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Research paper

Physicochemical properties and biocompatibility of *N*-trimethyl chitosan: Effect of quaternization and dimethylation

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ABSTRACT

The aim of this research was to investigate the effect of degrees of quaternization (DQ) and dimethylation (DD) on physicochemical properties and cytotoxicity of *N*-trimethyl chitosan (TMC). TMC was synthesized by reductive methylation of chitosan in the presence of a strong base at elevated temperature and polymer characteristics were investigated. The number of methylation process and duration of reaction were demonstrated to affect the DQ and DD. An increased number of reaction steps increased DQ and decreased DD, while an extended duration of reaction increased both DQ and DD. The molecular weight of TMC was in the range of 60–550 kDa. From the Mark–Houwink equation, it was found that TMC in 2% acetic acid/0.2 M sodium acetate behaved as a spherical structure, approximating a random coil. The highest solubility was found with TMC of an intermediate DQ (40%) regardless of DD and molecular weight. The effect of DD on the physicochemical properties and cytotoxicity was obviously observed when proportion of DD to DQ was higher than 1. TMC with relatively high DD showed reduction in both solubility and mucoadhesion and hence decreased cytotoxicity. However, the influence of DD was insignificant when DQ of TMC was higher than 40% at which physicochemical properties and cytotoxicity were mainly dependent upon DQ.

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1. Introduction

N,N,N-trimethyl chitosan (TMC) is a partially quaternized derivative of chitosan first synthesized by Muzzarelli and Tanfani [1] in an attempt to increase solubility of chitosan in water at neutral and basic pH values. The increase in solubility is achieved by replacing the primary amino group on the C-2 position of chitosan with quaternary amino groups [2]. It has been shown that TMC can decrease the TEER of Caco-2 cell monolayers and increase the transport of several hydrophilic compounds, peptide and protein drugs both *in vitro* (Caco-2 cells) [3–6] and *in vivo* (rats and pigs) [7–9]. Up to date, TMC has received considerable attention in drug and gene delivery not only in peroral route [10] but also in ocular [11], intranasal [12–14], buccal [15,16], pulmonary [17,18] and rectal [19] routes.

It is well known that polymer structure is a main factor influencing its physicochemical properties. Several research groups have studied the structure–physicochemical property relationship of TMC and reported that the properties of TMC depend on degree of quaternization (DQ) at 2-amino groups and degree of *O*-methylation at 3- and 6-hydroxy groups (DO_3 and DO_6, respectively) [6,20,21]. The best permeation enhancement of peptide and protein drugs is achieved when using TMC with DQ ca. 48% [6]. Moreover, high DO_3 and DO_6 found in TMC with high DQ decrease the solubility of the polymer [20]. Although mucoadhesive properties and cytotoxicity of TMC with different DQ have been explored, the results are controversial. Synman et al. [21,22] found that the mucoadhesive properties of TMC decreased with an increase in DQ, whereas Sandri et al. [15] reported the opposite results. Regarding the cytotoxicity, Thanou et al. [23], Amidi et al. [13] and Florea et al. [17] indicated that TMC was non-toxic even at high DQ. However, Mao et al. [24] found that TMC with DQ of 40% exhibited time- and dose-dependent cytotoxic responses which increased with increasing molecular weight. Similar results had been found by Kean et al. [25] who showed that the cytotoxicity of TMC increased with increasing DQ. These discrepancies may be attributed to different degrees of dimethylation (DD) of TMC. TMC with the same DQ but different in DD may show different properties in the mucoadhesion and cytotoxicity. In general, the mucoadhesion and cytotoxicity of TMC can probably be attributed to the interaction between positively charged groups of TMC and anionic components (sialic acid) of the glycoproteins in mucus layer and on the surface of epithelial cells [24]. We hypothesized

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that an increase in DD aside from DQ may affect the interaction of polymer and mucus layer/epithelial cells, resulting in the reduction in mucoadhesion and cytotoxicity. However, to the best of our knowledge, no experimental data are available to support this hypothesis. Therefore, the influence of the DQ together with the DD on the mucoadhesive properties and cytotoxicity was simultaneously elucidated.

In this present study, TMC with different DQ and DD was synthesized and characterized. The influence of synthesis process resulting in different DQ and DD was also investigated. The mucoadhesion of TMC was determined using a mucin particle method. The biocompatibility of TMC was characterized by MTT assay using a L929 fibroblast cell line.

2. Materials and methods

2.1. Materials

Chitosan (400 kDa, degree of deacetylation 84.7%) was purchased from Fluka (Steinheim, Germany). Type III mucin from procine stomach and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (Deisenhofen, Germany). Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco (Eggenstein, Germany). Tissue culture materials and plates were from Costar (Bodenheim, Germany). All other chemicals and solvents were of analytical purity.

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

TMC polymers differing in DQ and DD were prepared by reductive methylation of the parent chitosan with CH_3I in the presence of NaOH using the procedure described by Polnok et al. [26] with some modifications. Briefly, in the reaction step 1, a mixture of chitosan, NaI, 15% NaOH was mixed with *N*-methylpyrrolidinone while maintained in a water bath at 60 °C. Subsequently, CH_3I was added to the mixture and the reaction was carried out in the presence of a reflux condenser. Prior to the precipitation of the product from the solution mixture at the end of first reaction step, additional CH_3I and 15% NaOH were added. The reaction was further continued (addition step). In some instances, the precipitated polymer obtained from the first reaction step was mixed with NaI, NaOH in *N*-methylpyrrolidinone. CH_3I was added to the mixture and the reaction was carried out in the presence of a reflux condenser (second reaction step). The product (*N*-trimethyl chitosan iodide) was precipitated and converted to *N*-trimethyl chitosan chloride according to the previously described processes [20]. The reaction conditions of each polymer are summarized in Table 1. For ease of discussion, the abbreviation TMC*x*–*y* was used to describe the polymers where *x* represents the DQ in percent and *y*, the DD in percent.

