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Research paper

Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride

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Abstract

N-Trimethyl chitosan chloride (TMC) is a soluble chitosan derivative that shows effective enhancing properties for peptide and protein drug transport across mucosal membranes. TMC was synthesized by reductive methylation of chitosan in an alkaline environment at elevated temperature. The number of methylation process steps and the base used in the process was demonstrated to affect the degree of quaternization of the primary amino group and methylation of 3- and 6-hydroxyl groups. ^{1}H-Nuclear magnetic resonance spectra showed that the degree of quaternization of TMC was higher when using sodium hydroxide as the base compared to using dimethyl amino pyridine. The degrees of quaternization as well as *O*-methylation of TMC increased with the number of reaction steps. *O*-Methylation resulted in decreased solubility of TMC. The high degree of quaternization of TMC with a low degree of *O*-methylation was prepared by employing one reaction step with two subsequent addition steps and a controlled alkaline environment of the mixture reaction.

Keywords: Chitosan; *N*-Trimethyl chitosan chloride; Reductive methylation; Degree of quaternization; Degree of *O*-methylation

1. Introduction

Chitosan is a natural cationic polysaccharide consisting primarily of a repeating unit of β-(1–4)-2-amino-2-deoxy-D-glucose (D-glucosamine). It can be derived by partial deacetylation of chitin and is also naturally present in some microorganisms and fungi. Chitosan is available in different molecular weights (50–2000 kDa), viscosity grades, and degrees of deacetylation (40–98%) [1]. Chitosan is accepted as a biodegradable and non-toxic polymer and has been widely used as a pharmaceutical and cosmetic excipient [2,3].

Chitosan has been reported to enhance the absorption of peptide and protein drugs across mucosal membranes by opening the tight junctions [4–6]. Although, chitosan is soluble in aqueous acidic medium below pH 6.5, it precipitates above this pH. The application of chitosan was limited owing to its insolubility at physiological pH. Many efforts to prepare derivatives of chitosan by chemical modifications in order to increase the solubility in water have been reported [7–9]. Removal of the one or two hydrogen atoms of the amino group of chitosan and introduction of some hydrophilic substituent by chemical modification resulted in improvement of solubility in aqueous media [10].

N,*N*,*N*-Trimethyl chitosan chloride (TMC) is a partially quaternized derivative of chitosan and is freely soluble over a wide pH range. TMC has been proven to be a potent intestinal absorption enhancer for peptide drugs, even in neutral environments [11,12]. This polymer is able to open tight junctions, which seal the paracellular pathways, thereby facilitating the paracellular diffusion of peptide drugs. The process has the additional advantage of being reversible after removal of the polymer, leading to the resealing of the tight junctions [12]. The charge density of TMC, as determined by the degree of quaternization, has an important effect on the absorption enhancing properties of this polymer [13]. During the synthesis of TMC, the number of positive charges on the polymer chain is increased, causing the polymeric molecule to expand in solution due to repelling force between the functional groups [14].

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The methylation of the primary amine to the quaternary stage is accomplished by using a base to bind the acid, being generated during the reaction taking place and to avoid protonation of the unreacted primary amino groups. Although the high degree of substitution of the amino group of chitosan is difficult due to the presence of some acetyl group, the hydroxyl group of chitosan can be substituted in the methylation process. The high degree of *O*-methylation on the 3- and 6-hydroxyl groups will decrease the solubility of the polymer [15]. To tailor a TMC to satisfy the specific requirement would certainly need a careful synthesis process.

The specific aim of this study was to investigate the effect of the methylation process and type of base used on the degree of quaternization and *O*-methylation of the TMC polymer.

2. Materials and methods

2.1. Materials

Chitosan, high molecular weight (ChitoClear TD012, 97% deacetylated, viscosity 552 mPa s), and low molecular weight (ChitoClear TM568, 94.5% deacetylated, viscosity 12 mPa s) were obtained from Primex Biochemicals (Haugesund, Norway). The absolute molecular weight of high molecular weight chitosan was determined with a size exclusion chromatograph (SEC) connected to a multiple angle laser light scattering detector (MALLS) as previously described by Snyman et al. [15] and was found to be 260 400 g/mol. The molecular weight of low molecular weight chitosan was 8000 Da, as specified by the supplier. Methyl iodide, *N*-methylpyrrolidinone, and dimethylaminopyridine were obtained from Acros Organics (Geel, Belgium). All other chemicals were commercially available and used as received.

