Carbohydrate Polymers 87 (2012) 2399–2408

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Synthesis and characterization of chitosan-homocysteine thiolactone as a mucoadhesive polymer

Kotchakorn Juntapram[a], Nalena Praphairaksit[b], Krisana Siraleartmukul[c], Nongnuj Muangsin[d,*]

[a] Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[b] Department of Biology, Faculty of Science, Srinakarinwirot University, Bangkok 10110, Thailand
[c] Metallurgy and Materials Science Research Institute, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand
[d] Center for Petroleum, Petrochemicals and Advanced Materials, Department of Chemistry, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form 30 October 2011
Accepted 3 November 2011
Available online 12 November 2011

Keywords:
Chitosan
Mucoadhesive polymer
Thiolated chitosan
N,N,N-Trimethyl chitosan

ABSTRACT

Two mucoadhesive thiolated polymers were synthesized by the covalent attachment of homocysteine thiolactone (HT) to chitosan and *N,N,N*-trimethyl-chitosan (TM-chitosan) at various chitosan:HT ratios. The amount of thiol and disulphide groups immobilized on the chitosan influenced the polymer's mucoadhesion positively and negatively, respectively, with the optimal chitosan:HT (w/w) ratio being found to be 1:0.1. The interaction between mucin and chitosan and its three derivatives was highest for the thiolated chitosan derivatives but was pH dependent. HT-chitosan and TM-HT-chitosan, with the thiol groups of 64.15 and 32.48 μmol/g, respectively, displayed a 3.67- and 6.33-fold stronger mucoadhesive property compared to that of the unmodified chitosan at pH 1.2, but these differences were only ~1.7-fold at pH 6.4. The swelling properties of TM-HT-chitosan and HT-chitosan were higher than that of chitosan and TM-chitosan, attaining a swelling ratio of up to 240% and 140%, respectively, at pH 1.2 within 2 h.

1. Introduction

Chitosan or poly[β-(1–4)-2-amino-2-deoxy-D-glucopyronose] is a copolymer of glucosamine and *N*-acetylglucosamine. It has key properties that make it a good mucoadhesive polymer, including enzymatic biodegradability, non-toxicity and high biocompatibility (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Jayakumar, Deepthy, Manzoor, Nair, & Tamura, 2010; Jayakumar, Prabaharan, & Muzzarelli, 2011). Over the past few years, mucoadhesive chitosan have gained substantial interest for potential applications in drug delivery at mucosal membranes. It allows targeting and localization of the drug at a specific adsorption site and improves the effectiveness of relevant drugs by maintaining their plasma concentration at therapeutic levels for a prolonged period of time and inhibiting the dilution of the drug in the body fluids (Ludwig, 2005; Salamat-Miller, Chittchang, & Johnston, 2005).

The primary amino (NH_2) and hydroxyl (OH) groups of chitosan are considered to be responsible for mucoadhesive property via non-covalent bonds, e.g. hydrogen bonds and ionic interactions (Smart, 2005). In order to improve its mucoadhesive property, chitosan bearing thiol groups that can form covalent bonds with the mucus membrane via thio/disulfide exchange reaction has been synthesized and called thiolated chitosan (Bernkop-Schnurch, 2005; Leitner, Walker, & Bernkop-Schnurch, 2003). An example of thiolated chitosan is chitosan–TBA (4-thio-butyl-amidine). It exhibits over 100-fold higher mucoadhesiveness than that of unmodified chitosan (Bernkop-Schnurch, Hornof, & Zoidl, 2003; Bernkop-Schnurch, Kast, & Guggi, 2003) and can be used as platforms for oral controlled drug delivery (Roldo, Hornof, Caliceti, & Bernkop-Schnurch, 2004). More examples of thiolated chitosan include chitosan–thioglycolic acid conjugates (Hornof, Kast, & Bernkop-Schnurch, 2003; Kast & Bernkop-Schnurch, 2001; Kast, Frick, Losert, & Bernkop-Schnurch, 2003), chitosan–cysteine conjugates (Schmitz, Grabovac, Palmberger, Hoffer, & Bernkop-Schnurch, 2008) and chitosan-iminothiolane (Bernkop-Schnurch, Hornof, et al., 2003; Bernkop-Schnurch, Kast, et al., 2003).

Furthermore, thiolated chitosan has been extensively investigated as biomedical applications. For example, chitosan–thioglycolic acid (chitosan–TGA) conjugate nanoparticles as a biocompatible and easily uptake materials for normal cells and cancer cell lines (Anitha et al., 2011), thiolated chitosan in non-invasive drug delivery system (Bernkop-Schnurch, 2008), and thiolated chitosan–TGA nanoparticles as a drug delivery system for the urinary bladder (Barthelmes, Perera, Hombach, Dünnhaupt, & Bernkop-Schnurch, 2011).

The aim of this work was to prepare a mucoadhesive thiolated chitosan that would be potentially suitable for application in a mucoadhesive drug delivery system, especially in a low pH

* Corresponding author. Tel.: +66 02 2187596; fax: +66 02 2187598.
E-mail address: nongnuj.j@chula.ac.th (N. Muangsin).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.11.007

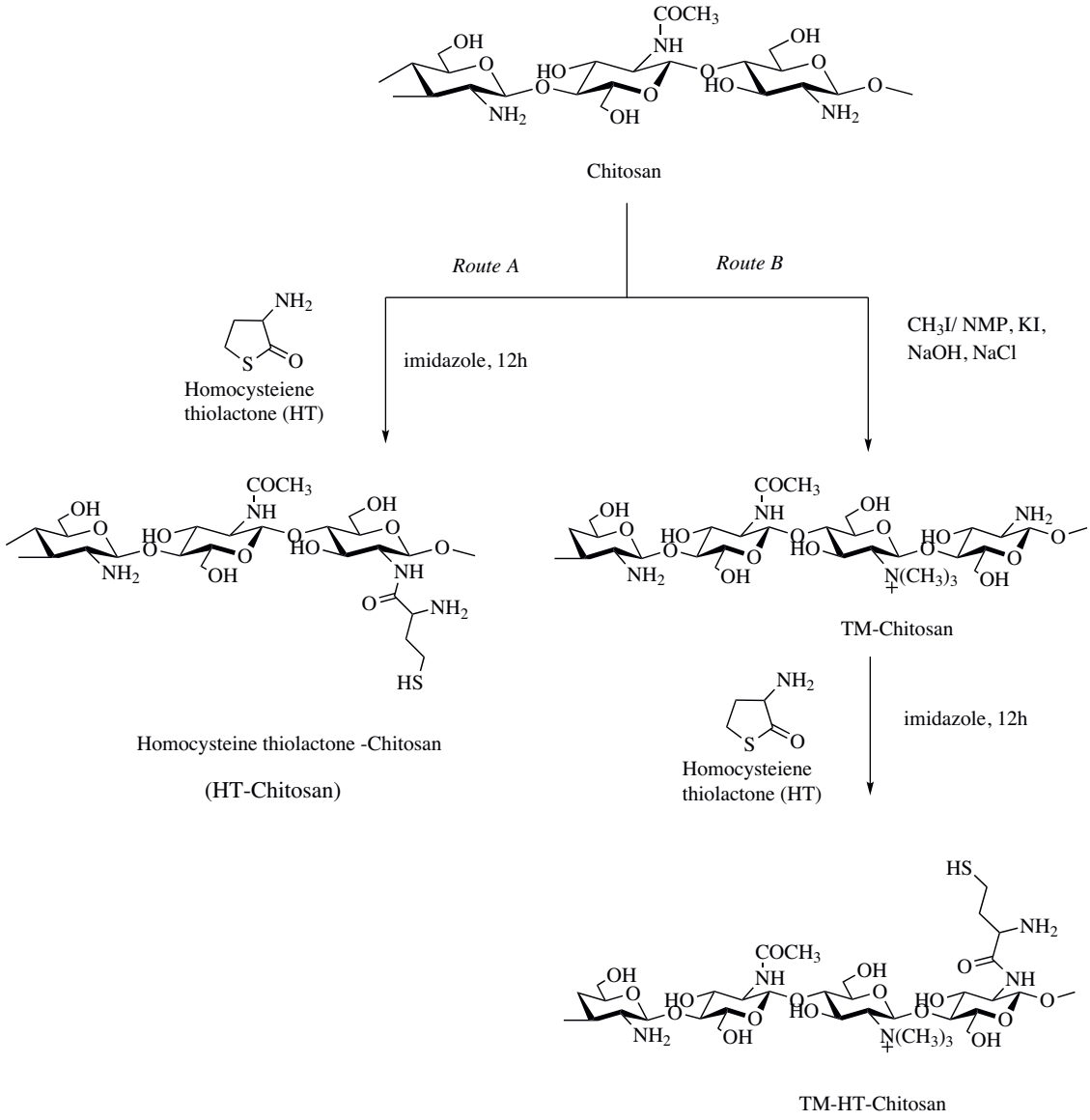

Fig. 1. Schematic representation of the reaction scheme for the covalent attachment of HT to chitosan (route A) and TM-chitosan (route B) to form HT-chitosan and TM-HT-chitosan, respectively.