Table 1
The reaction conditions in the synthesis of TMC polymers

Polymer	Reaction step 1[a] (h)	Reaction step 2[b] (h)	Addition step 1[c] (h)	Addition step 2[c] (h)
TMC10-40	1	–	–	–
TMC20-20	1	–	0.5	–
TMC20-60	1.5	–	–	–
TMC40-10	1	1	–	–
TMC40-40	1.5	–	1.5	–
TMC80-10	1	–	1	1

[a] Twelve milliliters of 15% NaOH added with 12 ml CH_3I in *N*-methylpyrrolidinone containing 2 g chitosan and 4.8 g NaI.
[b] Twelve milliliters of 15% NaOH and 0.6 g NaOH added with 9 ml CH_3I in *N*-methylpyrrolidinone containing precipitated product from first reaction step and 4.8 g NaI.
[c] Added with 12 ml of 15% NaOH and 6 ml CH_3I.

2.3. Characterization of TMC polymer

2.3.1. Degrees of substitution

^{1}H NMR spectra of TMC polymers were recorded on JEOL GX 400D (Tokyo, Japan) by dissolving samples in D_2O at 80 °C with suppression of the water peak. The degrees of substitution corresponding to %DQ, %DD, $\%DO_3$ and $\%DO_6$ were calculated from the data obtained from ^{1}H NMR spectra using the Eqs. (1)–(4) [20] as described below:

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

$$\%DD = \left[\frac{[(CH_3)_2]}{[H]} \times \frac{1}{3}\right] \times 100 \quad (2)$$

$$\%DO_3 = \left[\frac{[(3\text{-}OCH_3)]}{[H]} \times \frac{1}{3}\right] \times 100 \quad (3)$$

$$\%DO_6 = \left[\frac{[(6\text{-}OCH_3)]}{[H]} \times \frac{1}{3}\right] \times 100 \quad (4)$$

where $[(CH_3)_3]$ is the integral of trimethyl amino group at 3.3 ppm, $[(CH_3)_2]$ is the integral of dimethyl amino group at 3.0 ppm, $[(3\text{-}OCH_3)]$ is the integral of methyl group for 3-hydroxyl group at 3.5 ppm, $[(6\text{-}OCH_3)]$ is the integral of methyl group for 6-hydroxyl group at 3.4 ppm and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

2.3.2. Determination of molecular weight

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight distribution (M_w/M_n) of TMC were determined by a gel permeation chromatography (GPC) (Water Corporation, Washington, USA) at 30 °C. The GPC equipment consisted of ultrahydrogel linear column (MW resolving range = 1000–20,000,000), Waters 600E pump and Water 2410 refractive index detector. The eluent was 0.5 M acetate buffer. The standards used to calibrate the column were pullulans (MW 5900–788,000).

2.3.3. Intrinsic viscosity measurement

Intrinsic viscosities [η] of TMC were determined in 2% acetic acid/0.2 M sodium acetate (2% HAc/0.2 M NaAc) using an automated Ubbelohde capillary viscometer (Model Schott AVS-360, Germany) with a 0.63-mm capillary diameter at 25 ± 0.1 °C in triplicate. Solution concentrations were adjusted in order to obtain relative viscosity value in the range of 1.1–1.5 which was suitable for the calculation of [η] [27]. Six different concentrations were tested for each sample and each concentration was measured five times. The running times of solution and solvent were used to calculate the specific viscosity, reduced viscosity and inherent viscosity. In order to obtain the most accurate values, [η] was determined as an average of extrapolating both Huggins ($\eta_{sp}/c \sim c$) and Kraemer ($\eta_{inh} \sim c$) plots on the ordinate at $c = 0$ [28].

2.3.4. Determination of potentiometric titration curve

Potentiometric titration curve of TMC was constructed by dissolving 20 mg of polymer in 2 ml of 0.1 N HCl solution. A titrant was a solution of 0.1 N NaOH. Under continuous stirring, titrant was added stepwise and the volume of added NaOH and pH values of solution were recorded thoroughly [11].

2.4. Estimation of water solubility

The pH dependence of the water solubility of TMC was estimated using turbidity measurements. The test sample was dissolved in 0.1 N HCl solution. With the stepwise addition of 0.1 or 1.0 N NaOH solution, the transmittance of the solution was recorded on a Shimadzu UV-160 Spectrophotometer using a quartz cell with an optical path length of 10 mm at 600 nm. The test

was performed at room temperature [29]. In order to investigate the effect of ionic strength, the solutions of 0.1 N HCl and 1.0 N NaOH solutions were adjusted by sodium chloride (NaCl) to achieve the desired ionic strength of 0.05, 0.15 and 0.5 M.

2.5. Mucoadhesion by mucin particle method

Mucoadhesive properties of TMC were evaluated by using the mucin particle method developed by Takeuchi et al. [30]. Submicron-sized mucin (ss-mucin) suspension (1% w/v) was prepared by suspending and continuously stirring mucin type III powder in 10 mM Tris buffer, pH 6.8, for 10 h. Mucin suspension was then incubated at 37 °C overnight. The size of mucin was reduced by ultrasonication (Branson 1200, Connecticut, USA) until particle size was around 300–400 nm. It was then centrifuged at 4000 rpm for 20 min to extract submicron-sized mucin particles in the supernatant portion. The particle size and zeta potential of the precisely size-controlled ss-mucin were 400 ± 12 nm and −16.1 ± 1.8 mV, respectively.

