Synthesis and ^{13}C CP-MAS NMR Characterization of a New Chitosan-Based Polymeric Network

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ABSTRACT: New chemical hydrogels, potentially suitable for biomedical applications, have been synthesized and characterized by ^{13}C CP-MAS NMR spectroscopy. The polysaccharidic component of these hydrogels is chitosan, while the novel cross-linking agent is diethyl squarate (DES). ^{1}H and ^{13}C NMR spectra of chitosan allow a good characterization of the starting materials. In dried chitosan/DES networks, the structure of the chemical bridges (i.e., of the cross-links), has been investigated by ^{13}C CP-MAS NMR. The spectrum of the cross-linked material appears rather broad and poorly resolved. A large number of weak signals in the 160−190 ppm region is observed, all due to the squarate moiety, but none due to the original diethyl squarate. Squarate may be thought to act both as a bidentate cross-linking agent and as a monodentate substituent. A partial spectral assignment of the CP-MAS spectrum was obtained through a spectral "editing" sequence.

Introduction

Chitosan is obtained by *N*-deacetylation of chitin,[1] one of the most abundant naturally occurring polysaccharides. In fact, chitin can be treated in a such a way as to cause hydrolysis of most of the *N*−acetyl linkages, converting *N*−acetylglucosamine units into glucosamine ones. A relevant difference between chitin and chitosan is that, while chitin dissolves only in exotic/toxic solvents, nearly all aqueous acids dissolve chitosan.[2,3] For this property, chitosan[4−6] is suitable as a starting material for the synthesis of polysaccharide-based hydrogels.[7] Natural macromolecules-based networks have become increasingly important both as sorbents for water and as biocompatible materials. In fact, recent research has been devoted to synthesize highly hydrophilic chemical gels based on natural macromolecules.[8]

In particular, efforts have been made in order to obtain polymeric networks by a variety of chemical reactions involving nontoxic reagents. On the other hand, despite the interest in these biocompatible materials, few methods suitable for the characterization of such cross-linked polymers are currently available. Apart from IR spectroscopy, NMR offers the only nondestructive approach allowing for a better insight into the chemical structure and formation mechanisms of these amorphous, insoluble materials. Dissolution processes may be attempted as a result of chemical or enzymatic hydrolysis, or ultrasounds degradation. As a result, low-molecular-weight, soluble products may be obtained and studied. However, during the degradation process, the chemical bridges responsible for the cross-links may be heavily modified. As a consequence, soluble products might be not representative of the original structure of the network.

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Thus, the advantage of a direct solid-state investigation must be emphasized: this approach provides the opportunity to study insoluble compounds under nondestructive conditions. Since cross-linked polymers are involved in a multiplicity of industrial processes, many valuable studies have been recently carried out on the application of solid state ^{13}C NMR spectroscopy for studying and characterizing polymer networks.[9,10] Among other examples, are phenolic,[11] epoxy[12] resins, styrene-based[13,14] resins, methacrylate-based[15,16] resins, urea/formaldehyde condensates,[17,18] and cellulose-based insoluble polysaccharides[19].

In the present paper, ^{13}C CP-MAS NMR spectroscopy has been applied to new chitosan/squarate networks of different stoichiometric cross-linking degrees. Though the solid-state spectrum has a limited resolution, it contains sufficient resolved information to permit a detailed analysis.

Assignment was made easier by performing a spectral editing experiment which allows us to distinguish between quaternary carbon atoms, methyls, and methylenes. In this way, the possible chemical structure of the new hydrogels was investigated, shedding light on the cross-linking reactions.

Materials

Chitosan. Chitosan was purchased from Fluka. Since the degree of *N*-acetylation (DA) can markedly influence the properties of chitosan, different techniques have been applied to determine it.

Among others,[20] high-field ^{1}H NMR spectroscopy[21] is considered as a simple, rapid, and precise method. Therefore, high-resolution ^{1}H NMR spectroscopy, at 600.13 MHz, was employed. The DA value obtained in this way is (9.1 ± 0.5)%.

The molecular weight has been determined by viscosimetric measurements by applying the Mark−Houwink equation. It has been shown[22] that parameters in this equation depend on the DA of the chitosan. The molecular weight is $M_{chit} = (255 \pm 1)$ kD and the mean value of the polymerization degree X is ≈ 1500.

