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Synthesis of methylated chitosan containing aromatic moieties: Chemoselectivity and effect on molecular weight

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Abstract

N-Arylated chitosans were synthesized via Schiff bases formed by the reaction between the primary amino group of chitosan with aromatic aldehydes followed by reduction of the Schiff base intermediates with sodium cyanoborohydride. Treatment of chitosan containing *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl substituents with iodomethane under basic conditions led to quaternized *N*-(4-*N,N*-dimethylaminobenzyl) chitosan and quaternized *N*-(4-pyridylmethyl) chitosan. Methylation occurred at either *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl groups before the residual primary amino groups of chitosan GlcN units were substituted. The total degree of quaternization of each chitosan varied depending on the extent of *N*-substitution (ES) and the sodium hydroxide concentration used in methylation. Increasing ES increased the total degree of quaternization but reduced attack at the GlcN units. *N,N*-dimethylation and *N*-methylation at the primary amino group of chitosan decreased at higher ES's. Higher total degrees of quaternization and degrees of *O*-methylation resulted when higher concentrations of sodium hydroxide were used. The molecular weight of chitosan before and after methylation was determined by gel permeation chromatography under mild acidic condition. The methylation of the *N,N*-dimethylaminobenzyl derivative with iodomethane was accompanied by numerous backbone cleavages and a concomitant reduction in the molecular weight of the methylated product was observed. The antibacterial activity of water-soluble methylated chitosan derivatives was determined using *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria; minimum inhibitory concentrations (MIC) of these derivatives ranged from 32 to 128 μg/mL. The presence of the *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl substituents on chitosan backbone after methylation did not enhance the antibacterial activity against *S. aureus*. However, *N*-(4-*N,N*-dimethylaminobenzyl) chitosan with degree of quaternization at the aromatic substituent and the primary amino group of chitosan of 17% and 16–30%, respectively, exhibited a slightly increased antibacterial activity against *E. coli*.

Keywords: Chitosan; *N*-(4-*N,N*-Dimethylaminobenzyl) chitosan; *N*-(4-Pyridylmethyl) chitosan; *N*-(4-Methylbenzyl) chitosan; Methylation; Iodomethane; Antibacterial activity; ^{1}H NMR and GPC analysis

1. Introduction

Chitosan, derived from chitin, is a natural non-toxic biopolymer consisting of β-1-4 linked *N*-acetyl glucosamine (GlcNAc) and glucosamine (GlcN) repeating units. The application of chitosan is, however, limited owing to its solubility only in dilute organic acid solution. Many efforts to synthesize derivative of chitosan by chemical modification in order to increase the solubility in water have been reported (Domard, Rinaudo, & Terrassin, 1986; Loubaki, Ourevitch, & Sicsic, 1991; Muzzarelli & Ilari, 1994; Sashiwa & Shigemasa, 1999). Currently, many applications of water-soluble chitosan derivatives including: antibacterial activity (Jia, Shen, & Xu, 2001) anticoagulant activity (Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002),

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antioxidant activity (Xie, Xu, & Liu, 2001), absorption enhancer across mucosal surfaces (Kotze, Luessen, De Leeuw, De Boer, Verhoef, & Junginger, 1997), and gene delivery vehicles (Murata, Ohya, & Ouchi, 1996) have been reported. *N*,*N*,*N*-Trimethylammonium chitosan chloride (TMChC) is one of the water-soluble chitosan derivatives soluble over a wide pH range. Thus, much attention has been paid on its synthesis (Domard, Gey, Rinaudo, & Terrassin, 1987; Domard et al., 1986; Dung, Milas, Rinaudo, & Desbrieres, 1994; Elisabete, Douglas, & Sergio, 2003; Hamman & Kotze, 2001; Muzzarelli & Tanfani, 1985; Polnok, Borchard, Verhoef, Sarisuta, & Junginger, 2004; Sieval et al., 1998). Methylation of chitosan by a single treatment with iodomethane in *N*-methyl-2-pyrrolidone (NMP), NaOH and NaI, yielded low degrees of quaternization, DQ_{Ch}, while repeated methylation of chitosan lead to not only higher DQ_{Ch} but also higher degrees of *O*-methylation (DOM) (Elisabete et al., 2003; Hamman & Kotze, 2001; Polnok et al., 2004; Sieval et al., 1998). Due to methylation on the 3- and 6-hydroxyl groups of chitosan, the solubility of the higher DQ_{Ch} derivatives in water decreased (Sieval et al., 1998). Nevertheless, the methylated chitosan derivatives containing alkyl and furfuryl substituents were found to have higher antibacterial activity than chitosan in dilute acetic acid, and it increased with increasing chain length of the alkyl substituent (Jia et al., 2001; Kim, Choi, Chun, & Choi, 1997). Much less attention has been paid on the synthesis of methylated *N*-aryl chitosan derivatives. In this study, chitosans containing 4-methylbenzyl, 4-*N,N*-dimethylaminobenzyl and 4-pyridylmethyl substituents with various extents of substitution, ES, were synthesized then methylated by single treatment with iodomethane. The extent of *N*-methylation and *O*-methylation was determined by ^{1}H NMR and the influence of the sodium hydroxide concentration during methylation of *N*-(4-*N,N*-dimethylaminobenzyl) chitosan was investigated. Antibacterial activity of these methylated chitosan derivatives was also evaluated against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacteria at neutral pH.

2. Experimental

2.1. Materials

Chitosan with a weight average molecular weight, M_w, of 276 kDa was purchased from Seafresh Chitosan (Lab.) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of this material was determined to be 94% by ^{1}H NMR. A cellulose dialysis tubing (Aldrich) with molecular weight cut off 12,000–14,000 g/mol was used to purify all soluble modified chitosans. Sodium cyanoborohydride (Aldrich), 4-*N,N*-dimethylaminobenzaldehyde, and sodium iodide (Merck), 4-methylbenzaldehyde (Fluka), 4-pyridinecarboxaldehyde (Acros), iodomethane (Riedal-deHaen), 1-methyl-2-pyrrolidone, NMP (Fluka), were used as received, and all other reagents were distilled before use.

