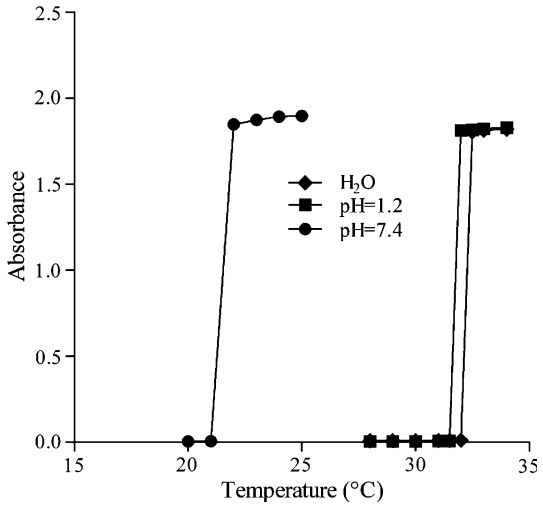

Fig. 1 LCST profile of poly(NIPAAm) in distilled water, acidic solution at pH = 1.2 and phosphate buffer at pH = 7.4 (determined by cloud point technique at 450 nm). The polymer concentration was 1% (w/v). The absorbance values are the mean of three independent experiments that deviated 0–5%. The *continuous lines* are "hand-drawn lines"

NIPAAm was copolymerized with MA and MM obtaining a pH-/temperature-responsive copolymer which was insoluble in the gastric juice, at pH = 1.2 and 37°C, but did dissolve at pH = 6.8 and 7.4, at the same temperature (Fig. 2). Secondly, NIPAAm was copolymerized with AAm obtaining a temperature-responsive copolymer (TP) with a sharp phase transition at ~37°C, at pH = 7.4 (phosphate buffer) (Fig. 3).

Therefore, poly(NIPAAm-co-MA-co-MM) appears to be a useful enterosoluble polymer (EP) for drug transport to the intestine; furthermore, poly(NIPAAm-co-AAm) acts

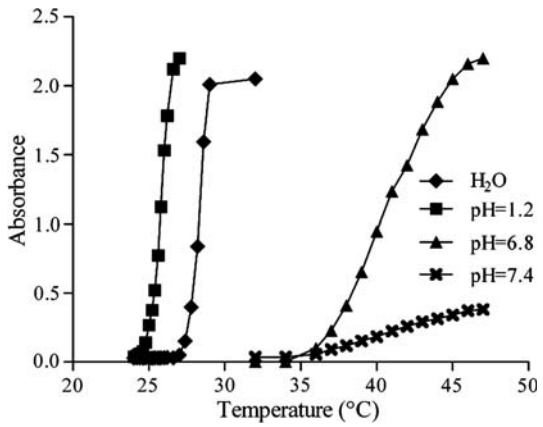

Fig. 2 LCST profile of poly(NIPAAm-co-MA-co-MM) in distilled water, acidic solution at pH = 1.2, and phosphate buffer at pH = 6.8, and 7.4 (determined by cloud point technique at 450 nm). The polymer concentration was 1% (w/v). The absorbance values are the mean of three independent experiments that deviated 0–6%. The *continuous lines* are "hand-drawn lines"

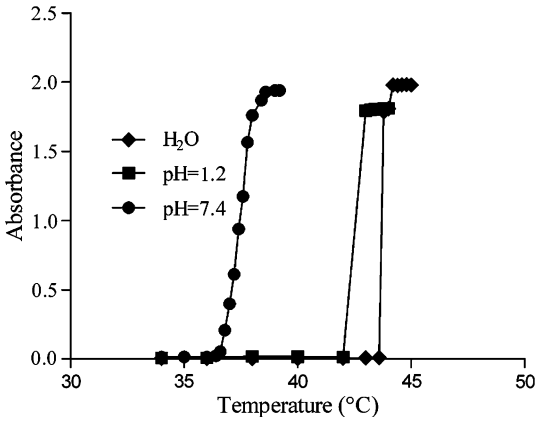

Fig. 3 LCST profile of poly (NIPAAm-co-AAm) in distilled water, acidic solution at pH = 1.2 and phosphate buffer at pH = 7.4 (determined by cloud point technique at 450 nm). The polymer concentration was 1% (w/v). The absorbance values are the mean of three independent experiments that deviated 1-5%. The *continuous lines* are "hand-drawn lines"

as a promoter of drug release by lowering the temperature slightly under 37°C.