Diethyl Squarate. Diethyl squarate was purchased from Fluka. Acetic acid and sodium acetate were purchased from

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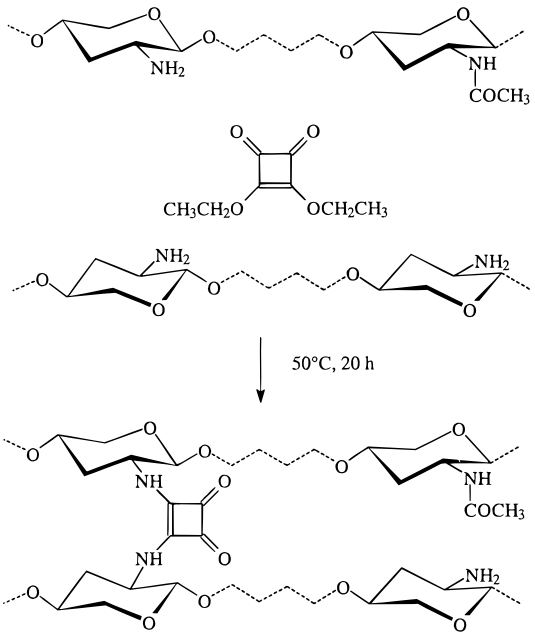

Figure 1. The reaction between chitosan and DES is sketched. The possible structure of the chemical bridges responsible for the cross-links is also shown.

Carlo Erba. All materials were used without further purification.

Stoichiometric Cross-Linking Degree. The stoichiometric degree of cross-linking is defined as R_s = DES equiv/NH_2 mol before gelation. Thus

$$R_s = (vrm_{chit}2)/(M_{DES}g)$$

where v = volume of DES, in mL; g = grams of chitosan; r = density of DES; M_{DES} = DES molecular weight; m_{chit} = molecular weight of chitosan repeating unit (calculated taking into account the sample degree of acetylation).

Synthesis of Hydrogels. Chitosan is solubilized in a CH_3COOH/CH_3COONa 1 M buffered solution, pH = 4.7. Chitosan concentration is 3.8% (w/v). In 5 or 6 days, chitosan is fully solubilized and a perfectly transparent solution is obtained. A known amount of DES is added to the solution under stirring at room temperature. During the stirring, bubble formation is observed in the viscous solution. The beaker containing the solution is then heated in an oven at 50 °C. After 20 h, a wall-to-wall, transparent gel containing some bubbles is obtained. The beaker is covered with Parafilm and kept at room temperature for 24 h: the bubbles progressively tend to disappear. The gel is then dialyzed against double-distilled water. After dialysis, a transparent, elastic gel is obtained. Its volume is about twice that before dialysis.

By adding variable, known amounts of DES to chitosan aqueous acidic solutions, gels differing in stoichiometric cross-linking degree, R_s, have been synthesized.

At $R_s \geq 0.86$, small opaque drops are observed close to the gel surface. To obtain perfectly transparent gels even at $R_s \geq 0.86$, a slightly different procedure was performed. Chitosan is solubilized in a CH_3COOH aqueous solution, 2% (w/v); the starting pH is $\approx$ 4.84; chitosan concentration is 3.8% (w/v). However, again at $R_s \geq 1.7$, the formation of small opaque drops is observed. Note that for obtaining gels with both procedures, the pH of the starting solution is very important and must be kept in the range 4.5−5.5. In fact, at pH = 3.94, a viscous solution is obtained without gel formation, while at pH = 5.7 upon heating the solution at 50 °C a precipitate is observed.

In Figure 1 the reaction between chitosan and DES is schematically shown along with the possible structure of the DES-based chemical bridges.

NMR Measurements. 1. NMR in Solution. High-resolution ^1H NMR spectrum, at 600.13 MHz, was performed on a Bruker AMX 600 spectrometer. A sample of chitosan was dissolved in 1 mL of a CD_3COOD/D_2O (5% (w/v)) solution. A solution was obtained with a chitosan concentration 0.1% (w/v). The suppression of the residual water signal was obtained applying a 1 D NOESY pulse sequence implemented with a low-power continuous wave presaturation during the relaxation delay ($D1$ = 2 s) and during the mixing time τ_m (τ_m = 200 ms).

A solution of DES in CD_3OD, 0.2 mol/L, was used for its $\{^1H\}$ ^{13}C NMR spectrum, performed at 75.48 MHz on a Bruker AC 300 spectrometer.

