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Synthesis of regioselective chitosan copolymers with β-cyclodextrin and poly(*N*-isopropyl acrylamide)

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ABSTRACT

This work aimed to design a synthetic route under mild conditions allowing the main chitosan chain to be grafted with β-cyclodextrin (β-CD) and poly(N-isopropyl acrylamide) (PNIPAm), at C2 and C6 positions, respectively. For this reason, the regioselectivity of proposed reactions is an important factor to be considered. β-CD is an oligosaccharide with a cyclic structure capable of forming inclusion complexes with hydrophobic molecules. Grafting β-CD onto the chitosan backbone by reductive *N*-alkylation at C2 position was carried out. With this purpose, the previous preparation of β-CD monoaldehyde was required. PNIPAm is a thermosensitive polymer with a transition temperature near 33 °C. To regioselectively anchor poly(*N*-isopropyl acrylamide) chains onto chitosan at C6 position, it was required to attach at the C6 position of chitosan an alkyl group for the subsequent grafting of PNIPAm-N_3 by means of copper-catalyzed azide-alkyne cycloaddition click reaction. To guarantee the regioselectivity of the functionalization of chitosan with a C6 terminal alkyne, its oxyalkylation with glycidyl propargyl ether in a solvent composed of LiOH/KOH/urea was used. The structure of all derivatives was confirmed by FT-IR and ^{1}H-NMR spectroscopy.

Keywords: chitosan copolymer, click chemistry, regioselectivity

INTRODUCTION

Natural polymers represent an alternative for the development of materials with biomedical applications. In the form of hydrogels, they can serve as support media for cell cultures, emulating properties of various tissues and serving as wound dressings. It is also possible to obtain nanoparticles that can be used as nanocarriers for controlled drug release [1].

Chitosan is a carbohydrate polymer obtained by the extensive deacetylation of chitin. It usually reaches degrees of deacetylation higher than 60-70%, but its solubility in dilute acid solutions is its most important characteristic [2]. Because of their nucleophilic nature, amino groups are susceptible to several modifications. The chemical structure of chitosan has been modified to improve its solubility, increase its affinity for certain surfaces and to confer it with additional interesting properties. Among all modifications, graft copolymerization is one of the most promising and versatile, since it allows enhancing or adding properties to polymer materials.

Cyclodextrins (CD) are cyclic oligosaccharides obtained by the enzymatic degradation of starch. By means of the action of cyclomaltodextrin glucanotransferase, the cyclic products of 6, 7 and 8 structural units could be obtained, denominated α-CD, β-CD, and γ-CD, respectively. Cyclodextrins are characterized by its relatively hydrophobic cavity that can form inclusion complexes with hydrophobic molecules. This feature can be exploited to load insoluble drugs into its cavity [3]. There are several methods to graft CD on chitosan [4, 5], mainly at the amino group, taking advantage of its reactivity. Formation of amide [6–12] nucleophilic substitution [13–19] and Schiff base reaction followed by reductive amination [20–23] have been used. Moreover, click chemistry methods have also been reported, with the aim to anchor CD at the C6 [24] or C2 [25] positions.

Poly(*N*-isopropyl acrylamide) is a well-known thermosensitive polymer, which has a lower critical solution temperature (LCST) around 33 °C [26, 27]. Above this temperature, polymer chains dehydrate abruptly, dominating hydrophobic interactions [28]. This thermosensitive polymer has been combined with chitin and chitosan to obtain interesting materials [29–32]. It is possible to graft pre-synthesized chains of PNIPAm-COOH into the amino group of chitosan by amidation [33]. In order to keep the polycationic character of chitosan, PNIPAm chains should be grafted at C6, making a previous modification of chitosan and followed by the Huisgen's cycloaddition "click" reaction [34, 35].

For the preparation of advanced chitosan-based materials with controlled and specific properties, it is necessary to have derivatives with defined molecular architecture. However, in general, it is difficult to prepare such derivatives with the necessary control of the structure. To this end, it is important to establish methods of synthesis with the desired chemo- and regioselectivity [4, 36]. This work is aimed to design a synthetic route under mild conditions allowing the grafting of β-CD at C2, and PNIPAm chains anchored at C6 position of chitosan. This procedure could allow synthesizing a double graft chitosan copolymer, which would be a convenient vehicle for the transport of hydrophobic molecules in the β-CD cavity, while exhibiting pH and temperature-sensitive behavior.