One milliliter of 1% w/v ss-mucin suspension was mixed with different volumes of 1 mg/ml polymer solutions under mild magnetic stirring. Then the particle size and zeta potential values were measured using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 4 mW HeNe laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173 °C backward scattering angle. The viscosity (0.88 mPa S) and refractive index (1.33) of water at 25 °C were used for data analysis. All experiments were performed in triplicate.

2.6. Cytotoxicity testing (MTT assay)

In vitro cytotoxicity of TMC was evaluated using a MTT assay according to the method described by Fischer et al. [31]. A mouse connective tissue fibroblast cell line, L929 (DSMZ, Braunschweig, Germany) was plated into 96-well microtiter plates at a density of 8000 cells/well. After 24 h incubation, culture medium was replaced by 100 μl of serial dilutions of the polymers in serum-supplemented tissue culture medium and the cells were incubated for 3 h. Subsequently, polymer solutions were aspirated and replaced by 200 μl DMEM without serum. Twenty microliters sterile-filtered MTT stock solution in phosphate-buffered saline (PBS), pH 7.4 (5 mg/ml) were added in each well reaching a final concentration of 500 g MTT/ml. After 4 h incubation, unreacted dye was aspirated and the formazin crystals were dissolved in 200 μl/well DMSO. Absorption was measured at 570 nm with a background correction of 690 nm using a Titertek Plus MS 212 ELISA reader (ICN, Eschwege, Germany). The relative cell viability (%) compared to control wells containing cell culture medium without polymer was calculated by $[A]_{test}/[A]_{control} \times 100$ ($n = 4$). The IC_{50} was calculated as a polymer concentration which inhibited growth of 50% of cells relative to non-treated control cells.

2.7. Statistical analysis

Results were recorded as means ± SD from at least three measurements. Significance between the mean values was calculated using ANOVA one-way analysis (SPSS 11.5.0 for windows). Probability values of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Synthesis and characterization of TMC polymers

3.1.1. The degrees of substitution

In this study, TMC was synthesized based on one methylation reaction step followed by subsequent addition steps because it had been demonstrated that the high DQ of TMC with a low degree of *O*-methylation could be achieved as compared to the use of multiple reaction steps [26]. Moreover, it was time-saving owing to the reduction of certain in-process procedures – precipitation, centrifugation and drying of the intermediate product. The degrees of substitution of various TMC polymers are listed in Table 2.

As seen in Table 2, using a one-step reaction, TMC10-40 was obtained with 13.9% DQ and the high substitution degree of DD at 39.1%. When extending the reaction duration from 1 to 1.5 h, DQ increased to 23.4% and DD significantly increased to 65.2% (TMC20-60). However, when increasing additional step for 0.5 h, DQ increased to 23.0% similar to TMC20-60, whereas DD decreased to 20.8% (TMC20-20). Similarly, it was observed that by extending duration of additional step of TMC20-20 from 0.5 to 1 h, DQ of TMC increased from 23.0% to 32.1% and DD from 20.8% to 33.0% (data not shown), therefore, an increase in reaction duration increased both DQ and DD. Comparing between TMC10-40 and TMC40-10 as well as between TMC20-60 and TMC40-40, it was obviously seen that an increase of the number of reaction step increased DQ but decreased DD.

Fig. 1 shows the ^1H NMR spectra of TMC20-20 and TMC20-60. The ^1H signal intensity of dimethylamino group ($-N(CH_3)_2$) of TMC20-60 was stronger than that of TMC20-20. The ratio between the integral of the *N*-trimethylamino group ($N^+(CH_3)_3$) and that of the *N*-dimethylamino group ($N(CH_3)_2$) was approximately 1:3 for TMC20-60 and 1:1 for TMC20-20. The results obtained may be explained by the less basic environment and reduction of methylating agent, CH_3I in the extended reaction step of TMC20-60 which would slow down conversion of an intermediate $N(CH_3)_2$ to a $N^+(CH_3)_3$. Similarly, Curti et al. [32] reported that the *N*-methylation of chitosan or the average DQ was strongly affected by the reaction conditions, i.e. the alkalinity of the medium and the availability of CH_3I.

From the results obtained, it could be suggested that high DQ of TMC with low DD could be obtained by increasing the number of reaction steps, whereas high DQ of TMC with high DD was resulted by extending the duration of the reaction.

3.1.2. Molecular weight

A summary of the M_w and M_w/M_n determined by GPC of TMC polymers is presented in Table 2. All TMC had a relatively wide molecular weight distribution with a polydispersity index in the range of 2.9–4.9.

Although a decrease in molecular weight of TMC with increasing DQ was not distinctly observed as reported by Snyman et al. [33], it was found that the molecular weight depended on the synthesis procedure. It increased with the extension of reaction duration correlated to an increase in DQ and DD as seen in TMC10-40/TMC20-60 and TMC20-20/TMC40-40. Therefore, an addition of methyl groups to the amino groups of chitosan resulted in TMC with high molecular weight.

On the contrary, the molecular weight decreased with increasing number of reaction steps or additional steps as seen in TMC10-40/TMC20-20 and TMC20-60/TMC40-40. Moreover, the molecular weight of TMC40-10 prepared by two reaction steps was markedly decreased in comparison with TMC40-40 prepared by one reaction step followed by one additional step. This was due to the degradation of the polymer in the strong basic environment. The results obtained are consistent with those previously reported by Hamman and Kotze [34] who observed that intrinsic viscosity, an indication of the molecular weight, of TMC increased with increasing reaction duration and decreased with increasing number of reaction steps. Therefore, it can be concluded that the molecular weight of TMC is affected by the addition of methyl groups to the amino groups of chitosan and the polymer degradation by the strong basic environment.

