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Coupling of amines with polyglucuronic acid: Evidence for amide bond formation

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ABSTRACT

Cellulose III was reacted with catalytic amounts of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), sodium hypochlorite and sodium bromide in order to selectively oxidize the cellulose primary hydroxyl groups to yield polyglucuronic acid. This product was further modified by a coupling of the carboxyl groups with a series of amines in the presence of carbodiimide. The samples were characterized by NMR and FTIR. These techniques allowed us to determine the degree of conversion (*DC*), which was deduced from the decrease of carboxyl and the increase in nitrogen content. In addition, the formation of an amide bond between both linear or cyclic amines and polyglucuronic acid was systematically observed. For this, the results of NMR spectroscopy experiments are of particular interest as they indicate clearly that a bond between the amines and polyglucuronic acid has been formed during the reaction. This bond was revealed by the presence of an extra signal near 170.5 ppm in the ^{13}C NMR spectra, characteristic of the acetamide moiety. In the case of coupling with 2-methoxy ethyl amine, the 2D ^{13}C–^{1}H HMBC spectrum revealed a set of cross-peaks between the C6 of the glucuronic residues and the methylenic protons H_a, H_b, thus confirming further the formation of the amide linkage. When the amidation was achieved with 4-amino TEMPO, the degree of conversion was further confirmed by electron paramagnetic resonance (EPR) spectroscopy.

1. Introduction

Large amount of natural polysaccharides such as cellulose, chitin and starch, which are produced annually, can be chemically modified to prepare new bio-based materials with end-use properties in the fields of adhesion, textile, detergent, paint, cosmetic, medicine, food, etc. Among the chemical modification, the selective oxidation of the primary alcohol group of polysaccharides, yielding polyuronic acids, has been studied for more than a half-century (Maurer & Reiff, 1943; Yackel & Kenyon, 1942). Polyuronic acids are of great interest since they yield valuable products possessing specific properties, ranging from gelation, complexation, anti-flocculation, adhesion, as well as a number of biological activity. Recently, a method for selectively oxidizing primary alcohol groups of polysaccharides has been described in literature. The technique is based on a reaction catalyzed by 2,2,6,6-tetramethyl-1-piperidine oxoammonium radical (TEMPO) in presence of NaOBr, generated *in situ* by NaOCl and NaBr, the catalyst being regenerated during the reaction. This method was firstly proposed for water-soluble polysaccharides (Chang & Robyt, 1996; de Nooy, Besemer, & van Bekkum, 1994, 1995; de Nooy, Besemer, van Bekkum, van Dijk, & Smit, 1996; Sierakowski, Milas, Desbrières, & Rinaudo, 2000) such as starch, inulin, amylodextrin, pullulan, alternan, amylopectin, chitosan and galactomannan. The method has been later extended to water-insoluble polysaccharides such as cellulose, amylose and chitin (Araki, Wada, & Kuga, 2001; Chang & Robyt, 1996; Isogai & Kato, 1998; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Sierakowski et al., 2000; Tahiri & Vignon, 2000).

The success of using the TEMPO–NaOCl–NaBr oxidation method for cellulose to produce water-soluble polyglucuronic acid seems to depend on the accessibility and on the crystalline state of the starting material (Chang & Robyt, 1996). In fact, the cellulose can be fully oxidized to yield pure polyglucuronic acid only if amorphous cellulose, cellulose II or cellulose III, are used as starting material (Isogai & Kato, 1998). With cellulose I, the oxidation proceeds only at the crystalline microfibril surfaces, a phenomenon that can be interesting for subsequent surface grafting or derivatization purposes (Araki et al., 2001).

The chemical modification is a way to modify and introduce specific functionalities leading to the development of new biopolymers. For preparing macromolecular prodrug carrier, the polymer must be biodegradable, biocompatible and must contain appropriate functional sites for chemical coupling. In this context, oxidized

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cellulose containing carboxylic groups represents an important class of biocompatible and bioresorbable products. Such materials are commercially available and they have been widely used in medicine for several decades to stop bleeding during surgery or to prevent the formation of adhesions following surgery (Stillwell, Marks, Sferstein, & Wiseman, 1997). Owing to the presence of carboxylic groups, an intensive investigation of oxidized cellulose as immobilizing matrix for a variety of amine-based drugs has been carried out.

In this work, we have selected a series of amines and used them for the amidation of polyglucuronic acid, resulting from the TEMPO-mediated oxidation of cellulose. For the amidation, we took advantage of the addition of carbodiimide in the reaction medium (Alinovskaya, Kaputskii, Yurkshtovich, Talapin, & Stel'makh, 1988; Firsov, Nazarov, & Fomina, 1987; Yasnitskii & Dol'berg, 1973). This diimide has indeed been extensively used to increase the reactivity of carboxyl groups toward amidation (Bulpitt & Aeschlimann, 1999; Danishefsky & Siskovic, 1971; Hoare & Koshland, 1967; Kuo, Swann, & Prestwich, 1991; Mauk & Mauk, 1989; Pouyani, Kuo, Harbison, & Prestwich, 1992; Taylor, 1991; Wang, Li, & Zhang, 1987; Zhu, Kumar, & Banker, 2001), but so far has been used rarely for polysaccharides holding carboxyl moieties. Using this technique, we report on the coupling of polyglucuronic acid with a range of linear and cyclic amines in the presence of carbodiimide. The extent of amidation was followed by FT-IR and NMR spectroscopies. Of particular interest was the coupling of 4-amino-TEMPO with polyglucuronic since the reaction could also be followed by electron paramagnetic resonance spectroscopy (EPR), taking advantage of the method developed by Irwin and collaborators for the interaction of nitroxide radicals with the galacturonic acid units from apple pectins (Chamulitrat, Irwin, Sivieri, & Schwartz, 1988; Irwin, Sevilla, Chamulitrat, Hoffman, & Klein, 1992; Irwin, Sevilla, & Osman, 1987).

2. Experimental section

2.1. Materials

Cotton linter cellulose samples used in this work, were purchased from Tubize Plastics, Rhodia (Belgium) and employed as received. TEMPO, sodium bromide and sodium hypochlorite from Aldrich were used as components into the oxidation reaction. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) and *N*-hydroxysuccinimide (NHS), components for the coupling reaction, were purchased from Sigma Chemical Co. The 4-amino TEMPO was obtained from Aldrich Chemical Co; *n*-butyl amine, *n*-octyl amine and 2-methoxy ethyl amine were purchased from Fluka.

2.2. Preparation of cellulose III by ammonia treatment

The protocol to prepare cellulose III (Da Silva Perez, Montanari, & Vignon, 2003) involved the use of exploded gaseous ammonia (EG-NH_3) and corresponded to samples processed by Rhodia Acetow, following their patented process, which consists of treating cellulose samples with gaseous ammonia under high pressure followed by a rapid decompression (Karstens & Steinmeier, 1995; Karstens, Stein, & Steinmeier, 1998). This particular cellulose preparation was obtained from Rhodia, company which developed the technology for its use. The scientific basis for the "ammonia explosion" process has been achieved by Dale and collaborators (Ferrer, Byers, Sulbaran-de-Ferrer, Dale, & Aiello, 2000; Holtzapple, Jun, Ashok, Patibandla, & Dale, 1991). This procedure that leads to cellulose III has been frequently used to improve the reactivity of crystalline cellulose for the preparation of derivatives in better yields (Klemm, Philipp, Heinze, Heinze, & Wagenknecht, 1998; Klenkova, 1967). Indeed, the conversion of cellulose to cellulose III is essentially a solid-state process that keeps the integrity of the cellulose microfibrils while achieving a substantial decrystallization and a reorganization of the intra-crystalline hydrogen bond pattern of cellulose (Chanzy, Henrissat, Vuong, & Revol, 1986; Wada et al., 2001).