2. Samples for Solid State NMR Measurements. Chitosan/DES gels were synthesized as previously described. They were extensively dialyzed against double-distilled water and finally cut in small pieces and freeze-dried. A white powder was obtained in all cases. Samples were packed into 4-mm zirconia rotors and sealed with Kel−F caps.

3. Solid-State NMR. Solid-state ^{13}C CP-MAS NMR spectra were performed on a Bruker AC-200 spectrometer, equipped with an HP amplifier ^1H 200 MHz, 120 W CW and with a pulse amplifier M3205. The spin rate was always kept at 8.0 kHz. The $\pi/2$ pulse was 3.5 μs, the contact time for the cross − polarization experiment was 1 ms, and the relaxation delay was 5 s. Spectra were obtained with 1024 words in the time domain, zero-filled, and Fourier-transformed with a size of 2048 words. A very low amount of CH_3COCH_3 in the sample of chitosan starting material was used as a reference for the spectral calibration; 8000 scans were performed for each sample.

Experiments were performed in the cross−polarization (CP) mode, with a simultaneous phase inversion (SPI); this method (CP-SPI) allows the selective observation of different types of carbons.

The contact time for the cross−polarization was 1 ms (τ_{CP} = 1 ms) while the length of the pulse used for the phase inversion was 26 μs (τ_{PI1} = 26 μs). The duration for τ_{CP1} and τ_{PI1} was respectively set equal to τ_{CP2} and τ_{PI2}. The setup requirement for these experiments was identical to that used in the CP-MAS experiments.

Analysis of NMR resonances was performed using a simulation program "GLINFIT".[23] This program can perform the full deconvolution of overlapped lines both with Gaussian and/or Lorentzian shapes. Errors in the integrals are less than 20% of their nominal value.

Results and Discussion

CP−MAS Spectral Editing Sequences. Spectral editing techniques are used to simplify complex spectra and to achieve the full resonance assignment. Nowadays, in liquid NMR spectroscopy, these techniques are routinely used for assigning signals due to chemically distinct nuclei.

Applying the DEPT[24,25] or INEPT[26] methods, the assignment of the carbon signal to a particular type of carbon can be established. In fact, by using these techniques, spectra may be edited by properly choosing the phase angle for one of the pulses in such a way that only one type of carbon signal appears in the spectra. Unfortunately, very few spectral editing methods have been developed which can be applied to rigid solids. The selective pulse sequences in high-resolution solid-state ^{13}C NMR are based either on the difference in strength of the dipolar carbon−proton couplings or on the difference in relaxation times.[27−29] In particular, the most used technique for selective observation of the quaternary and methyl carbons is the dipolar dephasing.[30] It is based on the observation that, in the absence of irradiation, methine and methylene carbon magnetizations are quickly dephased due to the large proton−

carbon dipolar interactions, while the magnetization of quaternary and methyl carbons decays more slowly.

However, this technique does not unambiguously distinguish between CH_2 and CH carbon signals. Recently, a new editing sequence has been proposed by Wu and Zilm.[31,32] The technique is the standard cross-polarization (CP) sequence combined with a simultaneous-phase inversion (SPI)[33] during the polarization inversion (PI). Properly choosing the pulse length for the simultaneous-phase inversion τ_{PI1} and τ_{PI2}, a carbon spectrum can be obtained where methine resonances disappear and methylene resonances are inverted, while signals due to methyls and nonprotonated C atoms are still positive.

Thus, a full spectral editing can be obtained. In Wu and Zilm's papers,[31,32] all details about the spin dynamics at low, moderate and high sample rotation rates are given.

In the present paper, the sequence has been applied both to the chitosan used as a starting material as well as to the chitosan networks for different stoichiometric cross-linking degrees, R_s.

In the first case, the SPI sequence is applied to confirm the spectral assignment, while, in the second case, resonances due to quaternary carbon atoms involved in the cross-links are brought to evidence. Moreover, by comparing SPI spectra obtained on cross-linked networks at different R_s, the effect of the cross-links is well observable and can be analyzed.

Chitosan−Diethyl Squarate Networks: NMR characterization. A new type of chemical network has been obtained by cross-linking chitosan in aqueous solution with a nonconventional reticulating agent, namely 3,4-diethoxy-3-cyclobutene-1,2-dione (diethylsquarate DES) (i.e., the diethyl ester of squaric acid).

