Carbohydrate Polymers 86 (2011) 1451–1460

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis of *N*,*N*,*N*-trimethyl chitosan homopolymer and highly substituted *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives with the aid of di-*tert*-butyldimethylsilyl chitosan

Berglind E. Benediktsdóttir[a], Vivek S. Gaware[a], Ögmundur V. Rúnarsson[a,b], Sigríður Jónsdóttir[c], Knud J. Jensen[d], Már Másson[a,*]

[a] Faculty of Pharmaceutical Sciences, School of Health Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavík, Iceland
[b] Center for Analysis and Synthesis, Department of Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden
[c] Science Institute, University of Iceland, IS-107 Reykjavik, Iceland
[d] Faculty of Life Sciences, Department of Basic Sciences and Environment, University of Copenhagen, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 12 May 2011
Received in revised form 30 May 2011
Accepted 4 June 2011
Available online 13 June 2011

Keywords:
Chitosan
tert-Butyldimethylsilyl
Deprotection
Stepwise reductive alkylation
Quaternary chitosan
Trimethyl chitosan

ABSTRACT

A highly chemoselective strategy for the synthesis of *N*,*N*,*N*-trimethyl chitosan (TMC) homopolymer and highly substituted *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives was achieved using di-*tert*-butyldimethylsilyl-3,6-*O*-chitosan (di-TBDMS chitosan) as a precursor. The influence of different solvents, reagents and other reaction conditions on the reduction, trimethylation and quaternization of these di-TBDMS chitosan derivatives was studied. Products were characterized by NMR after each step. Di-TBDMS chitosan was reacted with methyl iodide in NMP, giving a 100% substituted TMC with the trimethyl group appearing at 3.35 ppm in ^{1}H NMR spectrum. *N*-Propyl-, *N*-butyl- and *N*-hexyl-*N*,*N*-dimethyl chitosan derivatives were synthesized by stepwise reductive alkylation of di-TBDMS chitosan, followed by quaternization with dimethyl sulfate in dichloromethane, giving 65–72% substituted *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives under optimized conditions. Analysis of these water-soluble chitosan derivatives by FT-IR, ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC NMR enabled detailed structural characterization. All peaks could be assigned to *N*-modification, showing the selectivity of the di-TBDMS protection.

1. Introduction

Chitosan is a natural β-(1 → 4) linked glucosamine polymer, usually derived from chitin by deacetylation. Chitosan has many interesting properties which are useful in biological applications, including antimicrobial activity (Kong, Chen, Xing, & Park, 2010), and it has been studied as gene delivery agent (de la Fuente, Csaba, Garcia-Fuentes, & Alonso, 2008) and permeation enhancer for macromolecular drugs (Mourya & Inamdar, 2009). Furthermore, it is biodegradable and considered to be safe in non-parental dosage formulations (Baldrick, 2010). However, one of the main disadvantages of chitosan for many potential applications is its poor aqueous solubility at physiological pH. Therefore, synthetic strategies have been developed to modify the polymer backbone, in order to enhance aqueous solubility and biological activity (Alves & Mano, 2008). This can be achieved for example by trimethylating the free amino group (Runarsson, Holappa, Jonsdottir, Steinsson, & Masson, 2008), producing a derivative with permanent positive charge that is highly soluble independent of pH.

N,*N*,*N*-Trimethyl chitosan (TMC) is usually synthesized by dispersing chitosan in NMP with methyl iodide (CH_3I) in the presence of NaOH, either directly (Domard, Rinaudo, & Terrassin, 1986; Sieval et al., 1998) or by first forming *N*,*N*-dimethyl chitosan by reductive alkylation, followed by additional methylation using CH_3I (Muzzarelli & Tanfani, 1985). These reactions are not selective and produce a heterogenous mixture of *N*-monomethyl-, *N*,*N*-dimethyl-, *N*,*N*,*N*-trimethyl- and *O*-methyl chitosan (Domard et al., 1986; Ledung, Milas, Rinaudo, & Desbrieres, 1994; Muzzarelli & Tanfani, 1985; Sieval et al., 1998). Although modifications of this reaction have given higher degrees of trimethylation without *O*-methylation (Runarsson, Holappa, et al., 2008; Verheul et al., 2008), the resulting material is only partially trimethylated. Therefore, there is still a need for a selective approach to produce fully homogenous TMC materials.

Protecting groups have been used in chitosan chemistry to enable selective *N*-modifications and increase their solubility in organic solvents (Kurita, 2006). Although the most common strategy is to use a triphenylmethyl (trityl) group for *O*-protection

* Corresponding author. Tel.: +354 525 4463; fax: +354 525 4071.
E-mail address: mmasson@hi.is (M. Másson).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.06.007

(Holappa et al., 2004; Nishimura, Kohgo, Kurita, & Kuzuhara, 1991; Runarsson et al., 2007), the trityl group can only be introduced at the *O*-6 position, leaving the *O*-3 group unprotected. Furthermore, three synthesis steps are needed and the protection step must be performed at a temperature of approximately 100 °C. An alternative protection strategy is to use silyl groups to protect the alcohols. Silyl ethers are widely used as selective protective agents for hydroxyl moieties (Wuts & Greene, 2007) and protection of hydroxyl groups in chitosan with trimethylsilyl (TMS) groups has been investigated (Kurita, Hirakawa, Kikuchi, Yamanaka, & Yang, 2004). Although TMS protection provides somewhat improved solubility in organic solvents, the approach is not chemoselective and various degrees of substitution for the *O*-3, *O*-6 and amino group have been reported (Kurita et al., 2004). In addition, under acidic conditions, the TMS group is one of the most labile silyl groups (Wuts & Greene, 2007), which is a disadvantage when reaction conditions are slightly acidic.

The *tert*-butyldimethylsilyl (TBDMS) protection group is stable under a variety of reaction conditions, but can be easily removed under strongly basic or moderate acidic conditions without affecting other functional groups (Wuts & Greene, 2007). Recently we developed a synthetic strategy using TBDMS to protect hydroxyl groups in chitosan, in which the TBDMS group is introduced to the mesylate salt of chitosan in a single step, at room temperature. This synthetic method results in apparently 100% *O*-protected chitosan material, with both the *O*-3 and *O*-6 groups protected (di-TBDMS chitosan) (Runarsson, Malainer, Holappa, Sigurdsson, & Masson, 2008; Song, Gaware, Runarsson, Masson, & Mano, 2010). Furthermore, the resulting protected polymer has good solubility in a wide variety of organic solvents, such as DCM, NMP and EtOH (Runarsson, Malainer, et al., 2008), allowing for modification of the amino group on the chitosan backbone under conditions commonly used in organic chemistry. Previously, *N*-acylation of TBDMS-chitosan has been reported (Rúnarsson et al., 2010), showing that this material can also be used as precursor for complete and highly selective *N*-acylation.

Here, we report a detailed investigation of different *N*-alkylations of di-TBDMS chitosan. The aim of this study was to use di-TBDMS chitosan to obtain fully substituted *N*,*N*,*N*-trimethyl chitosan and highly substituted *N*-alkyl-*N*,*N*-dimethyl chitosan, avoiding *O*-methylation. Reactions were carried out in a stepwise manner, with intermediate products characterized by FT-IR, ^{1}H- and COSY NMR. The final products, *N*,*N*,*N*-trimethyl chitosan and *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives, were characterized in detail by FT-IR, ^{1}H and ^{13}C NMR and ^{1}H–^{1}H COSY- and ^{1}H–^{13}C HSQC NMR.

2. Experimental

2.1. Materials

Chitosan polymer HCl (G020102-1) was used with an average M_W of 8.1 kDa, as determined by end-reducing assay (Miller, 1959), and 8.5 kDa, as determined by viscometric methods (Ottoy, Varum, & Smidsrod, 1996). The degree of acetylation was 0.03, determined by ^{1}H NMR. Chitosan was obtained by donation from Genis EHF, Iceland. All other chemicals (obtained from Sigma–Aldrich®) were reagent grade or higher and used as received, with the exception of DMSO, DCM and NMP, which were stored over molecular sieves overnight before use. Dialysis membranes (RC, Spectra/Por, M_W cutoff 3500 Da) and Float-A-Lyzers (Spectra/Por, M_W cutoff 3.5–5 kDa, 5 ml sample volume) were purchased from Spectrum® Laboratories Inc. (Rancho Dominguez, USA).

2.2. Characterization and calculations

2.2.1. Equivalents

Equivalent quantities of reagents were calculated on the bases of one glucosamine unit.

2.2.2. NMR analysis

^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC spectra were recorded with either a Bruker Avance 400 instrument operating at 400.13 and 100.61 MHz at 300 K, or a Bruker Avance 300 operating at 300.13 MHz and 75.47 MHz at 300 K. $CDCl_3$, d_6-DMSO and D_2O were used as NMR solvents. Acetone was used as reference in D_2O NMR spectra for final compounds: proton (δ 2.22 ppm) and carbon (δ 30.9 ppm). Spectra were measured without water suppression. Sample concentrations ranged from 10 to 25 mg/ml.

