Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 69 (2007) 305–310
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

A novel method for obtaining a quaternary salt of chitosan

Douglas de Britto *, Odílio B.G. Assis

Embrapa Instrumentação Agropecuária Rua XV de Novembro, 1452 C.P. 741, 13560-970 São Carlos, SP, Brazil

Received 7 February 2006; received in revised form 24 August 2006; accepted 5 October 2006
Available online 22 November 2006

Abstract

A novel method for obtaining *N*,*N*,*N*-trimethylchitosan has been developed using dimethylsulfate as the methylant agent. Dimethylsulfate is not only more efficient but is less expensive than other commonly used agents. The resultant chitosan derivatives were characterized by FTIR, ^{1}H NMR, CP-MAS ^{13}C NMR, and a capillary viscometer. Films were processed by casting and then hydrophilic changes were assessed by water contact angle measurements. The highest degree of substitution ($\overline{DQ} = 52.5\%$) was obtained after a 6 h long-term reaction at room temperature. The use of higher temperatures helped to provoke polymeric thermal degradation and favor O-methylation over a *N*-methylation reaction.

Keywords: Chitosan; *N*,*N*,*N*-Trimethylchitosan; Dimethylsulfate; Quaternization degree; Hydrophilicity

1. Introduction

The intensive methylation of chitosan generates the *N*,*N*,*N*-trimethyl derivative (TMCh), characterized by possessing permanent positive charges in chains as a consequence of the quaternization of the amino groups in the C-2 position of the chitosan structure (Curti, Britto, & Campana-Filho, 2003; Dung, Milas, Rinaudo, & Desbriéres, 1994; Sieval et al., 1998). TMCh is a water-soluble polyelectrolyte with good intestinal absorption enhancing properties for hydrophilic and macromolecular drugs. It has been suggested as a soluble carrier for drug delivery (Kotzé et al., 1998; Thanou et al., 1998). Application of TMCh as an antibacterial agent has also been reported (Jia, Shen, & Xu, 2001; Kim, Choi, Chun, & Choi, 1997), with good activity described as the result of ionic interaction between chitosan positive charges and the negatively charged cell surface of bacteria.

TMCh can be synthesized by either covalent addition of a substituent containing a quaternary ammonium group, or by quaternization of the amino groups of the parent polymer (Curti et al., 2003). It is the latter method which has received most attention with alkylation agents such as alkyl halide usually employed. Degrees of quaternization ($\overline{DQ}$) superior to 53% have been achieved by reacting chitosan with iodomethane (an alkylation agent) in the presence of *N*-methyl-2-pyrrolidone (NMP). This process (Dung et al., 1994), has shown to be less severe than the usual quaternization reactions, this reducing the depolymerization intensity of the parent polymer. Despite being efficient, iodomethane is a highly volatile, carcinogenic and expensive reagent. In addition it offers limited control over a perilously chemical reaction.

In an attempt to overcome these disadvantages, an alternative sequence for the synthesis of chitosan quaternized derivatives is proposed using dimethylsulfate as the reactive agent. Dimethylsulfate is considerably less expensive than iodomethane and is less toxic. In addition it also has a high boiling point and no solvent is required for the reaction, unlike for NMP.

2. Experimental

The starting chitosan was purchased from Polymar (CE, Brazil) and was used "as supplied". Dimethylsulfate was

* Corresponding author. Tel.: +55 16 33742477; fax: +55 16 33725958.
E-mail addresses: dougbritto@ig.com.br (D. de Britto), odilio@cnpdia.embrapa.br (O.B.G. Assis).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.10.007

obtained from Vetec (R. Janeiro, Brazil) and other solvents used were supplied by Synth (S. Paulo, Brazil).

Five methylation reactions with varying time and temperature were carried out for comparison. Samples were identified as *T* when heated at 70 °C or R for room temperature, followed by the reaction time, in which 'H' indicates hours and 'M' minutes. The basic reaction sequence comprised a suspension of 1 g of chitosan (0.005 mol) in 16 cm^3 of dimethylsulfate and 4 cm^3 of deionized water. 1.2 g of NaOH (0.015 mol) and 0.88 g of NaCl (0.015 mol) were added and the solution mixed for the desired time using a magnetic stirrer. Reflux was applied when the reaction mixture was heated to 70 °C. The resulting derivatives were submitted to dialysis in a cellophane membrane (cut-off 12,000–14,000 g mol) for three days. The final products were obtained by precipitation with acetone. After exhaustive rinsing, the derivatives were filtered and vacuum dried.