2.3. Preparation of polyglucuronic acid by TEMPO-mediated oxidation

Oxidation experiments were carried out as previously published with minor modifications (Tahiri & Vignon, 2000). Cellulose III sample (1.95 g, 12.0 mmol glycosyl units) was dispersed in distilled water (180 mL) for 3 minutes with a high speed T25 basic Ultra-Turax homogenizer (Ika-Labortechnik, Staufen, Germany). A total of 90 mL of water was used to wash the homogenizer. TEMPO (30 mg, 0.19 mmol), NaBr (0.63 g, 6.1 mmol) and NaOCl (1.76 M solution, 1.5 mL, 2.64 mmol) was stirred in 20 mL of water until complete dissolution. This solution was then added to the cellulose suspension, which was mechanically stirred and maintained at 20 °C. The NaOCl (1.76 M solution, 11.5 mL, 20.24 mmol) was added dropwise to maintain the pH at 10 during the addition. After the total addition of NaOCl, the pH was maintained constant at 10 by adding 0.5 M NaOH solution until no more variation was observed, indicating the end of the reaction. Methanol (5 mL) was then added to destroy the residual NaOCl and the pH adjusted to 7 with 0.5 M HCl. A white suspension was obtained resulting from the oxidation of cellulose. After centrifugation, we separate the supernatant, corresponding to the water-soluble oxidized cellulose with glucopyranose units completely oxidized, from the water-insoluble fraction which corresponds to partially oxidized cellulose. The supernatant, referred to as polyglucuronic acid, was precipitated by adding an excess of ethanol, centrifuged, redissolved in water, dialyzed against water, and finally freeze-dried. The final yield of this process was between 90% and 95%.

2.4. Coupling reaction

The reaction of coupling between polyglucuronic acid and amine molecules was achieved in aqueous media. Amine molecules were added to the oxidized cellulose solutions (2.5 mmol amine/1 mmol glucuronic unit). The pH of the solution was adjusted to 7.5–8 with 0.5 M HCl. EDAC and NHS (ratios of 1.5 relative to glucuronic unit, as previously published (Roumani, 2004)), were dissolved in 2 mL of water, and added to the solution. The pH was adjusted and maintained to 8 by adding 0.5 M HCl and NaOH solutions. The solution was stirred during 24 h at 50 °C and finally precipitated by adding an excess of ethanol. After filtration on a 0.5 μm membrane and washing with ethanol (3 times), the precipitate was redissolved in water, evaporated again to remove any trace of ethanol, redissolved in water and finally freeze-dried.

2.5. NMR spectroscopy

Carbon-13 and proton NMR spectra were recorded with a BRUKER Avance 400 spectrometer operating at a frequency of 100.618 MHz for the ^{13}C and 400.13 MHz for the proton. Samples were studied as their sodium salt solutions in D_2O (6–10 mg in 500 μL of solvent) at 30 °C in 5 mm o.d. tubes. ^{13}C spectra were recorded using 90° pulses, 20,000 Hz spectral width, 65536 data points, 1.638 s acquisition time, 2 s relaxation delay. From 4096 up to 10240 scans were accumulated depending on the sample solubility. Proton spectra were recorded with 4006 Hz spectral width, 32768 data points, from 4.089 up to 7.497 s acquisition time, 0.1 s relaxation delay and up to 128 scans. The 2D ^{13}C–^{1}H experiments were performed with 4006 Hz spectral width, 2048

data points, 0.266 s acquisition time, 1 s relaxation delay and 128 scans.

2.6. Electron paramagnetic resonance (EPR)

EPR measurements were made with a Bruker EMX X-band continuous wave spectrometer equipped with a Bruker ER 4116 DM rectangular cavity operating at 9.658 GHz. Experiments were performed at room temperature (300 K) with a hyper frequency power of 1 mW and a modulation amplitude of 0.5 G. The amplitude of the magnetic field modulation and microwave power were adjusted so that no line-shape distortion was observable. The received gain was 63,200 and the sweep time was 42 s. Absolute quantification was obtained by comparison with a TEMPO sample of known concentration after double integration of EPR spectra.

Samples (2 mg), i.e. 4-amino TEMPO and polyglucuronic acid-4-amino TEMPO, were dissolved in H_2O (1 mL) and loaded into a closed capillary tube (o.d. 0.7 mm), which was introduced in a standard EPR tube (o.d. 3 mm).

In order to determine the coupling yield from EPR data, the average molecular weight of a glucosyl unit from the polyglucuronic acid-4-amino TEMPO derivative M was calculated as follows:

$$M = 329 \times DC + (1 - DC) \times 198 = 131 \times DC + 198 \quad (1)$$

Where 329 (g/mol) corresponds to the molar mass of a glucuronic unit coupled to a 4-amino TEMPO and 198 (g/mol) to the molar mass of polyglucuronic sodium salt, and where DC represents the degree of conversion.

The number of 4-amino TEMPO ($n_{4\text{-aminoTEMPO}}$) coupled with polyglucuronic acid, was determined by:

$$n_{4\text{-aminoTEMPO}} = \frac{DC \times m}{M} = \frac{DC \times m}{131 \times DC + 198} \quad (2)$$

which gives

$$DC = \frac{-198 \times n_{4\text{-aminoTEMPO}}}{131 \times n_{4-aminoTEMPO-\mathrm{m}}} \quad (3)$$

where m corresponds to the fraction of the dissolved sample which was introduced into the capillary tube.

2.7. Infrared spectroscopy

Infrared spectra were recorded with a FT-IR Perkin-Elmer 1720X spectrometer. Samples were studied as KBr pellets (1% in anhydrous KBr). Spectra were recorded using 3600 cm^{-1} spectral width (between 400 and 4000 cm^{-1}), 2 cm^{-1} resolution and 32 scans were accumulated. Samples were studied as acidic form to avoid the superposition of sodium carboxylate peak with hydrogen bonds. For this, few milligrams of sample were suspended in 1 mL of water, 1–2 drops of 1 M HCl were added and after stirring during 3–5 min the suspension was centrifuged and the precipitate was washed several times with water until neutralisation of sample.

2.8. Conductimetry

The residual carboxyl content of coupled oxidized cellulose samples or degree of oxidation (DO_1) was determined by conductometric titrations (Da Silva Perez et al., 2003). The samples (30–40 mg) were dissolved into 15 mL of 0.01 M hydrochloric acid solution. After 10 min of stirring, the suspensions were titrated with 0.01 M NaOH. As previously published (Da Silva Perez et al., 2003), the titration curves showed the presence of strong acid, corresponding to the excess of HCl and weak acid corresponding to the carboxyl content.

