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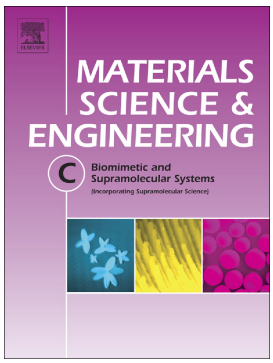

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Smart drug delivery system activated by specific biomolecules

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ABSTRACT

Essentially, the human body can release in different disease conditions specific biomolecules such as histamines when the body encounters a toxic substance, antibodies which are part of the body's natural immune response or nitric oxide as a cardiovascular signalling molecule. Design and development of "intelligent" delivery systems able to release the therapeutic agent only in the presence of bioactive compounds was presented here.

Poly(*N*-isopropylacrylamide-co-N-(3-aminopropyl)methacrylamide)) (poly(NIPAAm-co-APM)) was synthesized as an exciting pH/temperature sensitive copolymer. Under physiological conditions (pH = 7.4), the APM in copolymer is in the ionized state (pK_a = 8.7), highly hydrophilic and therefore the copolymer loses thermosensitive properties. Remarkably, after electrostatic interactions of APM with specific biomolecules, the copolymer restores the thermosensitive property. Thus, the microgels synthesized from this copolymer are in the "inactivated" state at normal physiological pH and temperature (pH = 7.4 and T = 36 ºC). In the presence of specific biomolecules, microgels undergo "activation", shrink and expel mechanically a certain amount of drug. It must be mentioned that the pH-sensitive component plays the role of a biosensor, the biomolecule acts as a triggering agent, and the poly(NIPAAm) represents the delivery component (actuator). MTT tests showed that poly(NIPAAm-co-APM) microspheres are completely devoid of toxicity; moreover, the rabbit dermal fibroblasts vastly adhere to the surface of microspheres.

Keywords: stimuli-sensitive polymer, poly(N-isopropylacrylamide), triggering agent, drug delivery, smart polymer

Abbreviations: AA, ascorbic acid; AIBN, *N,N'*-azobisisobutyronitrile; APM, N-(3-aminopropyl)methacrylamide; CA, cholic acid; CP, cloud point; DPH, diphenhydramine; ESEM, environmental scanning electron microscopy; Fmoc, 9-fluorenylmethoxycarbonyl; GA, glutaraldehyde; Ind, indomethacin; LCST, lower critical solution temperature; NA, nicotinic acid;

NIPAAm, *N*-isopropylacrylamide; PBS, phosphate buffer solution; SA, salicylic acid; VPTT, volume phase transition temperature.

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1. Introduction

The administration of drugs using controlled delivery formulations has represented an immense triumph of researchers and pharmaceutical companies by improving the efficiency of treatments and increasing patient comfort and compliance [1-4]. However, such an approach is neither sufficient nor effective in many clinical situations, including diabetes, heart rhythm disorders, angina pectoris, etc. In these cases, the drug must be given only when normal physiological parameters are disturbed. As a result, self-regulated drug delivery systems were proposed and most of them are based on intelligent polymers [5-7]. These polymers are materials which undergo a phase transition when small changes of the environmental parameters occur. Such parameters could be pH [8], temperature [9], electric or magnetic field [10,11], ionic strength [12], etc. Among these, temperature- and pH-responsive polymers have gained a special place in the area of biomedical applications because they use the variations of the pH and temperature of the human body to trigger the release of medicines [13,14]. Between thermoresponsive polymers, poly(N-isopropylacrylamide) (poly(NIPAAm)) is by far the most used for biomedical applications because in aqueous solution, it display a sharp phase transition at 32-34 ºC [15], a temperature which is close to that of the human body. The transition temperature is called "lower critical solution temperature" (LCST) [16] and "volume phase transition temperature" (VPTT) [17] when refer to linear and cross-linked polymers, respectively. Below the VPTT, the cross-linked hydrogel is in the swollen state while above the VPTT the hydrogel collapse expelling a large amount of water. pH-sensitive linear polymers [18] or cross-linked hydrogels [19] exploit the variation of pH of the fluids in human body. Usually, pH-responsive polymers contain weakly acidic (-COOH) [20] or weakly basic ($-NH_2$) [21] functional groups. The swelling and deswelling processes of pH-responsive hydrogels are the result of the protonation and deprotonation, respectively, of the pH-sensitive units. Notably, the copolymerization of NIPAAm with pH-sensitive monomers leads to copolymers possessing both temperature and pH sensitivity [22,23]. The pH-sensitive moieties can increase or decrease the LCST if they are in the un-protonated or protonated state [24]. However, in the ionized

state, the pH-sensitive co-monomer is more hydrophilic and therefore the copolymer may lose its thermosensitive properties [25]. Remarkably, the ionized co-monomer can interact electrostatically with specific biological molecules restoring the thermoresponsive properties of the copolymer. This behaviour is to some extent outstanding because in many pathological situations, the pH and temperature changes do not occur and therefore these systems are not suitable. Moreover, the human body can release under different pathological conditions specific active compounds such as histamines, antibodies or nitric oxide as a cardiovascular signalling molecule, which can induce the drug release.

Here, the design and development of "intelligent" delivery systems which release the therapeutic agent only in the presence of a bioactive compound is reported.

Poly(*N*-isopropylacrylamide-co-*N*-(3-aminopropyl)methacrylamide)) (poly(NIPAAm-co-APM)) with different co-monomer composition was synthesized as a dual pH/thermosensitive copolymer. Under simulated physiological conditions (phosphate buffer at pH = 7.4), the copolymer with the NIPAAm:APM co-monomer molar ratio of 89:11 is in the ionized state and is highly hydrophilic, therefore it loses thermosensitive properties. However, when the amino groups of the pH-sensitive units interact electrostatically with hydrophobic opposite charged biomolecules, the copolymer restores the thermosensitivity. The copolymer was transformed in microgels by chemical cross-linking of un-protected amino groups with glutaraldehyde. The poly(NIPAAm-co-APM) microgels, due to their reduced size swell but particularly collapse almost instantaneously. Moreover, swelling and collapsing processes occur only in the presence of hydrophobic bioactive drug (indomethacin) taken as model compound. The pH-sensitive amino group of the microgel is the biosensor, the indomethacin is the triggering agent and the thermosensitive poly(NIPAAm) network is the delivery component (i.e., the actuator).

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm), obtained from Aldrich Chemical Corp. (Milwaukee, WI, USA), was recrystallized with hexane. *N*-(3-aminopropyl)methacrylamide hydrochloride (APM·HCl), glutaraldehyde (GA) aqueous solution (25 %, w/v), *N*,*N*'-azobisisobutyronitrile (AIBN), 9-fluorenylmethoxycarbonyl chloride (Fmoc chloride), and methanol were supplied from Fluka AG (Buchs, Switzerland). AIBN was purified in methanol before use. Paraffin oil (d = 0.84 g ml^{-1}) was supplied by Sigma Chemical Co. (St Louis, MO, USA). Soybean lecithin was obtained from Iassyfarm (Iassy, Romania). Indomethacin (Ind), cholic acid (CA), salicylic acid (SA), nicotinic acid (NA), diphenhydramine (DPH), and ascorbic acid (AA) were obtained from Sigma-Aldrich GmbH (Buchs, Switzerland). All chemicals were of analytical or reagent grade and were used without purification unless stated. The following materials and reagents were supplied from Sigma-Aldrich and were used for the biocompatibility tests: Dulbecco's Modified Eagle Medium with Ham/F12 nutrient mix (DMEM/Ham-F12) with 1000 mg mL^{-1} glucose, 110 mg L^{-1} sodium pyruvate and 0.584 mg L^{-1} L-glutamine); Bovine Fetal Serum (BFS), heat inactivated, non-USA origin, sterile-filtered, suitable for cell culture; Penicillin/Streptomycin/Neomycin (P/S/N) solution with 5,000 units penicillin, 5 mg streptomycin and 10 mg neomycin mL^{-1}, sterile-filtered, suitable for cell culture; 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT); 2-propanol; Calcein AM reagent for live/dead assay; DAPI **(**2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; 4′,6-Diamidino-2-phenylindole dihydrochloride); Giemsa reagent.

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

The synthesis of linear poly(NIPAAm-co-APM) was carried out by free radical copolymerization in methanol using AIBN as the initiator. NIPAAm (1.13 g, 10 mmol), APM·HCl (0.179 g, 2 mmol),

and AIBN (0.010 g, 0.06 mmol) were solubilized in methanol (10 mL). Dried nitrogen was purged through the solution for 30 min prior to polymerization. The reaction mixture was allowed to react at 65 °C for 8 hours. The copolymer was precipitated into diethyl ether, and dried under vacuum. Finally, the copolymer was dissolved in 0.1M NaOH solution, dialysed for 5 days at 20 °C against distilled water (molecular weight cut off 10,000-12,000 Da; from Medicell International, London, United Kingdom), and recovered by freeze-drying in the un-protonated form.