The ^1H NMR spectra of chitosan and *N*,*N*,*N*-trimethyl derivatives were acquired at 353 K by using a 200 MHz spectrometer (Bruker AC200). For this analysis, samples were dissolved in D_2O/HCl (100/1 v/v) and in D_2O, respectively, at a concentration of 10 g/l. The parameters for the acquisition of the NMR spectra were as follows: a pulse of 90°, corresponding to a pulse width of 8.2 μm; LB = 0.3 Hz; NS = 16. The average degrees of acetylation ($\overline{DA}$) and quaternization ($\overline{DQ}$) were determined from the ^1H NMR spectra as described elsewhere (Britto & Campana-Filho, 2004; Curti et al., 2003; Hirai, Odani, & Nakajima, 1991; Sieval et al., 1998).

The solid-state CP-MAS ^{13}C NMR experiments were performed on a Varian Unity Inova 400 spectrometer operating at 400 MHz for ^1H frequency, using the combined techniques of proton dipolar decoupling (DD), magic angle spinning (MAS) and cross-polarization (CP). Contact time was 1 ms, acquisition time 51.2 ms and the recycle delay 4 s. The proton pulse width was 6 ms and an 18 kHz spectral window was used. Typically 2000 scans were acquired for each spectrum. The chemical shifts were externally referenced by setting the methyl resonance of hexamethylbenzene (HMB) to 17.3 ppm. The samples were contained in a SiN_4 cylindrical rotor which was spun at 5 kHz during measurements.

Viscosity measurements were carried out with an aqueous buffer solution for chitosan and an aqueous NaCl solution for quaternary derivatives. Chitosan solution was prepared by dissolving 50 mg into 50 cm^3 of buffer solution, followed by positive filtration through 0.45 μm membranes (Millipore®). The buffer solution comprised acetic acid (0.3 mol dm^{-3}) and sodium acetate (0.2 mol dm^{-3}) and was used as solvent for serial dilution. The TMCh solutions were prepared by dissolving 70 mg of the derivatives in 25 cm^3 of distilled water, followed by the addition of 25 cm^3 of a 0.2 mol dm^{-3} NaCl aqueous solution. Aqueous NaCl solution (0.1 mol dm^{-3}) was used as solvent for serial dilutions. The AVS-350 viscometer coupled to the AVS-20 automatic burette for serial dilution, both from Schott-Geräte was used for viscosity measurements. A glass capillary ($\phi = 0.53$ mm) containing 15 cm^3 of the polymer solution was immersed in a water bath maintained at 25.00 ± 0.01 °C. The intrinsic viscosities, $[\eta]$, were determined from curves of reduced viscosity, η_{sp}/C, vs. polymer concentration, *C*, for the relative viscosity, η_{rel}, in the range $2.0 > \eta_{rel} > 1.2$.

FTIR spectra were obtained from films ($\theta = 0.01$ mm thickness) cast in Petri dishes. Aqueous acetic acid solution (1%) and deionized water were used to prepare chitosan and derivative solutions respectively. The concentration of the polymeric solutions was 10 g dm^{-3}. A Perkin-Elmer spectrometer (model Paragon 1000) was also employed.

The hydrophilicity of the derivatives was estimated by static contact angle measurements using a sessile deionized water drop (around 5 μL). For this, the polymeric solutions were deposited onto a clean glass sheet (20 mm × 10 mm) and allowed to dry at room temperature and 40% RH gradient for 24 h. The solutions were prepared as described for FTIR analysis. Images of water droplets on the film surface were recorded using a video based optical device and the angles determined by FTA32 Image Software (First Ten Ångstroms). The recorded angle is the average of five measurements on each sample. All measurements were performed in air at room temperature.

