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Innovative antioxidant thermo-responsive hydrogels by radical grafting of catechin on inulin chain

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ABSTRACT

The synthesis of thermo-sensitive antioxidant hydrogels by grafting of an antioxidant molecule and a thermo-responsive monomer into inulin, in the presence of a crosslinker. In particular, catechin and NIPAAm, as antioxidant and thermo-responsive species, respectively, were inserted in a polymeric network employing a redox pair as an eco-friendly radical initiator system. Calorimetric, FT-IR, UV–Vis and fluorescence analyses were performed to verify both the formation of the inulin–antioxidant covalent bond and the insertion of bioactive molecules and monomers in the network. The hydrogels showed transition temperatures in the range 31.3–33.1 °C and a water affinity depending on the temperature of the surrounding medium. The antioxidant activity of the conjugates was evaluated measuring the scavenger properties of the hydrogels against the 2,2′-diphenyl-1-picrylhydrazyl radical and the total flavonoid content at different temperatures. The thermo-responsive antioxidant properties of the hydrogels confirmed the efficiency of the proposed synthetic strategy and demonstrated as the antioxidant–carbohydrate thermo-responsive conjugates possess peculiar features for specific applications in the food industry.

1. Introduction

Inulin is a dietary fiber composed of a mixture of oligo and/or polysaccharides consisting of fructose unit chains (linked by (2 → 1)-β-D-fructosyl-fructose bonds) of various lengths, terminated generally by a single glucose unit (linked by an a-D-glucopyranosyl bond) (Fig. 1) (Roberfroid & Delzenne, 1998). This natural polymer is widely distributed in some edible plants including asparagus, garlic, chicory, leek, onion and artichoke as storage carbohydrates (Kaur & Gupta, 2002). Inulin is a prebiotic, a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora, which confers benefits upon host well-being and health (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). It is well known that the colonic microflora has an important influence on human health (Steer, Carpenter, Tuohy, & Gibson, 2000). Consequently, there is a great interest in the use of prebiotics, such as inulin, as functional food ingredients to influence the composition of colonic microflora in order to improve health (Aryana & McGrew, 2007; Coppa, Zampini, Galeazzi, & Gabrielli, 2006; Manning & Gibson, 2004). The use of inulin in food formulations often it leads to significantly improved organoleptic characteristics, such as taste and mouth feel, and texture. Prebiotics show both important technological characteristics and interesting nutritional properties (Huebner, Wehling, & Hutkins, 2007) and, to serve as functional food ingredients, they must be chemically stable to food processing treatments such as heat. The insertion of a biocompatible antioxidant agent onto the structure of a prebiotic could be interesting to improve the stability of this kind of food ingredients.

Grafting polymerization is a well-known method to develop materials with a particular chemical and physical structure; this synthetic strategy allows the properties of natural and synthetic polymers to be improved, giving them new characteristics for specific applications (Dergunov et al., 2008; Joung, Choi, Bae, & Park, 2008). The grafting of molecules upon a natural polymer such as starch, cellulose and chitosan is of great importance for developing new materials by combining the properties of both grafted molecules and natural polymers (Meshram, Patil, Mhaske, & Thorat, 2009; Mishra, Tripathy, Srivastava, Mishra, & Behari, 2008). The possibility of grafting antioxidant moieties into a polysaccharide structure, by a radical procedure, represents an interesting innovation that could significantly improve the performance of the biomacromolecules, opening new applications in the biomedical and pharmaceutical fields. Polymeric antioxidants are a particular class of systems characterized by higher stability and slower degradation rate than compounds with low molecular weight and they could be applied in those fields in which the employment of a single molecule with antioxidant activity is prohibitive (Pan, Liu, & Lau, 1998; Puoci et al., 2008).

With the aim of imparting antioxidant controllable properties to the carbohydrate, our challenge was to synthesize a polymeric network containing (+)-catechin covalently bound to the inulin