EXPERIMENTAL PART

Reagents

Chitosan (Ch, M_w = 200 kDa, degree of acetylation: 82%) was acquired from Primex, Iceland. β-cyclodextrin hydrate (β-CD), Dess-Martin periodinane solution (0.3 M in methylene chloride, DMP), sodium cyanoborohydride ($NaBH_3CN$), glycidyl propargyl ether (GPE), LiOH, KOH, urea, $CuSO_4$-$5H_2O$, sodium ascorbate, PNIPAm azide-terminated (PNIPAm-N_3, M_n = 5,000) and 3;5,5′,5′-[2,2′,2″-nitrilotris(methylene)tris(1*H*-benzimidazole-2,1-diyl)tripentanoic acid tripotassium salt, $(BimC_4A)_3$, were purchased from Sigma-Aldrich and used as received. Throughout all experiments, Milli-Q water with a conductivity lower than 0.2 μS/cm was employed.

Synthesis of β-cyclodextrin monoaldehyde (β-CDma)

β-CDma was synthesized by the one-pot method [37]. Briefly, in a reaction vessel, β-CD hydrate (2.5 g, 1.98 mmol) was dissolved in 52.5 mL of DMSO, then 10 mL of DMP (3 mmol) was added and allowed to react for 2 hours at room temperature. The crude β-CDma was twice precipitated with acetone at -10 °C and separated by centrifugation. The rest of the impurities were removed by dissolving the product in water, filtered throughout 0.45 μm membrane and lyophilized. Yield: 81%.

Synthesis of chitosan-*g*-β-cyclodextrin (Ch-βCD)

A solution containing chitosan (150 mg in 15 mL 2% acetic acid) (pH ~ 5) and β-CDma (128 mg, 0.1 mmol) was prepared. Both reagents were allowed to react for one hour at room temperature. Then, a $NaBH_3CN$ solution (15.5 mg in 200 μL of water) was added

dropwise and left to react further for 1 hour. The product was precipitated with NaOH up to pH ~ 8 and carefully purified by successive washing with water and ethanol.

Synthesis of chitosan-GPE (Ch-GPE)

Three grams of chitosan (17.7 mmol) were dissolved in aqueous alkali/urea solvent (a mixture of LiOH: 7 g, KOH: 8 g, urea: 8 g and water: 79 g), frozen at -20 °C overnight, thawed and stirred [38]. The reaction was carried out with 4.5 mL of GPE (37.17 mmol) for 24 hours at room temperature. Then, the reaction product was precipitated in 6M HCl and dialyzed against deionized water (MWCO 1000 Da) until a residual conductivity lower similar to the original water. Subsequently, the product was lyophilized, obtaining 2.74 g of product. Yield: 91%.

Synthesis of chitosan-*g*-poly(*N*-isopropyl acrylamide) (Ch-PNIPAm)

Two solutions were prepared overnight: Ch-GPE (32 mg, 0.04 propargyl mmol), and PNIPAm-N_3 (200 mg, 0.04 azide mmol) in 10 mL and 20 mL of deionized water, respectively. Prior to mixing, some drops of HCl dilute solution were added to the Ch-GPE solution in order to keep the system homogeneous. Then, both polymer solutions were mixed in a reaction vessel, capped with a septa silicon rubber stopper, and bubbled with N_2 for 5 minutes. To start the reaction, 2 mL of water containing 32 mg (0.02 mmol) of $CuSO_4 \cdot 5H_2O$ and 9 mg (0.01 mmol) of $(BimC_4A)_3$ and 2 mL of sodium ascorbate (12 mg) solution were successively added to the reaction vessel, giving a slightly greenish color. Subsequently, N_2 was bubbled into the reaction medium for two minutes and the system

was allowed to react at room temperature during 72 hours. The reaction product was purified through dialysis (MWCO 15 kDa) with 2% acetic acid for 24 hours, and then with Milli-Q water, up to a conductivity close to the original water (circa 60 hours) and lyophilized.

Fourier Transformed Infrared Spectroscopy (FTIR)

The FTIR spectra were recorded on a Thermo Scientific Nicolet iS-50 (Madison, WI, USA), using the attenuated total reflection (ATR) mode by the accumulation of 64 scans with a resolution of 4 cm^{-1}.

Nuclear Magnetic Resonance (^{1}H-NMR)

High-resolution ^{1}H-NMR spectroscopy was carried out on a Bruker Avance 400 operating at 400 MHz. Unless otherwise specified, the spectrum was registered at 25 °C. Samples were solubilized in deuterium oxide or anhydrous dimethyl sulfoxide-d6 (99.9 % atom D) as indicated in the text (polymer concentration ≈ 6 mg/mL). TMS was used as an internal standard.

