Carbohydrate Polymers 108 (2014) 224–231

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Enhanced water-solubility and mucoadhesion of N,N,N-trimethyl-N-gluconate-N-homocysteine thiolactone chitosan

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ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 27 February 2014
Accepted 28 February 2014
Available online 11 March 2014

Keywords:
Mucoadhesive chitosan
Water-soluble chitosan
Cationic polymer

ABSTRACT

A water-soluble chitosan with improved mucoadhesion was prepared by modifying 19.4% of the amine groups of chitosan to trimethylammonium and conjugation of gluconolactone (GLU) and homocysteine thiolactone (HT) onto the remaining amine groups of the chitosan backbone. The derived trimethyl-gluconate-HT-chitosan (TM-GN-HT-chitosan) was confirmed by Fourier Transform Infrared spectroscopy, NMR and thermogravimetric analysis. The total thiol and disulfide group level on the TM-GN-HT-chitosan were17.96 ± 0.03 and 7.36 ± 0.03 μmol/g, respectively. The water solubility of the TM-GN-HT-chitosan conjugate was 79.0 ± 0.15%, more than that of TM-chitosan and chitosan, with an enhanced solubility over a broad pH range ranging from 85.6 ± 10.4% to 58.5 ± 1.1% maximal solubility at pH 2 to 11. Finally, TM-GN-HT-chitosan showed a nearly ~9.5-, 5.0- and 5.6-fold higher mucoadhesiveness than chitosan at pH 1.2, 4.0 and 6.4, respectively, and was optimal at pH 4.0.

1. Introduction

The mucoadhesive properties of chitosan have gained considerable interest due to its potential in drug delivery applications, since they improve the potential of chitosan for both drug localization at the mucosal target site and a prolonged residence time at the site of drug absorption with an increased drug concentration link with the mucosa (Sreenivas & Pai, 2009). However, the mucoadhesive properties of native chitosan are quite weak, due to the weak electrostatic interaction between the amino and hydroxyl groups on chitosan with the negatively charged mucin surface (Bernkop-Schnurch, 2005; Wagh, Joshi, Patel, & Jain, 2009). To address this problem, mucoadhesive chitosans have been developed based on the covalent attachment of thiolated chitosan onto the mucin surface by thiolated chitosan.

Thiolated chitosan consists of thiol group bearing side chains that can undergo covalent bond formation with the cysteine rich subdomains of the glycoproteins in the mucus layer via a thiol-disulfide exchange reaction (Anitha et al., 2011; Bernkop-Schnurch, 2005; Wagh et al., 2009). A diverse range of thiolated chitosans have been reported, such as chitosan conjugations with 4-thio-butyl-amidine (Deepak, Kumar, & Mahadevan, 2012), cysteine (Kurniawan, Fudholi, & Susidarti, 2012; Schmitz, Grabovac, Palmberger, Hoffer, & Bernkop-Schnurch, 2008), iminothiolane (Bernkop-Schnurch, Hormof, & Zoidl, 2003), 4-mercaptobenzoic acid (Millotti, Samberger, Frohlich, Sakloetsakun, & Bernkop-Schnurch, 2010), thioglycolic acid (Bernkop-Schnurch, Hormof, & Guggi, 2004; Hormof, Kast, & Bernkop-Schnurch, 2003), thioglycolic acid-3-methyl-1-phenylpyrazole-5-thiol (Muller, Ma, Gust, & Bernkop-Schnurch, 2013), and homocysteine thiolactone (HT) (Juntapram, Praphairaksit, Siraleartmukul, & Muangsin, 2012a, 2012b).

Recently, the synthesis of novel mucoadhesive *N,N,N*-trimethylchitosan-homocysteine thiolactone (TM-HT-chitosan), based on a thiol-disulfide exchange concept, was reported (Juntapram et al., 2012a, 2012b; Muzzarelli & Tanfani, 1985). Here, in this manuscript, the synthesis of a novel chitosan with a higher mucoadhesive level over pH 1.2–6.4 and a higher water-solubility over a broader pH range (pH 2–11) is reported. A three-step sequential approach was developed to synthesize water soluble mucoadhesive chitosan by first quaternization of chitosan

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http://dx.doi.org/10.1016/j.carbpol.2014.02.078
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to form cationic trimethyl chitosan. Secondly, the remaining amine groups of cationic trimethyl-chitosan were partially conjugated with gluconolactone (GLU) to form the water soluble trimethyl-gluconate-chitosan. Finally, homocysteine thiolactone was partially conjugated to introduce the thiol group and hence to enhance the mucoadhesive property.

2. Materials and methods

2.1. Materials

Chitosan, with a weight-average molecular weight (M_w) of 500 kDa and an 85% degree of deacetylation, was purchased from Seafresh Co., Ltd. (Thailand). The GLU, HT hydrochloride, 5,5′-Dithio-bis (2-nitrobenzoic acid) (DTNB), imidazole, iodomethane, 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDAC), N-methylpyrrolidone (NMP), porcin mucin (type II), basic fuchsin (pararosaniline), sodium metabisulfite, periodic acid, lactic acid, hydrochloric acid, sodium chloride and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich Co., Ltd. (USA) and used without further purification. Dialysis tubing (M_w cut-off of 12–14 kDa) was obtained from Membrane Filtration Products, Inc., (USA). All other chemicals were commercially available and used as received.

2.2. Synthesis of TM-chitosan

The chemical modification of chitosan was conducted in three consecutive steps and shown in Scheme 1. First, chitosan (1 g) was dissolved in 100 mL of 1% (v/v) lactic acid solution at room temperature (RT) overnight and then 5 mL of 15% (w/v) NaOH and 30 mL of a 1:1 (v/v) ratio of iodomethane: NMP were added. The reaction was refluxed for 45 min at 60 °C with stirring. The polymer was isolated by precipitation with 80% (v/v) ethanol and collected by centrifugation. The polymer precipitated cake was suspended in aqueous 5% (w/v) NaCl to exchange iodide by chloride ions and subsequently precipitated with 80% (v/v) ethanol and then 80% (v/v) acetone, and collected by centrifugation. Finally, the product was obtained after freeze-drying.