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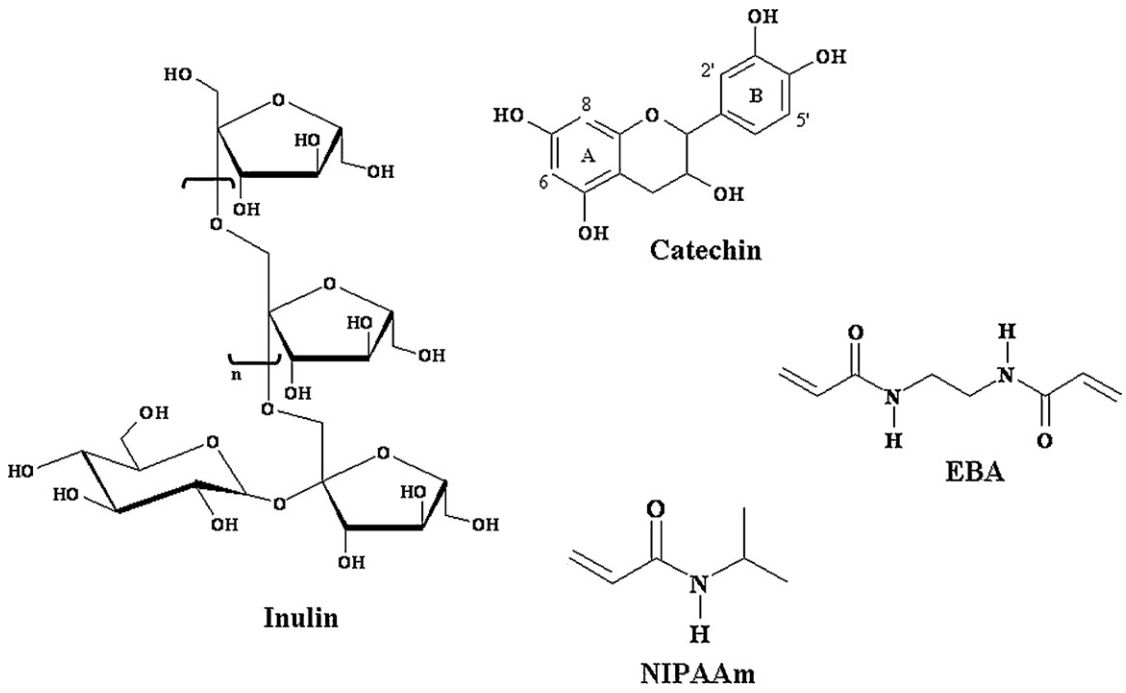

Fig. 1. Chemical structures of the reactive species in the polymerization feed.

chains (Fig. 2). In addition, the insertion in the polymeric backbone of stimuli-responsive moieties allows to modify the physico-chemical characteristics of the hydrogel modulating its antioxidant properties and tailoring the polymeric materials to specific applications.

Temperature is one of the most attractive physical parameter able to impart a reversible and yet discontinuous volume phase change to a polymeric network (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005; Iemma et al., 2009; Prabaharan & Mano, 2006). The polymer responds to this stimulus by changes in shape, surface characteristics, solubility, formation of an intricate molecular assembly or a sol-to-gel transition (Kumar, Srivastava, Galaev, & Mattiasson, 2007). A key temperature-responsive class is based on alkyl acrylamide polymers, especially poly(N-isopropylacrylamide) (PNIPAAm), which undergoes a sharp coil-globule transition and phase separation at its lower critical solution temperature (LCST) in water (Fujishige, Kubota, & Ando, 1989; Geever et al., 2006; Schild & Tirrell, 1990).

The thermo-responsive antioxidant–polysaccharide conjugates were synthesized by free radical grafting procedure of catechin, as antioxidant molecule, inulin, as biomacromolecule and N-isopropylacrylamide and N,N-ethylenebisacrylamide, as thermoresponsive and crosslinking agent, respectively (Fig. 1), employing ascorbic acid/hydrogen peroxide redox pair as water-soluble and biocompatible initiator system. Thermo-responsive antioxidant hydrogels were characterized by FT-IR spectroscopy, swelling behaviour, calorimetric, UV–Vis and fluorescence analyses. The antioxidant activity of the hydrogels was tested and the macromolecular networks were found to be able to interact with free radical species and to minimize the oxidative damage depending of the temperature of the surrounding medium. The strategy for synthesis proposed in the present paper appears suitable from an industrial point of view. It was planned as a one-step polymerization, without preventive functionalization of the reactive, in aqueous media; the initiator system allows the avoidance of high temperature, preserving both biomacromolecules and antiox-