The copolymer composition as well as values of the average molecular weights of poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm) used for the preparation of the "smart" microspheres are reported in Table 1.

^{1}H-NMR analysis confirms copolymer formation and the percentages of co-monomers in copolymer follows approximately those in the feed. The polymerization conditions such as the nature and amount of solvent and initiator were chosen in order to obtain copolymers with relatively low molecular weights. The purification by dialysis removes the oligomers and polymers with molecular weights smaller than 10,000 Da, obtaining relative low values of the polydispersity index (PI). The polymers with low molecular weights and narrow distributions undergo fast and homogeneous solubilization/precipitation and display low viscosities. These characteristics were successfully exploited in the preparation of microspheres and in the release studies.

3.2 Preparation of microspheres

Poly(NIPAAm-co-MA-co-MM) (EP) and poly(NIPAAm-co-AAm) (TP) are very soluble in methanol, therefore this solvent was chosen for the preparation of microspheres. Moreover, methanol is a good solvent for a large number of drugs such as propranolol, indomethacin, diclofenac, and tetracycline [20]. CAB is a hydrophobic polysaccharide, soluble in acetone, often used for the preparation of microspheres for controlled drug release [16, 17]. Therefore,

Table 1 Co-monomer composition (determined by ^{1}H-NMR) and average molecular weights (determined by GPC) of poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm)

Sample	Co-monomer composition								M_n (g/mol)	M_w (g/mol)	PI[c]
	In the feed (% mol ratio)				In copolymer (% mol ratio)						
	NIPAAm	MA	MM	AAm	NIPAAm	MA	MM	AAm			
EP[a]	80.6	8.1	11.3	–	78.99	7.65	13.34	–	37,824	90,720	2.4
TP[b]	83.3	–	–	16.6	83.0	–	–	16.0	52,647	137,812	2.61

[a] EP = poly(NIPAAm-co-MA-co-MM)

[b] TP = poly(NIPAAm-co-AAm)

[c] PI = polydispersity index. The molar masses are relative to the polystyrene calibration

Table 2 Influence of the CAB/EP ratio on microsphere characteristics

Sample	CAB (mg)	EP[a] (mg)	Vitamin B_{12} (mg)	Recovery (%)	Vitamin B_{12} in microspheres (%, w/w)	Encapsulation efficiency (%)	Mean diameter (μm)
CAB	300	0	10	98.4 ± 5	3.15 ± 0.1	97.8 ± 3.1	147 ± 43
CAB/EP_1	250	50	10	96.1 ± 3	3.11 ± 0.09	96.5 ± 2.8	125 ± 48
CAB/EP_2	200	100	10	93.4 ± 2	3.06 ± 0.01	95.0 ± 0.3	96 ± 52
CAB/EP_3	150	150	10	91.4 ± 5	2.89 ± 0.05	89.7 ± 1.5	72 ± 39
CAB/EP_4	100	200	10	–[b]	–	–	–

Solvent: methanol/acetone = 1 + 1 ml, stirring speed = 300 rpm, temperature = 25°C, reaction time = 10 h