The residual carboxyl groups content DO_1 or degree of oxidation after the coupling reaction is determined by the following equation:

$$DO_1 = \frac{[162 + (M_a\text{-}4)] \times n_{\mathrm{COONa}}}{m + [(M_a\text{-}40) \times n_{\mathrm{COONa}}]} \quad (4)$$

where M_a is the molar mass of amine molecule (mol/L), m is the weight of oven-dried sample (g) and n_{COONa} (mol) is the number of mol of carboxyl groups determined by the conductometric titration curve:

$$n_{\mathrm{COONa}} = (V_2 - V_1) \times c \quad (5)$$

where V_1 and V_2 are the amount of NaOH (in L) and c is the NaOH concentration (mol/L) (Da Silva Perez et al., 2003).

The degree of conversion (DC), which represents the number of coupled anhydroglucose units, is deduced by the following equation: $DC = 1 - DO_1$, where DO_1 is the residual degree of oxidation after the reaction of coupling.

2.9. Elemental analysis

The nitrogen content of the coupled samples was determined by elemental analysis. Indeed, the degree of conversion can also be calculated from the nitrogen content by using the following equation:

$$DC = \frac{198}{\frac{14 \times 100}{\%N} - M_a + 40} \quad (6)$$

where $\%N$ corresponds to the nitrogen content of the coupled samples determined by elemental analysis, 198 (g/mol) corresponds to the molar mass of glucuronic unit sodium salt and M_a is the molar mass of amine molecule (mol/L).

3. Results and discussion

Previous results (Da Silva Perez et al., 2003) have shown that a pretreatment of cellulose samples with ammonia which converted cellulose I into III improved its reactivity with respect to the TEMPO-mediated oxidation system. Therefore, in this study polyglucuronic acid was prepared by oxidation of cellulose III by the TEMPO-mediated method.

3.1. Coupling reaction with n-butyl amine, n-octyl amine and 2-methoxy ethyl amine

In this report, we have studied the coupling reaction of polyglucuronic acid with linear primary amines, such as *n*-butyl amine, *n*-octyl amine and 2-methoxy ethyl amine. After the coupling, the purification of the products included precipitation, dialysis and freeze-drying steps. The product samples were analyzed by conductimetry, FTIR spectroscopy, elemental analysis and NMR characterization. The coupling conditions of amines on oxidized cellulose or natural carboxylic acid polymers were recently studied (Araki et al., 2001; Bulpitt & Aeschlimann, 1999; Lillo & Matsuhiro, 2003; Novak, Banyai, Fleischer-Radu, & Borbely, 2007; Roumani, 2004; Zhu et al., 2001). Concerning the oxidized cellulose substrate, Araki and collaborators described a grafting of PEG-NH_2 onto cellulose according to the Bulpitt and Aeschlimann procedure (Bulpitt & Aeschlimann, 1999). The authors used a 1:2:1.5 mol ratio of carboxylated cellulose:PEG-NH_2:carbodiimide, respectively, leading to a degree of conversion of 26% (Araki et al., 2001). The yields of conversion were optimized by varying the relative ratios between polyglucuronic acid, amine and carbodiimide and the best degree of conversion was obtained with, respectively, 1:2.5:1.5:1.5 mol ratios of polyglucuronic acid:2-methoxy ethyl amine:carbodiimide:EDAC.

3.1.1. Conductimetry and elemental analysis

The degree of conversion was calculated from the residual carboxyl content (DO_1) of the coupled samples, and also from their amount of nitrogen determined by elemental analysis (Table 1). We can observe that there is a relatively significant agreement between the data obtained by conductimetry and elemental analysis (Table 1).

These results show that the coupling reactions occur with all the amine molecules, but the coupling yields depend on the carbon chain length of the amine molecule. In the case of *n*-octyl or *n*-butyl amine, coupling yields of 13% and 39% were obtained, respectively, by conductimetry (Table 1), indicating that the yield of coupling decreases when the length of the amine carbon chain is increased. This phenomenon may be due to the variable electronic distribution along the amine molecules, the steric effects and more probably to a decrease in solubility.

The *n*-butyl and 2-methoxy ethyl amines present different coupling yields. The 2-methoxy ethyl amine presents a methoxy group ($-OCH_3$) instead of an ethyl group ($-CH_2-CH_3$) for *n*-butyl amine.

Table 1
Degrees of conversion between polyglucuronic acid and amines

Amines	Weight yield (%)	Conductimetry	Elemental analysis	
		Degree of conversion	*N* (%)	Degree of conversion
n-Butyl amine	51.6	0.39	2.87	0.43
n-Octyl amine	70.3	0.13	1.0	0.15
2-Methoxy ethyl amine	72.4	0.56	3.8	0.59

This difference in the two amine structures leads to different inductive and mesomer effects. Furthermore the pK_a of the two amines has to be taken into account. According to the literature, the pK_a values of *n*-butyl amine and 2-methoxy ethyl amine are of 10.6 and 9.2, respectively (Bjerrum, Schwartzenbach, & Sillen, 1958; Hall, 1957). In aqueous medium, the protonation of amines provides ammonium salts (RNH_3^+). As during the coupling reaction the pH was maintained at around 8, the amount of ammonium ions in solution was lower for 2-methoxy ethyl amine ($pK_a = 9.2$) than for *n*-butyl amine ($pK_a = 10.6$). As a consequence, the 2-methoxy ethyl amine will be less protonated than *n*-butyl amine and thus should present a better reactivity (Table 1). Finally weight yields of 56% and 43% were obtained with 2-methoxy ethyl amine and *n*-butyl amine, respectively (Table 1). From these results it appears that the yields of coupling can be directly correlated with the amines reactivities.

3.1.2. FTIR spectroscopy

The infrared spectra of cellulose, polyglucuronic acid, polyglucuronic acid coupled with *n*-butyl amine and 2-methoxy ethyl amine are presented in Fig. 1. The FTIR spectrum of polyglucuronic acid shows a characteristic peak with at 1724 cm^{-1} corresponding to the (C=O) (free COOH) stretching vibration with strong intensity. In the spectrum of polyglucuronic acid-amine, the carboxyl acid peak appears at 1730 cm^{-1}. Two other distinct bands at 1650 and 1550 cm^{-1} were also observed, located in the zone related to the (–CONH–), corresponding, respectively, to the (C=O) stretching band and to the (–NH) bending vibration band (Williams & Fleming, 1966). The presence of these two bands indicates that an amide bond has been formed between polyglucuronic