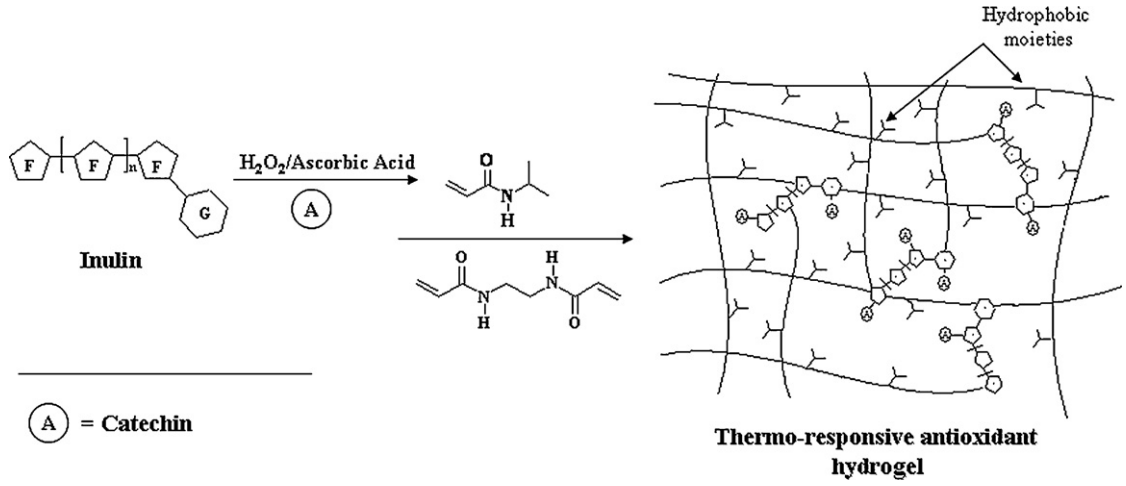

Fig. 2. Schematic representation of the synthesis thermo-responsive antioxidant–inulin conjugate.

idant integrity; finally, the copolymers were easily purified without organic solvent. The results of this research make the functionalized polymers useful materials in the optimization of food preservation and to help manufacturers in elaboration of new food products and packaging.

2. Experimental

2.1. Materials

Inulin from Dahlia tubers (MW ~5.000), N-isopropylacrylamide (NIPAAm), N,N′-ethylenebis(acrylamide) (EBA), (+)-catechin hydrate (CA), hydrogen peroxide (H_2O_2), ascorbic acid (AA), 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), sodium nitrite, aluminum chloride hexahydrate, sodium hydroxide and orthophosphoric acid were obtained from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Ethanol, methanol and water were reagent grade or HPLC-grade and provided by Carlo Erba reagents (Milan, Italy).

2.2. Instrumentation

The absorption spectra were measured on Jasco V-530 UV-Vis spectrophotomer and for measurement of the absorption spectra of the solid state samples, the diffuse reflectance spectra were recorded on the same instrument equipped with an integrating sphere accessory (Jasco ISN-470). The solid samples were placed between quartz plates (200 mm^2). The corrected emission spectra, all confirmed by excitation ones, were recorded with a Perkin Elmer LS-55 Luminescence spectrometer, equipped with Hamamatsu R928 photomultiplier tube. The fluorescence spectra in the solid state were recorded by using the front face accessory. The solid sample were placed between quartz plate (200 mm^2) on the sample holder. The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 210 nm. A 250 mm × 4 mm C-18 Hibar® Column, particle size 5 μm (Merck, Darmstadt, Germany) was employed. As reported in the literature (Wang, Helliwell, & You, 2000), the adopted mobile phase was methanol/water/orthophosphoric acid (20/79.9/0.1) and run isocratically at a flow rate of 1.0 mL min^{-1}. The column was operated at 30 °C. The sample injection volume was 20 μL. Freeze drier Micro Modulyo, Edwards was employed. Calorimetric analyses were performed employing a Netzsch DSC200 PC. The scanning electron microscopy (SEM) photographs were obtained with a Jeol JSMT 300 A; the surface of the samples was made conductive by deposition of a gold layer on the samples in a vacuum chamber.

2.3. Synthesis of antioxidant thermo-responsive hydrogels

The synthesis of antioxidant thermo-responsive hydrogels, by employing ascorbic acid/hydrogen peroxide redox pair as initiator system, was carried out as follows: in a 25 mL glass flask, inulin was dissolved in 6 mL of distilled water and then NIPAAm, EBA and CA (Table 1) were added. Finally, 1 mL of 1.0 M H_2O_2 and 0.054 g of ascorbic acid (Curcio et al., 2009) were introduced into the reaction flask and the mixture was maintained at 25 °C for 24 h under atmospheric air. The obtained hydrogels were washed with distilled water, frozen and dried with “freezing–drying apparatus” to afford vaporous solids. Hydrogels were checked to be free of unreacted antioxidant and any other compounds by HPLC analysis after purification step. Blank thermo-responsive hydrogels, that act as control, were prepared in the same reaction conditions but in the absence of the antioxidant.

2.4. Water content measurement

Aliquots (40–50 mg) of the polymeric particles 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 45 °C). After 24 h, the excess water was removed by filtration 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 weighed. The filter tare was determined after centrifugation with only water. The weights recorded were averaged and used to give the water content percentage (WR%) by the following Eq. (1):

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

where W_s and W_d are weights of swollen and dried polymeric particles, respectively. Each experiment was carried out in triplicate.

