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Quaternization of *N*-(3-pyridylmethyl) chitosan derivatives: Effects of the degree of quaternization, molecular weight and ratio of *N*-methylpyridinium and *N*,*N*,*N*-trimethyl ammonium moieties on bactericidal activity

Warayuth Sajomsang*, Uracha Rungsardthong Ruktanonchai, Pattarapond Gonil, Choochart Warin

National Nanotechnology Center, Nanodelivery System Laboratory, National Science and Technology Development Agency, Thailand Science Park, Pathumthani 12120, Thailand

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ABSTRACT

Methylated *N*-(3-pyridylmethyl) chitosan chloride (M3-PyMeChC) with various degrees of *N*-substitution (DS), degrees of quaternization (DQ), and molecular weights were synthesized by single methylation of *N*-(3-pyridylmethyl) chitosan with iodomethane under the basic condition. The DS and DQ were calculated by ^{1}H NMR, and they were in a range of $35 \pm 1\%$–$80 \pm 2\%$ and $72 \pm 1\%$–$80 \pm 2\%$, respectively. Depolymerization of the methylated chitosan derivatives was carried out by using 0.1–0.5% (w/v) of sodium nitrite solution and their molecular weights were determined by gel permeation chromatography. It was found that the concentration of sodium nitrite solution increased with a decrease in molecular weight. Bactericidal activity of these M3-PyMeChCs were determined by using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods against *Escherichia coli* ATCC 25922 (Gram-negative) and *Staphylococcus aureus* ATCC 6538 (Gram-positive) bacteria compared to the *N*,*N*,*N*-trimethyl chitosan chloride (TMChC) in accordance with the standard method of the National Committee for Clinical Laboratory Standards (NCCLS). It was found that the bactericidal activity was dependent on the chemical structure of quaternary ammonium moiety and the molecular weight, at constant DQ. The result revealed that the *N*,*N*,*N*-trimethyl ammonium group showed higher bactericidal activity than the *N*-methylpyridinium group at similar DQ and molecular weight. Moreover, the bactericidal activity of M3-PyMeChC tended to decrease with decreasing molecular weight from 82 kDa to 8 kDa, at DQ of $80 \pm 2\%$.

1. Introduction

It is known that pyridine is a simple aromatic heterocyclic organic compound used as a precursor to agrochemicals and pharmaceuticals in addition to being an important solvent and reagent. Since, the pyridine is a base with chemical properties similar to tertiary amines, it can be easily attacked by alkylating agents to obtain *N*-alkylpyridinium salts (Fujimoto et al., 2006). Pyridine derivatives were introduced into the polymer backbone in order to improve polymer properties including solubility, physicochemical and biological properties. Introduction of pyridine derivatives into the chitosan (Ch) backbone has recently major gained interest because it can be applied in metal absorption (Baba & Hirakawa, 1992; Inoue, Ohto, Yoshizuka, Yamaguchi, & Tanaka, 1997; Rodrigues, Laranjeira, de Favere, & Stadler, 1998), antimicrobial activity (Badawy, 2008; Kumar, Dutta, & Dutta, 2009; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008), gene delivery (Opanasopit et al., 2008; Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009), sensor application (Bao & Nomura, 2002) and biomedical application (Kumar et al., 2009; Sajomsang, Rungsardthong Ruktanonchai, Gonil, & Nuchuchua, 2009). Ch is a cationic polysaccharide obtained by deacetylation of naturally occurring polymer chitin. It consists of β-(1,4)-2-amino-2-deoxy-D-glucopyranose units (GlcN) and a small amount of 2-acetamido-2-deoxy-D-glucopyranose or *N*-acetyl-D-glucosamine (GlcNAc) residues. *N*-(2-Pyridylmethyl) Ch was firstly reported by Baba and Hirakawa (1992). It was prepared by a reductive amination reaction, which is based on the reaction between the primary amino group of Ch and an aldehyde group of pyridinecarboxaldehyde under mild acidic condition. The reaction is occurred through Schiff base intermediate following by the reduction with sodium borohydride or sodium cyanoborohydride. Subsequently, *N*-(2-pyridylmethyl) Ch and *N*-(4-pyridylmethyl) Ch have been synthesized by several research groups (Rodrigues et al., 1998; Sajomsang, Tantayanon, Tangpasuthadol, Thatte, & Daly, 2008; Sashiwa & Shigemasa, 1999). However, less attention has been paid on the synthesis of *N*-(3-pyridylmethyl) Ch.

* Corresponding author. Tel.: +66 2 564 7100; fax: +66 2 564 6981.
E-mail address: warayuth@nanotec.or.th (W. Sajomsang).

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Recently, *N*-(3-pyridylmethyl) Ch (3-PyMeCh) was synthesized by Badawy (2008). The fungicidal activity of 3-PyMeCh against soil-borne pathogenic fungi, *Pyricularia grisea*, *Pythium debrianum* and *Fusarium oxysporum*, was found with half maximal effective concentrations (EC_{50}) of 2.18, 0.80 and 2.23 mg/mL, respectively. Moreover, the 3-PyMeCh showed a growth inhibition and an antifeedant against the larvae of the cotton leafworm, *Spodoptera littoralis* at the measured concentration of 3 mg/g. When compared with Ch, the 3-PyMeCh showed higher fungicidal and insecticidal activities. However, its biological activity appears only in acidic medium because of its poor solubility in neutral and basic pH.

In order to enhance the water solubility, the methylation (quaternization) of 3-PyMeCh was performed to yield the *N*-methylpyridinium salt. The physicochemical properties of methylated *N*-(3-pyridylmethyl) Ch chloride (M3-PyMeChC) with various degrees of *N*-substitution (DS) and degrees of quaternization (DQ) were investigated. Previously, Holappa et al. (2006) have reported that Ch *N*-betainate with various DSs showed low antibacterial activity against *S. aureus* and *E. coli* at physiological pH and the antibacterial activity decreased with an increasing DS under acidic condition (pH 5.5). Moreover, they suggested that the position of the quaternary group is important factor for antibacterial activity of Ch. This suggestion is consistent with our recent report that the methylated *N*-(4-pyridylmethyl) Ch chloride (M4-PyMeChC) with DQ 80% did not enhance antibacterial activity against *S. aureus* and *E. coli* at neutral pH compared to the TMChC with DQ 64%. This could be due to differences in molecular weight of these compounds (Sajomsang, Gonil, & Saesoo, 2009). It is believed that the antibacterial action is due to adsorption of the positive charged of quaternary ammonium groups onto the negative charged of the bacterial cell surfaces and membrane with subsequent disruption of membrane integrity (Rabea, Badawy, Stevens, Smagghe, & Steurnaut, 2003). Therefore, the chemical structure of a quaternary ammonium groups is one of important factors that played an important role in antibacterial activity. In this study, the M3-PyMeChC was used as a model structure to study the effect of *N*-methylpyridinium group onto the Ch backbone on the bactericidal activity compared to the *N*,*N*,*N*-trimethyl group. We found that the M3-PyMeChC has higher molecular weight than M4-PyMeChC after methylation with iodomethane. Therefore, it can be easily compared to the *N*,*N*,*N*-trimethyl Ch chloride (TMChC) with similar molecular weight. Moreover, the bactericidal activity of the M3-PyMeChC has not been reported yet. The bactericidal activity was evaluated by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Staphylococcus aureus* ATCC 6538 (Gram-positive) and *Escherichia coli* ATCC 25922 (Gram-negative) bacteria at pH 7.2 compared to the TMChC in accordance with the standard method of the National Committee for Clinical Laboratory Standards (NCCLS). The effects of chemical structure of a quaternary ammonium groups and ratio between *N*-methylpyridinium and *N*,*N*,*N*-trimethyl ammonium moieties on the bactericidal activity were discussed at similar DQ and molecular weight. Furthermore, the effects of DQ and molecular weight of the M3-PyMeChC on the bactericidal activity were investigated.