3. Results and discussion

The average degree of acetylation, $\overline{DA}$, and the average degree of substitution (or quaternization), $\overline{DQ}$, are the most important characteristics of chitosan and its derivatives. A variety of techniques are available to determine both, however NMR spectroscopy is considered to yield the most reliable results. The ^1H NMR spectrum of chitosan (Fig. 1) indicated the following characteristic signals (Britto & Campana-Filho, 2004): (a) $\delta = 4.5 < \delta < 5.0$ attributed to hydrogen bonded to the anomeric carbon 1;

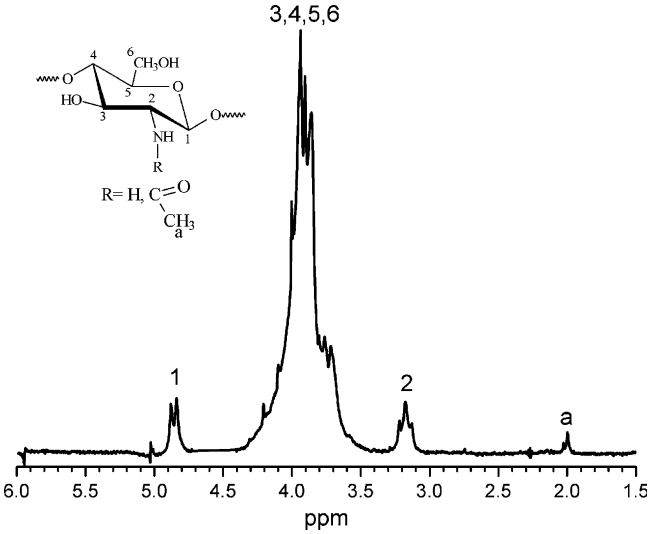

Fig. 1. ^1H NMR spectrum of chitosan dissolved in D_2O/HCl (100:1 v/v).

(b) $\delta = 3.4 < \delta < 4.0$ due to hydrogen bonded to the carbon atoms 3, 4, 5 and 6 of the glycopiranose unit; (c) $\delta \cong 3.18$ attributed to the hydrogen atom bonded to the carbon 2 of the glycopiranose ring and (d) $\delta \cong 1.99$ corresponding to the hydrogen atoms of the methyl moieties of the acetamido groups. The ratio between the intensity of the latter two signals mentioned above allowed the determination of the average degree of acetylation of the purified chitosan as $\overline{DA} = 9\%$ (Table 1).

Evidence for the occurrence of *N*-methylation is seen in the region $2.47 < \delta < 3.37$ in the ^{1}H NMR spectra of derivatives synthesized at room temperature for 6 h, R6H, (Fig. 2) and at 70 °C for 15 min, T15M, (Fig. 3). Despite these results, only the signal at 3.3 ppm is directly related to the occurrence of quaternization, corresponding to the hydrogen of a methyl group bonded to quaternary nitrogen (Britto & Campana-Filho, 2004; Curti et al., 2003). From the ratio of the area below this signal and that attributed to the hydrogen atom bonded to carbon C1, the values of $\overline{DQ}$ were calculated and are given in Table 1. The signal developed at 2.76 ppm is assumed to be referenced to the *N*,*N*-dimethylated sites (Britto & Campana-Filho, 2004) and its high intensity indicates a considerable number of *N*,*N*-dimethylated sites in the derivatives. The spectra of R6H and T15M also show two signals in the region $3.37 < \delta < 3.56$ both of which correspond to the *O*-methylated sites.

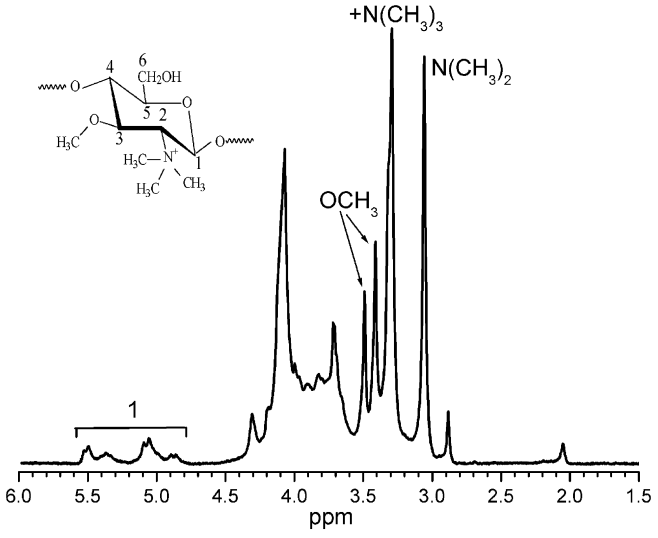

Fig. 2. ^{1}H NMR spectrum of the R6H derivative dissolved in D_2O.