2.3. ^{1}H-NMR analysis of poly(NIPAAm-co-APM)

The structure of the copolymer and the co-monomer molar ratio in the copolymer were determined by ^{1}H-NMR analysis. ^{1}H-NMR spectra of poly(NIPAAm-co-APM) were performed in deuterated water on a Varian Mercury Plus 400/Varian VXR 200 spectrometer operating at 400 MHz frequency. The co-monomer molar ratio in the poly(NIPAAm-co-APM) copolymer was calculated according to Equation (1) and (2):

$$\mathbf{x = 1} \qquad \mathbf{(1)}$$

$$\mathbf{4y = 0.49} \qquad \mathbf{(2)}$$

where x and y are the relative molar fractions of NIPAAm and APM, respectively. The first equation describes the peak area at 3.92 ppm of the methynic proton of NIPAAm and the second equation represents the area of the methylene protons of APM (e and f) at 2.72 and 3.20 ppm, respectively.

2.4. Potentiometric titration

Potentiometric titration was performed with an all-purpose Metrohm 716 DMS Titrino apparatus equipped with a dosing unit and a combined glass electrode. Poly(NIPAAm-co-APM) (0.25 g) in the deprotonated form was dissolved in distilled water (70 mL) and then titrated with 0.1 M HCl. The acid dissociation equilibrium constant K_a^{app} for the protonated APM was defined as:

$$\boldsymbol{K_a^{app} = [P]\,(a_H) / [PH^+]} \qquad \mathbf{(3)}$$

where *[P]* and *[PH^+]* represent the concentration of deprotonated and protonated primary amine residues in solution, respectively, and a_H is the activity of bulk protons.

The apparent dissociation equilibrium constant was calculated using the Henderson-Hasselbach Equation (4):

$$pK_a^{app} = pH + log(\alpha /(1-\alpha)) \quad (4)$$

where the degree of protonation α was calculated as the ratio between the equivalents of acid added and equivalents of amine groups in the copolymer sample. The value of the intrinsic dissociation equilibrium constant, pK_a^0, was obtained by extrapolation of $pK_a^{app} = f(\alpha)$ curve at $\alpha = 0$.

2.5. Determination of the lower critical solution temperature

The LCST was determined from the graphic representing dependence of the absorbance at 450 nm on temperature. The experiments were performed on a UV-Vis Specord 200 spectrophotometer (Analytic Jena, Jena, Germany) equipped with a temperature controller. The copolymer solutions (1 %, w/v) were prepared in distilled water, standard acidic solution (pH = 1.2; 64 mmol/L HCl + 50 mmol/L KCl), and standard phosphate buffer solution (PBS) (pH = 7.4; 50 mmol/L NaH_2PO_4 + 39 mmol/L NaOH). The heating rate was fixed at 0.2 or 1 °C every 10 min. The cloud point (CP) was taken as the temperature at which the absorbance reaches a value of 0.5.

2.6. Turbidimetric titration

To a solution of poly(NIPAAm-co-APM) in PBS (25 mL, 10 mg mL^{-1}) were added in small amounts (0.2 mL) 6 mL of Ind solution in PBS (10 mg mL^{-1}). The temperature of the cell was fixed at 36 ºC. After each addition, the solution was left to react for five minutes, and then the transmittance was measured with a Brinkmann PC 900 turbidimeter (USA) equipped with a 20-23-634-5 type fiber optic cell.

2.7. Protecting of copolymer amino groups with Fmoc chloride

Poly(NIPAAm-co-APM) (1 g, 0.94 mmol APM) were solubilized in 14 mL solution of dioxan/water (2/1, v/v) at 60 °C, then Na_2CO_3 (0.027 g, 0.25 mmol) were added (1.1 excess) to neutralize the HCl resulting from the blocking reaction between half of the amine groups of APM and Fmoc chloride. After complete solubilisation of the components, Fmoc chloride (0.164 g, 0.63 mmol; 1.35 excess relative to half of the amine groups) were added and the reaction lasts 6 hours. Finally, the copolymer solution was diluted with distilled water, dialysed for one week against distilled water at 4 °C ((molecular weight cut off 10,000-12,000 Da; from Medicell International, London, United Kingdom) and recovered by lyophilization as poly(NIPAAm-co-APM-co-APM-Fmoc).

2.8. Synthesis of poly(NIPAAm-co-APM-co-APM-Fmoc) microspheres

Copolymer microspheres were synthesized by chemical cross-linking reaction of amine groups of APM with glutaraldehyde. Typically, poly(NIPAAm-co-APM-co-APM-Fmoc) (0.5 g) were solubilized in DMF (3 mL), then 1M H_2SO_4 (0.2 mL) and glutaraldehyde solution (0.25 mL, 25 %, w/v) were added. The resulted solution was dispersed in paraffin oil (50 mL, d = 0.84 g mL^{-1}) containing soybean lecithin (0.25 g) as the dispersing agent. The mixture was stirred at 400 rpm by a three-blade turbine impeller for 20 hours at 45 °C. Finally, the cross-linked microspheres were washed successively with cyclohexane, methanol, water, and methanol, then they were dried from diethyl ether.

2.9. Deprotection of amino groups

Poly(NIPAAm-co-APM-co-APM-Fmoc) microspheres (0.4 g) were dispersed in piperidine solution in DMF (20 mL, 20%, v/v) at room temperature (24 ±1 °C) for 10 hours. Then, the deprotected microspheres were filtered and washed successively with DMF, methanol, water, methanol, and dried from diethyl ether.

2.10. Morphological analysis

The dimension and morphology of the microspheres were evaluated by optical and environmental scanning electron microscopy (ESEM). The morphological modifications of the microspheres below and above the VPTT were recorded by an optical microscope equipped with a digital camera. Before measurements with the optical microscope, the microspheres were swollen in PBS and stained with 0.1% (w/v) Congo Red.

2.11. Swelling degree

The volume change of microspheres, without or with an equimolar amount of Ind, at different temperatures was determined at equilibrium in PBS. The microspheres were placed in a graduated glass cylinder (i.d. = 12 mm) and equilibrated at the desired temperature. The swelling degree (q) was calculated according to Equation (5):

$$q = \frac{V_s}{V_d} \qquad \textbf{(5)}$$

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

2.12. Determination of the volume phase transition temperature

The VPTT of microgels was determined as the inflexion point in the curve *swelling degree-temperature* by Boltzmann fitting of the experimental data [26].

2.13. Swelling/collapsing tests

The swelling kinetic of poly(NIPAAm-co-APM) microgels was measured in PBS in the presence of an equimolar amount of Ind. The microgels were equilibrated at the desired temperature (45°C) in a graduated glass cylinder (i.d. = 12 mm), then the cylinder was transferred into a water bath at 4 °C.

The volume change was monitored periodically. The dynamic swelling factor (q_{ds}) was calculated according to Equation (6) [27].

$$q_{ds} = (V_t - V_d)/(V_{0\ (45\ °C)} - V_d) \quad (6)$$

where $V_{0\ (45\ °C)}$ is the volume of microgels at equilibrium at 45 °C, V_t is the volume of microgels at a given time, and V_d is the volume of dried microgels. To assess the collapsing kinetic, the microspheres equilibrated in PBS at 4 °C were moved into water bath at 45 °C. The dynamic collapsing factor (q_{dc}) was calculated according to Equation (7) [27]:

$$q_{dc} = (V_t - V_d)/(V_{0\ (4\ °C)} - V_d) \quad (7)$$

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

Kinetics of dynamic swelling ratio were calculated according to the "one phase association" Equation (8):

$$Y = Y_0 + (Plateau - Y_0) \times (1 - \exp(-k \times t)) + 1 \quad (8)$$

where *Plateau* is the top asymptote and *k* is the apparent first-order rate constant.

Kinetics of collapsing swelling ratio were calculated according to the "one phase decay" Equation (9):

$$Y = (Y_0 - Plateau) \times \exp(-k \times t) + Plateau \quad (9)$$

where *Plateau* is the bottom asymptote.

