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Research paper

Grafted thermo-responsive gelatin microspheres as delivery systems in triggered drug release

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ABSTRACT

In this paper, a novel class of microspheric hydrogels was synthesized by grafting of *N*-isopropyacrylamide (NIPAAm) with gelatin. The possibility of inserting commercial gelatin in a crosslinked structure bearing thermo-sensitive moieties, by radical process, represents an interesting innovation that significantly improves the device performance, opening new applications in biomedical and pharmaceutical fields. This synthetic approach allows a modification of the polymeric network composition, producing hydrogels with suitable physico-chemical properties and a transition temperature higher than NIPAAm homopolymers. The incorporation of monomers into the network was confirmed by infrared spectroscopy, and the composition of the polymerization feed was found to strictly influence the network density and the shape of hydrogels. Thermal analyses showed negative thermo-responsive behaviour with shrinking/swelling transition values in the temperature range 34.6–34.8 °C, according to the amount of the hydrophilic portions in the network. In order to test the preformed materials as drug carriers, diclofenac sodium salt was loaded into the spherical microparticles. After the determination of the drug entrapment percent, drug release profiles in media at different temperature were analysed. By using semi-empirical equations, the release mechanism was extensively studied and the diffusional contribution was evaluated.

1. Introduction

Gelatin (GL) is a proteic material obtained by hydrolytic degradation of naturally occurring collagen. It derives from the fundamental molecular unit of collagen, a triple helical structure, the tropocollagen rod [1]. Moreover, as gelatin is a byproduct of the meat-processing industry, it is a readily and economically available material, which has been used for decades in various forms in the food and pharmaceutical industries [2]. It is well known for its non-toxic, non-irritant and biodegradable properties, good living body compatibility and, because of its unique gelling properties, is an attractive candidate as starting material for preparing hydrogels [3].

Hydrogels are materials that, when placed in excess water, are able to swell and retain large volumes of water without dissolution. In recent years, smart hydrogels constitute a fast-growing area of polymer science because of the ability to rapidly respond to environmental stimuli, such as pH [4–6], electric current [7] and temperature [8,9]. Temperature is the most widely utilized triggering signal for a variety of triggered or pulsatile drug delivery systems [10–12]. The use of temperature as a signal has been justified by the fact that the body temperature often deviates from the physiological temperature (37 °C) in the presence of pathogens or pyrogens. This deviation sometimes can be a useful stimulus that activates the release of therapeutic agents from various thermo-responsive drug delivery systems for diseases accompanying fever [13]. Among others, hydrogels based on poly(*N*-isopropylacrylamide) (pNIPAAm) belong to the most intensively investigated thermo-reversible systems because of their unique phase transition at a lower critical solution temperature (LCST) in water around 32 °C, which is near the human body temperature [14,15]. The hydrogels based on pNIPAAm exhibit negative thermal response, which means that below its LCST, pNIPAAm chains hydrate to form an expanded structure with a large mesh size enabling the water diffusion, while above its LCST, these chains dehydrate to form a shrunken structure with a small mesh size. The change in the hydration state, which causes the volume phase transition, reflects competing hydrogen bonding properties, where intra- and intermolecular hydrogen bonding of the polymer molecules is favored compared to interaction with water molecules. [16,17]. This thermo-responsive behaviour has been applied in various monolithic

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on-demand drug delivery designs, where the release is determined either by a squeezing mechanism or by entrapment in the collapsed state, followed by enhanced permeation in the swollen state. However, many current thermo-responsive hydrogels based on pNIPAAm have problems in non-biodegradability and non-sustained drug release under physiological conditions. The degradation of the hydrogel matrix can not only circumvent removal of empty device but also be used to modulate drug release for a long period of time. Thus, there is still a need to develop non-toxic biodegradable hydrogels for specific biomedical applications.

As a biomaterial, gelatin displays several advantages: it is a natural polymer that has not shown antigenicity [18], it is completely resorbable *in vivo* and, due to the presence of the large number of functional groups in the side chain, can be easily modified [19,20] to form chemically crosslinked materials [21–25]. Moreover, chemical modification of the gelatin backbone overcomes the drawbacks related to its low stability at body temperature. In literature, a variety of hardening procedures to synthesize gelatin networks via physical (e.g., drying, heating, γ ray, electron beam and UV light exposure) [26–28] and chemical [29] crosslinking methods (e.g., formaldehyde, glutaraldehyde, polyepoxy compounds, tannic acid, dimethyl suberimidate, carbodiimide, acyl azide, transglutaminase and genipin) are reported. In addition, different methods to prepare gelatin thermo-sensitive hydrogels are proposed for tissue engineering [30,31] and drug delivery [32,33] applications. pNIPAAm was conjugated to chemically modified gelatin by radical polymerization to synthesize hydrophilic networks exhibiting LCST immediately below the physiological temperature. These materials can be used in tissue engineering field to induce cell adhesion at 37 °C but incomplete detachment at room temperature [34,35]. Chun et al. proposed the synthesis of a hydrogel-dispersed composite membrane based on pNIPAAm and crosslinked gelatin, and its thermally actuated transport characteristics of 4-acetamidophen were investigated in a diffusion cell [36]. Lee et al. studied the effect of gelatin on the release profile of anionic, cationic and neutral drugs from different organic hybrid gels, based on pNIPAAm and gelatin, crosslinked through a two-step process with genipin or glutaraldehyde [37].