DES is known to react with compounds having primary amino groups, including of course simple aminosugars.[34−36] With chitosan, the reaction takes place in a single step under mild conditions yielding wall-to-wall hydrogels[37] characterized by new types of chemical bridges between chitosan chains.

Before investigating the exact nature of the chemical bridges in such networks, the single reagents were separately studied by ^{13}C NMR spectroscopy.

Since, at room temperature, the cross-linking agent DES is a liquid soluble in methyl alcohol, a high-resolution ^{13}C NMR spectrum, at 75 MHz, was performed and all carbon resonances assigned. At 191.15 ppm, the resonance due to the carbonyl carbons A is observed, while a resonance at 185.74 ppm must be assigned to the quaternary carbon resonance B. The downfield shift of B is obviously due to a ring current originating from a strong[38] delocalization of π electrons on the four membered ring. At 75.04 (E) and 16.10 (F) ppm, resonances respectively, due to the methylene and methyl carbon atoms of the ethyl group, are observed.

Figure 2 reports the CP-MAS ^{13}C spectrum of a sample of chitosan and the relative assignments. For the sake of clarity, in the same figure both glucosamine as well as acetylglucosamine repetitive units (≈9%) are also sketched with the used labeling of C atoms. At 102.5 ppm, the resonance of the anomeric carbon C(1) of the glucosamine unit is observed, while at 95 ppm a much less intense signal due to C(1) of the acetylglucosamine unit is shown; in fact, due to the presence of a carbonyl in the acetoamide group bound to C(2), an upfield shift is induced on C(1) resonance. At 171.3 and

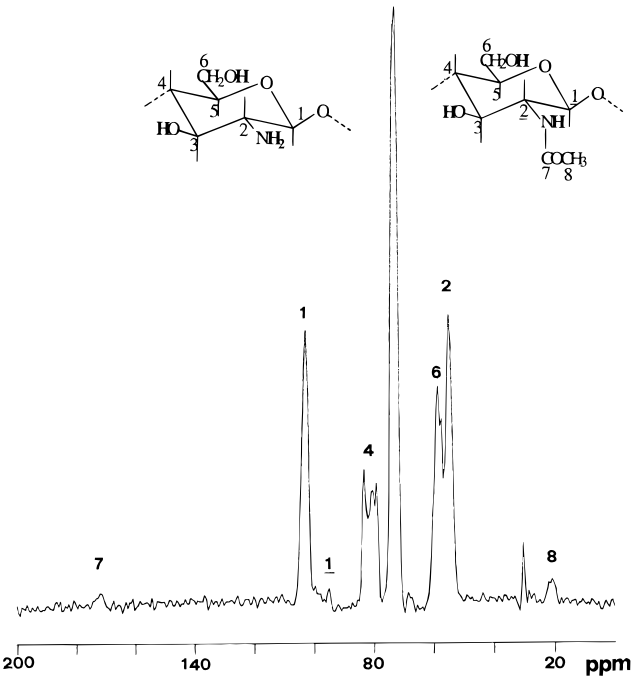

Figure 2. ^{13}C CP-MAS spectrum of a sample of chitosan. For the sake of clarity, both glucosamine and acetylglucosamine repetitive units are also sketched with the labeling for carbon atoms.

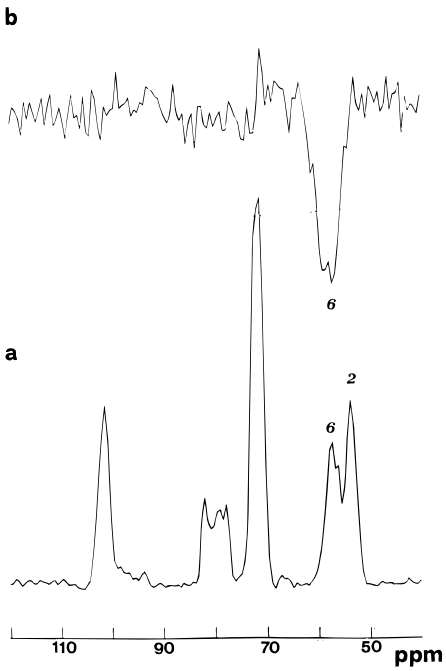

Figure 3. (a) ^{13}CP-MAS spectrum of a sample of chitosan. Only the range 40−120 ppm is shown. (b) The same spectral range is shown after applying the CP-SPI sequence. All resonances due to methine carbons are zeroed, while the inverted resonance due to the methylene carbon C(6) is very observable.