2. Experimental

2.1. Materials and reagents

Ch with average molecular weights (M_w) of 276 and 16 kDa were purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of Ch was determined to be 90% by ^{1}H NMR spectroscopy (Lavertu et al., 2003). A dialysis tubing with M_w cut-off of 12,000–14,000 and 3500 g/mol from Cellu Sep T4, Membrane Filtration Products, Inc. (Segiun, TX, USA) were used to purify all Ch derivatives. 3-Pyridinecarboxaldehyde and silver nitrate were purchased from Fluka (Deisenhofen, Germany). Sodium cyanoborohydride, sodium borohydride, iodomethane, and 1-methyl-2-pyrrolidone were purchased from Acros Organics (Geel, Belgium). Sodium iodide, sodium nitrite and hydrochloric acid were purchased from Carlo Erba Reagent (Italy). All chemicals and solvents were used as received without any further purification.

2.2. Instrumentation

All attenuated total reflectance fourier transform infrared (ATR-FTIR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA) using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at the ambient temperature (25 °C). These spectra were collected by using rapid-scan software in OMNIC 7.0 with 32 scans and a resolution of 4 cm^{-1}. The ^{1}H NMR spectra were measured on AVANCE AV 500 MHz spectrometer (Bruker, Switzerland). All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. D_2O/CD_3COOD and D_2O were used as the solvents for dissolving 5 mg of Ch or 3-PyMeCh and M3-PyMeChC, respectively.

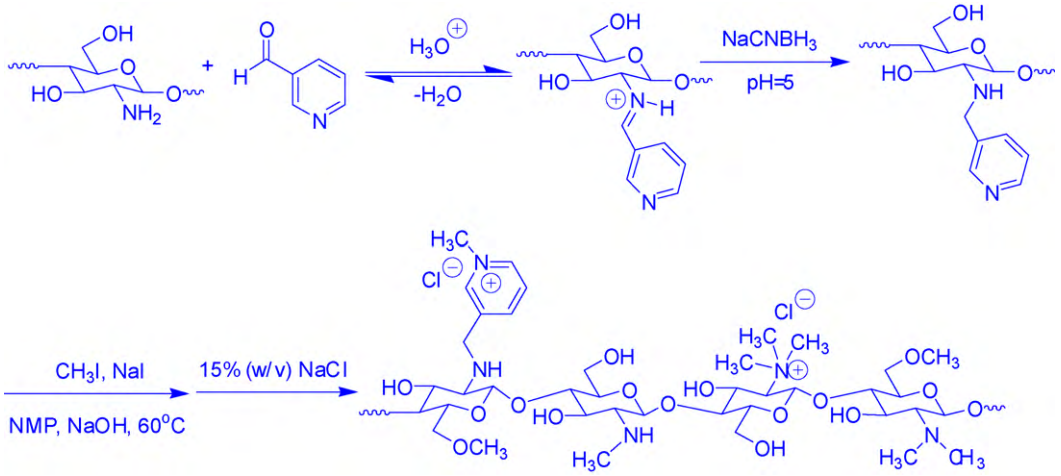
2.3. Synthesis of N-(3-pyridylmethyl) chitosan derivatives

Synthesis of the *N*-(3-pyridylmethyl) Ch (3-PyMeCh) was carried out in accordance with previously reported procedure shown in Scheme 1 (Sajomsang, Tantayanon, Tangpasuthadol, Thatte, et al., 2008). Ch (2.0 g, 12.22 mmol) was dissolved in 1% (v/v) of acetic acid (150 mL). The solution was then diluted with ethanol (100 mL). After that 3-pyridinecarboxaldehyde (0.4–2.0 mequiv./GlcN, 0.5–2.6 g) was added and stirred at the room temperature for 24 h. The pH of the solution was adjusted to 5 with 15% (w/v) of sodium hydroxide. Subsequently, sodium cyanoborohydride (3.08 g, 48.92 mmol) was added and stirred at room temperature for 24 h, followed by pH adjustment to 7 with 15% (w/v) of sodium hydroxide. The reaction mixture was then dialyzed in distilled water for 3 days in order to remove any impurity and freeze-dried to give powder 3-PyMeCh.

3-PyMeCh: ATR-FTIR; ν 3245, 2910, 2860, 1595, 1578, 1427, 1148, 1061, 1022, 788 and 707 cm^{-1}. ^{1}H NMR (D_2O/CD_3COOD): δ (ppm) 8.52–7.65 (m; 4H Py), 4.80 (s; 1H H1), 4.59 (s; 2H CH_2–NH) 4.20–3.0 (m, 6H H2–H6) and 1.89 (s; 3H NHAc).

2.4. Synthesis of methylated N-(3-pyridylmethyl) chitosan chloride

Previously, the methylation of Ch and its derivatives have been carried out by a single treatment with iodomethane in the presence of sodium hydroxide, sodium iodide and *N*-methyl pyrrolidone (NMP) (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008). A mixture of Ch (1.0 g) and NMP (50 mL) were stirred at the room temperature for 12 h. Then 15% (w/v) of sodium hydroxide (8.0 mL) and sodium iodide (3.0 g) were added and stirred at 60 °C for 15 min. Subsequently, iodomethane (8.0 mL) was added in three portions at 3 h intervals and stirred at 60 °C for 24 h. The reaction mixture was precipitated in acetone (600 mL). The precipitate was then dissolved in 15% (w/v) of sodium chloride in order to replace the iodide ions with chloride ions. After that the suspension was dialyzed with deionized water for 3 days to remove inorganic materials and then freeze-dried to give a cotton-liked powder of *N*,*N*,*N*-trimethyl Ch chloride (TMChC with DQ 30 ± 3%). Using the same technique described above, the 3-PyMeChs were used instead of Ch. In addition, a high DQ of TMChC was prepared by repeated methylation of TMChC similar to the procedure described above.



Scheme 1. Synthesis of methylated *N*-(3-pyridylmethyl) chitosan derivatives.

TMChC: ATR-FTIR; ν 3304 (O–H and N–H, GlcN), 2922, 2885, and 2834 (C–H, GlcN), 1470 (C–H, $N^+(CH_3)_3$), 1144 (C–O–C, GlcN), 1047, and 1029 cm^{-1} (C–O, GlcN). ^1H NMR (D_2O): δ (ppm) 5.4 (br. s; 1H H1′), 4.4–3.0 (br. m; 22H H2–H6, s; 6H *3*, *6-O*–CH_3; s, 9H $N^+(CH_3)_3$), 2.7 (br. m; 6H $N(CH_3)_2$), 2.3 (s; 3H $NHCH_3$), 1.9 (s; 3H NHAc). ^{13}C NMR (D_2O): δ (ppm) 96.5 (C1), 77.6 (C4), 74.7 (C5), 68.8 (C5), 60.0–55. (C2 and C6), 54.4 ($N^+(CH_3)_3$), 42.7 ($N(CH_3)_2$).

M3-PyMeChC: ATR-FTIR; ν 3349, 3056, 2924, 2877, 2815, 1654, 1633, 1506, 1472, 1195, 1106, 1028, 811 and 676 cm^{-1}. ^1H NMR (D_2O): δ (ppm) 8.70–7.90 (m; 4H Py), 5.40 (br. s; 1H H1′), 4.23–3.00 (br. m; 26H CH_2–NH, H2–H6, s, 6H *3*, *6-O*–CH_3, s; N^+CH_3 Py, s; 9H $N^+(CH_3)_3$), 2.60 (s; 6H $N(CH_3)_2$), 2.29 (s; 3H $NHCH_3$) and 1.92 (s; 3H NHAc).

