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Mucoadhesive 4-carboxybenzenesulfonamide-chitosan with antibacterial properties

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ABSTRACT

The mucoadhesive property of chitosan, especially in an acidic (<pH 6.0) environment, was increased by conjugating an aromatic sulfonamide group at the C2-N position of chitosan. Four different feeding ratios of 4-carboxybenzensulfonamide (4-CBS) to chitosan in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as a coupling agent were investigated. The 0.2:1 (w/w) ratio 4-CBS:chitosan revealed a 20-fold stronger mucoadhesion to mucin type II than the native chitosan in the simulated gastric fluid (SGF; pH 1.2), and a swelling ratio after 1 h in water, SGF and simulated intestinal fluid (pH 7.4) of about 2.9-, 3.0- and 3.4-fold higher than that of chitosan, respectively. In tissue culture, the 4-CBS-chitosan, like chitosan, were found to be non-cytotoxic to the Vero, KB, MCF-7 and NCI-H187 cell lines but showed potential antibacterial activity against *Escherichia coli* and *Staphlyococcus aureus* as model Gram-negative and Gram-positive bacteria, respectively.

1. Introduction

Mucoadhesive drug delivery systems are of increasing interest nowadays for transmucosal delivery routes, such as pulmonary, nasal and oral routes, due to their several potent advantages. For example, their increased specificity and localization at the target site gives a prolonged residence time at the site of required drug absorption, whilst the intensified contact with the mucosa increases the drug concentration gradient (Bernkop-Schnurch, 2005). The mucoadhesive ability of any given compound and dosage required is dependent upon a variety of factors, including the nature of the mucosal tissue and the physicochemical properties of the polymeric formulation, such as being predominantly hydrophilic with numerous hydrogen bonding groups, having a suitable surface for wetting the mucus membrane and possessing sufficient flexibility to penetrate the mucus network. Another factor is the type and density of the covalent and ionic interactions between the polymer and the mucosal membrane. As the number and strength of such interactions, and thus the net interaction strength density, increases then so the level of mucoadhesion also increases.

Various mucoadhesive polymers have been reported, such as chitosan (Sogias, Williams, & Khutoryanskiy, 2008), chitosan-poly(lactide-co-glycolide), carboxymethyl cellulose (NaCMC) and thiolated polymers. Thiolated polymers, as the new generation of strong mucoadhesive polymers, have a free thiol group in the polymeric backbone that can form disulfide bonds with the cysteine-rich sub-domains present in the mucin and so also improve the mucoadhesion of the polymer. A number of such thiolated polymers have been developed including chitosan-iminothiolane (Bernkop-Schnurch, Hornof, & Zoidl, 2003) and chitosan-thioglycolic acid (Chowdary & Rao, 2003; Grabovac & Bernkop-Schnurch, 2007; Kast & Bernkop-Schnurch, 2001). However, thiol groups are less stable above pH 5 as they can be oxidized to form disulfide bonds between themselves. Besides, at pH of less than 5.0 the level of thiolated anions, which is the functional group responsible for thiol/disulfide interactions with the mucus membrane, is decreased (Bernkop-Schnurch, Krauland, Leitner, & Palmberger, 2004), and accordingly their potential mucoadhesion in the acid environment of the stomach (~pH 1.2) is decreased.

It was, therefore, the aim of this study to design and synthesize a novel mucoadhesive polymer without thiol group showing similar properties as thiolated polymer but being stable in acid conditions.

As cationic polysaccharide, chitosan, the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan, is obtained from marine chitin (Muzzarelli et al., 2012). It is one of the obvious

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Table 1
Weight ratios of 4-CBS:chitosan, degree of substitution (% DS), the component of mucoadhesion (cps) in SGF (pH 1.2) and 0.1 N PB (pH 5.5), and the diameter of the inhibition zone against *E. coli* and *S. aureus* of chitosan and the four different 4-CBS-chitosan. Data are shown as the mean ± 1 SD. Means within a column with different letters differ significantly each other ($P < 0.1$; ANOVA).

