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Short communication

N-selective '*one pot*' synthesis of highly *N*-substituted trimethyl chitosan (TMC)

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ABSTRACT

Trimethylated chitosan (TMC) is a well-studied *N*-quaternary chitosan derivative that is promising for various pharmaceutical applications. The most commonly-used procedure today to obtain these derivatives is based on the reaction with methyl iodide as reagent and *N*-methylpyrrolidone as solvent. This procedure is not *N*-selective and significant *O*-methylation is observed in highly trimethylated material. The *O*-methylation reduces solubility of the TMC derivatives and therefore limits the use of highly *N*-quaternised products. Here we report a study of a '*one-pot*' synthesis procedure based on a new solvent system. We obtained readily-soluble TMC derivatives with a degree of *N*-quaternisation between 0.81 and 0.88 without any *O*-methylation. The uniformity of these compounds was confirmed with 1D, 2D NMR and IR spectroscopy.

1. Introduction

N-trimethyl chitosan (TMC) is one of the most commonly-studied chitosan derivatives. It was developed to improve the properties of chitosan and to overcome the main barrier in the use of chitosan in pharmaceutical applications, that is, its poor aqueous solubility at physiological pH. TMC has a fixed positive charge on the quaternary amino-group and the derivative is therefore highly soluble both in neutral and basic environments. The derivative has shown promising results when studied as a drug delivery system (Atyabi, Majzoob, Iman, Salehi, & Dorkoosh, 2005; Hamman, Stander, & Kotze, 2002; Jonker, Hamman, & Kotze, 2002), gene delivery material (Borchard, 2001) and antibacterial agent (Rúnarsson et al., 2007).

The commonly-used method for the synthesis of TMC was reported by Domard, Rinaudo, and Terrassin (1986) and further developed by Le Dung, Milas, Rinaudo, and Desbrieres (1994). Materials obtained by this procedure were fully characterised by Sieval et al. (1998). The Domard methylation method uses methyl iodide (MeI) as reagent, KI as catalyst and NaOH as base with *N*-methylpyrrolidone (NMP) as solvent. Some modifications of this synthesis procedure have been reported (Hamman & Kotze, 2001; Sieval et al., 1998) and used in several studies (Atyabi et al., 2005; Curti, de Britto, & Campana, 2003; Hamman et al., 2002; Kotze et al., 1998; Snyman, Hamman, & Kotze, 2003). Polnok, Borchard, Verhoef, Sarisuta, and Junginger (2004) investigated the effect of different bases on the degree of *N*-quaternisation. The study showed that NaOH is the most efficient base to give a high degree of *N*-quaternisation but the reaction was always accompanied with *O*-methylation, as Sieval et al. (1998) had previously shown. The *O*-methylation reduces the solubility of the TMC derivatives and therefore limits the use of highly *N*-quaternised products (Sieval et al., 1998). The main disadvantage of the Domard method is that selectivity for the amino-group is low and the substitution degree of the material obtained also appears to be dependent on the starting material and exact method used in the lab (Curti et al., 2003; Hamman & Kotze, 2001; Jonker et al., 2002; Kotze et al., 1998; Sieval et al., 1998; Snyman, Hamman, Kotze, Rollings, & Kotze, 2002; Suzuki, Oda, Shinobu, Saimoto, & Shigemasa, 2000). Another disadvantage of this procedure is that the compound needs to be worked up and the procedure repeated at least twice in order to obtain a high degree of *N*-quaternisation. Other synthetic strategies have been developed (Muzzarelli & Tanfani, 1985) to produce TMC derivatives but are not as widely used as the Domard reaction. De Britto and Assis (2007) recently suggested a new approach of producing TMC derivatives, using dimethylsulfate as reagent. Thus there is still an interest in developing a new synthesis method to obtain a more uniform material than the Domard methylation produces, and various other approaches have been tried. Hovewer, a suitable method to synthesise highly quaternised TMC derivatives without significant *O*-methylation is still lacking.

We have observed that using DMF/H_2O mixture as a solvent and performing the reaction without the aid of a catalyst can significantly reduce *O*-methylation. The degree of *N*-quaternisation,

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however, was always low in the materials obtained by the reported procedure (Rúnarsson et al., 2007). Here we report further development of this '*one pot*' *N*-selective synthesis procedure, based on this solvent system, to produce highly *N*-quaternised TMC derivatives. Three different chitosan starting materials were used. The compounds were analysed by use of 1D and 2D NMR and FT/IR spectroscopy to determine the relative degree of *N*-substitution and *O*-methylation.