Table 2
Intrinsic viscosity, molecular weight and substitution degrees of TMC polymers

Polymer	$[\eta]$[a] (dl/g)	M_w[b] ($\times 10^4$ g/mol)	M_w/M_n[b]	DQ (%)	DD (%)	3O-CH_3 (%)	6O-CH_3 (%)
TMC10-40	2.18	37.8	4.86	13.9	39.1	2.4	7.0
TMC20-20	2.09	28.6	3.91	23.0	20.8	11.3	16.7
TMC20-60	2.48	54.2	4.74	23.4	65.2	3.9	9.0
TMC40-10	1.06	6.1	3.25	42.4	12.4	6.1	8.0
TMC40-40	2.15	36.5	2.89	39.0	39.3	4.9	9.3
TMC80-10	2.01	26.6	3.50	76.6	8.5	58.0	52.0

[a] $[\eta]$ for the starting chitosan was 10.70 dl/g.
[b] M_w and M_w/M_n for the starting chitosan were 87.2 $\times$ 10^4 g/mol and 3.50, respectively.

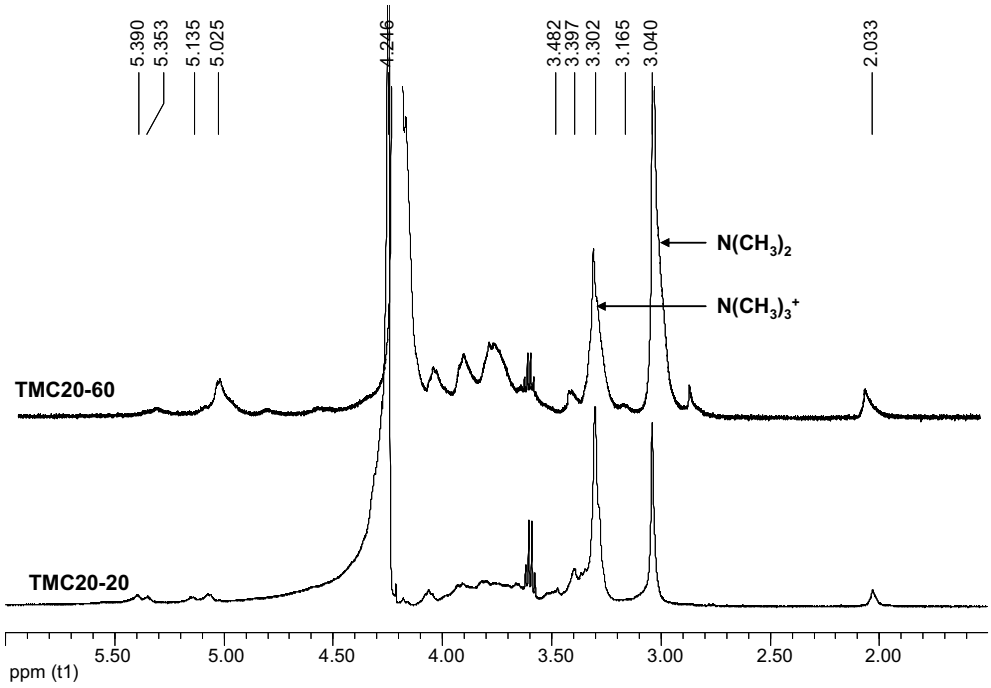

Fig. 1. ^{1}H NMR spectra of TMC20-60 and TMC20-20, prepared in one reaction step and one reaction step with one addition step, respectively.

3.1.3. Intrinsic viscosity

Based on the fact that $[\eta]$ is closely related to polymer-chain conformation, the dependence of $[\eta]$ upon the molecular weight gives information concerning the conformation and the extension of the polymer according to the Mark–Houwink equation

$$[\eta] = KM_v^a$$

where K and a are empirical constants for given solute–solvent system and temperature, $[\eta]$ is the intrinsic viscosity and M_v is the so-called viscosity-average molecular weight which can be substituted with the weight-average molecular weight, M_w. The Mark–Houwink exponent a is used as a parameter to determine the conformation of a polymer. Polymers in the shape of a sphere, random coil or rod have exponent a values of 0, 0.5–0.8 and 1.8, respectively [35,36].

The intrinsic viscosities of TMC in 2% HAc/0.2 M NaAc at 25 °C are given in Table 2. Regardless of the substitution degrees, it was found that the $[\eta]$ of polymer solution increased with an increase in the M_w of the polymer. This is consistent with the previous report by Snyman et al. [33] who observed that the decrease in absolute molecular weight was correlated well with the decreased $[\eta]$ of TMC polymers.

Fig. 2 shows the plot of log $[\eta]$ versus log M_w. The values of 0.39 and 2.14 $\times$ 10^{-4} were obtained for a and K, respectively. The value of Mark–Houwink exponent a suggested that TMC behaved like a spherical structure, approximating a random coil. The result is inconsistent with the observation of Synman et al. [22] who reported that TMC possessed a rod-shaped conformation. This discrepancy could be due to the difference in experimental conditions such as ionic strength, solvent, temperature, and pH value of solution [37,38].

