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NMR study of a novel chitosan-based hydrogel

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Abstract

Chemical gels are obtained by reacting chitosan with 1,1,3,3-tetramethoxypropane (TMP, a "masked" dialdehyde) and then reducing the polymeric Schiff-base networks with an excess of cyanoborohydride ($NaBH_3CN$). The gels have been characterized by means of three different ^{13}C magic angle spinning NMR techniques: cross-polarization, cross-polarization with a simultaneous phase inversion and single pulse excitation. In this way we obtained spectra containing sufficiently resolved information for structural analysis. A quantitative evaluation of the cross-linking degree of chitosan–TMP networks is thus attainable. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: NMR; Chitosan; Cross-linking degree

1. Introduction

Hydrophilic networks (hydrogels) are derivable via simple synthetic procedures from carbohydrate polymers (Crescenzi et al., 1997; De Angelis, Capitani & Crescenzi, 1998). These networks exhibit high water uptake and other physical properties that may be important for applications in the biomedical and sanitary areas. Pursuing our interest in such materials, we have recently prepared and characterized novel chitosan-based hydrogels.

We wish to report here on chemical gels obtained by reacting chitosan with 1,1,3,3-tetramethoxypropane (TMP, a "masked" dialdehyde) and then reducing the polymeric Schiff-base networks with an excess of cyanoborohydride ($NaBH_3CN$). Gels have been characterized by means of three different ^{13}C MAS (magic angle spinning) NMR techniques: CP (cross-polarization), CP-SPI (cross-polarization with a simultaneous phase inversion) and SPE (single pulse excitation). In this way we obtained spectra containing sufficiently resolved information for structural analysis. Additional spectral information was obtained by synthesizing soluble *N*-isopropyl chitosan derivatives, resulting from the reaction of the polysaccharide with 2,2-dimethoxypropane (DMP, a "masked" monocarbonyl compound).

On this soluble polymer an HMQC (hetero nuclear multiple quantum correlation) experiment was performed, allowing a full assignment of the ^{13}C spectrum.

A quantitative evaluation of the cross-linking degree of chitosan–TMP networks is thus attainable.

2. Materials and methods

Chitosan, cyanoborohydride, TMP and DMP were purchased from Fluka. Acetone, acetic acid and sodium acetate were purchased from Carlo Erba, Italy.

The degree of N-acetylation ($9.1 \pm 0.5\%$) and the molecular weight (255 ± 1 kDa) of chitosan have been previously reported (De Angelis et al., 1998).

2.1. Stoichiometric cross-linking degree of chitosan–TMP gels

The stoichiometric degree of cross-linking is defined as R_s = TMP equiv/NH_2 mol before gelation. Thus $R_s = (2v_{TMP} \times \rho_{TMP} \times m_{chit})/(g \times m_{TMP})$ where v_{TMP} = volume of TMP in ml; g = grams of chitosan; ρ_{TMP} = density of TMP, m_{TMP} = molecular weight of TMP and m_{chit} = molecular weight of chitosan repeating unit calculated taking into account the degree of N-acetylation.

2.2. Synthesis of chitosan–TMP hydrogels

Chitosan is solubilized in a 1 M CH_3COOH/CH_3COONa buffered solution, $pH = 4.7$, the concentration of chitosan being 3.8% (w/v). The dissolution of chitosan is quite slow; 5 or 6 days are needed for a perfectly transparent solution to

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be obtained. The chitosan solution is soaked in a water/ice bath. After few minutes a known amount of TMP is added to the solution under stirring. The temperature of the bath is slowly raised to 15°C in 8 h. The solution is then left to equilibrate at room temperature overnight. During this treatment, bubbles formed upon stirring the reagents are slowly eliminated. The temperature of the bath is then raised up to 40°C for a time t_r (time of reaction). At this temperature, a marked increase in viscosity of the solution is observed. The temperature of the bath is further raised up to 50°C. After 1 h the beaker is taken out the bath and equilibrated at room temperature. A wall-to-wall gel is obtained. The gel is then soaked in a $NaBH_3CN$ solution in water at room temperature. The ratio r is defined as $NaBH_3CN$ mol/NH_2 mol before gelation.

After 4 days of contact with this solution the gel becomes transparent and colorless. Finally, the gel is dialyzed against double-distilled water.

Three hydrogels S1, S2, S3 were synthesized with stoichiometric degree of cross-linking $R_s = 0.43$, 0.2, 0.6 and $t_r = 4$, 10 and 6 h, respectively. Another sample S4 corresponds to S3 before reduction.