2. Experimental section

2.1. Materials

Genis EHF (Iceland) provided the starting materials; chitosan I (average MW 8.1 kDa as determined with end reducing assay (Miller, 1959) and with a degree of *N*-acetylation of 0.05), chitooligomer II (average MW 775 Da determined with MALDI TOF, degree of polymerisation (DP) of 1–9 sugar units (Bahrke et al., 2002) and with degree of *N*-acetylation of 0.52), and then chitooligomer III (average MW ~775 Da and DP of 3–9 with *N*-acetylation of 0.18 (Rúnarsson et al., 2007)). All other chemicals used were commercially available and used as received. Dialysis membrane (Spectrapore@MW cutoff 500 and 3500 Da) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, USA).

2.2. Characterisation

All ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC measurements were done with Bruker AVANCE 400 (Bruker Biospin GmbH, Karlsruhe, Germany). For the ^{1}H and ^{13}C NMR the equipment operated at 400.13 and 100.61 MHz, respectively at 298 K. The *N*-acetyl peak was used as internal reference with D_2O or D_2O/DCl as solvents. The concentration of the samples was 40 mg/mL. The measurements were done without water suppression. ^{1}H–^{1}H COSY measurements were carried out in magnitude mode. The data matrix was 128 × 2 K and the spectra width was 2315 Hz. ^{13}C-^{1}H HSQC experiments were carried out in the phase-sensitive mode by means of the Echo/Antiecho gradient selection. The data matrix was 256 × 1K and the spectra width was 2815 Hz. The degree of substitution (ds) for the TMC derivatives were calculated by use of a previously-described method (Rúnarsson et al., 2007). All peaks were confirmed by use of COSY and HSQC correlation for the protons and carbons of the molecules. IR-spectras were recorded with AVATAR 370 FT-IR (Thermo Nicolet Corporation, Madison, USA).

2.3. The synthesis

The general procedure is based on Rúnarsson et al. (2007). The starting chitosan material (Scheme 1) was dissolved in DMF:H_2O [50:50] (v/v). Sodium hydroxide (3 equivalents (eq)) was added in equal portions with 5 min interval and then 6 eq MeI were added to the solution. Equivalent quantities were calculated on the basis of the number of free amino groups in the starting material. The reaction mixture was vigorously stirred for 48 h at room temperature (RT) before next addition of reagents (Scheme 1). This was repeated up to four times. When worked up the compounds were precipitated, ion exchanged, dialysed and freeze-dried like previously described by Rúnarsson et al. (2007).

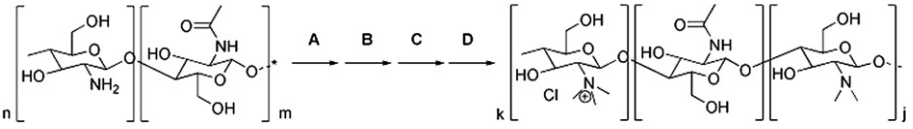
^{1}H NMR analysis of TMC I/II/III 21 C and D (400 MHz, D_2O δ ppm 2.01 (s, C**H**$_3$C=O), 2.50 (3.00 using D_2O/DCl (s, N–(C**H**$_3$)$_2$)), 2.60 (3.37 using D_2O/DCl (m, **H**-2 N–(CH$_3$)$_2$), 3.30 (s, N–(C**H**$_3$)$_3$), 3.5–4.0 (m, **H**-2, **H**-5, **H**-6, **H**-6́ (N–(CH$_3$)$_3$), **H**-3-**H**-6′ (N–(CH$_3$)$_2$), **H**-2-**H**-6′ (GluNAc), 4.30 (bs, **H**-4 N–(CH$_3$)$_3$), 4.40 (bs, **H**-3 N–(CH$_3$)$_3$), 4.9 (bs, **H**-1 N–(CH$_3$)$_2$), 5.3 (bs, **H**-1 GluNAc), 5.4 (bs, **H**-1 N–(CH$_3$)$_3$). ^{13}C NMR δ ppm 19 (**C**H$_3$C=O), 41 ((CH$_3$)$_2$), 54 ((CH$_3$)$_3$), 61 (C-6), 68 (C-3), 76 (C-5), 77 (C-4), 79 (C-2), 97 (C-1), and 173 (C=O). FTIR (KBr):ν 3423 (br, OH/NH), 2930 (m, C-H), 1625 (vs, C=O amide I) and 1476 (tertiary N–CH$_3$) cm^{-1}.

