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Synthesis of chitosan–PEO hydrogels via mesylation and regioselective Cu(I)-catalyzed cycloaddition

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ABSTRACT

In this work, a well-defined hydrogel was developed by coupling chitosan with PEO through "click chemistry". Azide functionalities were introduced onto chitosan, through mesylation of C-6 hydroxyl groups, and reacted with a di-alkyne PEO by a regioselective Cu(I)-catalyzed cycloaddition.

This synthetic approach allowed us to obtain a hydrogel with a controlled crosslinking degree. In fact, the extent of coupling is strictly dependent on the amount of azido groups on chitosan, which in turn can be easily modulated.

The obtained hydrogel, with a crosslinking degree of around 90%, showed interesting swelling properties. With respect to chitosan hydrogels reported in literature, a considerably higher equilibrium uptake was reached (940%).

The possibility to control the crosslinking degree of hydrogel and its capability to rapidly absorb high amounts of water make this material suitable for several applications, such as controlled drug release and wound healing.

1. Introduction

Chitosan, the linear cationic (1–4)-2-amino-2-deoxy-β-D-glucan with typical degree of acetylation ca. 0.25, is a most versatile polysaccharide that lends itself to countless chemical and biochemical modifications (Alves & Mano, 2008; Gomez d'Ayala, Malinconico, & Laurienzo, 2008; Muzzarelli and Muzzarelli, 2005; Qin, Li, Xiao, Liu, Zhu, & Du, 2006; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Kurita 2001). Among the latter, the combination with polyethyleneoxide (PEO) has been amply studied for a variety of purposes, in particular for the implementation of the performances of materials of biomedical interest (Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012; Xie et al., 2013) as well as for electrospinning of materials to be used in electrochemical sensors (Shukur, Ithnin, Illias, & Kadir, 2013).

Chitosan-based hydrogels for medical and pharmaceutical applications, as wound dressing, cartilage, tissue engineering and drug delivery systems, have been developed. Chitosan hydrogels are classified on the basis of the nature of network that can be created by either physical interactions or irreversible covalent linkages (Berger, Reist, Mayer, Felt, Peppas, & Gurny, 2004). Most of physical hydrogels are ionically crosslinked. Metallic anions, as polyoxyanions of Mo(VI) or Pt(II) (Brack, Tirmizi, & Risen, 1997; Draget, Varum, Moen, Gynnild, & Smidsrod, 1992), or molecules bearing phosphate groups, such as β-glycerophosphate and

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tripolyphosphate (Shu & Zhu, 2000), are particularly employed. As concerning covalent hydrogels, several dialdehydes, such as glyoxal (Khalid, Ho, Agnely, Grossiord, & Couarraze, 1999; Patel & Amiji, 1996) and glutaraldehyde (Dumitriu, Vidal, & Chornet, 1999; Yamada et al., 2000), are largely employed, as they react in aqueous media under mild conditions, and do not need addition of auxiliary molecules. However, a main disadvantage is their toxicity: indeed, glyoxal is known to be mutagenic (Murata-Kamiya, Kamiya, Kaji, & Kasai, 1997), while glutaraldehyde is demonstrated to be neurotoxic (Beauchamp, St Clair, Fennell, Clarke, & Morgan, 1992). Other compounds generally used for polysaccharide crosslinking such as epichlorohydrin (EPI) (Chiou, Ho, & Li, 2004; Wan Ngah, Endud, & Mayanar, 2002), ethylene glycol diglycidyl ether (Chiou & Li, 2003; Mi, Shyu, Chen, & Lai, 2002), diethyl squarate (De Angelis, Capitani, & Crescenzi, 1998) or genipin (Muzzarelli, 2009) exhibit direct crosslinking mechanisms of chitosan. The use of genipin is particularly interesting, since it is a naturally occurring material, commonly used in herbal medicine.

Another approach for the development of chitosan covalent hydrogels is the use of difunctional water soluble polymers as crosslinkers (Crescenzi, Paradossi, Desideri, Dentini, Cavalieri, Amici, & Lisi, 1997; Kim, Lee, & Cho, 1995). In this contest, PEO is particularly interesting due to high biocompatibility, pH independent swelling and drug release properties (Peppas, Keys, Torres-Lugo, & Lowma, 1999). PEO is a flexible polymer and can improve mechanical properties of hydrogels (Agnihotri & Aminabhavi, 2006). Chemically crosslinked chitosan–PEO hydrogels and films have been prepared by Michael addition type reactions between acrylated chitosan and PEO functionalized with hexa-thiol end groups (Kim, Choi, Noh, & Tae, 2007), or diacrylate polyethylene oxide and chitosan (Zhang, Yang, & Nie, 2008). Due to low reaction rate, Michael type addition is used to obtain in situ hydrogels. Nevertheless, the extent of reaction is hard to control, and the final crosslinking degree not predictable. Features of hydrogels, such as swellability, mass transport, biological interactions, strongly depend on the network characteristics, as length and density of crosslinking bridges. The application of highly selective, controlled and regioregular reactions is interesting to control structure/properties of final hydrogels, and in this context, "click chemistry" seems to be a perfect tool. "Click chemistry" is a general term that identifies a class of efficient and versatile chemical reactions with interesting features, including excellent functional group tolerance, high yields, good selectivity under mild conditions, absence of by-products and no need for tedious purification procedures (Hein, Liu, & Wang, 2008). In particular, the Cu(I)-catalyzed 1,3-dipolar cycloaddition of terminal alkynes with organic azides to give 1,4-regioselectively substituted triazoles (Hein et al., 2008; Rostovtsev, Green, Fokin, & Sharpless, 2002) has attracted great attention for modification of polysaccharides, as chitosan (Gruškienė, Čiuta, & Makuška, 2009), cellulose (Hafrén, Zou, & Córdova, 2006; Pohl, Schaller, Meister, & Heinze, 2008), dextran (Bernard, Save, Arathoon, & Charleux, 2008) and starch (Tankam, Müller, Mischnick, & Hopf, 2007). Hydrogels are obtained in a "one-pot" synthesis through reaction between a polysaccharide modified by insertion of alkyne or azido groups, and, respectively, a di-azido or di-alkyne crosslinker, resulting in formation of triazoles crosslinks. Hydrogels from hyaluronic acid (HA) (Crescenzi, Cornelio, Di Meo, Nardecchia, & Lamanna, 2007) or chitosan (Zampano, Bertoldo, & Ciardelli, 2010), and microparticles and block copolymers from dextran (De Geest et al., 2008; Schatz, Louguet, Le Meins, & Lecommandoux, 2009) have been described.