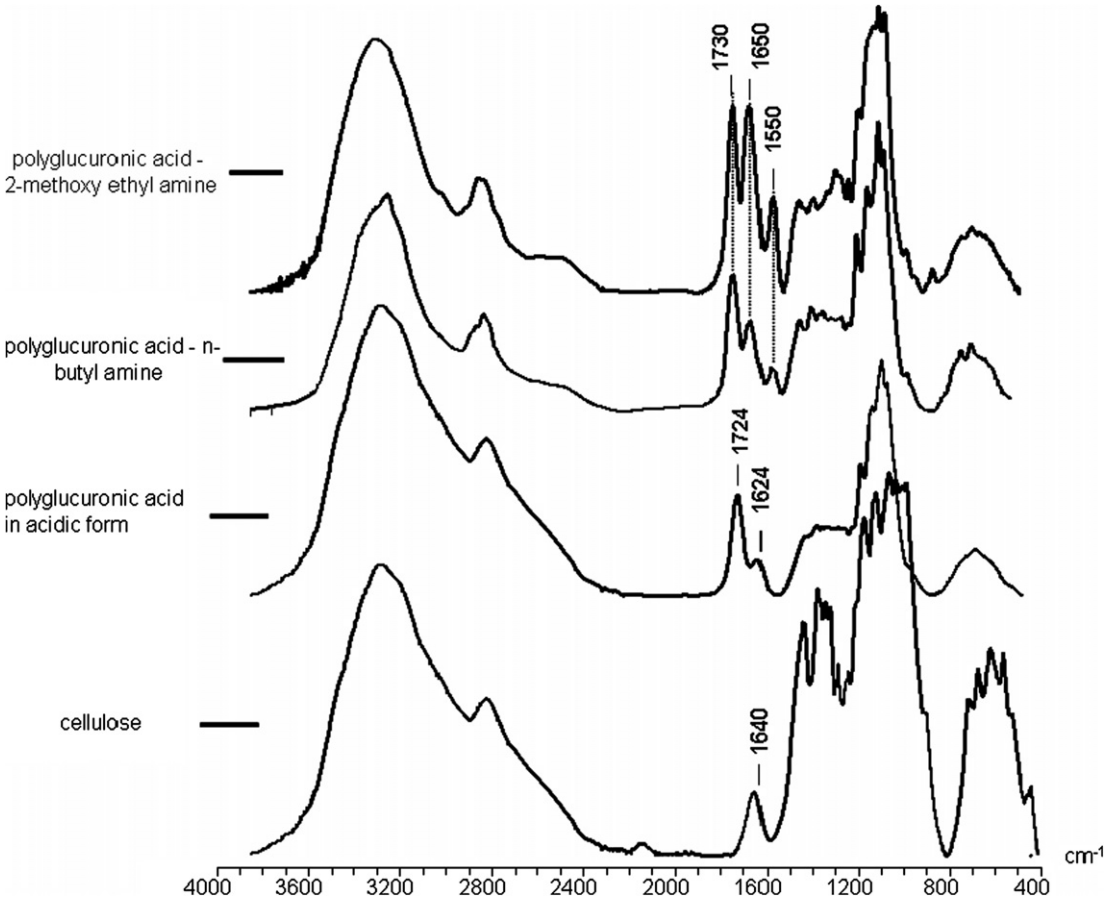

Fig. 1. FTIR spectra of cellulose, polyglucuronic acid, polyglucuronic acid-*n*-butyl amine and polyglucuronic acid-2-methoxy ethyl amine.

acid and the $-NH_2$ amine end group. Indeed, the carboxylic acid band is well separated from the amide group (–CONH–) bands at 1650 and 1550 cm^{-1} as already reported by several authors (Bulpitt & Aeschlimann, 1999; Deng, Liu, Du, Li, & Chen, 2007; Hu et al., 2006).

The FTIR spectroscopy appeared to be well adapted to measure the amount of amide bond within the product from to the presence of peaks assigned to amide formation. The FTIR analysis clearly shows that the coupling with the *n*-butyl amine is less important than with the 2-methoxy ethyl amine.

3.1.3. NMR characterization

The ^{13}C NMR spectrum of oxidized cellulose substrate has previously been reported in the literature and the different signals assigned (Bertocchi et al., 1995; Da Silva Perez et al., 2003; Tahiri & Vignon, 2000; Wada et al., 2001). The carbon resonances at 103.50, 73.75, 75.25, 81.95 and 76.30 ppm are assigned to C1, C2, C3, C4 and C5. The signal near 175 ppm is attributed to the C6 signal of the glucuronic unit (Da Silva Perez et al., 2003; Gomez-Bujedo, Fleury, & Vignon, 2004; Tahiri & Vignon, 2000). The ^{13}C NMR spectra of polyglucuronic acid-*n*-butyl amine and 2-methoxy ethyl amine derivatives are presented in Fig. 2. We observe in the two spectra the signals related to carbons of the polyglucuronic acid, and among them, several new resonances are detected corresponding to the coupled amines. The presence of an extra signal well separated from the (–COONa) C6 resonance was observed at 170.30 ppm in both cases – polyglucuronic acid-*n*-butyl amine and 2-methoxy ethyl amine derivatives (Fig. 2). The 170.30 ppm resonance can be assigned to (–CONH–) acetamide carbon