environment. Therefore, thiolated chitosan was developed from chitosan by the covalent attachment of homocysteine thiolactone (HT) onto the amino groups of chitosan, using imidazole as the reactive intermediate. To further improve the mucoadhesiveness of this thiolated chitosan, *N*,*N*,*N*-trimethyl-chitosan (TM-chitosan) (Sieval et al., 1998) were also synthesized and then grafted with HT, based on the strong positive charges and chain flexibility would be able to interact more strongly with the mucus glycoproteins (Andrews, Laverty, & Jones, 2009).

2. Materials and methods

2.1. Materials

Chitosan with 85% deacetylation (Mw of 100 kDa) was provided by Bonafides Co. Ltd. (Thailand). Homocysteine thiolactone (HT) hydrochloride, imidazole, mucin (type II) from porcine, iodomethane, *N*-methyl pyrrolidone, basic fuchsin (pararosaniline), sodium metabisuphite, periodic acid, acetic acid and lactic acid were obtained from Aldrich Co., USA and used without further purification. 5,5′-Dithio-bis(2-nitrobenzoic acid), used for quantitatively analyzing the thiol groups, was purchased from Aldrich Co., USA. Dialysis tubing (Mw cut-off 12–14 kDa) was obtained by Membrane Filtration Products, Inc., USA. All other chemicals were commercially available and used as received.

2.2. Synthesis of HT-chitosan

Chitosan were thiolated using HT by covalent attachment, as schematically summarized in Fig. 1A, using different (w/w) ratios of chitosan:HT to evaluate the effect of varying this ratio on the properties of the obtained HT-chitosan. Briefly, 100 mL of 1% (w/v) of chitosan in 1% (v/v) lactic acid was added to an aqueous solution of imidazole (0.68 g in 2.5 mL water), followed by the dropwise addition of HT (0.05, 0.1, 0.5 and 1.0 g in 100 mL water) and stirred at room temperature in a nitrogen atmosphere for 12 h. The reaction mixture was adjusted to pH 7, precipitated with excess acetone and harvested by centrifugation (12,000 rpm for 2 min). The pellet was re-dissolved in water and dialyzed (MW cut-off 12–14 kDa) against changes of 1 L of water for 2 days prior to being lyophilized

at −30 °C and 0.01 mbar. The dry product was stored at 4 °C before use.

2.3. Synthesis of TM-HT-chitosan

The TM-chitosan was first prepared from the corresponding chitosan by methylation as previously reported (Yin et al., 2009). TM-HT-chitosan was synthesized following the route schematically summarized in Fig. 1B. Firstly, 100 mL of 1% (w/v) of TM-chitosan in 1% (v/v) lactic acid solution was added to an aqueous solution of imidazole (0.68 g in 2.5 mL water), followed by the dropwise addition of HT (0.15 (w/v) aqueous) to the desired final concentration and stirred at room temperature under a nitrogen atmosphere for 12 h. The reaction mixture was adjusted to pH 7 and then precipitated, dialyzed, lyophilized and stored at 4 °C before use.

2.4. Characterization

2.4.1. Fourier transformed infrared spectroscopy (FT-IR)

The chitosan, HT and HT-chitosan and TM-HT-chitosan were analyzed (KBr) by FT-IR (Nicolet 6700) in the region from 4000 cm^{-1} to 400 cm^{-1}.

2.4.2. ^{1}H and ^{13}C Nuclear Magnetic Resonance spectroscopy (NMR)

^{1}H NMR and ^{13}C NMR spectra of HT-chitosan, TM-chitosan and TM-HT-chitosan in 2% (v/v) trifluoroacetic acid (CF_3COOH) in D_2O were recorded on Bruker NMR spectrometer operated at 400 MHz. The degree of quaternization (DQ) is one of the important characteristics of chitosan and its derivatives was calculated using the data obtained from the ^{1}H NMR spectra according to Eq. (1), as previously described (Sieval et al., 1998; Snyman, Hamman, Kotze, Rollings, & Kotze, 2002).

$$\%DQ = \left[\frac{[(CH_3)_3]}{[H3 - H6]} \times \frac{4}{9}\right] \times 100 \quad (1)$$

Here, $[(CH_3)_3]$ is the integral of the chemical shift of the trimethyl amino group at 2.96 ppm and [H3–H6] is the integral of the ^{1}H peak between 3.6 and 4.2 ppm.

2.4.3. Determination of the contents of thiol and disulfide groups

One of the important factors of these modified polymers is the level (density) of free thiol groups and disulfide bonds, which cannot be evaluated by NMR. Thus, the degree of modification was determined spectrophotometrically with Ellman's reagent as reported (Hornof et al., 2003). The amount of thiol moieties on the HT-chitosan and TM-HT-chitosan samples was calculated by reference to a standard curve, itself obtained from evaluation of a chitosan solution with increasing known amounts of cysteine HCl (0.001–0.01 g/L) standards at a wavelength of 450 nm. The amount of disulfide bonds was calculated by subtracting the quantity of free thiol groups, as determined above, from the total number of thiol moieties present on the polymer.

2.4.4. X-ray diffraction (XRD) pattern

The X-ray powder diffraction (XRD) pattern was performed on a Rigaku X-ray diffractometer Dmax 2200 Ultima at room temperature with a speed scan of 5°/min using CuKα radiation (λ = 1.5405 Å, 40 kV, 30 mA).

2.4.5. Thermogravimetric analysis (TGA)

The thermal stability of each of the samples was evaluated using TGA analysis. These experiments were performed on a PerKinElmer Pyris Diamond TG/DTA machine under a nitrogen flow at a rate of 30 mL/min. Approximately 5 mg of samples were placed in the alumina pan, sealed and heated at 10 °C/min from 25 to 500 °C.

2.5. In vitro bioadhesion of mucin to chitosan and the modified chitosans

2.5.1. Mucus glycoprotein assay

The Periodic Acid Schiff (PAS) method is widely used for both the quantitative and qualitative analysis of mucins, glycoproteins, glycogen and other polysaccharides in tissues and cells. The PAS colorimetric assay for the detection of glycoproteins was used as previously reported for the determination of the free mucin concentration, so as to evaluate the amount of mucin adsorbed onto the chitosan and its three derivatives. Schiff reagent contained 100 mL of 1% (w/v) basic fuchsin (pararosaniline) in an aqueous solution and 20 mL of 1 M HCl. To this was added sodium metabisuphite (1.67% (w/v) final) just before use, and the resultant solution was incubated at 37 °C until it became colorless or pale yellow. The PAS reagent was freshly prepared by adding 10 μL of 50% (v/v) periodic acid solution to 7 mL of 7% (v/v) acetic acid solution.

Standard calibration curves were prepared from the four mucin standard solutions (0.125, 0.25, 0.375 and 0.5 mg/mL). After adding 0.1 mL of periodic acid reagent, the solutions were incubated at 37 °C for 2 h before 0.1 mL of Schiff reagent was added and incubated at room temperature for 30 min. Next, 0.1 mL aliquots of the solution were transferred in triplicate into a 96-well microtiter plate and the absorbance at 555 nm was recorded. The mucin contents were then calculated by reference to the standard calibration curve.