2.2. Methods

2.2.1. Synthesis of N,N,N-trimethyl chitosan chloride

N,*N*,*N*-Trimethyl chitosan chloride (TMC) was synthesized by reductive methylation of chitosan based on the method previously described [9,15] with some modifications by varying the number of reaction steps and the type of base. The methylation of chitosan was successively repeated with the polymer obtained from the preceding step. TMC polymers of different degrees of methylation were synthesized. The reaction conditions of each step in the synthesis of the TMC polymers are described below and summarized in Table 1.

2.2.1.1. Reaction step 1. A mixture of 2 g of chitosan, 4.8 g of sodium iodide, 10 ml of 20% aqueous sodium hydroxide or 20 ml of 5% dimethylaminopyridine was mixed in *N*-methylpyrrolidinone in a water bath at 60 °C for 20 min. Subsequently, 12 ml of methyl iodide was added to the mixture and the reaction was carried out for 60 min in the presence of a Liebig's condenser. The polymer was collected by precipitation from solution using ethanol and diethyl ether. The product obtained from this step (*N*-trimethyl chitosan iodide) was washed twice with diethyl ether on a glass filter and dried under vacuum.

Table 1
Type of base and amount of methyl iodide used in sequence of reaction steps

Polymer no.	Reaction step 1[a]	Reaction step 2[a]	Addition step 1[b]	Addition step 2
1	NaOH	–	–	–
2	NaOH	NaOH	–	–
3	NaOH	–	NaOH	–
4	NaOH	NaOH	NaOH	–
5	NaOH	–	DMAP	–
6	DMAP	–	NaOH	–
7	DMAP	–	DMAP	–
8	DMAP	DMAP	DMAP	–
9	NaOH	–	NaOH[c]	NaOH[c]
10	NaOH	–	NaOH[d]	NaOH[d]
11[e]	NaOH	NaOH	NaOH	–
12[f]	NaOH	NaOH	NaOH	–

[a] 10 ml of 20% w/w NaOH or 20 ml of 5% w/w dimethylaminopyridine (DMAP) with 12 ml of methyl iodide.
[b] 10 ml of 20% w/w NaOH or 20 ml of 5% w/w DMAP with 5 ml of methyl iodide.
[c] 10 ml of 20% w/w NaOH with 5 ml of methyl iodide.
[d] 5 ml of 20% w/w NaOH with 5 ml of methyl iodide.
[e] Prepared from low molecular weight chitosan.
[f] Each steps was extended to 1.5 h.

2.2.1.2. Reaction step 2. The polymer obtained from the previous step was mixed with 4.8 g of sodium iodide, 10 ml of 20% sodium hydroxide or 20 ml of 5% dimethyl amino pyridine in *N*-methylpyrrolidinone at 60 °C for 20 min. Methyl iodide (12 ml) was added to the mixture and the reaction was carried out for 60 min in the presence of

Table 2
Degree of quaternization and *O*-methylation of the TMC polymers

Polymer no.	DQ (%)	3-OCH_3 (%)	6-OCH_3 (%)
1	29.7	17.9	25.5
2	35.4	35.0	32.3
3	40.3	22.6	31.9
4	77.6	62.2	62.4
5	25.3	17.31	23.7
6	23.1	25.6	21.8
7	20.5	23.7	27.3
8	23.7	25.9	18.1
9	72.1	48.1	43.3
10	43.1	24.7	35.3
11	77.9	66.7	68.7
12	90.5	82.8	98.5