2.5. X-ray diffraction

The X-ray diffraction (XRD) patterns of the Ch, 3-PyMeCh and M3-PyMeChC were obtained by JEOL JDX-3530 theta–2theta X-Ray diffractometer (Aremco Products, Inc., USA) with Cu Kα radiation (λ = 0.154 nm). The 2θ angle was scanned between 5° and 50° at 25 °C. The voltage was 40 kV and the intensity was 50 mA.

2.6. Estimation of water solubility

The water solubility of the TMChCs and M3-PyMeChCs with various pHs and concentrations was determined by using turbidity measurement. All samples were dissolved in deionized water. Then 0.1 or 1 M HCl solution and 0.1 or 1.0 M NaOH solution were slowly added. The transmittance of their solutions was recorded on a Lamda 650 UV/vis Spectrophotometer (PerkinElmer, USA) with an optical path length of 350 nm at 600 nm. The experiment was performed at 25 °C.

2.7. Determination of degree of N-substitution and degree of quaternization

In this study, the DS and DQ were generally determined by using ^1H-NMR spectroscopy as shown in Eqs. (1) (Crini et al., 1997) and (2), respectively (Sieval et al., 1998) while the degree of *O*-methylation (DOM) was determined by using equation 3 (Polnok, Borchard, Verhoef, Sarisuta, & Junginger, 2004)

$$\mathrm{DS}\,(\%) = \frac{\mathrm{Ar}/n}{[\mathrm{H}_2 + 1/3\,\mathrm{NHAc})]} \times 100 \quad (1)$$

where DS (%) is the degree of *N*-substitution, Ar is the integral area of aromatic protons, *n* is the number of aromatic hydrogen atoms per substituent, H_2 is the integral areas of the protons at C-2 carbon of GlcN, and NHAc is the integral area of GlcNAc protons

$$\mathrm{DQ}\,(\%) = \frac{(\mathrm{N}^+(\mathrm{CH}_3)_3/9)}{\mathrm{H1}'} \quad (2)$$

where DQ (%) is the degree of quaternization, the DQ at the primary amino groups of Ch is denoted as DQ_{Ch}, while the DQ at N atom of M3-PyMeChC is denoted as DQ_{Ar}. $N^+(CH_3)_3$ is the integral area of the *N*,*N*,*N*-trimethyl protons at δ 3.2 ppm, and H1′ is the integral area of both H1′ and H1 protons in the range of δ 5.3–5.0 ppm.

$$\mathrm{DOM}\,(\%) = \frac{\mathrm{O}\text{–}\mathrm{CH}_3/3}{\mathrm{H1}'} \quad (3)$$

where DOM (%) is the degree of *O*-methylation, *O*-CH_3 is the integral area of methoxy protons of either 3- or 6-hydroxy groups at δ 3.4 ppm or 3.3 ppm, respectively, and H1′ is the combined integral area of the H1′ and H1 protons in the range of δ 5.3–5.0 ppm.

2.8. Determination of chloride ion content

The chloride ion content of the methylated Ch derivatives was determined by potentiometric titration with silver nitrate ($AgNO_3$) solution. The titration curve was recorded on T50 Titrator using DM141-SC sensor (Mettler Toledo, USA). Briefly, 20 mg of each methylated Ch derivatives was dissolved in 50 mL of the deionized water. The sample solution was titrated by using 0.01 M silver nitrate solution. The volume of added silver nitrate solution and potential value of the solution were recorded.

2.9. Depolymerization of methylated chitosan derivatives

Methylated Ch derivatives were depolymerized by oxidative degradation using sodium nitrite ($NaNO_2$) solution according to the procedure described by Janes and Alonso, and Knight et al. (Knight, Shapka, & Amsden, 2007; Janes & Alonso, 2003). For this purpose, 0.1 mL of $NaNO_2$ (0.1–0.3%, w/v) was added to 2 mL of the methylated Ch derivatives solution (1%, w/v). Then 1 M hydrochloric acid was added to achieve solution at pH 5 and continue stirring at 25 °C. The reaction was left overnight to ensure completion of the degradation. Sodium borohydride was added and allowed to react for an additional 30 min to reduce the terminal aldehyde groups to hydroxyl groups. Then the solution was dialyzed with deionized water for 2 days to remove the impurity. Finally, the resulting methylated Ch derivative solution was then freeze-dried.

Table 1
N-arylation of chitosan with 3-pyridinecarboxaldehyde ($n = 3$).

Entries	Samples	Molar ratio (aldehyde:GlcN)	Targeted DS (%)	FW	Obtained DS (%)	Yield (%)
1	3-PyMeCh	0.4:1	40	195.04	35 ± 1	95 ± 2
2	3-PyMeCh	1:1	100	217.54	60 ± 1	93 ± 2
3	3-PyMeCh	2:1	200	235.54	80 ± 2	85 ± 1

FW is the formula weight of repeating unit = 12.2 + [FW of *N*-(3-pyridylmethyl) GlcN × DS] + [161 × (0.94 − DS)], DS is degree of *N*-substitution. Yield (%) = weight of *N*-(3-pyridylmethyl) chitosan derivatives (g) × [163.5/weight of chitosan (g) × theoretical calculated FW of *N*-(3-pyridylmethyl) GlcN] × 100.

2.10. Molecular weight determination

The weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of Ch and its derivatives were determined by using the gel permeation chromatography (GPC). It consists of Waters 600E Series generic pump, injector, ultrahydrogel linear columns (M_w resolving range 1 kDa to 20,000 kDa), guard column, pollulans as standard (M_w 5.9–788 kDa), and refractive index detector (RI). All samples were dissolved in acetate buffer pH 4 and then filtered through VertiPure nylon syringes filters 0.45 μm (Vertical chromatography Co., Ltd., Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4), were used at a flow rate of 0.6 mL/min at 30 °C. Then the injection volume 20 μL was used.

2.11. Bactericidal assessments

The bactericidal tests were performed in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) to determine broth microdilution minimum inhibitory concentration (MIC) values. The microorganisms used in the inhibitory test were obtained from the Department of Medical Sciences (Ministry of Public Health, Thailand). The microorganisms used in this study were *Staphylococcus aureus* ATCC 6538 as a model for Gram-positive bacteria and *Escherichia coli* ATCC 25922 as a model for Gram-negative bacteria. The strains were all cultured on Tryptic Soy Agar (TSA) (Difco, USA) and Mueller-Hinton Broth (MHB) (Difco, USA), incubated aerobically at 35.5 °C overnight. Water used for all experiments was distilled water obtained from the MilliQ Plus (Millipore, Schwalbach, Germany).

For the growth culture, one colony from culture on the TSA was inoculated into the MHB and incubated aerobically at 35.5 °C for 24 h. Then bacterial concentrations were determined by measuring optical density (OD) at wavelength of 600 nm at 0.2 (OD of 0.2 corresponded to a concentration of 10^8 CFU/mL) with a spectrophotometer (Spectronic G20, USA). The $MIC_{90\%}$ of the methylated Ch derivative solutions prepared as serial twofold dilutions for each tested organism was determined by modification of the broth dilution method in 96-well microtiter plate. The growth of bacteria was determined at the difference in absorbance after 24 h incubation at 35.5 °C. The absorbance at 600 nm was then determined by using microplate reader (Molecular Devices, SpectraMax M2, USA). All experiments were performed in triplicates against each tested microorganisms. The lowest concentration which inhibited microbial growth was reported as $MIC_{90\%}$, whereas minimal bactericidal concentration (MBC) was defined as the lowest concentration of the compound to kill the microorganisms (NCCLS, 2000, 2002).