2.5.2. Adsorption of mucin on chitosan and the three derivatives (TM-chitosan, HT-chitosan and TM-HT-chitosan)

A 0.5% (w/v) mucin solution in each of three broadly isoosmotic solutions that differ in pH, namely SGF (pH 1.2), 0.1 N sodium acetate buffer (pH 4.0) and SIF (pH 6.4) media, were prepared. Chitosan and its three derivatives were dispersed (at 20 mg/1.5 mL final) in the above mucin solutions and shaken at 37 °C for 2 h. Then the dispersions were centrifuged at 12,000 rpm for 2 min to pellet the chitosan–mucin or TM-HT-chitosan–mucin complex and the supernatant was harvested and used for the measurement of the free mucin content. The mucin concentration was calculated by reference to the calibration curve, and the amount of mucin adsorbed to the microspheres was calculated as the difference between the total amount of mucin added and the free mucin content in the supernatant.

2.6. Swelling study of chitosan and its three derivatives (TM-chitosan, HT-chitosan and TM-HT-chitosan)

Films of chitosan and the three chitosan derivatives were prepared as follows. A 2 g of chitosan or its derivatives (TM-chitosan, HT-chitosan and TM-HT-chitosan) was dissolved in 50 mL of 1% (v/v) aqueous lactic acid to yield a 1% (w/v) chitosan or chitosan derivative solution, poured into an 8 cm × 10 cm tray and air dried. The chitosan or chitosan derivative films were then cut into 5.0 mm diameter circles and each one was immersed in one of SGF, SIF or 0.1 N sodium acetate buffers (pH 4.0). The swelling properties were determined by measuring the change in the diameters of each film at various time intervals (0–8 h). Equilibrium was assumed to be attained when no further swelling (increase in disc diameter) was measured over time (i.e. at the asymptote of the swelling level *vs.* incubation time plot). The swelling ratio (S_w) for each sample determined at time t was calculated from Eq. (2) as previously reported (Muzzarelli & Tanfani, 1985).

$$S_w = \frac{D_t - D_0}{D_0} \times 100 \quad (2)$$

where D_t is the film diameter at time t and D_0 is the initial film diameter.

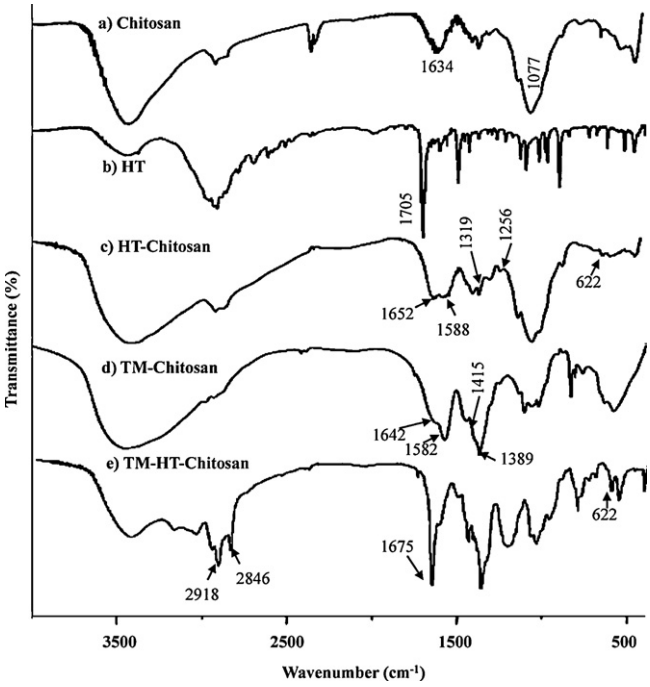

Fig. 2. Representative FTIR spectra of (a) chitosan, (b) HT, (c) HT-chitosan from a chitosan:HT (w/w) ratio of 1:1.0, plus (d) TM-chitosan and (e) TM-HT-chitosan (1:0.1 (w/w) TM-chitosan:HT).

2.7. Statistical analysis

All measurements were performed in triplicate in each experiment. The results are presented as the mean ± standard deviation (SD). To test for significant differences between means, statistical analysis was performed by one-way ANOVA, using Microsoft Excel (Microsoft Corporation). A value for $P < 0.01$ is considered to be statistically significant.

3. Results and discussion

3.1. Formation and characterization of HT-chitosan and TM-HT-chitosan

The reaction of chitosan or TM-chitosan and HT to form HT-chitosan or TM-HT-chitosan, respectively, was carried out via a ring-opened reaction. The sulfhydryl group of HT first formed the reactive intermediate HS–CH_2–CH_2–CH(NH_2)–C(O)–Im (where Im = imidazole) (Matsuda, Kobayashi, Itoh, Kataoka, & Tanaka, 2005), and then HT was covalently reacted at the amino group (–NH_2) of chitosan or TM-chitosan via the formation of amide bonds (Fig. 1).

3.2. Fourier transformed infrared spectroscopy (FTIR)

The FTIR spectra of chitosan and the three chitosan derivatives (TM-chitosan, HT-chitosan and TM-HT-chitosan) are shown in Fig. 2. The FTIR spectrum of chitosan showed the characteristic absorption bands of chitosan at 1634 cm^{-1} (C=O amide) and 1077 cm^{-1} (C–O stretching), whilst the HT spectrum showed the C=O ketone group absorption band at 1705 cm^{-1}. After grafting of the HT onto the C2-amine groups of chitosan, the characteristic signal at 1652 cm^{-1}, attributed to the stretching vibration of the C=O acetamide of HT-chitosan (amide I band) appeared. In addition, the absorption peak at 1588 cm^{-1} (amide II band) and 1319 cm^{-1} (amide III band) were stronger than those seen in chitosan, which can be attributed to the additional amide group of HT. The peaks at 1256 cm^{-1} and 622 cm^{-1} correspond to the disulfide and thiol groups, respectively (Bernkop-Schnurch, Kast, et al., 2003).

After quaternization of chitosan, the resultant TM-chitosan spectrum showed peaks at 1642 and 1582 cm^{-1} that are assigned to the C=O (amide) and N–H (amine) vibrations, respectively. For the representative TM-chitosan:HT sample (1:0.1 (w/w) ratio), a group of band at 2918 cm^{-1} and 2846 cm^{-1} assigned the vibration of CH_2(asym) and CH_2 (sym), respectively, attributed to the side chain of HT which are increased as compared with TM-chitosan. The new band at 1675 cm^{-1} is attributed to the stretching vibration of the C=O acetamide group of TM-HT-chitosan and no band around 1705 cm^{-1} (C=O of the lactone ring of the HT) presented, indicating that HT is grafted on to the chitosan backbone. Compared with chitosan, the absorption bands at 1230 cm^{-1} in both HT-chitosan and TM-HT-chitosan spectra correspond to an increased level of alkyl group C–C bonds. These results are all consistent with the successful preparation of HT-chitosan and TM-HT-chitosan conjugates.

3.3. ^1H and ^{13}C Nuclear Magnetic Resonance spectroscopy (NMR)

Representative ^1H NMR of chitosan, HT-chitosan and TM-HT-chitosan are shown in Fig. 3. The peaks for HT-chitosan at chemical shifts 3.4–4.0, 3.1 and 2.0 ppm were ascribed to the H3–H6, H2 (GluN) and acetyl (GluNAc) protons in the chitosan skeleton, respectively. The appearances of new proton positions from the ring opened side chain of HT were observed as at 2.8 and 2.5 ppm and are assigned to the Hd and He, respectively. The spectra of TM-HT-chitosan showed a chemical shift at 3.6–4.2, 3.2–3.4, 3.1, 3.0 and 2.0 ppm, which were attributed to H3–H6, 3-,6-OCH_3, H2, $^+N(CH_3)_3$ and acetyl (GluNAc) protons in the TM-chitosan backbone, respectively. The chemical shifts at 2.8 and 2.5 belonged to Hd overlapped with $(CH_3)_2$ and He, respectively. The DQ%, as calculated from Eq. (1), was 5.9%.