[a] *EP* enterosoluble polymer

[b] Aggregation occurred

Data are the results of three independent experiments ± SD

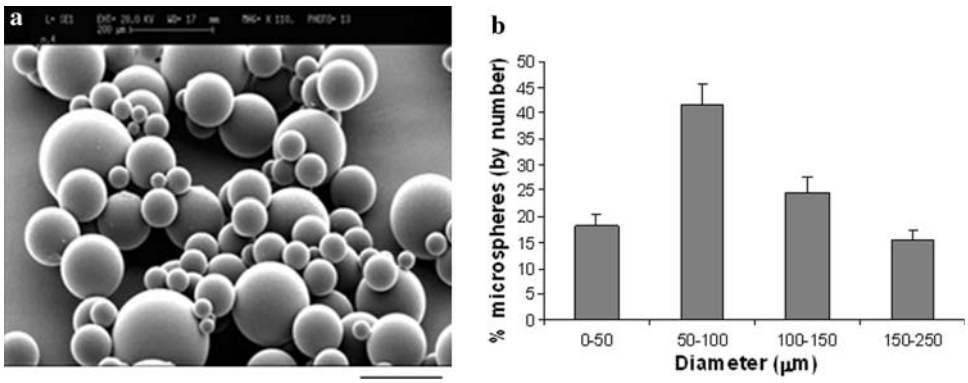

Fig. 4 SEM micrographs of CAB/EP_3 microspheres (**a**). The bar corresponds to 200 μm. Size distribution of CAB/EP_3 microspheres (**b**)

Table 3 Influence of the CAB/TP ratio on microsphere characteristics

Sample	CAB (mg)	TP[a] (mg)	Vitamin B_{12} (mg)	Recovery (%)	Vitamin B_{12} in microspheres (%, w/w)	Encapsulation efficiency (%)	Mean diameter (μm)
CAB/TP_1	250	50	10	97.2 ± 3	3.20 ± 0.05	99.3 ± 1.5	143 ± 51
CAB/TP_2	200	100	10	93.9 ± 5	2.98 ± 0.09	92.5 ± 2.8	114 ± 47
CAB/TP_3	150	150	10	94.2 ± 3	3.15 ± 0.13	97.8 ± 4.0	89 ± 38

Solvent: methanol/acetone = 1 + 1 ml, stirring speed = 300 rpm, temperature = 25°C, reaction time = 10 h