2.14. Biocompatibility tests

Cytotoxicity tests were evaluated on primary *Albino Rabbit* dermal fibroblasts, using MTT protocol, by extract method. A quantity of microspheres (50 mg) was sterilized by UV irradiation for 20 min and then mixed with 5 mL DMEM, supplemented with 10% BFS and 1% P/S/N. The microspheres suspension was incubated overnight at 37°C, 5% CO_2 and humidified atmosphere. Both fractions, the incubation medium and swollen microspheres, were used for biocompatibility experiments. The cytotoxicity of the microspheres was evaluated by MTT test, using the

microspheres extract. Thus, primary rabbit dermal fibroblasts were seeded on 48-well culture plates, at 1×10^4 cells/well, in DMEM/Ham-F12 medium supplemented with 10% BFS and 1% P/S/N. The cells were grown at 37°C, 5% CO_2 and humidified atmosphere. After the subconfluency of cells was reached, culture medium was changed with microspheres extract using 1/5, 1/10 and 1/20 extract dilutions. Only culture medium was added in the control wells. The MTT test was performed at 24, 48 and 72 hours of the extract incubation with the cells. Briefly, for MTT test, the culture media from the experimental wells and control were replaced with 500 µL of 0.25 mg mL^{-1} work solution and incubated for 3 hours at 37°C, humidity and dark condition. After the incubation period, the MTT solution from each well was replaced with 500 µL of isopropanol to solubilize the insoluble formazan crystals. The absorbance of the blue formazan solution was measured at 570 nm wavelength, using Tecan UV-Vis plate reader. The cell viability was calculated by normalizing absorbance results for experimental wells to the control, in which cells without any sample were incubated.

Also, the microspheres in direct contact with cells were analysed by fluorescence and phase-contrast microscopy. The microspheres suspension at 2 mg mL^{-1}, 1 mg mL^{-1}, and 0.5 mg mL^{-1} was added to primary rabbit dermal fibroblasts seeded in 6-well culture plates with 3×10^4/well. The cell morphology and cell adhesion on microspheres were analysed at 72 hours of incubation by phase-contrast and fluorescence microscopy. The Giemsa, Calcein-AM and DAPI were used as staining reagents.

2.15. 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 discussion

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

With the aim to increase the LCST towards the body temperature, NIPAAm is usually copolymerized with hydrophilic monomers [28]. If the hydrophilic co-monomer possesses positive or negative charges, the resulted copolymer displays both pH and temperature sensitive characteristics. In this study, NIPAAm was copolymerized with APM in methanol using AIBN as the initiator. ^{1}H-NMR analysis (Fig. 1a) was used to prove the copolymer generation and to determine the copolymer composition.

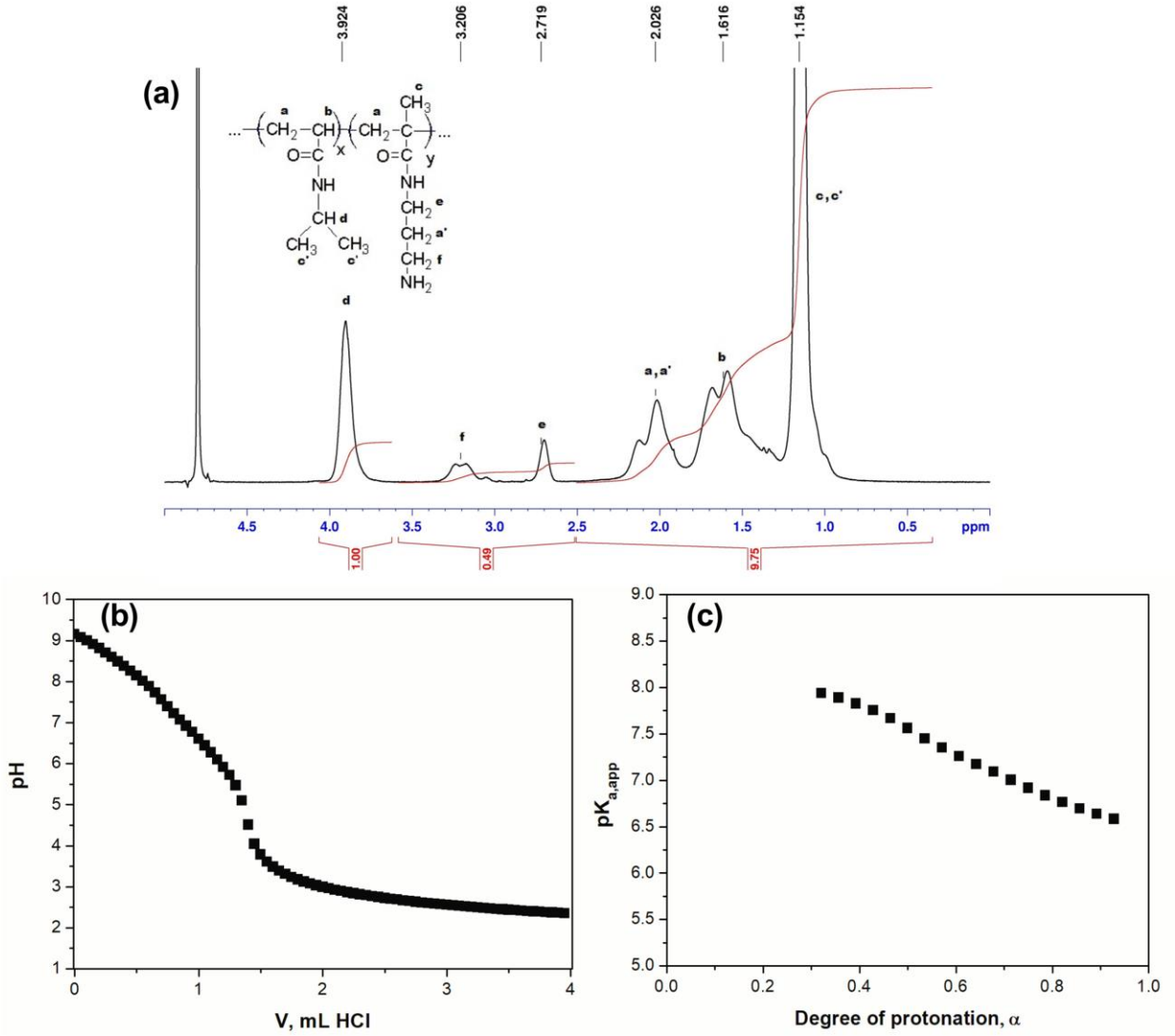

Fig. 1. (a) ^{1}H-NMR spectrum of poly(NIPAAm-co-APM) (sample C_3 in Table 1). (b) Potentiometric titration curve of poly(NIPAAm-co-APM) aqueous solution (sample C_3 in Table 1). (c) Dependence of pK_a^{app} function on α.

As shown in Table 1, the percentage of APM in copolymer is slightly higher than that in the initial mixture (samples C_2 and C_3). By increasing the amount of APM in the initial mixture a little decrease of APM in copolymer was noticed (sample C_4).

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

Sample	Comonomer composition				LCST (°C)		
	In the feed		In copolymer		pH=7.4	pH=1.2	H_2O
	$\times 10^{-3}$ M (% mol ratio)		(% mol ratio)				
	NIPAAm	APM	NIPAAm	APM			
C_0	10	0	100	0	29.2±0.2	30.8±0.3	32.0±0.2
C_1	10 (97.56)	0.25 (2.44)	n.d.[a]	n.d.	32.4±0.3	33.4±0.2	34.2±0.1
C_2	10 (95.24)	0.5 (4.76)	94.34	5.66	34.7±0.2	34.8±0.2	35.5±0.1
C_3	10 (90.91)	1 (9.09)	89.09	10.91	No LCST	n.d.	n.d.
C_4	10 (86.96)	1.5 (13.04)	88.50	11.50	No LCST	n.d.	n.d.

Data are the results of two independent experiments

[a]n.d.= not determined

3.2. Determination of the pK_a and its influence on LCST

Since the copolymer contains a monomer with ionisable amino groups that modulate the thermosensitivity of the system, it was imperious necessary to determine its pK_a. The pK_a value was determined by potentiometric titration of deprotonated poly(NIPAAm-co-APM) (sample C_3) with the HCl solution (Fig. 1b); then, the dependence of the pK_a^{app} on the ionization degree was analysed (Fig. 1c). As shown in Fig. 1c, a value of pK_a of 8.7 was found for APM in copolymer being

smaller than that of parent APM ($pK_a \approx 9.8$). Also, the pK_a values of similar monomers decrease upon polymerization [29,30].