3. Results and discussion

3.1. Synthesis of N-(3-pyridylmethyl) chitosan and its methylated derivatives

The *N*-(3-pyridylmethyl) Ch (3-PyMeCh) was carried out by reacting Ch with 3-pyridinecarboxaldehyde under mild acidic condition (Scheme 1). The reaction was occurred easily via Schiff base intermediate. Then the Schiff base was reduced with sodium cyanoborohydride. Previously, the *N*-pyridylmethyl Ch derivatives have been successfully synthesized by many research groups (Baba & Hirakawa, 1992; Badawy, 2008; Rodrigues et al., 1998; Sajomsang, Tantayanon, Tangpasuthadol, Thatte, et al., 2008). It was a versatile and specific method for creating a covalent bond between a substrate and the amine function of the Ch. The DS determined by the ^{1}H NMR spectroscopy (Eq. (1)), was dependent on the mole ratios of 3-pyridinecarboxaldehyde and the primary amino group of Ch. It was found that the DS increased with an increasing mole ratios of 3-pyridinecarboxaldehyde (Sajomsang, Tantayanon, Tangpasuthadol, Thatte, et al., 2008). The DS and yield of the 3-PyMeCh were in the range of 35 ± 1%–80 ± 2% and 85 ± 1%–95 ± 2%, respectively (Table 1). The single methylation (quaternization) of the 3-PyMeCh was carried out by using iodomethane as a methylating agent in the presence of sodium hydroxide, sodium iodide and NMP, which yielded *N*-methylpyridinium salt. Basically, the methylation was based on nucleophilic substitution of the primary amino group on the C-2 position of Ch to produce a permanent positively charges on the Ch backbone (Domard, Rinaudo, & Terrassin, 1986). In order to enhance the stability of the quaternary ammonium salts of Ch, the iodide counter-ion was replaced to the chloride ion by dissolving the TMChI and M3-PyMeChI (iodide formed) in an aqueous solution of 15% (w/v) sodium chloride and then dialyzed with deionized water. The ratio between iodide ion and chloride ion was determined by potentiometric titration with 0.01 M silver nitrate solution. It was found that the iodide ion was completely exchanged to the chloride ion as shown in Fig. 1. The titration curve showed that one end point of the chloride ion can be observed. If the chloride ion is partially exchanged, two end points would be obtained due to iodide and chloride ions, respectively (data not shown). The total DQ of M3-PyMeChC was in the range of 72 ± 1%–80 ± 2% as shown in Table 2. In comparison to the single methylation of Ch, the DQ of the TMChC was lower than that of the M3-PyMeChC by using the same condition (Table 2). This could be explained that *N*-methylation of the 3-PyMeCh led to quaternization of either *N*-

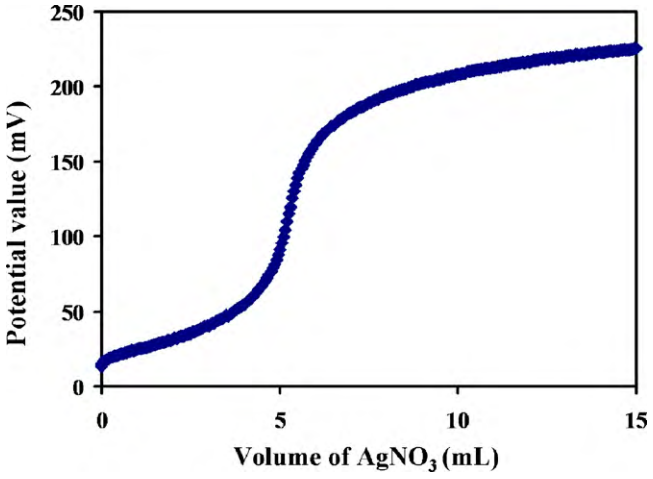


Fig. 1. Titration of the methylated *N*-(3-pyridylmethyl) chitosan chloride (M3PyMeChC4) with 0.01 M silver nitrate solution ($AgNO_3$).

Table 2
Methylation of chitosan and *N*-(3-pyridylmethyl) chitosan derivatives ($n = 3$).

Samples	DS (%)	DQ_T (%)		$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Total *O*-CH_3 (%)	Recovery (%)
		DQ_{Ar} (%)	DQ_{Ch} (%)				
TMChC	–	–	30 ± 3	40 ± 3	10 ± 3	16 ± 3	122 ± 1
TMChC	–	–	65 ± 2	20 ± 2	5 ± 2	35 ± 2	74 ± 2
TMChC	–	–	85 ± 1	Trace	Trace	167 ± 1	40 ± 3
M3-PyMeChC	35 ± 1	35 ± 1	45 ± 1	Trace	7 ± 1	40 ± 1	90 ± 1
M3-PyMeChC	60 ± 1	60 ± 1	12 ± 1	Trace	10 ± 1	12 ± 1	110 ± 2
M3-PyMeChC	80 ± 2	80 ± 2	Trace	Trace	5 ± 2	16 ± 2	100 ± 2

DS is the degree of *N*-substitution. DQ_{Ar} is the degree of quaternization at aromatic substituents. DQ_{Ch} is the degree of quaternization. $N(CH_3)_2$ is the *N*,*N*-dimethylation. $NHCH_3$ is the *N*-methylation. Total *O*-CH_3 is the total degree of *O*-methylation of 3-*O* and 6-*O* at 3-hydroxyl and 6-hydroxyl positions of GlcN of chitosan. Recovery (%) is weight of product (g)/weight of starting reactant (g) × 100.

pyridylmethyl moieties or the primary amino groups of Ch. This is the reason why the DQ of the M3-PyMeChC was higher than those of the TMChC at the same condition. Besides quaternization, the *N*,*N*-dimethylation, *N*-methylation and *O*-methylation at the primary amino groups and hydroxyl groups of Ch were also observed.

3.2. Characterization

The chemical structures of Ch and its derivatives were characterized by ATR-FTIR and ^{1}H NMR spectroscopy. In this study, the solid samples of Ch and its derivatives were characterized by using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE). Fig. 2a and b displays the ATR-FTIR of Ch and 3-PyMeCh, respectively. The characteristic ATR-FTIR pattern of Ch exhibited the absorption band at wavenumber 3357 cm^{-1} due to the OH and NH_2 groups. The absorption band at wavenumbers of 1639 and 1374 cm^{-1} corresponded to the C=O and C–O stretching of amide group, respectively. In addition, the absorption band at wavenumber 1583 cm^{-1} was due to the N–H deformation of amino groups, while the absorption band at wavenumbers 1147, 1056 and 1026 cm^{-1} corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching, respectively (Brugnerotto et al., 2001). The ATR-FTIR spectrum of the 3-PyMeCh was similar to that of Ch except the additional absorption bands at wavenumbers 1595, 1578, 1427, 788 and 707 cm^{-1}. These bands were assigned to the C=C stretching and C–H deformation (out of plane) of the aromatic group, respectively. The M3-PyMeChC exhibited the characteristic ATR-FTIR spectra at wavenumbers 1472 cm^{-1}, whereas TMChC exhibited the ATR-FTIR spectra at wavenumbers 1470 cm^{-1} due to the C–H symmetric bending of the methyl substituent of quaternary ammonium groups (Fig. 1c and d) (Kim, Choi, Chun, & Choi, 1997).