In general, the polymer conformation and the polymer–solvent interactions depend on the number of positive charges (NH_3^+) of chitosan which are related to the degree of deacetylation. Low value of deacetylation degree results in a rigid conformation, leading to a higher degree of expansion of chitosan [28]. In the buffer of 2% HAc/0.2 M NaAc (pH 4.5), chitosan with deacetylation degree of 85% exhibits random coil structure [39]. TMC being a cationic polyelectrolyte with pK_a value of about 6.5 (data shown below), all non-quaternized amino groups are protonated at low pH of the solvent. In this case, electrostatic repulsion forces of the protonated amino groups were hindered due to pendent methyl groups of TMC, leading to the condensed conformation.

3.1.4. Potentiometric titration curves

A potentiometric titration is one of the simplest methods used to determine the degree of deacetylation of chitosan [40,41]. Recently, it has been used with data from elemental analysis to determine DQ of TMC polymers [11].

In this study, a titration curve was generated by dissolving TMC in HCl solution and then titrating potentiometrically with NaOH

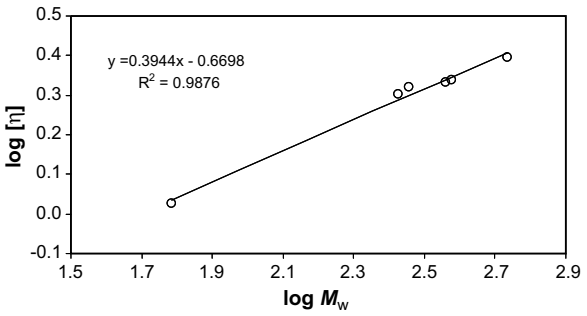

Fig. 2. Intrinsic viscosity of TMC in 2% HAc/0.2 M NaAc at 25 °C as a function of M_w determined by GPC.

solution. The potentiometric titration curve of TMC40-40, a representative example, is depicted in Fig. 3a exhibiting two inflection points. The first of which corresponds to the neutralization of the free acidity, while the second indicates the complete deprotonation of the protonated non-quaternized amine groups. The difference between the two inflection points along the abscissa (shown in Fig. 3b) yields the moles of OH^- required to deprotonate the protonated non-quaternized amino groups of TMC and reflects the amount of $-NH_2$, $-NH(CH_3)$ and $-N(CH_3)_2$ in the titrant solution. Assuming that the rest of the sample is $-N^+(CH_3)_3$ and $-NH-COCH_3$, the DQ value of the specimen could readily be obtained [11].

Since TMC was composed of not only quaternized amines but also mono- and di-methylated amines, it was found that the difference between the two inflection points was affected by DQ and DD. Number of OH^- required to deprotonate the protonated non-quaternized amino groups increased with increasing DD (2.47 mmol OH^-/g polymer for TMC20-20 compared to 2.63 mmol OH^-/g polymer for TMC20-60). When considering DD in the range 30–40%, number of OH^- required decreased linearly with the increase of DQ with regression coefficients of 0.98. Moreover, it was observed that at DD/DQ ratio <1, number of OH^- required decreased with increasing DQ with a linear correlation of 0.99. Therefore, DD

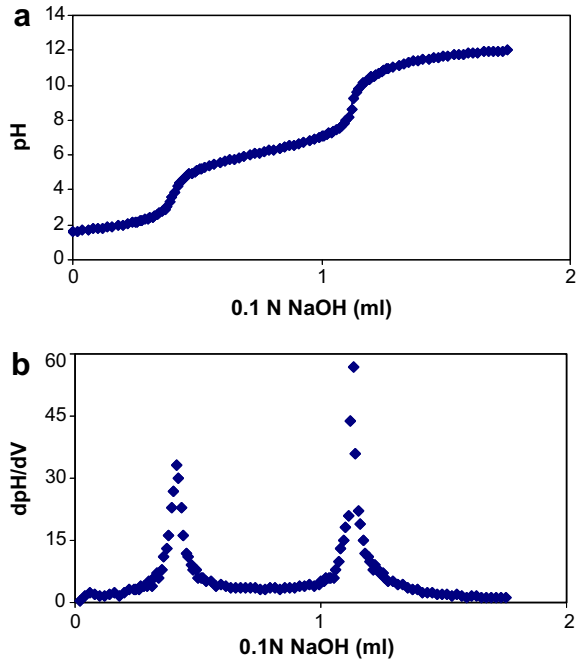

Fig. 3. The results from potentiometric titration of TMC40-40 showing (a) potentiometric titration curve and (b) first derivative curve.

would affect the number of OH^-, leading to an error for calculation of DQ. For an accurate result by this method, DQ should be determined and compared within almost the same value of DD or at DQ much higher than DD. In addition to the use of this method to determine DQ, the pK_a of all TMC polymers can be evaluated to be in the range of 6.1–6.4. During titration, the solution of TMC with DQ < 24% became cloudy when the pH >6, which was not found in higher DQ of TMC (discussed in detail in Section 3.2).

It is well documented in the literature that results obtained from pH-potentiometric titration are influenced by several factors. Balazs and Sipos [42] reported that the moisture content of the air-dry chitosan samples and the ash content caused variations in the values of degree of deacetylation. The precipitation of chitosan during titration also resulted in an error in the determination of deacetylation degree [40]. The precipitated chitosan reduced the concentration and could cover the surface of electrode, and thus the electrode would lose its accuracy. From the limitation of pH-potentiometric titration and the results obtained in this study, it was found that this method would not be suitable for characterization of TMC polymer.