Under simulated physiological conditions (PBS, pH = 7.4; gastric fluid, pH = 1.2) as well as in water, the LCST increases with the increase of the APM percentage in the copolymer (samples C_0, C_1 and C_2 in Table 1). However, at high values of APM, the copolymer loses the thermosensitive properties at physiological temperature (samples C_3 and C_4 in Table 1), due to the increased hydrophilicity of the copolymer. In fact, the hydrophilicity of the copolymer reflects the ionization of APM that occurs below its pK_a. It must be noticed that the LCST of poly(NIPAAm-co-APM) is higher at pH = 1.2 than at pH = 7.4, even though the ionic strength of the simulated gastric fluid is greater ($I_{pH=1.2}$ = 114 mM and $I_{pH=7.4}$ = 89 mM). At low pH, the degree of ionization of amino groups is higher, therefore the copolymer is more hydrophilic and a higher energy (temperature) is required for polymer collapsing. In pure water, the LCST is higher than those determined at pH = 1.2 and 7.4 because the amino groups of APM are protonated (pK_a = 8.7) and the ionic strength is zero.

3.3. Phase transition characterization

LCST is used to characterize the transition point of thermosensitive polymers and is either taken as the inflexion point of the curve obtained by plotting the absorbance versus temperature or as the temperature when the absorbance attains a given value [16,17]. However, the LCST values do not give some important characteristics such as the sharpness of the phase transition and the interval between the onset and the offset temperatures. Therefore, the allures of the curves obtained under simulated physiological conditions at pH = 7.4 are given in Fig. 2.

It is evident a decrease of sharpness of the phase transition and an increase of the interval between the onset and the offset temperatures with increasing the percentage of APM in the copolymer (Fig. 2a). Moreover, the copolymers with high amount of APM (C_3 and C_4) lose the thermosensitive characteristics at physiological pH and temperature. However, extraordinarily, this apparent

disadvantage becomes a benefit since these copolymers could restore their thermosensitivity only after electrostatic interactions of the amino groups of APM with certain biologically active compounds with particular properties (Fig. 2b).

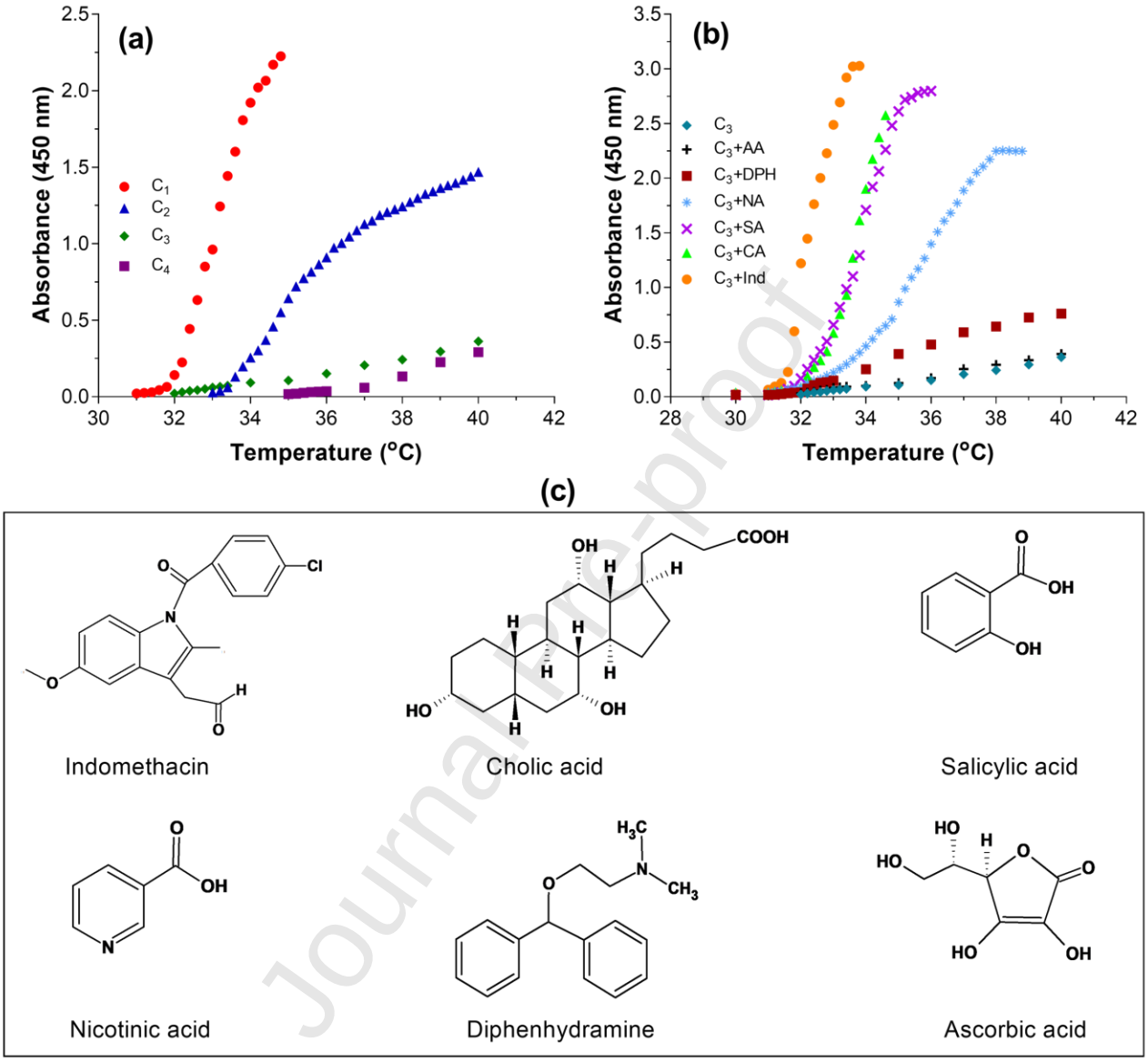

Fig. 2. (a) LCST profiles of poly(NIPAAm-co-APM) with different percentage of APM. (b) LCST profiles of sample C_3 in the absence and in the presence of stoichiometric amounts of different biologically active compounds. The absorbance values are mean of three independent measurements that deviated 0 –7 %. The experiments were performed at pH = 7.4 (PBS). The polymer concentration was 1% (w/v). (c) Chemical structure of bioactive compounds used in this study.

As shown in Fig. 2c and Table 2, Ind displays the highest hydrophobicity (log *P* = 4.27, log *S* = −4.62) and possesses a carboxylic group (pK_a = 4.5) that can interact electrostatically with opposite charged amino groups of APM in the copolymer. After binding, the copolymer restores the thermosensitive properties and displays the sharpest phase transition (Fig. 2b).

Table 2. Physico-chemical properties of biologically active compounds

Property / Drug	pK_a	Log P[a]	Log S[b]
Indomethacin (Na^+) (Ind)	4.5	4.27	−4.62
Cholic acid (Na^+) (CA)	4.98	2.02	−3.37
Salicylic acid (Na^+) (SA)	2.97	2.26	−1.82
Nicotinic acid (Na^+) (NA)	4.75	0.36	−0.84
Diphenhydramine (DPH)	8.98	3.27	−3.5
Ascorbic acid (AA)	4.7	−1.85	0.14

[a]Octanol/water partition coefficient

[b]Aqueous solubility coefficient

In addition, CA (log P = 2.02, log S = −3.37, pK_a = 4.98) and SA (log P = 2.26, log S = −1.82, pK_a = 2.97) are also hydrophobic compounds possessing carboxylic groups (Fig. 2c). After electrostatic interaction, they also restore the thermosensitivity of the copolymer, but the phase transition is not as abrupt as that obtained in the presence of Ind that is more hydrophobic. NA (log P = 0.36, log S = −0.84, pK_a = 4.75) displaying a weak hydrophobicity, restores the thermosensitivity of the copolymer but the phase transition is slow.

Therefore, biologically active compounds with high hydrophobicity and interacting electrostatically with the amino groups of APM (amino groups/carboxylic groups 1:1 molar ratio) in the copolymer are capable to restore the thermosensitive properties of the copolymer. In fact, the "complex"

between the drug and APM is a new co-monomer with a hydrophilic/hydrophobic balance different from that of the "un-complexed" co-monomer. To highlight this aspect, other two biologically active compounds were tested: DPH and AA (Fig. 2c). DPH was chosen because it displays a high hydrophobicity (log P = 3.27, log S = −3.5, pK_a = 8.98) (Table 2) but does not possess acidic groups able to interact electrostatically with the amino groups of APM. On the opposite, AA (log P = −1.85, log S = 0.14, pK_a = 4.7) possesses acidic groups but displays the highest hydrophilicity. After their addition in stoichiometric amounts, both are unable to restore the copolymer thermosensitivity. This exceptional finding could be exploited to produce devices with selective biosensor properties.