In the present work, Cu(I)-catalyzed cycloaddition was employed to develop a well-defined chitosan hydrogel by chemoselective coupling with PEO. Crosslinking of azide-functionalized chitosan was obtained by azide-alkyne "click" reaction with dialkyne–PEO. The final hydrogel was characterized in terms of swelling and crosslinking degree, and potential applications were discussed.

2. Materials and methods

2.1. Materials

Solvents and chemicals were purchased from Sigma-Aldrich. Trifluoroacetic anhydride, sodium azide (NaN_3), *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF anhydrous, inhibitor free), propargyl chloroformate (PCF), methane sulfonyl chloride (Mes-Cl), and hydrazine monohydrate were used as received. Bromotris(triphenylphosphine)Cu(I), (Br-Cu($P(Ph)_3)_3$) was stored under argon in a glove-box. Low-molecular-weight chitosan (50,000–65,000 Da, 75–85% deacetylated) was purchased by Sigma Aldrich. α-ω-Di-hydroxy-poly(ethylene-oxide) (HO–PEO_{6000}–OH, M_n = 6000 Da from Fluka) was dehydrated by azeotropic distillation with dry toluene in a Dean-Stark trap. Dry chloroform (Carlo Erba) was obtained by distillation over CaH_2 and stored over molecular sieves under inert atmosphere.

2.2. Synthesis of N-phthaloyl-chitosan (pht-Ch)

N-phthaloyl-chitosan was obtained by reaction of chitosan with phthalic anhydride, as described by Nishimura, Kohgo, and Kurita (1991). Briefly, chitosan (5.00 g, 31.0 mmol of monomeric unit) was dispersed in DMF and phthalic anhydride (11.50 g, 78.00 mmol) was added. The mixture was stirred for 6 h at 130 °C under nitrogen. Then, the reaction product was precipitated in ice–water, washed in ethanol and dried under vacuum for 24 h, till constant weight.

FT-IR (KBr, cm^{-1}): 3450 (bs, OH), 2920 (CH_2), 1776 and 1705 (s, imide C=O), 1283 and 1254 (C—O ester), 713 (aromatic ring). ^{1}H NMR (400 MHz, DMF-d7, δ): 1.8 (s, CH_3 acetamide), 3.2–5.4 (m, pyranose ring), 7.2–7.9 (m, phthaloyl group).

2.3. Functionalization of pht-Ch with azido (N_3) groups

Azido-functionalized derivative of chitosan was synthesized through a two-step process, involving *O*-mesylation of C-6 hydroxyl groups followed by nucleophilic substitution of *O*-mesylates with inorganic azide.

2.3.1. Activation of C-6 hydroxyl group of pht-Ch

Methanesulfonyl-chloride (mes-Cl) (3.78 g, 34.0 mmol) was added dropwise to a solution of pht-Ch (3.00 g, 11.0 mmol of monomeric unit) and DIPEA (4.38 g, 34.0 mmol) in dry DMF at 10 °C. The resulting gel was stirred for the first 4 h at 10 °C and for further 21 h at room temperature. Afterwards, it was transferred in a flask containing 300 mL of methanol and the obtained precipitate, pht-mesCh, was filtered on a glass porous filter and dried for 24 h under vacuum at 35 °C (yield: 2.89 g).

^{1}H NMR (400 MHz, D_2O/KOH, δ): 2.1 (s, CH_3 acetamide), 3.1 (s, CH_3 mesyl), 3.4–5 (m, pyranose ring), 7.2–7.9 (m, phthaloyl group).

2.3.2. Reaction with sodium azide

NaN_3 (1.30 g, 20.0 mmol) was added to a solution of pht-mesCh (1.30 g; 3.80 mmol of monomeric unit) in DMF/H_2O (25:1 v/v). The mixture was stirred at 80 °C for 12 h and then the polymer was precipitated in water and then repeatedly washed in methanol. The obtained pht-N_3Ch was recovered on a glass porous filter and dried for 24 h under vacuum at 35 °C (yield: 1.20 g).

^{1}H NMR (200 MHz, D_2O/KOH, δ): 2.1 (s, CH_3 acetamide), 3.4–5 (m, pyranose ring), 7.2–7.9 (m, phthaloyl group).

FT-IR (cm^{-1}): 3450 (bs, OH), 2110 (N_3), 1776 and 1712 (s, imide C=O), 1290 and 1260 (C—O ester), 1150–1000 (pyranose ring), 721 (aromatic ring).

2.4. Synthesis of di-alkyne–PEO

Di-alkyne–PEO derivative was synthesized through a two-step process. Briefly, a di-azido PEO was first obtained following the same reaction sequence used in the case of azido-chitosan synthesis. The azido groups of PEO were then reduced to amines with H_2/Pd. Finally the amino functionalized PEO was reacted in stoichiometric ratio with propargyl chloroformiate to give the di-alkyne–PEO.

2.4.1. Synthesis of di-azido PEO

Di-azido–PEO (N_3–PEO) was synthesized starting from α-ω-dihydroxyl–PEO through a two-step azidation process, analogously to azido functionalization of chitosan.

2.4.1.1. Activation of end hydroxyl groups of PEO. Methanesulfonyl-chloride (mes-Cl) (1.260 g, 11.00 mmol) was added to a solution of α-ω-dihydroxyl–PEO (6.00 g, 1.00 mmol) and DIPEA (1.418 g, 11.00 mmol) in 60 mL of dry chloroform at 0 °C. The mixture was stirred for 4 h at 0 °C and then for further 21 h at room temperature. Afterwards, it was concentrated and precipitated in a chilled diethyl ether/methanol 9/1 (v/v) mixture. The obtained solid, α-ω-di-mesyl-PEO, (mes-PEO) was recovered by filtration and dried for 24 h under vacuum at 30 °C. The conversion of terminal hydroxyl groups was evaluated by trifluoroacetic anhydride assay (5.82 g; 0.96 mmol; yield 97%).

^{1}H NMR (200 MHz, $CDCl_3$, δ): 3.06 (s, 6H), 3.64–3.70 (s, 545 H).

2.4.1.2. Reaction with sodium azide. Sodium azide (1.600 g, 24.60 mmol) was added to a solution of mes-PEO (5.50 g, 0.92 mmol) in DMF (55 mL). The mixture was stirred at 80 °C for 12 h, then dried and the solid residue was dissolved in chloroform. The resulting solution was filtered, concentrated and the product was recovered by precipitation in 300 mL of chilled diethyl ether. The obtained N_3–PEO was collected by filtration and dried for 24 h under vacuum at 30 °C (5.20 g; 0.86 mmol; yield 93%).