2.3. Synthesis of TM-GN-chitosan

TM-chitosan (1 g) was dissolved in 100 mL of 1% (v/v) lactic acid solution at RT overnight until completely dissolved. The required amount of GLU (0.25, 0.5, 0.75 or 1 g) was then added to give the final desired (w/w) ratio of TM-chitosan: GLU (1:0.25–1:1) and allowed to react for 24 h at 60 °C with stirring. The reaction mixture was then neutralized with 1 M NaOH and precipitated with an excess volume of acetone. The product was filtered and residual free lactic acid and unreacted GLU were removed by dialysis against distilled water. The product was obtained after freeze-drying.

2.4. Synthesis of TM-GN-HT-chitosan

TM-GN-chitosan (1 g), derived from a 1:1 (w/w) ratio of TM-chitosan: GLU, was dissolved in 100 mL of 1% (v/v) lactic acid solution, and then an aqueous solution of imidazole (0.68 g in 2.50 mL water) was added to the reaction flask followed by the dropwise addition of an aqueous solution of HT (0.125% (w/v) in 100 mL water). A carbonyl group was activated by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and left with stirring at RT under a nitrogen (N_2) atmosphere for 24 h without exposure to light. The solution was neutralized with 1 M NaOH, precipitated with excess acetone, washed with distilled water and filtered. The residual free lactic acid and unreacted HT were then removed by dialysis in distilled water. The product was obtained after freeze-drying.

2.5. Characterization of the chitosan and chitosan conjugates

2.5.1. Analytical instruments

2.5.1.1. Fourier transform infrared spectroscopy (FT-IR). Chitosan and the obtained products after chemical derivatization were analyzed in solid state by FT-IR (Nicolet 6700) in the region from 400 to 4000 cm^{-1}, to provide information on the functional chemical groups through their characteristic frequencies and intensities. All samples were prepared as KBr pellets.

2.5.1.2. ^1H NMR spectroscopy. ^1H NMR spectra were taken in a Mercury Varian NMR spectrometer operated at 400 MHz (Agilent Technologies, CA, USA), using a pulse accumulation of 64 scans. The chitosan and three chitosan conjugates were each dissolved in deuterium oxide (D_2O) and acidified with 2% (v/v) trifluoroacetic acid (CF_3COOH).

2.5.1.3. Thermogravimetric analysis (TGA). The thermogravimetric analysis (TGA) of the chitosan and three chitosan conjugates were measured as a function of increasing temperature using a PerkinElmer Pyris Diamond TG/DTA machine under a N_2 atmosphere at a flow rate of 30 mL/min. Samples (~5 mg) were heated in the aluminum oxide pan in the balance system at a rate of 25 °C/min from 50 to 600 °C.

2.5.2. Determination of the degree of quaternization (%DQ) on the TM-chitosan by ^1H NMR

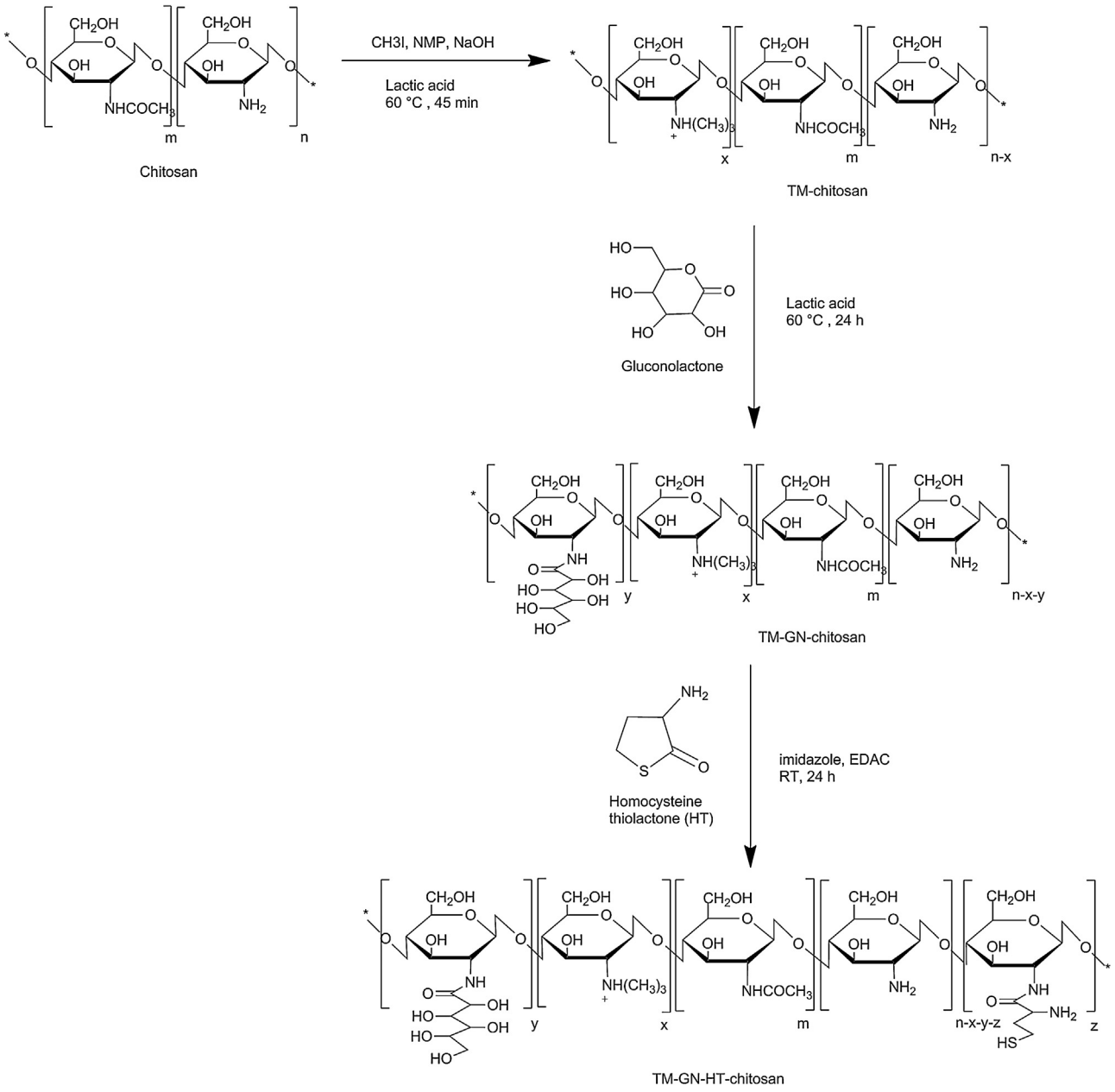
^1H NMR spectroscopy was used to measure the degree of quaternization (DQ) of chitosan in the TM-chitosan, which is one of the most important characteristics of TM-chitosan. The DQ was calculated using the data obtained from the ^1H NMR spectra according to Eq. (1), as previously described (Jia, Shen, & Xu, 2001);