Representative ^{13}C NMR spectra of chitosan, HT-chitosan and TM-HT-chitosan are shown in Fig. 4, where the chemical shifts at δ 97.6, 59.9, 69.9, 76.4, 74.8 and 55.7 ppm are assigned to the C1, C2, C3, C4, C5 and C6, respectively. The signal at 161.6 is attributed to the carbonyl group of chitosan (Sun, Du, Fan, Chen, & Yang, 2006). The spectrum of HT-chitosan is broadly similar to that of chitosan, except for the new high intensity peaks at around 24.5–35.7 ppm that are assigned to the methylene group of the grafted cysteine side chain (Cd, Ce). In addition, the peak at δ 50.0 ppm is assigned to the Cb alkyl carbon, and the characteristic peak at δ 161–163 ppm is assigned to the two types of carbonyl group, the C=O carbonyl group of the acetyl GluNAc of chitosan and the HT-chitosan side chain at 161 and 162.6 ppm, respectively. In the TM-HT-chitosan spectra of TM-HT-chitosan, peaks related to C1, C2, C3, C4, C5 and C6 of the saccharide ring and the peak of dimethyl (overlapped with the trimethyl group) at chemical shifts of 97.8, 59.8, 70.2, 76.7, 75.1, 55.8 and 41.9 ppm, respectively, were all observed. The appearance of the peak at δ 182.0 ppm, assigned to the C=O carbonyl of the amide group, verified the covalent attachment of TM-chitosan and HT via an amide bond. The ^{13}C NMR spectrum confirmed that HT-chitosan and TM-HT-chitosan was successfully prepared.

3.4. Quantification of the thiol levels in HT-chitosan and TM-HT-chitosan

The amount of free thiol groups and disulfide bonds immobilized in the HT-chitosan and TM-HT-chitosan derived polymers are summarized in Table 1. The HT-chitosan derived from a 1:0.1 (w/w) ratio of chitosan:HT exhibited the highest amount of free thiol groups (64 μmol/g), which implies that this polymer will be the strongest mucoadhesive compared to the three other HT-chitosan derived from the other chitosan:HT ratios. In addition, at

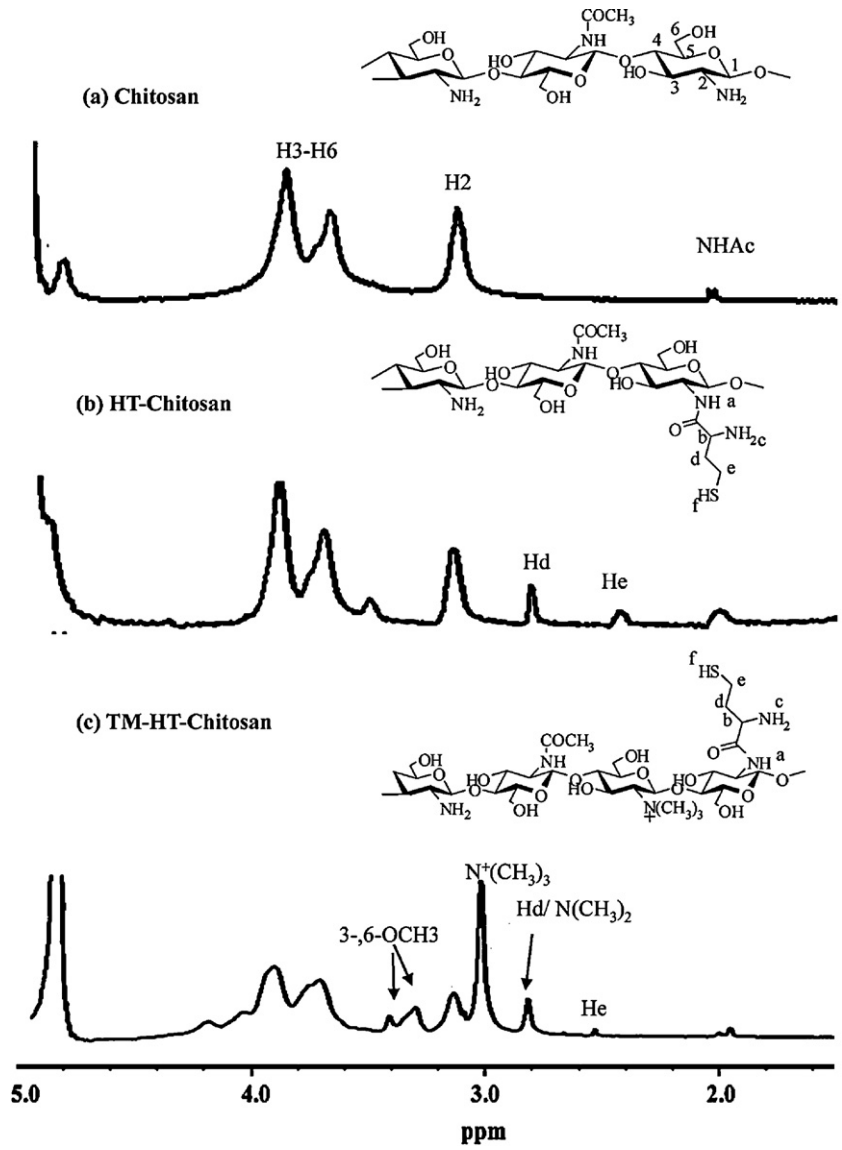

Fig. 3. Representative ^{1}H NMR spectra of (a) chitosan, (b) HT-chitosan (1:0.1 (w/w) chitosan:HT) and (c) TM-HT-chitosan (1:0.1 (w/w) TM-chitosan:HT).

the highest tested HT proportion (a 1:1 (w/w) ratio of chitosan:HT) the highest level of total disulphide groups (~220 μmol/g) was observed, some 3.59- to 5.48-fold higher than the other HT-chitosan derived from the lower HT proportions. The results imply that the optimum proportion of HT should not exceed that of 1:0.1 (w/w) chitosan:HT, since an excess HT only yields a higher level of disulfide bonds.

Therefore, TM-HT-chitosan was synthesized at the 1:0.1 (w/w) ratio of TM-chitosan:HT, and was then evaluated compared to HT-chitosan to ascertain the potential effect of the permanent

Table 1
Comparison of the different chitosan:HT mass ratio and the levels of their free thiol and disulfide groups.

Batch	Chitosan/HT ratio	Total thiol groups (μmol/g) (±SD, $n=3$)	Total disulfide groups (μmol/g) (±SD, $n=3$)	Adsorbed mucin at pH 1.2 (mg) (±SD, $n=3$)	Adsorbed mucin at pH 4.0 (mg) (±SD, $n=3$)	Adsorbed mucin at pH 6.4 (mg) (±SD, $n=3$)
Chitosan	–	–	–	0.06 ± 0.01	0.31 ± 0.05	0.42 ± 0.03
Chitosan/HT	1.0:0.05	35.13 ± 0.05	61.32 ± 0.12	0.22 ± 0.01[a]	0.55 ± 0.02[a]	0.65 ± 0.01[a]
Chitosan/HT	1.0:0.10	64.15 ± 0.04[*]	40.15 ± 0.03	0.22 ± 0.02[a,b]	0.60 ± 0.03[a]	0.72 ± 0.01[a,d]
Chitosan/HT	1.0:0.50	49.99 ± 0.05	45.69 ± 0.08	0.25 ± 0.03[a]	0.54 ± 0.03[a]	0.67 ± 0.02[a]
Chitosan/HT	1.0:1.00	50.56 ± 0.05	220.05 ± 0.14[*]	0.26 ± 0.01[a]	0.51 ± 0.02[a]	0.62 ± 0.04[a]
TM-Chitosan/HT	1.0:0.10	32.48 ± 0.03	38.74 ± 0.72	0.38 ± 0.01[a,c,e]	0.79 ± 0.03[a,c,e]	0.75 ± 0.01[a,c,f]

[*] The mean difference is significant ($P<0.01$) compared to Chitosan/HT 1.0:0.05 using LSD method.
[a] The mean difference is significant ($P<0.01$) compared to Chitosan using LSD method.
[b] The mean difference is insignificant ($P>0.01$) compared to Chitosan/HT 1.0:0.05 using LSD method.
[c] The mean difference is significant ($P<0.01$) compared to Chitosan/HT 1.0:0.05 using LSD method.
[d] The mean difference is significant ($P<0.01$) compared to Chitosan/HT 1.0:0.05 using LSD method.
[e] The mean difference is significant ($P<0.01$) compared to Chitosan/HT 1.0:0.1 using LSD method.
[f] The mean difference is insignificant ($P>0.05$) compared to Chitosan/HT 1.0:0.1 using LSD method.