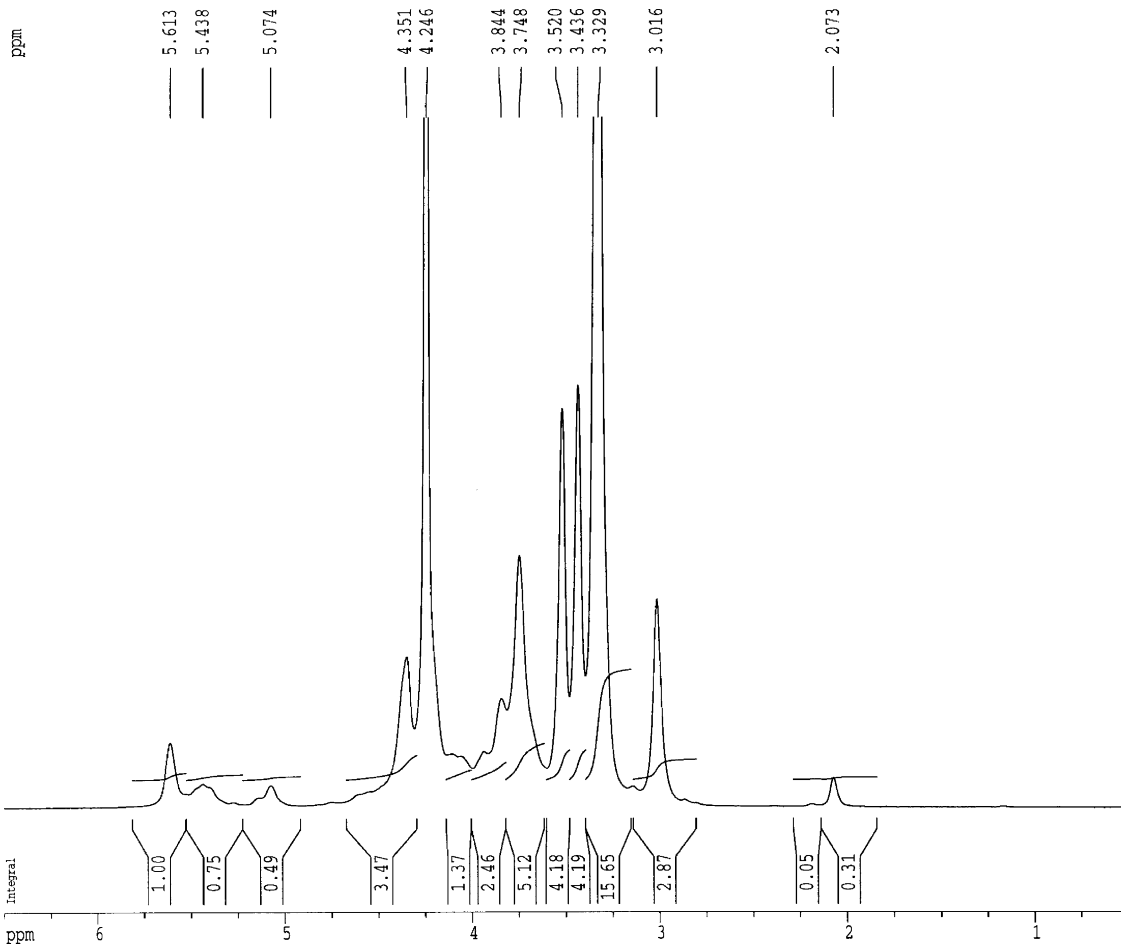

Fig. 1. ^{1}H-NMR spectrum of TMC polymer no. 4 prepared in two reaction steps and one addition step with sodium hydroxide as base.

a Liebig's condenser. The product was precipitated, washed and dried as described above.

2.2.1.3. Addition step. Prior to precipitation of the product from the solution mixture at the end of the previous reaction step, an additional 5 ml of methyl iodide and 10 ml of 20% sodium hydroxide or 20 ml of 5% dimethylaminopyridine were added. The reaction was further continued for another 60 min at 60 °C. The product (*N*-trimethyl chitosan iodide) was precipitated from solution using ethanol and diethyl ether, washed twice with diethyl ether on a glass filter, and finally dried in a vacuum chamber.

2.2.1.4. Ion-exchange step. The products prepared as described above were dissolved in 80 ml of 5% (w/v) sodium chloride solution to exchange the iodide ion with chloride, and were subsequently precipitated by using ethanol and diethyl ether. The products were repeatedly dissolved in 40 ml of water and precipitated with the use of ethanol and diethyl ether to remove the remaining sodium chloride. The final products were dried in the vacuum chamber for at least 12 h prior to further characterization.