2.5. Calorimetric analysis

In a standard procedure for the determination of the transition temperature of antioxidant thermo-responsive hydrogels (Table 2), the sample was immersed in distilled water at room temperature for at least 2 days and allowed to reach the equilibrium state. Then, about 10 mg of the swollen sample were placed inside an aluminum pan and sealed tightly by a perforated aluminum lid. The thermal analyses were performed in the range of temperature from 25 to 55 °C with a heating rate of 3 °C min^{-1} on the swollen hydrogel samples under a dry nitrogen atmosphere with a flow rate of 25 mL min^{-1}. In order to verify the covalent insertion of CA, about 6.0 mg of dry sample was placed in a hermetic aluminum pan and then sealed. In this case, the thermal analyses were performed from 25 °C to 400 °C under a dry nitrogen atmosphere with a flow rate of 25 mL min^{-1} and a heating rate of 5 °C min^{-1}.

2.6. Evaluation of the antioxidant activity: scavenging activity on DPPH radicals

In order to evaluate the free radical scavenging properties of the synthesized hydrogels, their reactivity toward a stable free radical, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), was evaluated according to the literature with some modifications (Spizzirri et al., 2009). For this purpose, 25 mg of each polymer were dispersed in 1 mL of distilled water in a volumetric flask (25 mL). The samples were incubated in a water bath at 25 °C and 45 °C, respectively and allowed to stand for 16 h. Then, maintaining these temperature conditions, 4 mL of ethanol and 5 mL of ethanol solution of DPPH (200 μM) were added obtaining a solution of DPPH with a final concentration of 100 μM. After 10 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied on the blank hydrogels in order to evaluate the interference of polymeric material on DPPH assay. The scavenging activity of the tested materials was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according the following Eq. (2):

$$\mathrm{Inhibition\%} = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymers, and A_1 is the absorbance of polymeric samples. The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical, according to Eq. (2). Each measurement was carried out in

Table 1
Polymerization feed composition of thermo-responsive antioxidant hydrogels.

Hydrogel code	Inulin (mg)	NIPAAm (mg/mmol)	EBA (mg/mmol)	CA (mg/mmol)
I-B	250	500/4.42	100/0.88	–
I-CA	250	500/4.42	100/0.88	50/0.17
II-B	500	500/4.42	100/0.88	–
II-CA	500	500/4.42	100/0.88	50/0.17

Initiator system: H_2O_2 (1 mL 1.0 M); ascorbic acid (0.054 g/0.03 mmol).

quintuplicate, and data were expressed as means (±SEM) and analysed using ANOVA.

2.7. Determination of total flavonoid content

A slightly modified version of the spectrophotometric method was used to determine the flavonoid contents of samples (Dewanto, Wu, Adom, & Liu, 2002). Briefly, in a test tube 20 mg of each polymer were dispersed in 2 mL of distilled water and allowed to stand for 16 h, respectively at 25 °C and 45 °C. Then, maintaining these temperature conditions, 150 μL of a 5% $NaNO_2$ solution were added followed, after 6 min, by addition of 300 μL of a 6% $AlCl_3{\cdot}6H_2O$ solution. After another 5 min 1 mL of 1 M NaOH was added, the mixture was brought to 5 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nm against a control prepared using the blank polymers under the same reaction conditions. The amount of total flavonoids in the hydrogels was expressed as mean (micrograms of catechin equivalents per gram of polymer) ± SD for five replications, by using the equation obtained from the calibration curve of the antioxidant. This one was recorded by employing five different catechin standard solutions with the same procedure. The final concentrations of catechin in the test tubes were 10, 25, 50, 75, 100 μM, respectively.