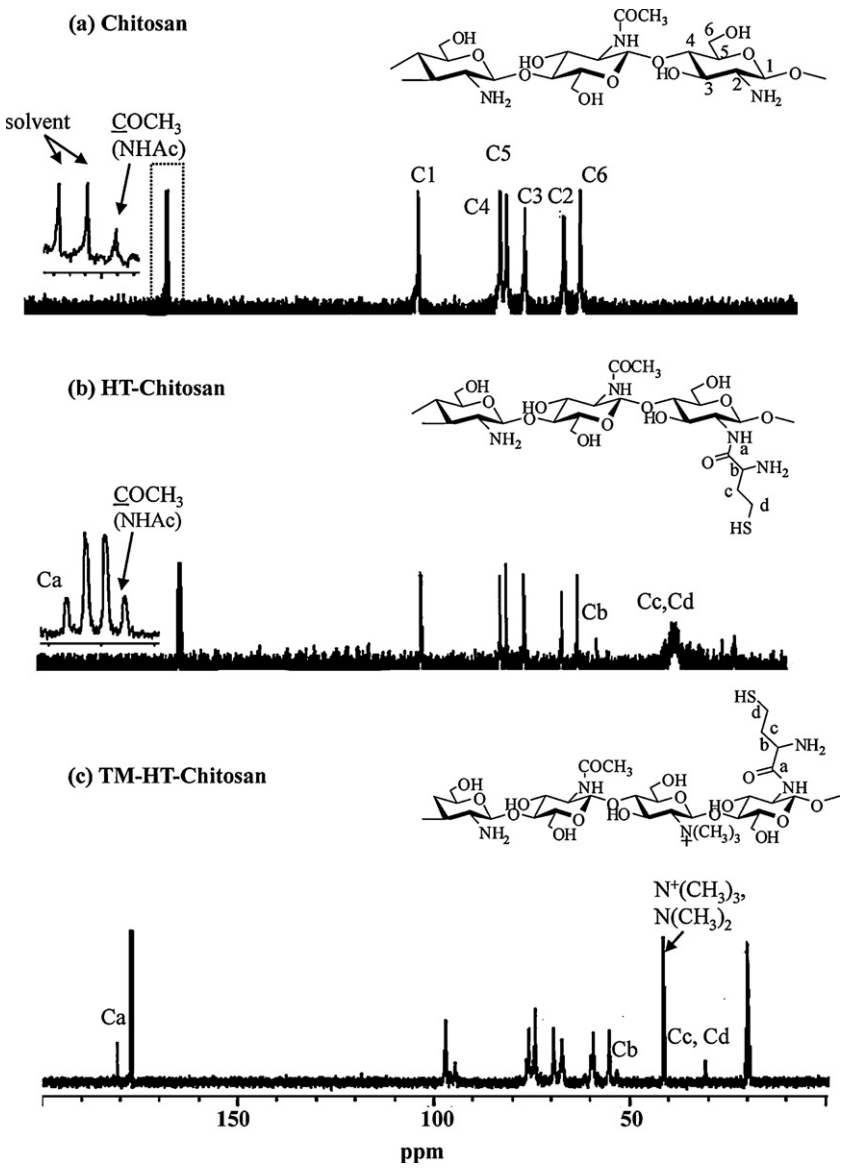

Fig. 4. Representative ^{13}C NMR spectra of (a) chitosan, (b) HT-chitosan (1:0.1 (w/w) chitosan:HT) and (c) TM-HT-chitosan (1:0.1 (w/w) chitosan:HT).

pH-independent positive charges on the mucoadhesive property of the polymer.

3.5. X-ray diffraction (XRD) analysis

Representative X-ray diffractograms for the chitosan, HT-chitosan, TM-chitosan and TM-HT-chitosan polymers (data not shown) where some differences in the peak height, width and position between the four compounds were observed. Chitosan powder exhibited two typical peaks at (2θ) 10.6° and 20.0°, which corresponded to crystal forms I and II, respectively (Dung, Milas, Rinaudo, & Desbrires, 1994). However, after HT substitution, the peak at 10.6° 2θ was significantly weaker (almost absent) in the HT-chitosan and TM-HT-chitosan spectra, suggesting that the original crystallinity of chitosan was destroyed. The HT-chitosan (and TM-HT-chitosan) was more amorphous in nature, which may well improve their biodegradability and mucoadhesive properties (Prabaharan & Gong, 2008). The XRD pattern exhibiting a characteristic peak at 20° 2θ was shifted to 21.6° 2θ for TM-chitosan that corresponds to the crystal form II was also altered. Compared with chitosan, the intensities of these peaks were decreased. It is possible that the present of the HT moiety resulted in a change in the crystallinity of the chitosan and TM-chitosan backbone. Thus, the three derivatives of chitosan (HT-chitosan, TM-chitosan and TM-HT-chitosan) were more amorphous than that of chitosan. The results also supported that HT was successfully introduced into the chitosan and TM-chitosan backbone.

3.6. Thermogravimetric analysis (TGA)

The thermal properties of the polymers were investigated by TGA. The TG curves and the corresponding DTG curves of chitosan, HT-chitosan, TM-chitosan and TM-HT-chitosan are displayed in Fig. 5. The TG curve of the unmodified chitosan showed two stages of weight loss, the first being water loss at 26–160 °C, whilst the second stage was from ~208 °C to 288 °C, accounting for a loss of 34.6% of the total weight and was ascribed to the degradation of chitosan backbone. In contrast, the HT-chitosan sample (1:0.1 (w/w) ratio of chitosan:HT) showed three stages of weight loss. The first and the third staged are attributed to the loss of water

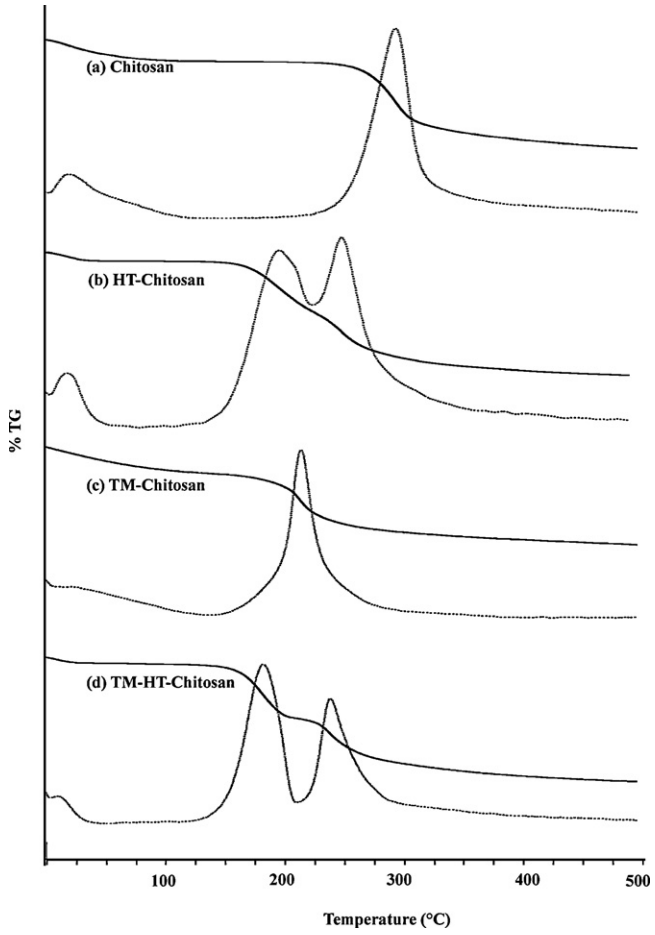

Fig. 5. Representative TGA thermograms (TG and TGA curves) of (a) chitosan, (b) HT-chitosan (1:0.1 (w/w) chitosan:HT), (c) TM-chitosan and (d) TM-HT-chitosan (1:0.1 (w/w) TM-chitosan:HT).

and chitosan backbone degradation, respectively. The second stage with the sharp weight loss in the 196–262 °C range is accounted for 34.1% of the total weight and ascribed to the thermal degradation of the HT grafted side chain.