20.9 ppm, weak resonances respectively due to the carbonyl C(7) and to the methyl C(8) of *N*-acetylglucosamine units are observable.

By applying the selective CP-SPI sequence, the resonance due to methylenes C(6) can be easily assigned (see Figure 3). In Figure 3a, resonances in the range 40−120 ppm are shown, while in Figure 3b, the same spectral range is shown after applying the SPI sequence. All signals due to methine carbon atoms are zeroed

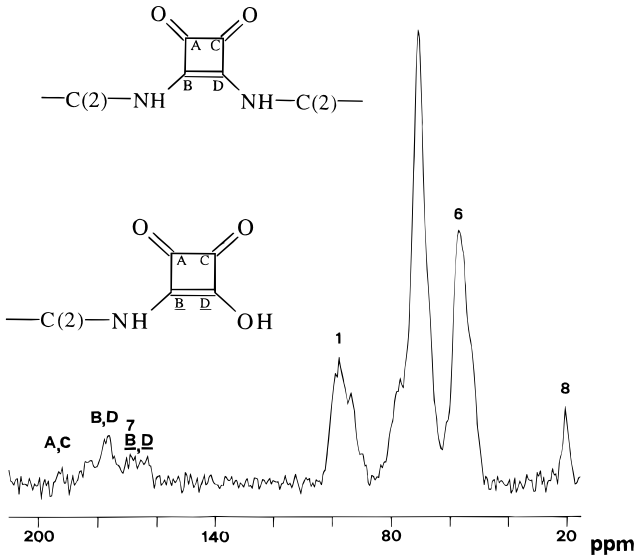

Figure 4. ^{13}C CP-MAS spectrum of a dried chitosan/squarate network at $R_s = 0.6$.

whereas the inverted resonance due to the methylene carbon C(6) is very observable. Thus, C(6) resonates at 58.6 ppm while the nearby resonance at 54.9 ppm is due to C(2). At ≈72.2 ppm, a set of overlapped resonances is due both to C(3) and C(5).

In the frequency range 79−83 ppm, the resonance due to C(4) is split in different peaks. Splittings of the order of 1−2 ppm observed in carbon resonances may be ascribed either to differences in the packing of the polymeric chains or to different internal torsion angles. In this case the splittings observed are possibly due to both effects. In fact, the resonance due to C(4) is sensitive to the ω torsion angle, and it is also well-known that chitosan exhibits polymorphism.[39]

Generally, the chains assume an essentially linear structure which undergoes a full twist every 10.1−10.5 Å along the axis of the chain, giving rise to γ-gauche effects.[40]

The ^{13}C CP-MAS spectrum of a dried chitosan/DES gel, at $R_s = 0.6$, is shown in Figure 4. In Figure 5, ^{13}C CP-MAS spectra of a sample of chitosan (top) and of a chitosan/squarate network (bottom) are reported for comparison (only the range 40−110 ppm is shown).

The spectrum of the network appears broad and poorly resolved with full disappearance of the resonance due to C(2). The residual signal of C(2) is observable only as a shoulder on the C(6) resonance. This shoulder is probably ascribable to C(2) carbon atoms of the glucosamine units far from the chemical bridges. The resonance of C(2) carbons involved in cross-links is probably hidden in the broad resonance due to C(3), C(4), and C(5), centered at ≈71 ppm. The downfield shift of resonance C(2) may be attributed to an additive β-effect on ^{13}C chemical shift[40] which generally is of the order of at least 10 ppm downfield.