^{1}H NMR (200 MHz, $CDCl_3$, δ): 3.38 (s, 4H), 3.64–3.70 (s, 545 H).

2.4.2. Synthesis of di-amino–PEO (NH_2–PEO)

Amounts of 5.17 g N_3–PEO (0.86 mmol), 1.0 g Pd/C and a volume of 30 mL chloroform-–ethanol mixture (1:1, v/v) were put in a two-necked 100 mL flask under nitrogen atmosphere. The mixture was kept under hydrogen flow for 24 h at room temperature, then centrifuged for 35 min at 8000 rpm to remove the suspended Pd/C. The solution was precipitated with 300 mL of ethyl ether/hexane 9:1 (v/v). The obtained precipitate, di-amino–PEO, was filtered and dried under vacuum at 30 °C for 24 h. The presence of aminic groups on NH_2–PEO was confirmed by the ninhydrin test (4.85 g; 0.81 mmol; yield 93%).

^{1}H NMR (200 MHz, $CDCl_3$, δ): 3.20 (4H, t), 3.64–3.70 (545 H, s), 3.91 (4H, m).

2.4.3. Synthesis of di-alkyne–PEO

Propargyl chloroformate (PCF) (0.900 g, 7.890 mmol) was added to a solution of NH_2–PEO (4.85 g, 0.81 mmol) and anhydrous Na_2CO_3 (3.00 g, 28.3 mmol) in 40 mL of dry chloroform at 0 °C. The resulting mixture was stirred for 4 h at 0 °C and then for 21 h at room temperature. The reaction mixture was concentrated, filtered and precipitated in 300 mL of chilled diethyl ether. The obtained product, di-alkyne–PEO, was dried for 24 h under vacuum at 30 °C. (4.60 g; 0.76 mmol; yield 95%).

^{1}H NMR (200 MHz, $CDCl_3$, δ): 2.50 (2H, t), 3.36 (4H, t), 3.64 (545H, s), 4.65 (2H, t), 5.59 (2H, b).

2.5. 1,3-Huisgen cycloaddition ("click" reaction) between pht-N_3Ch and di-alkyne–PEO

DIPEA (65.52 mg, 0.520 mmol) was added to a solution of di-alkyne–PEO (1.360 g, 0.230 mmol) in dry DMF (18 mL) under inert atmosphere. The mixture was degassed by three cycles of freeze-thawing, transferred under argon in a bottle containing pht-N_3Ch (530 mg, 1.68 mmol of monomeric units, corresponding to around 0.42 mmol of azido groups) and Bromo-tris (triphenylphosphine) Cu(I) (48.37 mg, 0.052 mmol) and stirred for 48 h at 35 °C. Copper was removed through a neutral alumina column, then the solution was concentrated and the product precipitated in 300 mL of chloroform/methanol 1:1 (v/v). The obtained product, phtCh-crosslinked–PEO, was collected on a glass porous filter and dried for 24 h under vacuum at 35 °C (yield: 1.231 g; theoretical yield: 1.538 g).

^{1}H NMR (200 MHz, D_2O/KOH, δ): 2.1 (s, CH_3 acetamide), 2.5 (alkyne proton PEO), 3.4–5 (m, pyranose ring and PEO), 7.2–7.9 (m, aromatic).

2.6. Deprotection of amines

A mixture of phtCh-crosslinked–PEO (3.70 g, 6.93 mmol), hydrazine monohydrate (20 mL), and water (40 mL) was stirred for 15 h at 100 °C under a nitrogen atmosphere. After cooling, the mixture was further diluted with 50 mL of water and dried. This procedure was repeated three times. The residue was suspended in deionized water, the white powder was filtered, washed with ethanol and ether and finally dried under vacuum at 35 °C.

^{1}H NMR (200 MHz, D_2O/KOH, δ): 2.1 (CH_3 acetamide), 2.5 (alkyne proton PEO), 3.4–5 (pyranose ring and PEO).

2.7. Crosslinking degree

Crosslinking degree was determined gravimetrically by extraction of the soluble phase with water/acetic acid (20%, v/v acetic acid) for 12 h at 25 °C, according to the following equation:

$$\%\,\text{crosslinking} = \frac{W_0 - W_{\text{extr}}}{W_0} \times 100 \quad (1)$$

where W_0 is the initial weight and W_{extr} is the residual weight after the extraction process.

2.8. Equilibrium swelling

The amount of water uptake (equilibrium swelling) was evaluated according to a conventional tea bag method (Pourjavadi, Ghasemzadeh, & Soleyman, 2007). In particular, a tea bag (100-mesh nylon screen), containing an accurately weighed amount of powder sample was soaked in distilled water at room temperature for 2 h. The tea bag was then hung up for 15 min to remove the excess of water and then weighed again. The equilibrium swelling (ES) was calculated as a mean of three measurements according to the following equation:

$$\%\ \text{equilibrium swelling} = \frac{W_e - W_0}{W_0} \times 100 \quad (2)$$

where W_e is the weight of the swollen hydrogel and W_0 is the weight of dried sample.