2.2.3. Calculations of substitution degrees

Integral values from ^{1}H NMR were used to determine the degree of substitution (DS) for *N*-substituted chitosan derivatives (Appendix A).

2.2.4. FT-IR analysis

Samples were mixed thoroughly with KBr and then pressed into pellets using a Specac compressor (Specac Inc., Smyrna, USA). FT-IR measurements were performed with an AVATAR 370 FT-IR instrument (Thermo Nicolet Corporation, Madison, USA) with 32 scans and resolution of 4 cm^{-1}.

2.3. Synthesis

2.3.1. Chitosan mesylate (**1**)

Synthesized according to a published procedure (Runarsson, Malainer, et al., 2008; Song et al., 2010). FT-IR (KBr): ν 3376 (O–H), 2880 (C–H), 1677 (C=O amide I), 1586 (C=O amide II), 1384 (C–H), 1156–1074 (C–O) cm^{-1}. ^{1}H NMR (400 MHz, D_2O): δ 2.10 (CH_3, GlcNAc), 2.84 (CH_3S), 3.21 (H-2), 3.7–4.1 (H-3, H-4, H-5, H-6), 4.9 ppm (H-1, partially overlapped with the water peak).

2.3.2. 3,6-O-di-TBDMS-chitosan (di-TBDMS chitosan, **2**)

Synthesized according to a published procedure (Runarsson, Malainer, et al., 2008; Song et al., 2010). FT-IR (KBr): ν 3469 (N–H), 2957–2859 (C–H), 1705 (C=O amide I), 1573 (C=O amide II), 1474 (C–H), 1390–1362 (C–H), 1109–1050 (C–O), 836–776 (Si–CH_3) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.05 ((CH_3)$_2$Si), 0.89 ((CH_3)$_3$C), 2.71 (H-2), 3.33 (H-5), 3.50 (H-3), 3.68 (H-4), 3.84–3.89 (H-6), 4.30 (H-1), 8.12–8.20 ppm (NH_2).

2.3.3. N,N,N-Trimethyl-di-TBDMS chitosan (**3**)

Di-TBDMS chitosan (**2**) (1.42 g, 3.6 mmol di-TBDMS-glucosamine units) was dissolved in dry NMP (20 ml). Cesium carbonate (Cs_2CO_3, 4.63 g, 14.2 mmol, 4 equiv.) was added and the solution stirred for 3 h, followed by addition of CH_3I (1.11 ml, 17.8 mmol, 5 equiv.). The reaction was carried out in a closed reaction vial at 50 °C. *Note*: extreme caution must be taken when handling CH_3I; a special chemical vapor-proof mask should be used when appropriate. After 24 and 48 h, additional CH_3I (1.11 ml, 17.8 mmol, 5 equiv.) was added to the brown reaction solution, and the reaction was carried out for a total of 96 h. The solution was then dialyzed against deionized water for 4 days and freeze-dried overnight, giving a dark red product. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.01–0.31((CH_3)$_2$Si), 0.86–0.90 ((CH_3)$_3$C), 3.64 ppm (N^+–(CH_3)$_3$ partially overlapped by H-1 to H-6). Yield: 1.856 g (93%).

2.3.4. N,N,N-Trimethyl chitosan (**4**)

The trimethylated protected product (**3**) (1.856 g, 3.30 mmol *N*,*N*,*N*-trimethyl-di-TBDMS-glucosamine units) was deprotected

by treatment with 1 M tetrabutylammonium fluoride (TBAF) solution in NMP (10 ml) at 50 °C for 72 h. The resulting solution was dialyzed for 4 days against deionized water, then ion-exchanged against 5% aq. NaCl (w/v) overnight followed by dialysis against deionized water for 2 days. The resulting compound was then freeze-dried, giving deprotected, trimethylated chitosan. In cases where NMR analysis showed that the trimethylated chitosan was not fully deprotected, the deprotection process was repeated. The material was then dissolved in 5% aq. NaCl (10 ml) and shaken overnight. The solution was centrifuged and the liquid decanted from grey precipitates, dialyzed for 3 days against deionized water and freeze-dried overnight, resulting in white and fluffy trimethylated chitosan. FT-IR (KBr): ν 3422 (O–H), 2923 (C–H), 1653 (C=O amide I), 1488 (C–H), 1051 (C–O) cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 2.08 (CH_3, GlcNAc), 3.35 (N^+–$(CH_3)_3$, H-14), 3.75 (H-2), 3.90 (H-6), 3.99 (H-5), 4.36 (H-4), 4.47 (H-3), 5.49 ppm (H-1). ^{13}C NMR (300 MHz, D_2O): δ 54.6 (C-14), 61.6 (C-6), 68.7 (C-3), 76.2 (C-5), 77.9 (C-4), 79.4 (C-2), 97.2 ppm (C-1). Yield: 238 mg (29%).

*2.3.5. General procedure for N-alkylimine-di-TBDMS chitosan (**5**)*

Compound **2** (1.50 g, 3.80 mmol di-TBDMS-glucosamine units) was dissolved in DCM (15 ml). Triethylamine (0.575 ml, 4.1 mmol, 1.1 equiv.) was added and the solution stirred for 15 min. Then, 5 equivalents of the corresponding aldehyde (propyl, butyl or hexyl) was added to the solution, along with molecular sieves, and stirred overnight at 45 °C in a closed reaction vial. After 24 h of reaction, another 5 equivalents of aldehyde were added and the reaction carried out for a total of 96 h at 45 °C. The solvent was then partially evaporated under reduced pressure and the resulting material precipitated in ACN (150 ml). The white solid was filtered off and washed with ACN (3 × 30 ml), allowed to air-dry and then further dried in a vacuum oven at 40 °C overnight, giving an off-white compound. ***N*-Propylimine-di-TBDMS chitosan (5a)**: FT-IR (KBr): ν 3462 (N–H), 2957–2858 (C–H), 1707 (C=O, amide I), 1675 (N=C), 1473 (C–H), 1390–1362 (C–H), 1257 (C–N), 1116–1052 (C–O), 836–777 (Si–CH_3) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.05 ((CH_3)$_2$Si), 0.86 ((CH_3)$_3$C), 1.06 (H-9), 2.23 (H-8), 2.82 (H-2), 3.13–4.21 (H-3, H-4, H-5, H-6), 4.50 (H-1), 7.50 ppm (H-7). Yield: 1.276 g (76%) starting from 1.200 g of (**2**). ***N*-Butylimine-di-TBDMS chitosan (5b)**: FT-IR (KBr): ν 3467 (N–H), 2958–2858 (C–H), 1705 (C=O, amide I), 1673 (N=C), 1473 (C–H), 1389–1362 (C–H), 1256 (C–N). 1113–1056 (C–O), 837–777 (Si–CH_3) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.05 ((CH_3)$_2$Si), 0.86 ((CH_3)$_3$C and H-10) 1.53 (H-9), 2.19 (H-8), 2.81 (H-2), 3.08–4.21 (H-3, H-4, H-5, H-6), 4.52 (H-1), 7.48 ppm (H-7). Yield: 1.433 g (84%) starting from 1.500 g of (**2**). ***N*-Hexylimine-di-TBDMS chitosan (5c)**: FT-IR (KBr): ν 3480 (N–H), 2958–2858 (C–H), 1704 (C=O, amide I), 1674 (N=C), 1473 (C–H), 1389–1362 (C–H), 1257 (C–N), 1117–1061 (C–O), 837–777 (Si–CH_3) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.05 ((CH_3)$_2$Si), 0.86 ((CH_3)$_3$C and H-12), 1.32 (H-10, H-11), 1.51 (H-9), 2.19 (H-8), 2.79 (H-2), 3.12–3.90 (H-3, H-4, H-5, H-6), 4.45 (H-1), 7.44 ppm (H-7). Yield: 1.169 g (98%) starting from 1.000 g of (**2**).