4-CBS:chitosan (w/w) ratio	DS (%)[a]	The component of mucoadhesion (η_b)[b]		Diameter of inhibition zone (mm)[c]	
		SGF (pH 1.2)[d]	0.1 N PB (pH 5.5)[e]	*E. coli*	*S. aureus*
0:1	–	14.6 ± 0.4	36.3 ± 2.6	6.0 ± 0.0	6.3 ± 0.1
0.05:1	4.1	44.5 ± 0.9	6.2 ± 0.1	ND	ND
0.2:1	4.5	285.9 ± 0.7	32.8 ± 0.5	9.0 ± 0.0	8.0 ± 0.0
0.5:1	6.8	125.0 ± 3.5	29.0 ± 2.3	ND	ND
1:1	13.3	2.3 ± 1.1	6.2 ± 0.1	ND	ND

ND is not determined. Mean ± SD ($n = 3$).
[a] Degree of substitution, as determined by ^{1}H NMR absorption spectroscopy.
[b] As determined by Oswald viscometer.
[c] As determined by the agar diffusion method.
[d] Simulated gastric fluid (0.1 N HCl, pH 1.2).
[e] 0.1 N phosphate buffer (PB, pH 5.5).

choices for pharmaceutical application due to its properties such as non-toxicity, enzymatic biodegradability and high biocompatibility, plus its antimicrobial activity (Jayakumar, Prabaharan, & Muzzarelli, 2011). Furthermore, the free amino groups in chitosan, which carry a positive charge at pH < 6.0, interact with many negatively charged surface groups of the mucus membrane providing a good mucoadhesive property that is suitable for drug delivery systems in the intestinal tract (Sogias et al., 2008). Therefore, not many cases of chitosan and its derivatives have been reported as suitable for drug delivery in the stomach (pH 1.2). One example of the chitosan applications in low pH condition is the study effect of the chemical crosslink of glyoxal on chitosan microsphere for tetracycline delivery in stomach-specific anti-*Helicobacter pylori* therapy (Hejazi & Amiji, 2004). The nanoparticles of pH-responsive chitosan/heparin prepared by a simple gelation method were stable at pH 1.2–2.5 that was useful for protecting drugs in the gastric acid (Lin et al., 2009). Moreover, these nanoparticles can adhere to and infiltrate to cell–cell junction to treat *H. pylori* infection in stomach. Although, highly acidic condition in stomach would be an obstacle to use modified chitosan due to highly dissoluble polymer, some researchers try to overcome that obstacle by improving modified appropriate functional group on chitosan and further use as mucoadhesive polymer. The aim of this work was to develop a mucoadhesive polymer for use in a mucoadhesive delivery system with a good swelling property and resistance under acidic conditions so as to be suitable for orally administered and poorly absorbed drugs, especially at the gastrointestinal tract. Therefore, a novel mucoadhesive chitosan derivative was developed from chitosan by the covalent attachment of 4-carboxybenzenesulfonamide (4-CBS), which was tested as inhibitors of β-carbonic anhydrase enzyme activity and the antimicrobial activity against *H. pylori* (Gao et al., 1996), onto the amino groups of chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as a coupling agent results in a mucoadhesive 4-CBS-chitosan that is not only a potential carbonic anhydrase inhibitor (Nishimori et al., 2007) but is also stable in acid conditions. The obtained polymer is characterized by ^{1}H NMR, FT-IR and TGA analysis. As a preliminary work before going in to biomedical applications, the swelling behavior, mucoadhesive properties and cytotoxicity were tested. In addition, the anti-bacterial activities were also investigated in terms of the inhibition of bacterial growth using the agar diffusion assay.

2. Materials and methods

2.1. Materials

Chitosan with an average molecular weight >500 kDa was provided by the Bonafide Co., Ltd. in Thailand. The degree of deacetylation of chitosan was determined to be 81% by ^{1}H NMR analysis. Mucin from porcine stomach (type 2), 4-CBS and EDAC were obtained from Sigma (St. Louis, USA). Cellulose dialysis tubing (Membrane Filtration Products Inc., USA) with a 12–14 kDa molecular weight cut-off was used to purify all modified chitosan. All other chemicals were obtained commercially as reagent grade and used as supplied without purification.

2.2. Synthesis of 4-CBS-chitosan

The covalent attachment of chitosan and 4-CBS was achieved as follows. Chitosan (1 g) was dissolved in 100 mL of 1% (v/v) acetic acid solution at room temperature overnight and then 4-CBS and EDAC at a mole ratio of 1.2:1 EDAC:4-CBS were added at four different (w/w) ratios of 4-CBS:chitosan, ranging from 0.05:1 to 1:1, as detailed in Table 1. The reaction was refluxed for 6 h to form the 4-CBS-chitosan using EDAC as the coupling agent (Fig. 1). Excess EDAC was removed by precipitation following the addition of 1 M HCl to the reaction mixture and then centrifugation. The supernatant was then neutralized with 1 N NaOH and the residual free acetic acid, NaCl, *o*-acylurea derivative and unreacted 4-CBS were eliminated by dialysis against three changes of 1000 mL of ethanol. The reaction mixture was then filtered, washed with water and lyophilized at 198 mbar and −45 °C.