3. Result and discussion

3.1. Synthesis of antioxidant thermo-sensitive network

Inulin, because of its biodegradability and biocompatibility properties, was chosen as polymer backbone to be functionalized with CA, to obtain macromolecular system showing raised antioxidant properties for food and food packaging applications. In addition, the presence in the polymerization feed of NIPAAm and EBA, as stimuli-responsive monomer and crosslinker, respectively, allows to synthesize a polymeric network able to modulate their antioxidant properties in response to the temperature of the surrounding environment. The employed synthetic strategy involved the use of the ascorbic acid/hydrogen peroxide redox pair, a biocompatible and water soluble system, as radical initiator (Spizzirri et al., 2010). Comparing to conventional initiator systems, like azo compounds, which require relatively high reaction temperature to ensure their rapid decomposition, the afore mentioned redox pair shows several advantages. First of all, this kind of system does not generate toxic reaction products; moreover, it is possible to perform the reaction processes at room temperatures, to avoid antioxidant degradation (Kitagawa & Tokiwa, 2006). On the other hand, to activate inulin toward radical reactions and, thus, to promote the insertion of antioxidant molecules (avoiding self-reactions), radical initiators should preferably react with the macromolecule before adding CA. A possible mechanism to synthesize antioxidant thermo-sensitive hydrogels is proposed in Fig. 2. The hydroxyl radicals, generated by the interaction between redox pair components, attack the reactive residues in the side chains of polysaccharide chain, producing radical species on the sugar structure. These react with the antioxidant molecules inducing an antioxidant–inulin covalent bond. Literature data suggest that on the phenolic ring free radical species attack at the ortho- and para-positions relative to the hydroxyl group (Kobayashi & Higashimura, 2003). This findings support the hypothesis that the binding sites involved in the antioxidant–sugar conjugation are the positions 2′and 5′(B ring) and 6 and 8 (A ring) for CA (Fig. 1). The heteroatom-centered radicals in the side chains of inulin preferentially react in some of the above-mentioned positions.

The proposed synthetic strategy permitted the preparation of two biomacromolecular networks with antioxidant activity labelled I-CA and II-CA, respectively, as reported in Table 1. The inulin was functionalized with CA by introducing in the reaction feed 0.68 mmol antioxidant per grams of carbohydrate for hydrogel I-CA and 0.34 mmol per grams of inulin for II-CA and the macromolecular moieties were chemically inserted in a polymeric structure built using a molar ratio NIPAAm/EBA equal to 5.0 for all formulations. To remove unreacted antioxidant and monomers, physically incorporated in the carbohydrate structure, the hydrogels were extensively washed with water and washing media were analysed by HPLC. Finally, the hydrogels were frozen and dried with freez-drier to obtain a porous and vaporous solid extensively characterized. Blank thermo-responsive hydrogels, that act as control, were prepared in the same reaction conditions but in the absence of the antioxidant.

3.2. Characterization of antioxidant stimuli-responsive hydrogel

In order to verify the covalent insertion of macromolecule, antioxidant and monomers onto the polymeric backbones, the hydrogels were characterized by Fourier Transform IR spectrophotometry, calorimetric, UV–Vis and fluorescence analyses. The FT-IR spectra of both I-CA and II-CA conjugates shown the appearance of new peaks at 1557 and at 1525 cm^{-1}, respectively, awardable to carbon to carbon stretching within the aromatic ring of catechin. The incorporation of the monomers in the hydrogels was confirmed by Fourier transform infrared spectroscopy. The FT-IR spectra of thermo-responsive hydrogels show the disappearance of bands at 990–918 and 980–954 cm^{-1}, awardable to C–C double bounds of NIPAAm and EBA confirming the absence of unreacted

Table 2
Swelling behaviour, transition temperature, inhibition (%) of DPPH radical and total flavonoid content of thermo-responsive antioxidant hydrogels.

Sample	LCST (°C)	WR (%)			Inhibition of DPPH radical (%)		Total flavonoid content (mg/g)	
		25 °C	45 °C	S_r	25 °C	45 °C	25 °C	45 °C
I-B	30.5 ± 0.1	592 ± 5	359 ± 4	1.1	22 ± 2	12 ± 1	0	0
I-CA	31.3 ± 0.2	558 ± 4	450 ± 2	1.3	74 ± 3	55 ± 2	3.73 ± 0.05	1.63 ± 0.02
II-B	31.2 ± 0.2	656 ± 3	380 ± 3	1.8	32 ± 2	21 ± 1	0	0
II-CA	33.1 ± 0.1	818 ± 6	377 ± 3	2.2	80 ± 3	25 ± 1	4.85 ± 0.07	3.67 ± 0.03

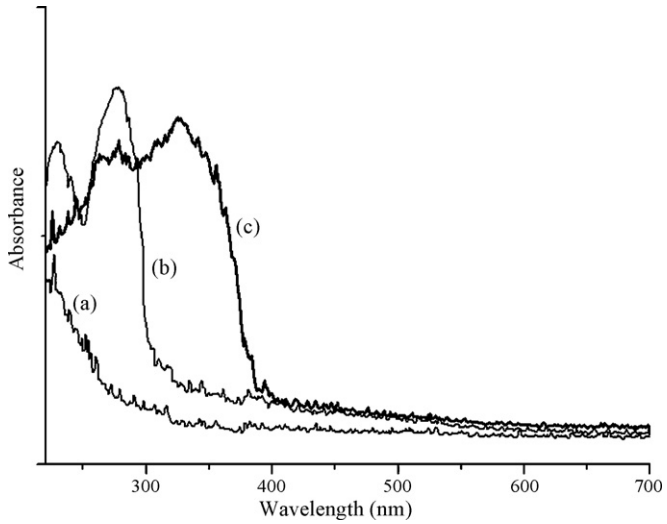

Fig. 3. UV–Vis spectra of II-B (a), CA (b) and II-CA (c).

monomers in the polymeric networks. In addition, the characteristic absorption bands of all the reagents in the polymerization feed are evident: 3280 (stretching vibrations of NH of amidic co monomers); 2940 (C–H stretching of CH_3, CH_2 and CH groups); 1647 cm^{-1} (C=O stretching amidic groups of NIPAAm and EBA).