In this study, thermo-responsive microspheres based on commercial gelatin were obtained by graft polymerization of NIPAAm, gelatin and *N*,*N*′-methylenebisacrylamide as crosslinking agent. Graft polymerization is a synthetic methodology to chemically modify of the biomacromolecules for specific applications, by inserting in the same polymer chain functionalities with selected properties, [38]. It is well known that proteins are major targets for photo-oxidation within cells, due to their high abundance, the presence of endogenous chromophores within the protein structure (amino acid side chains), their ability to bind exogenous chromophoric materials and their rapid rates of reaction with other excited state species. In particular, Met, Trp, Tyr, Cys and His residues have been shown to be the most vulnerable to modification by photo-oxidation [39,40]. The residues in the side chains of gelatin able to undergo oxidative modifications represent suitable target groups to prepare composite materials showing both protein and grafted molecule characteristics. The graft copolymerization of gelatin with various monomers is an effective method to improve its properties [41].

The possibility to insert commercial gelatin in a crosslinked structure bearing thermo-sensitive moieties by reverse phase suspension radical polymerization represents an interesting innovation that significantly improves device performance, opening new applications in biomedical and pharmaceutical fields [42]. Moreover, the introduction of hydrophilic moieties (gelatin) on a polymeric backbone bearing hydrophobic portion (the isopropyl groups of the NIPAAm) allows to raise LCST values of the hydrogel close to the body temperature, extending the applicability of the hydrogels. In comparison with pNIPAAm gels, the gelatin-grafted microspheres provide many others advantages, such as increased water content and improved mechanical properties. In addition, hydrogels characterized by a spherical shape are ideal vehicles for many pharmaceutical applications due to their ability to encapsulate a wide variety of drugs and to better modulate the release profile.

The microparticles were characterized by Scanning Electronic Microscopy (SEM), Fourier Transform Infrared spectroscopy, particle size distribution, calorimetric and swelling analyses. In order to verify the suitability of these materials as thermo-responsive devices for drug delivery, a commonly used anti-inflammatory drug, diclofenac sodium salt (DC), was loaded in the polymeric structures. DC is a potent non-steroidal anti-inflammatory drug (NSAID) with analgesic effects and may cause side effects [43]. Thus, the identification of strategies to reduce toxicity and to increase the pharmacological effect of the NSAID may be highly relevant. Several diclofenac carriers formulations have been developed to improve the pharmacological efficiency of the therapeutic reducing the side effects [44]. The release profiles were first evaluated at 25 and 40 °C; furthermore, pulsatile drug release experiments were performed by temperature cycling around the LCST value of the hydrogels to test the reversible thermo-responsive switching behaviour of the materials. Finally, to estimate the diffusional contribute on the delivery of the drug, semi-empirical equations were employed [45,46].

2. Materials and methods

2.1. Materials

Gelatin (Ph Eur, Bloom 160), *N*-isopropylacrylamide (NIPAAm), *N*,*N*′-methylenebisacrylamide (MEBA), sorbitan trioleate (Span 85), polyoxyethylene sorbitan trioleate (Tween 85), *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TMEDA), ammonium persulfate, sodium hydrogen phosphate, disodium hydrogen phosphate, ammonium acetate and diclofenac sodium salt (DC) were provided from Sigma–Aldrich (Sigma Chemical Co, St. Louis, MO). Acetonitrile, methanol and water were from Carlo Erba Reagents (Milan, Italy) and all of HPLC grade. 2-Propanol, ethanol, acetone, glacial acetic acid and diethyl ether were from Carlo Erba Reagents (Milan, Italy) and all of analytical grade. *n*-hexane and chloroform, purchased from Carlo Erba Reagents, were purified by standard procedures.

2.2. Microsphere preparation (standard procedure)

Microspheres based on GL, NIPAAm and MEBA were produced by radical copolymerization technique [42]. Briefly, a mixture of *n*-hexane and chloroform was placed in a round-bottomed cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 30 °C and then treated, after 30 min of N_2 bubbling, with an aqueous solution of GL, NIPAAm, MEBA and ammonium persulfate as radical initiator. The density of the organic phase was adjusted by the addition of chloroform or *n*-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span 85 and Tween 85, then after 10 min with TMEDA, and stirring was continued for another 60 min. The Table 1 reports the experimental conditions of each polymerization reaction. The microparticles were filtered by a sintered glass filter (Ø10 mm; porosity, G3), serially washed with 50 ml of 2-propanol, 50 ml of ethanol, 50 ml of acetone and 50 ml of diethyl ether and dried overnight under vacuum at 40 °C.

Table 1
Experimental conditions for stimuli-responsive microsphere synthesis.