2.3. Characterization

2.3.1. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR)

For the characterization of chitosan and the four different 4-CBS-chitosan, 30 mg of each compound were dissolved in 2% (v/v) trifluoroacetic acid (CF_3COOH) in deuterium oxide (D_2O). ^{1}H NMR spectra was recorded by a Mercury Varian NMR spectrometer operated at 400 MHz (Agilent Technologies, CA, USA), using pulse accumulating of 64 scans.

2.3.2. Fourier transformed infrared spectroscopy (FT-IR)

The chitosan and the four different 4-CBS-chitosan were dried and ground into a fine powder. The powder then was mixed with KBr at 1:100 (w/w) and pressed into a disc. Analysis was performed a Perkin-Elmer Spectrum RX-1 FT-IR spectrometer system, scanning from 600 to 4000 cm^{-1}.

2.3.3. Thermogravimetric analysis (TGA)

Samples of 3–6 mg were assayed by TGA. The aluminum oxide pan, placed in the balance system, was continuously recorded as a function of temperature using a METTLER STAR SW 9.01 TGA under a nitrogen atmosphere, a temperature range of 30–600 °C and a heating rate of 10 °C/min.

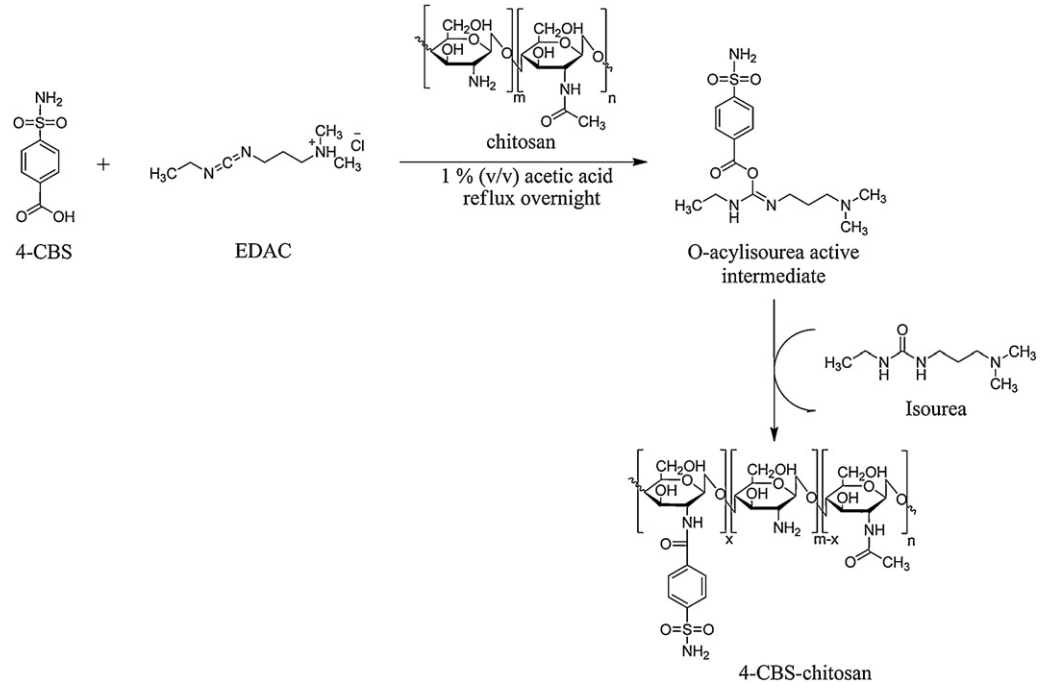

Fig. 1. Reaction scheme for the covalent attachment of 4-CBS to chitosan.

2.4. Determination of the degree of deacetylation (% DD) and degree of 4-CBS substitution (% DS) on the chitosan by ^{1}H NMR

Chitosan, the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan, is obtained from marine chitin. Most properties, such as the solubility, biodegradability, pK_a, and self-aggregation of chitosan, depend on the proportion of acetylated and non-acetylated GluN units, so the called degree of deacetylation (% DD) (Kasaai, 2010). The % DD is the proportion of the acetyl groups that have been removed, and it is essential information to study their chemical structures, properties and structure–properties relationships. The degree of substitution (% DS) is the proportion of amino groups in the chitosan backbone that have been covalently linked to the 4-CBS group, and is of interest as it is related to the mucoadhesive property.