3.2. Solubility of TMC polymers

3.2.1. Effect of degree of quaternization

Fig. 4 shows the pH dependence of the transmittance of the TMC with different DQ. As seen in Fig. 4a, the water solubility of TMC with DQ of 13.9% (TMC10-40) was high at acidic pH but decreased at pH a little over neutrality. The solubility of TMC10-40 in basic pH was abruptly decreased when increasing concentration. In addition, it was observed that the solubility decreased with increasing DD in the basic region, especially when DD/DQ > 1 (data not shown). In contrast, the solubility of TMC with DQ of 42.4% (TMC40-10) was high and retained over a wide pH range (Fig. 4b). The water solubility of TMC was substantially decreased with DQ of 76.6% (TMC80-10), as seen by the low % transmittance (Fig. 4c). However, the solubility of the high DQ of TMC was not pH-dependent (Fig. 4b and c).

The results were comparable to the finding obtained from potentiometric titration mentioned before. The lower solubility of the high DQ of TMC (TMC80-10) was expected due to high degree of *O*-methylation at the 3- and 6-hydroxyl groups [20]. However, the opposite result was found in the low DQ of TMC as reported by Kotze et al. [43] who indicated that TMC with low DQ of 12.6% was highly soluble over a wide pH range even at high concentration of 10% w/v. This discrepancy may be from different DD of TMC. It is possible that the water solubility of TMC polymers with low DQ and relatively high DD decreased in an basic solution because they included about 65–75% of non-quaternized residues, mainly in forms of $-N(CH_3)_2$, $-NH(CH_3)$ and $-NH_2$. The high pendent methyl groups hinder intra- and/or intermolecular interactions resulting in the decreased solubility of the TMC with low DQ.

3.2.2. Effect of ionic strength

The effect of ionic strength on the water solubility of TMC was also investigated. Fig. 5 shows pH dependence of water solubility of TMC10-40 as a function of ionic strength. For TMC with low DQ (<24%), ionic strength did not affect the solubility of polymers at pH lower than their pK_a 6.5, after that the solubility decreased with increasing pH and ionic strength of the medium and the decrease was more pronounced in the higher ionic strength solution. On the other hand, the ionic strength did not affect the solubility of TMC with DQ higher than 40% (data not shown).

It is known that ionic strength affects the hydrodynamic behavior of chitosan and its derivatives. Yang et al. [44] reported that viscosities of *N*-alkylated mono-/disaccharide chitosans with low substitution degree decreased with an increase in ionic strength,

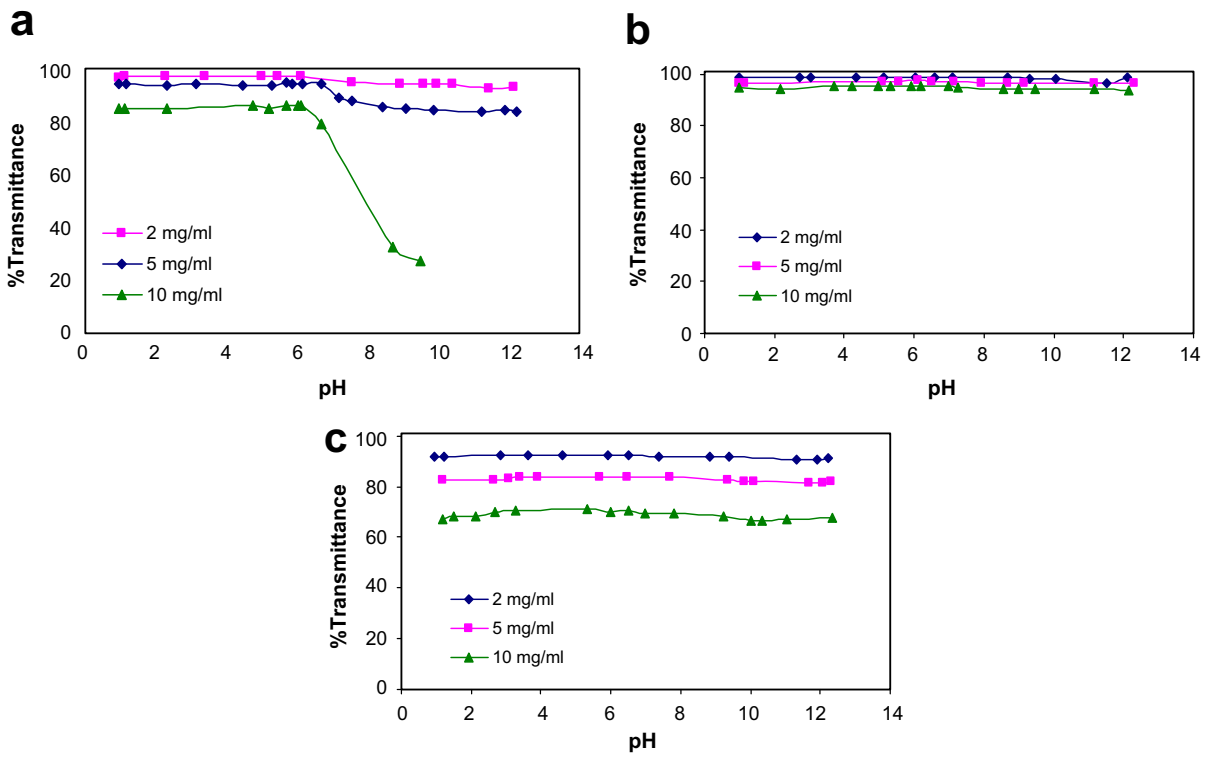

Fig. 4. The pH dependence of water solubility of (a) TMC10-40, (b) TMC40-10 and (c) TMC80-10.

while change in solubility of high substitution degree of chitosan with ionic strength was not marked. Holme and Perlin [45] also observed that ionic strength had an effect on the solubility of *N*-sulfated chitosan. Generally, in high ionic strength solutions, the concentration of the counter-ions is raised which screens the protonated amino group of chitosan and in turn the solubility becomes reduced [46]. This may provide some evidence to support the findings in our experiment.