Aqueous dispersed phase			Organic continuous phase	Surfactants mixture	Initiator system	Hydrogel	
GL (mg)	NIPAAm (mg/mmol)	MEBA (mg/mmol)	$CHCl_3$/*n*-hexane (ml/ml)	Span 85/Tween 85 (μl/μl)	$(NH_4)_2S_2O_8$/TMEDA (mg/μl)	mg conv.%	ID
100	400/4.04	120/0.78	16/22	140/30	100/150	503 81%	GM-1
150	400/4.04	120/0.78	17/23	140/30	110/160	487 73%	GM-2
200	400/4.04	120/0.78	16/23	140/30	110/160	500 69%	GM-3

GL = Gelatin, NIPAAm = *N*-Isopropylacrylamide, MEBA = *N*,*N*′-methylenbisacrylamide, Span 85 = sorbitan trioleate, Tween 85 = polyoxyethylene sorbitan trioleate, TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylendiamine. For all polymerizations, the amount of aqueous phase is 2.5 ml.

2.3. FT-IR spectroscopy

Fourier-Transmission IR (FT-IR) spectra of starting monomers and hydrogels were measured as pellets in KBr with a FT-IR spectrophotometer (model Jasco FT-IR 4200) in the wavelength range of 4000–400 cm^{-1}. Signal averages were obtained for 100 scans at a resolution of 1 cm^{-1}.

2.4. Shape and surface morphology

The shape and surface morphology of the microspheres were studied using scanning electron microscopy. The samples were prepared by lightly sprinkling the microsphere powder on a double adhesive tape, which was stuck on aluminum stub. The stubs were then coated with gold to thickness of about 300 Å using a sputter coater and then viewed under scanning electron microscopy (Leo stereoscan 420) and shown in photomicrographs.

2.5. Dimensional distribution

The particle size distribution was carried out using an image processing and analysis system, Leica DMRB equipped with a Leica Wild 3D stereomicroscope. This image processor calculates the particle area and converts it to an equivalent circle diameter.

2.6. Water content of gelatin microspheres

The swelling characteristics were determined to check hydrophilic affinity of microparticles. Briefly, aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted and left to swell by immersing the filter plus support in a beaker containing the swelling media (PBS solution, pH = 7.0, at 25 °C and 40 °C). After 24 h, the excess of water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighted. The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water content percent (WR%) by the following Eq. (1):

$$\mathrm{WR}\ (\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (1)$$

where W_s and W_d are weights of swollen and dried microparticles, respectively. The WR (%) for all prepared materials are reported on Table 1.

2.7. Thermo-behaviour of GL microspheres

The DSC thermograms of microspheres were recorded on Netzsch DSC200 PC, and the LCST values for all polymers are reported on Table 2. In a standard procedure, the sample was immersed in distilled water at room temperature for at least 2 days to reach a swollen state. About 10 mg swollen sample was placed inside a hermetic aluminum pan and then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25 °C to 55 °C on the swollen hydrogel samples under a dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and heating rate 3 °C/min.

2.8. Drug loading by soaking procedure

Incorporation of DC into microspheres was performed as follows: 200 mg of preformed empty microspheres were wetted with 2.0 ml in a concentrated drug solution (10 mg/ml). After 3 days, under slow stirring at 25 °C, the microspheres were filtered and dried at reduced pressure in presence of P_2O_5 to constant weight. The loading efficiency percent (LE %) of all samples was determined by HPLC analysis of filtered solvent according to Eq. (2):

$$\mathrm{LE}\ (\%) = \frac{C_i - C_0}{C_i} \times 100 \quad (2)$$

Here, C_i was the concentration of drug in solution before the loading study and C_0 the concentration of drug in solution after the loading study. The calculated LE (%) values of different copolymers are listed on Table 2. In addition, the drug-loaded percent (DL%) in each matrix was calculated, and the values were listed on Table 2, according to Eq. (3):

$$DL(\%) = \frac{\text{Amount of drug in the beads}}{\text{Amount of beads}} \times 100 \quad (3)$$

2.9. Drug stability at pH 7.0 at 25 and 40°C

The DC stability was studied at pH 7.0 and at different temperatures (25 and 40 °C). Aliquots of drug (10 mg) were incubated at 25 and 40 °C in phosphate buffer solution (PBS) 10^{-3} M at pH 7.0. At scheduled time intervals, corresponding to the condition of the drug release experiments, the samples were withdrawn and assayed by HPLC, in order to determine the drug concentra-

Table 2
Thermal analysis, swelling behaviours and drug loading parameters of hydrophilic stimuli-responsive microspheres.

ID	Calorimetric analysis	Swelling behaviour			Drug loading parameters	
	LCST (°C)	WR_{25} (%)	WR_{40} (%)	S_r	LE (%)	DL (%)
GM-1	34.7	250 ± 4	53 ± 2	4.7	99.5 ± 0.2	9.9 ± 0.1
GM-2	34.6	194 ± 3	79 ± 3	2.5	98.5 ± 0.9	9.8 ± 0.2
GM-3	34.8	171 ± 4	71 ± 3	2.3	99.2 ± 0.4	9.9 ± 0.1

LCST = lower critical solution temperature, WR_{25} and WR_{40} = water content percent at 25 and 40 °C, S_r = swelling ration WR_{25}/WR_{40}, LE = loading efficiency, DL = drug loading.