RESULTS AND DISCUSSION

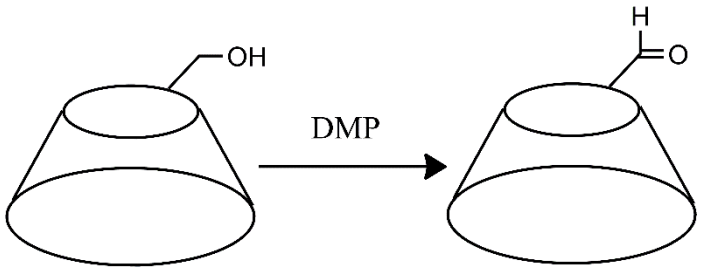
Chitosan is a polysaccharide susceptible to chemical modifications, due to the reactivity of the hydroxyl groups at C6 and C3 positions, respectively, and the highly reactive amino group at C2. Among the different chemical approaches to modify chitosan, grafting procedures are of particular interest to design polymers with a controlled molecular architecture for the development of new materials with specific applications.

A graft copolymer contains a long macromolecular chain, in this case, chitosan, with grafts of other anchored polymer chains. Graft copolymers can be synthesized primarily by three strategies: grafting *through*, grafting *from* and grafting *onto*, depending on the method of synthesis. Grafting *onto* is an interesting technique based on the coupling between terminal functional groups of the graft chains *onto* pendant functional groups of the backbone polymer.

Using this approach, we proceeded to graft β-cyclodextrin and poly(*N*-isopropyl acrylamide) at C2 and C6 positions, respectively.

Grafting β-cyclodextrin onto chitosan at C2 position (Ch-βCD)

The insertion of β-CD onto the chitosan backbone was carried out via the reductive amination procedure. With this purpose, β-CD was formerly modified to β-CD monoaldehyde (β-CDma) by the well-known reduction of one of the primary hydroxyl groups of β-CD with Dess-Martin periodinane (Scheme 1) [37]. This reaction, which takes place under very mild conditions, could guarantee that only the monoaldehyde derivative is obtained if some specific conditions are kept.



Scheme 1. Synthesis of β-cyclodextrin monoaldehyde.

The FTIR spectra of β-CD and β-CDma are shown in Figure 1. It could be seen the appearance of the carbonyl absorption band at 1700 cm^{-1}, due to the C=O stretching vibration. The low intensity of the signal is in accordance with the low percentage of substitution as compared to the remaining hydroxyl groups in the structure of β-CD (one aldehyde per six primary hydroxyl groups).

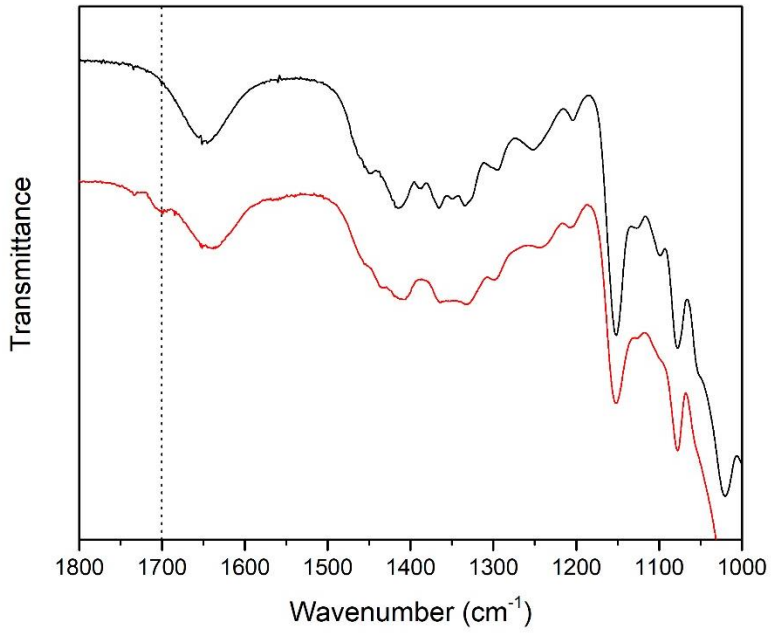


Figure 1. FTIR spectra of β-CD (black) and β-CDma (red).

^{1}H NMR spectra of these compounds are presented in Figure 2. Notice a new signal at 9.7 ppm assigned to the C6 formyl proton of the aldehyde. Besides, another new signal in the β-CDma spectrum can be observed at 4.93 ppm (B, anomeric proton from substituted units), which has a 1:6 integration ratio as compared to the signal at 4.82 ppm (A, anomeric proton from unsubstituted units), thus confirming that a successful monosubstitution of the β-CD was achieved during the oxidation.