For TM-chitosan and TM-HT-chitosan, the thermal decompositions showed two stages, the stage from 160 to 225 °C is due to the degradation of the cleavage of the substituent groups. The weight loss of TM-HT-chitosan (23.3% total weight) was lower than that of TM-chitosan (35.9% total weight), suggesting that TM-HT-chitosan is more thermal stable than TM-chitosan. Overall, the TGA analysis demonstrated the loss of the thermal stability for HT-chitosan and TM-HT-chitosan compared to the original chitosan. Introduction of the HT side chain into the polysaccharide structure should disrupt the crystalline structure of chitosan, especially through the loss of the hydrogen bonding (Zhang, Ping, Zhang, & Shen, 2003).

3.7. Mucoadhesive properties

3.7.1. Assessment of the mucoadhesive behavior of chitosan and the three derivatives (TM-chitosan, HT-chitosan and TM-HT-chitosan) by mucus glycoprotein assay

The mechanism of mucoadhesion has been theoretically reported to be based on the six general components of electrostatic, wetting, adsorption, diffusion, mechanical and fracture theories (Smart, 2005). Many methods have been employed to evaluate these interactions *in vitro* and *in vivo*. In this study, we selected a commercial powder preparation of porcine mucin type

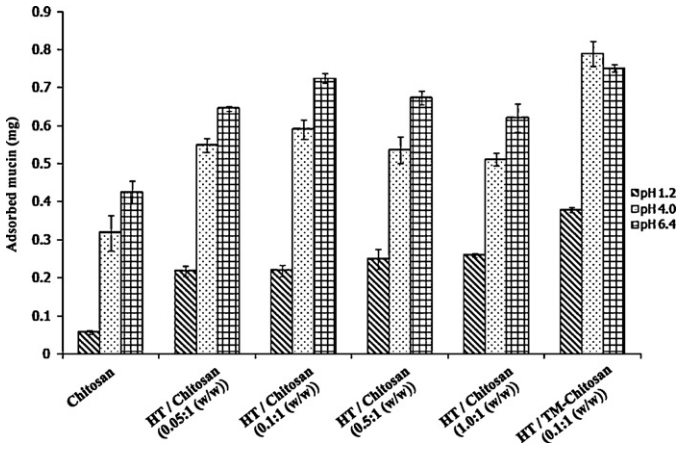

Fig. 6. Adsorption of mucin on chitosan, HT-chitosan of different chitosan:HT (w/w) ratios and TM-HT-chitosan at pH 1.2, 4.0 and 6.4. Data are shown as the mean ± SD and are derived from three independent repeats. Means with a different lower case letter are significantly different ($P < 0.01$).

II, which is typically used in mucoadhesion assays due to its lower batch-to-batch variability and higher between assay reproducibility (Rossi, Ferrari, Bonferoni, & Caramella, 2001). As a strong interaction exists between mucin and chitosan or its derivatives, mucin should be spontaneously adsorbed onto the surface of the chitosan or its three derivatives (CS, HT-chitosan and TM-HT-chitosan). Therefore, the mucoadhesive property of the chitosan and its three derivatives was assessed by suspension of mucin in their aqueous solutions at room temperature. The spectrophotometric detection method used here (Section 2.5) allowed relatively very dilute solutions of mucin (125–500 μg/mL) to be measured and showed a linear relationship between the amount of mucin and the absorbance at 555 nm, with the linear regression equations obtained by the least square method being $y = 0.5629x - 0.0452$, $y = 1.41x + 0.0832$ and $y = 1.9268x + 0.0415$ for the assay in pH 1.2 (SGF), pH 4.0 and pH 6.4 (SIF), respectively (see support information). As the mucin concentration increased, so the amount of mucin adsorbed also increased.

3.7.2. Adsorption of mucin on polymer

The amount of mucin that was adsorbed onto the polymer (chitosan or its three derivatives) decreased at lower pH values, being maximal at pH 6.4 (SIF) and minimal at pH 1.2 (SGF) (Fig. 6), because the degree of the ionization of sialic acid or the different forms of the glycoprotein will be influenced by the pH value of the environment. Sialic acid is a saccharide acid, and mucin is a glycoprotein. The values of pK_a and p*I* for sialic acid and mucin are 2.6 (Johnson & Rainsford, 1972) and ~3–5, respectively. Hence, the ionization of the sialic acid and the glycoprotein will be more sensitive to pH, in the acidic environment. As the pH value decreases, the amount of ionized sialic acid also decreases (He, Davis, & Illum, 1998), and so reduces the potential for interaction with chitosan or its three derivatives.

Chitosan and TM-chitosan, along with their thiolated derivatives (HT-chitosan and TM-HT-chitosan, respectively), were evaluated for their mucin adsorption ability as a measure of their mucoadhesiveness. The thiolated derivative polymers dramatically and significantly increased the mucin adsorption level above that seen with chitosan or TM-chitosan in all three pH mediums evaluated (Fig. 6). The results revealed that the representative HT-chitosan (derived from a 1:0.1 (w/w) ratio of chitosan:HT) absorbed mucin about 3.9-, 1.9- and 1.7-fold more than chitosan at pH 1.2, 4.0 and 6.4, respectively, whilst the corresponding TM-HT-chitosan sample showed some ~6.7-, 2.5- and 1.8-fold higher mucoadhesion

than TM-chitosan at pH 1.2, 4.0 and 6.4, respectively. Comparing the two corresponding (i.e. 1:0.1 (w/w) ratio (TM-chitosan:HT)) thiolated chitosan, a higher mucoadhesion level was seen for TM-HT-chitosan than HT-chitosan at all three pH values, but this difference was more marked in the more acidic media, being about 1.72-, 1.37- and 1.04-fold higher at pH 1.2, 4.0 and 6.4, respectively.

At the low pH range (pH 1.2 and 4.0), when the proportion of HT in the chitosan:HT (w/w) ratio increased from 1:0.05 to 1:0.1, a statistically significant increase in the level of thiol groups was observed (Table 1), but the slight numerical changes in the mucoadhesion level were not statistically significant (Fig. 6). This might be due to the reactivity of the thiol groups on the polymer. In the lower pH, the thiol group in the thiolated are less reactive, hence oxidation of thiol groups occurs before contact with the mucus gel layer (Palmberger, Hombach, & Bernkop-Schnurch, 2008). Hence, the mucoadhesive ability of chitosan was increased by the addition of the thiol groups because of the electrostatic and hydrophobic effects. With respect to the electrostatic effect, this is due to the remaining NH_3^+ moieties of both the chitosan backbone and the HT side chain being able to interact with either the COO^- or SO_3^- groups on the mucin carbohydrate side chain in an acidic media. For the hydrophobic effect, the $-CH_2$ moieties of HT interact in part with the $-CH_3$ groups on the mucin side chains which, lead to a high mucoadhesive adsorption. Therefore, both electrostatic and hydrophobic effects on the mucoadhesion of chitosan and its three derivatives are in the lower pH range.