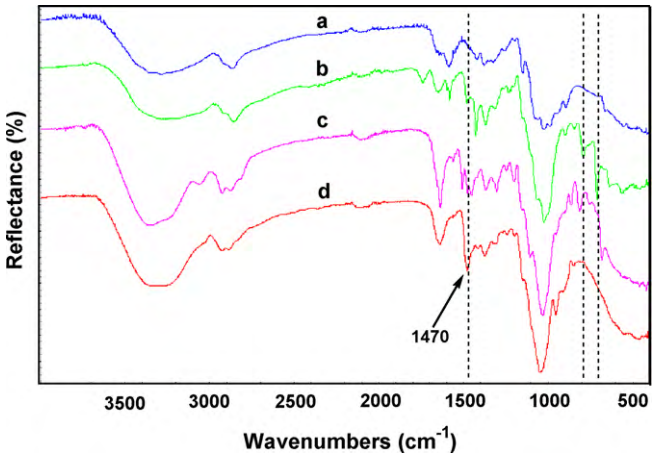


Fig. 2. ATR-FTIR spectra of chitosan (a), *N*-(3-pyridylmethyl) chitosan derivatives (b), methylated *N*-(3-pyridylmethyl) chitosan derivatives (c), and *N*,*N*,*N*-trimethyl chitosan chloride (d).

Fig. 3 shows ^{1}H NMR spectra of Ch (a) and 3-PyMeCh (b). All spectra exhibited the characteristic ^{1}H NMR pattern of Ch, i.e., the multiplet at δ 4.0–3.0 ppm due to H3, H4, H5, H6 and two singlets at δ 3.1 and 2.1 ppm due to the H2 proton of the GlcN, and the *N*-acetyl protons of GlcNAc, respectively (Lavertu et al., 2003). The ^{1}H NMR spectrum of the 3-PyMeCh exhibited the multiplet proton signals at δ 8.52–7.65 ppm due to the aromatic protons, while the singlet proton signal at δ 4.59 ppm was assigned to the methylene protons adjacent N atom of Ch (Sajomsang, Tantayanon, Tangpasuthadol, Thatte, et al., 2008). Fig. 4 shows ^{1}H NMR spectra of the TMChC1 with DQ_{Ch} 30 ± 3% (a), MPyMeChC1 with DQ_{Ch} 45 ± 1% and DQ_{Ar} 35 ± 1% (b), and MPyMeChC3 with DQ_{Ch} 12 ± 1% and DQ_{Ar} 60 ± 1% (c). The ^{1}H NMR spectra of TMChC1 had all signals belonging to Ch and the additional signals due to *N*-methylation and *O*-methylation similar to the ones reported by Sieval et al. (1998). The ^{1}H NMR spectrum of the M3-PyMeChC exhibited the proton signal at δ 5.40 ppm due to the H1′ proton of the GlcN of M3-PyMeChC. The proton signals at δ 4.23, 3.20, 2.60 and 2.30 were assigned to the *N*-methyl protons of pyridinium moiety, *N*,*N*,*N*-trimethyl protons, *N*,*N*-dimethyl protons, and *N*-methyl protons of GlcN, respectively (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008). Furthermore, *O*-methylation was also observed at δ 3.30 and 3.40 ppm, which was assigned to 6-*O*-methylated and 3-*O*-methylated protons, respectively (Polnok et al., 2004; Sieval et al., 1998).

3.3. X-ray diffraction

Fig. 5 shows the X-ray diffraction (XRD) patterns of Ch and its derivatives. The XRD patterns of Ch exhibited three characteristic peaks around $2\theta = 11.2°$, 20.8° and 22.9° (Zhang & Neau, 2001). The reflection fall at $2\theta = 11.2°$ corresponded to the crystal form I and the strongest reflection appeared at $2\theta = 20.8°$ corresponding

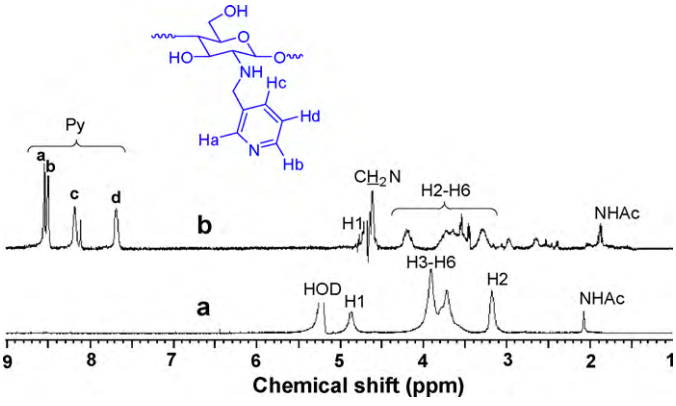


Fig. 3. ^{1}H NMR spectra of chitosan (a), and *N*-3-pyridylmethyl chitosan derivatives (b).

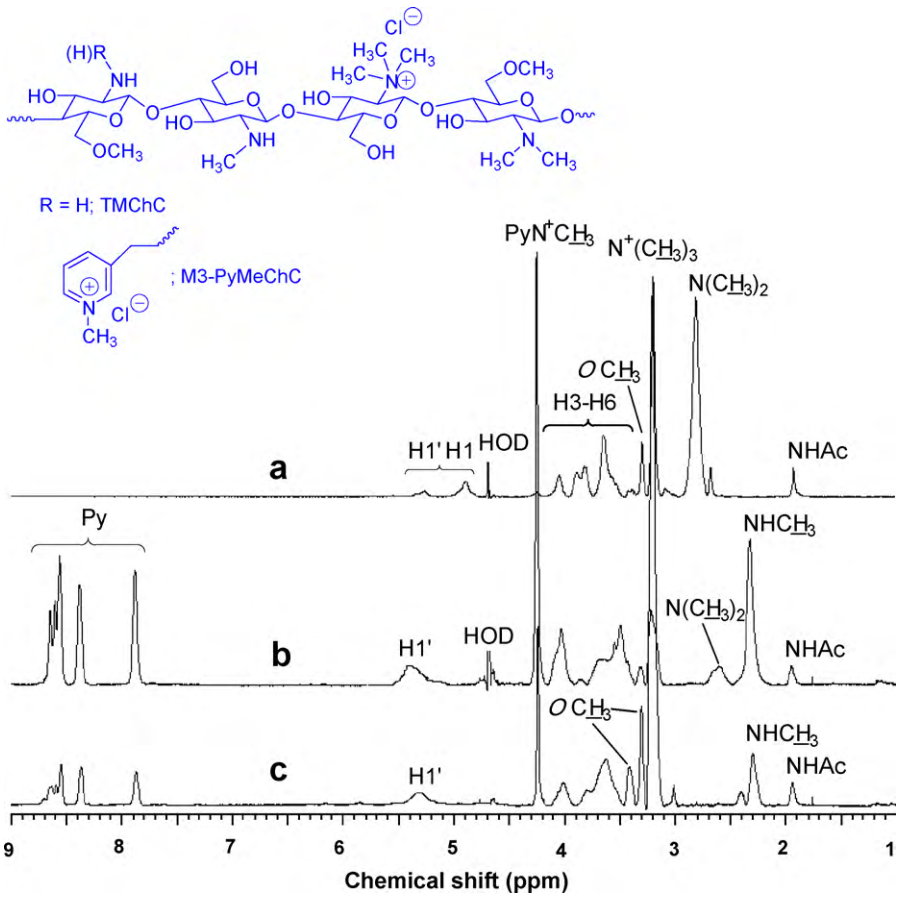


Fig. 4. ^{1}H NMR spectra of *N*,*N*,*N*-trimethyl chitosan chloride (TMChC1) with DQ_{Ch} 30% (a), methylated *N*-(3-pyridylmethyl) chitosan chloride (MPyMeChC1) with DQ_{Ch} 45% and DQ_{Ar} 35% (b), and methylated *N*-(3-pyridylmethyl) chitosan chloride (MPyMeChC3) with DQ_{Ch} 12% and DQ_{Ar} 60% (c).

to crystal form II (Fig. 5a). In comparison to the Ch, the 3-PyMeCh showed the single XRD pattern at 20.6° (Fig. 5b), while the M3-PyMeChC showed very broad XRD pattern (Fig. 5c). A decrease in crystallinity of the 3-PyMeCh could be ascribed to the presence of 3-pyridylmethyl moiety. It is possible that the steric hindrance of the 3-pyridylmethyl group obstructed the formation of inter- and extra-molecular hydrogen bonds of the Ch backbone. Therefore, the 3-PyMeCh can be partially dissolved in NMP or DMSO solvent (data not shown), while the Ch cannot be dissolved in all organic solvents. It was noted that the M3-PyMeChC showed lower crystallinity than that of the 3-PyMeCh and Ch. The low crystallinity of the M3-PyMeChC was resulted from *N*-methylpyridinium moiety leading to destruction of the strong hydrogen bonds in the parent Ch. Moreover, the M3-PyMeChC can be dissolved at all pH ranges. This result revealed that the qauternization of the 3-PyMeCh led to a decrease in crystallinity and an increase in water solubility (Sajomsang, Rungsardthong Ruktanonchai, et al. 2009).