$$\%DQ = \left[\frac{[(CH_3)_3]}{[H3 - H6]} \times \frac{5}{9}\right] \times 100, \quad (1)$$

where, $[(CH_3)_3]$ is the integration area of the trimethyl amino group at 3.02 ppm and [H3–H6] is the total integration area of the proton peaks of H3–H6 of the chitosan ring between 3.62 and 4.15 ppm.

2.5.3. Determination of the thiol and disulfide groups content in the TM-GN-HT-chitosan

Ellman's reagent (DTNB) was used to quantify the concentration of thiol groups in the TM-GN-HT-chitosan. The reagent reacts with the sulfhydryl groups on the TM-GN-HT-chitosan, cleaving the disulfide bond to give the yellow-colored 2-nitro-5-thiobenzoate (NTB^{2-}) ion that can be evaluated by measured the absorbance at 450 nm (Ellman, 1959). Briefly, 5 mg of TM-GN-HT-chitosan or 5 mg of each respective control (chitosan, TM-chitosan and TM-GN-chitosan) were hydrated in 250 μL deionized water. Then, 250 μL 0.5 M phosphate buffer pH 8.0 and 500 μL Ellman's reagent were added and incubated for 2 hat RT. The supernatant was separated from the precipitated polymer by centrifugation (3200 rpm, 5 min) and the absorbance was measured at a wavelength of 450 nm. L-Cysteine hydrochloride standards (10–70 μmol/L) were used to determine the quantity of thiol moieties on the TM-GN-HT-chitosan, whilst the amount of disulfide bonds were quantified with Ellman's reagent after reduction with $NaBH_4$ by subtracting the quantity of free thiol groups as previously reported (Xie, Liu, & Chen, 2007). The linear range for L-cysteine hydrochloride standards were obtained at 100–1000 μmol/L, with a linear equation (square method) of $y = 0.001x - 0.186$.



Scheme 1. Synthesis of TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan.

2.6. *In vitro bioadhesion of mucin to chitosan and the three chitosan conjugates*

2.6.1. *Mucin determination by the periodic acid Schiff (PAS) method*

The periodic acid Schiff (PAS) colorimetric method was used to measure the concentration of free mucin. The Schiff reagent was prepared as previously reported (Kafedjiiski, Krauland, Hoffer, & Bernkop-Schnourch, 2005). To 100 mL of 1% (w/v) basic fuchsin (pararosaniline) in deionized water was added 20 mL of 1 M HCl and 1.67% (w/v) sodium metabisufite and incubated at 37 °C until pale yellow. The periodic acid reagent was freshly prepared by the addition of 10 μL of 50% (v/v) periodic acid in water to 7 mL of 7% (v/v) acetic acid solution.

Standard calibration curves of mucin were prepared from mucin standard solutions in the range of 0.2–1.0 mg/mL. First, 100 μL of periodic acid reagent was added to 20 μL of sample solution and then incubated at 37 °C for 2 h before 100 μL of Schiff reagent was added and incubated at RT for 30 min. Finally, the absorbance was measured at a wavelength of 555 nm. The mucin content was then calculated by reference to the standard calibration curve.

2.6.2. *Adsorption of mucin on chitosan and the three chitosan conjugates*

Mucin was prepared in 0.1 M phosphate buffer pH 1.2, pH 4.0 and pH 6.4 at 0.5% (w/v). Suspensions of sample stock solution were mixed with type II mucin (1 mg/mL), vortexed, and incubated at 37 °C for 2 h. After that the dispersions were centrifuged at 12,000 rpm for 2 min and the residual free mucin was measured in the supernatant using the PAS assay. The mucin concentration was calculated by reference to the calibration curve and the amount of mucin adsorbed to the polymer was calculated as the difference between the total amount of mucin added and the free mucin content in the supernatant.