2.3. Synthesis of chitosan–DMP derivatives

Chitosan is solubilized in a CH_3COOH/CH_3COONa 1 M buffered solution, pH = 4.7, the polysaccharide concentration being 3.8% (w/v). A known stoichiometric amount of DMP ($S = 0.43$) is added to the solution under stirring. The solution is kept at 40°C for 4 h, then the temperature is raised to 50°C. After 1 h, the solution is allowed to cool down to room temperature.

A $NaBH_3CN/H_2O$ (0.074% w/v) solution is added to the chitosan–DMP solution. The mixture is kept under stirring for 4 days and then exhaustively dialyzed against double-distilled water. An *N*-isopropyl chitosan derivative is obtained.

2.4. Stoichiometric degree of substitution of chitosan–DMP derivatives

The stoichiometric degree of substitution (DS) has been defined as DS = (DMPequiv/NH_2mol), thus $DS = (v_{DMP} \times \rho_{DMP} \times m_{chit})/(g \times m_{DMP})$, where v_{DMP} = volume of DMP in ml; ρ_{DMP} = density of DMP and m_{DMP} = molecular weight of DMP.

2.5. Samples for solid state NMR measurements

Chitosan–TMP gels were extensively dialyzed against double-distilled water, cut in small pieces and freeze-dried. A powder was obtained in all cases. Powdered samples were packed in 4 mm zirconia rotors and sealed with Kel-F caps.

2.6. Solid state NMR

Solid state ^{13}C MAS NMR spectra at 50.28 MHz were performed on a Bruker AMX-200 spectrometer equipped with an HP amplifier ^1H 200.13 MHz, 120 W CW.

In all measurements the spin rate was 8 KHz. The $\pi/2$ pulse width was 3.5 μs, the contact time for the cross-polarization experiment was $\tau = 1$ ms.

In order to obtain the best signal-to-noise ratio on all C signals and to minimize artifacts, the proper contact time was carefully chosen according to a previously reported procedure (Harris, 1988; Voelkel, 1988). The recycle time was 4 s.

SPE experiments were also performed. In order to avoid signals saturation a recycle time $D_1 = 40$ s and a ^{13}C pulse width of 3 μs, corresponding to a flip angle of 60°, were used.

All spectra were obtained with 512 complex data points in the time domain, zero filled to 1024 points prior to Fourier transformation.

Experiments were also performed in the CP mode, with SPI (Wu & Zilm, 1993a,b); this method (CP-SPI) allows the selective observation of different types of carbon. The contact time for the cross-polarization was 1 ms while the length of the pulse used for the phase inversion was 25 μs.

2.7. NMR in solution of chitosan–DMP polymers

About 10 mg of chitosan–DMP were dissolved in 1 ml of CD_3COOD/D_2O (5% w/v). The temperature was kept at 333 K.

^1H and ^{13}C NMR spectra in water solution are referenced with respect to 1,4 dioxane, assumed at 3.75 and 67.4 ppm, respectively.

^1H, with water suppression (Bax & Davis, 1985) and ^{13}C NMR spectra of chitosan–DMP polymers have been run at 600.13 and 150.13 MHz, respectively, on a Bruker AMX-600 spectrometer.

^1H–^{13}C correlated map (Hurd & John, 1991) (HMQC) was obtained with proton detection and gradients selection. The contact time was chosen to optimize a $J_{H-X} = 150$ Hz. Gradients have been applied along the *z*-axis. Experiments were performed with 1000 data points in F2 and 512 experiments in F1.

3. Results and discussion

Aldehydes readily condense with primary amines in aqueous media to give the corresponding Schiff bases whose reduction finally leads to the formation of stable secondary amines (Tennant, 1979). In the condensation reaction, the aldehyde can often be replaced with "masked" carbonyl reagents, particularly when the parent carbonyl compound is unstable.

In our case, chitosan is the primary amine reacting with the methyl acetal of 1,3-dipropanale (TMP). In fact acetal compounds are particularly suitable to give acid scission, readily transforming into the corresponding aldehyde. $NaBH_3CN$ is finally used in the reduction step.