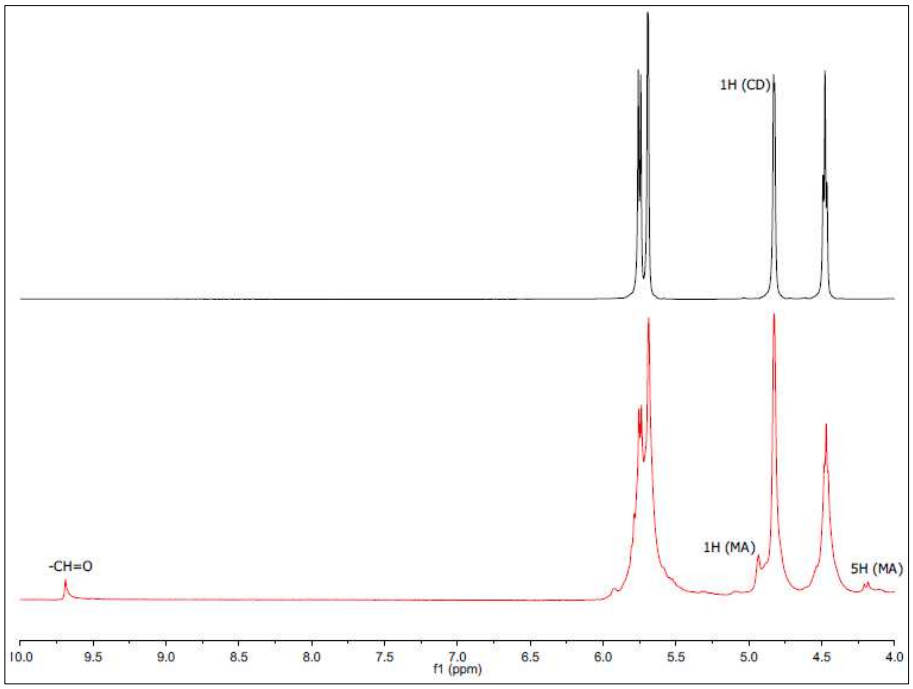
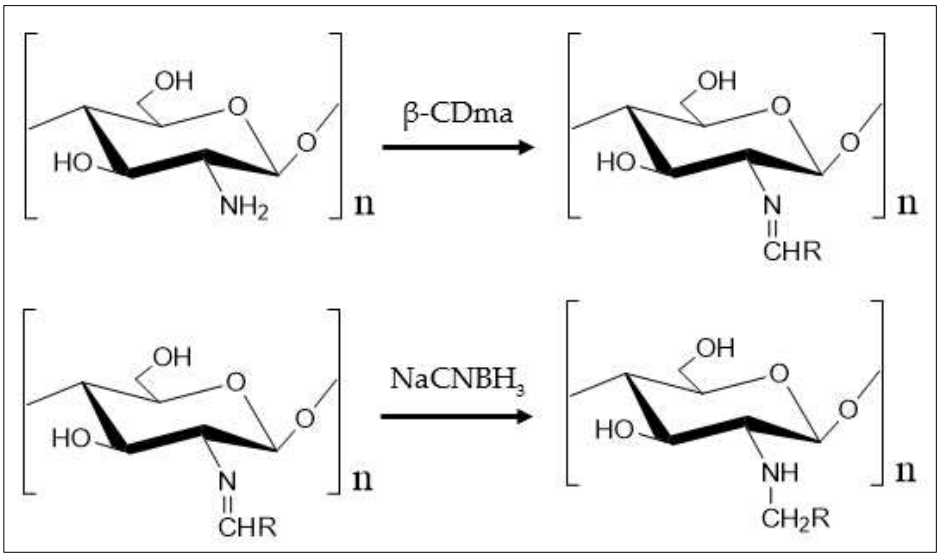


Figure 2. ^{1}H-NMR spectra of β-CD (black) β-CDma (red), dissolved in anhydrous dimethyl sulfoxide-d6.

The reaction between the aldehyde group of β-CDma and amino groups of chitosan results in the Schiff base formation [39], which could be afterward reduced with $NaBH_3CN$ as depicted in Scheme 2. This is a chemoselective and efficient reaction taking place only with the amino groups of chitosan. As a result, the grafting of β-CD moieties onto the chitosan backbone is obtained. Like the pristine chitosan, the Ch-βCD copolymer is soluble in aqueous acidic solutions.



Scheme 2. Schiff base formation and reduction.

Figure 3 shows the appearance of a band at 1558 cm^{-1} (wagging vibration of -NH-) in the FT/IR spectrum, corresponding to the secondary amine of the Ch-βCD copolymer. There is also an attenuation of the band at 659 cm^{-1} (bending vibration outside the plane of $-NH_2$), which corresponds to the primary unreacted amino groups of chitosan.