2.2.2. Determination of degree of quaternization

^{1}H-Nuclear magnetic resonance (NMR) spectra of the TMC polymers were obtained with an NMR spectrometer (AV-400, Bruker, Switzerland) by dissolving samples of the polymers in D_2O at 80 °C with suppression of the water peak. The degree of quaternization was calculated using data obtained from the ^{1}H-NMR spectra according to the previously described method [9,15] using Eq. (1):

$$\%\mathrm{DQ} = \left[[(\mathrm{CH_3})_3]/[\mathrm{H}] \times 1/9\right] \times 100 \quad (1)$$

where %DQ is the degree of quaternization in percentage, $[(CH_3)_3]$ is the integral of the chemical shift of the trimethyl amino group at 3.3 ppm, and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

The degrees of substitution at the 3- and 6-hydroxyl groups of chitosan were calculated using data from the same ^{1}H-NMR spectra. The chemical shifts of 3- and 6-hydroxymethyl groups were assigned to be at 3.5 and 3.4 ppm, respectively. The degree of *O*-methylation of the polymer was calculated using Eq. (2):

$$\%\mathrm{DOM} = \left[[(\mathrm{CH_3})_3]/[\mathrm{H}] \times 1/3\right] \times 100 \quad (2)$$

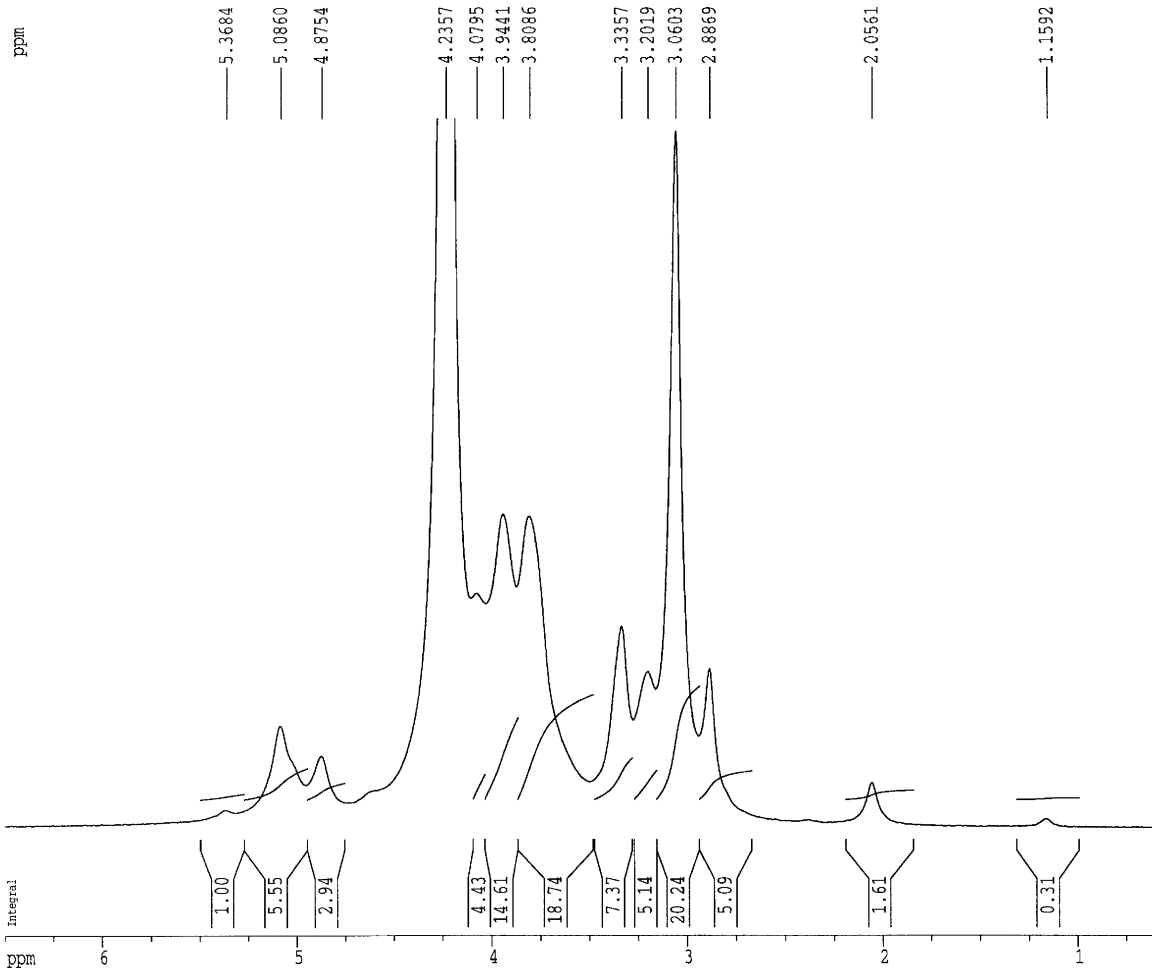

Fig. 2. ^{1}H-NMR spectrum of TMC polymer no. 8 prepared in two reaction steps and one addition step with dimethyl amino pyridine as base.