3.4. Protecting of the copolymer amino groups with Fmoc chloride

For biomedical applications such as drug delivery systems, the poly(NIPAAm-co-APM) copolymer could be converted to a three-dimensional hydrogel. The hydrogel could be synthesized by chemical cross-linking of the amino groups of the copolymer with chemicals with bi-functional moieties such as glutaraldehyde. However, since the amino groups are responsible for restoring the thermosensitive properties of the copolymer, the most part must be protected during the cross-linking reaction. Since the percentage of amino groups in the copolymer is enough low and they are randomly distributed on the copolymer chain, the cross-linking reaction takes place with difficulties; therefore, high amounts of the cross-linking agent are necessary. However, since most of the amino groups could be blocked with the cross-linker, Fmoc chloride was used as the protecting agent of the amino groups.

The percentage of the amino groups protected with Fmoc chloride was determined from the ratio between integral of the aromatic protons signals (h, h') from Fmoc chloride (divided to the total number of aromatic protons) and the integral of methylene aliphatic protons signals (e, e', f) from APM (divided to the total number of methylene protons) (Fig. 3), according to the Equation (10):

$$\%\text{Fmoc} = \frac{\frac{\int \text{h,h'}}{8}}{\frac{\int \text{e,e',f}}{4}} \times 100 \qquad (10)$$

The reaction parameters were varied in such a manner that at the end of the reaction 35.5 to 52.5% of the amino groups were protected (Fig. 3). The equimolar amount of Fmoc chloride relative to half of the amino groups was used to block half of the total amount of free amino groups but it was not sufficient (35.5 %; Fig. 3b). In contrast, the excess of 1.35 Fmoc chloride leaded to a protection of 52.5 % (Fig. 3c). The resulted copolymer (poly(NIPAAm-co-APM-co-APM-Fmoc)) contained NIPAAm and both free (APM) and blocked amino groups (APM-Fmoc).

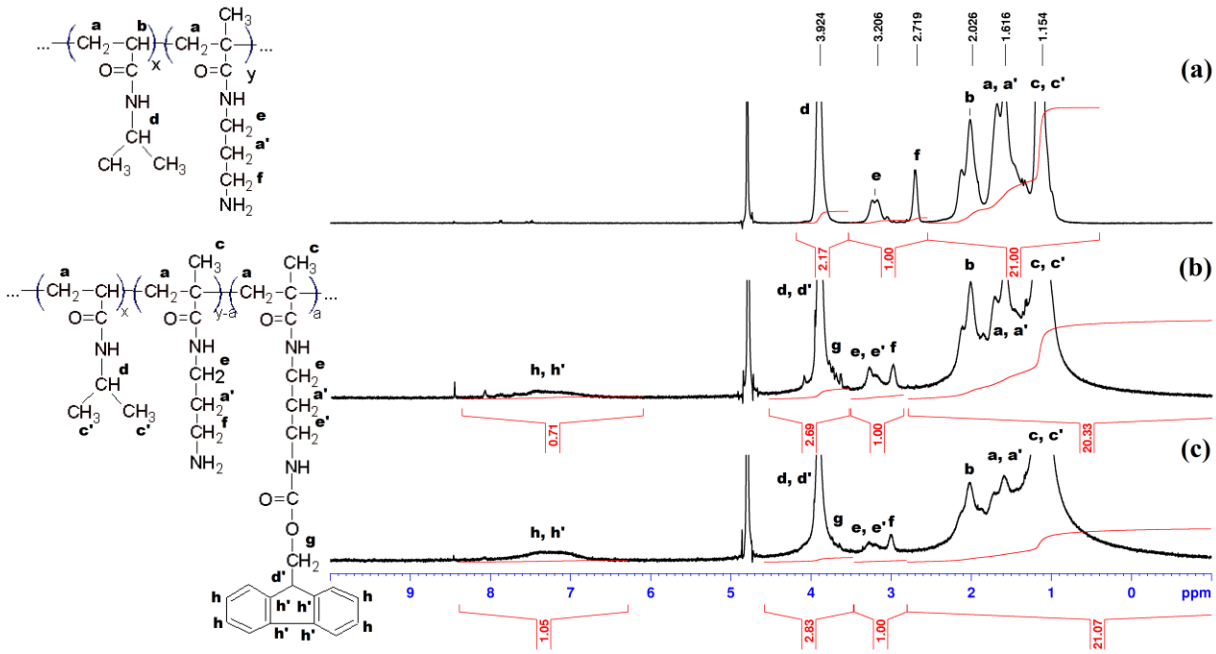

Fig. 3rev. ^{1}H-NMR spectra of poly(NIPAAm-co-APM) before (a) and after protection with either an equimolar amount of Fmoc chloride (b) or with a 1.35 excess of Fmoc chloride (c).

3.5. Synthesis of poly(NIPAAm-co-APM-co-APM-Fmoc) microspheres

Poly(NIPAAm-co-APM-co-APM-Fmoc) microspheres were synthesized by cross-linking of the un-protected amino groups with glutaraldehyde. Usually, the copolymer and the cross-linker are solubilized in acidic aqueous solution, then they are dispersed in an immiscible continuous phase

(paraffin oil) [17]. Interestingly, in this study, DMF was chosen as the solubilizing agent for poly(NIPAAm-co-APM-co-APM-Fmoc). First, after protection with hydrophobic Fmoc, the copolymer has a limited solubility in water. Second, in an aqueous solution, the cross-linking reaction must be performed under the LCST of the copolymer. In concentrated copolymer solution, the LCST decreases to very low temperatures prolonging the reaction time [31]. Moreover, the DMF is not miscible with paraffin oil, allowing a good dispersion of polymer solution. In DMF, the cross-linking reaction has been performed at 45 ºC and thus, the reaction time has been shortened to 20 hours. The resulted microparticles display a round shape, a relative large size distribution (Fig. 4a) and a smooth surface (Fig. 4b). It must be underlined that microspheres are completely un-swellable in aqueous solution due to the presence of hydrophobic F-moc moieties.