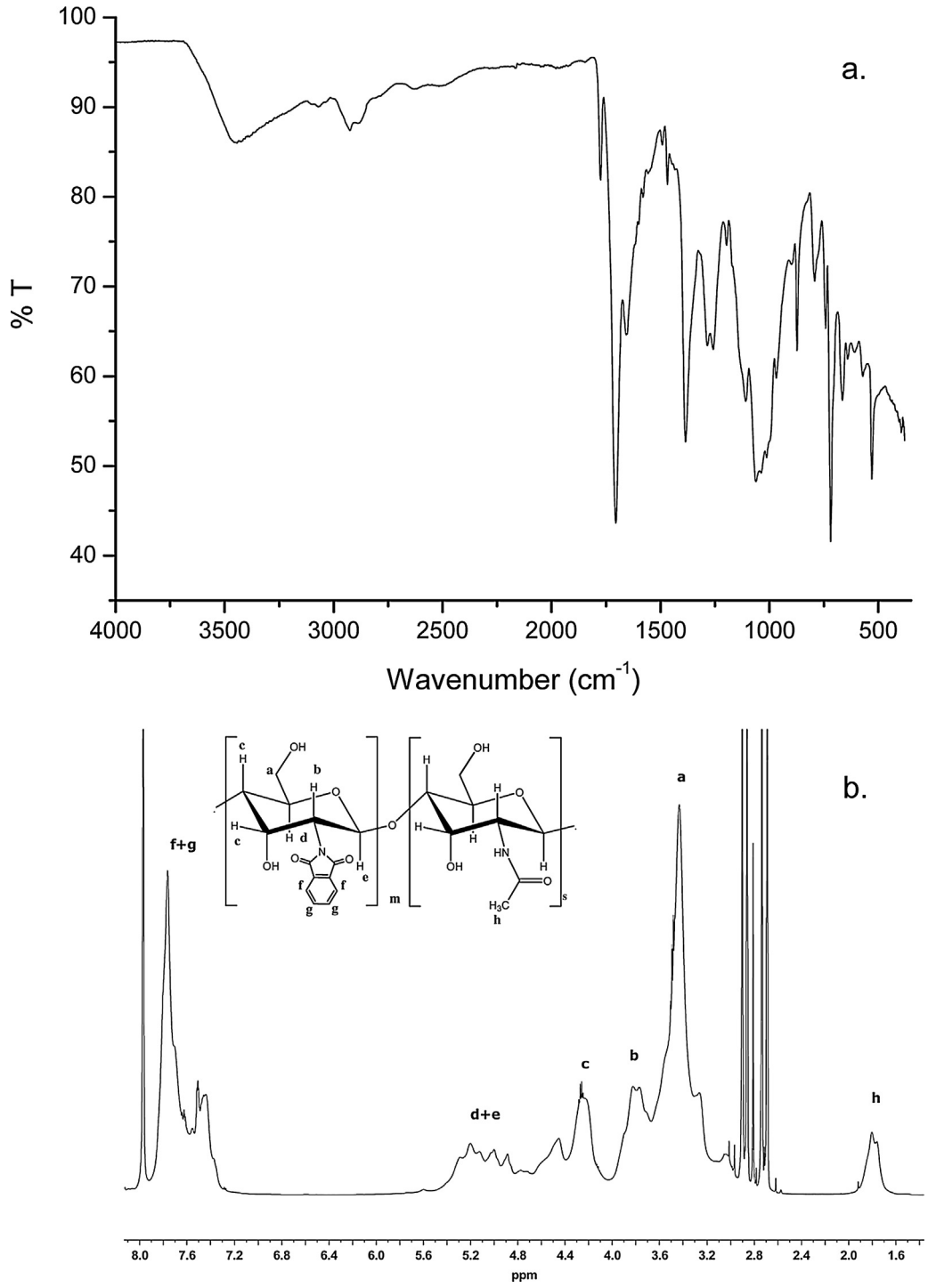
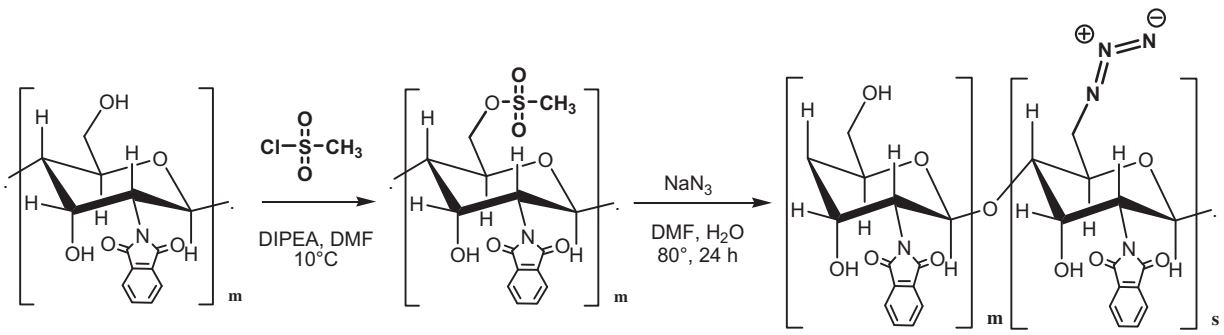


Fig. 1. FT-IR spectrum (a) and ^{1}H NMR spectrum in DMSO-d_6 (b) of pht-Ch.

2.9. Characterization techniques

Differential scanning calorimetry (DSC) measurements were performed by a Mettler-Toledo mod. STAR 822e calorimeter (Greifensee, Switzerland), using 10 ± 2 mg of sample. The samples were heated from room temperature up to 150 °C, left to this *T* for 5 min, then cooled to room temperature and reheated to 150 °C. The heating/cooling rate was 10 °C min^{-1}. The analysis was performed on chitosan-crosslinked–PEO hydrogels before and after extraction with acetic acid and on plain PEO, in order to quantify the amount of PEO in the hydrogel.

^{1}H NMR spectra were recorded at 25 °C on Varian Gemini VXR-200 (200 MHz) and Bruker DRX (400 MHz) spectrometers using deuterated solvents and TMS as internal standard.



Scheme 1. Activation of chitosan primary hydroxyl groups through reaction with mesyl chloride followed by nucleophilic substitution with sodium azide.

FTIR spectra were obtained using a Jasco FT-IR-430 spectrometer. The samples were analyzed as thin films obtained by solution casting onto a KBr disk.

Thin layer chromatography (TLC) was performed on silica gel support Merck 60 F254 using UV lamp, with iodine and/or ninhydrin as detector and a chloroform/methanol mixture 9/1 (v/v) as solvent.

3. Results and discussion

3.1. Functionalization of chitosan with N_3 groups

Due to the higher reactivity of aminic group compared to hydroxyl group, a preliminary protection of aminic moieties was necessary before chitosan functionalization with azide. Reaction with phthalic anhydride was chosen as the most suitable method to both preserve the aminic functionalities and to impart solubility in organic solvents. Moreover, phthaloyl groups can be easily removed to regenerate the free amino groups. Phthaloylation reaction was carried out in absence of water in order to promote a partial *O*-phthaloylation on C-6 and to get a modified chitosan with reduced crystallinity and improved solubility (Nishimura et al., 1991).

The characteristic absorptions attributable to phthalimide groups at 1770 and 1710 cm^{-1} were observed in the IR spectrum (Fig. 1a). ^1H NMR spectrum of pht-Ch (Fig. 1b) showed a multiplet in the aromatic region (δ = 7.8–7.6 ppm), which confirms the presence of *N*-phthaloyl groups onto chitosan chains, and a second multiplet centered at around 7.46 ppm related to aromatic protons of *O*-phthaloyl groups (Zampano et al., 2010). The degree of NH_2-protection was estimated by the ratio between integral of aromatic peaks and those relative to the acetyl group (1.8 ppm) and it was found to be around 75%.