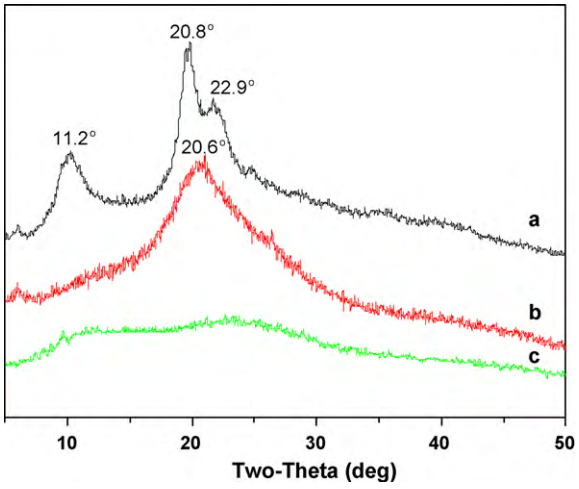


Fig. 5. X-ray diffraction patterns of chitosan (a), *N*-(3-pyridylmethyl) chitosan (b) and methylated *N*-(3-pyridylmethyl) chitosan chloride (c).

3.4. Water solubility

Fig. 6 exhibits the concentration and pH dependence of the transmittance of the TMChC and M3-PyMeChC at various DQs and molecular weights. The water solubilities of the TMChC and M3-PyMeChC decreased with an increasing concentration from 1 mg/mL to 5 mg/mL (Fig. 6a and b). The TMChC4 with DQ 60 ± 2% showed high water solubility, and it can be retained over a wide pH range. On the other hand, the water solubility of the TMChC1 with DQ 30 ± 3% was abruptly decreased particularly in a basic pH and high concentration. It was found that the MPyMeChC1 showed a little bit higher solubility than MPyMeChC3. This is due to the lower ratio of $CH_3N^+Py/N^+(CH_3)_3$ of MPyMeChC1 comparing to the MPyMeChC3. This result was consistent with the MPyMeChC4, which had the highest ratio of $CH_3N^+Py/N^+(CH_3)_3$. Therefore, the water solubility of the MPyMeChC4 was significantly decreased with an increasing in pH values. It was also confirmed that the ratio of $CH_3N^+Py/N^+(CH_3)_3$ increased with decreasing water solubility. This could be due to an increase in ratio of $CH_3N^+Py/N^+(CH_3)_3$ that leads to increase hydrophobicity of the pyridylmethyl moiety. However, the water solubility is dependent

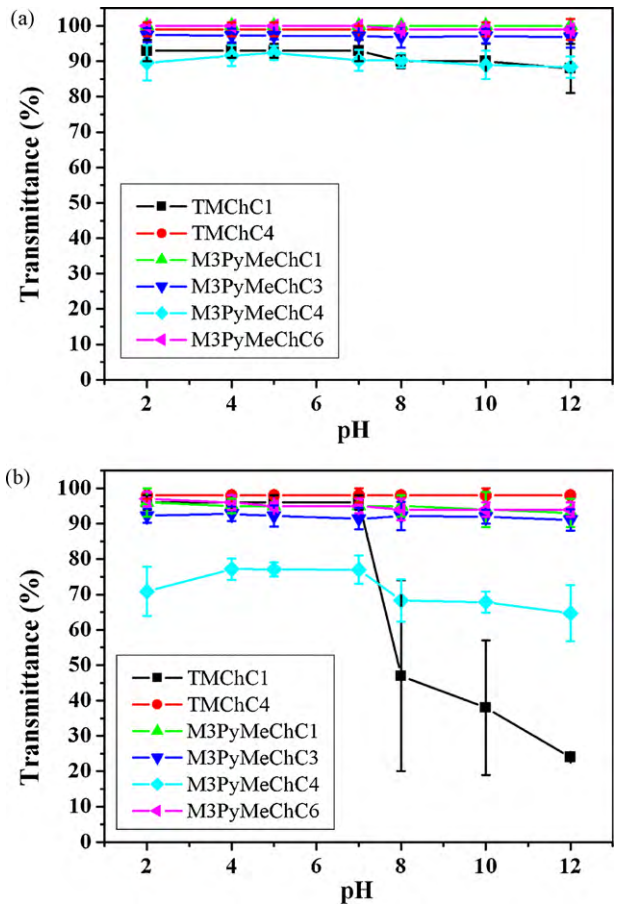


Fig. 6. The pH dependence of water solubility of *N*,*N*,*N*-trimethyl chitosan chloride (TMChC), and methylated *N*-(3-pyridylmethyl) chitosan chloride (M3PyMeChC) at 1 mg/mL (a) and 5 mg/mL (b).

on the molecular weight. Basically, low molecular weight showed higher water solubility than high molecular weight. It was found that the MPyMeChC6 with M_w 8 kDa showed higher water solubility than those of the MPyMeChC4 with M_w 82 kDa at similar DQ level and ratio of $CH_3N^+Py/N^+(CH_3)_3$.

3.5. Determination of molecular weight

The weight average molecular weight (M_w), number average molecular weight (M_n) and M_w/M_n of Ch and its methylated derivatives were determined by gel permeation chromatography (GPC) as shown in Table 3. The molecular weight average of the native Ch was found to be M_n 48 kDa, M_w 276 kDa and M_w/M_n 5.67. A relatively wide molecular weight distribution with a polydispersity index (PDI) of Ch was observed. The methylation of Ch and 3-PyMeCh led to a decrease in the molecular weight. This was similar to the finding of Snyman et al., who found that the decrease in molecular weight of Ch depended on the methylation procedure, and the molecular weight was much more decreased with the extension of reaction duration correlated to an increase in the DQ (Snyman, Hamman, Kotze, Rollings, & Kotźe, 2002). Similar result has been observed in our research group (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008). We found that the methylation step leading to methylated Ch derivatives was accompanied by a significant molecular weight decrease. This was plausible that an oxidative degradation process and alkaline depolymerization occurred. However, the molecular weight of the methylated Ch derivative products during the methylation reaction cannot be controlled. One approach examined to reduce the molecular weight was diazotization of the primary amine through its reaction with nitrous acid as proposed by Allan and Peyron (Allan & Peyron, 1975). The advantage of the nitrosating specie is that it can be selectively attacked the amine groups of the Ch. Subsequently, the glycosidic linkages of the Ch are cleaved and a 2,5-anhydro-D-mannose unit is formed at the reducing end of the cleaved polymer. Normally, nitrous acid was prepared from nitrite salt such as sodium and potassium with hydrochloric acid or acetic acid. The degradation was occurred at glycosidic linkages of the Ch backbone and related stoichiometric with the mole of sodium nitrite added. Therefore, the degree of depolymerization can be controlled (Janes & Alonso, 2003; Peniston & Johnson, 1975). However, it was difficult to predict the percentage of linkages that needed to be cleaved since sodium nitrite is not capable of cleaving linkages adjacent to *N*-acetyl-D-glucosamine (GlcNAc) residues.