Both the % DD and % DS were determined by ^{1}H NMR spectra (400 MHz), as outlined above and previously reported (Hamman & Kotze, 2001) using Eqs. (1) and (2), respectively.

$$\%\,\mathrm{DD} = 100 - \left[\frac{I_{(\mathrm{COCH_3})}}{I_{(\mathrm{H_{3-6}})}} \times \frac{5}{3}\right] \times 100 \quad (1)$$

where $I_{(\mathrm{COCH_3})}$ is the integral of the methyl protons of the acetylated chitosan at 1.78 ppm and $I_{(\mathrm{H_{3-6}})}$ is the integral of the H3–H6 protons of chitosan at 3.44–3.63 ppm.

$$\%\,\mathrm{DS} = \left[\frac{I_{(\mathrm{SO_2NH_2})}}{I_{(\mathrm{H_{3-6}})}} \times \frac{5}{2}\right] \times 100 \quad (2)$$

where $I_{(\mathrm{SO_2NH_2})}$ is the integral of the benzene ring of 4-CBS-chitosan protons at 7.78–7.76 ppm and $I_{(\mathrm{H_{3-6}})}$ is the integral of the H3–H6 protons of 4-CBS-chitosan at 3.44–3.63 ppm.

2.5. Determination of the mucoadhesiveness of chitosan and the 4-CBS-chitosan

In this work, the amine (—NH_2) groups of 4-CBS-chitosan was partially protonated to the ammonium cation (—NH_3^+) groups by dissolving the 4-CBS-chitosan in 1% (v/v) acetic acid in order to increase the positive charges in the system and, hence, increase the mucoadhesion force. The mucoadhesive properties of 4-CBS-chitosan were determined based on the viscometric changes of porcine gastric mucin and selected polymers in SGF (pH 1.2). The viscosities of a 0.1% (w/v) solution of each polymer, 15% (w/v) mucin and a 15% (w/v) mucin–0.1% (w/v) polymer mixture in SGF were performed at 37 °C on an Oswald viscometer (Tamson, TV 4000), using the procedure described by Hassan and Gallo (1990). Briefly, dried mucin was hydrated with SGF by gentle stirring for 3 h at 25 °C to yield a 20% (w/v) dispersion. Chitosan was dissolved in 10% (v/v) acetic acid to yield a 4% (w/v) stock chitosan solution that was then diluted by SGF to yield a 0.1% (w/v) chitosan solution. The four different of 4-CBS-chitosan were likewise each dissolved in SGF to yield the respective 0.1% (w/v) 4-CBS-chitosan solutions. The viscosities of the 15% (w/v) mucin–0.1% (w/v) polymer mixtures were then measured in SGF for 15 min. The mucoadhesive properties of the 4-CBS-chitosan in SGF were compared with that in PB (pH 5.5) using same method.

The viscosity coefficient was then determined by Eq. (3) as follows:

$$\eta_t = \eta_m + \eta_p + \eta_b \quad (3)$$

where η_t is the viscosity coefficient of the system, η_m and η_p are the individual viscosity coefficients of the mucin and polymer, respectively, and η_b is the viscosity component due to the mucoadhesive.

2.6. Swelling of chitosan and the 4-CBS-chitosan

The swelling of the chitosan and the 4-CBS-chitosan films was studied by observing the change in the diameter of the films. Each polymer (1 g) was dissolved in 50 mL of 1% (v/v) acetic acid, and the solution was then poured into a plastic plate (8 cm × 10 cm) and left at ambient conditions until dry. The dried films were cut into 6.0 mm diameter discs using a paper punch and placed into water, SGF or SIF, as appropriate, at room temperature. The swelling ratios were measured at particular predetermined time points after immersion in the respective solutions, and were evaluated by measuring the change in the diameter of the flat discs

using a micrometer scale. The swelling ratio (S_w) of each film was determined by Eq. (4):

$$S_w(\%) = \frac{D_t - D_0}{D_0} \times 100 \quad (4)$$

where D_t is the film diameter at time t and D_0 is the initial film diameter (Muzzarelli, 1996).