As expected, the partial protection of primary amino groups led to a chitosan derivative with greatly improved solubility in organic solvents, in particular, polar aprotic solvents, such as DMF and DMSO.

The azidation of pht-Ch was performed through a two-step process, involving the *O*-mesylation of C-6 hydroxyl groups followed by nucleophilic substitution of *O*-mesylated chitosan with sodium azide (Scheme 1).

The first step provided activation of hydroxyl groups by mesyl chloride, through an efficient method developed by Hoffmann

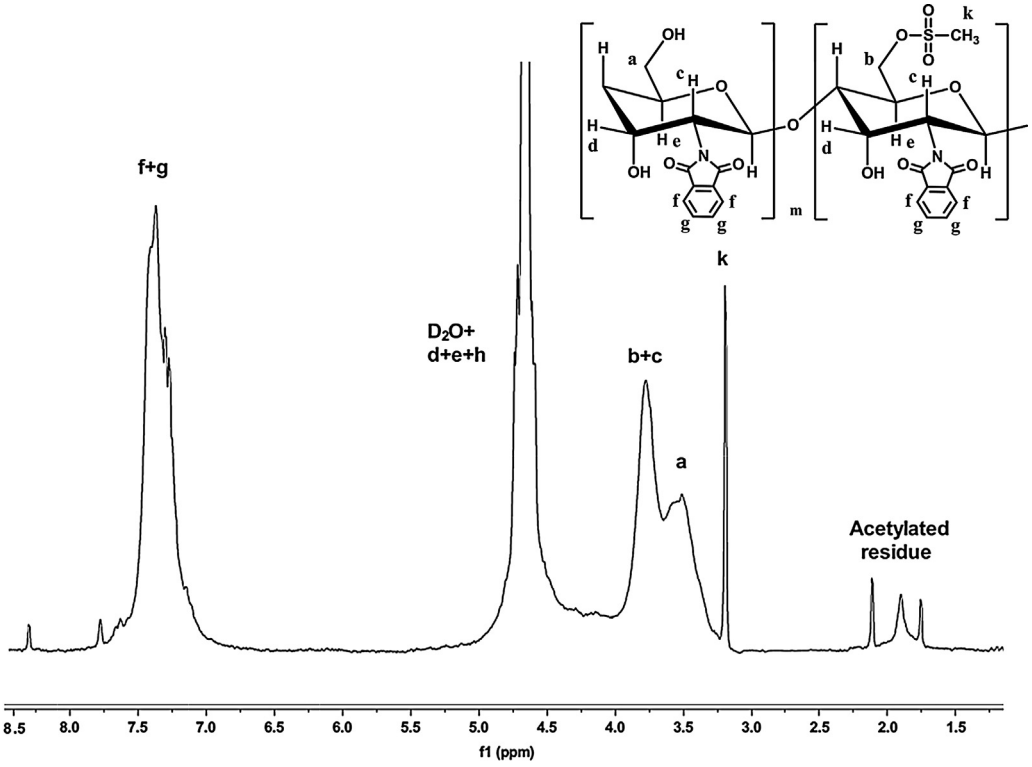


Fig. 2. ^1H NMR spectrum of pht-mesCh after second reaction cycle with mesyl chloride in D_2O/KOH 37%.

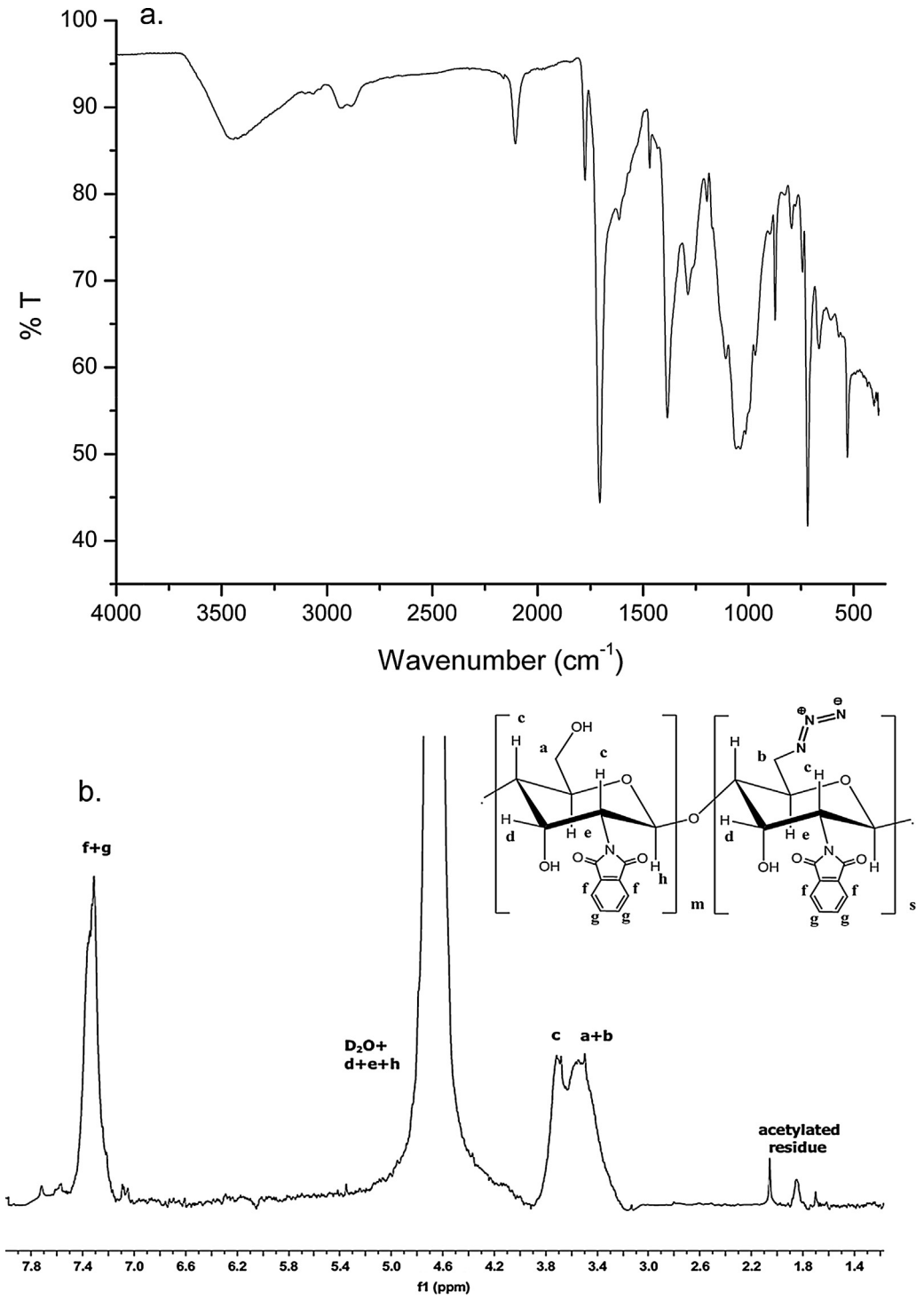
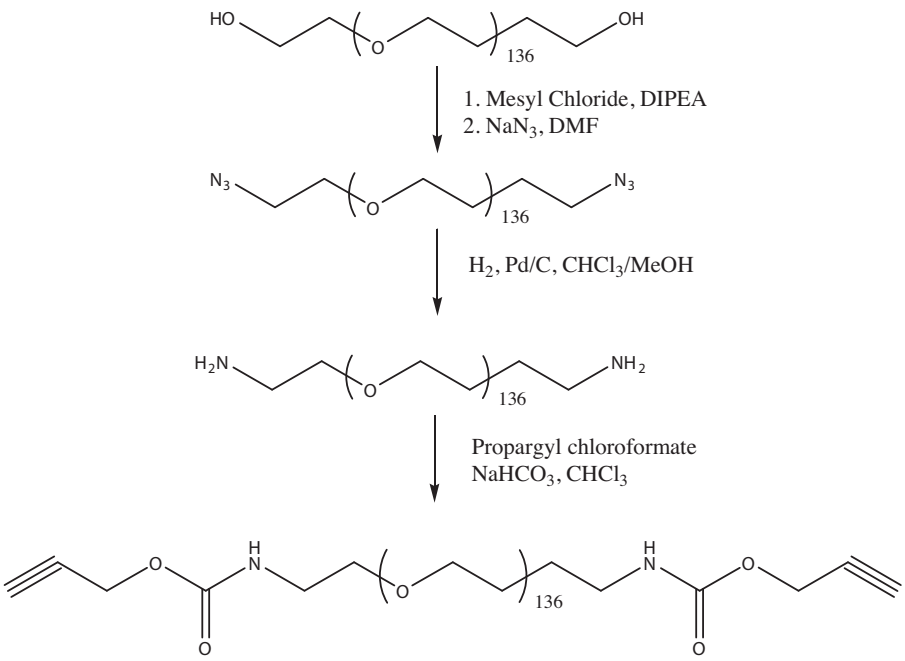