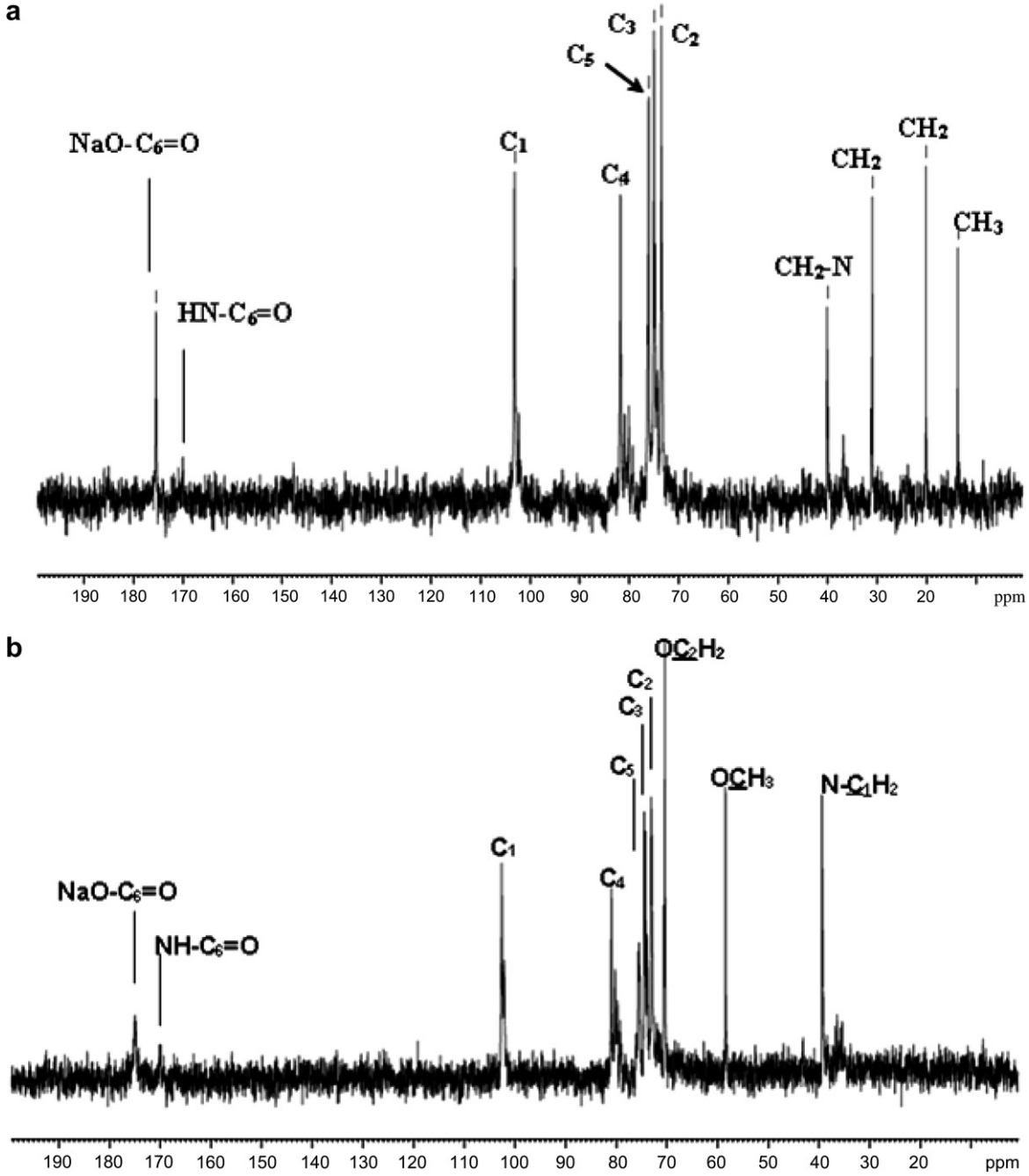

Fig. 2. ^{13}C NMR spectra in D_2O: (a) of polyglucuronic acid-*n*-butyl amine; (b) of polyglucuronic acid-2-methoxy ethyl amine.

according to ^{13}C NMR data already published by several authors (Jiang, Drouet, Milas, & Rinaudo, 2000; Novak et al., 2007; Toffey, Samaranayake, Frazier, & Glasser, 1996). The presence of this signal supported the evidence for amide bond formation.

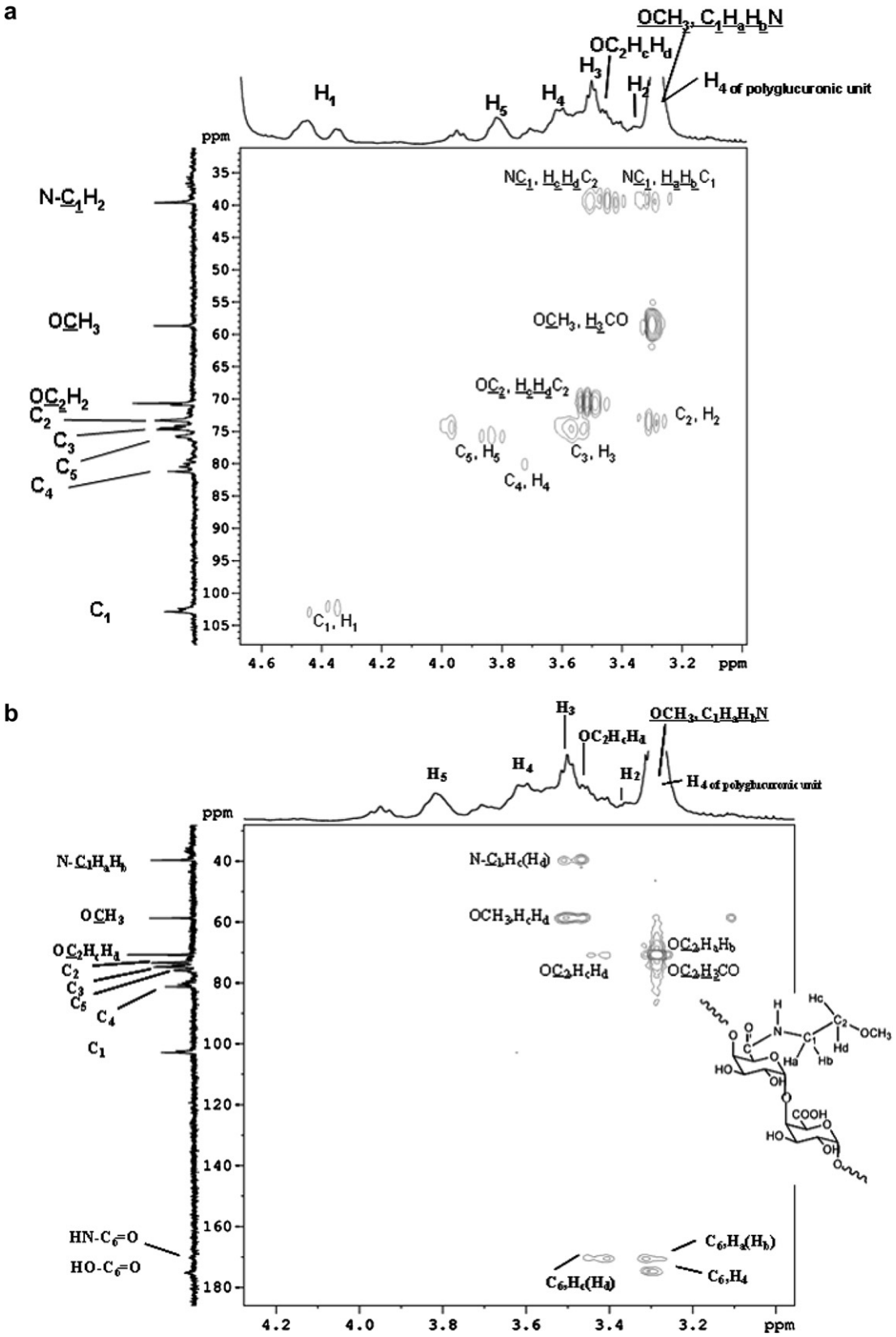

Fig. 3. 2D ^{13}C–^{1}H spectra in D_2O: (a) HMQC of polyglucuronic acid-2-methoxy ethyl amine; (b) HMBC of polyglucuronic acid-2-methoxy ethyl amine.

Several new resonances in the spectra of polyglucuronic acid coupled with the amines were attributed. In Fig. 2a, signals at 13.90, 20.35, 31.20 and 40.25 ppm were observed corresponding to CH_3 and CH_2 groups of the *n*-butyl amine molecule, respectively. Concerning the polyglucuronic acid coupled with 2-methoxy ethyl amine (Fig. 2b), the signals assigned to 2-methoxy ethyl amine were observed at 39.60, 58.65 and 70.60 ppm corresponding to $N–C_1H_2$, $O–CH_3$ and $O–C_2H_2$ groups, respectively.

The ^1H spectra of polyglucuronic acid coupled with *n*-butyl amine and 2-methoxy ethyl amine were analyzed and the different proton signals assigned by 2D-COSY (spectrum not presented) and 2D $^{13}C–^1H$ HMQC experiments. The 2D $^{13}C–^1H$ HMQC spectrum of polyglucuronic acid-2-methoxy ethyl amine was presented in Fig. 3a. All the characteristic proton signals of oxidized cellulose (H1, H2, H3, H4, H5) were easily identified and are in agreement with assignment already published by Heyraud and collaborators (Heyraud, Courtois, Dantas, Colin-Morel, & Courtois, 1993) and Tahiri and Vignon (Tahiri & Vignon, 2000). The attribution of proton signals corresponding to the amine molecules are reported in Table 2. The presence of characteristic signals both in the ^1H and ^{13}C NMR spectra confirmed the coupling of 6-carboxycellulose with 2-methoxy ethyl amine and *n*-butyl amine via an amide linkage.