In this study, depolymerized TMChCs and M3-PyMeChCs were performed by using 0.1% (w/v) to 0.5% (w/v) of sodium nitrite in aqueous hydrochloric acid and stirred for 24 h (Table 3). The result revealed that the molecular weight of TMChCs with DQ 30 ± 3% was reduced from 281 kDa to 95 kDa, 48 kDa and 35 kDa by using 0.1% (w/v), 0.3% (w/v) and 0.5% (w/v) of sodium nitrite, respectively (entries 2, 3 and 4). This indicated that the concentration of sodium nitrite increased with a decrease in molecular weight. Moreover, the molecular weight of M3-PyMeChC1 and M3-PyMeChC4 with DQ 80 ± 2% was decreased from 58 kDa to 39 kDa and 82 kDa to 56 kDa, respectively when treated with 0.1% (w/v) of sodium nitrite (entries 8 and 9, and 11 and 12). From ^{1}H NMR spectroscopy, the primary amino groups of the Ch were methylated or pyridylmethylated, indicating the presence of both secondary and tertiary amines. Therefore, the remained primary amino groups of

Table 3
Weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of chitosan and *N*-(3-pyridylmethyl) chitosan derivatives after methylation.

Entries	Samples	DS (%)	DQ_T (%)	M_n (kDa)	M_w (kDa)	M_w/M_n
1	Chitosan (Ch)	–	–	48.71	276.06	5.67
2	TMChC1	–	30 ± 3	56.10	281.07	5.01
3	TMChC2	–	30 ± 3	25.71	95.76	3.72
4	TMChC3	–	30 ± 3	9.37	35.42	3.92
5	TMChC4	–	65 ± 2	26.59	84.10	3.16
6	TMChC5[a]	–	65 ± 2	14.04	42.78	3.04
7	TMChC6	–	85 ± 1	22.30	67.36	3.02
8	M3-PyMeChC1	35 ± 1	80 ± 1	13.96	58.01	4.15
9	M3-PyMeChC2	35 ± 1	80 ± 1	10.48	39.58	3.95
10	M3-PyMeChC3	60 ± 1	72 ± 1	21.04	114.15	5.42
11	M3-PyMeChC4	80 ± 2	80 ± 2	17.49	82.18	4.69
12	M3-PyMeChC5	80 ± 2	80 ± 2	15.80	56.62	3.58
13	M3-PyMeChC6[a]	80 ± 2	80 ± 2	3.50	8.44	2.41

DQ_T is the total degree of quaternization ($DQ_{Ar} + DQ_{Ch}$).
[a] Started from chitosan with M_w = 16 kDa.

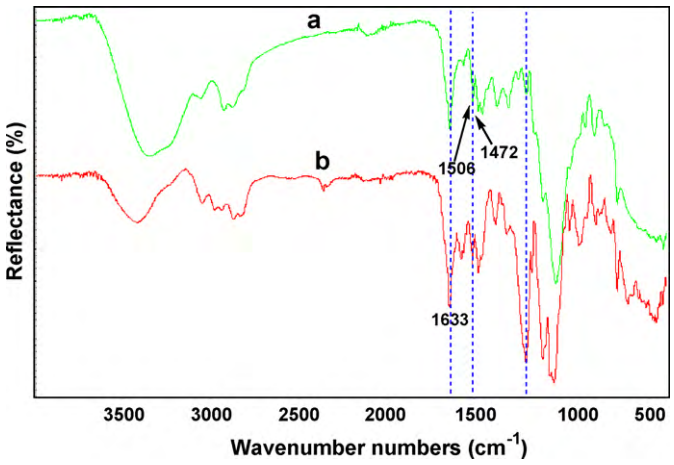


Fig. 7. ATR-FTIR spectra of methylated *N*-(3-pyridylmethyl) chitosan chloride (MPyMeChC4) with molecular weight 82 kDa (a) and methylated *N*-(3-pyridylmethyl) chitosan chloride (MPyMeChC5) with molecular weight 56 kDa (b).

the Ch can be reacted with nitrous acidium ion, then the glycosidic linkages of the Ch backbone were cleaved. Moreover, the side reaction, which is *N*-nitrosamines, might occur because the nitrosation of secondary amines are quite stable and can be formed under milder conditions (Knight et al., 2007). However, the reaction of secondary amines with nitrosating agents was limited at pH 5 since the nitrosating agents are changed to nitrosyl chloride or possibly nitrous anhydride at this pH, which are not as effective nitrosating agents (Digenis & Issidorides, 1979). Moreover, the depolymerization of the methylated Ch derivatives might occur due to acidic condition used. It is possible that the *O*-glycosidic bond of the Ch backbone can be cleaved by acid hydrolysis. In order to confirm the chemical structure of the M3-PyMeChC after depolymerization with nitrous acid, the M3-PyMeChC5 with molecular weight of 56 kDa was determined by using ATR-FTIR spectroscopy compared with M3-PyMeChC4 with the molecular weight of 82 kDa (Fig. 7). The absorption band at wavenumber at 1633 cm^{-1} was corresponded to the C=O stretching of amide group, whereas the absorption band at wavenumber 1583 cm^{-1} cannot be seen due to the N–H deformation of amino group. This was due to the substitution of pyridylmethyl moiety onto the primary amino groups of the Ch backbone. The absorption band at wavenumber at 1506 cm^{-1} was corresponded to the C=C stretching of aromatic, while the absorption band at wavenumber at 1472 cm^{-1} was corresponded to the C–H symmetric bending of the methyl substituent of quaternary ammonium group. It can be observed that the chemical structure of the M3-PyMeChC5 was similar to the M3-PyMeChC4. Therefore, it was confirmed that the chemical structures of the methylated Ch derivatives did not change after the molecular weight was reduced by depolymerization with a nitrous acid.