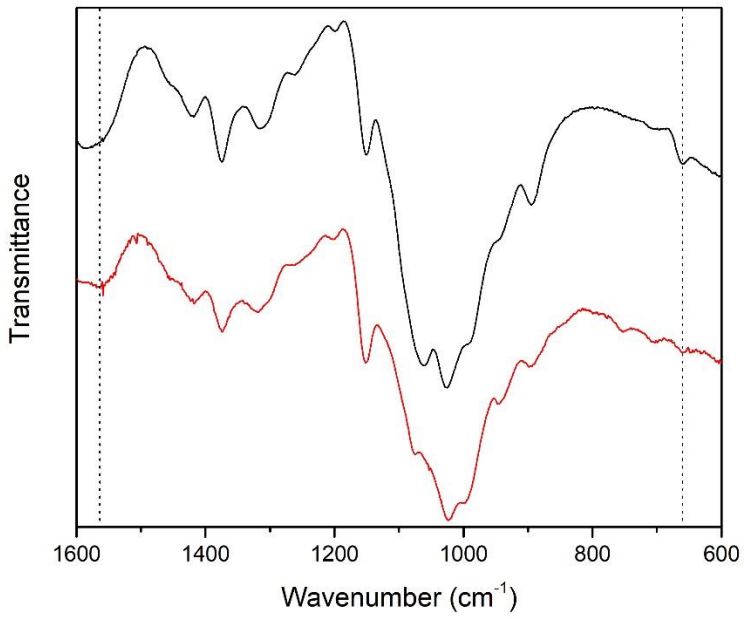


Figure 3. FTIR spectra of chitosan (black) and Ch-βCD (red).

Grafting poly(*N*-isopropyl acrylamide) onto chitosan at C6 position (Ch-PNIPAm)

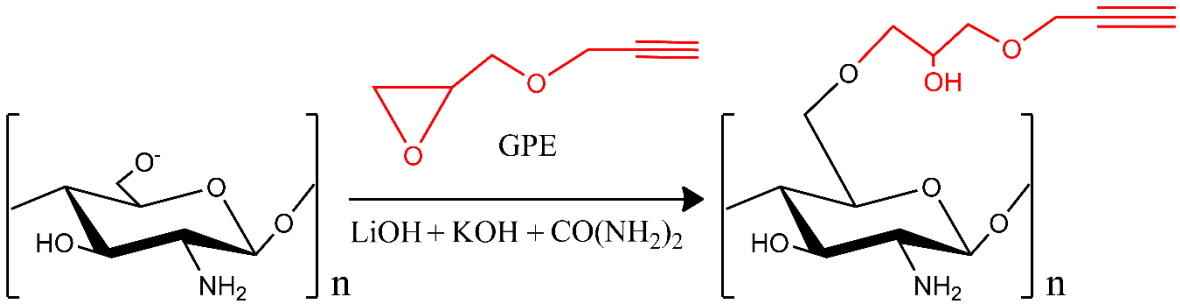
In order to achieve chitosan grafted at C6 position, it was decided to use a click chemistry approach. In particular, the copper-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction is a precise way to regioselectively attach poly(*N*-isopropyl acrylamide) onto the C6 position under very mild experimental conditions. This reaction could be verified between a C6-alkyne chitosan derivative and an azide-terminated PNIPAm.

For this purpose, a pre-click reaction is necessary to regioselectively functionalize chitosan on the C6 position with an alkyne group. Commonly used strategies include the protection of amino groups, followed by functionalization on C6 and, subsequently, deprotecting the amino groups. Among the approaches proposed to protect amino groups, the *N*-

phthaloylation [40, 41], Schiff base formation [42] and polymer-surfactant complexes [43] should be mentioned. All these procedures were unsuccessfully tested, the main disadvantages being the limited solubility of the protected derivatives in aqueous media. Indeed, this is a must in order to achieve strong basic conditions to obtain the alkoxide to react with propargyl bromide via the Williamson ether reaction [44]. Moreover, the remarkable degradation of the chitosan backbone during the deprotection of the *N*-phtatloyl derivative is also an important drawback to be taken into account [45].

Recently, a novel solvent system has been developed, that allows chitosan to be solubilized in alkaline aqueous media in the presence of urea at low temperatures [46]. These conditions proved to be convenient for etherification reactions under homogeneous conditions with very low polymer degradation. Regarding the oxyalkylation of chitosan with propylene epoxide, it was demonstrated by ^{13}C NMR that, under these conditions, the etherification reaction gives O-(2-hydroxypropyl) derivatives at C6 hydroxyl groups and, to a lesser extent, at the C3 -OH groups [38]. Moreover, it was demonstrated that there is no substitution with amino groups at C2 position. These results open the possibility of regioselectively controlling the etherification of chitosan at C6, through a one-step method, without amino group protection.