3. Results and discussions

It has been reported that the degree of *N*-quaternisation of TMC can be increased by repeating the reaction steps in the Domard synthetic method (Domard et al., 1986; Le Dung et al., 1994; Polnok et al., 2004; Sieval et al., 1998), but this will also increase the *O*-methylation considerably. In our previous study (Rúnarsson et al., 2007), we observed very little *O*-methylation by using the relatively inexpensive solvent mixture of DMF/H_2O. Now we have taken this method further by repeated additions of reagents until the material was nearly fully *N*-quaternised (Scheme 1). The effect of reaction time and temperature on the selectivity of reaction was also investigated.

When analysing the substitution degree of the Domard method (Fig. 1B) we can see that the *N,N,N*-trimethylation increases rapidly in this two-step synthesis but the degree of *N,N*-dimethylation starts to drop accordingly in the final step. What is interesting is that the *O*-methylation increases in correlation with *N,N,N*-trimethylation. When DMF/H_2O was used as solvent (Fig. 1A, C and D) the two-step procedure reported in our previous publication (Rúnarsson et al., 2007) yielded considerable *N,N*-dimethylation with up to 0.60 ds and without any detectable *O*-methylation but when *N,N*-dimethylation is up to this level by using the Domard reaction *O*–methylation is significant. To investigate the *N*-selectivity of our *'one pot'* reaction further we added two additional steps to our *'one pot'* synthesis method. In Fig. 1A, C and D it is clear that the *N,N,N*-trimethylation increases in the same manner as the Domard reaction and again the *N,N*-dimethylation decreases in the same ratio, showing that almost all the free amino groups have been methylated. In contrast with the Domard reaction the hydroxyl groups were not methylated. As seen in Fig. 1 this was the case for all three starting materials, indicating that the method is not dependent on the starting material and resulting in a final degree of *N*-quaternisation between 0.81–0.92 (calculated on the basis of the number of free amino groups). Increasing the temperature 50 or 75 °C in the *'one pot'* reaction did not improve the results and thus it appears that the relative rate of hydrolysis of MeI is increased more than the rate of *N*-methylation therefore lowering the *N*-quaternisation.

At higher temperature the reactivity of the MeI will increase, generally, making the risk of *O*-methylation higher (de Britto & Assis, 2007). The DMF/H_2O solvent system on the other hand



Scheme 1. Synthetic route of the methylated chitosan derivatives. Reagents and conditions: chitosan I, II or III used as starting material, '*one pot*' additions: A, B, C and D; DMF/water, 3 eq NaOH, 6 eq MeI, RT, 48 h.