3.6. Bactericidal activity

The *in vitro* bactericidal activity of the methylated Ch derivatives was evaluated by using a minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) procedure against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria at pH 7.2 in accordance to methods of the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000, 2002). The result revealed that the MICs and MBCs were dependent on the type of bacterium, DQ, molecular weight, the chemical structure of a quaternary ammonium group and ratio of $CH_3N^+Py/N^+(CH_3)_3$ group. The TMChC markedly showed bactericidal activity against both bacteria with MIC values ranging from 31 μg/mL to >2000 μg/mL, whereas MBC value was 125 to >2000 μg/mL depending on its DQ and molecular weight (Table 4). The bactericidal activity of the TMChC increased with an increase in DQ from 30 ± 3% to 85 ± 1% when compared the molecular weight ranging from 67 kDa to 95 kDa (Table 4 and entries 2, 4 and 6). However, the bactericidal activity decreased with decreasing molecular weight from 281 kDa to 35 kDa and from 84 kDa to 42 kDa at DQ30 ± 3% and DQ65 ± 2%, respectively (Table 4 and entries 1–5). The result indicated that high DQ and molecular weight of the TMChC showed higher bactericidal activity than low DQ and molecular weight. This was attributed to at high DQ the polymer has higher permanent positively charged density of the quaternary amino groups than at low DQ. Therefore, higher positively charged density was obtained against both bacteria more than lower positively charged density of the Ch backbone or some neutral Ch derivatives such as hydroxypropyl Ch (Peng, Han, Liu, & Xu, 2005). When considering the bactericidal activity of the M3-PyMeChC against both bacteria, it was found that the MIC values were in the range of 125–250 μg/mL, whereas MBC values were in the range of 125–2000 μg/mL depending on the DQ, the chemical structure of a quaternary ammonium group, ratio of $CH_3N^+Py/N^+(CH_3)_3$ and molecular weight (Table 4). It was noted that the differences found between MICs and MBCs of *S. aureus* (Gram-positive) were more substantial than those of *E. coli* (Gram-negative). Even though Gram-positive cell walls are much thicker (20–80 nm) and far less structured than Gram-negative ones (7–8 nm). However, the outer membrane (OM) of Gram-negative bacteria has lipopolysaccaride (LPS) that contained endotoxin as a toxic substance (Chen et al., 2000; Franklin & Snow, 1981). Therefore, Gram-negative bacteria showed less effective to the methylated Ch derivatives than Gram-positive bacteria. Moreover, it can be observed that the MIC values of M3-PyMeChC1-5 were similar at 125 μg/mL except M3-PyMeChC6 was 250 μg/mL in both bacteria due to the lowest molecular weight. However, their MBC values were different. It is important to note that lower ratio of $CH_3N^+Py/N^+(CH_3)_3$ indicated lower MBC values, whereas the DQ and the molecular weight were similar (Table 4 and entries 7 and 11). Furthermore, the M3-PyMeChC1-2 with molecular weight 58 kDa and 39 kDa showed the lowest MBC values compared to other M3-PyMeChCs. It might be possible that the difference in chemical structure of the quaternary ammonium groups, *N*,*N*,*N*-trimethyl ammonium group and *N*-methylpyridinium group, led to difference in bactericidal activity. In comparison to the MPyMeChC5 with DQ_{Ar} 80 ± 2% and MPyMeChC1 with DQ_{Ar} 35 ± 1% and DQ_{Ch} 45 ± 1%, the TMChC6 with DQ_{Ch} 85 ± 1% showed the highest bactericidal activity against both bacteria, in which their sequences were as follows: TMChC6 (DQ_{Ch} 85 ± 1%) > MPyMeChC1 (DQ_{Ar} 35 ± 1% and DQ_{Ch} 45 ± 1%) > MPyMeChC5 (DQ_{Ar} 80 ± 2%), when similar molecular weight was compared in the range of 56–67 kDa (Table 4 and entries 6, 7 and 11). The result revealed that the *N*,*N*,*N*-trimethyl ammonium group was more active than the *N*-methylpyridinium group. Previously, Holappa et al. (2006) have reported that an introduction of the cationic betainoyl group on the primary amino group of Ch was negatively correlated with antibacterial activity. Therefore, the optimal positioning of the positive charge in relation to the Ch backbone, which is the key issue, is proposed (Rúnarsson et al., 2007).

It is well known that the molecular weight of Ch is one of the most important factors affecting on the bactericidal activity. However, the results published so far have shown contradictory conclusions. Jeon et al. reported that 10 kDa is the minimum molecular weight required for inhibition of bacteria (Jeon, Park, & Kim, 2001). Zheng and Zhu showed that a mixture of 0.25% (w/v) chitooligosaccharide with molecular weight <5 kDa yielded the highest inhibition over *E. coli*, whereas a 305 kDa fraction had the highest effect against *S. aureus*, at a similar concentration (Zheng & Zhu, 2003). No, Park, Lee, and Meyers (2002) and Qin et al. (2006) reported that Ch showed higher antibacterial activity

Table 4
Bactericidal activity of methylated chitosan and its derivatives.

Entries	Samples	M_w (kDa)	DS (%)	DQ_{Ar} (%)	DQ_{Ch} (%)	DQ_T (%)	MIC/MBC (μg/mL)	
							S. aureus	*E. coli*
1	TMChC1	281.07	–	–	30 ± 3	30 ± 3	1000/2000	2000/2000
2	TMChC2	95.76	–	–	30 ± 3	30 ± 3	2000	2000
3	TMChC3	35.42	–	–	30 ± 3	30 ± 3	>2000	>2000
4	TMChC4	84.10	–	–	65 ± 2	65 ± 2	125/1000	250/1000
5	TMChC5[a]	42.78	–	–	65 ± 2	65 ± 2	500/1000	500/1000
6	TMChC6	67.36	–	–	85 ± 1	85 ± 1	31/125	31/250
7	M3-PyMeChC1	58.01	35 ± 1	35 ± 1	45 ± 1	80 ± 1	125/125	250/500
8	M3-PyMeChC2	39.58	35 ± 1	35 ± 1	45 ± 1	80 ± 1	125/125	250/500
9	M3-PyMeChC3	114.15	60 ± 1	60 ± 1	12 ± 1	72 ± 1	125/1000	250/1000
10	M3-PyMeChC4	82.18	80 ± 2	80 ± 2	Trace	80 ± 2	125/500	250/1000
11	M3-PyMeChC5	56.62	80 ± 2	80 ± 2	Trace	80 ± 2	125/2000	250/1000
12	M3-PyMeChC6[a]	8.44	80 ± 2	80 ± 2	Trace	80 ± 2	250/2000	250/1000

[a] Started from chitosan with M_w = 16 kDa.

than Ch oligomer. Moreover, they reported that the most effective molecular weight against *S. aureus* and *E. coli* bacteria is 470 kDa, when the testing range was 1–1671 kDa. On the other hand, Ch oligomer or low molecular weight Ch showed higher antibacterial activity than Ch against *E. coli* (Fernandes et al., 2008; Liu et al., 2006). Therefore, it is still difficult to compare antibacterial activities reported in these studies as the assay conditions have not been identical. In this study, the differences in molecular weight of the MPyMeChC4-6 ranging from 82 kDa to 8 kDa on bactericidal activity against *S. aureus* and *E. coli* bacteria were evaluated, at similar DQ of 80 ± 2% (Table 4 and entries 10 and 11). It was found that the polymer at molecular weights of 82 kDa and 56 kDa were slightly active more than molecular weight 8 kDa against *S. aureus* bacteria, but they were not different in bactericidal activity against *E. coli*. So far, it has been reported that the antibacterial activity of Ch occurred as a result of several mechanisms. It is postulated that the positively charged density of the Ch absorbed onto the negatively charged cell surface of bacteria leads to the leakage of proteinaceous and other intracellular constituents. However, the primary amino groups of Ch are modified to two different quaternary ammonium groups, which are *N*-methylpyridinium and *N*,*N*,*N*-trimethyl ammonium groups. Therefore, different quaternary nitrogen functionalities on the Ch backbone might lead to different mechanisms absorbed onto the negatively charged cell surface of bacteria, leading to different bactericidal activities. Our result revealed that besides the DQ and molecular weight, the chemical structure of a quaternary ammonium groups and ratio between *N*-methylpyridinium and *N*,*N*,*N*-trimethyl ammonium moieties has affected the bactericidal activity.

4. Conclusion

N-(3-pyridylmethyl) Ch derivatives (3-PyMeCh) were prepared by reductive amination then methylated with iodomethane in the presence of sodium hydroxide, sodium iodide and *N*-methyl pyrrolidone to produce the quaternary ammonium salts at *N*-pyridylmethyl substituents and the primary amino groups of Ch. These products can be easily dissolved in the water at all pH ranges. The bactericidal activity of all methylated *N*-(3-pyridylmethyl) Ch chlorides (M3-PyMeChCs) was performed by using the standard method of the National Committee for Clinical Laboratory Standards (NCCLS). Ch containing the *N*,*N*,*N*-trimethyl ammonium moiety at the primary amino group exhibited more active than *N*-methylpyridinium moiety with similar degree of quaternization and molecular weight. The ratio of *N*,*N*,*N*-trimethyl ammonium moiety increased with an increase in bactericidal activity. The molecular weight of the M3-PyMeChCs decreased from 82 kDa to 8 kDa with reducing the bactericidal activity. The result revealed that the chemical structure of the quaternary ammonium moiety and the molecular weight played an important role on the bactericidal activity.

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