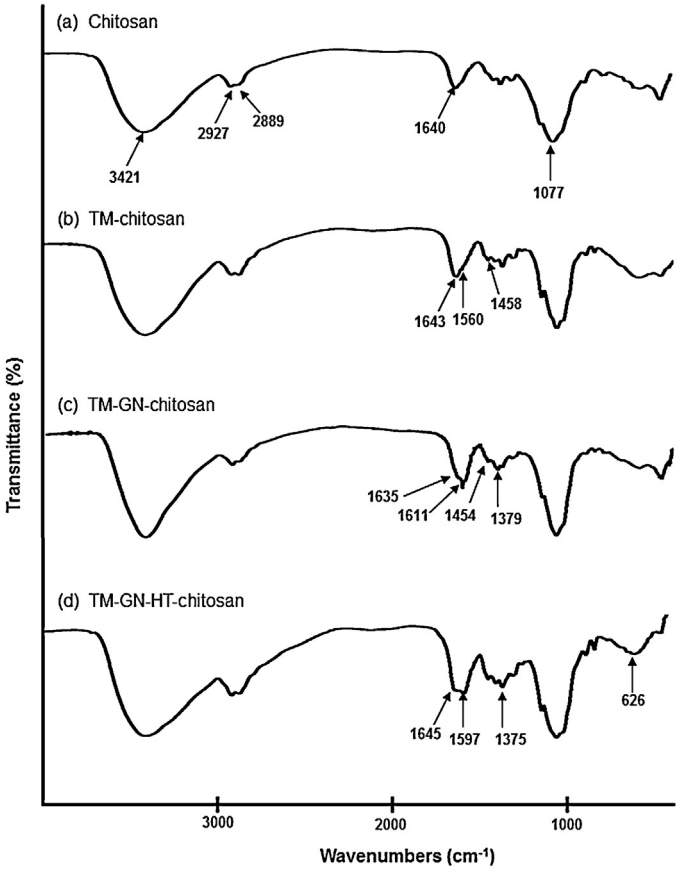


Fig. 1. Representative FT-IR spectra of (a) chitosan, (b) TM-chitosan, (c) TM-GN-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU), and (d) TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU).

2.7. Solubility of chitosan and the three chitosan conjugates

The solubility of chitosan and the three chitosan conjugates in distilled water at room temperature was calculated as reported (Kilcoyne & Gerlach, 2011; Ping, Stanley, & Lisbeth, 1998). Briefly, 0.1 g of sample was suspended in 10 mL distilled water and stirred at 25 °C for 5 h to give a saturated solution and insoluble remains. The undissolved solids were then collected by gravity filtration, washed with acetone and dried at 40 °C under vacuum overnight. The solubility (% *Sa*) was calculated using Eq. (2);

$$\%Sa = \left[\frac{0.1 - W_1}{0.1}\right] \times 100 \quad (2)$$

where W_1 is the weight of undissolved polymers (g). Thus, a 100% *Sa* represents a solubility of almost 10 g/L.

Moreover, the relative solubility of chitosan and the three chitosan conjugates was assessed at RT over a pH range of 2 to 11. The samples were dissolved in 0.1 M HCl solution to make 1% (w/v) of chitosan, followed by the dropwise addition of a 0.1 M NaOH solution until reach the desired pH. The transmittance of the solution was measured using a microtiter plate reader (Biotex powerwave XS2) at 600 nm.

3. Results and discussion

3.1. FT-IR analysis

The FT-IR spectra of the unmodified chitosan and its three modified chitosan are shown in Fig. 1. The spectrum of the unmodified chitosan (Fig. 1a) showed a strong broad band at 3421 cm^{-1}, which corresponded to the stretching vibration of O—H and N—H bending. The characteristic chitosan peak belonging to the C=O (amide) stretching vibration was observed at 1640 cm^{-1}, along with a broad absorption peak at 1077 cm^{-1} due to the symmetric C—O—C and C—O stretching vibrations. After, quaternization of chitosan, the FT-IR spectrum of TM-chitosan (Fig. 1b) showed a peak at 1643 cm^{-1} corresponding to the C=O (amide) stretching vibration, a second band at 1560 cm^{-1} that arose from the angular deformation of N—H in the amino group, and a third band at 1458 cm^{-1} that was assigned to the asymmetric stretching of C—H in the methyl groups.

After conjugation of gluconolactone onto some of the remaining amino groups of TM-chitosan, the resultant TM-GN-chitosan gave a group of FT-IR bands at 1635 cm^{-1} and 1379 cm^{-1} (Fig. 1c), which were attributed mainly to $-NH_2$ bending and C—N stretching, respectively. In addition, the absorption band near 1454 cm^{-1} was attributed to the asymmetric stretching of C—H in the methyl groups, whilst those at 1065 and 1023 cm^{-1} were assigned to the C—O stretching vibration of C3—OH and C6—OH, respectively. The increased intensity of the C—O stretching bands is due to the substitution of gluconate into the chitosan structure.

After conjugating homocysteine thiolactone onto the TM-GN-chitosan at a 1:0.1 (w/w) TM-GN-chitosan: HT ratio, the observed FT-IR signals of the obtained TM-GN-HT-chitosan at 2923 cm^{-1} and 2877 cm^{-1}, which were assigned to the vibration of CH_2 (asym.) and CH_2 (sym.), respectively, of the side chain of HT (Fig. 1d), were increased in peak height compared with that of the TM-chitosan and TM-GN-chitosan. The absence of a band around 1700 cm^{-1} (C=O of the lactone ring of the HT) indicated that HT was conjugated onto the chitosan backbone. Moreover, the synthesis of TM-GN-HT-chitosan was confirmed by the presence of a thiol absorption band at 626 cm^{-1}.

3.2. ^1H NMR spectroscopic analysis of the samples

The representative ^1H NMR spectra of chitosan and the three modified chitosans are shown in Fig. 2. The internal standard used for assigning the chemical shifts of the protons was D_2O/CF_3COOH, which appears as a singlet at 4.79 ppm.