*2.3.6. General procedure for N-alkyl-di-TBDMS chitosan (**6**)*

The corresponding imine (2.13 mmol *N*-alkylimine-di-TBDMS-glucosamine units, compounds **5a**–**5c**) was dissolved in DCM (20 ml). Sodium triacetoxyborohydrate (STAB-H, 1.81 g, 8.5 mmol, 4 equiv.) was added to the solution, followed by addition of AcOH (488 μl, 8.5 mmol, 4 equiv.). The solution was then stirred for 24 h at room temperature. Finally, the compound was precipitated in ACN (200 ml). The white solid was filtered off and washed with water (2 × 10 ml) and ACN (3 × 30 ml), air-dried and then further dried in a vacuum oven at 40 °C overnight, yielding an off-white compound. ***N*-Propyl-di-TBDMS chitosan (6a)**: FT-IR (KBr): ν 3468 (N–H), 2958–2858 (C–H), 1708 (C=O amide I), 1473 (C–H), 1389–1362 (C–H), 1256 (C–N), 1110–1047 (C–O), 837–777 (Si–CH_3) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.07 ((CH_3)$_2$Si), 0.9 ((CH_3)$_3$C overlapped with H-9), 1.42 (H-8), 2.39 (H-2), 2.58 and 2.72 (H-7), 3.32–3.97 (H-3, H-4, H-5, H-6), 4.21 ppm (H-1). Yield: 827 mg (88%) starting from 935 mg of **5a**. ***N*-Butyl-di-TBDMS chitosan (6b)**: FT-IR (KBr): ν 3467 (N–H), 2958–2858 (C–H), 1706 (C=O, amide I), 1473 (C–H), 1389–1362 (C–H), 1257 (C–N), 1113–1056 (C–O), 837–777 (Si–CH_3) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.07 ((CH_3)$_2$Si), 0.90 ((CH_3)$_3$C overlapped with H-10), 1.33 (H-8, H-9), 2.38 (H-2), 2.59 and 2.77 (H-7), 3.25–3.99 (H-3, H-4, H-5, H-6), 4.21 ppm (H-1). Yield: 1.388 g (98%) starting from 1.405 g of **5b**. ***N*-Hexyl-di-TBDMS chitosan (6c)**: FT-IR (KBr): ν 3466 (N–H), 2958–2858 (C–H), 1709 (C=O, amide I), 1473 (C–H), 1390–1362 (C–H), 1257 (C–N), 1115–1053 (C–O), 837–777 (Si–CH_3) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.07 ((CH_3)$_2$Si), 0.90 ((CH_3)$_3$C overlapped with H-12), 1.27 (H-8, H-9, H-10, H-11), 2.38 (H-2), 2.58 and 2.76 (H-7), 3.40–3.98 (H-3, H-4, H-5, H-6), 4.21 ppm (H-1). Yield: 837 mg (97%) starting from 856 mg of **5c**.

*2.3.7. General procedure for N-alkyl-N,N-dimethyl chitosan (**7**)*

The corresponding *N*-alkyl-di-TBDMS chitosan (1.6 mmol *N*-alkyl-di-TBDMS-glucosamine units; compounds **6a**–**6c**) was dissolved in dry DCM (15 ml). Then, Li_2CO_3 (0.47 g, 6.4 mmol, 4 equiv.) was added to the solution and the mixture stirred for 1 h, followed by addition of dimethyl sulfate (DMS, 1.21 ml, 12.8 mmol, 8 equiv.). Note: Extreme caution must be taken when handling DMS; a special chemical vapor-proof mask should be used when appropriate. The reaction was carried out at 45 °C in a closed reaction vial under vigorous stirring. After 24 h reaction time, the addition of DMS (1.21 ml, 12.8 mmol, 8 equiv) was repeated. The reaction was stopped after 96 h by dialyzing the mixture against saturated $NaHCO_3$ solution for 1 day and then against deionized water for 4 days, followed by freeze-drying overnight. The quaternized protected material was then dissolved in 1 M TBAF solution in NMP (10 ml) and stirred at 50 °C for 48 h. The yellow solution was then dialyzed against deionized water for 1 day, ion exchanged against 5% aq. NaCl (w/v) solution for 1 day and then against deionized water for 2 days. The resulting material was freeze-dried overnight, yielding a deprotected quaternized material. In cases where NMR analysis indicated that the material was not fully deprotected, the deprotection process was repeated. Finally, the material was dissolved in 5% aq. NaCl (10 ml) and shaken overnight. The solution was centrifuged and the liquid decanted from grey precipitates and dialyzed for 3 days against deionized water, followed by freeze-drying overnight, giving a white and fluffy quaternized material. ***N*-Propyl-*N*,*N*-dimethyl chitosan (7a)**: FT-IR (KBr): ν 3416 (O–H), 2970–2880 (C–H), 1635 (C=O, amide I), 1480 (C–H), 1051 (C–O) cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 0.88 (H-9″), 0.99 (H-9), 1.50 (H-8″), 1.89 (H-8), 3.27 (H-13), 3.35 (H-14), 3.46 and 3.57 (H-7), 3.79 (H-2), 3.90–3.95 (H-5, H-6), 4.37 (H-4), 4.49 (H-3), 4.89 (H-1″), 5.45 (H-1′), 5.53 ppm (H-1). ^{13}C NMR (300 MHz, D_2O): δ 10.5 (C-9), 16.5 (C-8), 51.1 and 51.5 (C-13), 54.6 (C-14), 61.8 (C-6), 67.5 (C-7), 68.5 (C-3), 76.3 (C-5), 78.0 (C-2 and C-4), 96.0 (C-1′), 97.2 ppm (C-1). Yield: 211 mg (46%) starting from 800 mg of **6a**. *N*-Butyl-***N*,*N*-dimethyl chitosan (7b)**: FT-IR (KBr): ν 3405 (O–H), 2961–2876 (C–H), 1647 (C=O, amide I), 1474 (C–H), 1386 (C–H), 1257 (C–N), 1057 (C–O) cm^{-1}. ^1H NMR (400 MHz, D_2O): δ 0.97 (H-10, H-10″), 1.41 (H-9, H-9″, H-8″), 1.85 (H-8), 3.17 (H-15), 3.27 (H-13), 3.34 (H-14), 3.49 and 3.61 (H-7), 3.78 (H-2), 3.87 (H-6), 3.94 (H-5), 4.35 (H-4), 4.49 (H-3), 5.11 (H-1″), 5.46 (H-1′), 5.53 ppm (H-1). ^{13}C NMR (400 MHz, D_2O): δ 10.5 (C-10″), 13.6 (C-10, C-9″), 19.8 (C-9), 24.7 (C-8, C-8″), 47.3 (C-15), 51.2 and 51.6 (C-13), 54.6 (C-14), 61,8 (C-6), 66.2 (C-7), 68.5 (C-3), 76.3 (C-5), 77.7 (C-2), 77.9 (C-4), 96.0 (C-1′), 97.2 ppm (C-1). Yield: 92 mg (11%) starting from 1.381 g of **6b**. ***N*-hexyl-*N*,*N*-dimethyl chitosan (7c)**: FT-IR (KBr): ν 3418 (O–H), 2956–2860 (C–H), 1645 (C=O, amide I), 1470 (C–H), 1380 (C–H),

1225 (C–N), 1056 (C–O) cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 0.90 (H-12), 1.36 (H-9, H-10, H-11), 1.86 (H-8), 3.27 (H-13), 3.34 (H-14), 3.46 and 3.62 (H-7), 3.76 (H-2), 3.89–3.92 (H-6), 3.97 (H-5), 4.32 (H-4), 4.50 (H-3), 5.51 ppm (H-1). ^{13}C NMR (300 MHz, D_2O): δ 13.5 (C-12), 21.8 (C-8), 22.0 (C-11), 25.3 (C-9), 30.5 (C-10), 50.8 and 51.1 (C-13), 54.0 (C-14), 61.3 (C-6), 65.8 (C-7), 67.9 (C-3), 76.0 (C-5), 77.0 (C-2), 77.4 (C-4), 96.7 ppm (C-1). Yield: 238 mg (49%) starting from 766 mg of **6c**.

2.3.8. *N,N-Dimethyl chitosan (**8**)*

Prepared according to a published procedure (Verheul et al., 2008). ^1H NMR (300 MHz, D_2O/d-acetic acid): δ 2.06 (CH_3, GlcNAc), 3.06 (N–(CH_3)$_2$), 3.39 (H-2), 3.75–3.97 (H-5, H-6), 4.10 (H-4), 4.23 (H-3), 5.10 ppm (H-1). ^{13}C NMR (300 MHz, D_2O): δ 42.0 (N–(CH_3)$_2$), 60.5 (C-6), 67.5 (C-3), 68.3 (C-2), 74.6 (C-5), 75.8 (C-4), 95.2 (C-1) ppm. Yield: 198 mg (82%).

2.3.9. *N,N,N-Trimethyl chitosan (**9**)*

Partially trimethylated chitosan was prepared according to a published procedure (Verheul et al., 2008). ^1H NMR (300 MHz, D_2O): δ 2.10 (CH_3, GlcNAc), 2.78 (N–(CH_3)$_2$), 3.35 (N^+–(CH_3)$_3$), 3.75 (H-2), 3.82–3.97 (H-5, H-6), 4.16–4.25 (H-3, H-4, dimethyl), 4.37 (H-4, trimethyl), 4.47 (H-3, trimethyl), 5.42 (H-1, dimethyl), 5.49 ppm (H-1, trimethyl). ^{13}C NMR (300 MHz, D_2O): δ 42.3 (N–(CH_3)$_2$), 54.6 (N–(CH_3)$_3$), 61.7 (C-6), 68.7 (C-3), 75.1 (C-2, dimethyl), 76.2 (C-5), 77.8 (C-4), 79.3 (C-2, trimethyl), 96.2 (C-1, dimethyl), 97.2 ppm (C-1, trimethyl). Yield: 98.5 mg (63%).