[a] *TP* thermoresponsive polymer

Data are the results of three independent experiments ± SD

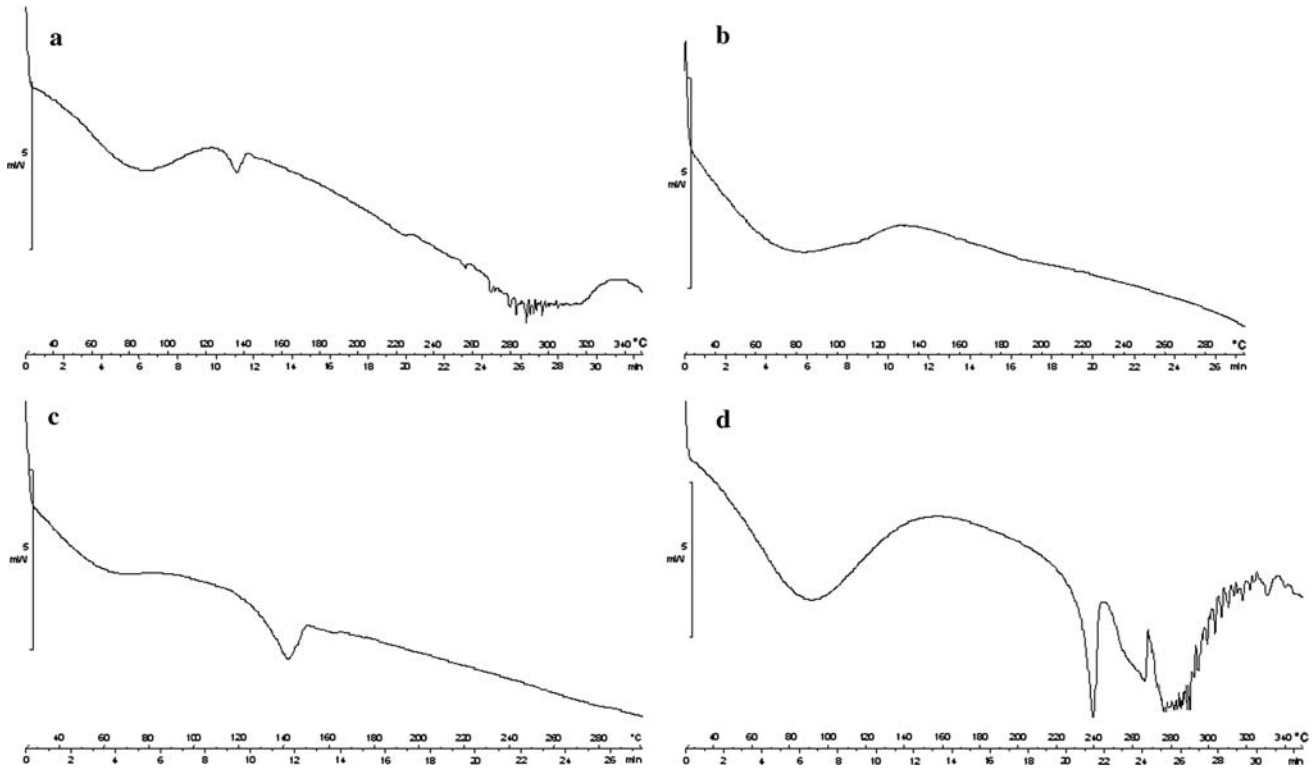

Fig. 5 DSC thermograms of CAB/EP_3 microspheres containing vitamin B_{12} (Table 2) (**a**) and pure EP (**b**), CAB (**c**), and vitamin B_{12} (**d**). Scanning rate = 10°C/min

these two polymers were solubilized in different proportions (see Table 2) in a methanol/acetone mixture and dispersed in paraffin oil containing soybean lecithin as the emulsifier.

By a progressive evaporation of the solvents, the precipitation of the polymer inside the droplets occurred obtaining spherical and discrete microparticles (Fig. 4a).

Table 4 Glass transition temperatures (determined by DSC) of the pure polymers and of the microspheres obtained from polymer mixtures

Code	Sample	Tg_1(°C)[a]	Tg_2(°C)[a]	Note
S#0	CAB	114	–	–
S#1	EP	138	–	–
S#2	CAB/EP	112	137	CAB/EP_3 without vit B_{12}
S#3	CAB/EP +vit B_{12}	113	138	CAB/EP_3 with vit B_{12}
S#4	TP	145	–	–
S#5	CAB/TP	115	146	CAB/TP_3 without vit B_{12}
S#6	CAB/TP +vit B_{12}	115	145	CAB/TP_3 with vit B_{12}

[a] Tg_1, Tg_2 are the glass transition temperatures of the first, respectively the second polymeric component of microspheres

The microspheres were characterized by a large size distribution, the diameter varying between 20 and 250 μm (Fig. 4b).

In order to obtain microspheres with a different internal morphology, affecting the release rate of drugs, the ratio between the two polymers (CAB and SP) was modified. As shown in Table 2, a high percentage of recovery was obtained at different CAB/EP ratios.

When the amount of CAB is smaller than the amount of EP (CAB/EP_4), aggregation occurred, due probably to the sticky nature of EP, therefore the CAB acts also as a support for microsphere building and separation. The encapsulation efficiency of vitamin B_{12} was very high and slightly decreased with increasing the amount of EP. In fact, an increased amount of EP was accompanied by a decrease in the amount of the more viscous CAB. Therefore, the viscosity of the polymer solution decreased ($[\eta]_{CAB} = 58.83 \pm 3.1$ (ml/g) and $[\eta]_{CAB\ +\ EP} = 39.07 \pm 3.6$ (ml/g), where $[\eta]_{CAB}$ and $[\eta]_{CAB\ +\ EP}$ represent the intrinsic viscosities of sole CAB and CAB + EP (1:1, w/w), respectively, dissolved in a mixture of methanol/acetone (1:1, v/v). Consequently the size of microspheres decreased because the higher the viscosity of the polymer solution, the higher the forces that need to be overcome to form fine particles. Smaller microspheres are characterized by a higher contact