The ^1H NMR spectra of chitosan (Fig. 2a) revealed the H3–H6 protons in the pyranose ring at around 3.57–3.76 ppm. The signal at 3.03 ppm was attributed to the H2 of the glucosamine (GlcN) units, whilst that at 1.92 ppm was assigned to the acetyl protons of the N-acetylglucosamine (GlcNAc) units in the skeleton. After quaternization of chitosan, the presentation of new protons positions in TM-chitosan in Fig. 2b, explained the characteristic peaks of TM-chitosan at 4.15–3.69, 3.62, 3.29, 3.14, 3.02, 2.83, 2.18 and 2.03 ppm due to the N-methylation and O-methylation of chitosan. These were attributed to the H3–H6, 3-,6-OCH_3, H2, $N^+(CH_3)_3$, $N(CH_3)_2$, $NH(CH_3)$ and acetyl (GlcNAc) protons in the TM-chitosan backbone, respectively. The DQ, as calculated from Eq. (1) using the integration areas from the ^1H NMR spectra, was 19.44%.

The ^1H NMR spectrum of TM-GN-chitosan (Fig. 2c) showed the characteristic peaks of both TM-chitosan and the gluconate segments with chemical shifts of 4.40 and 4.33 ppm that were assigned to the H7 and H8 protons respectively, observed as new proton positions from the ring opened side chain of gluconolactone. The residual chemical shift of the gluconate protons overlaps with the backbone protons of chitosan. After conjugation of the homocysteine thiolactone hydrochloride onto the amino groups of TM-GN-chitosan, the ^1H NMR spectrum of the TM-GN-HT-chitosan (Fig. 2d) revealed signals from the new protons from the ring opened side chain of homocysteine thiolactone at 2.80 and 2.41 ppm, which were attributed to H13 overlapped with $N(CH_3)_2$ and H14, respectively. Furthermore, the protons at 4.08 and 1.37 ppm showed the reacted chitosan with lactic acid. The degree of conjugated GN, and the level of thiol groups and disulfide

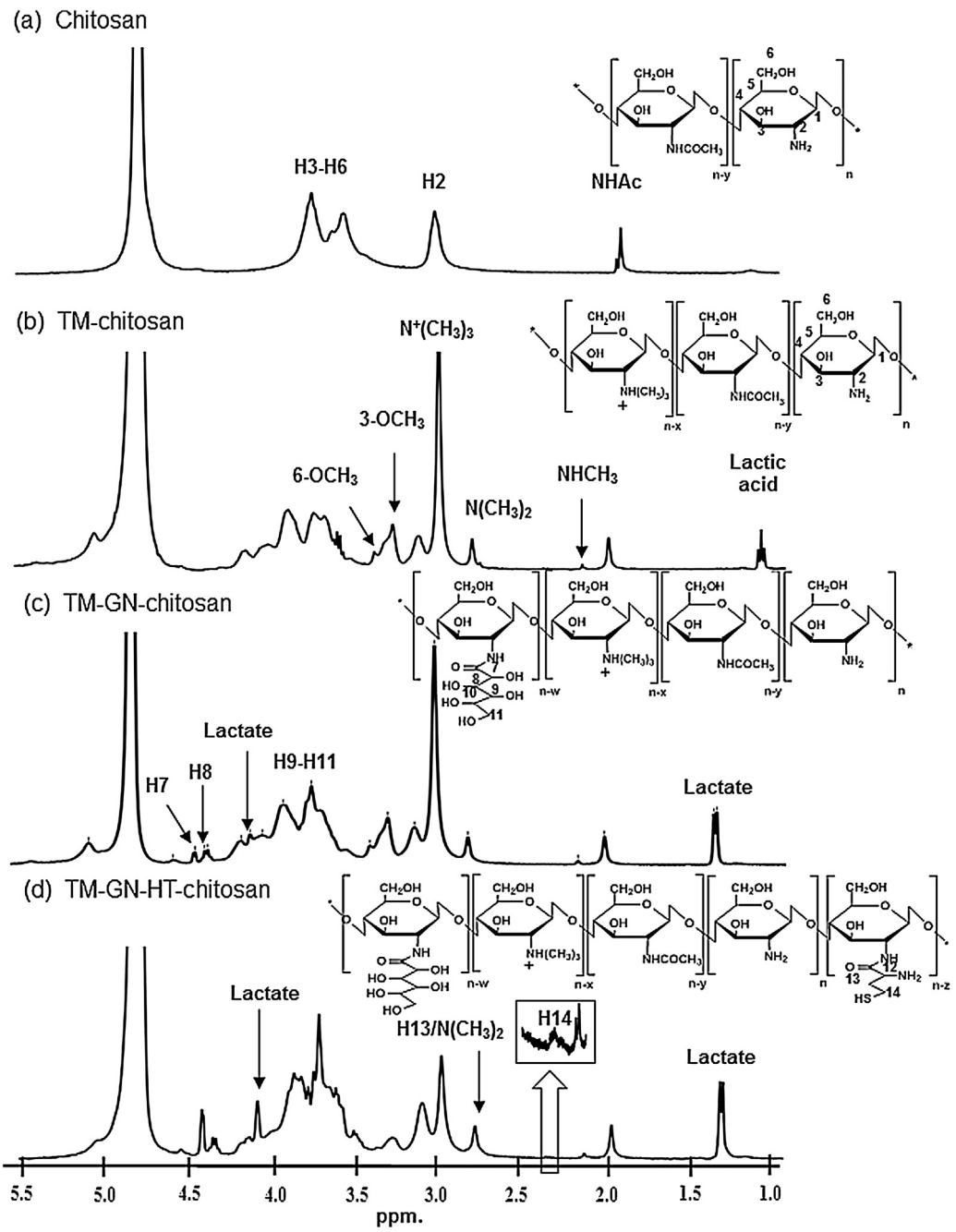


Fig. 2. Representative ^{1}H NMR spectra of (a) chitosan, (b) TM-chitosan, (c) TM-GN-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU), and (d) TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU).

bonds, could not be evaluated by NMR, and so were determined by the solubility of the conjugated products.and Ellman's methods, respectively.