UV–Vis spectra of the antioxidant hydrogels show covalent bond formation between antioxidant moieties and polymeric backbone. In the spectrum of conjugates, the presence of two absorption peaks at 278 and 327 nm in the aromatic region is related to the presence of CA covalently bonded to the polymeric network. In addition, in the free antioxidant the wavelength of the aromatic peaks appear at 229 and 275 nm, lower than grafted hydrogels, as depicted in Fig. 3.

The emission spectra of free antioxidant and conjugates also confirm the covalent functionalization of the polysaccharides. In the spectra of I-CA and II-CA conjugates, bathochromic shift of the emission peaks of CA from 381 to 433 nm, was detected (Fig. 4). These spectral red shifts are due to the covalent conjugation, because no emission peak is detected in the same wavelength range for blank hydrogels.

Thermal characterization of prepared conjugates was performed by recording of DSC thermograms of dried antioxidant hydrogel (I-CA and II-CA), blank hydrogel (I-B and II-B), and pure

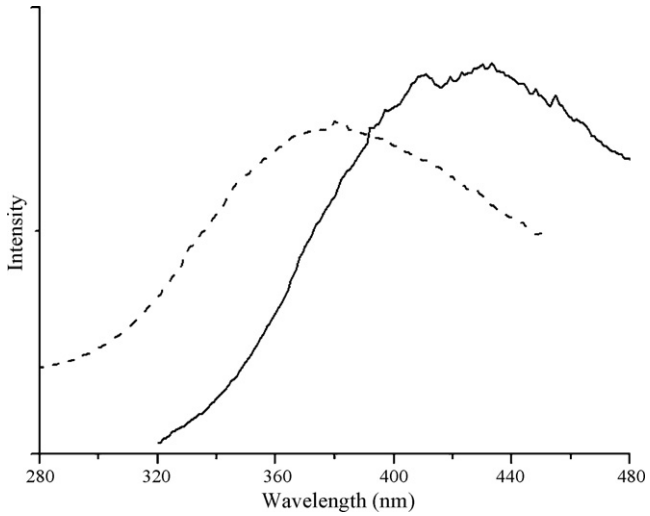

Fig. 4. Emission spectra of CA (- - -) and II-B (—).

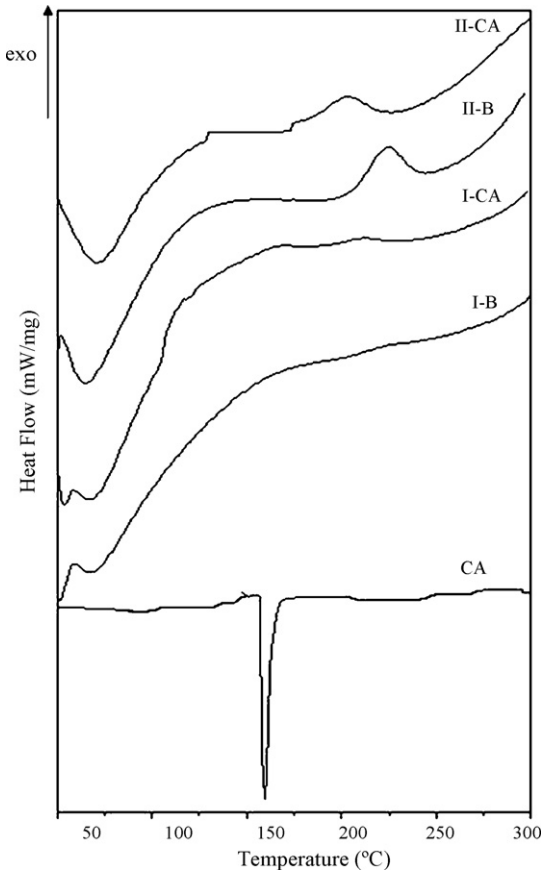

Fig. 5. Calorimetric analyses of and thermo-responsive antioxidant hydrogels (I-CA, II-CA), pure antioxidant (CA) and blank hydrogels (I-B, II-B).