Table 1
Average degree of acetylation ($\overline{DA}$) of starting chitosan and average degree of quaternization ($\overline{DQ}$) of TMCh samples determined by ^{1}H NMR

Sample	$\overline{DA}$ (%)	$\overline{DQ}$ (%)
Chitosan	4.0	–
R6H	–	52.5
T15M	–	36.8
T1H	–	15.8
T3H	–	27.0

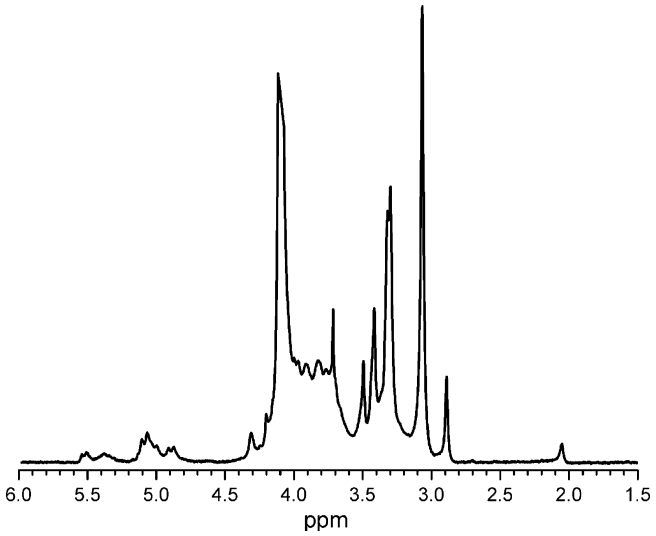

Fig. 3. ^{1}H NMR spectrum of the T15M derivative dissolved in D_2O.

From Table 1 it is clear that the different conditions of reaction resulted in products with differing $\overline{DQ}$, in which high values of $\overline{DQ}$ was achieved for salts synthesized at room temperature. It was first expected that derivatives with a higher level of substitution could be accomplished by increasing the temperature. Unfortunately, due to the presence of H^+ in the reactive medium, higher temperatures appear to accelerate the polymeric chain degradation that is mainly the depolymerization process. Depolymerization generates small chains that are dragged out during the dialysis sequences, explaining the low values of $\overline{DQ}$ observed for derivatives obtained at 70 °C. Thermal degradation can be also assessed by means of a numerical reduction over reaction yield: at higher temperatures the final amount of derivative is less than 40% of the starting amount of chitosan. In comparison it is as high as 90% when the reaction occurs at room temperature.

The solid-state CP-MAS ^{13}C NMR technique has mostly been applied to characterize chitosan and its derivatives, and largely in the determination of the degree of acetylation (Cervera et al., 2004; Duate, Ferreira, Marvão, & Rocha, 2001; Holappa et al., 2004; Nunthanid et al., 2004; Ottøy, Vårum, & Smidsrød, 1996; Velde & Kienkens, 2004). This has proved to be a powerful tool since no prior sample preparation is required. The CP-MAS ^{13}C NMR spectrum for chitosan (Fig. 4) is very similar to that reported in the literature (Cervera et al., 2004; Duate et al., 2001; Ottøy et al., 1996) and the following characteristic signals can be identified: (a) $\delta = 24.4$ ppm attributed to the carbon atom of the methyl moieties of the acetamido groups; (b) $\delta = 60$ ppm two convoluted signals are observed and attributed to carbon C6 and C2; (c) $\delta = 76.4$ ppm due the carbon C5 and C3; (d) $\delta = 84$ ppm corresponding to carbon atom C4 and finally (e) $\delta = 106.2$ ppm corresponding to carbon atom C1.

For samples with a high degree of acetylation, the signal at 24 ppm grows in intensity (Cervera et al., 2004; Duate