Fig. 3. FT-IR spectrum (a) and ^{1}H NMR spectrum in D_2O/KOH 37% (b) of pht-N_3Ch.

(Franz, Roper, Wartchow, & Hoffmann, 2004; Roper, Franz, Wartchow, & Hoffmann, 2003). Mesylation of hydroxyl groups requires mild conditions and it is an almost quantitative reaction, with high selectivity. With respect to the widely reported reaction with halides, such as carbon tetrabromide, the present methodology has the advantage that the reaction product can be easily characterized. Methyl group of mesyl adduct shows, indeed, a clear singlet in ^{1}H NMR spectrum at around 3 ppm. Therefore, the presence of mesyl groups onto the polymeric chains can be confirmed through this signal in the ^{1}H NMR spectrum and, moreover, the subsequent azidation can be followed by the disappearance of the singlet at 3 ppm, as well as by the appearance of azide stretching band in the FT-IR spectrum.

Pht-Ch was dissolved in dry DMF at 80 °C to get a homogeneous solution. After the addition of DIPEA and mesyl chloride the viscosity significantly increased till the formation of a gel, probably due to hydrogen and polar interactions between chitosan and solvent (Lei, Li, Pan, & Han, 2003). The reaction product, recovered by precipitation with methanol, was analyzed by ^{1}H NMR spectroscopy using alkaline D_2O (pH 12) as solvent. The presence of a peak at 3.20 ppm, characteristic of mesyl group protons (CH_3-OSO), confirmed the occurring of activation reaction. The conversion degree



Scheme 2. Synthetic pathway for α-ω-dialkyne–PEO preparation.

was calculated by the ratio between the integral of mesyl signal and those of acetyl group and was found to be 10% by mol.

In order to get a higher degree of mesylation, the reaction was repeated twice, according to the procedure as described above. ^{1}H NMR analysis of final purified product revealed a conversion degree higher than 25% (Fig. 2).

It was observed that the reaction is extremely selective toward primary hydroxyl group (Fleet, Nicholas, Smith, Evans, Fellows, & Nash, 1985; Scriven & Turnbull, 1988). Repeated cycles led to an enhanced functionalization degree, even if gel formation in DMF represents a limit. Thanks to this methodology it is possible to modulate and control the functionalization degree and, consequently, the resulting crosslinking degree. Nucleophilic substitution of mesyl groups was carried out with a large excess of sodium azide in a refluxing mixture of DMF/water. The FT-IR spectrum of C(6)-azidated chitosan (Fig. 3a) shows a strong absorption at 2110 cm^{-1} typical of the azide moiety, thus confirming that the reaction was successful.

The conversion degree was estimated through NMR analysis (Fig. 3b). The complete disappearance of signal related to mesyl group CH_3-OSO protons (δ = 3.20) allows to presume that a quantitative conversion of mesylic groups occurred. On the basis of this evidence, the degree of azidation was assumed to be equal to the mesylation degree, namely 25%.

3.2. Synthesis of α-ω-dialkyne–PEO_{6000}

The synthetic pathway to prepare α-ω-dialkyne–PEO starting from α-ω-dihydroxyl–PEO is illustrated in Scheme 2.

Terminal hydroxyl groups were first converted into azide groups following the same synthetic route previously described in the case of chitosan, i.e. mesyl activation of hydroxyl moieties, and then nucleophilic substitution of mesyl groups by azide. The azide functionalities were then converted into amine groups by H_2/Pd reduction, and finally the amine groups reacted with propargyl chloroformate to get the di-alkyne terminated PEO.

The whole reaction pathway allows a full conversion of —OH end groups of PEO to alkyne groups as confirmed by ^{1}H NMR analysis. In turn, the product, used without further purification allows a fine control of final crosslinking of chitosan molecules and absence of side reactions. This method was chosen as an alternative strategy with respect to the more usual esterification reaction with 4-pentynoic acid that presents several drawbacks, as incomplete reagents conversion. In our approach, the conversion degree of intermediate and final products was found to be higher than 95%, as evaluated by ^{1}H NMR analysis. In particular, for the final product a quantitative conversion of NH_2–PEO amines was estimated by the ratio between signal related to PEO protons (CH_2—NH—CO, δ = 4.0 ppm) and those of chloroformate protons (HC≡C—CH_2—OCO—, δ = 4.65 ppm) (Fig. 4).