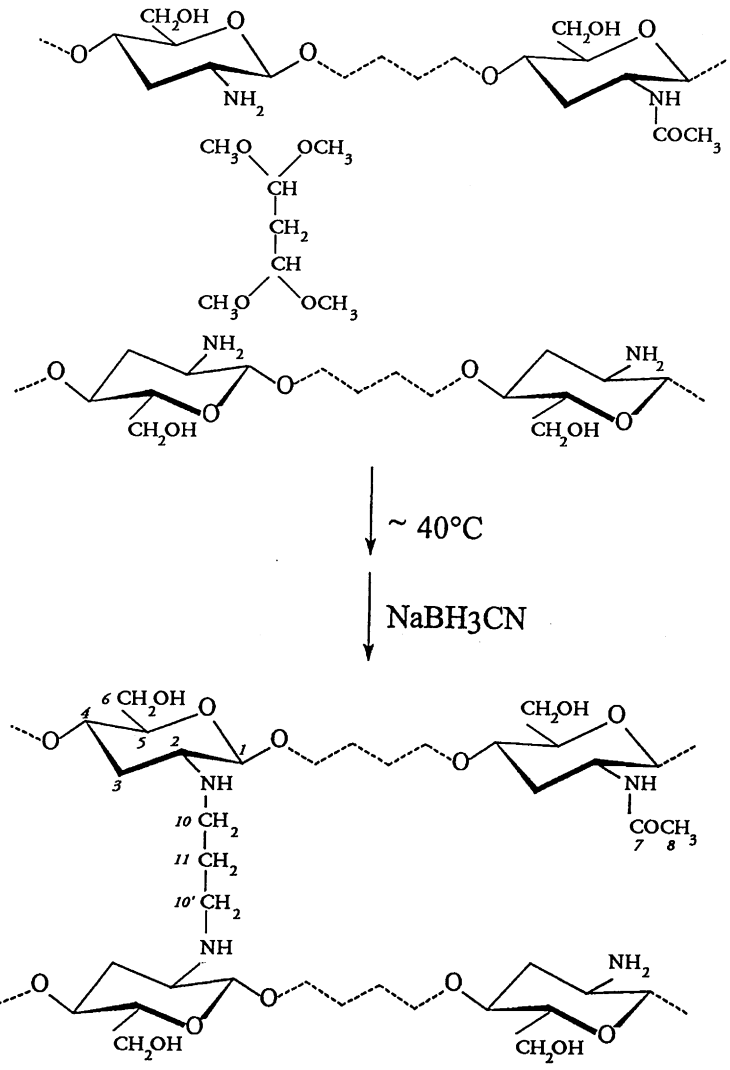


Fig. 1. Scheme of the reaction between chitosan and 1,1,3,3,tetramethoxypropane (TMP) with the assumed structure of the chemical bridges (cross-links).

In Fig. 1 the reaction is sketched along with the possible structure of the network. Starting from the observation that a low degree of cross-linking is associated with high water uptake, we note that the degree of cross-linking of this network should be rather low. In fact the freeze-dried hydrogels are capable of absorbing an amount of water as high as about 98 times their dry weight (Crescenzi et al., 1997).

The actual cross-linking degrees of the networks have been obtained by ^{13}C CP-MAS NMR spectroscopy. In Fig. 2a the spectrum of the starting chitosan is shown with the spectral assignment. In Fig. 2b it is shown the spectrum of the network S1 ($R_s = 0.43$ and $t_r = 4$ h). Comparing Fig. 2a and b, we observe a marked broadening of all resonances after cross-linking. This broadening is particularly evident on the C-4 resonance.

In Fig. 2b we can observe a weak, broad resonance at ≈ 26 ppm, partially overlapped to the methyl resonance of acetylated units of chitosan. This resonance, absent in the spectrum of chitosan, has been assigned to the methylene carbon atoms C-11 (see Fig. 1). Unfortunately the resonance of methylene carbons C-10 and C-10′ (see Fig. 1), i.e. methylene directly bound to the nitrogen in the secondary amine, lies at a lower frequency and is hidden under the very intense resonances due to the C-2 and C-6 of chitosan.

No resonances are observed above180 ppm. Therefore carbon atoms in an aldehydic environment can be excluded. As a consequence, the presence of 1,1-dipropanale moieties bound as a pendant group, i.e. R–NH–CH_2–CH_2–*C*H=O can be ruled out.

Another important feature of Fig. 2b is the very intense shoulder at ≈ 93 ppm. Since the area of this shoulder is about 30% of the area of the C-1 resonance, its presence must be carefully considered. In fact, it must be remembered that the degree of cross-linking of the network is quite low, as shown by the presence of the very weak resonance at ≈ 26 ppm. Before assigning the 93 ppm resonance we wish to point out that a γ-gauche effect (Tonelli, 1989), such as that encountered in the bending of a sugar residue, produces