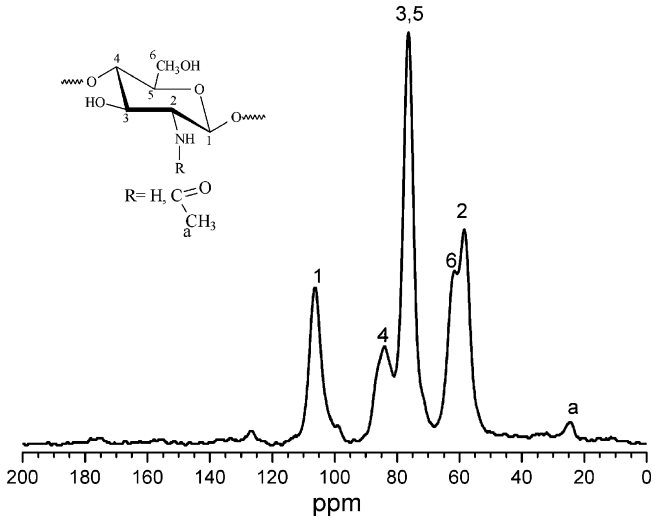

Fig. 4. Solid state CP-MAS ^{13}C NMR spectrum of chitosan.

et al., 2001). Furthermore an additional signal appears at 175 ppm, pointing to the presence of a carbon atom in carbonyl group (C=O). Such a signal was not observed in our spectrum due to the low degree of acetylation, whose value was $\overline{DA} = 9\%$, calculated according to method proposed by Ottøy et al. (1996). This figure however is greater than that determined by ^{1}H NMR (Table 1). According to Ottøy et al. (1996), for samples with $\overline{DA} = 20$–50%, both CP-MAS ^{13}C NMR and ^{1}H NMR techniques are in good agreement. For $\overline{DA}$ lower than 20% however it appears that the value of $\overline{DA}$ calculated by both techniques differ.

After the quaternization reaction, changes are introduced in the CP-MAS ^{13}C NMR derivatives spectra (Fig. 5). The signals at 40 and 48.5 ppm can be attributed to carbon atoms of *N*-monomethylated and *N*,*N*-dimethylated groups, respectively. In previous work (Curti et al., 2003), analysis by ^{13}C NMR (liquid state) revealed the existence of a signal at 44 ppm that was attributed to *N*,*N*-dimethylated groups. In fact, due to the low resolution attained in that spectrum from the liquid state it was not possible to distinguish the signal of the *N*-monomethylated group. The values found here are in close accordance with values reported (Sohár, 2000) for secondary (35.9 ppm) and tertiary (47.5 ppm) amines.

The occurrence of quaternization was demonstrated by the signal at 55.6 ppm, equivalent to three carbon of *N*,*N*,*N*-trimethylated groups. The position of this carbon signal was previously confirmed by Holappa et al. (2004) in the analysis of *N*-betainates chitosan by CP-MAS ^{13}C NMR technique. Holappa identified the signal at 57.27 ppm. Similarly, as reported by Curti et al. (2003), an intense signal at 56 ppm, due to the *N*,*N*,*N*-trimethylated groups could be identified as well at spectra derivatives with higher degree of quaternization.

It is worth noticing (Fig. 5) that for the sample R6H the intensity of the peak at 55.6 ppm is comparatively higher than that for the sample T15M, meaning a greater $\overline{DQ}$ achieved when chitosan was reacted at room temperature for 6 h. Conversely as a consequence of its low degree of quaternization, the corresponding T15M quaternary signal is almost merged with glucopyranose C2 and C6 carbon signals that are seen overlapped at 60 ppm. These results are in good agreement with those obtained above by means of the ^{1}H NMR technique.

Another important observation concerning the derivative spectrum is the presence of a signal at 70 ppm (Fig. 5). This peak is considerably more intense for the T15M sample and can be attributed to the carbon atom of *O*-methylated sites. In this case temperature is critical in facilitating the *O*-methylation. Such a feature can be also found in the T1H sample spectrum (Fig. 6) in which this signal is also intense. Such results stress the negative effect of the use of temperature in the reaction, where 15 min showed to be sufficient for the occurrence of undesirable O-methylation, detrimental to the *N*,*N*,*N*-trimethylation, comparatively to samples reacted at room temperature.

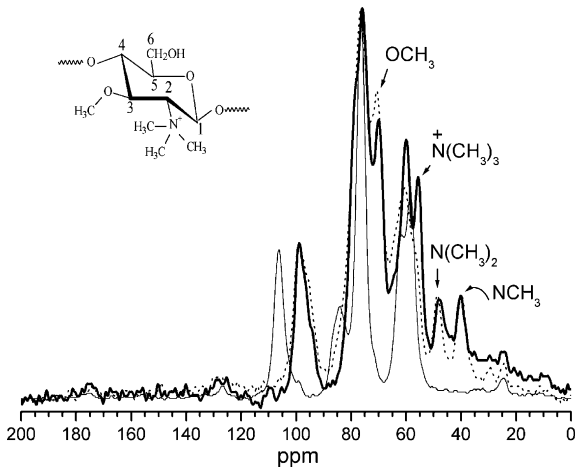

Fig. 5. Solid state CP-MAS ^{13}C NMR spectra of R6H (—) and T15M (···) samples. The spectrum of chitosan (—) is added for comparison.