2.7. Cytotoxic activity

In this work, the 0.2:1 (w/w) ratio 4-CBS:chitosan was selected for evaluation of its *in vitro* cytotoxic activity against four mammalian cell lines (the primate Vero), and the human KB (epidermoid carcinoma of oral cavity), MCF-7 (breast adenocarcinoma), and NCL-H187 (small cell lung carcinoma cell lines) as it showed the strongest mucoadhesive property. The cytotoxic activity tests were performed in Bioassay laboratory, National Center for Genetic Engineering and Biotechnology, Thailand with screening code V5962, the brief procedures were as follows:

The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney Vero cell line (ATCC CCL-81), with the pEGFP-N1 plasmid (Clontech) under geneticin resistance selection. The cell line was maintained in complete medium (CM), comprised of minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.8 mg/mL geneticin, at 37 °C in a humidified incubator with 5% (v/v) CO_2.

The cytotoxicity assay for the Vero cells was carried out by adding 45 μL of a cell suspension (3.3×10^4 cells/mL) into each well of 384-well plates containing 5 μL of the test compound previously diluted in 0.5% (v/v) DMSO, and then incubating for four days at 37 °C in a humidified 5% (v/v) CO_2 incubator. Fluorescence signals were then measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA), in the bottom reading mode with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The fluorescence signal attained at day four was subtracted from the background fluorescence, as reported elsewhere (Hunt, Jordan, Jesus, & Wurm, 1999).

The cytotoxicity test was also performed based on the resazurin microplate assay (REMA), as described elsewhere (Brien, Wilson, Orton, & Pongnan, 2000), on the KB, MCF-7 and NCL-H187 cell lines. Late log phase growth cells were harvested and diluted to 7×10^4 cells/mL (KB) or 9×10^4 cells/mL (MCF-7 and NCL-H187) in fresh CM. The test sample of chitosan or 4-CBS-chitosan (5 μg) diluted in 0.5% (v/v) DMSO, and 45 μL of the appropriate cell suspension were added to each well of a 384-well plate and incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator. After incubation for three (KB and MCF-7) or five (NCL-H187) days, 12.5 μL of a 62.5 μg/mL resazurin solution was added to each well, and the plates incubated at 37 °C for 4 h. The fluorescence was then measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) with excitation and emission wavelengths of 530 nm and 590 nm, respectively.

The percent inhibition of cell growth was then calculated from Eq. (5):

$$\%\,\text{inhibition} = 1 - \left(\frac{FU_t}{FU_c}\right) \times 100 \quad (5)$$

where FU_t and FU_c are the fluorescence levels from the treated and untreated conditions, respectively.

Dose response curves were plotted from six concentrations of 2-fold serially diluted test compounds, and the sample concentration that inhibited cell growth by 50% (IC_{50}) was derived using the SOFTMax Pro Software (Molecular Devices, USA).

2.8. Antimicrobial activity of chitosan and 4-CBS-chitosan against Escherichia coli and Staphlyococcus aureus bacteria

The antibacterial activity of the chitosan and the 4-CBS-chitosan derived from a 0.2:1 (w/w) ratio of 4-CBS:chitosan against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) was evaluated using the agar well diffusion method. The *E. coli* and *S. aureus* strains were obtained from the Department of Medical Sciences (Thailand). A representative single colony was picked off, placed in nutrient broth (NB; peptone 5 g/L, beef extract 3 g/L, agar 17 g/L; pH 7.0–7.2) and incubated at 37 °C for 24 h. Cells were then harvested by centrifugation and resuspended in fresh NB to and 20 μL spread evenly over the surface of a 5-mm diameter NB-agar plate. The 4-CBS-chitosan derived from a 0.2:1 (w/w) ratio of 4-CBS:chitosan was dissolved in 1% (v/v) acetic acid to 2.5 mg/mL and then 20 μL was applied to the 5 mm diameter well in the NB agar plate and incubated at 37 °C for 24 h prior to examination of the bacterial growth (as a confluent lawn) and the zone of inhibition around the well. A solution of 1% aqueous acetic acid was used as a control group to verify the response of both bacteria in the presence of acidic solution with above method (data not shown). The diameter of the inhibition zone was then measured and reported in the triplicate repeat set.

2.9. Statistical analysis

All measurements were performed in triplicate in each experiment with the results presented as the mean ± 1 SD. Statistical analysis was performed by one-way ANOVA using Microsoft Excel (Microsoft Corporation) with $P < 0.05$ considered to indicate statistical significance.

3. Results and discussion

3.1. Synthesis and structural analysis of 4-CBS-chitosan

The covalent attachment of 4-CBS to chitosan was achieved by the coupling reaction of the carboxylic acid groups (—COOH) of 4-CBS to the primary amine groups ($—NH_2$) of chitosan *via* the coupling agent EDAC, as shown schematically in Fig. 1.