These results supported that the TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan conjugates have been obtained.

3.3. Thermogravimetric analysis (TGA)

Thermal analysis has been widely used for the characterization of polymeric materials. The thermal properties of chitosan and the three modified chitosans and revealed two distinct stages in their thermal degradation (Fig. 3).

During the first stage (50–110 °C) of chitosan degradation (Fig. 3a) a slight loss of mass (4%) was observed and was attributed to water evaporation. This indicated a direct dependence of the water content on the number of charges on the polymer chains (Ali & Singh, 2009). The second decomposition stage, with a significant loss of mass at 325 °C (55%), was due to the decomposition of the chitosan backbone.

The TG curve for TM-chitosan (Fig. 3b) showed a 7% weight loss during the first degradation stage from 50 to 200 °C. This is due to the greater hydrophilicity of TM-chitosan. The second decomposition stage (305 °C) occurred at a lower temperature than that of chitosan, and accounted for a 50% weight loss due to the deacetylation of chitosan and the decomposition of the substituted sites in the methylated derivatives (Mourya & Inamdar, 2009). Thus, the thermal stability of chitosan was decreased after quaternization to form TM-chitosan, which is consistent with the previously reports (Xu, Xin, Li, Huang, & Zhou, 2010; Juntapram et al., 2012a, 2012b).

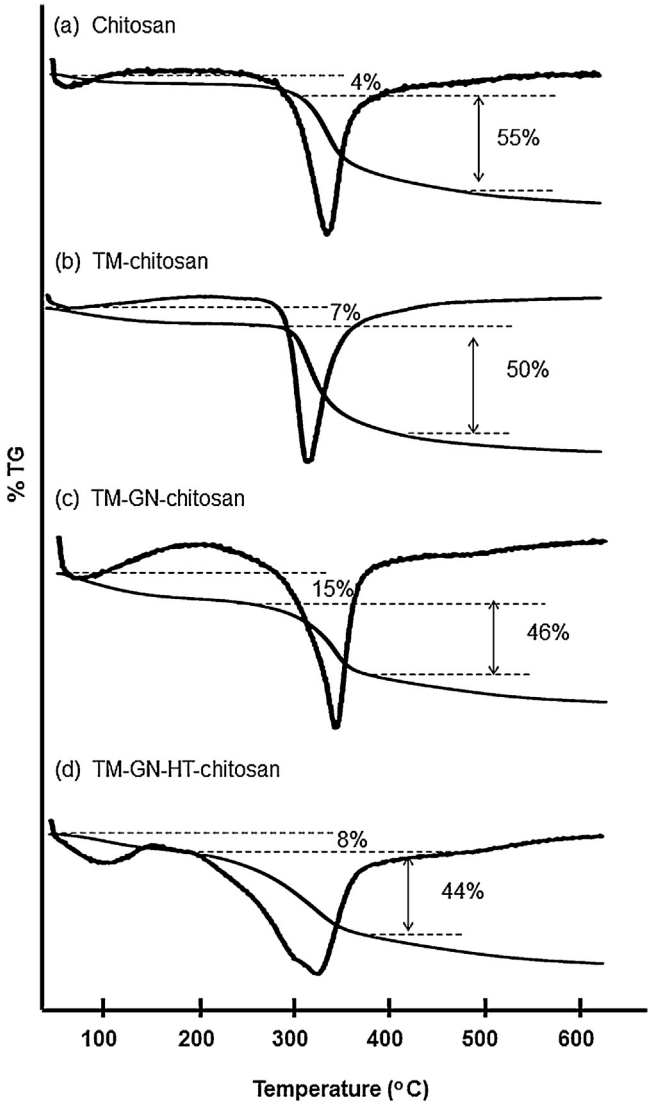


Fig. 3. Representative TG and DTG curves of (a) chitosan, (b) TM-chitosan, (c) TM-GN-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU), and (d) TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU).

For the TM-GN-chitosan (Fig. 3c) and TM-GN-HT-chitosan (Fig. 3d), the first thermal decomposition stage, representing the water loss, occurred in the range of 50–200 °C and 50–150 °C with a 5% and 8% weight loss, respectively. The second decomposition stage was observed at 317 °C and 315 °C, respectively, in between that of chitosan and TM-chitosan, and accounted for a smaller weight loss of 46% and 44%, respectively. This might be due to the cleavage of the substituent groups (Viviane, Mauro, & Valfredo, 2004). Regardless, the three chitosan conjugates all had a lower thermal stability compared to the unmodified chitosan. The loss of thermal stability upon the incorporation of GLU and HT side chains onto the chitosan backbone could be due to the changed crystalline structure of chitosan, especially through the loss of hydrogen bonding between chitosan chains.

3.4. Determination of the thiol and disulfide groups content

The amount of thiol groups and disulfide bonds immobilized in the conjugated TM-GN-HT-chitosan was 17.96 ± 0.03 μmol/g and 7.36 ± 0.03 μmol/g for the thiol and disulfide groups, respectively.

3.5. Solubility of chitosan and the three chitosan conjugates

3.5.1. Solubility in water

The solubility of chitosan and the three chitosan conjugates in distilled water at RT is summarized in Fig. 4a.