TLC analysis, carried out with ninhydrin as detector, confirmed the total absence of residual amine groups.

3.3. 1–3 Huisgen cycloaddition between pht-N_3Ch and α-ω-dialkyne–PEO

The coupling between pht-N_3Ch and α-ω-dialkyne–PEO was achieved by means of "click chemistry" and a crosslinked copolymer was obtained. Clicking reaction was performed in dry DMF, using $BrCu(P(Ph)_3)_3$ as catalyst, with a 10% molar excess of alkyne groups with respect to azide. During the reaction, a gradual precipitation of chitosan was observed, following the formation of a crosslinked network. The resulting dispersion was precipitated with chloroform, repeatedly washed, and the final product was analyzed by FT-IR and ^{1}H NMR spectroscopies. The FTIR spectrum showed the complete disappearance of the characteristic azide signal at 2110 cm^{-1}, suggesting that a quantitative coupling reaction occurred (Fig. 5a). ^{1}H NMR analysis (Fig. 5b) showed the characteristic signals of PEO protons O—CH_2—CH_2 (δ = 3.60) and of phthalimide aromatic protons (δ = 7.30), which confirmed the presence of both polymers in the network. The appearance of the signal related to the alkyne proton of α-ω-dialkyne–PEO (δ = 2.50) highlighted the presence of a low amount of residual alkyne groups, due to the excess used with respect to azide, likely attributable to PEO chains grafted onto chitosan (see Scheme 3).

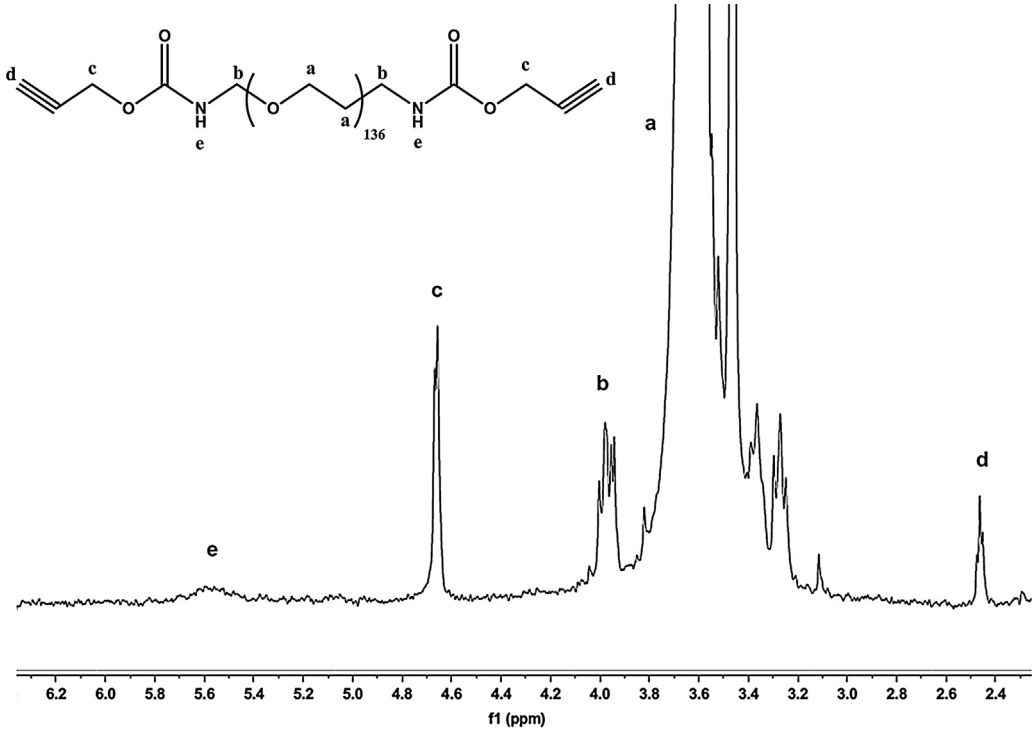


Fig. 4. ^{1}H NMR spectrum of α-ω-dialkyne–PEO_{6000} in $CDCl_3$ with proton assignment.

3.4. NH_2 deprotection of phtCh-crosslinked–PEO

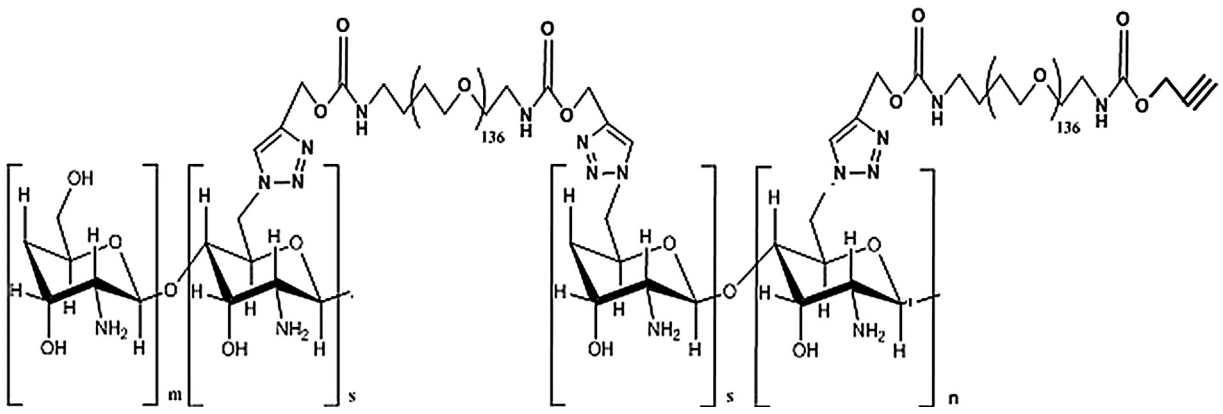
PhtCh-crosslinked–PEO was treated with hydrazine monohydrate in order to restore the free amino groups of chitosan. The resulting product, in form of powder, was analyzed by ^{1}H NMR. The spectrum (not shown) evidenced the complete disappearance of aromatic proton peaks, as a proof that a full deprotection of aminic groups was realized. The chemical structure of the final Ch-crosslinked–PEO is sketched in Scheme 3.