The carboxylic acid moieties of 4-CBS were activated by EDAC (coupling agent) to form an *o*-acylurea derivative as the intermediate product. The *o*-acylurea then reacts with the primary amine groups of chitosan to form the amide 4-CBS-chitosan. The resulting 4-CBS-chitosan appeared as a white, odorless, fibrous polymer and was easily dissolved in 1% (v/v) acetic acid solution to form a high viscosity pale yellow gel. The obtained 4-CBS-chitosan was characterized by ^{1}H NMR, FT-IR and TGA analysis.

3.2. Characterization of the 4-CBS-chitosan

3.2.1. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR)

The chemical structures of chitosan, 4-CBS and the four different 4-CBS-chitosan derived from different 4-CBS:chitosan weight ratios were characterized by ^{1}H NMR. Representative results are shown in Fig. 2, and for the 4-CBS-chitosan derived from a 0.2:1 (w/w) ratio of 4-CBS:chitosan are as follows: ^{1}H NMR (D_2O/CF_3COOH): δ (ppm) 7.96 (d, 2H, J = 8.0 Hz, Ph), 7.77 (d, 2H, J = 8.0 Hz, Ph), 4.60 (s, 1H, H1), 4.31 (s, 1H, H1′), 3.64–3.43 (m, 4H, H3, H4, H5 and H6), 2.92 (s, 1H, H2) and 1.81 (s, 3H, N*H*COCH$_3$).

The ^{1}H NMR spectrum of chitosan (Fig. 2a) showed a signal at 1.78 ppm (s, 3H) due to the acetyl protons of the GluNAc units, a singlet at 2.89 ppm (s, 2H) attributed to the H at the C2

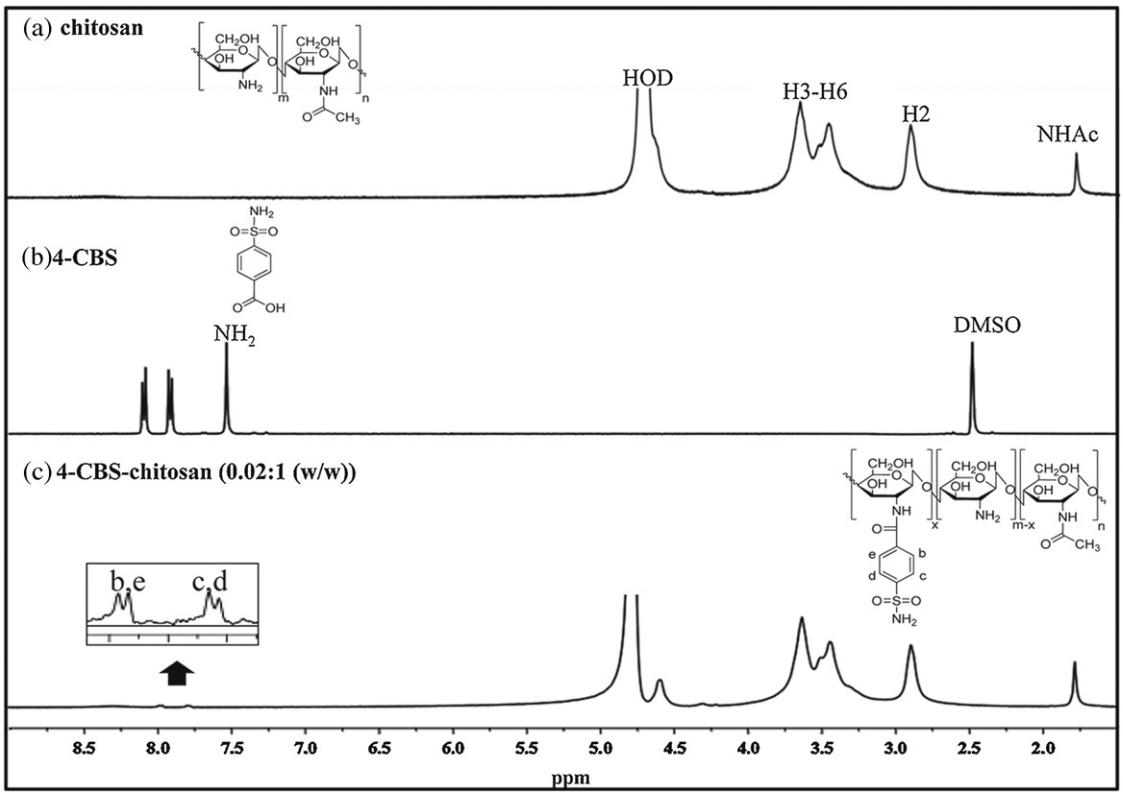

Fig. 2. ^{1}H NMR spectra of the (a) chitosan, (b) 4-CBS and (c) the representative 4-CBS-chitosan derived from a 0.2:1 (w/w) ratio of 4-CBS:chitosan. Spectra shown are representative of those seen from four independent samples.