2.2. Characterizations

FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared (FT-IR) spectrometer; all samples were prepared as potassium bromide pellets. The ^{1}H, ^{13}C NMR spectra were measured on a Mercury Varian 300 MHz spectrometer. All measurements were performed at 300 K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. D_2O/CF_3COOD, 1% (v/v) and D_2O were the solvents for 5 mg chitosan and its derivatives, respectively. The molecular weights of chitosan and its derivatives were determined using a gel permeation chromatography laser light-scattering (GPC-LS) system consisting of Agilent 1100 Series generic pump and injector, three Viscotek Columns (ViscoGEL Poly-CAT™), Wyatt Optilab rEX differential refractive index detector (DRI), Wyatt Dawn Heleos laser light-scattering detector (LS) and a Wyatt viscometer detector. The mobile phase was 5% acetic acid (pH 4) at a flow rate of 1 mL/min at 20 °C. The chromatograms were collected by Astra V software, and analyzed with the Astra 5.3.1.5 program.

2.3. Preparation of N-aryl chitosans

Chitosan (1.00 g, 6.11 meq GlcN) was dissolved in 0.2 M acetic acid (pH 4, 70 mL). The solution was diluted with ethanol (70 mL), and then aromatic aldehyde (0.1–3.0 meq/GlcN) was added to the solution. The reaction mixture was stirred at room temperature for 1 h. At this point the pH of the solution was adjusted to 5 by adding 1 M NaOH. Then, $NaCNBH_3$ (1.54 g, 24.46 meq/GlcN) was added, and the resulting solution was allowed to stir at room temperature for 24 h, followed by adjusting the pH to 7 with 15% (w/v) NaOH. If precipitation occurred, the precipitate was continuously extracted (Soxhlet) with ethanol:ether (1:1 v/v) for 2 days and washed with ethanol several times followed by an acetone wash prior to drying at room temperature under nitrogen. In case of a homogeneous solution, the aqueous solution was dialyzed in distilled water for 4 days and then freeze-dried.

N-(4-Methylbenzyl) chitosan (Me-BzCh). FT-IR (KBr): ν 3430 (O—H and N—H stretching of alcohol and amine), 1514, 1452, (C=C stretching of aromatic), 1155, (C—O—C asymmetric stretching of GlcN), 1081 1033 (C—O stretching of GlcN), and 807 (C—H out of plane bending of aromatic) cm^{-1}. ^{1}H NMR (D_2O/CF_3COOD): δ (ppm) 7.18 (s; 4H Ph), 4.48–3.51 (br m; 7H $NHCH_2$, H3, H4, H5, H6 and H6′), 2.97 (br s; 2H H2 and H2′), 2.21 (s; 3H CH_3Ph), 1.91 (s; 3H $NHCOCH_3$).

N-(4-N,N-Dimethylaminobenzyl) chitosan ($N(CH_3)_2$-BzCh). FT-IR (KBr): ν 3430, 1602, 1526, 1155, 1081, 1033, and 811 cm^{-1}. ^{1}H NMR (D_2O/CF_3COOD): δ (ppm) 7.52 (s; 4H Ph), 4.93 (s; 1H H1), 4.42–3.53 (br m; 7H $NHCH_2$, H3, H4, H5, H6 and H6′), 3.12 (s; 6H $N(CH_3)_2$ Ph), 2.97 (br s; 2H H2 and H2′), 1.91 (s; 3H $NHCOCH_3$).

N-(4-Pyridylmethyl) chitosan (PyMeCh). FT-IR (KBr): *v* 3430, 1605, 1470, 1155, 1081, 1033, and 818 cm^{-1}. ^1H NMR (D_2O/CF_3COOD): δ (ppm) 7.22–6.95 (dd; 4H Py), 6.1 (s; 1H $CH{=}NH$), 3.51–4.48 (br m; 7H $-NHCH_2$, H3, H4, H5, H6 and H6′), 2.97 (br s; 2H H2 and H2′), 1.91 (s; 3H $NHCOCH_3$).

2.4. Regenerated chitosan and N-aryl chitosans

Chitosan and *N*-aryl chitosans (0.50 g) were regenerated by dissolving in 1% (w/v) aqueous acetic acid (100 mL). This solution was added dropwise into 2% (w/v) Na_2CO_3, (H_2O:MeOH; 40:60 v/v), (100 mL). The pH of the solution was adjusted to 9 by adding 15% (w/v) NaOH. The regenerated chitosan and *N*-aryl chitosans were then recovered by filtration and used while still moist for subsequent synthesis steps.

2.5. Isolation and purification

After methylation, methylated chitosan and its derivatives were precipitated in acetone (300 mL). The precipitate was dissolved in 15% (w/v) NaCl solution in order to replace the iodide counterion with a chloride counterion. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials. The dialyzed solution was then concentrated under vacuum using a rotary evaporator. The concentrated solution was added into acetone (100 mL) to precipitate the product, which was collected and dried overnight at room temperature under a stream of nitrogen.

2.6. Preparation of N,N,N-trimethylammonium chitosan chloride (TMChC)

Regenerated chitosan (about 0.50 g) was dispersed in NMP (25 mL) at room temperature and the mixture was stirred for 12 h. Then sodium iodide (1.5 g) and 15% (w/v) aqueous sodium hydroxide (3.0 mL) were added and the mixture was stirred at 50 °C for 15 min. Subsequently, iodomethane (1 mL) was added every 4 h; finally the mixture was stirred at 50 °C for 12 h. The isolation and purification of product was performed as described in Section 2.5.

TMChC. FT-IR (KBr): *v* 3444, 1475, 1107, 1071, and 1057 cm^{-1}. ^1H NMR (D_2O): δ (ppm) 5.42 (br s; 1H H1, H1′), 4.40–3.01 (br m; 23H $NHCH_2-$, H2, H3, H4, H5, H6 and H6′, s; OCH_3, br s; $N^+(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H $NHCH_3$), 1.97 (s; 3H $NHCOCH_3$). ^{13}C NMR (D_2O): δ (ppm) 96.55 (C1), 77.62 (C4), 74.74 (C5), 68.85 (C5), 60.0–55.5 (C2 and C6), 54.43 ($N^+(CH_3)_3$), 42.71 ($N(CH_3)_2$).

2.7. Preparation of high degree of quaternization of TMChC (HDQ-TMChC)

N,*N*,*N*-Trimethylammonium chitosan chloride (about 0.50 g) was further methylated as described in Section 2.6. Then a third methylation was carried out in the presence of iodomethane (2 mL) and a sodium hydroxide pellet and the stirring was continued for 6 h. The isolation and purification of product was performed as described in Section 2.5.

HDQ-TMChC. FT-IR (KBr): *v* 3438, 1475, 1108 1085, and 1057 cm^{-1}. ^1H NMR (D_2O): δ (ppm) 5.59 (br s; 2H H1, H1′), 4.64–3.13 (br m; 23H $NHCH_2$, H2, H3, H4, H5, H6 and H6′, s; OCH_3, br s; $N^+(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H $NHCH_3$), 1.97 (s; 3H $NHCOCH_3$).