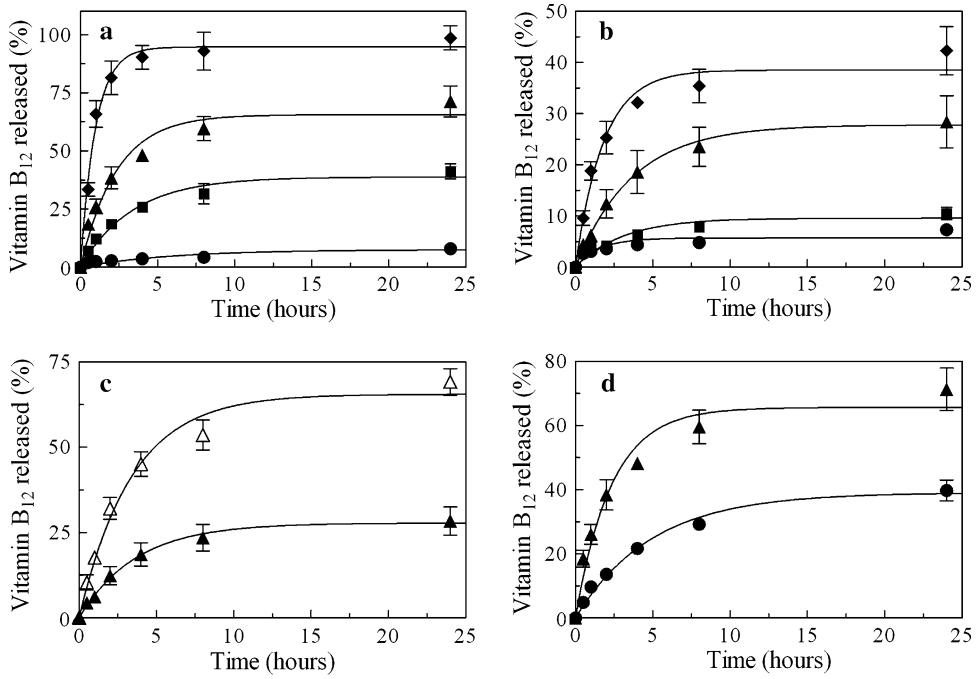

Fig. 6 Time course of vitamin B_{12} release at pH = 7.4 (**a**) and pH = 1.2 (**b**) (37°C) from CAB/EP microspheres with different amounts of EP: 16.6% (*filled square*), 33.3% (*filled triangle*), and 50%, w/w (*filled diamond*). For comparison, the release profile of vitamin B_{12} from sole CAB microspheres is reported (*filled circle*). **c** Time course of vitamin B_{12} release from CAB/EP_2 microspheres at pH = 1.2, and temperature under (20°C; *open triangle*) and above (37°C; *filled triangle*) the LCST. **d** Time course of vitamin B_{12} release from CAB/EP_2 microspheres at pH = 6.8 (*filled circle*) and pH = 7.4 (*filled triangle*) (37°C). The *continuous lines* were calculated according to the following equation: $Y = Y_{max} \times (1 - e^{-kt})$ with values of Y_{max} and k given in Table 5. Y_{max} indicates the percentage of vitamin B_{12} released at time ∞, and k is the first-order rate constant for vitamin B_{12} release

surface with the paraffin oil, and therefore a higher amount of vitamin B_{12} diffuses into the continuous phase. High levels of encapsulation efficiency of vitamin B_{12} were also obtained when poly(NIPAAm-co-AAm) was used in a mixture with CAB (Table 3).

The DSC diagram of CAB/EP_3 microspheres containing vitamin B_{12} (Fig. 5a) shows a water loss at ~90°C, the melting peak of CAB occurring at ~140°C. The lack of the melting point of vitamin B_{12} at 242°C evidenced a molecular dispersion of the vitamin in the matrix of microspheres [21, 22]. Also the DSC diagram of EP (Fig. 5b) showed no melting point suggesting that EP is in the amorphous state in microspheres [22].