In contrast, however, at pH 6.4 a statistically stronger mucoadhesiveness was observed with the HT-chitosan sample derived from the lower HT proportion (1:0.1 (w/w) chitosan:HT), with a ~1.1-fold higher level of adsorbed mucin being observed that from the HT-chitosan derived from a 1:0.05 (w/w) ratio. However, since these experiments were performed at a pH above 6, which will result in an increased concentration of the reactive form of thiolate anions, $-S^-$, this may have lead to a greater extent of oxidation and nucleophilic attack (Leitner et al., 2003). Regardless, the potential influence of the level of thiol groups on the mucoadhesive properties of the polymer could clearly be observed, and so not only electrostatic and hydrophobic effects are at play but also the level of covalently linked thiol groups in the polymer is an important determinant of the polymer's mucoadhesiveness. The higher the density of thiol groups covalently attached to the copolymer, the higher the amount of mucin was bound onto the polymer (Table 1 and Fig. 6). This can be explained by the formation of covalent disulfide bonds between the thiol-bearing side chains of the thiolated polymer and cysteine-rich subdomains of the mucus glycoprotein (Grabovac, Guggi, & Bernkop-Schnurch, 2005).

With respect to the level of disulfide bonds, the HT-chitosan sample derived from a 1:1 chitosan:HT (w/w) ratio showed a significant increase in the level of disulfide groups (Table 1), but no statistically significant increase in the mucoadhesion ability (Fig. 6). Thus, it is possible that the level of total disulfide bonds is not a principal factor influencing the mucoadhesive properties of the thiolated chitosan.

For TM-HT-chitosan a significant reduction in the total thiol group level was seen (Table 1) because the quaternization of chitosan leads to a steric inhibition effect from the fixed positively charged quaternary ammonium group charges making it difficult for the HT groups to interact with the chitosan amine group. However, a statistically ($P > 0.01$) higher mucoadhesion level for the TM-HT-chitosan was observed compared to that for chitosan and HT-chitosan at pH 1.2 and 4.0 being ~1.7- and 1.3-fold higher than that seen in the HT-chitosan (derived from a 1:0.1 (w/w) chitosan:HT ratio) at pH 1.2 and 4.0, respectively. This was expected since the $^+N(CH_3)_3$ group found in TM-HT-chitosan would be able to interact with the COO^- or the SO_3^- groups on the mucin glycoprotein side chain given that most mucin glycoproteins have a high sialic acid and sulfate content, and so a strongly negative surface charge (Lai, Wang, Wirtz, & Hanes, 2009).

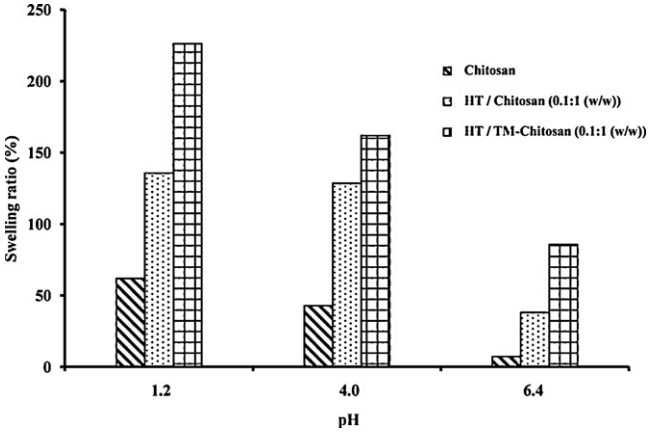

Fig. 7. Swelling behavior of the chitosan and the thiolated chitosans (HT-chitosan and TM-HT-chitosan, both from a 1:0.1 (w/w) ratio of chitosan:HT) as a function of the media pH. Data are shown at equilibrium (60 min swelling time) as the mean ± SD and are derived from three independent repeats. Means with a different lower case letter are significantly different ($P < 0.01$).

On the other hand, TM-HT-chitosan showed only a slightly (1.04-fold) numerically larger (and not statistically significant) mucoadhesion level at pH 6.4 compared to the corresponding (1:0.1 (w/w) ratio chitosan:HT) HT-chitosan, which is likely to be due to the fact that the influence of the positive charge was not enough to increase the mucoadhesion when compared with the effect of the thiol group at a higher pH (pH 6.4).

3.8. Swelling study

The swelling properties are of paramount importance in any prospective evaluation of biomaterials, and they can be affected by many factors, such as the cross-linking density and the hydrability of the materials, and the ionic strength and pH value of the media (Yu, Song, Shi, Xu, & Bin, 2011). In this research the swelling ratio was measured in three broadly isoosmotic buffer solutions that differ in their pH, being SGF (pH 1.2), 0.1 M acetate buffer (pH 4.0) and SIF (pH 6.4) and the swelling ratio was calculated using Eq. (2) as detailed in Section 2.6. The results, as the variation in the swelling ratio with time, for the four HT-chitosan that varied in chitosan:HT ratios, showed essentially the same pattern (data not shown) and so that for the HT-chitosan derived from a 1:0.1 (w/w) ratio of chitosan:HT is shown as an example, along with the data for the chitosan and TM-HT-chitosan in Fig. 7. All three polymers swelled rapidly (within 30 min) in all three different pH media, with the HT-chitosan and TM-HT-chitosan presenting a higher swelling ratio than that of chitosan. This may be attributed to the fact that the hydrophility of the thiolated chitosan was greater than that for chitosan (Wu et al., 2009).

In addition to the influence of HT grafting upon the polymer swelling, a clear pH dependence was also noted. As the pH value increased the degree of observed swelling decreased, especially from pH 4 to pH 6.4 (Fig. 7). This could be explained from the pH-dependent charge balance of HT-chitosan, TM-HT-chitosan and chitosan, and so the degree of interaction between these three polymers is modified in accord with their charge balance. The TM-HT-chitosan sample is selected here as an example to explain the potential mechanism due to the statistically significant changes in the swelling behavior with pH compared to that for the control chitosan, with the discussion schematically.

In strongly acidic medium (pH 1.2), the high swelling behavior can be explained by the amine group of the TM-chitosan

being protonated which then favors chain expansion, through the electrostatic repulsions between the like-charged polymer segments (Khalid, Agnely, Yagoubi, Grossiord, & Couarraze, 2002). Furthermore, there is an overall increase in the swelling ratio after grafting HT side chains onto chitosan. This is in good agreement with the literature, where it has been reported previously that adding more hydrophilic groups to chitosan increases its water absorption (Lee, Kim, & Lee, 2000). In order to investigate the effect of the thiol group on the degree of swelling at the lower pH of the thiolated polymer, the thiol groups may be oxidized (Palmberger et al., 2008) leading to less reactive of the thiol groups. Hence, the influence of the thiol groups was attributed to the effect of the interruption of the dominant amine groups.

At a high pH (pH 6.4), the swelling ratio is strongly reduced due to the almost complete deprotonization of the chitosan amine group (Khalid et al., 2002) leading to a re-association of the interchain hydrogen bonds and consequently to weaker interactions between the polymer chains and the aqueous media. It is known that a low concentration of charged ionic groups in the polymer decreases swelling behavior. On the other hand, at pH levels above 6 the thiolate groups become charged anions, $-S^-$, which represents the reactive oxidation form (Leitner et al., 2003), and thereby leads to electrostatic repulsion between the polymer segments. However, the polymers have only a relatively low density of thiol groups compared to that for the amine groups, and so the affects of the amino group are dominant.

4. Conclusions

In this study, mucoadhesive thiolated chitosan have been successfully synthesized by covalent attachment of HT onto the amino group of chitosan under mild conditions. The chemical structures and physical properties of thiolated chitosan were investigated. The chitosan:HT with the mass ratio of 1:0.1 resulted in strongly improved mucoadhesive properties and a good swelling behavior at low pH. Compared to HT-chitosan, TM-HT-chitosan exerted higher mucoadhesive property. The results suggested that increasing of cationic charges in the TM-chitosan backbone can increase the mucoadhesiveness of thiolated chitosan.