tion. The High-Pressure Liquid Chromatography (HPLC) conditions were a mixture of aqueous solution of ammonium acetate, methanol and acetonitrile (40/30/30, v/v/v). The pH of the aqueous mobile phase portion of ammonium acetate buffer (pH 7.0, 10^{-3} M) was adjusted with glacial acetic acid. The mobile phase was filtered, degassed and pumped isocratically at a flow rate of 0.6 mL min^{-1}; UV detection at 284 nm [47]. The HPLC analyses were carried out using a Jasco PU-2080 liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μl loop), a Jasco UV-2075 HPLC detector and Jasco-Borwin integrator. A reversed-phase C18 column (μBondapak, 10 μm of 150 × 4.6 mm internal diameter obtained from Waters) was used. Retention time 4.2 min; Limit of Detection (LOD) 0.7 μM; Limit of Quantification (LOQ) 14 μM.

2.10. In vitro release studies at 25 °C and 40 °C

Release studies were carried out using the dissolution method described in the USP XXIV (Apparatus 1-basket stirring element). Aliquots (10 mg) of drug-loaded microparticles were dispersed in flasks containing PBS solution (pH 7.0) and maintained at 25.0 ± 0.1 and 40.0 ± 0.1 °C in a water bath. At suitable time intervals, an aliquot of the release medium was withdrawn, filtered (Iso-Disc™ Filters PTFE 25-4 25 mm × 0.45 μm, Supelco) and the solutions were analysed by HPLC.

2.11. In vitro pulsatile drug release from 25 °C to 40 °C

Oscillatory drug release profile of the materials was investigated by immersing the microspheres in a solution at pH 7.0 (10^{-3} M, phosphate saline buffer). The dissolution tube was alternatively placed in water baths thermostated at 25 and 40 °C, and, at suitable time intervals, an aliquot of the release medium was withdrawn, filtered (Iso-Disc™ Filters PTFE 25-4 25 mm × 0.45 μm, Supelco) and the solutions were analysed by HPLC. The release period was extended over several cycles until no further drug was released (5 h). Two different experiments were performed: the first starting from 25 °C and the second from 40 °C. The larger temperature difference was used to help increase the speed of collapse, since DC is a small molecule.

2.12. Statistical analysis

All of the experiments were done in triplicate, and the results were in agreement within ±5% standard error. One-way analysis of variance was performed to assess the significance of the differences among data. Turkey–Kramer post-test was used to compare the means of different treatment data. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis of gelatin-grafted microspheres

Gelatin is a mixture of high molecular weight and water-soluble proteins extensively used in food, adhesives and pharmaceutical fields. Because of the various potential uses of gelatin, it is useful to investigate its modification to develop new materials with improved properties. The graft copolymerization of gelatin with various monomers is an effective method to improve the properties of the protein. The residues in the side chains of gelatin, in particular Met, Trp, Tyr, Cys and His residues, represent suitable target groups to prepare composite materials showing both protein and grafted molecule characteristics because of their susceptibility to undergo oxidative modifications.

Grafting reaction was carried out by chemical means, and the initiators producing free-radical species after warming at 40 °C, such as ceric ammonium nitrate, ammonium or potassium persulfate (KPS), were employed. In this study, free-radical suspension polymerization was employed as polymerization technique to covalent insert NIPAAm and gelatin in a polymeric network. A possible mechanism of NIPAAm and gelatin grafting by means of KPS-initiated radical polymerization is proposed in Fig. 1. After thermal dissociation of the initiator, the formed anionic radicals attack H-atoms in hydroxyl, thiolic or amino groups in the side chain of gelatin, forming a macroradical with several active sites. At those sites, polymer chain of NIPAAm starts and propagates as regular radical polymerization of polyacrylates. Varying the amount of gelatin in the polymerization feed, three different hydrogels were prepared (Table 1), and the optimization of the polymerization method required several attempts to produce devices showing a spherical shape. It was observed that hydrophilic/lipophilic bal-