Based on the above analysis, it was decided to introduce the alkyne terminal group via pre-click reaction with glycidyl propargyl ether in aqueous KOH/LiOH/urea solution at room temperature (Scheme 3).



Scheme 3. Pre-click reaction (terminal alkyne functionalization).

In the FT-IR spectrum of the pre-click derivative Ch-GPE (Figure 4), the weak absorption band characteristic of the alkyne group can be appreciated at 2115 cm^{-1}. This signal is attributed to the C≡C stretching vibration in monosubstituted terminal alkynes, thus suggesting the successful pre-click reaction [47]. Other authors also reported a similar absorption band when introducing alkyne groups on chitosan [48, 49].

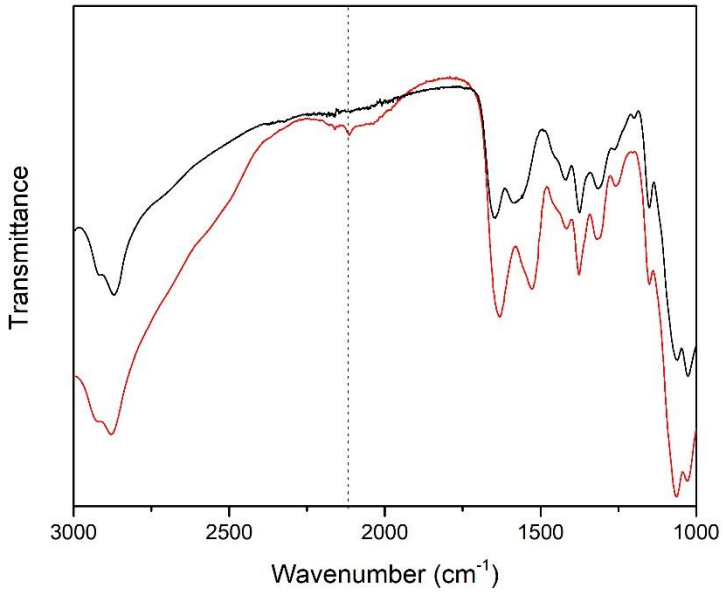


Figure 4. FTIR spectra of chitosan (black) and Ch-GPE (red).

As shown in the ^{1}H-NMR spectrum of Ch-GPE (Figure 5), it is noteworthy the appearance of two signals due to the presence of terminal alkyne groups on the pre-click derivative. The peaks at 2.85 and 4.15 ppm, assigned to $-O-CH_2-C\equiv C\underline{\mathbf{H}}$ and $-O-C\underline{\mathbf{H}}_2-C\equiv CH$ of the propargyl group, respectively, unequivocally confirms the formation of O6 alkynyl substitution.