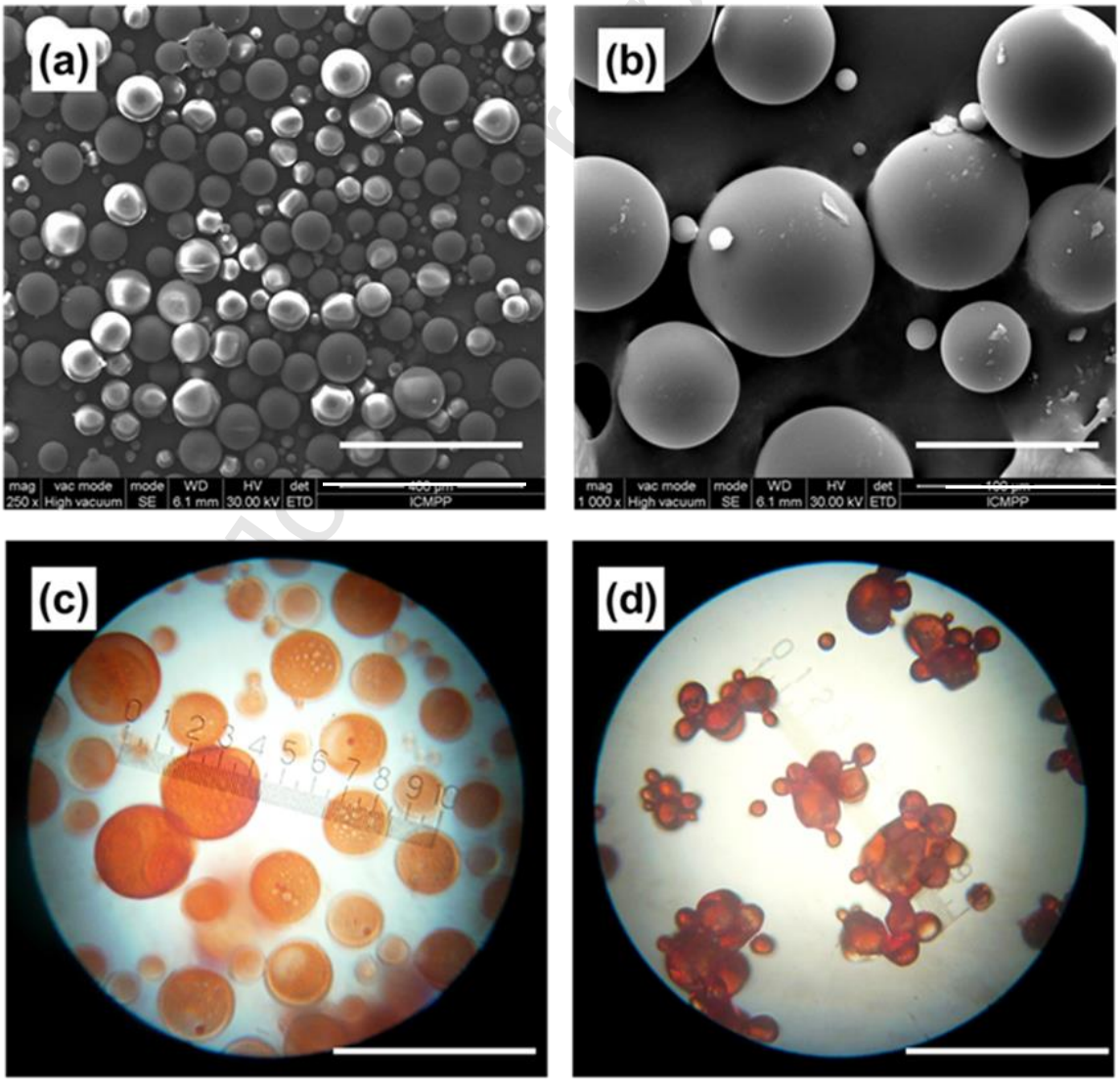

Fig. 4. Scanning electron microscopy of poly(NIPAAm-co-APM-co-APM-Fmoc) microspheres in the dried state: general view (a) and surface details (b). Optical photomicrographs of stained microspheres after the de-protection step, taken in the swollen state at pH = 7.4 (PBS), equilibrated with an equimolar amount of Ind, below the LCST value at 25 ºC (c), and above the LCST value at 45 ºC (d). The bars correspond to 400 μm in panel (a) and (c), 100 μm in panel (b), and 600 μm in panel (d).

As it previously stated, since the free amino groups are responsible for the thermosensitive properties as well as for the swelling characteristics of microspheres, the removal of Fmoc was performed after the cross-linking process. The reaction took place in a solution of piperidine in DMF (20 %, v/v) for 6 hours at room temperature. After de-protection, the amino groups were determined by titration and an exchange capacity of 0.68 meq amino groups/1 gram copolymer was calculated (the starting copolymer (sample C_3 in Table 1) has an exchange capacity of 0.94 meq g^{-1}).

After the de-protection step, the microspheres show significant swelling properties which reflect their porous structure and the loose internal configuration resulting from the removal of water after freezing (Fig. 4c,d).

3.6. Phase transition of microspheres

One of the most important questions after microspheres synthesis is whether they maintain the thermosensitive properties of the linear copolymer under physiological conditions. The temperature at which the phase transition of the swollen microspheres takes place is called "volume phase transition temperature" (VPTT). The VPTT is usually determined as the inflection point of the curve obtained by plotting the weight of the swollen hydrogel at equilibrium against temperature [32]. This method could be applied to large piece of hydrogels but not to microgels containing a large amount of water between them. The VPTT of the microspheres was determined by measuring

the swelling ratio at different temperatures situated below and above the LCST of the linear copolymer. As it was stated, the linear copolymer with 10.91% (mol) APM (sample C_3 in Table 1) does not display thermosensitive properties in the absence of biologically active compounds. As follows, the microspheres were swollen at equilibrium under simulated physiological conditions (PBS at pH = 7.4) and then complexed with a stoichiometric amount of Ind. Finally, the swelling ratio of microgels was determined at different temperatures after the equilibrium has been reached (Fig. 5a).

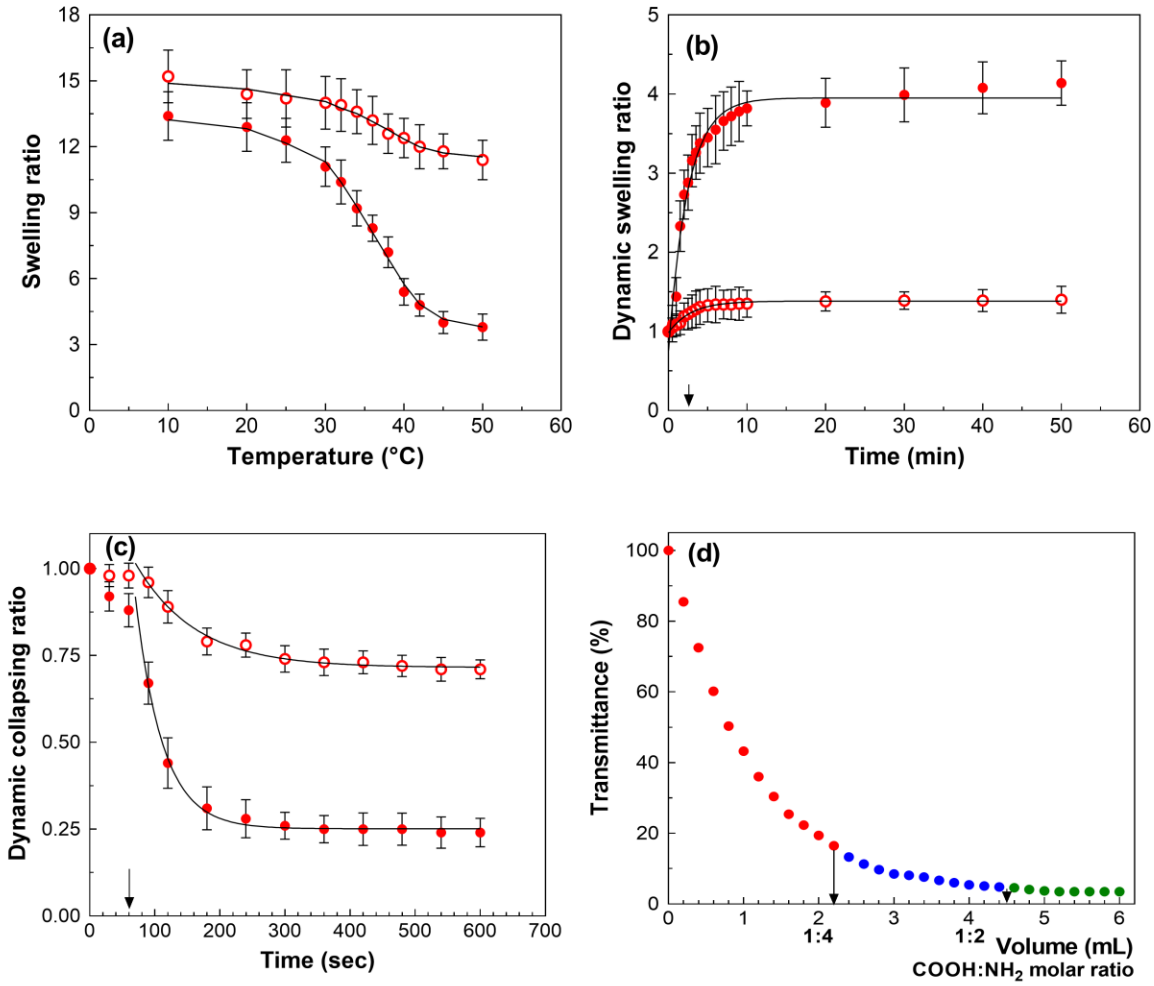

Figure 5. Swelling ratio of poly(NIPAAm-co-APM) microspheres at different temperatures in the absence (empty circles) and in the presence (filled circles) of stoichiometric amounts of Ind (a). Kinetics of swelling (b) and collapsing (c) of poly(NIPAAm-co-APM) microspheres in the absence (empty circles) and in the presence (filled circles) of stoichiometric amount of Ind. The lag time (= 60 s; arrow) corresponds to the time interval needed to change the sample temperature. The continuous lines in Figs 5b and 5c were calculated according to Equations 8 and 9, respectively, with values of Plateau and *k* shown in Table 3. The measurements were performed under simulated

physiological conditions (PBS at pH = 7.4). (d) Turbidimetric titration of poly(NIPAAm-co-APM) linear copolymer with Ind in PBS at 36 ºC.

It must be noticed that a sharp phase transition occurred only for "complexed" microspheres with Ind at a temperature close to the LCST of the linear copolymer. However, a slight decrease of the swelling ratio with the increase of temperature in the absence of Ind was noticed.