where %DOM is the degree of *O*-methylation in percentage, [(CH_3)] is the integral of the chemical shift of methyl substitution for 3- or 6-hydroxyl groups at 3.5 ppm or 3.4 ppm, respectively, and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

3. Results and discussion

The calculated degrees of quaternization and *O*-methylation of various TMC polymers are shown in Table 2. Obviously, the degree of quaternization increases significantly with the number of reaction steps when using sodium hydroxide as the base (polymers 1–4). When dimethylaminopyridine was used as the base, the degrees of quaternization of the polymers were lower than when using sodium hydroxide (polymers 5–8). The ^{1}H-NMR spectra of TMC polymers obtained from two reaction steps followed by the last addition step synthesis with sodium hydroxide (polymer 4) and dimethyl amino pyridine (polymer 8) as the base are shown in Figs. 1 and 2, respectively. The degree of quaternization of polymer 1 is 77.6%, whereas that of polymer 8 is 23.7%. These results indicate that the degrees of quaternization of TMC polymers obtained from the processes using dimethylaminopyridine as the base are lower than those using sodium hydroxide as the base. This may be explained by the weaker alkaline properties of dimethylaminopyridine compared to sodium hydroxide.

Similar to the degrees of quaternization, the degrees of *O*-methylation at 3- and 6-hydroxyl groups of chitosan were also found to increase with the increasing number of reaction steps. The degrees of *O*-methylation of TMC polymers obtained from the processes using sodium hydroxide are also higher than those using dimethyl amino pyridine. This can also be explained by the alkaline properties of the base used. However, the degrees of *O*-methylation appeared not to increase significantly when a methylation reaction was conducted in the addition step. Comparisons between polymers 1 and 3 and between polymers 2 and 4 would obviously clarify this. These results can be attributed to the less basic environment in the methylation solution of the addition step when compared to the preceding reaction step.

Highly substituted polymers (degree of quaternization $> 70\%$) could be obtained not only with the high molecular weight chitosan by means of two methylation steps followed