In the 2D $^{13}C–^1H$ HMBC spectrum (Fig. 3b), two and three-bond $^{13}C,^1H$ couplings can be observed. Strong cross-peaks between C6 (O═C–NH) and H_a and/or H_b protons of amine carbon C_1, confirmed the formation of a chemical bond between the amine molecule and the carboxyl group of the glucuronic unit. An additional strong connectivity between C6 (O═C–NH) and H_c and/or H_d protons of amine carbon C2 across 4 linkages suggesting a "w" conformation, confirmed the coupling between the carboxy group and the 2-methoxy ethyl amine.

In conclusion, all the FTIR and NMR experiments confirmed the formation of a covalent bond between polyglucuronic acid and amine molecules via an amide bond in aqueous medium using carbodiimide as a coupling agent.

3.2. Coupling reaction with a cyclic amine containing a radical: 4-Amino TEMPO

In the second part of this report, in order to support additional evidence for the amide bond formation on C6 carbon of polyglucuronic acid, we decided to study the coupling reaction of polyglucuronic acid with a paramagnetic amine nucleophile as a mean to assess the coupling reaction by a spin-labelling method. The 4-amino TEMPO has been chosen as label and the electron paramagnetic resonance (EPR) spectroscopy will allow to follow the coupling (Table 3). This reaction specifically labels acid glycans at the C6 position because only carboxyl functional groups can form carbodiimide activated ester or inter-/intra-molecular lactones with the primary amine group of the 4-amino TEMPO to produce the amide of polyglucuronic acid. This type of reaction was already described by Irwin et al. for polygalacturonic acid from apple fruits (Chamulitrat et al., 1988; Irwin, Pfeffer, Gerasimowicz, Pressey, & Sams, 1984; Irwin, Sevilla, & Stoudt, 1985; Irwin et al., 1987, 1992).

Table 3
Degrees of conversion between polyglucuronic acid and 4-amino TEMPO

	DO_1	N (%)	Degree of conversion
Conductimetry	0.67	–	0.33
Elemental analysis	0.67	4.27	0.37
EPR analysis	0.67	–	0.36

$^*DO_1$: residual carboxyl content of coupled oxidized cellulose samples.

3.2.1. EPR characterization

In general, the EPR spectroscopy has been shown to be an excellent technique to characterize the microstructural and dynamic properties of various species. Nitroxyl spin labels have been widely used as probes to obtain information about the nature of localized molecular properties such as conformation, flexibility and solute–solvent interactions in diverse systems such as gels, liquid crystals, vesicles, nucleic acids, lipids and proteins (Chamulitrat et al., 1988; Gaffney & Marsh, 1998; Kurad, Jeschke, & Marsh, 2004; Mchaourab, Lietzow, Hideg, & Hubbell, 1996).

The EPR spectra of polyglucuronic acid-4-amino TEMPO and of an aqueous solution of 4-amino TEMPO as standard reference are presented in Fig. 4. Their spectra contained three well-resolved derivatives of Lorentzian lines satisfactorily explained by the Kivelson theory (Kivelson, 1960). These three symmetrical lines or triplet are generally characteristic of the presence of free radical in solution.

For the aqueous solution of 4-amino TEMPO, the general shape of the EPR spectrum is characteristic of a radical fast motion while for polyglucuronic acid-4-amino TEMPO spectrum, the line broadening (particularly the line at high field) clearly shows a decrease in the radical correlation time (Fig. 4). This decrease can be attributed to the restrictive motion of the spin label linked on a polymer molecule, which is an indication that the nitroxide moiety has been successfully coupled with the polyglucuronic acid.

Irwin and collaborators reported that when plant homogalacturonans, either in solution or as solid suspensions were reacted with a carbodiimide reagent in the presence of a paramagnetic nucleophile, the nitroxyamide EPR powder patterns were significantly broadened when as few as 2.5% of the carboxyl functional groups had been labeled (Irwin et al., 1987). This broadening effect was the same for reactions occurring in either solution or the solid state. The same behavior can be observed in the polyglucuronic acid-4-amino TEMPO spectrum with a more important effect.

It was possible to obtain by double integration (area) of the labeled polyglucuronic acid spectrum the absolute number of

Table 2
Chemical shift data of polyglucuronic acid-*n*-butylamine and polyglucuronic acid-2-methoxy ethyl amine

Polyglucuronic acid-*n*-butyl amine				Polyglucuronic acid-2-methoxy ethyl amine			
^1H δ (ppm)		^{13}C δ (ppm)		^1H δ (ppm)		^{13}C δ (ppm)	
H1	4.69	C1	103.5	H1	4.44	C1	102.85
H2	3.56	C2	73.75	H2	3.36	C2	73.20
H3	3.81	C3	75.25	H3	3.51	C3	75.60
H4	3.83	C4	81.95	H4	3.60	C4	81.15
H5	4.05	C5	76.30	H5	3.81	C5	75.70
CH_3	1.08	CH_3	13.90	$O–CH_3$	3.28	$O–CH_3$	58.65
CH_2	1.51, 1.69	CH_2	20.35, 31.20,	$H_aH_b(C_1)$	3.28	$O–CH_2$	70.60
		$N–CH_2$	40.25	$H_cH_d(C_2)$	3.45	$N–C_1H_2$	39.60
		O═C6–ONa	175.65			O═C6–ONa	175.25
		O═C6–NH	170.30			O═C6–NH	170.25

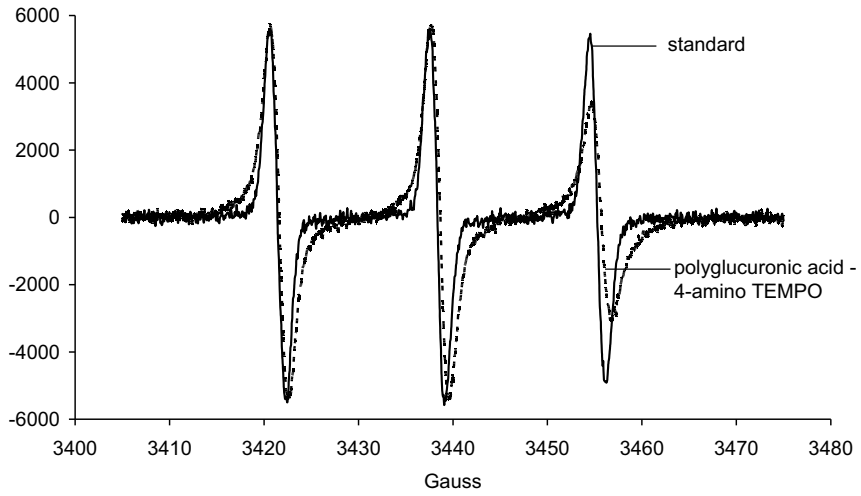

Fig. 4. EPR spectra of 4-amino TEMPO (standard) and polyglucuronic acid-4-amino TEMPO.

coupled radicals and thus the fraction of labels along the cellulosic chain. Thus, the coupling yield can be calculated (Table 3).