Taken together, the data from Figs. 4 and 5 imply that the charge density of TMC, represented by DQ, would be an important factor determining its water solubility and the optimum value was an intermediate DQ of 30–40%. In addition, DD obviously affected the solubility properties of TMC when DQ was lower than 24%.

3.3. Polymer–mucin interactions

In this work, mucoadhesive properties of TMC were evaluated by using the mucin particle method based on the change in surface properties of mucin particle, particle size and zeta potential, by the adhesion of the polymer. It was expected that the suspension of ss-mucin particles when mixed with a polymer solution, would induce the ss-mucin particles to aggregate, if the polymer had a strong affinity to them. Procine gastric mucin type III, a commercially available mucin, was chosen in this study. Leitner et al. [47] stated that there is no significant difference in the results obtained with native mucus and hydrated commercial mucin.

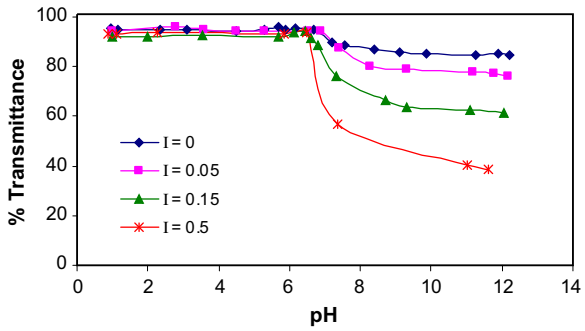

Fig. 5. Effect of ionic strength on the water solubility of 5 mg/ml TMC10-40.

The interaction was determined at pH 6.8 in Tris buffer where chitosan was insoluble and lost mucoadhesive properties. Fig. 6 shows evolution of particle size and zeta potential of ss-mucin particles versus added volume of 1 mg/ml TMC40-40 solution. Two regions could be defined. In region I, polymer did not affect the size and zeta potential of ss-mucin. Increases in size and zeta potential were observed in region II where the aggregation occurred after the zeta potential of ss-mucin exceeded the critical zeta potential of ss-mucin (ca. −7 mV). This finding can be explained by DLVO theory [48]. It was also found that all polymers with different DQ and DD exhibited equal volume of 0.4 ml in region I, indicating that this region was polymer structure independent. This could be explained from the same conformation of TMC polymers. The slope of zeta potential profiles in region II and an extrapolated critical volume (V_c) of polymer used to neutralize negative charge of ss-mucin to zero could be used as indices of mucin–polymer adhesive bond strength of TMC polymers. The stronger the mucoadhesive bond strength, the higher the value of slope as well as the lower the V_c value was observed.

By referring to the results of ss-mucin–polymer interaction studies (Table 3), it can be deduced that TMC exhibited mucoadhesive characteristic and the rank order of mucoadhesive bond strength of TMC was TMC80-10 > TMC20-20 > TMC40-40 > TMC40-10 > TMC10-40 > TMC20-60. Within the same molecular weight of polymer, it was found that the mucoadhesion of TMC depended on the proportion of DD to DQ. The mucoadhesive bond strength of TMC linearly decreased with increased ratio of DD/DQ, as shown in Fig. 7. At the same DD of 40%, the mucoadhesive

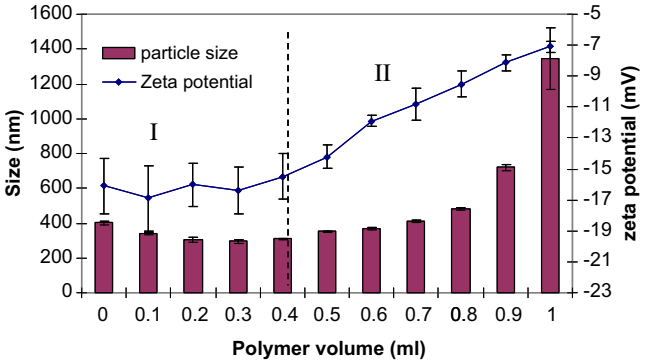

Fig. 6. Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml TMC40-40 solution. Concentration of ss-mucin suspension was 1% w/v at pH 6.8.

bond strength of TMC40-40 was twofold higher than that of TMC10-40. Similarly, at the same DQ of 20%, the mucoadhesive bond strength of TMC20-60 was threefold lower than that of TMC20-20. The results obtained could be explained by the electrostatic interaction between positively charged amino groups of TMC and the negatively charged sialic acid residue of mucus glycoproteins or mucins. When increasing DD, the high number of methyl pendent groups acted to shield the positive charges of TMC which reduced the interaction between polymer and mucin and hence the decreased mucoadhesive properties.

Furthermore, it was observed that the interaction between ss-mucin particles and TMC was molecular weight-dependent. The interaction decreased with decreased molecular weight (TMC40-40 and TMC40-10). No apparent change in surface properties of ss-mucin was detected with 40% DQ TMC derived from chitosan 25 kDa (data not shown). Indeed, the molecular weight of polymer is one of the important factors on mucoadhesive property which has been found in the polymer having the molecular weight above 100,000 g/mol [49]. Taken together, the data obtained suggest that mucoadhesive properties of TMC were influenced by the combination of positive charge density, steric hindrance of pendent groups on polymer and molecular weight.