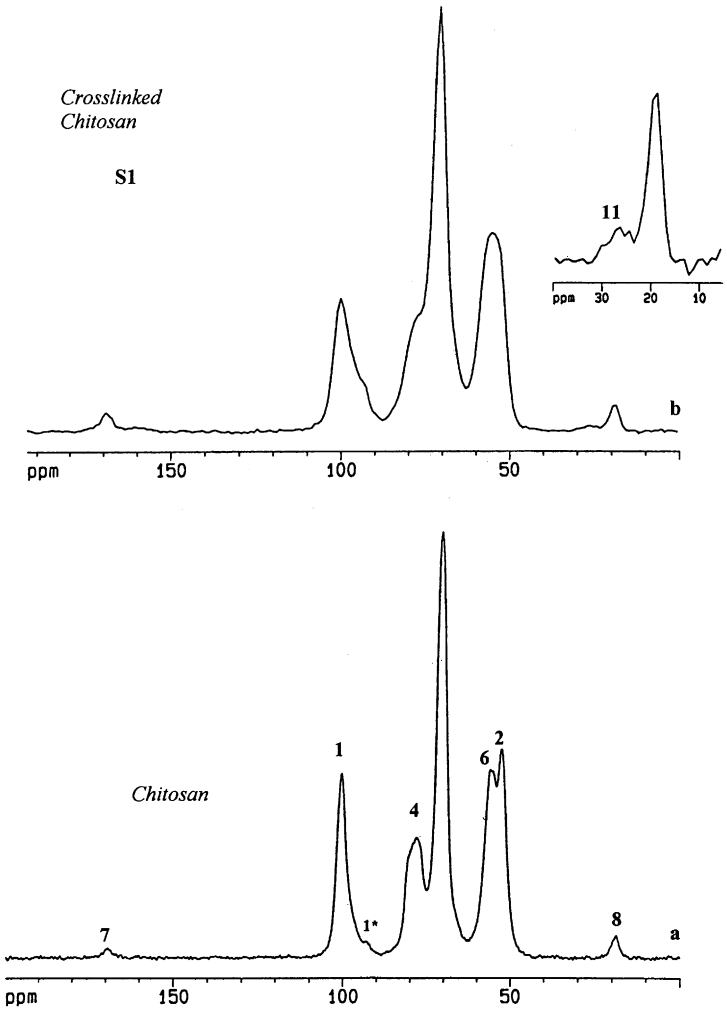


Fig. 2. ^{13}C CP-MAS spectra of: (a) chitosan; and (b) chitosan–TMP network with a very low degree of cross-linking (sample S1 with $R_s = 0.43$ and $t_r = 4$ h : see experimental part). The broad resonance of C-11 is also clearly evident.

exactly this type of upfield shifts. This effect was previously observed in oligomeric products of cellulose (Gast, Atalla & McKelvey, 1980).

Thus the presence of the resonance at ≈93 ppm can be rationalized if we admit the occurrence of a conformational change that follows the cross-linking reaction even when the degree of cross-linking is low. This interpretation agrees with a number of observations reported in the literature. In fact, X-ray and electron diffraction studies (Mazeau, Winter & Chanzy, 1994; Yui, Imada, Okuyama, Obata, Suzuki & Ogawa, 1994) have shown that inter-rings hydrogen bonds (N-2–O-6 and also N-2–O-5) contribute to the three-dimensional stabilization of the crystal structure of chitosan. Moreover, it has been observed that complex-formation or protonation (Saito, 1986; Saito, Tabeta & Ogawa, 1987; Ogawa, Oka, Miyanishi & Hirano, 1984) induce conformational changes of chitosan. Hence, it is not surprising that a low amount of cross-linking in N-2 is able to induce a different conformation of the nearby chitosan un-bridged units.

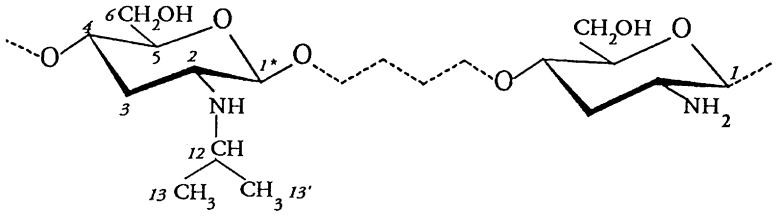


Fig. 3. Schematic structure of the chitosan–DMP derivative.