Note that the cross-linking reaction may occur only on nonacetylated C(2) positions. All other resonances either remain in the original position or are shifted upfield. This is most evident for the C(1) resonance which moves from 102.5 to a broad resonance centered at ≈98 ppm. This upfield shift might be due to the ring current of squarate bound in C(2). Note that at 20.9 ppm the weak resonance due to methyls of acetylglucosamine units is still very observable. In the range 160−200 ppm, many resonances can be observed due

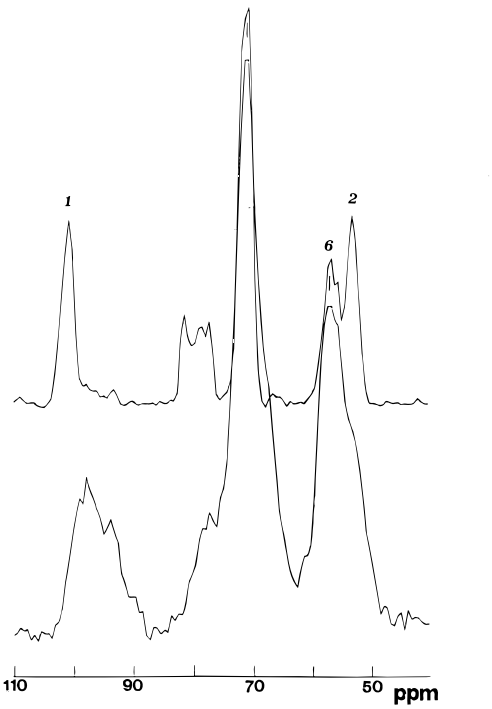

Figure 5. ^{13}C CP-MAS spectra of a sample of chitosan (top) and of a chtiosan/squarate network at $R_s = 0.6$ (bottom). Only the spectral range 40−120 ppm is shown.

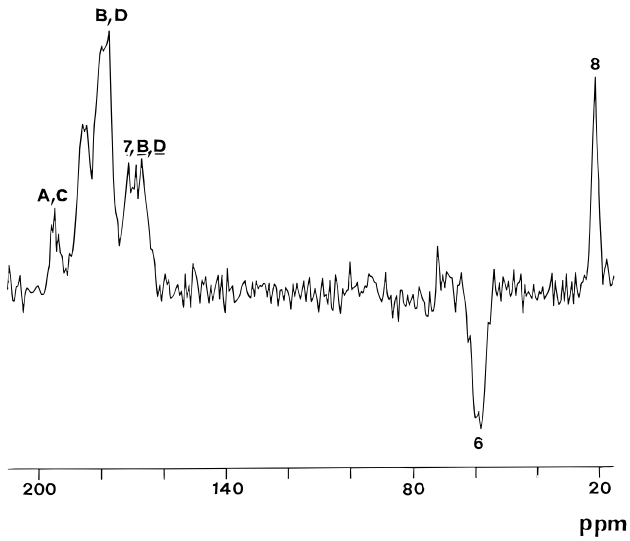

Figure 6. After applying the CP-SPI sequence, the spectrum of a chitosan/squarate network, at $R_s = 0.6$, is shown. All resonances due to methine carbons are zeroed, while the inverted resonance due to the methylene carbon C(6) is very observable. Resonances due to methyl, carbonyl, and quaternary carbon atoms are positive and well-observed.

to carbonyl and quaternary carbon atoms. By applying the selective CP-SPI sequence, these signals appear to increase their resolution and can be tentatively assigned (see Figure 6).

Here all resonances due to methine carbon atoms are zeroed while, at 57 ppm, with the phase fully reversed, the methylene C(6) resonance is unequivocally assigned.

The resonance due to methyl C(8) is still observed at 20.1 ppm.

Figure 7 shows the resonances of the CP-SPI experiment in the range 160−200 ppm, where in the bottom spectrum of uncross-linked chitosan in the same spectral range the resonance of the carbonyl C(7) of the