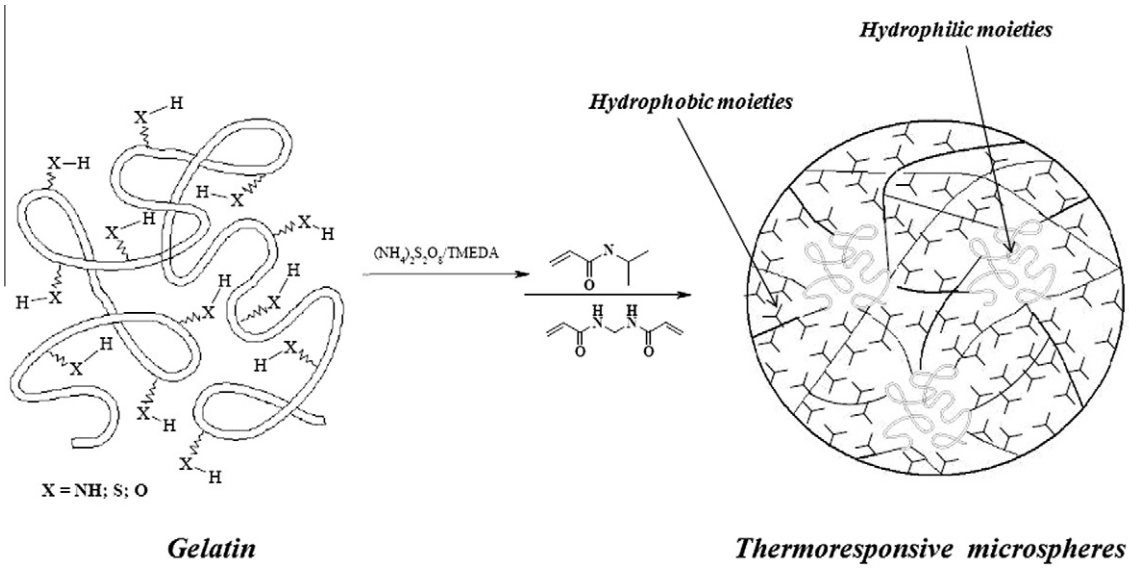

Fig. 1. Schematic representation of the free-radical grafting polymerization.

ance (HLB) of surfactants is important. Many tests were carried out to determine the correct ratio of Span 85 (HLB = 1.8) and Tween 85 (HLB = 11). Finally, a system with a total HLB = 3.4 was able to stabilize the aqueous dispersed phase.

3.2. Characterization of gelatin microspheres

The materials were characterized by FT-IR spectrophotometry, swelling behaviour, particle size distribution, morphological and calorimetric analyses.

The FT-IR spectra of all samples showed the disappearance of bands at 944 and 921 cm^{-1} ascribable to carbon–carbon double bond of NIPAAm and the appearance of the typical absorption bands of the commercial gelatin. In particular, the peaks at 3450 and 3423 cm^{-1} due to N–H stretching of the secondary amide, carbonyl groups stretching at 1680 and 1640 cm^{-1}, N–H bending between 1550 and 1500 cm^{-1}, N–H out of plane wagging at 670 cm^{-1} and C–H stretching at 2922 and 2850 cm^{-1} are visible in the spectra of the hydrogels.

The thermo-responsive characteristics of the hydrogels were tested by studying their swelling behaviour. The values of contained water percentages were determined in aqueous media (PBS solution pH = 7.0, 10^{-3} M) at 25 °C and 40 °C, respectively. The data, reported in Table 2, illustrate the water uptake (WR (%)) at different temperature, in grams per gram of dry copolymer, for each studied composition. In addition, to better highlight the thermo-responsive behaviour of the hydrogels, the S_r parameter was introduced and calculated as the ratio between the WR (%) value at 25 °C and 40 °C. The presence of pendant hydrophobic groups in the polymeric chains produced a different water affinity of the hydrogels at 25 °C and 40 °C. In particular, at 40 °C, a considerable lowering of the water content was observed, due to the predominance of the hydrophobic interactions between the hydrocarbon moieties on the polymeric chains, which causes the solvent diffusion outside the polymeric network. When the temperature decreases to 25 °C, the water affinity of the microparticles is improved, and the swelling degree is greater according to the gelatin amount in the polymerization feeds: this behaviour can be ascribable to the enhanced crosslinking density of the hydrogels, due to the increased gelatin content. In particular, for the sample GM-1, the WR (%) changed from 53% to 250% (S_r = 4.7) when the temperature decreases from 40 °C to a temperature below the LCST of the hydrogel. Increasing the amount of gelatin in the polymerization feed, the S_r values tend to be lower (2.5 and 2.3 for GM-2 and GM-3, respectively) as a consequence of reduced WR (%) difference between the experimental temperatures. Thus, it can be assumed that the effect of the hydrogel crosslinking degree is predominant with respect to the hydrophilic/hydrophobic balance in the network.

The shape and the surface of the microparticles were evaluated by using scanning electron microscopy. In particular, Fig. 2A showed the spherical shape of sample GM-2, while in Fig. 2B, the high porosity of the outside surface of GM-1 is evident. Similar results were obtained for all samples, suggesting their potential use as drug delivery devices. The spherical shape, indeed, allows the elimination of the anisotropic swelling normally associated with others geometries, while the micropores facilitate drug diffusion through the polymeric network.

The diameter of the hydrogels was in the dimensional range 80–100 μm for GM-1, 90–120 μm for GM-2 and 110–130 μm for GM-3. The microsphere diameters were strictly dependent on the crosslinker amount in the polymeric networks; the values of mean diameter, in general, decrease as the crosslink density increases.

The DSC thermograms reported in Fig. 3 showed the LCST values of GM polymers. The hydrogels GM-1, GM-2 and GM-3 showed LCST values ranged from 34.6 to 34.8 °C, higher than pNIPAAm homopolymer (Table 2). The different GL contents in the hydrogels did not significantly modify the LCST because the greater amount of GL in the network enhances the crosslinking density of the microparticles. Thus, although the gelatin moieties increase the hydrophilic properties of polymeric network, the crosslinking degree opposes to the swelling of the matrices, decreasing the freedom degrees of the polymeric chains. Thus, the gelatin moieties did not provide enough interactions to inhibit the collapse and entanglement of NIPAAm networks in the hydrogels when the temperature raises the LCST.