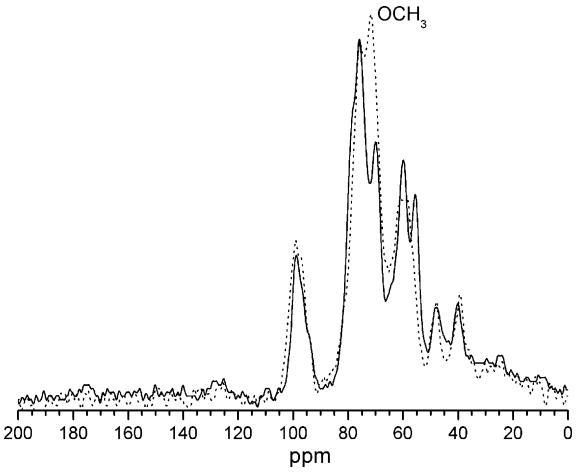

Fig. 6. Solid state CP-MAS ^{13}C NMR spectrum of T1H (···) sample. The spectrum of R6H (—) is added for comparison.

Another important feature of these spectra is that after quaternization the C1 carbon signal shifted from 106.2 ppm for chitosan to 98.9 ppm for TMChs samples, due to the substitution of nitrogen atoms.

Methylation is also observed by means of FTIR spectroscopy. Comparing the spectrum of chitosan and the derivatives R6H and T3H in the interval of 1700–1200 cm^{-1} (see Fig. 7) the following differences were observed (Britto & Campana-Filho, 2004): (a) in the derivative's spectra, the band centered at 1475 cm^{-1}, corresponding asymmetrical stretching of C–H in the methyl groups is not present in the spectrum of the starting chitosan and conversely in the chitosan spectrum (b), the band due to angular deformation of N–H in amino groups assigned near 1590 cm^{-1} are quite reduced for the derivatives, being overlaid by the signal at 1630 cm^{-1}.

Degradations which took place during the reaction can be assessed by measuring the intrinsic viscosity, $[\eta]$, since $[\eta]$ is directly related to the average molar mass. The viscometric data of chitosan and its derivatives were calculated using the Huggins and Kraemmer equations, the results are summarized in Table 2.

The samples R6H and T15M, presented both similar reduction of $[\eta]$ of about 50%. However, for derivatives processed by using the temperature over a period higher than 15 min it was not possible to calculate $[\eta]$. This is due to the relative viscosity falling to values $\eta_{rel} < 1.2$, despite the preparation of a more concentrated polymeric solution. This confirms degradation during the quaternization reaction.

From the Mark Houwink equation ($[\eta] = KM_v^a$) and empiric values of K and a for chitosan reported in the literature (Rinaudo, Milas, & Dung, 1993; Wang, Bo, Li, & Qin, 1991), the average viscosity molecular weight of the chitosan was calculated as $M_v \cong 65{,}000$ g mol^{-1}.

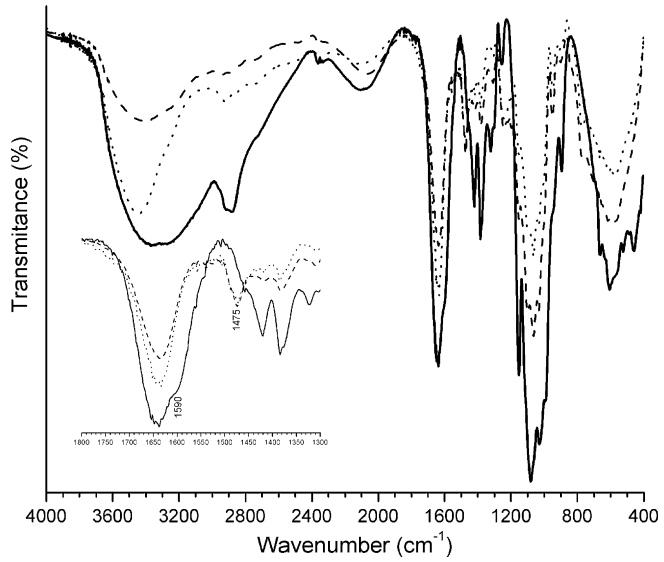

Fig. 7. FTIR spectra of the chitosan (—), R6H (···) and T3H (- - -).