3.2.2. Degree of conversion

The coupling yield was determined by three different methods, conductimetry, elemental analysis and EPR spectroscopy, the data are reported in Table 3. A very good correlation is obtained between the three methods, indicating that the amide bond occurred on one third of the available acid functions. This yield is relatively significant, as the cyclic 4-amino TEMPO could induce a steric hindrance and thus a weak coupling yield. Concerning the regularity of the coupling along the glucuronic chain, different structures could be expected: (i) a block grafting along the chain, (ii) an uniform distribution (a chemical bond grafted on three), or (iii) an ability to randomly graft the available near-neighbor sites.

3.2.3. FTIR spectroscopy

The FTIR spectra of original polyglucuronic acid, 4-amino TEMPO and polyglucuronic acid-4-amino TEMPO are reported in Fig. 5. As already observed above for polyglucuronic acid coupled with

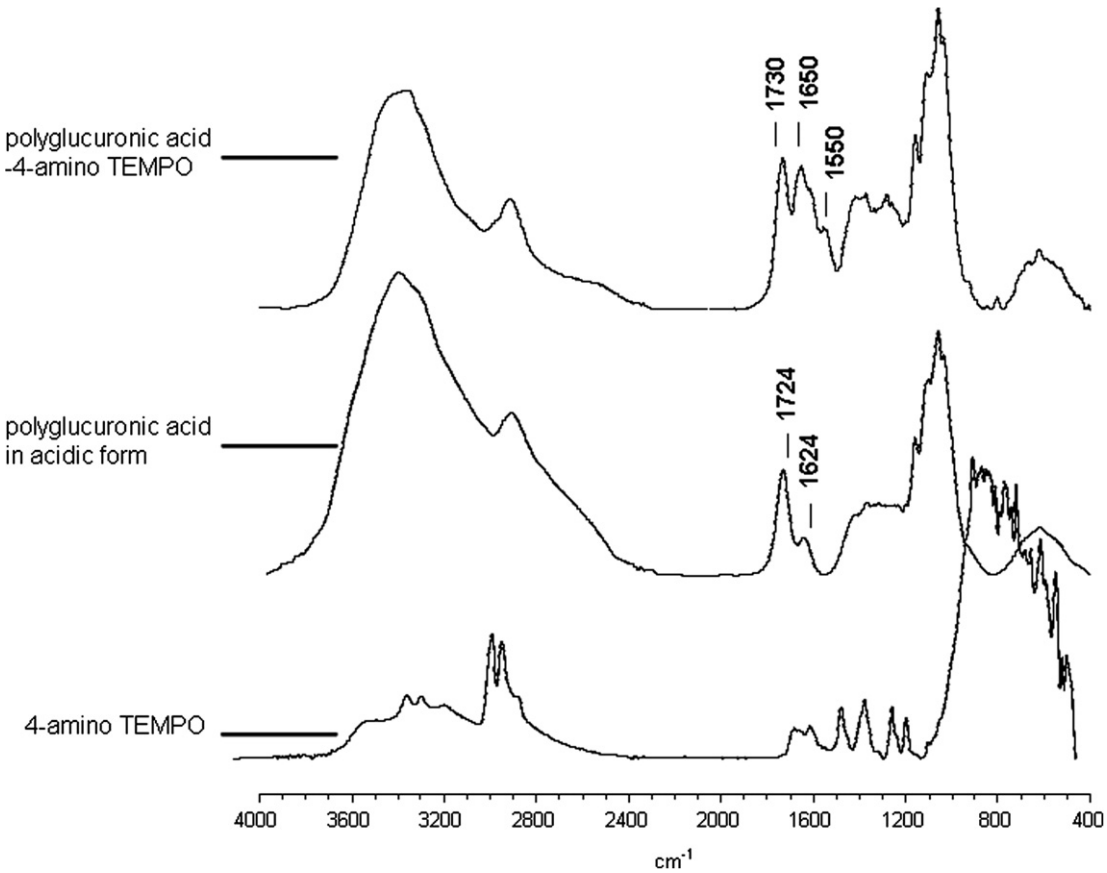

Fig. 5. FTIR spectra of 4-amino TEMPO, polyglucuronic acid and polyglucuronic acid-4-amino TEMPO.

Table 4
Chemical shift data of polyglucuronic acid-4-amino TEMPO

^{1}H δ (ppm)		^{13}C δ (ppm)	
H1	4.69	C1	103.35
H2	3.55	C2	73.75
H3	3.82	C3	75.25
H4, H4*	3.85	C4, C4*	81.95
H5	4.04	C5	76.30
H4′	2.60	CH_3	15.35, 25.90
CH_2	1.68	C4′	39.45
CH_3	1.43	C3′, C5′	43.75
		C2′, C6′	56.40
		O=C6–O$^-$	175.65
		O=C6–NH	174.75

linear amines, the two peaks corresponding to a C=O stretching band at 1650 cm^{-1} and a –NH deformation band at 1550 cm^{-1} are observed and confirmed the coupling with the 4-amino TEMPO.

3.2.4. NMR characterization

The proton spectrum of the polyglucuronic acid coupled with 4-amino TEMPO was analyzed and the different protons assigned by 2D-COSY experiment (especially focused on correlations at long distance) and the data reported in Table 4. In the ^{13}C spectrum (Fig. 6), six major signals easily assigned to polyglucuronic acid were observed at 175.65 ppm (C6), 76.30 ppm (C5), 81.95 ppm (C4), 75.25 ppm (C3), 73.75 ppm (C2) and 103.35 ppm (C1). The presence of another signal well separated from the (–COONa) C6 resonance was observed at 174.75 ppm (Table 4) and corresponded to (–CONH–) acetamide C6 carbon. Other ^{13}C signals were detected and attributed to the nitroxide radical, as already published by

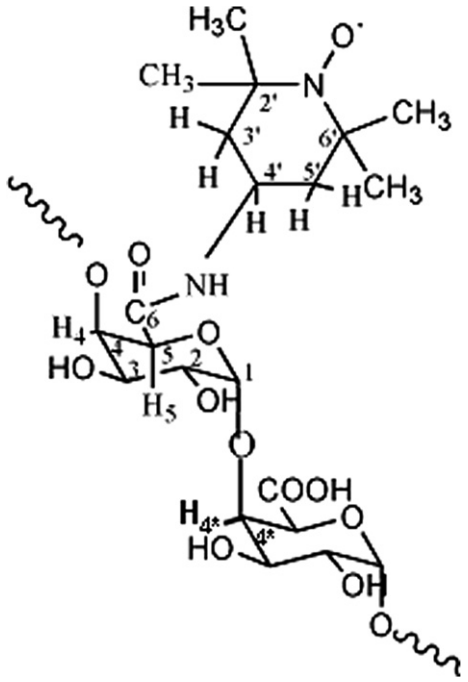

Fig. 7. Possible chemical structure of polyglucuronic acid-4-amino TEMPO.