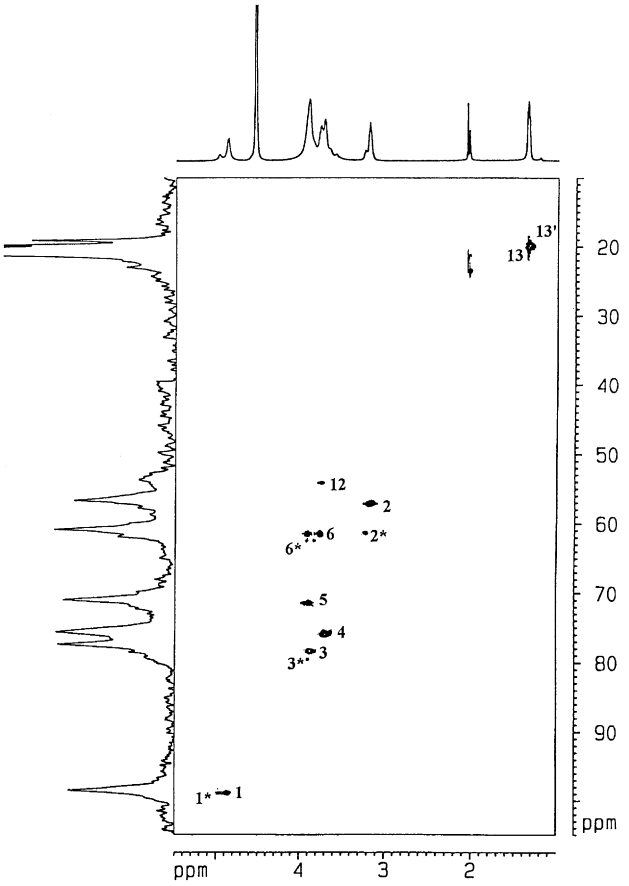


Fig. 4. Two-dimensional HMQC of N-2-substituted chitosan is shown along with the resonances assignment; ^{13}C and ^{1}H spectra are also shown.

To confirm this interpretation, a soluble model compound, chitosan–DMP, was prepared and characterized by NMR spectroscopy both in the solid state and in solution. Fig. 3 shows a schematic structure of chitosan–DMP. Let us mark with a $*$ the C atoms of a unit in which position 2 is substituted; thus units still acetylated and units with a substitution in 2 are evidenced (Capitani, Porro & Segre, 2000a). Since this model compound dissolves easily in acidic aqueous solutions, the resonances assignment and the degree of substitution have been obtained using standard solution methods.

In Fig. 4 the HMQC is shown along with carbon and proton spectra and with the resonance assignment. In the ^{1}H spectrum, at 4.915 and 5.016 ppm, respectively, two resonances of anomeric protons on C-1 and C-1* are observable. The degree of substitution DS is easily obtained by integrating the methyl resonances with respect to resonances of anomeric protons; DS turns out to be about 30%. Note that in solution the anomeric C atom shows only one resonance centered at 99.2 ppm. In Table 1 we report ^{1}H and ^{13}C chemical shifts and relative assignment of chitosan–DMP.

Fig. 5 shows the solid state ^{13}C CP-MAS spectrum of chitosan–DMP. Let us compare Fig. 5 and 2b. Both spectra show the same shape of the anomeric carbon resonance. In both cases the intense shoulder centered at ≈ 95 ppm is present. This observation strongly supports the interpretation that the ≈ 95 ppm resonance is due to the anomeric carbon of chitosan, upfield shifted by a γ-gauche effect due to the presence of a bent conformation. As previously mentioned, intermolecular N-2–O-6 and N-2–O-5 hydrogen bonds contribute to the three-dimensional stabilization of the crystal structure of chitosan. Thus, a few cross-linked units might induce a rearrangement of the hydrogen bond network, causing the occurrence of a stable conformation characterized by the presence of *gauche* (or *gauche**) interring angles.

3.1. Samples with different degrees of cross-linking

In order to obtain a network with a high swelling capability, the degree of cross-link should be quite low. However in any polymer the higher the degree of cross-linking, the easier is its characterization by solid state NMR. Hence, to confirm our previous resonance assignment, two samples S2 and S3 were synthesized, carefully changing the reaction parameters R_s and t_r (see Section 2). These samples have a quite poor swelling capability but present a relatively high degree of cross-linking, i.e. with well observable ^{13}C resonances. Moreover, to ascertain the presence of aldehydic groups, the non-reduced sample S4 was also characterized.

Fig. 6 shows the ^{13}C solid state NMR spectra of these samples. The assignment of the resonance due to methylene carbon atoms C-11 (see Fig. 1) can be safely confirmed. Moreover, a broad resonance at ≈ 43 ppm due to methylene carbons C-10 and C-10$'$ (see Fig. 1) is well observable.

Table 1
^{1}H and ^{13}C spectral assignment of chitosan and of chitosan–DMP in solution (C2* marks any substitution in position 2)

C1	C2	C3	C4	C5	C6		C12	CH_3 (13)	CH_3 (13$'$)
H1	H2	H3	H4	H5	H6	H6$'$	H12	CH_3	CH_3
98.46	56.80	78.06	75.56	70.98	61.16				
4.89	3.19	3.91	3.72	3.91	3.94	3.78			
	C2*								
98.46	60.55	79.21			62.21		53.70	19.50	18.78
4.99	3.22	3.96			3.94	3.85	3.77	1.39	1.37

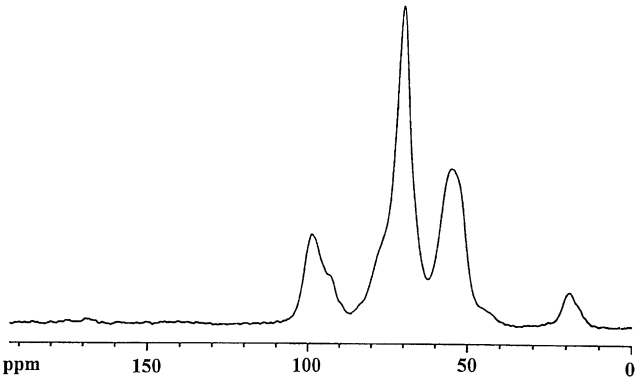


Fig. 5. ^{13}C CP-MAS spectrum of N-2-substituted chitosan–DMP derivative.