In order to determine the compatibility between the two components of the microspheres (CAB with EP and CAB with TP) and the affinity of vitamin B_{12} for the polymeric components, the glass transition temperatures (Tg) of separated polymers and of microspheres in the absence and presence of vitamin B_{12}, were determined [23]. Given the importance of the plasticizing effect of water [24], all the samples were initially heated at 150°C, this allowed the removal of any residual moisture/solvent that could obscure the interpretation of Tg; the results are shown in Table 4.

The presence of two Tg values for the microspheres obtained from a mixture of CAB with EP or with TP (samples S#2 and S#5) demonstrated that there is no interaction between the two components, they precipitated separately during microsphere preparation forming small microdomains (see also SEM micrograph shown in Fig. 7a). The Tg values of these microspheres in the absence and presence of vitamin B_{12} give important information concerning the distribution of the vitamin in one or another polymeric phase. It was found that the interactions between the drug and polymer decrease the Tg of the polymer (plasticizing effect of drug) [25]. The Tg values of the two polymeric components of microspheres were not modified in the presence of vitamin B_{12} when compared to the Tg values of polymeric microspheres obtained in the absence of vitamin B_{12} (pairs S#2, S#3 and S#5, S#6). These results indicate that vitamin B_{12} does not bind selectively one or another polymeric component and it is molecularly dispersed in the whole network of microspheres.

3.3 Release studies

Most of the drug release studies take into account pH- and temperature-responsive polymers in the hydrogel form, as obtained by cross-linking of the linear species [11–13]. The release of entrapped drugs reflects the matrix swelling/shrinkage due to the change of pH or temperature. However, few researchers investigated the solubility and hydrophilic/hydrophobic properties of polymers and drugs for controlled drug release [26, 27].

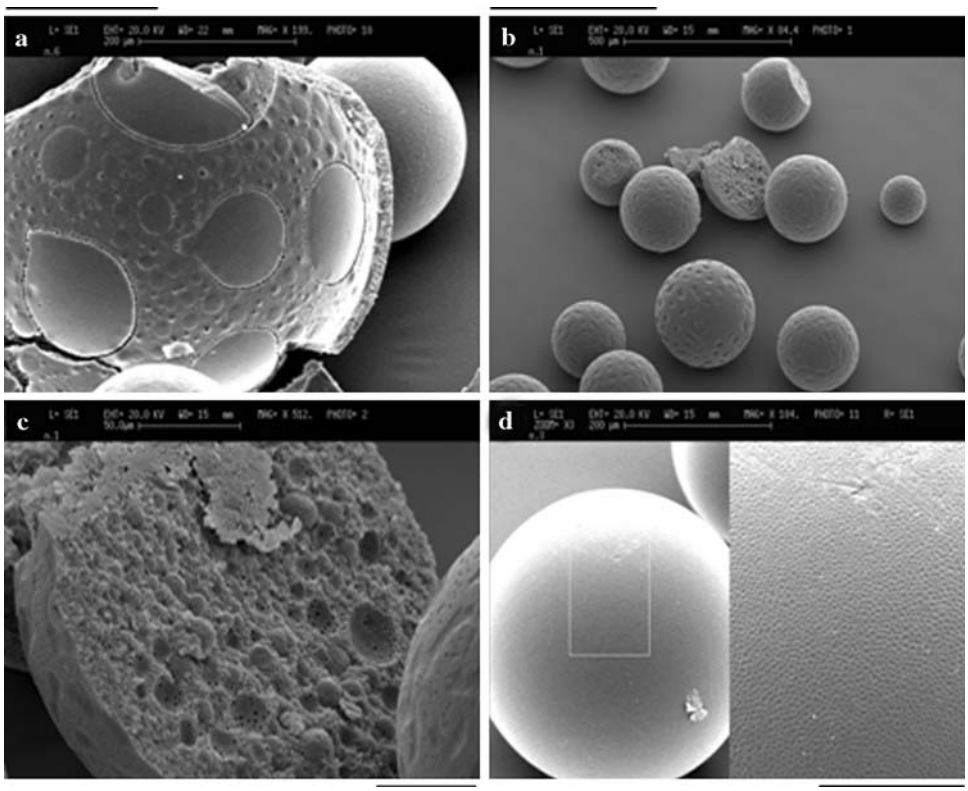

Fig. 7 SEM micrographs of CAB/EP_3 microspheres before (**a**) and after (**b** and **c**) release studies at pH = 7.4. For comparison, a CAB microsphere without EP is shown in **d** (sample CAB in Table 2, after release). The bars correspond to 200, 500, 50 and 200 μm in panel a, b, c and d, respectively