3.4. Cytotoxicity

The effects of polymer structure on L929 cells were investigated by testing cell viability via MTT assay. The concentration of TMC resulting in 50% inhibition of cell growth, IC_{50} value was evaluated. The results are summarized in Table 4. TMC80-10 was particularly toxic with an IC_{50} of 10 μg/ml. For 40% DQ TMC, a decrease of molecular weight of TMC has caused reduction in toxicity of TMC40-10 compared to TMC40-40. However, TMC10-40 and TMC20-60 were shown to be completely non-toxic with IC_{50} > 1 mg/ml. These appear consistent with the conclusion earlier drawn by Mao et al. [24] and Kean et al. [25] who reported that cytotoxicity of TMC increased with increasing molecular weight and DQ of the TMC. Haas et al. [50] reported that the cytotoxicity on COS-1 cells of TMC with a low DQ (4%) and intermediate DQ (10% and 18%) was less than that of chitosan, meanwhile TMC with high DQ of 66% appeared to be more toxic.

Table 3
Characteristics of the interaction between ss-mucin particle and TMC polymers (mean ± SD, n = 3)

Polymer	Slope (mV/ml)	V_c[a] (ml)
TMC10-40	5.8 ± 0.4	3.0 ± 0.2
TMC20-20	13.1 ± 1.4	1.4 ± 0.1
TMC20-60	4.6 ± 0.4	3.7 ± 0.5
TMC40-10	7.6 ± 0.9	2.1 ± 0.1
TMC40-40	11.3 ± 0.3	1.7 ± 0.0
TMC80-10	15.3 ± 2.0	1.3 ± 0.1

[a] The extrapolated volume of 1 mg/ml polymer solution used to neutralize negative charge of 1% w/v ss-mucin to zero.

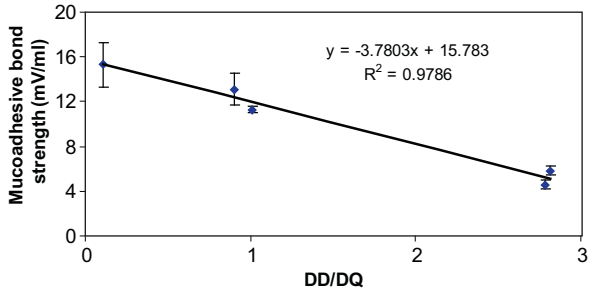

Fig. 7. Correlation between the ratio of DD/DQ of TMC and mucoadhesive bond strength measured by the mucin particle method. Each point represents mean ± SD of three experiments.

Table 4
Cytotoxicity of TMC polymers on L929 fibroblast cells following 3 h incubation as determined by MTT assay (n = 4)

Polymer	IC_{50} (μg/ml)
TMC10-40	>1000
TMC20-20	24
TMC20-60	>1000
TMC40-10	14
TMC40-40	12
TMC80-10	10

Considering the effect of DQ and DD, it was found that cytotoxicity of TMC was influenced by the proportion ratio of DD to DQ. The cytotoxicity of TMC began to decrease when DD/DQ ratio was higher than 1 and TMC showed non-toxic property when ratio of DD to DQ was about 3:1. Since the cytotoxicity of TMC would probably be a consequence of its relatively positive charge [31,51], this phenomenon could be explained by the steric effect of methyl pendent groups of dimethylamino groups which shielded a proportion of the positive charges present on TMC decreasing the interaction of the positively charged amino groups of TMC with the anionic compartments of glycoproteins on the cell membrane. Due to high positive charge density of TMC40-40, the amount of methyl pendent groups from dimethylamino groups was insufficient to shield its positive charges, leading to low cell viability. Similarly, Mao et al. [24] reported that grafting PEG (polyethylene glycol) on TMC polymer chain can improve the biocompatibility of TMC, and the extent of which is substitution degree and PEG molecular weight-dependent.

Taking data from cytotoxicity and mucoadhesion in consideration, it was observed that cytotoxicity data fairly correlated with mucoadhesive bond strength. These confirmed that the electrostatic interaction between the positively charged amino groups of TMC and the negatively charged residues in mucus layer and on cell membrane was the predominant mechanism for mucoadhesion and cytotoxicity of TMC.

4. Conclusions

This work reports the effects of DQ and DD on the physicochemical properties of TMC in terms of solubility, mucoadhesive properties and cytotoxicity. The synthesis of TMC polymers by using one methylation reaction step with subsequent multiple addition steps resulted in the high DQ and large molecular weight. Furthermore,

it was found that an increase in the number of reaction steps increased DQ and decreased DD, whereas an extended duration of reaction increased both DQ and DD. The results also showed that M_w of TMC was in the range of 60–550 kDa and TMC in 2% HAc/0.2 M NaAc was present in a spherical structure, approximating a random coil. The charge density of TMC, represented by DQ, was an important factor to determine its water solubility and the optimum value was an intermediate DQ of 40% regardless of DD and molecular weight. However, DD obviously affected the solubility properties of TMC when DQ was lower than 24%. Cytotoxicity of TMC correlated with mucoadhesive bond strength showed to be dependent upon the ratio of DD to DQ. The high DD in TMC led to a decrease in both mucoadhesivity and cytotoxicity and the effect of DD was evidently observed when DD/DQ > 1. Non-toxic TMC was observed at DD ca. threefold higher than DQ.

In summary, the effect of DD on the physicochemical properties and cytotoxicity was obviously observed when DD/DQ > 1. TMC with relatively high DD possessed reductions in solubility, mucoadhesive properties and hence decreased cytotoxicity. However, the influence of DD was marginal when DQ of TMC was larger than 40% at which physicochemical properties and cytotoxicity was mainly affected by DQ. The results from this study represent helpful information on the synthesis of suitable properties of TMC.

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