3.3. In vitro release studies

Thermally responsive drug delivery systems have attracted ever-increasing attention because they can control the release of drug in response to change in body temperature and therefore act as self-regulating systems. In order to estimate the potential application of prepared matrices as drug delivery devices, the

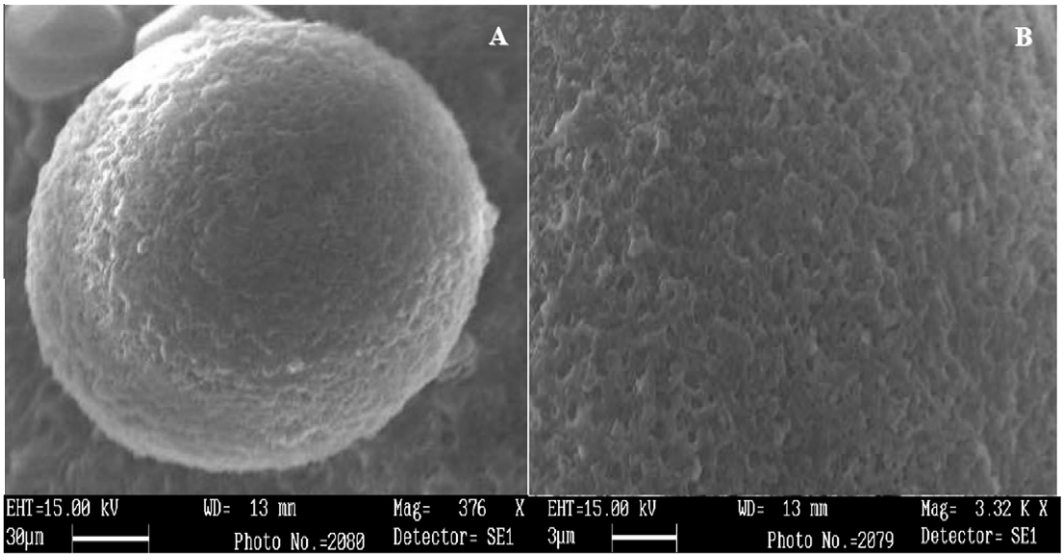

Fig. 2. SEM micrographs of GM-2 (A) and GM-1 outside surface (B).

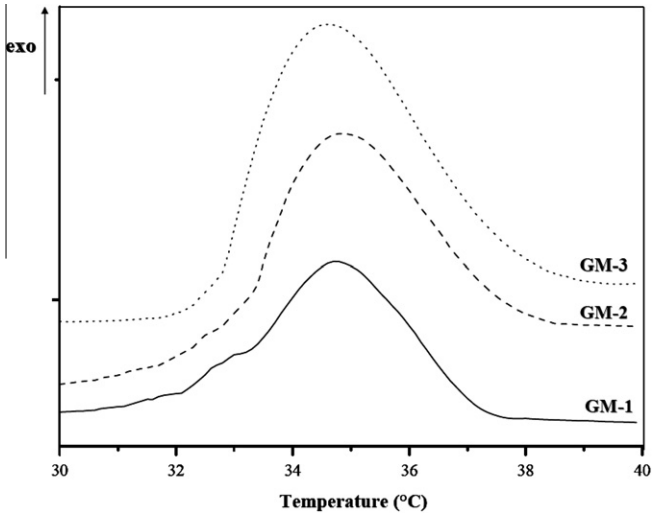

Fig. 3. DSC thermograms of the swollen stimuli-responsive hydrogels GM-1 (—), GM-2 (- - -) and GM-3 (···) at a heating rate of 3 °C/min (the temperatures at the maximum points of the exotherms were referred as volume phase transition temperature of the hydrogels).

microparticles were loaded with one of the most commonly used anti-inflammatory drugs, diclofenac sodium salt (DC), by soaking procedure, and the loading efficiency of all samples (LE %) was determined by HPLC analysis (Table 2). The DC was loaded in the microparticles with a LE (%) > 98% for all grafted hydrogels. DC release experiments were carried out in PBS solution (pH 7.0, 10^{-3} M) at 25 and 40 °C, and the amount of drug released was expressed as drug delivered (M_t) related to the effectively entrapped total mass (M_0), as a function of time. As reported in Fig. 4, for all samples and at each experimental time, at 40 °C, a higher amount of drug molecules diffuses through the network in the surrounding media with respect to 25 °C. In addition, at 25 °C, a slow release was observed, while at 40 °C, a significant burst effect after 30 min was recorded. It has been suggested that burst release is mainly caused by drug present on the surface of microspheres [48,49]. Clearly, this is not the case here. The major cause of burst release is the rapid collapse of the hydrogels from swelled to collapsed state.