2.8. Preparation of methylated N-aryl chitosans

About 0.50 g each of regenerated *N*-aryl chitosans, *N*-(4-methylbenzyl) chitosan, *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan and *N*-(4-pyridylmethyl) chitosan, was methylated as described in Section 2.6. A second set of reactions at 5%

Table 1
Methylation of chitosan and *N*-aryl chitosans

Samples	ES (%)	NaOH (w/v) (%)	DQ_T (%)		$N(CH_3)_2$ (%)	$NHCH_3$ (%)	3-*O* (%)	6-*O* (%)	Recovery (%)
			DQ_{Ar} (%)	DQ_{Ch} (%)					
TMChC	–	15	–	31	23	Trace	10	23	82
HDQ-TMChC	–	15	–	89	Trace	Trace	80	97	28
MMe-BzCh1	11	5	–	ND	32	10	ND	ND	80
MMe-BzCh2	11	15	–	11	61	ND	ND	Trace	76
$MN(CH_3)_2$-BzCh1	3	15	3	27	48	Trace	Trace	7	86
$MN(CH_3)_2$-BzCh2	10	5	10	12	65	Trace	ND	7	74
$MN(CH_3)_2$-BzCh3	17	5	17	16	28	13	ND	7	80
$MN(CH_3)_2$-BzCh4	17	15	17	30	20	17	Trace	15	68
$MN(CH_3)_2$-BzCh5	42	5	42	5	Trace	7	ND	Trace	78
$MN(CH_3)_2$-BzCh6	42	15	42	14	Trace	12	Trace	17	84
MPyMeCh1	12	5	12	18	45	Trace	ND	Trace	86
MPyMeCh2	12	15	12	36	22	7	3.3	7	76

ES is the extent of *N*-substitution; DQ_{Ar} is degree of quaternization at aromatic substituents; DQ_{Ch} is degree of quaternization; $N(CH_3)_2$ is *N*,*N*-dimethylation; $NHCH_3$ is *N*-methylation; DOM is total degree of *O*-methylation of 3-*O* and 6-*O* at 3-hydroxyl and 6-hydroxyl positions of GlcN of chitosan, respectively; recovery (%) is weight of product (g)/weight of starting reactant (g) × 100; ND is non- detectable.

(w/v) aqueous sodium hydroxide concentration (3.0 mL) was also performed. The results are summarized in Table 1.

Methylated N-(4-methylbenzyl) chitosan (MMe-BzCh). ^{1}H NMR (D_2O): δ (ppm) 7.36 (br s; 4H Ph), 5.40, 4.96 (s; 2H H1, H1′), 4.42–3.13 (br m; 23H $NHCH_2$—, H2, H3, H4, H5, H6 and H6′, s; OCH_3, br s; $N^{+}(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H CH_3 Ph), 1.97 (s; 3H $NHCOCH_3$).

Methylated N-(4-N,N-dimethylaminobenzyl) chitosan (MN(CH_3)$_2$-BzCh). FT-IR (KBr): ν 3442, 1559, 1475, 1147, 1104, 1059, and 850 cm^{-1}. ^{1}H NMR (D_2O): δ (ppm) 7.75–7.50 (dd; 4H Ph), 5.40, 4.96 (s; 2H H1, H1′), 4.42–3.13 (br m; 32H —$NHCH_2$, H2, H3, H4, H5, H6 and H6′, br s; $N^{+}(CH_3)_3$ Ph, s; OCH_3, br s; $N^{+}(CH_3)_3$), 2.71 (br m; 6H $N(CH_3)_2$), 2.31 (s; 3H $NHCH_3$), 1.97 (s; 3H $NHCOCH_3$). *^{13}C NMR (D_2O):* δ (ppm) 145.52, 141.53, 130.98, 119.56 (C-Ph), 96.55 (C1), 77.13–58.67 (C2, C3, C4, C5, and C6), 56.98 ($N^{+}(CH_3)_3$ Ph), 53.88 ($N^{+}(CH_3)_3$), 41.77 ($N(CH_3)_2$), 36.30 (NCH_3).

Methylated N-(4-pyridylmethyl) chitosan (MPyMeCh). FT-IR (KBr): ν 3453, 1474, 1103, and 1078 cm^{-1}. ^{1}H NMR (D_2O): δ (ppm) 8.60–7.95 (dd; 4H Py), 5.36, 4.96 (s; 2H H1, H1′), 4.42–3.15 (br m; 35H $NHCH_2$, H2, H3, H4, H5, H6 and H6′, s; OCH_3, s; $N^{+}CH_3$ Py, s; $N^{+}(CH_3)_3$), 2.83 (s; 6H $N(CH_3)_2$), 2.37 (s; 3H $NHCH_3$), 1.98 (s; 3H $NHCOCH_3$). ^{13}C NMR (D_2O): δ (ppm) 174.99, 160.69, 144.82, 127.45 (C-Py), 96.55 (C1), 78.8–60.9 (C2, C3, C4, C5, and C6), 68.31 ($N^{+}CH_3$ Py), 54.43 ($N^{+}(CH_3)_3$), 42.71 ($N(CH_3)_2$).

2.9. Antibacterial assessments

Antibacterial activity of methylated chitosan and its derivatives was assessed using *E. coli* ATCC 25911 (Gram-negative) and *S. aureus* ATCC 29113 (Gram-positive). The strains were kept frozen in tryptose-glycerol freezing media at −80 °C. These assessments were carried out in 50 mM phosphate buffer solution at pH 7.0. Bacterium was incubated in sterile nutrient broth (5 mL) for 12 h at 37 °C. Then 1 mL of this culture was transferred into a fresh sterile nutrient broth (25 mL) and incubated on a shaker for ca. 3.5 h at 37 °C. Following this, 2 mL of this solution was diluted with sterile nutrient broth such that the optical density (absorbance) of the resulting solution was either 0.200 (for *E. coli*) or 0.400 (for *S. aureus*). Finally, this solution was further diluted to a volume four times that of the original one using sterile nutrient broth. The resulting dilute solutions appeared clear to the eye and were used for antibacterial assessment against each bacteria. The cell count of bacteria in either solution was predetermined to be 4×10^{7}cells/mL. In order to test the antibacterial activity due to the chitosan derived agents, the diluted bacterial culture was incubated at 37 °C for 14 h in a 96-well plate in the presence of the agent with final concentrations ranging from 2 to 256 μg/mL in multiples of 2, in separate wells. After this period, individual results were visually assessed; the wells where bacterial growth occurred turned visibly turbid, indicating no activity against the microorganism growth. MIC (minimum inhibitory concentration) values were assigned as the lowest possible concentration of the agent that inhibited growth (indicated by a clear well). Control tests were simultaneously run to ensure proper bacterial growth within the diluted bacterial culture in absence of any agent and indication of no growth in solutions of the agent in absence of any bacterial culture. Each assessment was performed at least two times to ensure reproducibility of results (Daly & Manuszak-Guerrini, 1998).