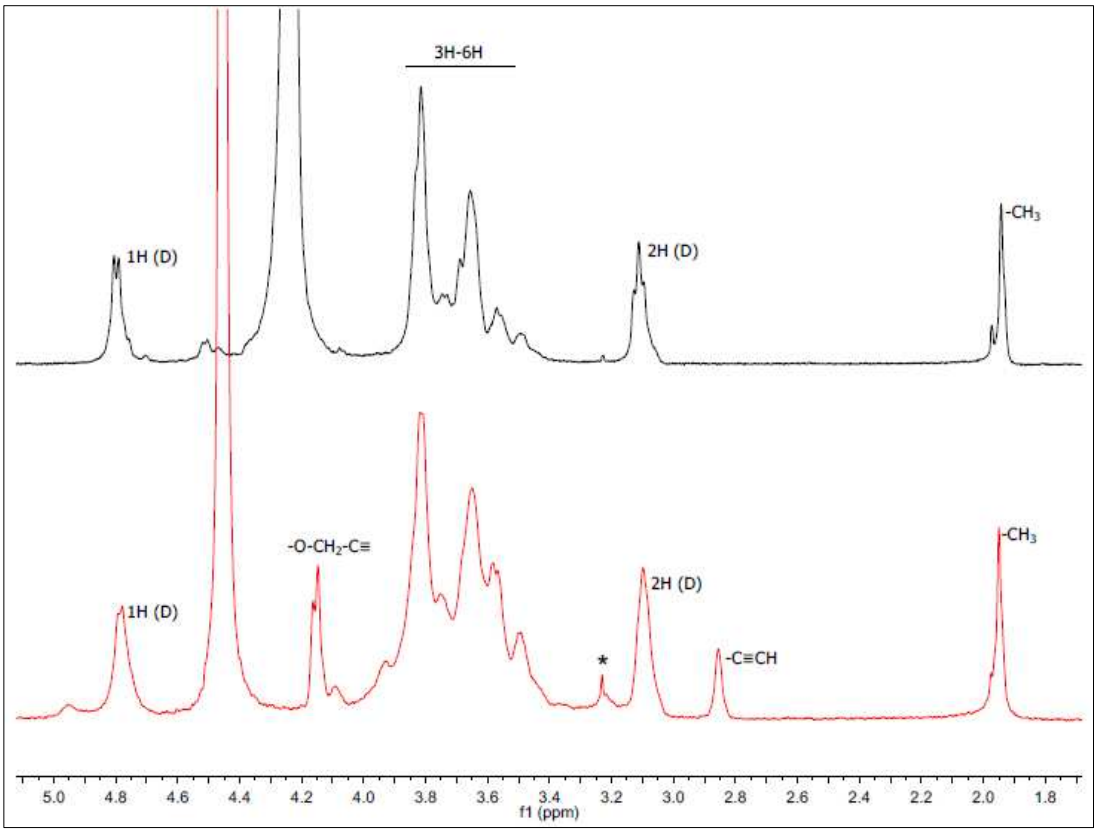


Figure 5. ^{1}H-NMR spectra of chitosan (black) and Ch-GPE (red) recorded in D_2O.

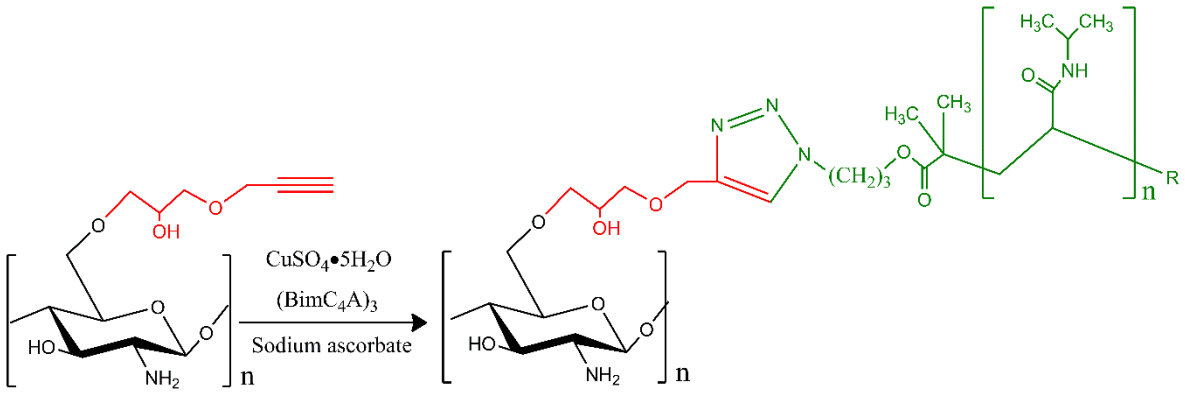
The degree of substitution per chitosan glucopyranose unit was calculated to be 0.26, using the following equation:

$$DS = \frac{(\int H_a + \int H_b)/3}{\int H}$$

where H_a and H_b are the integral intensities corresponding to the protons of −O−CH_2−C≡C**H** and −O−C**H**$_2$−C≡CH of the propargyl group, and H those corresponding to both anomeric protons.

After alkynyl terminal functionalization of chitosan at C6 position, click reaction was carried out with PNIPAm azide-terminated, using Cu(I) as catalyst, as shown in Scheme 4. This strategy allows the synthesis of chitosan-*g*-PNIPAm copolymer to give a 1,2,3-triazole by Huisgen cycloaddition. It is worth noting that chitosan-*g*-PNIPAm copolymer was readily soluble in water. Similar behavior was observed for chitosan-*g*-poly(*N*-vinyl caprolactam) [50].

It was possible to confirm this reaction by the disappearance of the absorption bands attributed to the azide group and terminal alkyne on the copolymer, both centered around 2100 cm^{-1}. In addition, a new absorption band corresponding to isopropyl group (1390 cm^{-1}), secondary amine vibration (1457 cm^{-1}) and strengthening of the amide I (1615 cm^{-1}) from PNIPAm grafted chains can be also observed in Figure 6.



Scheme 4. Copper-catalyzed azide-alkyne cycloaddition click reaction.