Since the microparticles have a well-defined geometry and a narrow dimensional distribution, we determined the mechanism of drug release (Fickian or non-Fickian). In particular, the kinetics

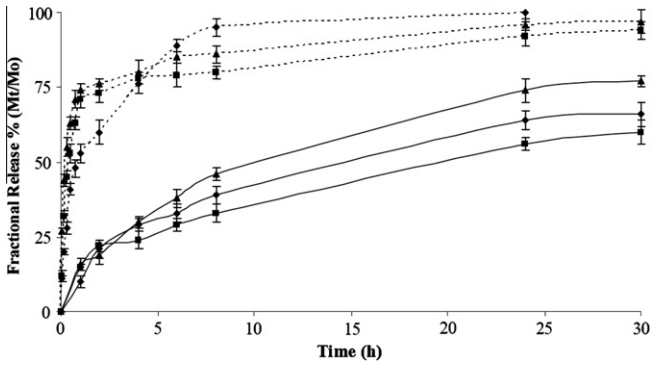

Fig. 4. Drug release expressed as percent of DC delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for microspheres GM-1 (♦), GM-2 (■) and GM-3 (▲) at 25 °C (solid lines) and 40 °C (dashed lines) and at pH 7.0 (PBS solution 10^{-3} M).

of DC release at 25 °C, under LCST value, was analysed by the semi-empirical Eq. (4) for $M_t/M_0 \leqslant 0.6$. [45]

$$\frac{M_t}{M_0} = Kt^n \tag{4}$$

where M_t/M_0 is the drug fraction released at time t and K and n are a constant and the kinetic exponent of drug release, respectively. Although the use of this equation requires detailed statistical analysis, the calculated exponent, n, gives an indication of the release kinetics. If $n = 0.43$, the drug diffuses and releases out of the polymer matrix following a Fickian diffusion. For $n > 0.43$, an anomalous or non-Fickian type drug diffusion occurs. If $n = 0.85$, a completely non-Fickian or Case II release kinetics is operative. The intermediary values ranging between 0.43 and 0.85 are attributed to anomalous type diffusive transport. The least-squares estimations of the fractional release data along with the estimated correlation coefficient values, r, are presented in Table 3. As the results shown in Table 3, in the experiments at 25 °C, the exponents n ranges from 0.43 to 0.58, indicating that the polymers at this temperature mainly followed a Fickian diffusion way.

A more informative analysis can be obtained by fitting the data with the model proposed by Peppas and Sahlin. [46] The equation for this model is as follows:

$$\frac{M_t}{M_0} = K_1 \cdot^{1/2} + K_2 \cdot t \tag{5}$$

with $M_t/M_0 \leqslant 0.95$. In this equation, the first term is the Fickian contribution and the second term is the Case II relaxational contribution. Table 3 reports K_1 and K_2 values according to Eq. (5). For all investigated samples, the term $K_1 t^{1/2}$ is greater than the term $K_2 t$, indicating that the predominant mechanism, at 25 °C, for DC release is the Fickian diffusion through the swollen microparticles. Thus, the drug release was determined by two factors, the swelling rate of polymer and the diffusivity of the drug through the network. Because, at the same temperature, there are no marked differences of the diffusivity of drug in each polymer, the swelling rate of the polymer was the dominating factor. When the dried gels are placed in the release media, water molecules diffuse into the gel network and the matrix is swelled. At the same time, drug molecules diffuse through the gel layer and enter in the medium.

When the polymers were at 40 °C, above their LCST, the release profile of DC is affected not only by the diffusion of the drug through the polymer network, but also by the squeezing effect of the swelled polymer. Thus, the hydrogels probably release the drug by a combination of convective forces of the drug out of the hydrogel due to physical collapse of the hydrogel as well as to diffusive forces. The n values for all samples are between 0.35 and 0.48, indicating a prevalent Fickian diffusion transport for all polymers. Nevertheless, fitting the experimental data using the Peppas–Sahlin equation, more detailed information can be obtained; in particular, at 40 °C, it is possible to observe a greater contribution due to K_2 (non-Fickian release) respect to the fitting at 25 °C, as a consequence of the reduced role of the diffusional component in the drug release mechanism.

3.4. Pulsatile drug release experiments

The reversible volume phase transition of the hydrogels was evaluated cyclically modifying the medium temperature and determining the drug release in each experimental condition. Pulsatile devices may have many applications in areas of medicine where a constant rate of drug release does not match the physiological requirements of the body. To demonstrate reversible on/off-switching of DC release, the microspheres were repeatedly heated above and cooled below their LCST in in vitro pulsatile release experiments, and the release profiles were reported in

Table 3
Release kinetics parameters of different formulations.

ID	$\frac{M_t}{M_0} = Kt^n$ (6)					
	$K \times 10^3$ (min^{-n})		n		r	
	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
GM-1	14.15 ± 0.99	9.26 ± 2.01	0.47 ± 0.03	0.41 ± 0.05	0.99	0.94
GM-2	14.15 ± 0.98	10.24 ± 0.97	0.43 ± 0.03	0.48 ± 0.03	0.98	0.99
GM-3	13.59 ± 0.10	20.06 ± 2.60	0.58 ± 0.02	0.35 ± 0.04	0.98	0.97
	$\frac{M_t}{M_0} = K_1 \cdot t^{1/2} + K_2 \cdot t$ (7)					
	$K_1 \times 10^3$ ($min^{-1/2}$)		$K_2 \times 10^3 s(min^{-1})$		r	
	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
GM-1	13.67 ± 0.83	7.50 ± 0.33	−0.01 ± 0.17	−0.15 ± 0.02	0.99	0.99
GM-2	12.46 ± 1.21	11.00 ± 0.49	−0.05 ± 0.26	−0.35 ± 0.03	0.98	0.96
GM-3	15.05 ± 0.74	12.68 ± 0.93	−0.10 ± 0.16	−0.42 ± 0.05	0.99	0.86

Fig. 5, as a function of the temperature cycling at fixed pH value (PBS solution 10^{-3} M, pH 7.0). In these conditions, the release profiles during 5 h were found to have good sustaining efficacy, and the effect of temperature cycling on drug release may reflect a response rate to various environments. The experiments were started placing the samples both in a swelling (25 °C) and in a collapsed (40 °C) state and recording the amount of DC released, expressed as M_t/M_0 percent, in the surrounding environment after each temperature change.