3. Results and discussion

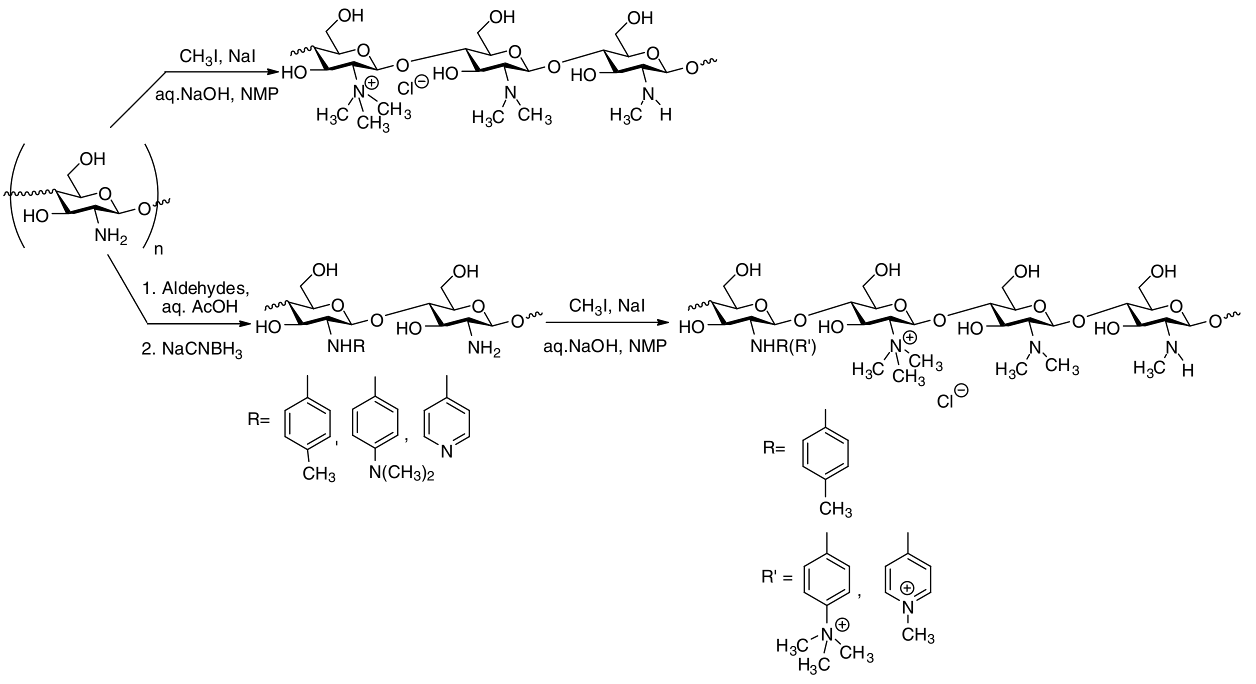
3.1. Synthesis and characterization of methylated chitosans

In this study, the procedure for methylation of chitosan derivatives with iodomethane in the presence of sodium hydroxide and sodium iodide in NMP at 50 °C (Scheme 1) was slightly modified from the method described by Elisabete et al. (2003). To achieve maximum DQ_{Ch}'s, the *N*,*N*,*N*-trimethylammonium chitosan chloride (TMChC) isolated in the first step was further methylated; the resultant derivative is identified as high degree of quaternization TMChC (HDQ-TMChC). Due to extensive *O*-methylation, the HDQ derivative was less soluble in water than the product obtained with a single methylation step.

The ^{1}H NMR spectra of TMChC and HDQ-TMChC (Fig. 1) had all the signals belonging to chitosan and the additional signals due to *N*-methylation and *O*-methylation similar to the ones reported by Sieval et al. The higher DQ_{Ch}'s (89%) and DOM's (177%) obtained after two sequential treatments with iodomethane. are easily observed in the spectra. Only traces of *N*,*N*-dimethylation and *N*-methylation were observed following the sequential treatments (Hamman & Kotze, 2001; Sieval et al., 1998).

3.2. Synthesis and characterization of methylated *N*-aryl chitosans

The syntheses of Me-BzCh, $N(CH_3)_2$-BzCh and PyMeCh carried out by reductive amination of the corresponding Schiff base intermediates (Scheme 1) have already been reported (Sajomsang, Tangpasuthadol, Tantayanon, Thatte, & Daly, 2006). The extent of *N*-substitution, ES, determined by ^{1}H NMR spectroscopy, varied as the different ratios of aldehyde to GlcN of chitosan were used. A Me-BzCh with ES 11% was methylated with iodomethane using a single treatment under the same conditions as used for the synthesis of TMChC. The ^{1}H NMR spectrum of methylated *N*-(4-methylbenzyl) chitosan, MMe-BzCh2, is shown in Fig. 2. The signal at δ 5.59 ppm is unambiguously assigned to H1′ proton of the GlcN of MMe-BzCh2. The signals at δ 7.2 and 2.2 ppm are due to aromatic protons and the methyl protons on aromatic substituents, respectively, and the signals at δ 3.2, 2.7, and 2.3 are assigned to *N*,*N*,*N*-trimethyl protons, *N*,*N*-dimethyl protons, and *N*-methyl protons of GlcN, respectively. In this case,



Scheme 1. Synthesis of methylated chitosan and *N*-aryl chitosans.