3.5. Hydrogel characterization

Crosslinking degree of Ch-crosslinked–PEO was determined gravimetrically by extraction with a water/acetic acid mixture. A weight loss of 10% was detected, corresponding to a high crosslinking degree (90%). The soluble phase was recovered by removing the solvent under vacuum and analyzed by ^{1}H NMR in D_2O solution. The presence of both signals related to PEO and chitosan in the spectrum (not shown) was attributed to the presence in the soluble fraction of a certain amount of non crosslinked chitosan–*g*–PEO chains, as a consequence of the molar excess of di-alkyne PEO.

A preliminary swelling study showed that the new crosslinked chitosan is able to absorb as much as 940% of water. Water uptake kinetics is not described, since the equilibrium swelling in water was achieved in a few minutes, as found in the case of films of chitosan crosslinked with PEG-diacrylate (Zhang et al., 2008). These characteristics are appealing for wound dressing, particularly in the case of highly exuding lesions.

With respect to chitosan hydrogels crosslinked with glutaraldehyde (Rohindra, Nand, & Khurma, 2004) or PEG-diacrylate (Zhang et al., 2008), a considerably higher equilibrium uptake was reached (940% against 100–160%). This significant improvement can be ascribed to the different molecular weight of crosslinker. When the crosslinker molecular weight is high, as in the present work (PEO 6000), a loose polymeric network is formed, resulting in highly swollen and porous hydrogel. On the contrary, at low crosslinker molecular weight (PEG 600), the network is tighter, resulting in lower porosity and, consequently, lower swelling. Furthermore,



Scheme 3. Chemical structure of Ch-crosslinked–PEO.

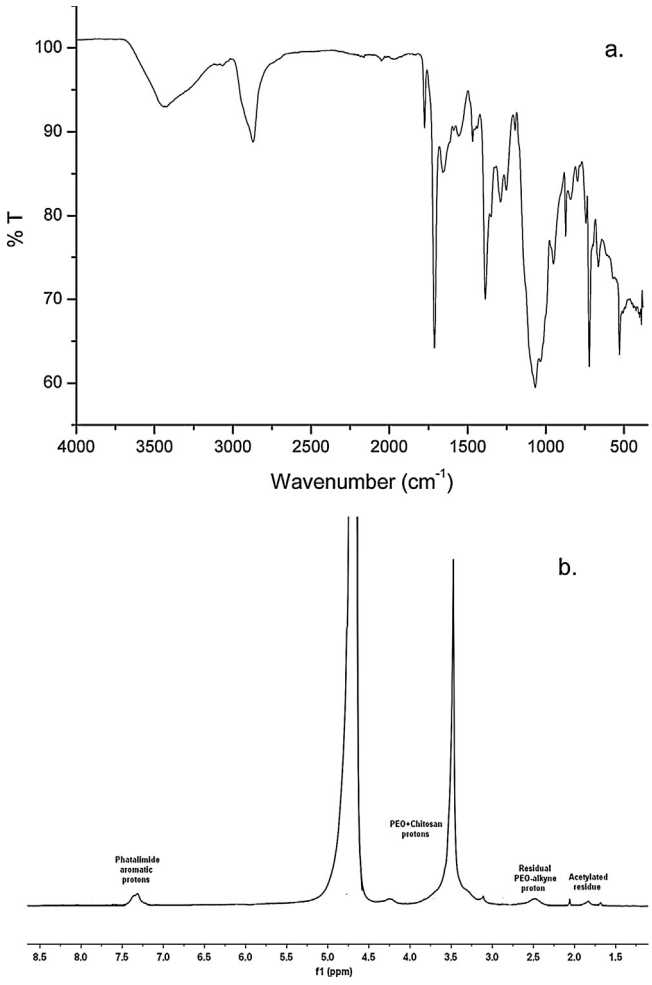


Fig. 5. FT-IR spectrum (a) and NMR spectrum in D_2O/KOH 37% (b) of pht-Ch–PEO hydrogel.

in comparison with a chitosan/PEO network characterized by a similar molecular weight of PEO but a more complex architecture (Kim et al., 2007), the swelling of the chitosan/PEO hydrogel here described is definitely larger (940% against around 100%).

Thermal analysis was performed by DSC in order to quantify the amount of PEO within the network. Measurements were carried out on Ch-crosslinked–PEO sample and on plain PEO (Fig. 6).

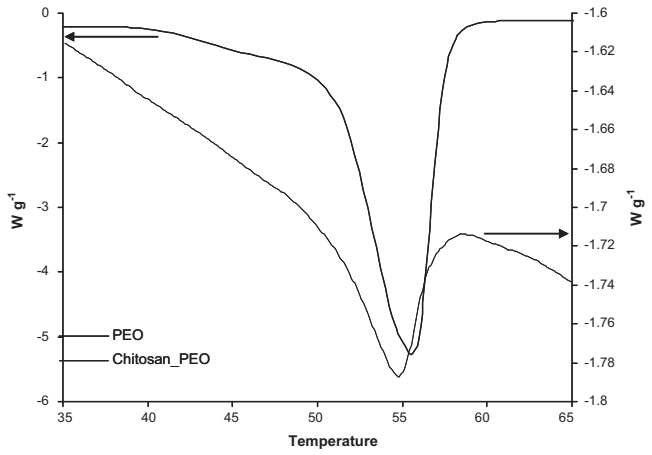


Fig. 6. Thermograms of plain PEO and Ch–PEO hydrogel.

The thermogram of Ch-crosslinked–PEO shows an endothermic peak at around 55 °C, related to the melting process of PEO chains. Assuming that the melting enthalpy of PEO in the network is the same of plain PEO, the amount of PEO in the hydrogel was evaluated from ΔHm and found to be around 45% (w/w).

4. Conclusions

A well-defined chitosan hydrogel was developed through a chemoselective coupling with PEO, achieved by "click chemistry". This synthetic pathway has the advantage to allow a control of final crosslinking degree of hydrogels. In fact, due to the high selectivity of click reaction, the crosslinking extent is strictly dependent on the amount of azido groups of chitosan, which can be modulated through successive cycles of mesylation reactions, as established.

This is of interest for all applications in which a fine control over crosslinking extent and swelling properties is required, as for example for controlled drug release. Furthermore, in comparison with different chitosan/PEO hydrogels described in the literature, a greater amount of absorbed water associated to a rapid swelling was found. These characteristics make the material suitable for wound dressing, in cases where a quick absorption of exudate is required.

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