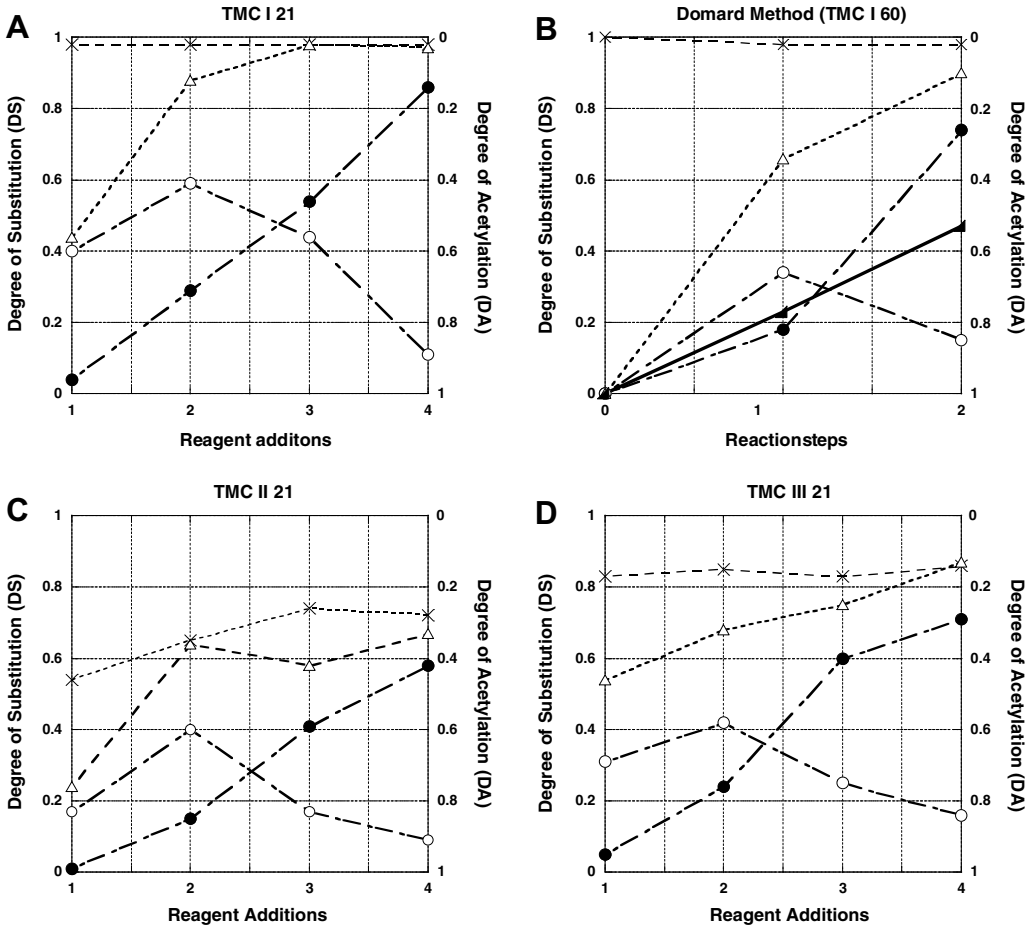


Fig. 1. The degree of substitution after each addition of reagents as determined by NMR of the isolated product were *N,N,N*-trimethylation (●), *N,N*-dimethylation (○), *N*-acetylation (×), total *N*-substitution (△) and *O*-methylation (◢) is shown for: (A) the TMCI reaction with (four additions), (B) the Domard reaction, starting material chitosan I, (C) the TMCII reaction (four additions) and (D) the TMCIII reaction (four additions). *Note: N*-monomethylation of the reactions is not shown on the graphs. *N*-monomethylation ranged between 0 and 0.14 after the first two additions of reagents but was not present after three and four additions. The data points for the first two steps are based on data previously published compounds (Rúnarsson et al., 2007).

seems to be the key factor in this reaction. DMF is a common solvent for SN2 reactions, hence by using aqueous conditions the reactivity can decrease 10^5 fold (Chandrasekhar & Jorgensen, 1985). Here, the ratio of DMF/water seems to lowers the reactivity of the hydroxyl group enough to keep the *O*-methylation down. It is also noted that precipitation increases after each addition of reagents, indicating that the highly *N*-quaternised material precipitates before *O*-methylation takes place. High selectivity was achieved on all three chitosan starting materials. When the Domard method is used the results can vary depending on starting materials. A one-step reaction gave Kotze et al. (1998) a 0.13 degree *N,N,N*-trimethylation but no *O*-methylation was reported. A one-step reaction gave Sieval et al. (1998) a 0.35 degree *N,N,N*-trimethylation with some *O*-methylation but they did not calculate the *O*-substitution degree. After three steps a 0.43 *N*-quaternised material with *O*-methylation (Hamman and Kotze, 2001; Snyman et al., 2002) has been reported but Sieval and co-workers obtained a 0.85 *N*-quaternised substance which was almost fully *O*-methylated.

The chemical structure of the new material produced from chitosan I and with four additions of reagents was confirmed by use of H^1–H^1 COSY and H^1–C^{13} HSQC NMR spectroscopy (Fig. 2). In the 2-D spectras we can see three monomeric units, *N,N,N*-trimethyl- (0.86 ds), *N,N*-dimethyl- (0.11 ds) and *N*-acetyl chitosan (0.02 ds). The H-1, H-2, H-3, H-4 and methyl peaks are well resolved in the COSY and HSQC spectras. Hence when analysing TMC materials formed by the Domard method from our previous study (Rúnarsson et al., 2007) we obtain a very heteromeric material where protons H-2–H-6 form a broad multiplet in the range 3.6–4.6 ppm representing at least five monomeric units (*N,N,N*-trimethyl- and *N,N*-dimethyl chitosan with *O*-6 and/or *O*-3 methylation and more). All the possible monomeric units have a slightly different shift depending on the substitutients (Fig. 2), making the spectra more complex. The aqueous solubility of the new product with *N,N,N*-trimethylation between 0.81–0.88 was very good, making it possible to dissolve at least 80 mg per mL water after ion exchange, but the Cl^- form of the polymer derivatives had much better solubility than the corresponding I^- form. Thus it was difficult to determine the structure by NMR spectroscopy before ion exchange. One of the good qualities of the new highly *N*-quaternised material was its uniformity, which made it easier to obtain good quality 2D NMR spectra and simplified the characterisation. The uniformity therefore makes it possible to eliminate all doubts about the actual structure of the compound when used for pharmaceutical applications.