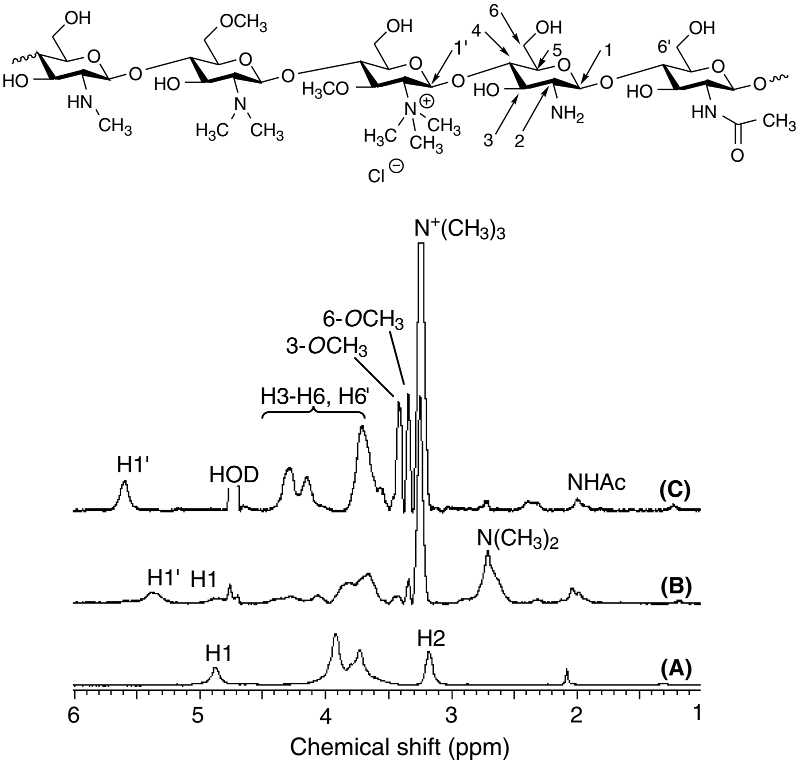


Fig. 1. ^{1}H NMR spectra of (A) chitosan (D_2O/CF_3COOD), (B) TMChC (D_2O), and (C) HDQ-TMChC (D_2O).

O-methylation at the GlcN of chitosan is also observed by the appearance of small signal at δ 3.3 ppm.

The typical ^{1}H NMR spectra of MN(CH_3)$_2$-BzCh1 and MPyMeCh2 are shown in Figs. 3 and 4, respectively. Both spectra are similar to those of the corresponding *N*-aryl chitosans except for the additional signal of H1′ proton of the GlcN of methylated chitosan at δ 5.40 ppm. The other additional signals at δ 3.5 ppm are due to the *N,N,N*-trimethyl protons at the aromatic substituent, and the signals at δ 3.2, 2.7, and 2.3 ppm are assigned to *N,N,N*-trimethyl protons, *N,N*-dimethyl protons, and *N*-methyl protons of the GlcN of chitosan, respectively.

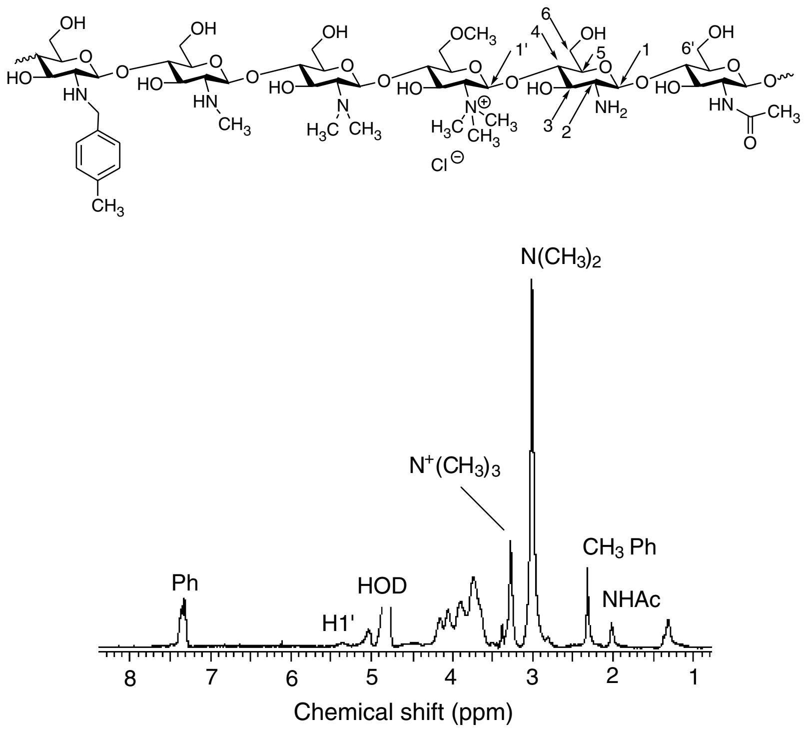


Fig. 2. ^1H NMR spectrum of methylated *N*-(4-methylbenzyl) chitosan (CF_3COOD/D_2O) with ES 11% using 15% (w/v) NaOH.

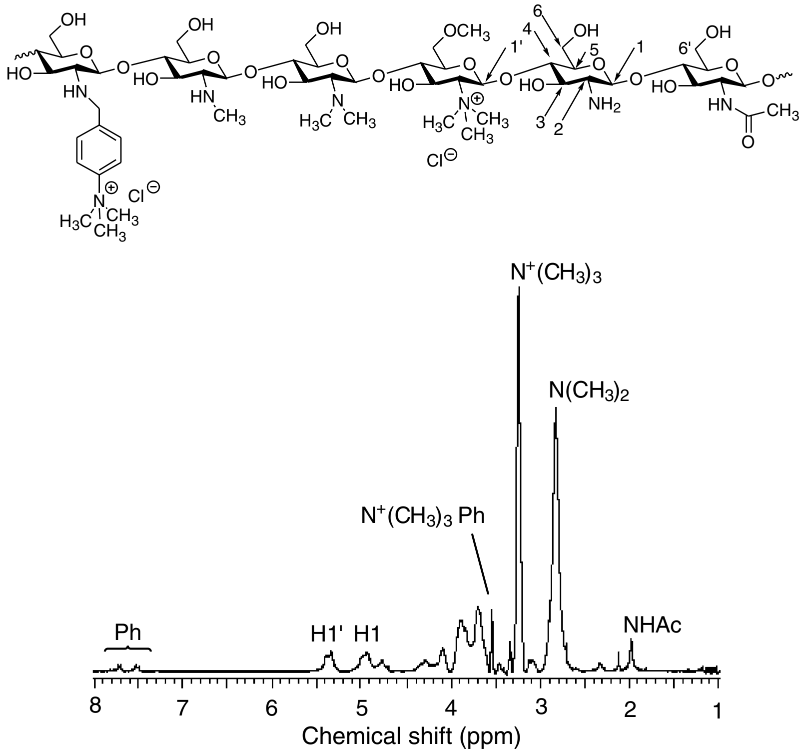


Fig. 3. ^1H NMR spectrum of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (D_2O) with ES 3% using 15% (w/v) NaOH.