position of the GluN units, whilst the signals due to the hydrogen atoms (H3–H6) in the chitosan ring were observed at around 3.63–3.44 ppm.

The ^{1}H NMR spectrum of 4-CBS (Fig. 2b) showed the signals at 7.91 ppm (d, 2H, J = 8.0 Hz) and 8.08 (d, 2H, J = 8.0 Hz), which were assigned to the aromatic protons of the sulfonamide ring, respectively.

The representative ^{1}H NMR spectra of the 0.2:1 (w/w) 4-CBS:chitosan (Fig. 2c) showed the characteristic peaks of both chitosan and 4-CBS segments. The aromatic proton position of 4-CBS showed at 7.77 ppm (d, 2H, J = 8.0 Hz) and 7.96 ppm (d, 2H, J = 8.0 Hz). Both the GluNAc and GluN groups contributed to the multiple peak region of numeric carbons (H3–H6) from 3.64 to 3.43 ppm and a singlet peak at 2.92 ppm from the C2-H position of GluN. The chemical shift at 1.81 ppm (s, 3H) was assigned to the acetyl proton of GluNAc. These results support that the all ratio of 4-CBS-chitosan were successfully synthesized chitosan. The ^{1}H NMR spectra of the other (w/w) ratios of 4-CBS:chitosan were similar to that of the 0.2:1 ratio (data not shown). Increase in 4-CBS:chitosan made the peak at 7.77–7.96 ppm to increase. Consequently, % DS would be increasing.

3.2.2. Determination of the degree of deacetylation (% DD) and the degree of 4-CBS substitution (% DS) on the chitosan by ^{1}H NMR

The % DD and % DS play an important role in characterizing most of the physical and chemical properties of chitosan. ^{1}H NMR spectra analysis was used to analyze the % DD and % DS of chitosan because the sample preparation is simple, accurate and the purity of samples does not need to be calculated as long as the impurity peaks do not overlap with the sample peaks (Lavertu et al., 2003). The % DD of chitosan was determined to be 81% using Eq. (1). The % DS increased with increasing 4-CBS:chitosan (w/w) ratios (Table 1), as expected, up to a maximum of 13.3% in the 1:1(w/w) ratio 4-CBS:chitosan.

3.2.3. Fourier transformed infrared spectroscopy (FT-IR)

FT-IR analysis was performed to determine the 4-CBS-chitosan formed between chitosan and 4-CBS. Representative results for the 4-CBS-chitosan derived from the 0.2:1 (w/w) ratio of 4-CBS:chitosan are shown in Fig. 3.

The FT-IR spectra of the 0.2:1 (w/w) ratio 4-CBS:chitosan (Fig. 3c), shown as a representative of the different (w/w) ratio 4-CBS-chitosan, revealed the characteristic peaks of both chitosan

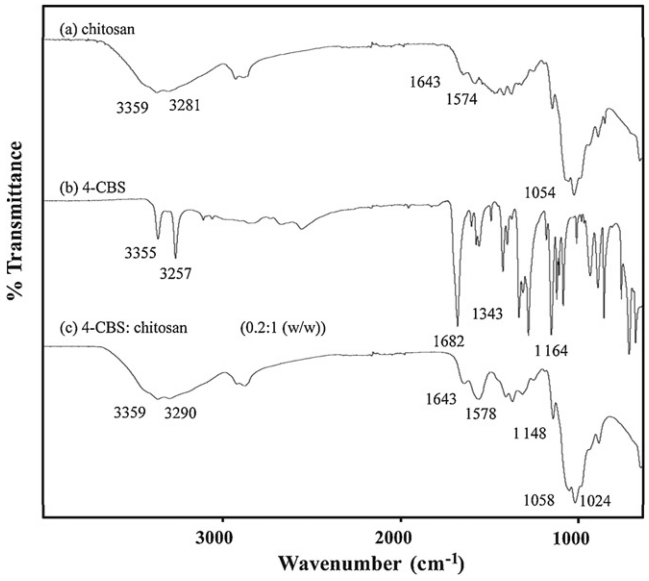

Fig. 3. FT-IR spectra of the (a) chitosan, (b) 4-CBS and (c) the representative 4-CBS-chitosan derived from a 0.2:1 (w/w) ratio of 4-CBS:chitosan. Spectra shown are representative of those seen from four independent samples.