4. Conclusion

The present synthetic procedure enables us to make a highly uniform and almost fully *N*-quaternised TMC material without *O*-methylation. The degree of quaternisation was not dependent on

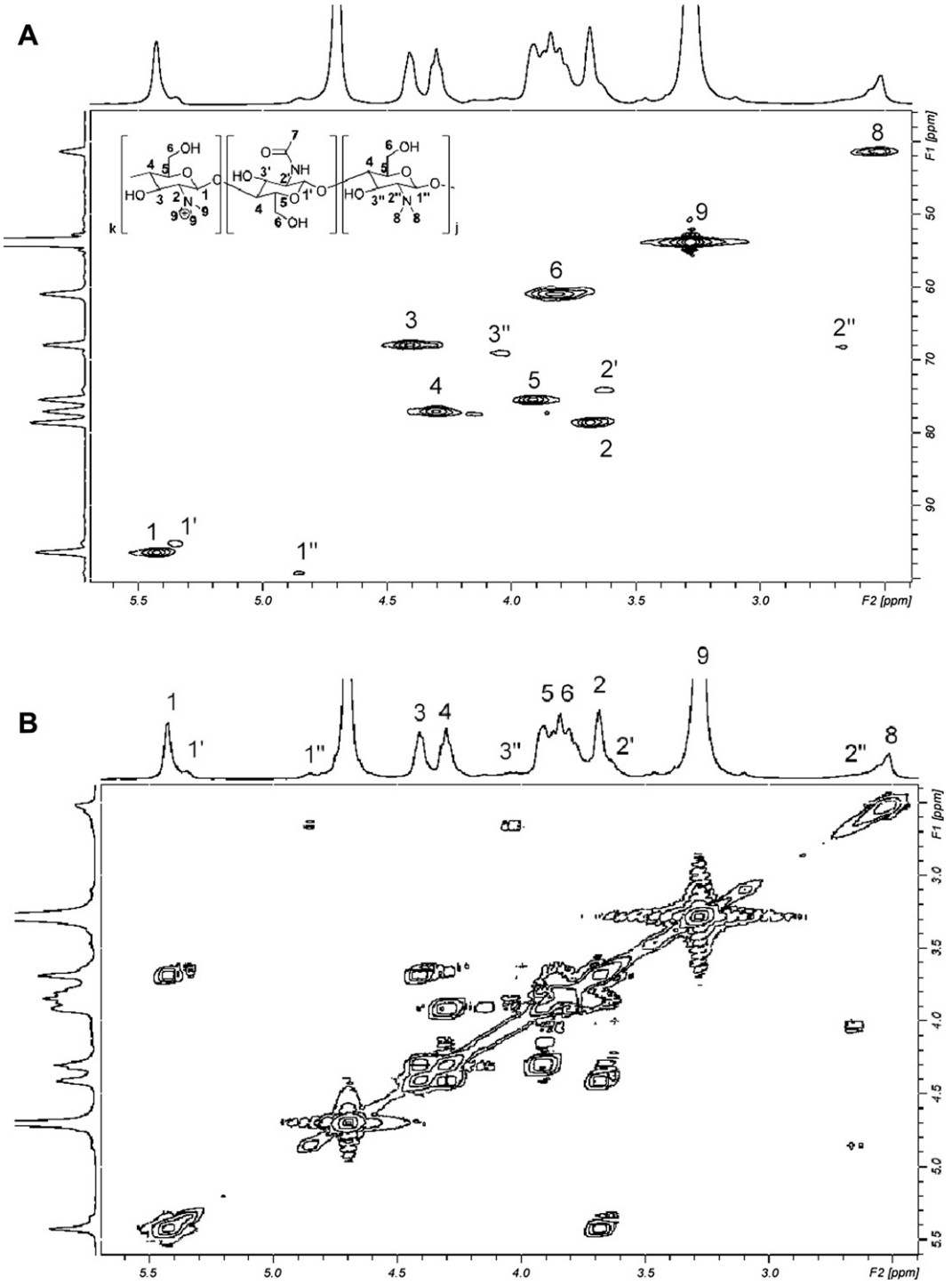


Fig. 2. COSY and HSQC NMR spectras of TMCI 21D after four additions of reagent.

the starting material. The uniform product was fully characterised by 2D NMR.

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