The ^{13}C NMR spectrum of $MN(CH_3)_2$-BzCh3 is shown in Fig. 5. The aromatic carbon signals are exhibited at δ 145, 141, 131, and 119 ppm. The carbon signals at δ 96.3–58.7 and 57.0 ppm are due to C1—C6 and *N,N,N*-trimethyl carbon of the aromatic substituent, and those at δ 53.8, 41.8, and 36.3 ppm are due to *N,N,N*-trimethyl carbon, *N,N*-dimethyl carbon, and *N*-methyl carbon of the GlcN of chitosan, respectively.

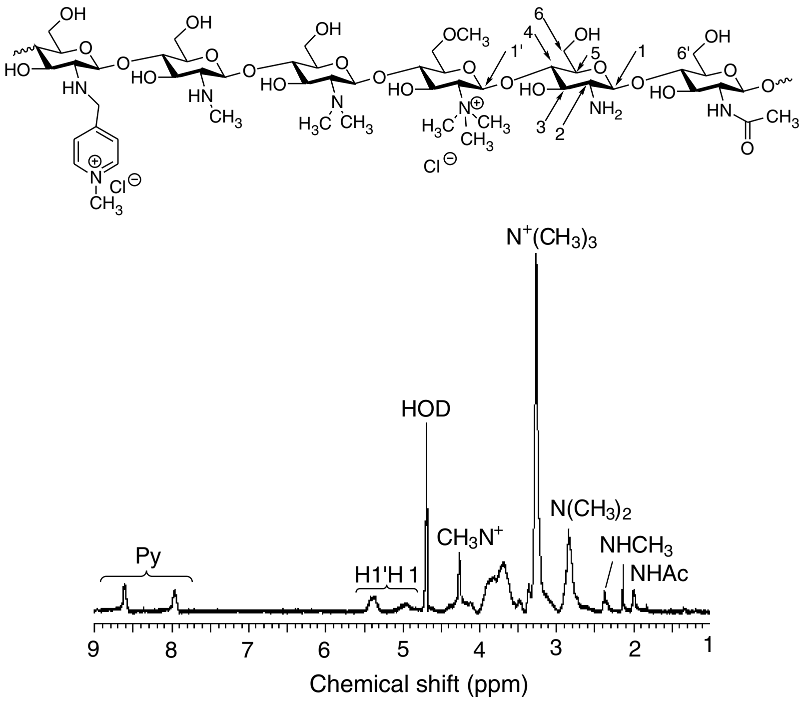


Fig. 4. ^{1}H NMR spectrum of methylated *N*-(4-pyridylmethyl) chitosan (D_2O) with ES 12% using 15% (w/v) NaOH.

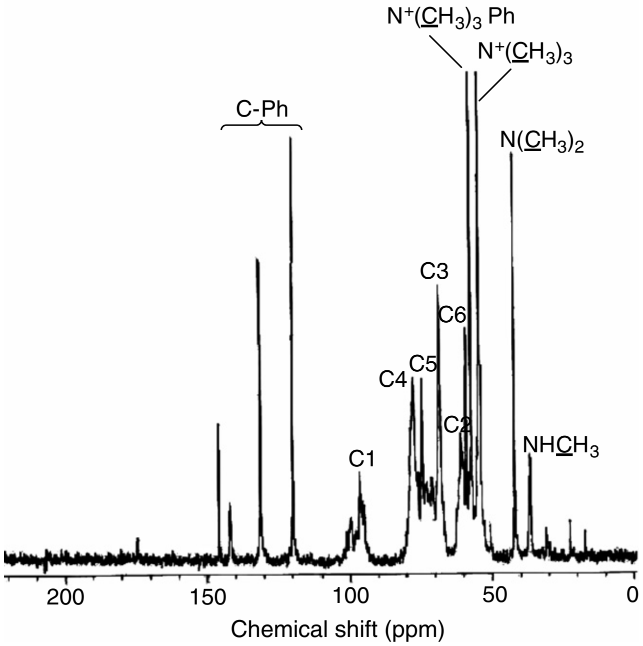


Fig. 5. ^{13}C NMR spectrum of methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (D_2O) with ES 17% using 5% (w/v) NaOH.

3.3. Chemoselectivity of methylation of N-(4-N,N-dimethylaminobenzyl) chitosans and N-(4-pyridylmethyl) chitosans

Table 1 showed the results of the methylation of chitosan containing aromatic moieties. The methylation of Me-BzCh by a single treatment with iodomethane yielded MMe-BzCh (Scheme 1). When 5% (w/v) sodium hydroxide solution was used, neither the formation of quaternary ammonium salts nor *O*-methylated products was observed. However, the presence of *N*,*N*-dimethylation and *N*-methylation were detected. In the presence of 15% (w/v) sodium hydroxide, MMe-BzCh was obtained with DQ_{Ch} 11%; only trace DOM was observed. The results indicated that the methylation requires a certain concentration of sodium hydroxide which must be high enough to prevent a protonation of unreacted primary amino groups from hydroiodic acid generated as the methylation proceeds. Interestingly, the DQ_{Ch} and DOM of MMe-BzCh were lower than TMChC when using 15% (w/v) sodium hydroxide in methylation. This could be attributed to the bulkiness of *N*-methylbenzyl substituent at a given GlcN causing steric hindrance to the *N*-methylation and *O*-methylation of neighbouring GlcN's of chitosan. Accordingly, after introducing the aromatic substituent at the primary amino groups of chitosan, the DQ_{Ch} and DOM is dependent not only on sodium hydroxide concentration but also the ES.

On the other hand, the methylation of $N(CH_3)_2$-BzCh with various ES's by single treatment with iodomethane yielded the corresponding quaternary ammonium salt, $MN(CH_3)_2$-BzCh. In this case, *N*-methylation could occur at both the aromatic substituent and primary amino group of chitosan. By comparison, $MN(CH_3)_2$-BzCh2 had DQ_{Ch} of 12% while none was detected for MMe-BzCh1 under the same conditions. Apparently the formation of the quaternary ammonium salt at *N*,*N*-dimethylaminobenzyl substituent, which made $MN(CH_3)_2$-BzCh2 more soluble in water, facilitated further reaction. The Me-BzCh was not water soluble. At 5% (w/v) sodium hydroxide, the DQ_{Ch} of $MN(CH_3)_2$-BzCh with various ES's was in the range of 5–16%. The DOM at 3-hydroxyl group was not observed while DOM at 6-hydroxyl group was 7%. *N,N,N*-Trimethylation, *N,N*-dimethylation and *N*-methylation at the primary amino group of GlcN of chitosan were also detected. The GlcN *N,N*-dimethylation decreased when higher ES were present. This reduction could be attributed to the steric effect of the aromatic substituent as well as the lower numbers of amino groups of GlcN available to react with iodomethane. With 15% (w/v) sodium hydroxide, the DQ_{Ch} increased to 14–30% and the DOM also increased to a trace at the 3-hydroxyl group and 17% at the 6-hydroxyl group.