Acknowledgments

This research was financially supported by The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) to K.J., The Center for Petroleum, Petrochemicals and Advanced Materials, Program of Petrochemistry and Polymer Science, plus the Center of Innovative Nanotechnology (CIN) and the National Research University Project of CHE and the Ratchadaphiseksomphot Endownment Fund (AS613A) to N.M. The comments of three anonymous reviewers are gratefully appreciated. Thanks are also given to Dr. Robert Butcher of the PCU, Faculty of Science, Chulalongkorn University, for constructive comments and English corrections.

References

Andrews, G. P., Laverty, T. P., & Jones, D. S. (2009). Mucoadhesive polymeric platforms for controlled drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, *71*(3), 505–518.

Anitha, A., Deepa, N., Chennazhi, K. P., Nair, S. V., Tamura, H., & Jayakumar, R. (2011). Development of mucoadhesive thiolated chitosan nanoparticles for biomedical applications. *Carbohydrate Polymers*, *83*, 66–73.

Barthelmes, J., Perera, G., Hombach, J., Dünnhaupt, S., & Bernkop-Schnurch, A. (2011). Development of a mucoadhesive nanoparticulate drug delivery system for a targeted drug release in the bladder. *International Journal of Pharmaceutics*, *416*(1), 339–345.

Bernkop-Schnurch, A. (2005). Thiomers: A new generation of mucoadhesive polymers. *Advanced Drug Delivery Reviews*, *57*(11), 1569–1582.

Bernkop-Schnurch, A. (2008). Thiolated chitosan in non-invasive drug delivery. In *Natural-based polymers for biomedical applications*. Cambridge: Woodhead Publ. Ltd., pp. 624–643.

Bernkop-Schnurch, A., Hornof, M., & Zoidl, T. (2003). Thiolated polymers–thiomers: Synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates. *International Journal of Pharmaceutics*, *260*(2), 229–237.

Bernkop-Schnurch, A., Kast, C. E., & Guggi, D. (2003). Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: Thiomer/GSH systems. *Journal of Controlled Release*, *93*(2), 95–103.

Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, *36*, 981–1014.

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

Grabovac, V., Guggi, D., & Bernkop-Schnurch, A. (2005). Comparison of the mucoadhesive properties of various polymers. *Advanced Drug Delivery Reviews*, *57*(11), 1713–1723.

He, P., Davis, S. S., & Illum, L. (1998). In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *International Journal of Pharmaceutics*, *166*(1), 75–88.

Hornof, M. D., Kast, C. E., & Bernkop-Schnurch, A. (2003). In vitro evaluation of the viscoelastic properties of chitosan–thioglycolic acid conjugates. *European Journal of Pharmaceutics and Biopharmaceutics*, *55*(2), 185–190.

Jayakumar, R., Deepthy, M., Manzoor, K., Nair, S. V., & Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydrate Polymers*, *82*, 227–232.

Jayakumar, R., Prabaharan, M., & Muzzarelli, R. A. A. (Eds.). (2011). *Chitosan for biomaterials. Advances in polymer sciences* (p. 244). Springer.

Johnson, P., & Rainsford, K. D. (1972). The physical properties of mucus: Preliminary observations on the sedimentation behaviour of porcine gastric mucus. *Biochimica et Biophysica Acta (BBA) – General Subjects*, *286*(1), 72–78.

Kast, C. E., & Bernkop-Schnurch, A. (2001). Thiolated polymers–thiomers: Development and in vitro evaluation of chitosan–thioglycolic acid conjugates. *Biomaterials*, *22*(17), 2345–2352.

Kast, C. E., Frick, W., Losert, U., & Bernkop-Schnurch, A. (2003). Chitosan–thioglycolic acid conjugate: A new scaffold material for tissue engineering? *International Journal of Pharmaceutics*, *256*(1–2), 183–189.

Khalid, M. N., Agnely, F., Yagoubi, N., Grossiord, J. L., & Couarraze, G. (2002). Water state characterization, swelling behavior, thermal and mechanical properties of chitosan based networks. *European Journal of Pharmaceutical Sciences*, *15*(5), 425–432.

Lai, S. K., Wang, Y.-Y., Wirtz, D., & Hanes, J. (2009). Micro- and macrorheology of mucus. *Advanced Drug Delivery Reviews*, *61*(2), 86–100.

Lee, S. J., Kim, S. S., & Lee, Y. M. (2000). Interpenetrating polymer network hydrogels based on poly(ethylene glycol) macromer and chitosan. *Carbohydrate Polymers*, *41*(2), 197–205.

Leitner, V. M., Walker, G. F., & Bernkop-Schnurch, A. (2003). Thiolated polymers: Evidence for the formation of disulphide bonds with mucus glycoproteins. *European Journal of Pharmaceutics and Biopharmaceutics*, *56*(2), 207–214.

Ludwig, A. (2005). The use of mucoadhesive polymers in ocular drug delivery. *Advanced Drug Delivery Reviews*, *57*(11), 1595–1639.

Matsuda, A., Kobayashi, H., Itoh, S., Kataoka, K., & Tanaka, J. (2005). Immobilization of laminin peptide in molecularly aligned chitosan by covalent bonding. *Biomaterials*, *26*(15), 2273–2279.

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

Palmberger, T. F., Hombach, J., & Bernkop-Schnurch, A. (2008). Thiolated chitosan: Development and in vitro evaluation of an oral delivery system for acyclovir. *International Journal of Pharmaceutics*, *348*(1–2), 54–60.

Prabaharan, M., & Gong, S. (2008). Novel thiolated carboxymethyl chitosan-g-[beta]-cyclodextrin as mucoadhesive hydrophobic drug delivery carriers. *Carbohydrate Polymers*, *73*(1), 117–125.

Roldo, M., Hornof, M., Caliceti, P., & Bernkop-Schnurch, A. (2004). Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: Synthesis and in vitro evaluation. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*(1), 115–121.

Rossi, S., Ferrari, F., Bonferoni, M. C., & Caramella, C. (2001). Characterization of chitosan hydrochloride–mucin rheological interaction: Influence of polymer concentration and polymer:mucin weight ratio. *European Journal of Pharmaceutical Sciences*, *12*(4), 479–485.

Salamat-Miller, N., Chittchang, M., & Johnston, T. P. (2005). The use of mucoadhesive polymers in buccal drug delivery. *Advanced Drug Delivery Reviews*, *57*(11), 1666–1691.

Schmitz, T., Grabovac, V., Palmberger, T. F., Hoffer, M. H., & Bernkop-Schnurch, A. (2008). Synthesis and characterization of a chitosan-N-acetyl cysteine conjugate. *International Journal of Pharmaceutics*, *347*(1–2), 79–85.

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

Smart, J. D. (2005). The basics and underlying mechanisms of mucoadhesion. *Advanced Drug Delivery Reviews*, *57*(11), 1556–1568.

Snyman, D., Hamman, J. H., Kotze, J. S., Rollings, J. E., & Kotze, A. F. (2002). The relationship between the absolute molecular weight and the degree of quaternisation of N-trimethyl chitosan chloride. *Carbohydrate Polymers*, *50*(2), 145–150.

Sun, L., Du, Y., Fan, L., Chen, X., & Yang, J. (2006). Preparation, characterization and antimicrobial activity of quaternized carboxymethyl chitosan and application as pulp-cap. *Polymer*, *47*(6), 1796–1804.

Wu, Z. M., Zhang, X. G., Zheng, C., Li, C. X., Zhang, S. M., Dong, R. N., et al. (2009). Disulfide-crosslinked chitosan hydrogel for cell viability and controlled protein release. *European Journal of Pharmaceutical Sciences*, *37*(3–4), 198–206.

Yin, L., Ding, J., He, C., Cui, L., Tang, C., & Yin, C. (2009). Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. *Biomaterials*, *30*(29), 5691–5700.

Yu, Q., Song, Y., Shi, X., Xu, C., & Bin, Y. (2011). Preparation and properties of chitosan derivative/poly(vinyl alcohol) blend film crosslinked with glutaraldehyde. *Carbohydrate Polymers*, *84*(1), 465–470.

Zhang, C., Ping, Q., Zhang, H., & Shen, J. (2003). Synthesis and characterization of water-soluble O-succinyl-chitosan. *European Polymer Journal*, *39*(8), 1629–1634.