For most biomedical applications, the thermoresponsive hydrogels should display high swelling/deswelling rates as response to the small changes of external temperatures. Taking into account the intimate mechanism, the swelling and deswelling processes of thermoresponsive hydrogels are the result of water diffusion within/from polymer matrix. Generally, the diffusion rate of water from a three-dimensional network depends to the size and porosity of the network. Low size and high porous hydrogels allow a rapid diffusion of water and therefore are characterized by high swelling/collapsing rates. The microparticles here synthesized are no larger than 100 μm in the dried state (Fig. 4a,b) and display a very porous structure in the swollen state. However, these characteristics are not sufficient since these microspheres swell and collapse only after electrostatic interactions of amino groups with carboxylic groups of Ind. Therefore, the microparticles were initially equilibrated in PBS in the presence of a stoichiometric amount of Ind and subsequently the swelling/collapsing experiments were performed by changing the temperature from below to above VPTT and vice versa. As shown in Fig. 5, the swelling (Fig. 5b) and deswelling (Fig. 5c) processes occurred relatively quickly after each temperature change. The collapsing process is faster ($k = 0.018\ s^{-1}$, Table 3) than the swelling process ($k = 0.41\ min^{-1}$), because during the microgels collapse a large amount of water is pushed out mechanically from microgels. In fact, the collapsing process takes place almost instantaneously, the delay occurred at the beginning of the process was due to the slower transfer of heat from outer bath to the microspheres in the cylinder. In the absence of the complexing agent, the microgels exhibit low swelling ($k = 0.35\ min^{-1}$) and especially collapsing rates ($k = 0.0083\ s^{-1}$) because they have almost lost their thermosensitivity.

Table 3. Values of kinetic parameters of dynamic swelling ratio and dynamic collapsing ratio of poly(NIPAAm-co-APM) microspheres in the absence and in the presence of Ind (PBS at pH 7.4)

Dynamic swelling ratio			Dynamic collapsing ratio		
	Plateau	k (min^{-1})		*Plateau*	k (s^{-1})
Un-complexed	1.38	0.35	Un-complexed	0.71	0.0083
Complexed[a]	3.95	0.41	Complexed	0.24	0.018

[a]The poly(NIPAAm-co-APM):Ind molar ratio = 1:1

The thermosensitivity tests were performed in the presence of stoichiometric amounts of biologically active compounds, however, these microgels could be also thermosensitive at lower degrees of complexation with bioactive substances. To investigate this hypothesis, a turbidimetric titration of the linear copolymer was performed at 36 ºC. This temperature was chosen because it is situated above the LCST of the polymer:Ind complex (1:1 molar ratio) when the complex is fully opaque, but below the LCST of the un-complexed linear copolymer when it is fully transparent. As it can be seen in Fig. 5d, the gradual addition of Ind determines a progressive reduction of transmittance. Remarkably, a reduction of transmittance with about 85% was achieved only after the complexation with Ind of 25% of free amino groups of copolymer indicating a very high sensitivity. Correspondingly, the microgels obtained from this copolymer collapse almost totally in the presence of merely 25 % of the complexing agent.

3.7. The operation mechanism of the sensor-actuator system

The operating mechanism of the here-reported self-regulated drug delivery system is shown in Fig. 6. Since the pK_a of the copolymer is approx. 8.7, under simulated physiological conditions (PBS at pH = 7.4), the amino groups are protonated, more hydrophilic and consequently the microgels are in the swollen state and lose the thermosensitive properties (Fig. 6a). Under the same conditions (PBS at pH = 7.4), Ind showing pK_a = 4.5 is in the ionized form and highly reactive. After electrostatic

interactions with the amino groups of APM, the hydrogel restores its thermosensitive properties (Fig. 6b). At the human body temperature, the microgels collapse releasing a certain dose of drug, the dose depending to the degree of complexation with Ind (Fig. 6c,d). An animated video showing how this system works is given in the Supplementary Material.

It is evident that the self-regulated drug delivery system developed here is based on a sensor (pH-sensitive amino groups) that could detect the presence of a biologically active compound released from the organism under pathological conditions. The bioactive compound (Ind is taken as model) plays the role of a triggering agent because it triggers the collapsing of the hydrogel after electrostatic interaction with the amino groups. In addition, the thermosensitive hydrogel acts as an actuator since it collapses and expels a certain amount of drug.

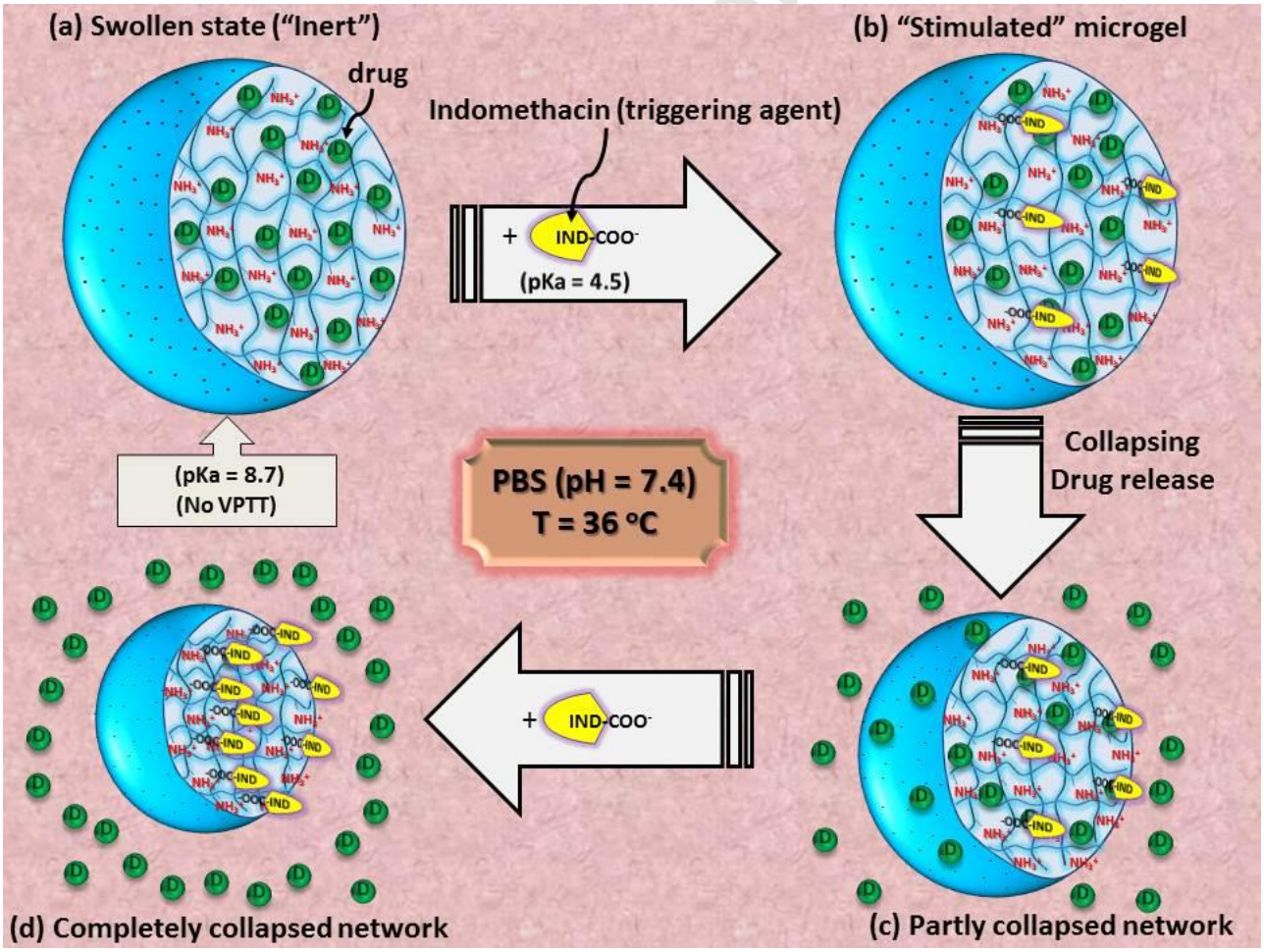

Fig. 6. Schematic representation of the operating mechanism of poly(NIPAAm-co-APM) microgels under simulated physiological conditions: ionized state, no VPTT (a); "complexed" with Ind and "activated" (b); partially (c) and completely (d) collapsed.

3.8. Biocompatibility assay

It is well-known that the cytotoxicity of a biomaterial is highly important for its biomedical applications. In this study, the cytotoxicity of poly(NIPAAm-co-APM) microspheres is investigated by MTT assay. MTT assay, developed for the first time by Mosmann in 1983 [33], is based on the capacity of a mitochondrial dehydrogenation enzyme in viable cells to split the tetrazolim rings of the MTT and form formazane crystals with a dark blue color. Consequently, the amount of the resulted formazane is directly proportional to the number of surviving cells.

The extract resulted from microspheres incubation at different concentration of particles (0.5, 1.0, and 2 mg mL^{-1}) and tested for cytotoxicity through MTT method shows that poly(NIPAAm-co-APM) microspheres are completely devoid of toxicity (Fig. 7a).