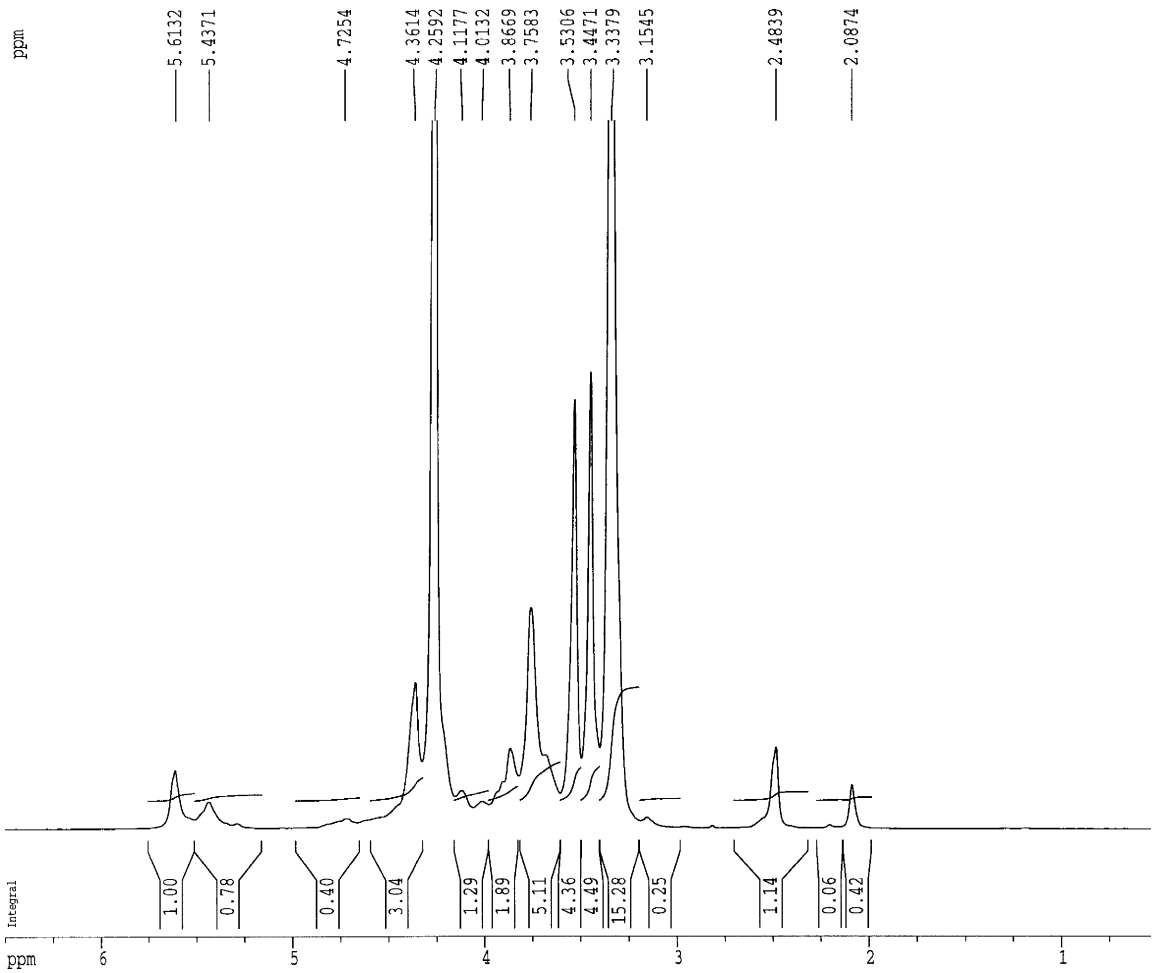

Fig. 3. ^{1}H-NMR spectrum of TMC polymer no. 11 prepared in two reaction steps and one addition step with sodium hydroxide as base (from low molecular weight chitosan).

by the addition step (polymer 4) as described above, but also with the low molecular weight chitosan. The degree of quaternization of the low molecular weight one (polymer 11) was 77.9% and the degrees of methylation of 3- and 6-hydroxyl groups were 66.7 and 68.7%, respectively. The ^{1}H-NMR spectrum of this methylated polymer is depicted in Fig. 3. The degree of quaternization and *O*-methylation of this TMC polymer is similar to that derived from the high molecular weight chitosan.

By extending the duration of all steps from 1 to 1.5 h, a much higher degree of quaternization, as high as 90.5%, could be achieved (see polymer 12). Such conditions, resulted in very high degrees of *O*-methylation at 3- and 6-hydroxyl groups of 82.8 and 98.5%, respectively, as evident from the spectrum shown in Fig. 4. However, such high degrees of *O*-methylation have been reported to decrease the solubility of this substituted polymer [9].

Generally, it is desirable to prepare TMC polymers with a high degree of quaternization but with a low degree of *O*-methylation. This could possibly be accomplished by modification of the methylation processes from two reaction steps and one addition step to one reaction step and two addition steps. The first reaction step was the same as described above. On the other hand, the two addition steps were varied in the amount of 20% sodium hydroxide used, i.e. 10 ml of 20% sodium hydroxide (polymer 9) and 5 ml of 20% sodium hydroxide (polymer 10). Methyl iodide (5 ml) was used for both batches in both addition steps. The degrees of quaternization and methylation at 3- and 6-hydroxyl groups of TMC polymer 9 were found to be 72.1, 48.1 and 43.3%, respectively, while those of polymer 10 were found to be 43.1, 24.7 and 35.3%, respectively. It is clear that the degrees of substitution of TMC polymer 9 are higher than those of the polymer 3 and polymer 10, whose degrees of substitution are almost similar. This may probably be due to the stronger alkaline environment in the reaction mixture of the polymer 9 than that of the polymers 3 and 10.