The results clearly exhibited that the DQ_T, the sum of DQ_{Ar} and DQ_{Ch}, increased with increasing ES of $N(CH_3)_2$-BzCh. It also indicated that *N,N*-dimethylamino group on the aromatic substituent is more reactive than the primary amino group of chitosan. In other words, all *N,N*-dimethylamino groups were completely methylated giving DQ_{Ar} values equal to the corresponding ES's. This is confirmed by the ^1H NMR spectra shown in Fig. 6. The signal at δ 3.1 ppm of *N,N*-dimethyl protons was shifted to δ 3.5 ppm, corresponding to *N,N,N*-trimethyl protons on aromatic substituent (Poucher & Campbell, 1974). Moreover, increasing the sodium hydroxide concentration led to the increase in DQ_{Ch} and DOM (Fig. 7). However, increasing ES did not increase DQ_{Ch}, because of lower numbers of unsubtituted GlcN units. In addition, it was found that 6-*O*-methylation occurs more readily than 3-*O*-methylation (Table 1). This is expected because the intermolecular hydrogen bonds of the 6-hydroxyl groups are disrupted by the aryl substitution but the 3-hydroxyl groups retain their intramolecular hydrogen bonding among GlcN units. This effect is already evident at an ES of 3% ($MN(CH_3)_2$-BZCh1), the higher concentration of sodium hydroxide favors 6-*O*-methylation. In case of methylation of PyMeCh with iodomethane, the similar result is observed.

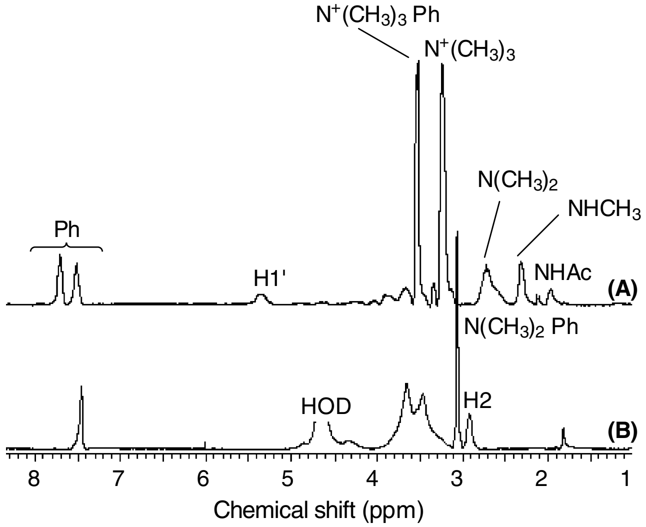


Fig. 6. ^1H NMR spectra of (A) methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (D_2O) and (B) *N*-(4-*N,N*-dimethylaminobenzyl)chitosan (D_2O/CF_3COOD) with ES 17% using 15% (w/v) NaOH.

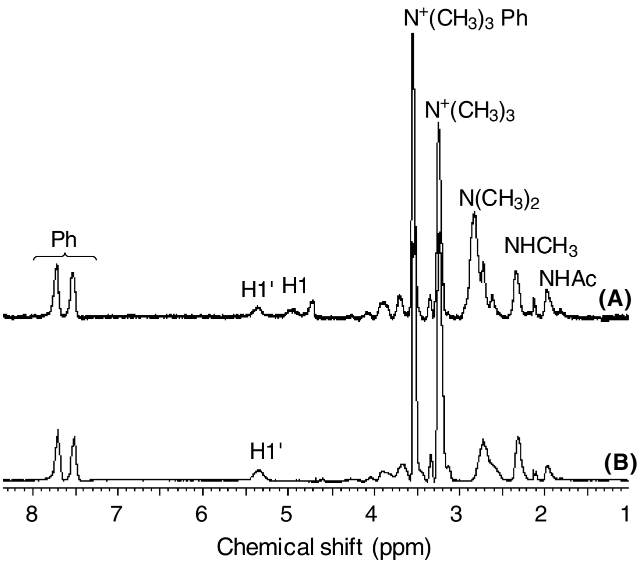


Fig. 7. ^1H NMR spectrum of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (D_2O) with ES 17% using (A) 5% (w/v) and (B) 15% (w/v) NaOH.

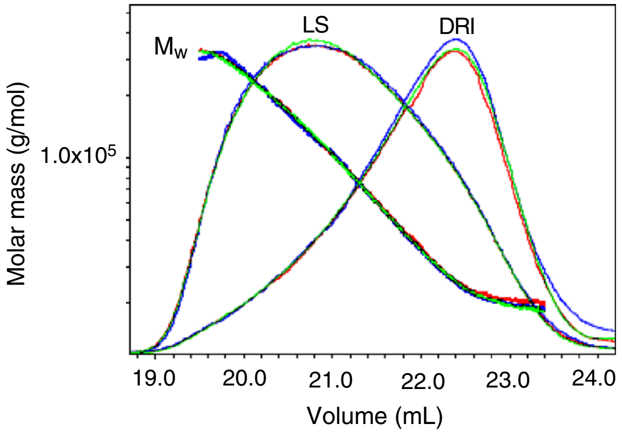


Fig. 8. Example of distribution of molar mass obtained by GPC-LS of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan with ES 17% and 5% (w/v) NaOH.

3.4. Reduction in molecular weight of chitosan after arylation and methylation

The molecular weight of chitosan and methylated chitosan derivatives were determined by gel permeation chromatography (GPC) with ViscoGEL Poly-CAT™ column that can be used under mild acidic conditions. In this study, multiple detection by differential reflective index (DRI), laser light-scattering (LS) and viscosity was used. Fig. 8 shows the molar mass distribution of $MN(CH_3)_2$-BzCh5 using LS and DRI detectors. Fig. 9 showed the relationship between RMS radius and molecular weight in logarithmic

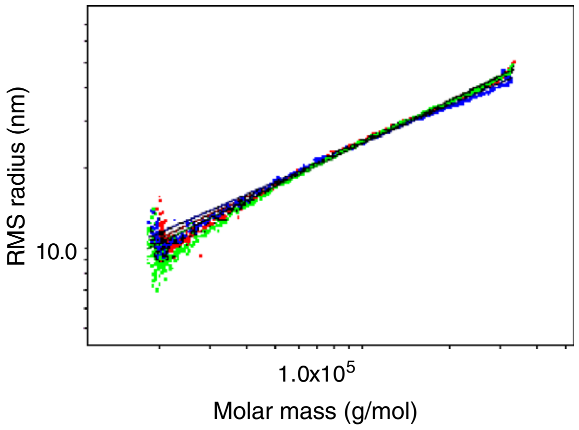


Fig. 9. Relationship between RMS radius values and the molar mass (g/mol) of methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan with ES 17% and 5% (w/v) NaOH.