Chitosan is insoluble in water and the aqueous solution at neutral pH. However, it ionizes at acidic pH and becomes soluble. The formation of a quaternary salt and hydrophilic groups onto the chiotosan backbone greatly improved its solubility. The results demonstrate that all conjugated chitosan can be dissolved in distilled water at room temperature with various solubility values. After quaternization of chitosan, the solubility of TM-chitosan was 24.0 ± 3.8% in water, based on the initial dried weight. The water solubility of TM-gluconate-chitosan increased from 40.5 ± 5.7% to 84.5 ± 3.2% as the TM-chitosan: GLU (w/w) ratio increased from 1:0.25 to 1:1. After conjugation of gluconate onto the chitosan backbone, the level of internal hydrogen bonding between the amine groups and the crystallinity of the native chitosan were both reduced. Although the TM-GN-chitosan still has a number of —OH groups, the water solubility was still high because the GN chains can interrupted the strong hydrogen bonds between the amine groups of chitosan. With respect to the TM-GN-HT-chitosan, the solubility was numerically slightly, but not statistically significantly, lower than that for the TM-GN-chitosan from the same TM-chitosan: GLU (w/w) ratio of 1:1. Presumably, this is accounted for as that the —SH group in the TM-GN-HT-chitosan was only slowly and incompletely associated with hydrogen bonding and so the TM-GN-HT-chitosan was less soluble in water than the TM-GN-chitosan.

3.5.2. Solubility of chitosan and the three chitosan conjugates at pH 2–11

The solubility of chitosan and its conjugated derivatives are expected to be dependent on their respective pKa values and concentrations (Chung, Kuo, & Chen, 2005). Chitosan has a pKa of ~6.5, which leads to chitosan precipitation in neutral and basic solutions.

Accordingly, the solubility of chitosan and the three chitosan conjugates were evaluated at RT over a pH range of 2–11 (Fig. 4b). The relative solubility of chitosan, measured as the turbidity of the solution by relative transmittance, was abruptly decreased from its initial level (100–92.5% at pH 2–6) down to almost insoluble levels at pH values of higher than 6.5 (23.2–3.1% at pH 6.5–11). In contrast, the TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan solutions exhibited a higher relative solubility over the entire pH range. Rather, the relative solubility (as the optical transmittance) of the TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan solutions were only slightly decreased from that at pH 2–6.5 (94.0–90.1%, 97.1–94.1% and 85.6–79.8% respectively), but did not decrease as dramatically at alkaline pH values (67.1–56.9%, 81.6–67.6% and 66.5–58.5% at pH 7–11, respectively) as did chitosan. Thus, the modification of chitosan by introducing hydrophilic functional groups, via quaternization and conjugation with GN and HT, improved the solubility at all pH values, but markedly so in the neutral to alkaline range (pH 6.5–11) compared to the unmodified chitosan.

3.6. Mucoadhesive properties of chitosan and the three chitosan conjugates

The mucoadhesive properties of chitosan and the three chitosan conjugates were evaluated in terms of their *in vitro* binding to mucin in solution, and the results are summarized in Fig. 4c.

The adsorption of mucin was maximal at pH 4.0 and minimal at pH 1.2 for the chitosan and all three chitosan conjugates, being some four-fold higher at pH 4.0 than at 1.2 for chitosan and ~2.0-fold higher for the three chitosan conjugates. This is likely to be because although at pH 1.2 the amine groups of chitosan are protonated and so charged ($-NH_3^+$; p*K*a 6.5–6.8), a significant amount