3. Results and discussion

Di-TBDMS chitosan (**2**) was used as the starting material for selective modification of the amino group (Scheme 1). Di-TBDMS not only protects chitosan's hydroxyl groups, but also dramatically enhances the solubility of chitosan in common organic solvents, providing homogenous conditions (Runarsson, Malainer, et al., 2008).

3.1. *N,N,N-Trimethylation of di-TBDMS chitosan (**3**)*

Different reaction conditions were explored for the trimethylation process (Table 1). Performing the reaction with CH_3I in DCM, an aprotic solvent of medium polarity, with diazabicycloundecene (DBU) or Cs_2CO_3 did not lead to any detectable trimethylation. Although normally, trialkylation of primary amines would be possible under these conditions, nucleophilic substitution reaction between the two uncharged reactants generally proceeds faster in solvents with high dipole moments and dielectric constants (Reichardt, 2004). Similar observations for the quaternization of poly(urethaneimide) with methyl halides revealed that methyl halides do not react or react slowly with poly(urethaneimide) in chloroform but proceed to completion with NMP as solvent (Jonquieres, Awkal, Clement, & Lochon, 2006). This was confirmed in the current procedure, since performing the reaction with CH_3I in DMF lead to 90% trimethylation. Full *N,N,N*-trimethylation of chitosan could finally be achieved when NMP was used as solvent. Although it can be difficult to remove solvents with high boiling points, such as NMP, from polymeric products, these solvents were compatible with the dialysis membranes used (Spectrum® Laboratories, 2011) and could therefore be removed by dialysis. Trimethylation of di-TBDMS chitosan lead to peak broadening in ^1H NMR spectra and although a large peak could be detected at 3.64 ppm (Fig. 1A.a), the degree of trimethylation could only be determined after deprotection.

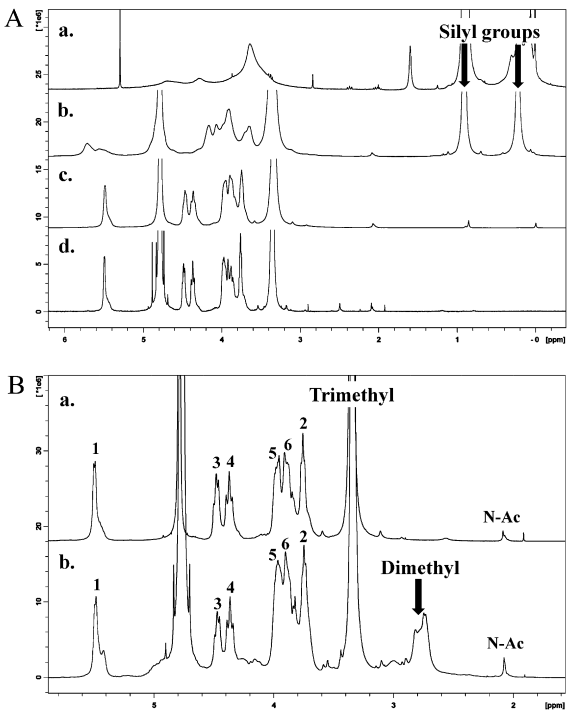
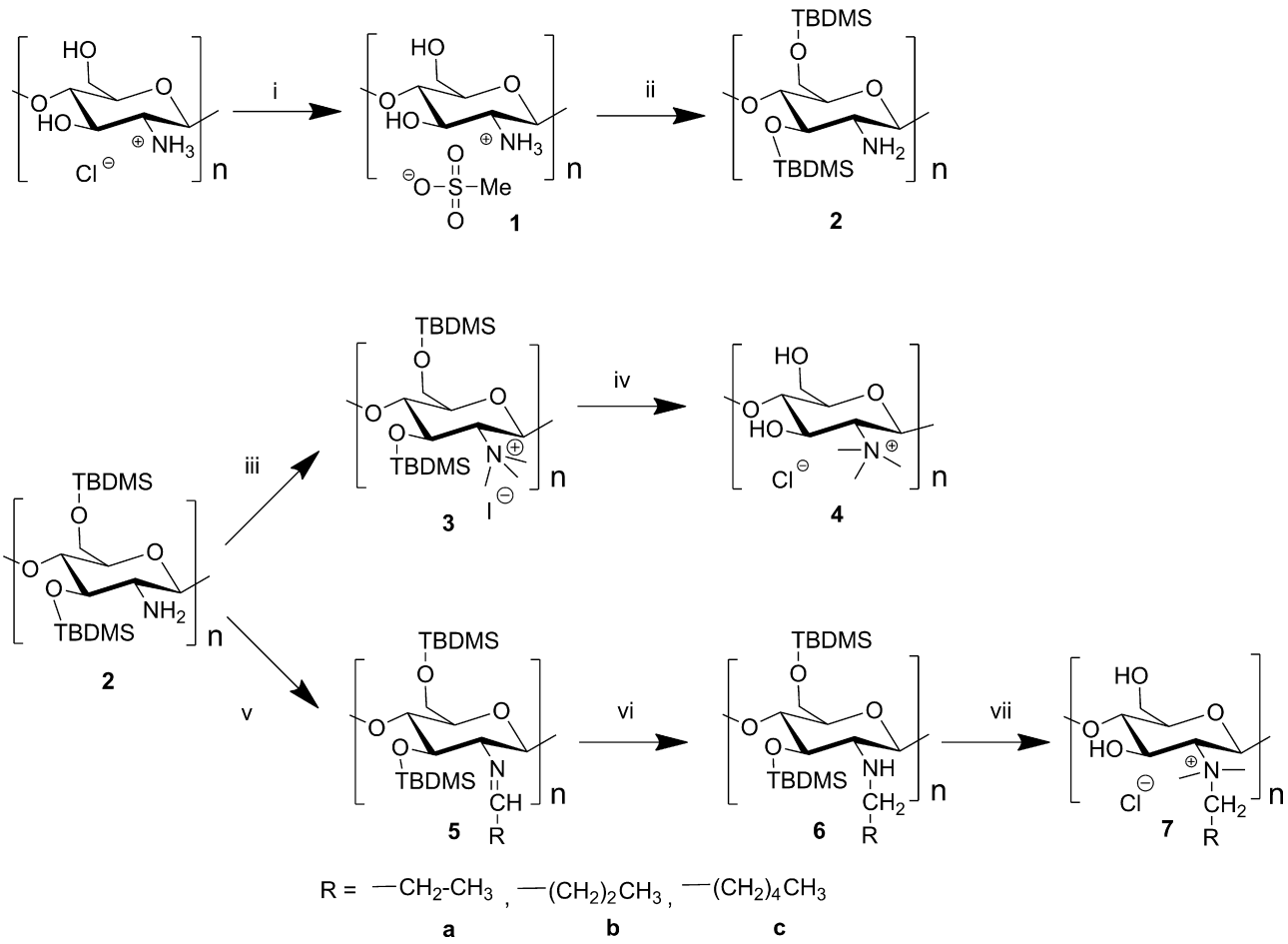


Fig. 1. (A) ^1H NMR analysis of the deprotection process of *N,N,N*-trimethyl-di-TBDMS chitosan. (a) *N,N,N*-Trimethyl-di-TBDMS chitosan (**3**). (b) TMC after 24 h deprotection with 1% HCl in EtOH. (c) TMC after 48 h deprotection at 50 °C with TBAF. (d) TMC after 24 h subsequent second deprotection at 50 °C with TBAF (**4**). (B) Comparison of *N,N,N*-trimethyl chitosan (TMC) synthesized by two different methods. (a) TMC (**4**) synthesized from the current method. (b) TMC (**9**) synthesized from the method of Verheul et al. (2008).

3.2. *Deprotection of N,N,N-Trimethyl-di-TBDMS-chitosan (**3**)*

Previously, the TBDMS group was cleaved from the chitosan backbone using 1% HCl in EtOH (Runarsson, Malainer, et al., 2008). Since depolymerization can occur from acid hydrolysis of the *O*-glycosidic bond (Vårum, Ottøy, & Smidsrød, 2001), it is desirable to avoid prolonged treatment in acid. Furthermore, after 24 h of treatment of **3** in 1% HCl, 33% of the TBDMS groups were still present (Fig. 1A.b). In a search for alternative conditions, we found that deprotection of **3** with 1 M TBAF in NMP (Scheme 1iv) was more efficient than HCl (Fig. 1A.c), with the formation of a strong Si–F bond being the driving force in the deprotection (Wuts & Greene, 2007). Still, traces (~0.4%) of TBDMS protection groups remained on the polymer backbone after the first deprotection step. The deprotection was then repeated to give ≥99.8% desilylation of TMC (Fig. 1A.d), as has been reported in the synthesis 3-mono-*O*-ethyl-cellulose, where the deprotection step was repeated to fully remove the thexyldimethylsilyl protection group (Koschella, Fenn, & Heinze, 2006).