Table 2
Values of the intrinsic viscosity, $[\eta]$, for chitosan (0.3/0.2 mol dm^{-3} CH_3COOH/CH_3COONa aqueous solution) and TMCh (0.1 mol dm^{-3} NaCl aqueous solution)

Sample	$[\eta]$ cm^3 g^{-1}
Chitosan	322
R6H	169
T15M	161
T1H	–
acT3H	–

The derivatives show good film forming ability by casting from aqueous solution. For a low degree of substitution the resultant films were transparent and turned slightly yellow or off-white in color for higher substitutions. The recording contact angles indicated that the higher angle is measured on the precursor chitosan film (Table 3). The water contact angle of around 100° for chitosan is in accordance with the literature (Hsieh, Tsai, Wang, Chang, & Hsieh, 2005; Tangpasuthadol, Pongchaisirikul, & Hoven, 2003). Hydrophobicity is usually expressed in terms of wet ability which involves the interaction of a liquid to the surface, where a reduction on the contact angle corresponds to an increase in the degree of hydrophilicity. From the measured angles it is evident that the affinity towards water has increased in the quaternized derivatives. The sample T3H reacting at 70 °C for 3 h was the most hydrophilic derivative. For this set of experiments, a relation between $\overline{DQ}$ and contact angle was not clearly established. There are two opposing phenomena that occur during the reaction: (i) the quaternization of nitrogen and (ii) the *O*-methylation and *N*,*N*-dimethylation. The first increases the hydrophilicity due to the formation of permanent positive charges whilst the second reduces hydrophilicity due to the introduction of hydrophobic CH_3 groups. In addition the molecular weight of the polymer plays an important role on its hydrophilicity. Consequently the degree of hydrophilicity of the derivatives will depend on a balance of these phenomena.

Of significance is the effect of the precursor polymeric solution initial pH on the wettability of the cast film. For films processed from acid solution as the starting chitosan and the sample (T3H), the recorded contact angles are

Table 3
Values of contact angle for chitosan and TMCh samples

Sample	Contact angle (°)	Initial pH of the solution
Chitosan	102 ± 2	7.0[a]
Chitosan	89 ± 1	3.9
R6H	79 ± 3	4.3
T15M	71 ± 2	4.0
T1H	68 ± 3	6.6
T3H	43 ± 1	7.0
T3H	33 ± 3	4.0

[a] This film was obtained by neutralization of dry acid film with diluted NaOH solution, washed with deionized water and dried at room temperature.

inferior to the same material when processed from a more alkaline solution. This can be explained by the function of the intensity of the protonation of the amino groups performed by the acidic medium, resulting in a more hydrophilic polymer. Indeed, any solid sample of chitosan obtained from an acid media, e.g., chitosan chloridrate, is a polyelectrolyte with positive charges that has more affinity to water and consequently is easily hydrated.

4. Conclusions

The use of dimethylsulfate as a methylant agent for synthesizing *N*,*N*,*N*-trimethylchitosan has been demonstrated as a simple and an effective alternative to more expensive and often more toxic agents . The quaternization intensity was found to be time and temperature dependent, with final degrees varying from 15.8% to 52.5%. NMR and FTIR spectroscopy was used to describe the main alterations occurring in the starting chitosan, where undesirable O-methylation was observed to take place for reactions carried out above room temperature. Evidence of polymeric degradation during quaternization reaction was confirmed by viscosity measurement. An improvement of the hydrophilicity in the derivatives was apparent from the measurements of contact angle.

Acknowledgments

The authors wish to thank Dr. Sergio P. Campana Filho for the viscometric measurements in his laboratory at Instituto de Química de São Carlos, USP. This work was supported by CNPq and Embrapa.

References

Britto, D., & Campana-Filho, S. P. (2004). A kinetic study on the thermal degradation of *N*,*N*,*N*-trimethylchitosan. *Polymer Degradation and Stability, 84*, 353–361.