At ≈160 ppm carbon bound to nitrogen in the imine environment is observed. In Fig. 6b an aldehydic carbon resonance at ≈189 ppm is also observed. This resonance has been assigned to 1,3-dipropanale pendant groups with one non-reacted terminal aldehyde, i.e. R–NH–CH_2–CH_2–*C*H=O.

By observing the spectrum of the non-reduced sample S4

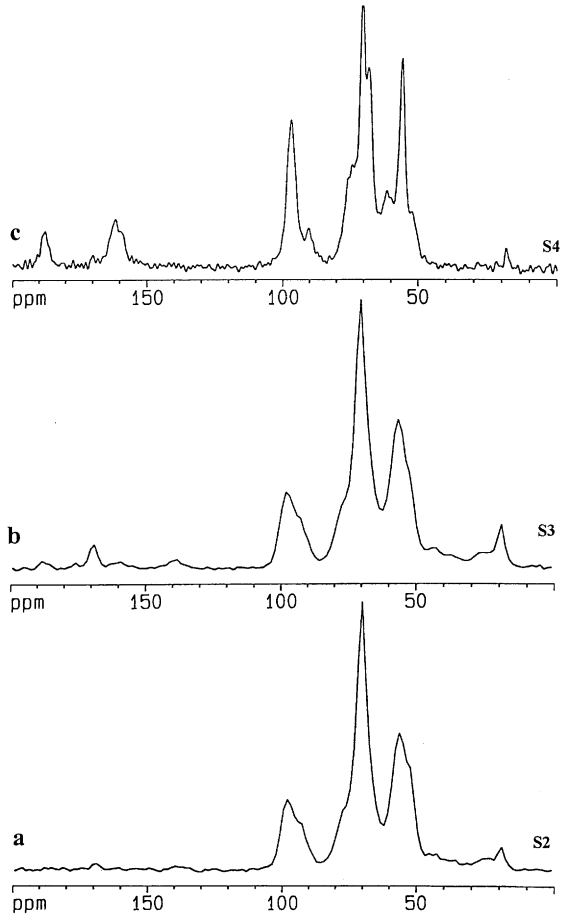
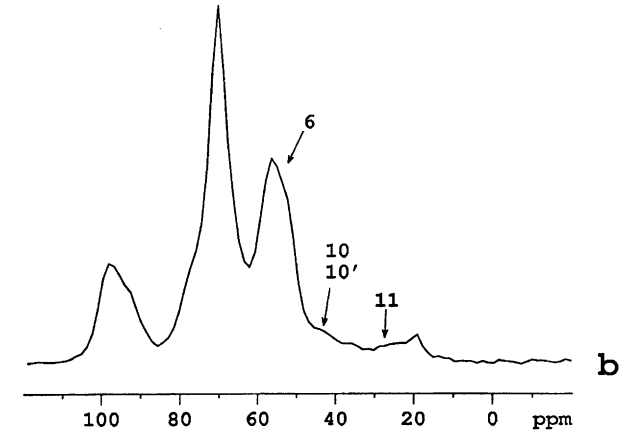


Fig. 6. ^{13}C CP-MAS spectra of: (a) sample S2 ($R_s = 0.2$, $t_r = 10$ h); (b) sample S3 ($R_s = 0.6$, $t_r = 6$ h); and (c) sample S4 (S3 before reduction).