After desilylation, ^1H NMR revealed a TMC homopolymer (Fig. 1B.a). The trimethyl peak was observed at 3.35 ppm, with no indications of *O*- or *N,N*-dimethylation. The highest previously reported degree of trimethylation without *O*-methylation was approximately 80%, achieved by adding CH_3I and NaOH in four portions to chitosan dissolved in a DMF/water mixture (Runarsson, Holappa, et al., 2008). Although higher degrees of trimethylation have been reported (90.5%), these were accompanied by high degrees of *O*-methylation resulting in reduced water solubility (Polnok, Borchard, Verhoef, Sarisuta, & Junginger, 2004). By using di-TBDMS protected chitosan, *O*-methylation was avoided and good solubility in organic solvents was maintained, which is important for obtaining full *N,N,N*-trimethylation. Further-



Scheme 1. Synthetic route for the preparation of *N*,*N*,*N*-trimethyl chitosan (**4**) and *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives (**7a**–**7c**) *Note*: Reagents and conditions: (i) methanesulfonic acid, water, 10 °C; (ii) TBDMS chloride (5 equiv.), imidazole (10 equiv.), DMSO, N_2, room temperature; (iii) CH_3I (15 equiv.), Cs_2CO_3 (4 equiv.), NMP, 60 °C; (iv) TBAF (1 M), NMP, 50 °C; (v) propyl aldehyde, hexyl aldehyde or dodecyl aldehyde (10 equiv.) respectively, triethylamine (1.1 equiv.), DCM, 45 °C; (vi) STAB-H (4 equiv.), AcOH (4 equiv.), DCM, room temperature; (vii) DMS (16 equiv.), Li_2CO_3 (4 eq), NMP, 45 °C then TBAF (1 M), NMP, 50 °C. Chitosan has 3% *N*-acetylation that has been omitted for clarity.

more, the absence of *O*-methylation in this product now confirms that the hydroxyl groups are fully protected in the di-TBDMS chitosan precursor. As part of the present work, a methylation reaction was carried out according to the procedure recently reported (Verheul et al., 2008) which confirmed that significant *O*-methylation was avoided. However, only 64% trimethylation was achieved with the remaining amino groups dimethylated (Fig. 1B.b).

The chemical structure of the fully substituted TMC and the assignment of individual peaks was further confirmed by 2D HSQC and COSY NMR analysis (Fig. 2A and B, respectively), revealing a homogenous polymer with clearly resolved peaks for all atoms of the polymer, with no indication of *N*,*N*-dialkylation, *N*-monoalkylation or *O*-alkylation.

3.3. *N*-Alkylimine formation of di-TBDMS chitosan protected polymer (***5a***–***5c***)

N-Alkylation of di-TBDMS chitosan was achieved by stepwise reductive alkylation, where the imine was isolated after condensation of an aldehyde to the amino group of chitosan. This approach can be used to eliminate the risk of possible dialkylation (AbdelMagid, Carson, Harris, Maryanoff, & Shah, 1996). For example, significant *N*,*N*-dialkylation was reported as a side-product in the *N*-monoalkylation of *O*-acetyl protected glucosamine (Liberek et al., 2005), where imine formation and reduction were performed simultaneously. Although conventionally, the primary amine is used in excess to avoid dialkylation (AbdelMagid et al., 1996), this approach cannot be used with polyamines such as chitosan, if a

Table 1
Reaction conditions and results for the trimethylation reaction of di-TBDMS chitosan (**2**).

Methylating reagent (equiv.)	Base (equiv)/solvent	Temp (°C)	Time (h)	ds (%)[a]
CH_3I (10)	DBU (10)/DCM	RT	48	nd[b]
CH_3I (10)	DBU (10)/DCM	40	72	nd[b]
CH_3I (10)	Cs_2CO_3 (1.2)/DCM	RT	48	nd[b]
CH_3I (10)	Cs_2CO_3 (3)/DMF	50	96	90
CH_3I (15)	Cs_2CO_3 (3)/NMP	50	96	100

[a] Degree of substitution calculated from ^{1}H NMR data, substitution excludes 3% *N*-acetylation.
[b] nd: not detected.
^{1}H NMR of protected chitosan did not indicate trimethylation and deprotection was therefore not performed.

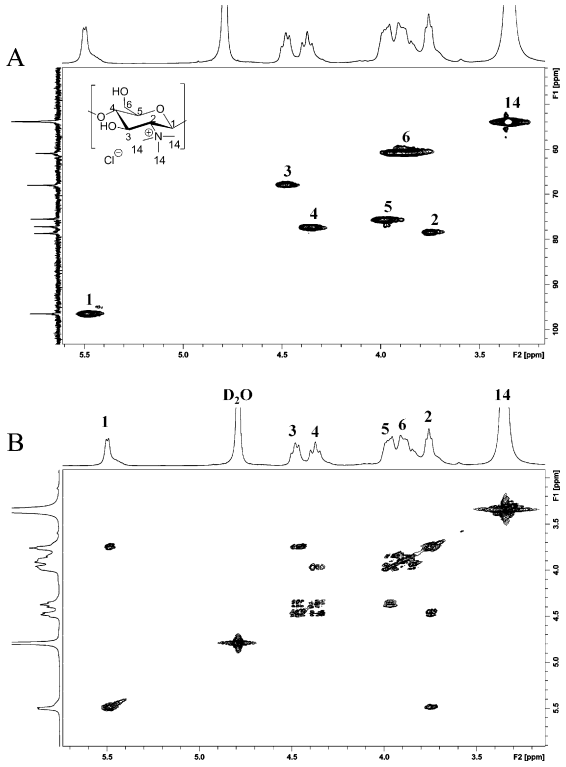


Fig. 2. NMR analysis of TMC (**4**). (A) ^{1}H–^{13}C HSQC. (B) ^{1}H–^{1}H COSY spectra.

high degree of substitution is desired. Furthermore, the risk of aldehyde reduction, which can take place when reducing agents such as borohydrides are used, is also circumvented (Gribble & Ferguson, 1975).

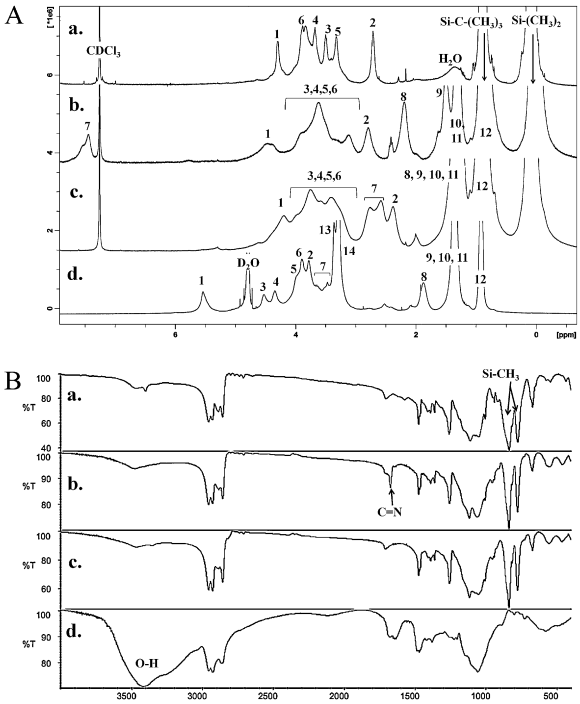


Fig. 3. ^{1}H NMR (A) and FT-IR spectra (B) of the main chitosan compounds in the synthesis of *N*-hexyl-*N*,*N*-dimethyl chitosan. (a) Di-TBDMS chitosan (**2**). (b) *N*-Hexylimine-di-TBDMS chitosan (**5c**). (c) *N*-Hexyl-di-TBDMS chitosan (**6c**). (d) *N*-Hexyl-*N*,*N*-dimethyl chitosan (**7c**).

Table 2
Different reducing conditions for *N*-alkylimine-di-TBDMS chitosan derivatives.

Reducing agent (equiv.)	Solvent	Temp (°C)	Time (h)	rd (%)[a]
H_2/Pd	DCM	RT	24	52
$NaBH_4$ (1.6)	MeOH/DCM (50:50)	RT	24	29
STAB-H (1.6)	MeOH/DCM (50:50)	RT	24	20
STAB-H (1.6)	MeOH/DCM (20:80)	40	24	26
STAB/AcOH (2/4)	DCM	RT	48	95
STAB/AcOH (4/4)	DCM	RT	24	100

[a] rd: reduction degree determined by ^{1}H NMR.