The captures (Figure 7b) show the microscopic images of the fibroblasts in the absence (A) and in contact with microspheres (B–F). The living cells from capture A and B (phase contrast) and D (Calcein AM) as well those fixed and stained from capture C (Giemsa staining), E and F (DAPI staining of the cell nuclei) revealed cytocompatible properties of the microspheres. The direct contact of the microspheres with cells does not modify cell shape and morphology as well as their behaviour in culture media (B, C). Moreover, the rabbit dermal fibroblasts adhere in abundance to the surface of microspheres, which is highlighted in the capture E and F.

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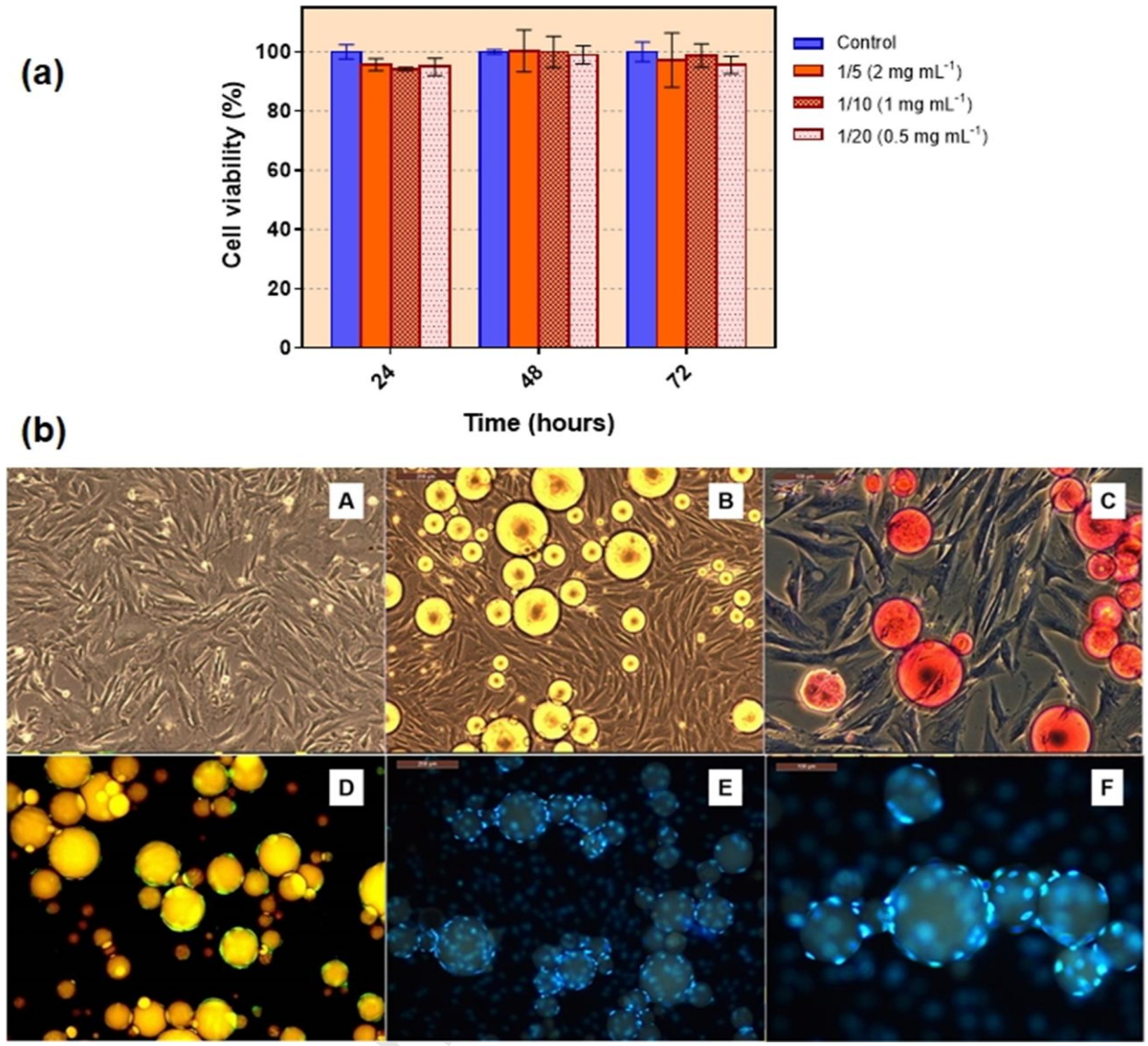

Fig. 7. (a) Viability of the primary rabbit dermal fibroblasts cultured in microspheres extract at different microspheres concentration. (b) Phase-contrast (A,B) and fluorescence micrographs (C, Gielsa; D, Calcein AM; E and F, DAPI) of the dermal fibroblasts without (A) and in the presence of poly(NIPAAm-co-APM) microspheres (B,C,D,E,F).

4. Conclusions

Here, the design and the development of an "intelligent" delivery system releasing the therapeutic agent only in the presence of bioactive compounds are reported. This system does not exploit the change of temperature and pH, but use the normal physiological temperature of the human body to produce the hydrogel collapse. This ternary system involves a triggering agent (bioactive

compound), a sensor capable to interact electrostatically with the bioactive compound (APM) and an actuator (poly(NIPAAm)) that pushes the drug upon collapsing. MTT tests proved that poly(NIPAAm-co-APM) microspheres are not only devoid of toxicity but, on the contrary, the rabbit dermal fibroblasts adhere substantially to their surface. While the basic concepts of the stimuli-sensitive hydrogels are sound, the practical applications require significant improvements in the hydrogel properties. For example, drug loading and drug retention within microgels for a long period of time could be a major drawback. Also, the selectivity of these systems must be substantially improved.

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Conflict of interest

The authors declare that they have no conflict of interest.

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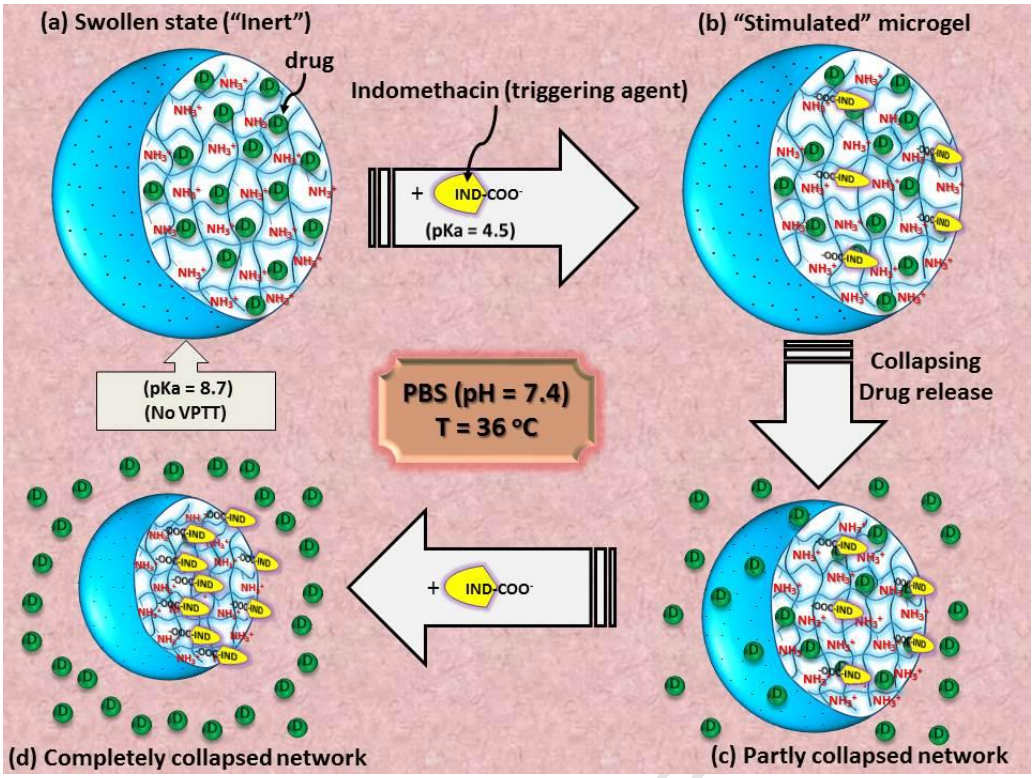

Graphical abstract

Highlights

- A biosensor, an actuator activated by a triggering agent is developed.
- The hydrogel is "inert" at normal physiological conditions (PBS at pH = 7.4).
- The shrinkage of hydrogel is triggered by selected biomolecules.

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