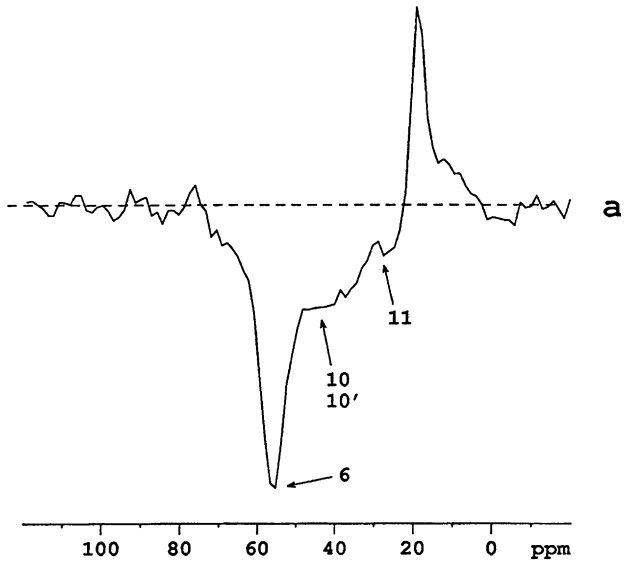


Fig. 7. (a) ^{13}C CP-MAS-SPI spectrum of network S2 ($R_s = 0.2$, $t_r = 10$ h) : all resonances of methine carbons are zeroed, the resonance due to the methyl group is positive, while the resonances due to methylene carbons are inverted. Note that the weak and broad resonances assigned to C-11 and C-10 (C-10′) are inverted. Only the 0–120 ppm range is shown. (b) ^{13}C CP-MAS spectrum of the same sample.

(Fig. 6c), the assignment of resonances at ≈160 and ≈189 ppm can be easily confirmed (Alcazar, Almena, Begtrup & de la Hoz, 1995; Gómez-Sanchez, Maya & Hermosin, 1990). In fact, S4 should contain iminium cross-linking bridges, i.e. $-NH^+=C_IH-C_{II}H=C_{III}H-NH-$. Thus the very intense resonances observed at ≈160 ppm are undoubtedly due to the C_I and C_{III} carbons, while the C_{II} carbon signal is clearly observable at ≈90 ppm. Moreover, since the reduction has not been performed, no resonances are detectable at 26 and 43 ppm, while the aldehydic carbon resonance is well observable at ≈189 ppm.

We note that by increasing the amount of cross-linking agent (TMP) and/or the time of reaction (t_r), some secondary reactions occur. According to the literature, the reaction

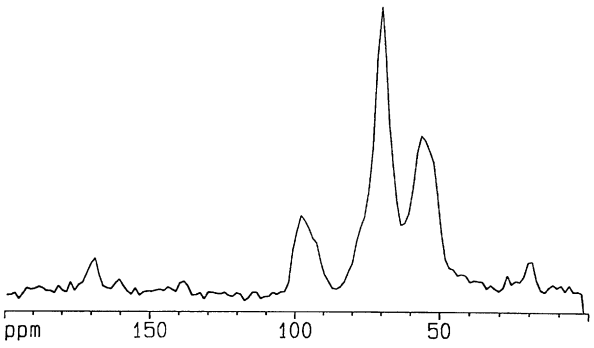


Fig. 8. ^{13}C MAS SPE spectrum of sample S2 ($R_s = 0.2$, $t_r = 10$ h).

between aldehydes and primary amines might proceed beyond the first stage of alkylation to give a mixture of primary, secondary and tertiary amines. The intermediate in the alkylation of a secondary amine might be, for instance, an enamine RCH=CHNR′$_2$ which should resonate at ≈140 ppm (see the weak resonance in Fig. 6b).

Fig. 7a shows the 0–120 ppm region of the sample S2 spectrum ($R_s = 0.2$, $t_r = 10$ h), obtained by the CP-SPI spectral editing sequence. With this sequence, it is possible to confirm the assignment of the weak and broad resonances centered at ≈26 and ≈43 ppm to methylene carbon atoms. In fact the ^{13}C spectrum in Fig. 7a shows that methine carbon resonances are nicely zeroed and methyl and quaternary carbon resonances are positive, while methylene carbon resonances are inverted. The ^{13}C CP-MAS spectrum in the same region is also shown in Fig. 7b.

3.2. Degree of cross-linking

For a polymeric network the characterization of the chemical bridges is fundamental. The other important question concerns the evaluation of the actual degree of cross-linking.

Since CP is not a linear technique, the quantitative reliability of intensity of resonances in CP-MAS spectra must be interpreted with caution. In fact the efficiency of the CP-process depends on the number of abundant spin H close to dilute spin C and on their distance from C.

To avoid this problem, at least partially, and to choose the best contact time we investigated the dynamics of CP (Mehring, 1983). As reported in the experimental part, the best contact time (1 ms) was found following a previously described method (Capitani, Del Nobile, Mensitieri, Sannino & Segre, 2000b) Note that CP-MAS spectra run at 210 K (spectra not shown) do not appreciably differ from spectra run at room temperature.

Finally, with the purpose of improving the linearity of our data an SPE experiment (Mehring, 1983) was performed. The major difficulty in SPE experiments is the long recycle time needed. In fact the recycle time must be long enough to allow all carbon resonances to relax back to their equilibrium values, depending on both T_1 (^{13}C) and on the flip angle of the exciting pulse. Thus, as described in the experimental section, the proper recycle time and the proper flip angle were used.