Di-TBDMS chitosan (Fig. 3A.a and B.a) was reacted with either propyl-, butyl- or hexyl aldehyde (Scheme 1v). This reaction yielded *N*-alkylimine-di-TBDMS chitosan, as evidenced by the appearance of an imine proton at 7.4 ppm by ^{1}H NMR analysis (Fig. 3A.b) and confirmed by COSY NMR (Appendix B). IR spectra also showed a characteristic stretch for the imine C=N group at 1673 cm^{-1} (Fig. 3B.b). Calculations from the ^{1}H NMR peaks indicated a partial *N*-substitution of 74–80%. Although various reaction conditions were investigated, including extended heating, prolonged reaction times and varying amounts of aldehyde, this did not significantly affect the degree of substitution (data not shown), which remained in the previously stated range. Molecular sieves were used to drive the reaction to completion by water removal. Full substitution was however not established, probably because of the instability of the *N*-alkyl imine, which can convert back to a primary amine and the corresponding aldehyde (Layer, 1963). Also, the possibility that steric hindrance of the di-TBDMS protection groups prevented full substitution could not be excluded.

3.4. Reduction to N-alkyl-di-TBDMS chitosan derivatives (**6a**–**6c**)

Typical reducing agents, such as sodium borohydrate and catalytic hydrogenation with H_2/Pd, were tested for reduction of the imine to amine, but resulted in only a partial conversion of 29% and 52%, respectively (Table 2). Thus, STAB-H, a sodium borohydride derivative, was chosen for reduction of *N*-alkylimine-di-TBDMS chitosan to *N*-alkyl-di-TBDMS chitosan. This reducing agent is mild and has given better yields and faster reactions than other reducing agents, including $NaBH_4$ or catalytic hydrogenation (AbdelMagid et al., 1996). Only a 26% reduction was observed with MeOH/DCM as the solvent. STAB-H can degrade in protic solvents such as MeOH (Gribble, 2007), and this could have contributed to the partial conversion of the imine to amine. Because STAB-H is partially soluble in DCM, this solvent was used as the solvent of choice. Acids can also be used to facilitate complete conversion from imine to amine (AbdelMagid et al., 1996). This was observed in the present study, where addition of 4 equivalents of STAB-H and acetic acid to the reaction in DCM resulted in complete conversion from the imine to the amine (Scheme 1vi). ^{1}H NMR spectra revealed the disappearance of the N=C–H proton, confirming successful reduction (Fig. 3A.c), while COSY spectra showed a distinct correlation for reduced *N*-alkylated chitosan (Appendix C). Interestingly, prochiral H-7 protons in the *N*-alkyl chain were observed as two peaks at 2.6 and 2.7 ppm. Similar observations were made for the N–CH_2 peaks of *N*-alkyl-D-glucosamine, where the two protons were also found to have slightly different chemical shifts (Liberek et al., 2005). Reduction was also confirmed by IR, where the imine stretch at 1673 cm^{-1} was absent (Fig. 3B.c).

3.5. N,N-Dimethylation and deprotection of N-alkyl-chitosan derivatives (**7a**–**7c**)

N,*N*-Dimethylation of *N*-propyl-, *N*-butyl- and *N*-hexyl-di-TBDMS chitosan with CH_3I, in NMP with Cs_2CO_3 as base, was investigated because this procedure was successful for the produc-

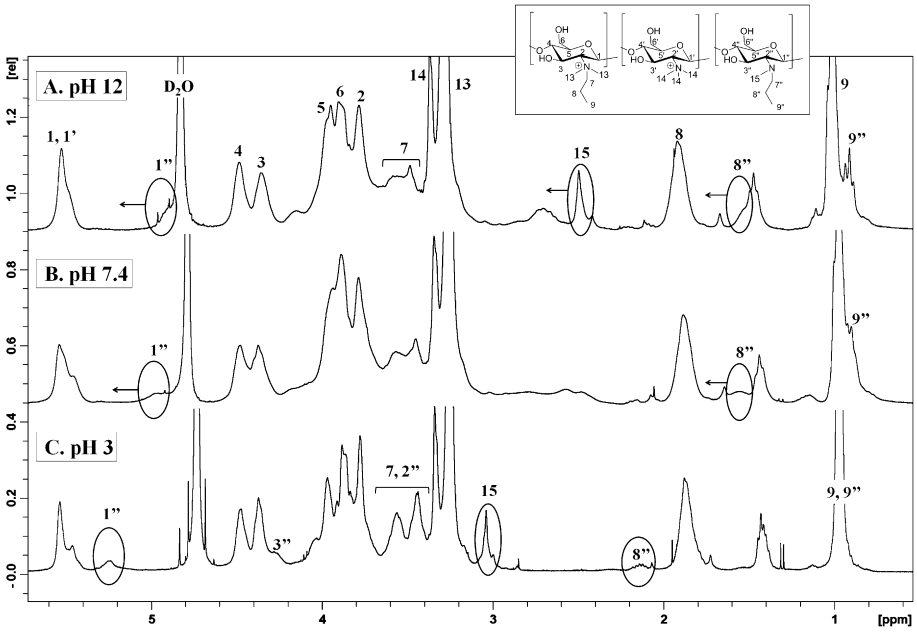


Fig. 4. ^{1}H NMR spectra of *N*-propyl-*N*,*N*-dimethyl chitosan at (A) pH 12, (B) pH 7.4 and (C) pH 3.

Table 3
Degree of *N*,*N*-dimethylation for *N*-propyl-di-TBDMS chitosan.

Methylating reagent (equiv.)	Base (equiv.)/solvent	Temp (°C)	Time (h)	*N*,*N*-Dimethylation (%)[a]
CH_3I (10)	Cs_2CO_3 (2)/DMF	50	72	<5[b]
CH_3I (10)	Cs_2CO_3 (3)/NMP	60	48	17
CH_3I (10)	Cs_2CO_3 (5)/NMP	100	72	19
CH_3I (10)	Li_2CO_3 (4)/NMP	80	72	15
DMS (16)	Li_2CO_3 (8)/NMP	80	96	<5[b]
DMS (16)	Li_2CO_3 (4)/DCM	45	96	63

[a] Degree of substitution for *N*,*N*-dimethylation of *N*-propyl-di-TBDMS chitosan determined by ^{1}H NMR.
[b] Determined in D_2O/DCl.

tion of TMC. However, this reaction gave low quaternization values (Table 3). Since cesium base has been reported to suppress over-alkylation (Salvatore, Nagle, & Jung, 2002), lithium carbonate was chosen instead, but the reaction still resulted in low quaternization. Dimethyl sulfate (DMS), a more reactive methylating agent than CH_3I (Friedl, 1990), was therefore used for *N*,*N*-dimethylation. When NMP was used as solvent, the reaction was <5% but when DCM was used as solvent (Scheme 1vii), the reaction was successful, with a degree of *N*,*N*-dimethylation of 65% for *N*-propyl-, 72% for *N*-butyl- and 68% for *N*-hexyl chitosan derivatives (Fig. 3A.d).

Previous syntheses of quaternized chitosan derivatives such as *N*-ethyl-*N*,*N*-dimethyl chitosan have been reported, where the *N*-monoethyl material was produced in one step, followed by dimethylation with CH_3I (Bayat et al., 2006). However, the possibility of *N*,*N*-diethylation or *O*-methylation was not excluded and detailed NMR characterization of the derivative was not reported. Therefore, a more detailed investigation of the synthesis of similar quaternary compounds was needed.

Very broad peaks were observed for the *N*,*N*-dimethyl-*N*-alkyl-di-TBDMS chitosan derivatives that were poorly resolved in ^{1}H NMR, and were therefore not characterized before deprotection. Deprotection by 1 M TBAF was confirmed by the disappearance of the silyl peaks in the ^{1}H NMR spectra (Fig. 3A.d), the disappearance of the Si–CH_3 band at 837–777 cm^{-1} and the appearance of a large band at 3418 cm^{-1} in FT-IR spectra, corresponding to the O–H bond in the IR spectra (Fig. 3B.d). A peak corresponding to the dimethyl peak at 3.27 ppm for *N*-propyl-, *N*-butyl-, and *N*-hexyl-*N*,*N*-dimethyl chitosan appeared (Fig. 3A.d), which did not shift when NMR samples were acidified with DCl. A small peak assigned

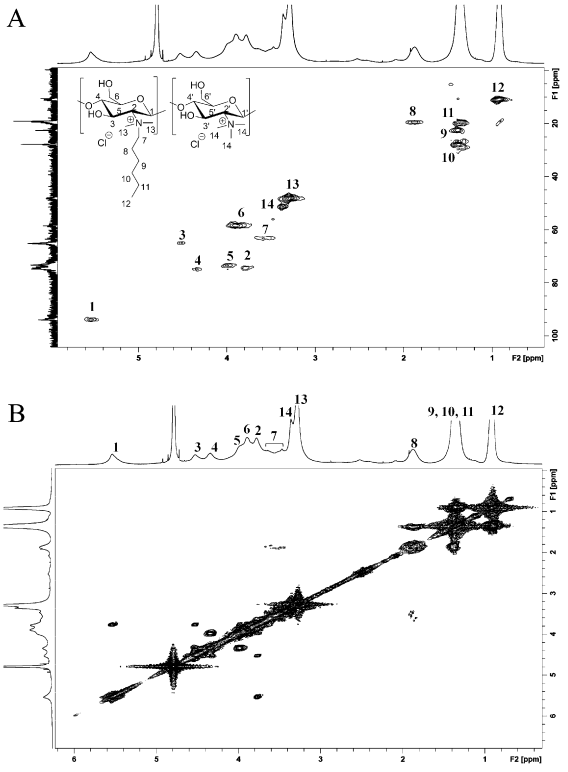


Fig. 5. NMR analysis of *N*-hexyl-*N*,*N*-dimethyl chitosan (**7c**). (A) ^{1}H–^{13}C HSQC. (B) ^{1}H–^{1}H COSY spectra.