Cervera, M. F., Heinämäki, J., Räsänen, M., Maunu, S. L., Karjalainen, M., Acosta, O. M. N., et al. (2004). Solid-state characterization of chitosan derived from lobster chitin. *Carbohydrate Polymers, 58*, 401–408.

Curti, E., Britto, D., & Campana-Filho, S. P. (2003). Methylation of chitosan with iodomethane: Effect of reaction conditions on chemoselectivity and degree of substitution. *Macromolecular Bioscience, 3*(10), 571–576.

Duate, M. L., Ferreira, M. C., Marvão, M. R., & Rocha, J. (2001). Determination of the degree of acetylation of chitin materials by ^{13}C CP/MAS NMR spectroscopy. *International Journal of Biological Macromolecules, 28*, 359–363.

Dung, P., Milas, M., Rinaudo, M., & Desbriéres, J. (1994). Water soluble derivatives obtained by controlled chemical modifications of chitosan. *Carbohydrate Polymers, 24*, 209–214.

Hirai, A., Odani, H., & Nakajima, A. (1991). Determination of degree of deacetylation of chitosan by ^{1}H NMR spectroscopy. *Polymer Bulletin, 26*, 87–94.

Holappa, J., Nevalainen, P., Savolainen, J., Soininen, P., Elomaa, M., Safin, R., et al. (2004). Synthesis and characterization of chitosan N-betainates having various degrees of substitution. *Macromolecules, 37*, 2784–2789.

Hsieh, C.-H., Tsai, S.-P., Wang, D.-M., Chang, Y.-N., & Hsieh, H.-J. (2005). Preparation of γ-PGA/chitosan composite tissue engineering matrices. *Biomaterial, 26*, 5617–5623.

Jia, Z., Shen, D., & Xu, W. (2001). Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research, 33*, 1–6.

Kim, C. H., Choi, J. W., Chun, H. J., & Choi, K. S. (1997). Synthesis of chitosan derivatives with quaternary ammonium salt and their antibacterial activity. *Polymer Bulletin, 38*, 387–393.

Kotzé, A. F., Lueben, H. L., Leeuw, B. J., Boer, B. G., Verhoef, J. C., & Junginger, H. E. (1998). Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Journal of Controlled Release, 51*, 35–46.

Nunthanid, J., Laungnat-anan, M., Sriamonsak, P., Limmatvapirat, S., Puttipipatkhachorn, S., Lim, L. Y., et al. (2004). Characterization of chitosan acetate as a binder for sustained release tablets. *Journal of Controlled Release, 99*, 15–26.

Ottøy, M. H., Vårum, K. M., & Smidsrød, O. (1996). Compositional heterogeneity of heterogeneously deacetylated chitosans. *Carbohydrate Polymers, 19*, 17–24.

Rinaudo, M., Milas, M., & Dung, P. L. (1993). Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. *International Journal of Biological Macromolecules, 15*, 281–285.

Sieval, A. B., Thanou, M., Kotzé, A. F., Verhoef, J. C., Brusse, J., & Junginger, H. E. (1998). Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride. *Carbohydrate Polymers, 36*, 157–165.

Sohár, P. (2000). *Nuclear magnetic resonance spectroscopy* (Vol. 2). Boca Raton, FL: CRC Press.

Tangpasuthadol, V., Pongchaisirikul, N., & Hoven, V. P. (2003). Surface modification of chitosan films. Effects of hydrophobicity on protein adsorption. *Carbohydrate Research, 338*, 937–942.

Thanou, M., Sieval, A. B., Kotzé, A. F., De Boer, A. G., Verhoef, J. C., & Jungiger, H. E. (1998). *N*,*N*,*N*-trimethyl chitosan chloride (TCM) of high degree of substitution as a potential absorption enhancer for hydrophilic drugs. In A. Domard, G. A. F. Roberts, & K. M. Vårum (Eds.). *Advance in chitin science* (Vol. 2, pp. 384–389). Lyon: Jacques Andre Publisher.

Velde, K. V., & Kienkens, P. (2004). Structure analysis and degree of substitution of chitin, chitosan and dibutyrylchitin by FT-IR spectroscopy and solid state ^{13}C NMR. *Carbohydrate Polymers, 58*, 409–416.

Wang, W., Bo, S., Li, S., & Qin, W. (1991). Determination of the Mark-Houwink equation for chitosan with different degrees of deacetilation. *International Journal of Biological Macromolecules, 13*, 281–285.