The synthesis procedure using one methylation reaction step with subsequent multiple addition steps is much more

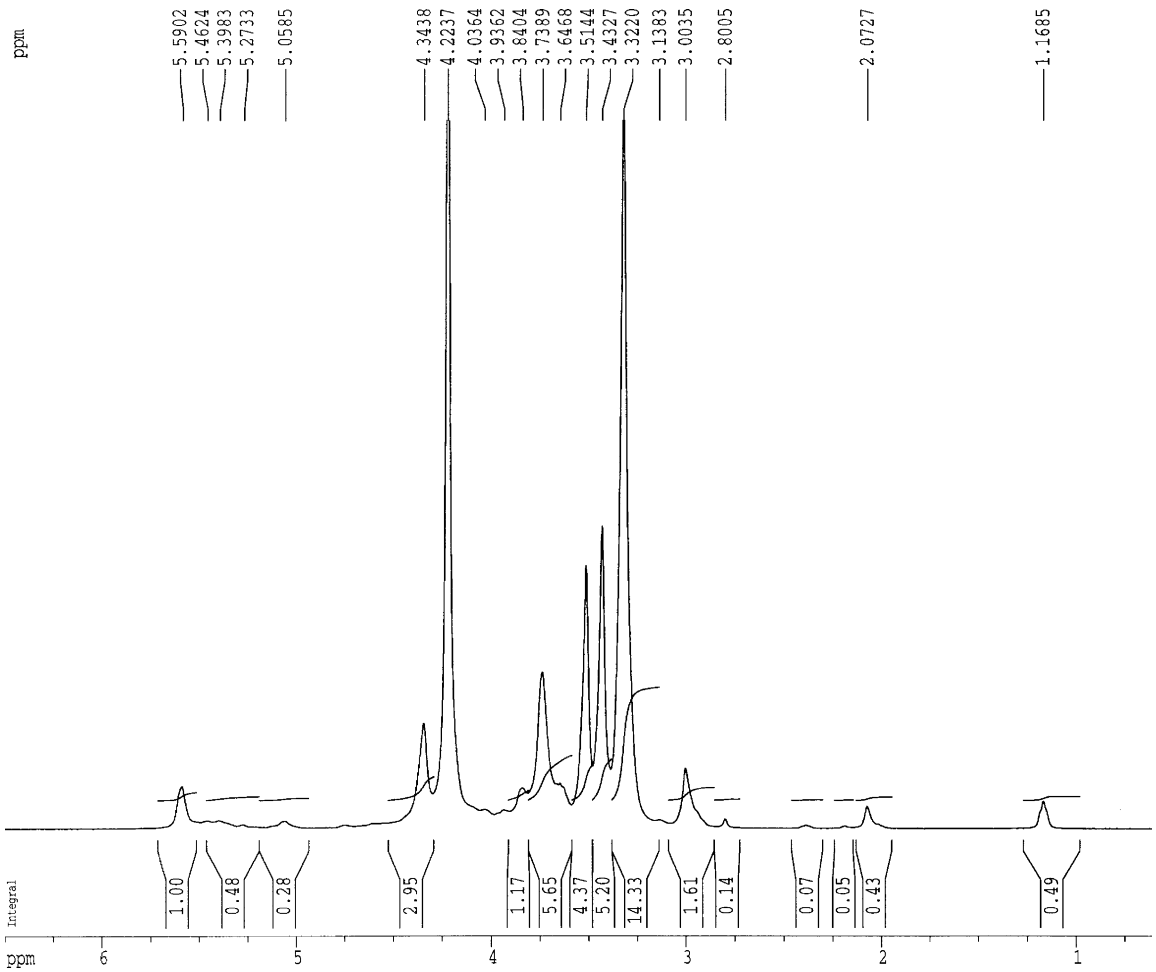

Fig. 4. ^{1}H-NMR spectrum of TMC polymer no. 12 prepared in two reaction steps and one addition step with sodium hydroxide as base; each step was 1.5 h.

convenient to prepare TMC than multiple reaction steps. This not only results in higher yields (data not shown), but also in the reduction of certain in-process procedures such as precipitation, centrifugation, and drying of the intermediate product.

4. Conclusions

N-Trimethyl chitosan derivatives with controllable degrees of substitution were prepared by alterations of the number of the reaction and addition steps, including the type and concentration of base used in the reaction mixture. Furthermore, a higher degree of quaternization of the TMC polymer (more than 90%) was obtained. However, this also resulted in high degrees of *O*-methylation, which would decrease the aqueous solubility of the polymer. The synthesis of TMC polymers with a high degree of quaternization but a low degree of *O*-methylation requires several steps of the methylation process.

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