The time courses of vitamin B_{12} release from CAB/EP microspheres, containing different percentage of EP, are shown in Fig. 6.

The inclusion of EP in CAB microspheres increases the hydrophylicity of the whole matrix at temperatures lower than LCST. The release buffer (pH = 7.4) diffuses from the peripheral to the inner part of the microspheres solubilizing EP. Therefore, vitamin B_{12}, that is molecularly dispersed in the matrix as proven by DSC diagrams, diffuses more rapidly from microspheres containing a high amount of EP (Fig. 6a). SEM micrographs taken before the release studies (Fig. 7a) indicate that EP forms small microdomains during the preparation process, leading to a porous inner structure after their dissolution (Fig. 7b, c).

At pH = 1.2 and 37°C, EP is not soluble, therefore the release of vitamin B_{12} reduces (Fig. 6b). When the temperature is lowered to 20°C, EP solubilizes and the percentage of the release of vitamin B_{12} increases (Fig. 6c), even if the diffusion rate is lower at this temperature. In phosphate buffer at pH = 6.8, EP is less soluble than at pH = 7.4 (see Fig. 2), therefore the release rate is lower (Fig. 6d).

Data reported in Fig. 6 and Table 5 indicate that values of the rate constant for vitamin B_{12} release from CAB microspheres (i.e., k) increases with EP % at pH = 7.4.

On the other hand, k is EP-independent at pH = 1.2. Moreover, k is temperature-independent between 20 and 37°C, at pH = 1.2, and pH-independent between pH = 6.8 and 7.4, at 37°C. Furthermore, the total amount of vitamin B_{12} released from CAB microspheres (i.e., indicated by the Y_{max} value at time ∞) increases with EP %, at pH = 1.2 and 7.4 at 37°C. Lastly, the Y_{max} value (at time ∞) increases on lowering the temperature from 37 to 20°C, at pH = 1.2.

The preparation of microspheres by mixing CAB with TP was aimed to modulate the release rate of drugs by a slight change of temperature below and above the body temperature. TP was designed to show a LCST value of ~37°C at pH = 7.4. The time courses of vitamin B_{12} release at temperatures below and above 37°C are reported in Fig. 8.

Below LCST, i.e., at 35°C, TP is in the hydrated state and solubilizes increasing the hydrophylicity of hydrophobic CAB microspheres. Above LCST, i.e., at 39°C, TP looses the water, becoming hydrophobic and insoluble. Therefore, the release rate is higher at lower temperature.

Data reported in Fig. 8 and Table 6 indicate that values of the rate constant and of the molar fraction (at time ∞) for vitamin B_{12} release from CAB microspheres (i.e., k and Y_{max}, respectively) increase with TP %, below and above LCST (i.e., at 35 and 39°C, respectively), at pH = 7.4.

Table 5 Values of Y_{max} and k for vitamin B_{12} release from CAB/EP and CAB microspheres

Microspheres	pH	Temp. (°C)	EP (%)	Y_{max}	k (h^{-1})
CAB/EP_1	7.4	37	16.6	38.7 ± 2.1	0.29 ± 0.4
CAB/EP_2			33.3	65.6 ± 3.8	0.43 ± 0.07
CAB/EP_3			50.0	94.6 ± 2.3	1.0 ± 0.09
CAB			0.0	7.5 ± 1.2	0.17 ± 0.07
CAB/EP_1	1.2	37	16.6	9.6 ± 1.0	0.33 ± 0.10
CAB/EP_2			33.3	27.8 ± 0.7	0.27 ± 0.02
CAB/EP_3			50.0	38.5 ± 1.3	0.55 ± 0.08
CAB			0.0	5.7 ± 0.7	0.65 ± 0.27
CAB/EP_2	1.2	20	33.3	65.6 ± 3.1	0.29 ± 0.04
		37	33.3	27.8 ± 0.7	0.27 ± 0.02
CAB/EP2	6.8	37	33.3	39.1 ± 1.7	0.20 ± 0.02
	7.4	37	33.3	65.6 ± 3.8	0.43 ± 0.07