to *N*-monomethylation of *N*-alkyl chitosan was not always clearly present in the NMR spectra at neutral pH (Fig. 4B) but became apparent when the solution was acidified with DCl at ~3.05 ppm (Fig. 4C) or the material deprotonized at pH 12 (Fig. 4A). The degree of *N*-monomethylation of *N*-alkyl chitosan derivatives was 15–18%. Furthermore, when acidified, the *N*-alkyl peaks of the *N*-monomethylated residue shifted downfield towards the values of their *N*-alkyl-*N*,*N*-dimethyl counterparts. Trimethylation was also detected at 3.34–3.35 ppm, as the result of partial *N*-alkylation, with the degree of trimethylation of 13–17%. The structure was further investigated by detailed 2D COSY and HSQC NMR, which confirmed the positions of the different protons on the chitosan backbone, as well as protons on the alkyl chain (Fig. 5A and B).

Steric hindrance appears to be involved in the reactivity of di-TBDMS chitosan. While, *N*,*N*,*N*-trimethylation of di-TBDMS chitosan by CH_3I proceeded to completion, giving 100% trimethylated material, *N*,*N*-dimethylation by the more reactive DMS of *N*-propyl-, *N*-butyl- and *N*-hexyl-di-TBDMS chitosan was approximately 70%. In fact, a complete absence of *N*,*N*-dimethylation was observed in the case of *N*-dodecyl-di-TBDMS chitosan (data not shown).

4. Conclusions

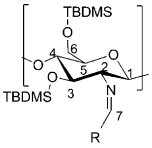
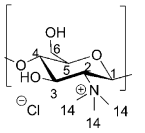
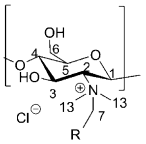
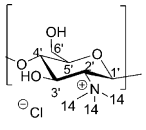
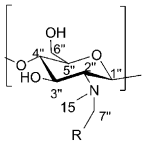
In this study, the advantages of a di-TBDMS chitosan protection strategy for the synthesis of *N*,*N*,*N*-substituted chitosan have been demonstrated as an addition to previously achieved *N*-acylation and *N*-acetylation of di-TDMS chitosan. Full *N*,*N*,*N*-trimethylation of chitosan polymer is reported for the first time. Furthermore, highly substituted *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives were synthesized and characterized in detail by NMR, confirming the assignments of each peak. Partial *N*-alkylation (propyl, butyl or hexyl) of di-TBDMS chitosan was found to be likely due to the instability of the imine bond and/or steric hindrance from the protecting groups. Subsequent *N*,*N*-dimethylation of the *N*-alkylated material required DMS, a more reactive methylating agent than CH_3I. This could be attributed to steric hindrance from the TBDMS group and the existing *N*-alkyl chain, which can reduce the reactivity of the amino group. Deprotection of the quaternized derivatives required alternative conditions than those previously published, with TBAF providing efficient deprotection of the bulky TBDMS protected chitosan derivatives. The resulting *N*,*N*,*N*-trimethyl- and *N*-alkyl-*N*,*N*-dimethyl chitosan derivatives were water soluble, with no evidence of *O*-methylation. This also confirmed that the hydroxyl groups are fully protected in the di-TBDMS chitosan precursor used in the synthesis.

In the present study, we demonstrate that di-TBDMS chitosan polymers can be chemically modified under homogenous conditions in common organic solvents at moderate temperatures under different reaction conditions. Di-TBDMS chitosan thus represents an ideal precursor for further *N*-selective modifications.

Acknowledgements

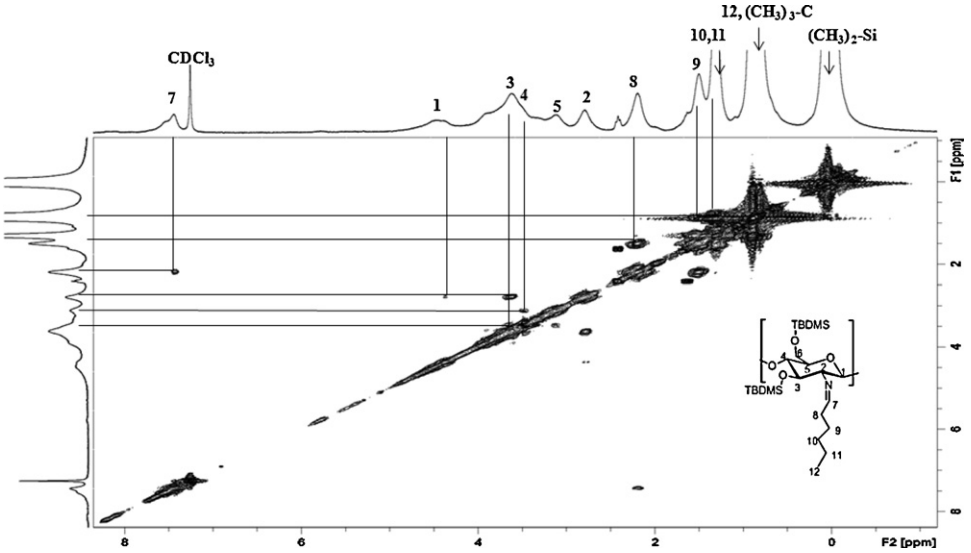
The project was supported by the Icelandic Science and Technology Policy Council Research Programme for Nanotechnology, the Eimskip Fund of the University of Iceland, the Bergþóru and Þorsteins Scheving Thorsteinssonar Fund and the University of Iceland Research Fund. We are grateful for the donation of chitosan starting material provided by Genis EHf.

Appendix A. Equations used for the calculation of *N*-substituted chitosan derivatives

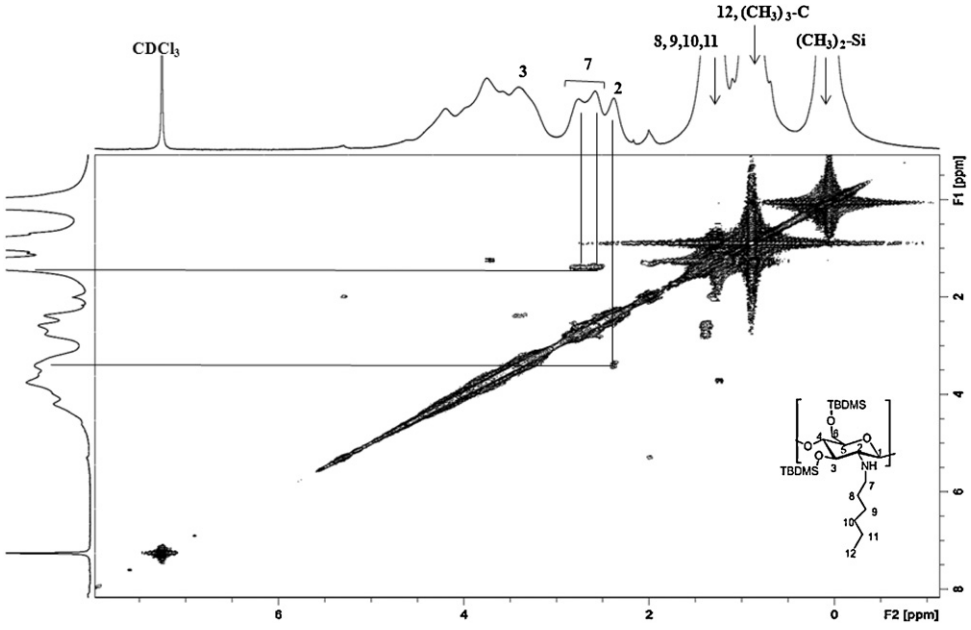
Chitosan derivative	Structure	Equation	Substitution
***N*-Alkylimine-di-O-TBDMSchitosan (5)** R = –CH_2CH_3(**a**), –$CH_2CH_2CH_3$ (**b**) or –$CH_2CH_2CH_2CH_2CH_3$ (**c**)		$A = \left(\frac{\int \text{H-7}}{\int (\text{S-}(CH_3)_2)_2} \times \frac{12}{1} \right)$	*N*-Alkylation
***N*,*N*,*N*-Trimethyl chitosan (4)**		$B = \left(\frac{\int \text{H-14}}{\int \text{H-1}} \times \frac{1}{9} \right)$	*N*,*N*,*N*-Trimethylation
		$C = \left(\frac{\int (CH_3 \text{ in alkyl chain})}{\int (\text{H-1},1',1'')} \times \frac{1}{3} \right) - E$	*N*-Alkyl-*N*,*N*-dimethyl
***N*-Alkyl-*N*,*N*-dimethyl chitosan (7)**[a] R = –CH_2CH_3(**a**), –$CH_2CH_2CH_3$(**b**) or –$CH_2CH_2CH_2CH_2CH_3$(**c**)		$D = \left(\frac{\int (\text{H-1},1')}{\int (\text{H-1},1',1'')} \times \frac{1}{1} \right) - C$	*N*,*N*,*N*-Trimethylation
		$E = \left(\frac{\int (\text{H-15})}{\int (\text{H-1},1',1'')} \times \frac{1}{3} \right)$	*N*-Alkyl-*N*-monomethyl

[a] Substitution determined at pH 3.