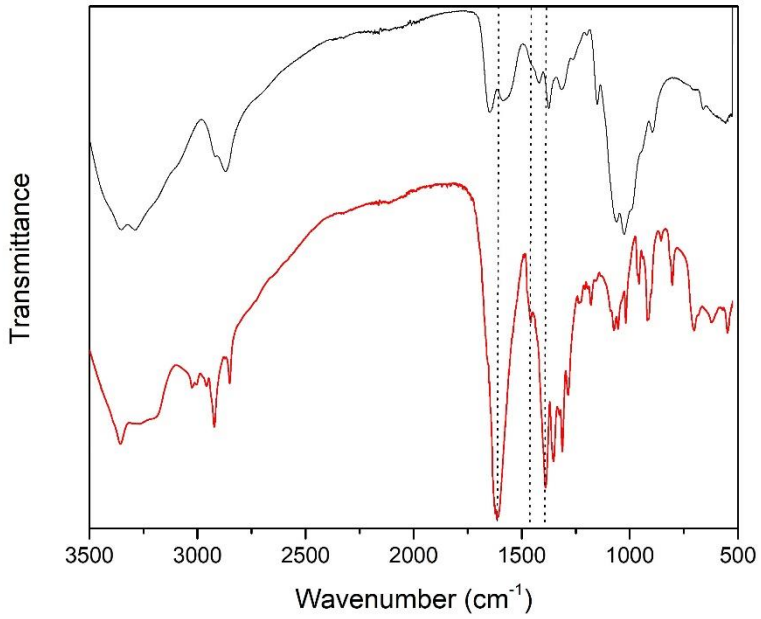


Figure 6. FTIR spectra of chitosan (black) and Ch-PNIPAm (red).

The ^{1}H-NMR spectrum of Ch-PNIPAm is shown in Figure 7. It could be noted the appearance of a new signal corresponding to the methine proton of the 1,2,3-triazole ring at 7.4-7.6 ppm. All signals of the *N*-isopropyl acrylamide structure can also be noticed.

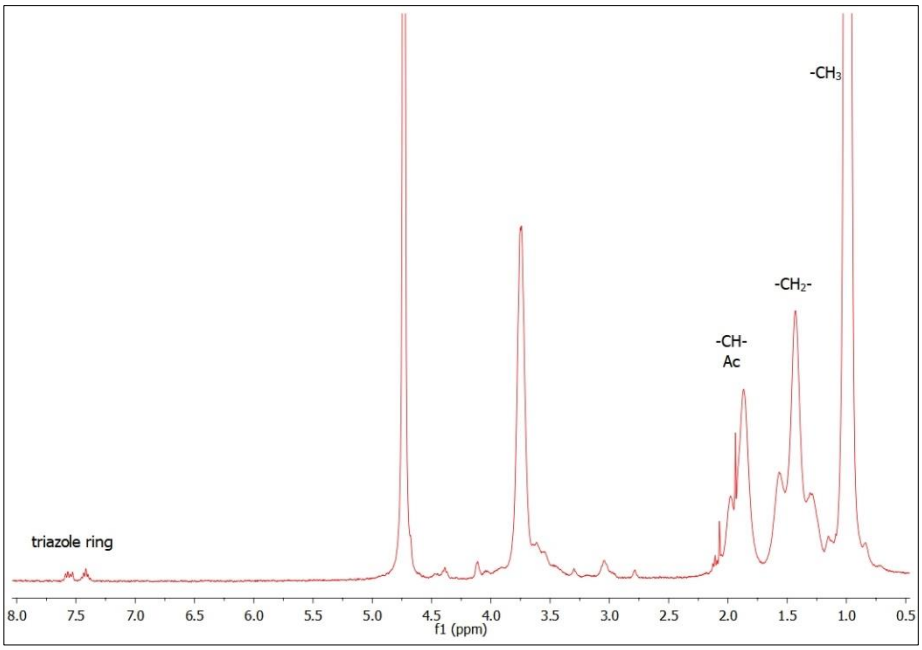


Figure 7. ^{1}H-NMR spectrum of Ch-PNIPAm recorded in D_2O.

CONCLUSIONS

Chitosan-g-β-cyclodextrin and chitosan-g-poly(*N*-isopropyl acrylamide) copolymers were synthesized in a regioselective way. On the one hand, the former copolymer was synthesized by linking β-cyclodextrin monoaldehyde with the amino groups of chitosan by Schiff base formation/*N*-alkylation at C2 position.

On the other hand, the anchoring of PNIMAm at C6 position of chitosan was achieved by the copper-catalyzed azide-alkyne cycloaddition click reaction. With this aim, a C6-alkynyl derivative was prepared in aqueous KOH/LiOH/urea solution by oxyalkylation of

chitosan with glycidyl propargyl ether. Subsequently, the Cu-catalyzed Huisgen cycloaddition with PNIPAm-N_3 was conducted in order to obtain the grafted PNIPAm copolymer at C6 position.

All reactions were carried out under very mild conditions at room temperature, and products with defined molecular architecture were obtained. The developed methodology and findings of the present study can now be combined in order to prepare a double-grafted chitosan copolymer, which can be of great interest in designing advanced thermosensitive biopolymeric materials and hydrogels as potential carriers of hydrophobic molecules; further investigations in our laboratory are ongoing.

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