Values of Y_{max} and k (±SD) (n = 3) were calculated according to the following equation: $Y = Y_{max} \times (1 - e^{-k \times X})$

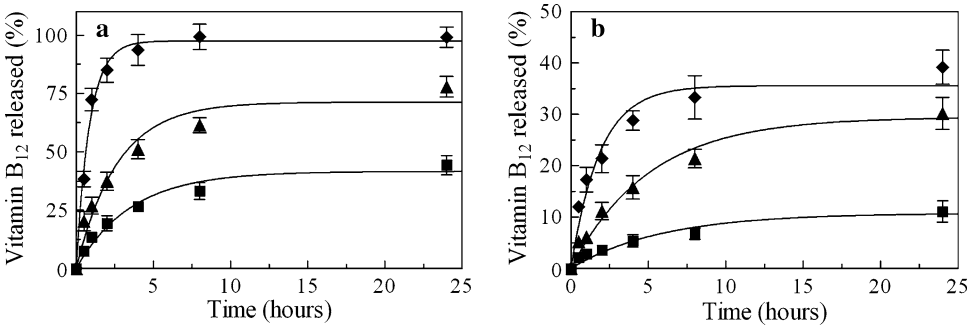

Fig. 8 Time courses of vitamin B_{12} release from CAB/TP microspheres at pH = 7.4, below and above LCST (i.e., at 35°C (**a**) and 39°C (**b**), respectively). The proportion of TP in microspheres is 16.6% (*filled square*) (CAB/TP_1), 33.3% (*filled triangle*) (CAB/TP_2), and 50%, w/w (*filled diamond*) (CAB/TP_3). The continuous lines were calculated according to the following equation: $Y = Y_{max} \times (1 - e^{-kt})$ with values of Y_{max} and k given in Table 6. Y_{max} indicates the percentage of vitamin B_{12} released at time ∞, and k is the first-order rate constant for vitamin B_{12} release

Table 6 Values of Y_{max} and k for vitamin B_{12} release from CAB/TP microspheres at pH 7.4

Temperature (°C)	TP (%)	Y_{max} (%)	k (h^{-1})
35	16.6	41.6 ± 2.7	0.28 ± 0.05
	33.3	71.3 ± 5.0	0.38 ± 0.08
	50.0	97.6 ± 2.2	1.1 ± 0.1
39	16.6	10.8 ± 1.1	0.17 ± 0.04
	33.3	29.5 ± 1.7	0.20 ± 0.03
	50.0	35.6 ± 2.2	0.54 ± 0.10

Values of Y_{max} and k (±SD) (n = 3) were calculated according to the following equation: $Y = Y_{max} \times (1 - e^{-k \times X})$

4 Conclusions

Smart polymers based on poly(NIPAAm-co-MA-co-MM) and poly(NIPAAm-co-AAm) were prepared as new materials with pH-/temperature-sensitive properties. These polymers were mixed with CAB and vitamin B_{12}, taken as a molecular model of drugs, and transformed into microspheres by the oil/oil solvent evaporation method. Vitamin B_{12} was found to be molecularly dispersed in the polymer matrix. The release of vitamin B_{12} was modulated by pH, temperature, and the proportion of the smart polymers in microspheres. Taking into account the vitamin B_{12} release characteristics, poly(NIPAAm-co-MA-co-MM) could be used as an enterosoluble polymer (EP) for drug transport to the intestine, and poly(NIPAAm-co-AAm) as a promoter of drug release by lowering the temperature below 37°C.

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