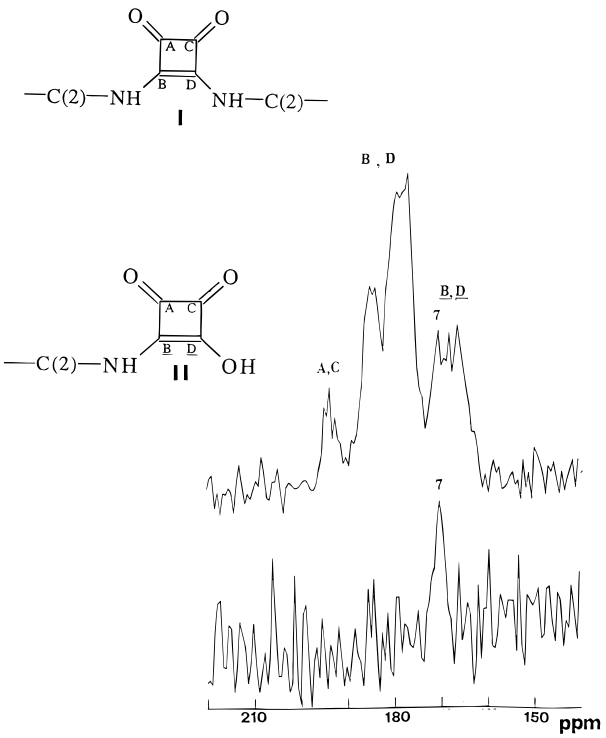

Figure 7. CP-SPI spectrum of a chitosan/squarate network, at R_s = 0.6 (top), compared with the CP-SPI spectrum of chitosan (bottom). Only the range 140−210 ppm is shown. Two possible structures of squarate bound to the chitosan as a bidentate cross-linking agent (structure I) as well as a pendant group (structure II) are sketched.

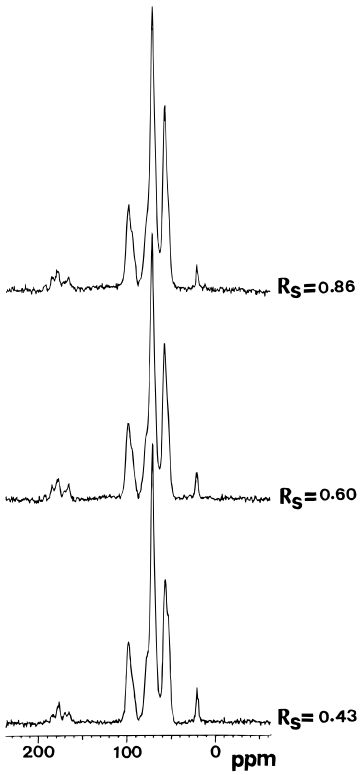

Figure 8. ^{13}C CP-MAS spectra of three chitosan/squarate networks at different R_s: R_s = 0.43 (bottom); R_s = 0.6 (middle); R_s = 0.86 (top).

acetylglucosamine appears at 171.3 ppm.

Two possible structures of a squarate bound to the chitosan are also sketched: in these the squarate is represented both as a bidentate cross-linking moiety (structure I), as well as a pendant group (structure II).

A tentative assignment of the spectrum might be as described in the next few lines.

It is clear that since acetylated units are not involved in the cross-links, the chemical shift of C(7) must be relatively unaffected. Since in the ^{13}C NMR spectrum of DES, carbonyl (A) and quaternary (B) carbon resonances are respectively at 191.2 and 186 ppm, in the spectrum of the cross-linked polymer, carbon signals having a chemical shift higher than 190 ppm may be attributed to the carbonyls A and C of squarate involved in the cross-links. In turn, signals in the range 165−188 ppm may be ascribed to quaternary carbons of squarate moieties acting both as a bidentate cross-linking agent (see structure I in Figure 7) as well as a monodentate substituent (see structure II in the same figure).

In fact, on the basis of the reaction mechanism between chitosan and DES, the possible reaction of some molecules of DES with only one amine group of chitosan must be taken into account, giving rise to squarate residues as pendant groups. Moreover, since DES in aqueous solutions slowly hydrolyzes to squaric acid, after extensive dialysis pendant groups should have the same structure but the ethyl group may be substituted with an −OH group (see structure II in Figure 7). Hence, unequivalent quaternary carbon signals are observed. Resonances B and D at ≈180 ppm are relative to squarate as a bidentate cross-linking agent, while in the range 165 to 170 ppm, B and D are probably due to squarate as a monodentate substituent. The upfield shift might be due to a loss of ring current occurring in nonsymmetric squarate molecules.

Chitosan-Based Networks at Different Stoichiometric Degrees of Cross-Linking. A solid-state ^{13}C NMR investigation has also been performed on cross-linked chitosan networks at different stoichiometric cross-linking degrees R_s.

Figure 8 shows the spectra of three cross-linked polymers differing in R_s (R_s = 0.43, 0.6, and 0.86). Note that in the range 10−110 ppm all resonances seem rather insensitive to R_s. In turn, in the frequency range 160−200 ppm, upon increasing R_s, major differences are present, which are better observable in SPI experiments (see Figure 9, on the left).