For all samples, it can be observed a higher DC amount released at 40 versus 25 °C. This behaviour is due to the strong hydrophobic interactions between the isopropyl groups of NIPAAm moieties, as the temperature reached the LCST, so that a significant amount of aqueous drug solution was dispelled from the collapsed hydrogels. As a consequence, exposing the DC-loaded samples to a temperature of 40 °C, a significant burst effect was observed for all samples, with M_t/M_0 percent values ranged from 45.0 to 60.0 after 30 min. These percentages decrease to values between 11.0 and 18.0 when the microspheres are placed to a temperature of 25 °C. The results showed that the hydrogels based on gelatin can be used to obtain an effective modulation of the release rate of DC.

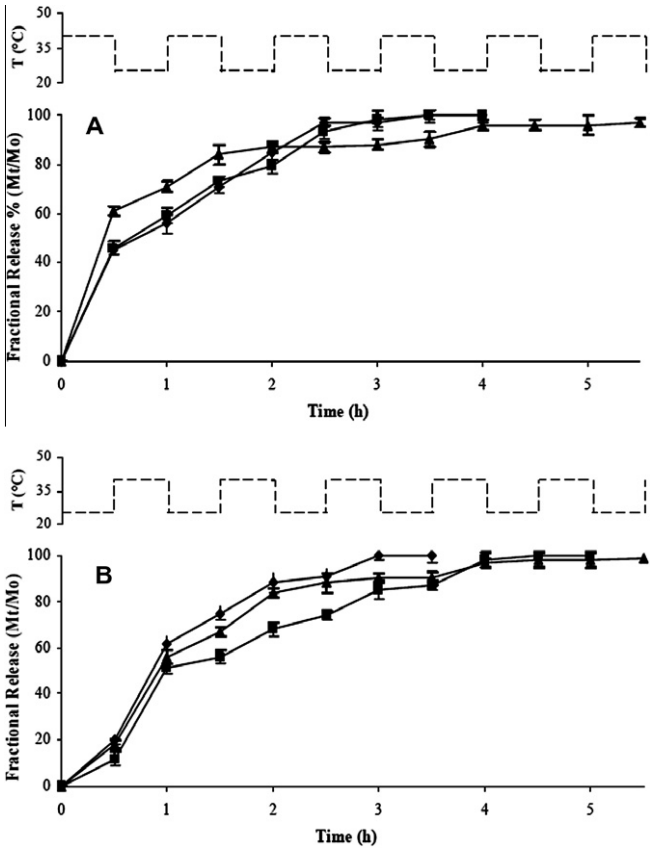

Fig. 5. Temperature-dependent stepwise DC release profile expressed as percent of drug delivered (M_t) related to the effectively entrapped total dose (M_0), as a function of time for microspheres GM-1 (♦), GM-2 (■) and GM-3 (▲) hydrogels first exposed to a temperature of 40 °C (A) and 25 °C (B), with alternating temperature between 25 °C and 40 °C at pH 7.0 (PBS solution 10^{-3} M).

4. Conclusions

Thermo-responsive microspheres were designed and synthesized by free-radical grafting of NIPAAm and commercial gelatin. MEBA was employed as co-crosslinking agent, while reverse phase suspension polymerization was the selected method to obtain the microspheres. The grafting procedure allows the insertion of commercial gelatin in a crosslinked structure without any other derivatization reaction, obtaining materials characterized by improved mechanical properties, LCST values close to body temperature and a spherical shape, the most suitable geometry for a drug delivery device. In addition, varying the gelatin amount in the feed composition, hydrogels with different hydrophobic/hydrophilic balance were synthesized. The hydrogels were characterized by morphological analyses showing a spherical shape and a highly porous surface. All samples showed negative thermo-responsive behaviour and different affinity to aqueous media depending on the external temperature. Moreover, in our experiment, introducing the hydrophilic moieties in the polymeric network, higher LCST values than pNIPAAm hydrogels were recorded. The materials were tested as drug carriers for DC release, which was found to be greatly influenced by the hydrogels crosslinking degree and the drug–polymer interactions. Depending on temperature of the surrounding environment, the DC release takes place by abrupt volume changes of the hydrogels and by diffusion of the therapeutic through the polymeric network. In order to estimate the diffusional contribute on the drug delivery, semi-empirical equations were employed, showing the enhanced role of the diffusional component in the release mechanism at temperature below the LCST. Finally, the reversible on/off-switching behaviour of the microspheres was demonstrated by performing in vitro pulsatile release experiments.

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