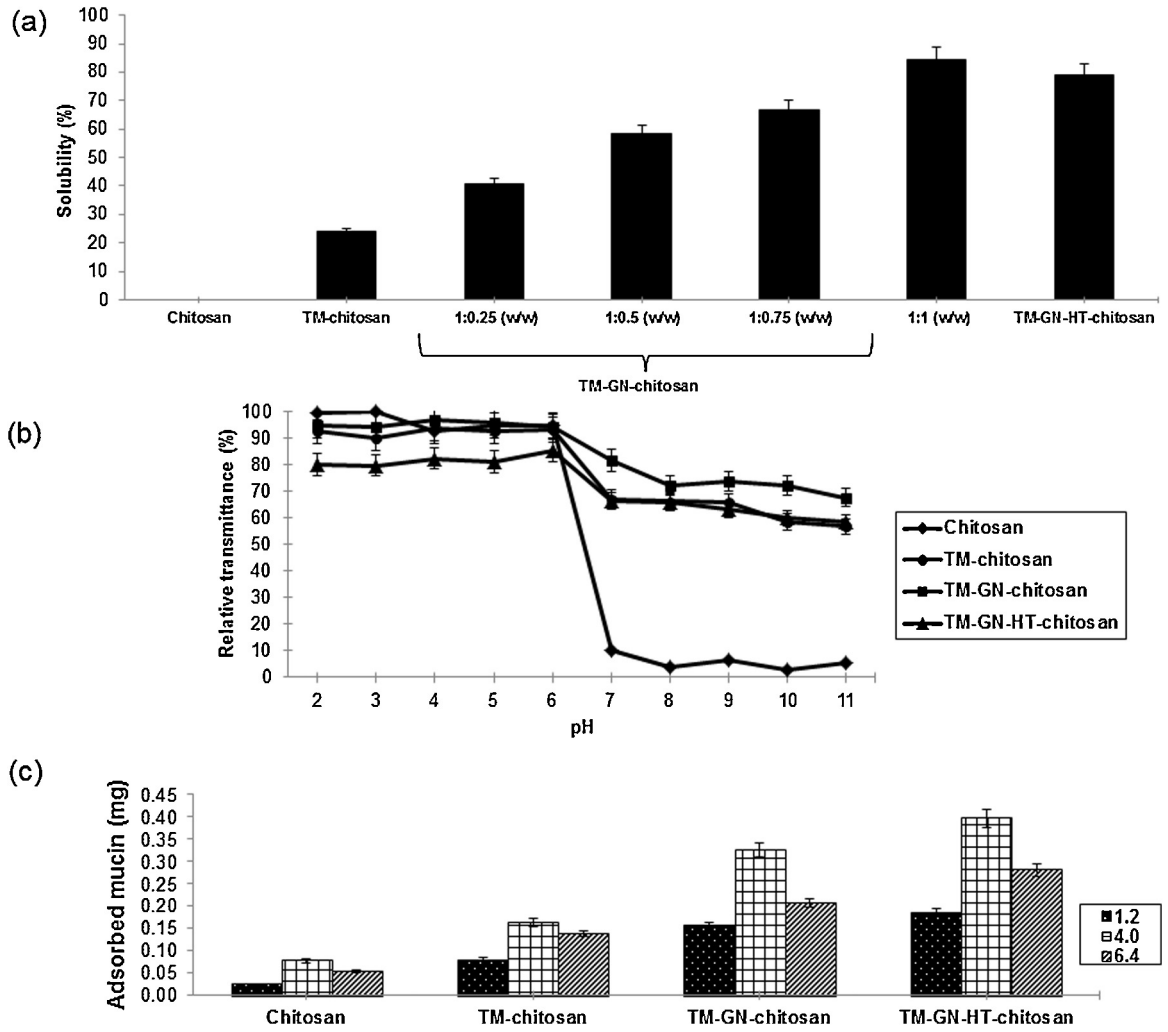


Fig. 4. (a) Water solubility of chitosan, TM-chitosan, TM-GN-chitosan from different TM-chitosan: GLU (w/w) ratios, and TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU). (b) The pH solubility of chitosan, TM-chitosan, TM-GN-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU) and TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU). (c) The adsorption of mucin on chitosan, TM-chitosan, TM-GN-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU) and TM-GN-HT-chitosan (1:1 (v/v) ratio of TM-chitosan: GLU) at pH 1.2, 4.0 and 6.4. Data are shown as the mean ± SD and are derived from three independent repeats.

of the sialic acid and carboxyl groups of mucin (—SO_3H and —COOH, average p*K*a of 2.6) will also be protonated and so not charged, reducing the electrostatic interaction of the mucus glycoproteins and polymers. In addition, the steric conformation of the aggregated mucin fibers and increased viscoelasticity of the mucin at low pH values would reduce the interaction. Indeed, mucus pH is highly dependent on the mucosal surface, and strongly acidic conditions promote the aggregation of mucin fibers and greatly increase the mucus viscoelasticity (Samuel, Wang, & Hanes, 2009). At pH 4.0, a larger proportion of the sialic acid and carboxyl groups of mucin were deprotonated and so charged (—SO_3^- and —COO^-), whilst most of the chitosan amine groups remain protonated and charged, which allowed a stronger electrostatic interaction, whilst the mucin fibers are less compacted allowing more access to its surface area including the charged groups.

However, whilst the same pH-dependent trend was seen in the three chitosan conjugates, the amount of mucin bound by them was significantly greater at each pH than that bound by chitosan. For the TM-chitosan, 4.0-, 2.0- and 2.8-fold more mucin was bound than chitosan at pH 1.2, 4.0 and 6.4, respectively, whilst it was 8.0-, 4.13- and 4.2-fold higher for the TM-GN-chitosan and 9.5-, 5.0- and 5.6-fold higher for the TM-GN-HT-chitosan, respectively. This reflects the less pH-dependent (over this pH range) and higher density of positive charges on the TM-chitosan than the chitosan, and so the enhanced electrostatic interaction with the negatively charged mucin. Conjugating GLU onto the TM-chitosan backbone further enhanced (1.5- to 2.0-fold) the level of absorbed mucin over that on the TM-chitosan since the hydroxyl groups of GN that can hydrogen bond with the —COOH and —SO_3H groups of the mucin glycoproteins. Conjugating HT onto the TM-GN-chitosan further slightly (1.19- to 1.33-fold) increased the mucin binding over that of the TM-GN-chitosan, which can be explained by formation of covalent bonds between the thiol groups of the conjugate and the cysteine rich subdomains of glycoproteins in the mucin layer (Sreenivas & Pai, 2008). Moreover, the hydrophobic effect of the —CH_2 moieties in HT interact with the —CH_3 groups on the native mucin.

At pH 6.4, the mucoadhesive properties of chitosan and the three conjugates (TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan) were decreased slightly compared to that at pH 4.0, but were still higher than that at pH 1.2. This is because at pH values above 6, the amino groups of chitosan became largely deprotonated and so uncharged (—NH_2), as were the $^+N(CH_3)_3$ groups of the TM-chitosan too. At the same time, the —OH and —SH groups of the conjugates were able to form hydrogen and covalent bonds, respectively, with the native mucin. In addition, the side chain of the HT (—CH_2 groups) could interact with the side chain of native mucin (–CH_3 groups) to increase the van der Waals' interactions.

In conclusion, electrostatic and hydrophobic interactions and hydrogen and covalent bonding all had an effect on the mucoadhesive properties of chitosan, TM-chitosan, TM-GN-chitosan and TM-GN-HT-chitosan at all pH values, but especially at a neutral to alkaline pH.

4. Conclusion

A TM-GN-HT-chitosan polymer was successfully synthesized by covalent conjugation of HT onto the primary amine groups ($-NH_2$) of TM-GN-chitosan. This modified polymer had an enhanced water solubility, especially at a neutral to basic pH, and exhibited higher mucoadhesive properties than that of chitosan or TM-GN-chitosan over the tested pH range (pH 1.2–6.4). Increasing the number of cationic groups, hydroxyl groups and thiol groups increased the water-solubility and mucoadhesiveness of the TM-GN-HT-chitosan.

Acknowledgements

The authors gratefully acknowledge funding from the Asia Research Center and the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University to N.M. (RES560530064-FW). The authors also thank Dr. Robert Butcher of the PCU, Faculty of Science, Chulalongkorn University for constructive comments and English corrections.

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