In fact, by increasing R_s, major differences can be observed on the resonances centered at ≈168 and ≈185 ppm. To clarify this point, a full deconvolution of the resonances in the 160 to 200 ppm range was performed and the area of each resonance was evaluated (see Figure 9, right side). It is worth noting that the area of the resonance at 171.3 ppm may be used as a reference. In fact, this resonance is due to the carbonyl carbon of acetylglucosamine units. As previously mentioned, these units are present in a known amount, about 9% of the total units; moreover acetylated units are not involved in the cross-linking reaction and their resonances are quite insensitive to variations in R_s. Thus, the area of the 171.3 resonance can be used for a calibration of the percent of squarate effectively used in the reaction.

Since the assignment of the resonances in the 160−200 ppm range is rather questionable, the second column of Table 1 shows only the ratio between carbons of acetylated units and all quaternary C atoms due to squarate bound to the chitosan network.

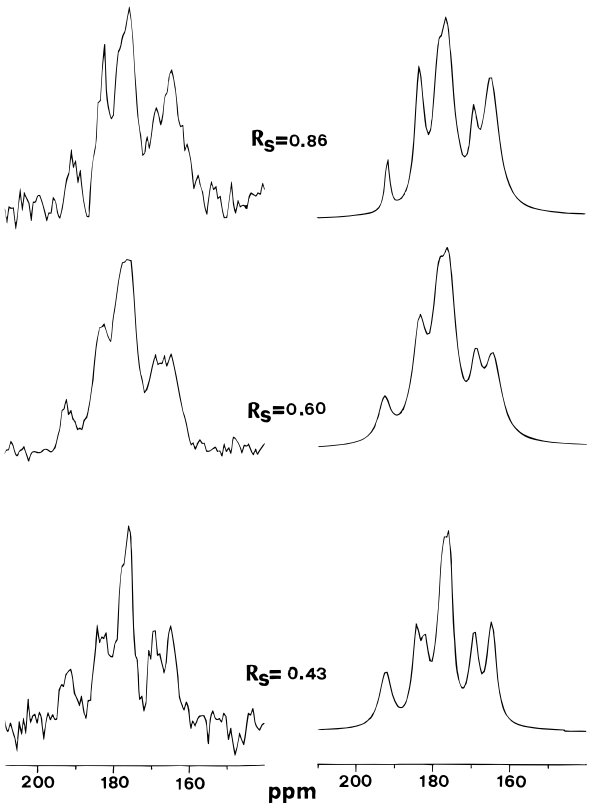

Figure 9. CP-SPI spectra of three chitosan/squarate networks at different R_s: $R_s = 0.43$ (bottom); $R\mathbf{s} = 0.60$ (middle); $R_s = 0.86$ (top). Only the range 160−200 ppm is shown. On the left, experimental spectra are shown while, on the right, the simulated spectra are reported.

Table 1. For Samples at a Different Cross-Linking Degree R_s, the Percentage of Squarate Bound to the Chitosan Is Reported

R_s	% of squarate bound to the chitosan
0.43	0.15
0.6	0.23
0.86	0.32

Data reported in Table 1 shows that by increasing R_s, an increasing amount of bound squarate is indeed measured.

Conclusions

New chitosan-based polymeric networks have been synthesized using a nonconventional cross-linking reagent, DES. This reagent has been recently used in glycoproteins synthesis. Since chitosan and DES react in a single step, under mild conditions, no reductive, toxic reagent is needed for obtaining the irreversible formation of secondary amine groups.

^{13}C solid-state NMR spectroscopy is a powerful tool for studying under nondestructive conditions the chemical bridges responsible for the cross-linking. The spectrum of the network is rather broad. The cross-links strongly affect some resonances, resulting in a deshielding or in a shielding. The upfield shift has been attributed to the ring current due to the delocalization of π electrons on a DES ring, while the downfield shift may be ascribed to the well-known additive β effect.

A number of quaternary carbon signals is also observed. All these features of the carbon spectra have been attributed to the presence of DES bound to one or two deacetylated units of chitosan. Since the resonance of C(6) is almost unaffected by the cross-link, C(6) carbon atoms should be oriented in a such a way to be as far as possible from the chemical bridges originating the network. Hence, a degree of order might be supposed to exist within the network.

Properly chosen spectral "editing" sequences and full simulation allow us to determine the amount of DES bound to the chitosan as a bidentate substituent as well as a pendant group.

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