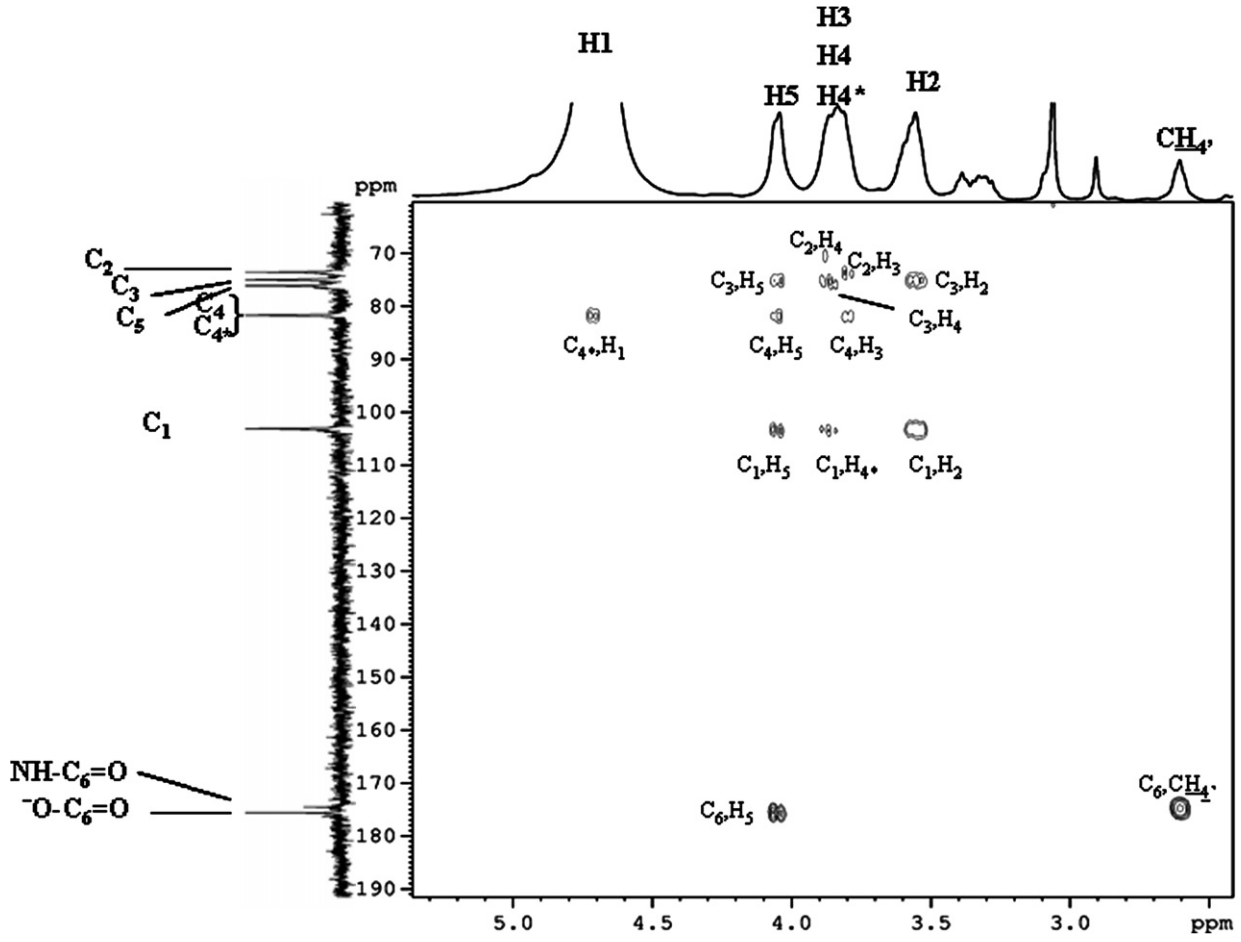

Fig. 6. 2D ^{13}C–^{1}H HMBC spectrum of polyglucuronic acid-4-amino TEMPO in D_2O.

Irwin et al. (1987). The resonances at 56.40, 43.75, 39.45, 25.90, 15.35 ppm were assigned, respectively, to quaternary, tertiary, secondary and primary (25.90 and 15.35 ppm) carbons of 4-amino TEMPO. The ^{13}C NMR data were reported in Table 4.

The evidence of coupling between polyglucuronic acid and 4-amino TEMPO was supported and highlighted by the 2D ^{13}C–^{1}H HMBC NMR experiments (Fig. 6) where long range coupling constant could be observed via cross-peak correlations.

The main observation in the ^{13}C NMR spectrum is the presence at 174.75 ppm of a carbonyl peak corresponding to a carbonyl amide function (Fig. 6) in addition to the carboxyl signal (COOH) at 175.65 ppm. We observe the presence of a strong cross-peak between C6 of polyglucuronic acid and $H_{4'}$ of nitroxide radical as shown in Fig. 6. This correlation corresponds to a connectivity across three bonds and clearly indicates the formation of the amide bond. Strong connectivities between C1 and H4* and H1 and C4* corresponding to inter-residual three-bond correlations over the glycosidic linkages are observed. One of the probable chemical structures of the polyglucuronic acid-4-amino TEMPO is represented in Fig. 7.

4. Conclusion

The TEMPO-mediated oxidation of cellulose III samples is highly selective for primary hydroxyl groups, while secondary hydroxyl groups were not oxidized. In the present study, we describe a method of coupling amines to oxidized cellulose (polyglucuronic samples) via carbodiimide sites activation of carboxylic groups. The degree of conversion was determined by different methods, namely conductometric titration, elemental analysis, ^{13}C NMR and FTIR spectroscopies, and EPR in the case of coupling with a nitroxyl amine radical. We showed that the coupling of amines, linear and cyclic structures, could be realized with significant yields.

The yield of formation of the amide bond depends on the reactivity of amines chains. This was illustrated with various amine structures which present differences in the carbon chain lengths and in different inductive and mesomeric effects. The degree of conversions showed that the yields of coupling increased when going from *n*-octyl amine to *n*-butyl amine and 2-methoxy ethyl amine. The FTIR analysis confirmed the chemical reaction between the glucuronic units and the amine by the presence of two distinct bands at 1652 and 1550 cm^{-1} attributed to (C=O) stretching band and (–NH) deformation band, respectively.

The ^{13}C NMR spectra also confirmed that the coupling was realized. In the case of linear amines, the presence of an extra signal around 170.25 ppm, characteristic of the acetamide moiety, in addition to the carboxyl signal (–COONa) usually observed near 175.65 ppm, corroborated the formation of the amide bond. In the case of the coupling of 2-methoxy ethyl amine, a set of strong cross-peak between C6 of the glucuronic residues and methylenic protons of amine was revealed by the 2D ^{13}C–^{1}H HMBC spectrum, thus confirming the coupling.

EPR measurements realized on polyglucuronic acid-4-amino TEMPO confirmed the amide bond formation with nitroxyl amine radical, covalently attached to polyglucuronic acid. In fact, a restrictive motion of the spin label linked on the coupled polymer molecule was observed indicating that the nitroxide moiety was successfully attached. A very good correlation in the coupling yield measurement was observed between the three different methods, showing that the bond occurred on one third of the available acidic functions. In the 2D ^{13}C–^{1}H HMBC spectrum, the presence of strong cross-peaks between C6 of polyglucuronic acid and NH and $H_{4'}$ of nitroxide radical was observed corresponding to connectivities across two and three bond and indicating clearly the amide bond formation.

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