Appendix B. COSY spectra of *N*-hexylimine-di-TBMDS chitosan (5c)



Appendix C. COSY spectra of *N*-hexyl-di-TBMDS chitosan (6c)



References

AbdelMagid, A. F., Carson, K. G., Harris, B. D., Maryanoff, C. A., & Shah, R. D. (1996). Reductive amination of aldehydes and ketones with sodium triacetoxyborohydride. Studies on direct and indirect reductive amination procedures. *Journal of Organic Chemistry*, *61*(11), 3849–3862.

Alves, N. M., & Mano, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. *International Journal of Biological Macromolecules*, *43*(5), 401–414.

Baldrick, P. (2010). The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology and Pharmacology*, *56*(3), 290–299.

Bayat, A., Sadeghi, A. M. M., Avadi, M. R., Amini, A., Rafiee-Tehrani, M., Shafiee, A., Majlesi, R., & Junginger, H. E. (2006). Synthesis of N,N-dimethyl N-ethyl chitosan as a carrier for oral delivery of peptide drugs. *Journal of Bioactive and Compatible Polymers*, *21*(5), 433–444.

de la Fuente, M., Csaba, N., Garcia-Fuentes, M., & Alonso, M. J. (2008). Nanoparticles as protein and gene carriers to mucosal surfaces. *Nanomedicine*, *3*(6), 845–857.

Domard, A., Rinaudo, M., & Terrassin, C. (1986). New method for the quaternization of chitosan. *International Journal of Biological Macromolecules*, *8*(2), 105–107.

Friedl, F. E. (1990). Rate of quaternization as a function of the alkylating agent. In T. H. Applewhite (Ed.), *World conference on oleochemicals into the 21st centaury*. Kuala Lumpur, Malaysia: American Oil Chemists' Society.

Gribble, G. W. (2007). *Sodium triacetoxyborohydride. e-EROS encyclopedia of reagents for organic synthesis [Online] March 15, 2007*. John Wiley & Sons, Inc. Accessed 31.03.2011. http://onlinelibrary.wiley.com/o/eros/articles/rs112/frame.html

Gribble, G. W., & Ferguson, D. C. (1975). Reactions of sodium–borohydride in acidic media – Selective reduction of aldehydes with sodium triacetoxyborohydride. *Journal of the Chemical Society – Chemical Communications*, *13*, 535–536.

Holappa, J., Nevalainen, T., Savolainen, J., Soininen, P., Elomaa, M., Safin, R., Suvanto, S., Pakkanen, T., Masson, M., Loftsson, T., & Jarvinen, T. (2004). Synthesis and characterization of chitosan N-betainates having various degrees of substitution. *Macromolecules*, *37*(8), 2784–2789.

Jonquieres, A., Awkal, M., Clement, R., & Lochon, P. (2006). Synthesis and characterization of film-forming poly(urethaneimide) cationomers containing quaternary ammonium groups. *Polymer*, *47*(16), 5724–5735.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, *144*(1), 51–63.

Koschella, A., Fenn, D., & Heinze, T. (2006). Water soluble 3-mono-O-ethyl cellulose: Synthesis and characterization. *Polymer Bulletin*, *57*(1), 33–41.

Kurita, K. (2006). Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology*, *8*(3), 203–226.

Kurita, K., Hirakawa, M., Kikuchi, S., Yamanaka, H., & Yang, J. (2004). Trimethylsilylation of chitosan and some properties of the product. *Carbohydrate Polymers*, *56*(3), 333–337.

Layer, R. W. (1963). The chemistry of imines. *Chemical Reviews*, *63*(5), 489–510.

Ledung, P., Milas, M., Rinaudo, M., & Desbrieres, J. (1994). Water-soluble derivatives obtained by controlled chemical modifications of chitosan. *Carbohydrate Polymers*, *24*(3), 209–214.

Liberek, B., Melcer, A., Osuch, A., Wakiec, R., Milewski, S., & Wisniewski, A. (2005). N-Alkyl derivatives of 2-amino-2-deoxy-D-glucose. *Carbohydrate Research*, *340*(11), 1876–1884.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*(3), 426–428.

Mourya, V., & Inamdar, N. (2009). Trimethyl chitosan and its applications in drug delivery. *Journal of Materials Science: Materials in Medicine*, *20*(5), 1057–1079.

Muzzarelli, R. A. A., & Tanfani, F. (1985). The N-permethylation of chitosan and the preparation of N-trimethyl chitosan iodide. *Carbohydrate Polymers*, *5*(4), 297–307.

Nishimura, S., Kohgo, O., Kurita, K., & Kuzuhara, H. (1991). Chemospecific manipulations of a rigid polysaccharide: Syntheses of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications. *Macromolecules*, *24*(17), 4745–4748.

Ottoy, M. H., Varum, K. M., & Smidsrod, O. (1996). Compositional heterogeneity of heterogeneously deacetylated chitosans. *Carbohydrate Polymers*, *29*(1), 17–24.

Polnok, A., Borchard, G., Verhoef, J. C., Sarisuta, N., & Junginger, H. E. (2004). Influence of methylation process on the degree of quaternization of N-trimethyl chitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*(1), 77–83.

Reichardt, C. (2004). *Solvent effects on the rates of homogeneous chemical reactions*. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.

Runarsson, O. V., Holappa, J., Jonsdottir, S., Steinsson, H., & Masson, M. (2008). N-selective 'one pot' synthesis of highly N-substituted trimethyl chitosan (TMC). *Carbohydrate Polymers*, *74*(3), 740–744.

Runarsson, O. V., Holappa, J., Nevalainen, T., Hjalmarsdottir, M., Jarvinen, T., Loftsson, T., Einarsson, J. M., Jonsdottir, S., Valdimarsdottir, M., & Masson, M. (2007). Antibacterial activity of methylated chitosan and chitooligomer derivatives: Synthesis and structure activity relationships. *European Polymer Journal*, *43*(6), 2660–2671.

Runarsson, O. V., Malainer, C., Holappa, J., Sigurdsson, S. T., & Masson, M. (2008). tert-Butyldimethylsilyl O-protected chitosan and chitooligosaccharides: Useful precursors for N-modifications in common organic solvents. *Carbohydrate Research*, *343*(15), 2576–2582.

Rúnarsson, Ö. V., Holappa, J., Malainer, C., Steinsson, H., Hjálmarsdóttir, M., Nevalainen, T., & Másson, M. (2010). Antibacterial activity of N-quaternary chitosan derivatives: Synthesis, characterization and structure activity relationship (SAR) investigations. *European Polymer Journal*, *46*(6), 1251–1267.

Salvatore, R. N., Nagle, A. S., & Jung, K. W. (2002). Cesium effect: High chemoselectivity in direct N-alkylation of amines. *The Journal of Organic Chemistry*, *67*(3), 674–683.

Sieval, A. B., Thanou, M., Kotze, A. F., Verhoef, J. E., Brussee, J., & Junginger, H. E. (1998). Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride. *Carbohydrate Polymers*, *36*(2–3), 157–165.

Song, W. L., Gaware, V. S., Runarsson, O. V., Masson, M., & Mano, J. F. (2010). Functionalized superhydrophobic biomimetic chitosan-based films. *Carbohydrate Polymers*, *81*(1), 140–144.

Spectrum® Laboratories. (2011). *Chemical compatibility*. Accessed 29.03.2011. http://www.spectrumlabs.com/dialysis/Compatibility.html

Vårum, K. M., Ottøy, M. H., & Smidsrød, O. (2001). Acid hydrolysis of chitosans. *Carbohydrate Polymers*, *46*(1), 89–98.

Verheul, R. J., Amidi, M., van der Wal, S., van Riet, E., Jiskoot, W., & Hennink, W. E. (2008). Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan. *Biomaterials*, *29*(27), 3642–3649.

Wuts, P. G. W., & Greene, T. W. (2007). *Protection for the hydroxyl group, including 1,2- and 1,3-diols. Greene's protective groups in organic synthesis*. New Jersey: John Wiley & Sons, Inc. (pp. 189–198).