scale measured by GPC-LS of MN(CH_3)$_2$-BzCh5. From GPC-LS data, the function radius of gyration (R_g) can be obtained directly. The increment of refractive index (dn/dc) was determined based on $\Delta n = c(dn/dc)$, where Δn is the refractive index of polymer in dilute 5% acetic acid, c is the concentration of polymer, and n is the solution refractive index. The weight average molecular weight of chitosan and its derivatives were determined by the GPC-LS system (Table 2) based on Zimm plots processed by Astra V software. Every sample measurement was repeated three times. In our previous work, we found that the treatment of chitosan with benzaldehyde and Borch reduction caused a decrease in the molecular weight of chitosan (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2006). The molecular weight of chitosan in the *N*-arylation step decreased slightly. The methylation step leading to MN(CH_3)$_2$-BzCh3 is accompanied by a significant molecular weight decrease. The M_w reduction suggests that numerous backbone cleavages are occurring during the combined *N*-arylation and methylation steps (Table 2). After methylation of N(CH_3)$_2$-BzCh, the weight average molecular weight of the chitosan derivative decreased from 276 to 62 kDa. It was plausible that an oxidative degradation process and alkaline depolymerization occurred. These results demonstrate that estimating molecular weights of chitosan products based upon the initial molecular weight of the starting chitosan can be misleading (Holappa et al., 2006). The low value of the R_g indicated that the methylated derivatives might be able to diffuse more easily through bacterial cell walls, and thus they would be more effective biocides.

3.5. Antibacterial activity of methylated chitosan and methylated N-aryl chitosans

Antibacterial activities of methylated chitosans and their derivatives were evaluated using MIC values against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria at pH 7.0 (Table 3). Their MIC values were in the range of 32–128 μg/mL against both bacteria. Generally, the antibacterial activity at neutral pH was affected by DQ_{Ch}. The higher DQ_{Ch} would enhance antibacterial activity due to larger number of positive charges encountered in electrostatic interactions between the positively charge of quaternary ammonium group of chitosan macromolecule and negatively charged bacterial cell wall which led to the leakage of proteinaceous and other intracellular constituents (Rabea,

Table 2
Molecular weight, molecular size, increment of refractive index, and distribution of chitosan and methylated chitosan derivatives from GPC-LS

Samples	ES (%)	DQ_{Ch} (%)*	dn/dc (mL/g)	M_n (kDa)	M_w (kDa)	M_w/M_n	η (mL/g)	R_g (nm)
Ch	–	–	0.165	87	276	3.2	434.5	56.4
TMChC	–	31	0.128	71	162	2.3	121.7	28.4
MN(CH_3)$_2$-BzCh3	17	16	0.151	35	62	1.8	90.1	27.9

* DQ_{Ch} was determined by ^{1}H NMR (Sieval et al., 1998).

Table 3
Antibacterial activity of methylated chitosan and methylated *N*-aryl chitosans

Samples	ES (%)	Water solubility[a]	DQ_T (%)		MIC (μg/mL)	
			DQ_{Ar}	DQ_{Ch}	*S. aureus*	*E. coli*
TMChC	–	++	–	31	32	128
HDQ-TMChC	–	+	–	89	64	128
MN(CH_3)$_2$-BzCh1	3	++	3	27	32	128
MN(CH_3)$_2$-BzCh2	10	++	10	12	64	128
MN(CH_3)$_2$-BzCh3	17	++	17	16	32	64
MN(CH_3)$_2$-BzCh4	17	++	17	30	32	64
MN(CH_3)$_2$-BzCh5	42	+	42	5	64	128
MN(CH_3)$_2$-BzCh6	42	+	42	14	64	128
MPyMeCh1	12	++	12	18	128	128
MPyMeCh2	12	++	12	36	64	128

++, totally soluble in one minute; +, partially soluble.
[a] 10 mg/mL.

Badawy, Stevens, Smagghe, & Steurbaut, 2003). It should be noted that HDQ-TMChC had higher QD_{Ch} than TMChC and its derivatives but its antibacterial activity against *S. aureus* was decreased probably due to its high DOM, which reduced its solubility in water (Sieval et al., 1998). Introducing the *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl substituents into chitosan backbone did not contribute to the antibacterial activity against *S. aureus*. Although DQ_T was higher than that of TMChC, the antibacterial activity of the corresponding methylated chitosans against *S. aureus* did not dramatically increase. However, it was noted that with DQ_{Ar} 17%, $MN(CH_3)_2$-BzCh3 and $MN(CH_3)_2$-BzCh4 exhibited a slightly higher antibacterial activity against *E. coli* while with DQ_{Ar} 42%, $MN(CH_3)_2$-BzCh5 and $MN(CH_3)_2$-BzCh6 did not enhance the antibacterial activity against either bacteria relative to the activity exhibited by TMChC. The high ES (42%) of *N,N*-dimethylaminobenzyl substituents significantly changed the hydrophilic/hydrophobic balance and reduced the potential interaction of these derivatives with the bacterial cell wall.

4. Conclusion

Methylated *N*-(4-methylbenzyl) chitosan was insoluble in water. Methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan and methylated *N*-(4-pyridylmethyl) chitosan yielded derivatives with a high total degree of quaternization which were soluble in water. *N*-Methylation of *N*-aryl chitosans containing *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl substituents produced quaternary ammonium salts of both the aryl substituents and primary amino group of GlcN. The presence of the amino aromatic substituents increased the total degree of quaternization of chitosan. The concentration of sodium hydroxide controlled the chemoselectivity of the methylation. The methylated *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl chitosan derivatives were water soluble over an entire range of pHs and displayed antibacterial activity against *S. aureus* and *E. coli*. They did not, however, show enhanced antibacterial activity against *S. aureus* when compared to that of *N*-trimethylated chitosan. Methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan with ES 17% exhibited high antibacterial activity against *E. coli*. At ES higher than 17%, the antibacterial activity against both bacteria was reduced, possibly due to the lower hydrophilic/hydrophobic balance. However, methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan and methylated *N*-(4-pyridylmethyl) chitosan derivatives will be useful as potential new polycationic materials.

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