antioxidants (CA), as depicted in Fig. 5. As far as DSC of I-B and II-B is concerned, a glass transition was recorded; the ΔC_p values associated to these transitions were respectively 0.63 and 0.66 $J\,g^{-1}\,K^{-1}$. The calorimetric analysis of pure CA shows a melting endotherm at 155.8 °C. Since the grafting of CA produces structural modification onto the polysaccharide chains, in the DSC thermogram of antioxidant hydrogels marked differences appear. The calorimetric analysis displays the absence of melting endotherm of CA, while, the ΔC_p values associated to the glass transition in the conjugates were 1.73 and 1.72 $J\,g^{-1}\,K^{-1}$ for I-CA and II-CA, respectively, probably as consequence of more rigidity of polymeric chains. This discrepancy suggests that the antioxidant hydrogels have a different thermal behaviour respect to unmodified polysaccharide probably due to the covalent bond of the bioactive molecule on the polysaccharide chain.

Using scanning electron microscopy, information about the surface properties of the microparticles were obtained. Fig. 6 clearly depicts the outside surface of the antioxidant thermo-responsive hydrogels I-CA and II-CA, characterized by a high porosity suitable for a rapid swelling/deswelling associated with temperature changes.

Thermal analyses were performed on the swollen samples from 25 °C to 55 °C and the LCST values were collected on Table 2. These values were strictly dependent on the hydrophobic/hydrophilic balance in the polymerization feed and on the chemical and structural properties of hydrophilic monomer/crosslinker. The data indicate that all the copolymers are characterized by a LCST higher than the pure PNIPAAm hydrogel (Geever et al., 2006) as consequence of the increased hydrophilic/hydrophobic balance in the polymeric structure (Fig. 7). Thermo-responsive behaviour of PNIPAAm hydrogel is strongly influenced by polymer–water affinity;

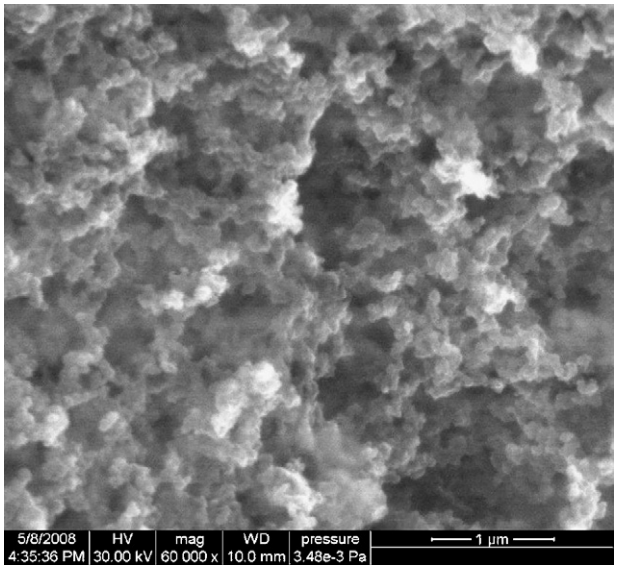

Fig. 6. SEM micrograph of outside surface of conjugate I-CA.

at temperature below its LCST, the hydrophilic groups (amide groups) in the side chains of the hydrogel interact with water molecules by hydrogen bonds. However, as the external temperature increases, the copolymer–water hydrogen bonds are disrupted and the water molecules, rigidly structured around the polymer chains, gain more freedom degrees and can rapidly diffuse across the bulk phase. As a result, hydrogen bonds between solvent molecules in the continuous phase are formed; while, inside the polymeric network, hydrophobic interactions among the isopropyl groups become dominant. When in the polymeric chains hydrophilic groups are randomly inserted, polymer–water interactions significantly increase and more energy is required to destroy hydrogen bond, allowing solvent diffusion. The transition temperatures of the thermo-responsive hydrogels ranged from 30.5 to 33.1 °C. The insertion of the antioxidant moieties in the polymeric backbone, by modification of hydrophilic/hydrophobic balance of the network, produces an increase of the transition temperature in respect to the blank polymer of 0.8 °C for hydrogel I-CA and 1.9 °C for II-CA.