The SPE spectrum of sample S2 ($R_s = 0.2$, $t_r = 10$ h) is shown in Fig. 8. Even if almost identical to the CP-MAS spectrum, more noise is present. Thus, due to the similarity between CP and SPE spectra, we assume that data of CP-MAS spectra are reasonably quantitative and can be used for the determination of the degree of cross-linking.

As measured by ^1H NMR in solution, the amount of acetylated units is ≈9%; hence in sample S2, to obtain a quantitative analysis we can compare the acetyl resonance to that of methylene C11. The obtained cross-linking degree turns out to be ≈5% with an error estimated ≈20% of the nominal value.

The same procedure cannot be applied to the noisy MAS spectrum of S1 sample. In fact in this sample we are barely able to observe the weak resonance at ≈26 ppm. Thus in this sample the degree of cross-linking should be of the order of 2–3%.

4. Conclusions

A new, highly hydrophilic chitosan-based network has been prepared. Samples synthesized in different conditions as well as relevant model polymeric chitosan derivatives have been studied by ^{13}C solid state NMR spectroscopy.

The chemical units originated by the reaction between chitosan and TMP have been characterized by solid state NMR.

An interesting finding is that even a low amount of cross-linking bridges is able to induce a notable conformational change of chitosan chains.

Since CP is not a linear technique, for the evaluation of the degree of cross-linking an SPE experiment has been performed using a highly cross-linked sample, and the result compared with the corresponding values obtained with an optimized CP-MAS spectrum. We were thus able to evaluate the error in CP-MAS experiments. This information was translated for evaluating the degree of cross-linking in a sample only lightly cross-linked.

A full ^1H relaxation study as a function of temperature as well as additional physico-chemical characterization work (swelling and compression moduli as a function of pH and ionic strength) on the new hydrogels are in progress.

References

Alcazar, J., Almena, I., Begtrup, M., & de la Hoz, A. (1995). *Journal of Chemical Society, Perkin Transactions 1*, *21*, 2773–2781.

Bax, A., & Davis, D. G. (1985). *Journal of Magnetic Resonance*, *65*, 355–360.

Capitani, D., Porro, F. & Segre, A.L. (2000a). *Carbohydrate Polymers 42*, 283–286.

Capitani, D., Del Nobile, M. A., Mensitieri, G., Sannino, A., & Segre, A. L. (2000b). *Macromolecules*, *33*, 430–437.

Crescenzi, V., Paradossi, G., Desideri, P., Dentini, M., Cavalieri, F., Amici, E., & Lisi, R. (1997). *Polymer Gels and Networks*, *5*, 225–239.

De Angelis, A. A., Capitani, D., & Crescenzi, V. (1998). *Macromolecules*, *31*, 1595–1601.

Gast, J. C., Atalla, R. H., & McKelvey, R. D. (1980). *Carbohydrate Research*, *84*, 137–146.

Gómez-Sanchez, A., Maya, I., & Hermosin, I. (1990). *Carbohydrate Research*, *200*, 167–180.

Harris, R. K. (1988). *Multinuclear magnetic resonance in liquids and solids — chemical application.* In: P. Granger & R. K. Harris (Eds.), NATO ASI Series (Vol. 322). Berlin: Springer Verlag. Chapter XVI, pp. 291.

Hurd, R. E., & John, B. K. (1991). *Journal of Magnetic Resonance*, *91*, 648–653.

Mazeau, K., Winter, W. T., & Chanzy, H. (1994). *Macromolecules*, *27*, 7606–7612.

Mehring, M. (1983). *Principles of high resolution NMR in solids*, Berlin: Springer.

Ogawa, K., Oka, K., Miyanishi, T., & Hirano, S. (1984). In J. P. Zikakis, *Chitin, chitosan and related enzymes* (pp. 327–345). Orlando, FL: Academic Press.

Saito, H. (1986). *Magnetic Resonance Chemistry*, *24*, 835–852.

Saito, H., Tabeta, R., & Ogawa, K. (1987). *Macromolecules*, *20*, 2424–2430.

Tennant, G. (1979). Imines. Nitrones, nitriles and isocyanides. In I. O. Sutherland (Ed.), *Comprehensive organic chemistry, the synthesis and reactions of organic compounds* Chap. 8, p. 385.

Tonelli, A. E. (1989). *NMR spectroscopy and polymer structure: the conformational connection*, New York: VCH (chap. 4).

Voelkel, R. (1988). *Angewandte Chemie International Edition in English*, *27*, 1468–1483.

Wu, X., & Zilm, K. W. (1993a). *Journal of Magnetic Resonance A*, *102*, 205–213.

Wu, X., & Zilm, K. W. (1993b). *Journal of Magnetic Resonance A*, *104*, 119–122.

Yui, T., Imada, K., Okuyama, K., Obata, Y., Suzuki, K., & Ogawa, K. (1994). *Macromolecules*, *27*, 7601–7605.