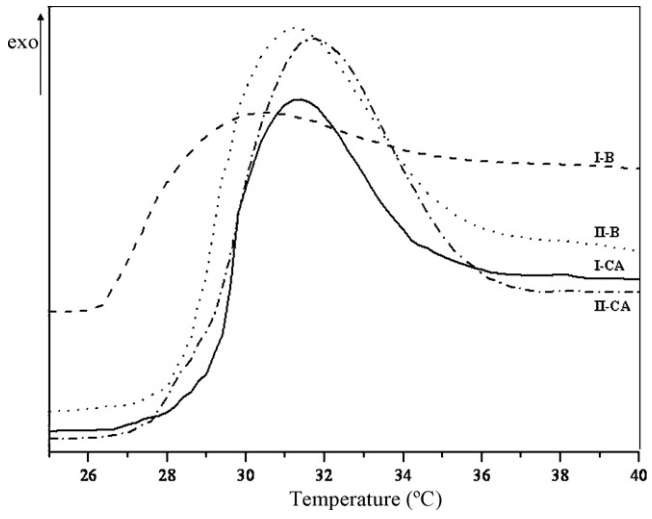

Fig. 7. DSC thermograms at a heating rate of 3 °C min^{-1} of the blank hydrogels (I-B, II-B), and conjugates (I-CA, II-CA).

Investigation of the water affinity of the hydrogels was carried out by studying their swelling behaviour in media (phosphate buffer solution 10^{-3} M) at 25 and 45 °C and the results are reported in Table 2. The data illustrate the water uptake, in grams per gram of dry copolymer, for each studied composition, and the ratio between the swelling at 25 °C and 45 °C at fixed pH values (S_r) was reported for all samples. The hydrogels showed different water affinity at 25 and 45 °C due to the pendant hydrophobic groups in the polymeric chains. In particular, at 45 °C, there is a considerable lowering of the water content, due to solvent diffusion outside the polymeric network and to resultant hydrophobic interactions between hydrocarbon moieties on the polymeric chains as illustrated in Fig. 2. When the temperature decreases to 25 °C, below the transition temperature of the hydrogels, the water content is greater than that found at 45 °C for all copolymers and the S_r values ranged from 1.1 to 1.8 for I-B and II-B and from 1.3 to 2.2 for I-CA and II-CA.

3.3. Evaluation of the antioxidant activity

3.3.1. Determination of scavenging activity on DPPH radicals

The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of antioxidant properties of compounds. In the DPPH assay, the antioxidant reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen donating ability of the antioxidant. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities (Spizzirri et al., 2009). Conjugates scavenger ability were evaluated in term of DPPH reduction using catechin as reference compound and data are expressed as inhibition (%). As reported in Table 2, in our experimental conditions, antioxidant polymers showed a scavenging activity strictly correlated to the experimental temperature and to the crosslinking degree of the hydrogels. In particular, DPPH reduction of 74% was recorded for conjugate I-CA in the swollen state at 25 °C, while the same hydrogel produce in the shrinked state at 45 °C a DPPH reduction of 55%. Increasing the amount of inulin in the polymerization feed (hydrogel II-CA) remarkable differences of DPPH reduction between the swollen (80%) and the shrinked (25%) states were recorded.

3.3.2. Determination of total flavonoid content

It has been frequently reported that both phenolic and flavonoids compounds are closely associated with antioxidant activity (Dewanto et al., 2002). Thus, $AlCl_3$ assay was employed to have a direct determination of the total flavonoid content, expressed as mg of CA per g of hydrogel. By comparing the obtained data with the CA calibration curve, the amount of catechin equivalent at different temperatures around the transition temperature was determined. In particular, for the conjugates I-CA and II-CA these values in the swollen state were 3.73 mg/g and 4.85 mg/g of hydrogels, while the recorded total flavonoid content in the shrinked state were 1.63 mg/g and 3.86 mg/g for I-CA and II-CA, respectively.

4. Conclusions

The synthetic procedure proposed in this paper involves one pot reaction consisting of direct polymerization of the inulin with an antioxidant molecule in the presence of a thermo-responsive co monomer and a crosslinking agent to obtain hydrogels with controllable antioxidant properties depending of the external temperature. In particular, CA was chosen as antioxidant agent

and the insertion of the reactive species onto the networks was confirmed by FT-IR. In addition, the covalent bond between the carbohydrate chain and the antioxidant was confirmed by calorimetric, UV–Vis and fluorescence analyses. The thermal analyses of the hydrogels showed their negative thermo-responsive behaviour with LCST values in the range 31.3–33.1 °C, as confirmed by water affinity measurements determined at temperatures around swelling–shrinking transition temperatures. The antioxidant activity of the thermo-responsive conjugates were evaluated by different assays and compared to a control, treated in the absence of antioxidant molecule. In particular, the scavenging activity on DPPH radicals and the total flavonoid content in polymeric matrices were performed at different temperatures. The results confirmed the controllable antioxidant properties of the hydrogels in response to the thermo-sensitive water affinity of the network. The planned strategy for synthesis is a very simple approach to prepare macromolecular systems with high antioxidant power, which could be successfully applied in all the fields in which a consistent reduction of oxidative stress is required.

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