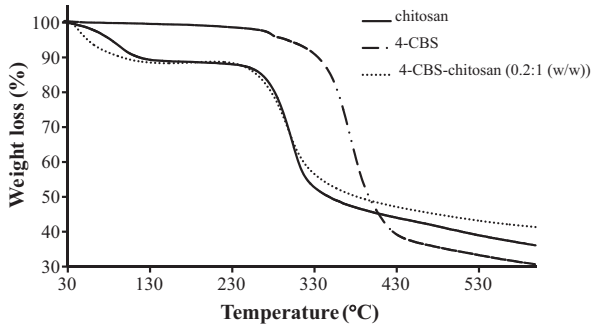

Fig. 4. Representative TGA thermogram of chitosan, 4-CBS and the representative 4-CBS-chitosan derived from the 0.2:1 (w/w) ratio of 4-CBS:chitosan. Graphs shown are representative of those seen from four independent samples.

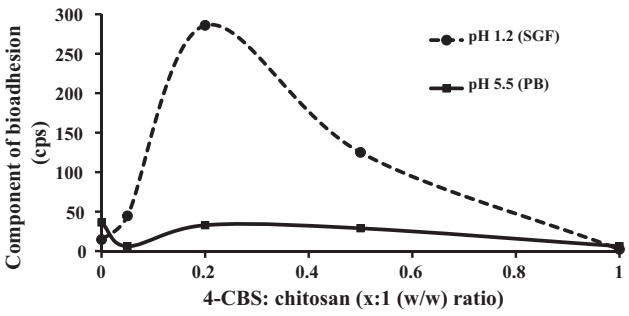

Fig. 5. The component of mucoadhesion (cps) of the 0.2:1 (w/w) ratio of 4-CBS:chitosan in SGF (0.1 N HCl; pH 1.2) and PB (0.1 N phosphate buffer; pH 5.5). Data are shown as the mean ± 1 SD and are derived from three independent repeats.

(Fig. 3a) and 4-CBS (Fig. 3b). An increase in the absorbance of the peak at 1617–1501 cm^{-1} was also observed when compared with that at 1148–1024 cm^{-1} of the C—O—C cyclic ethers stretching. This is in accordance with that the substitution of the C=C aromatic ring stretching of 4-CBS at 1682 cm^{-1} possibly overlapped the amide I band stretching of chitosan. Moreover, that the intensity peak of CH_2OH at the range of 1140–1080 cm^{-1} did not change reflects the fact that no substitution at the hydroxyl group of chitosan occurred.

3.2.4. Thermogravimetric analysis (TGA)

TGA is a simple analytical technique which is used to evaluate the thermal stability of a compound and its decomposition temperature, including that of polymers. The TG thermograms of the chitosan, 4-CBS and 4-CBS-chitosan are summarized in Fig. 4. The degree of weight loss (slope) of the samples increased with increasing temperature and was different for the different samples, reflecting the degradation of each component in the materials.

The TG curve of the 4-CBS-chitosan (the 0.2:1 (w/w) ratio 4-CBS:chitosan is shown as a representative case in Fig. 4) had two stages of weight loss and was broadly similar to that for chitosan. The first stage ranged between 30 °C and 160 °C and is attributed to moisture vaporization, whilst the second stage ranged between 240 °C and 310 °C, with a 40% total weight loss, and is likely to be the degradation of 4-CBS from the 4-CBS-chitosan backbone. The total weight loss of the 4-CBS-chitosan at 600 °C was 55%. Therefore, the coupling of 4-CBS onto the chitosan backbone may partially decrease the crystalline nature and small specific volume of the rigid chitosan backbone chain (Yanming, Ruan, Wang, Zhao, & Bi, 2004), resulting in a lower weight loss than the chitosan. Although the 4-CBS-chitosan showed similar characteristics with chitosan, they have a higher thermal stability and decomposition temperature than chitosan, and so may be more suitable as a polymer for drug delivery systems.

3.3. Mucoadhesive property of chitosan and the 4-CBS-chitosan in SGF

The viscosity of the 4-CBS-chitosan is an important factor in influencing their mucoadhesive properties. A good mucoadhesion has advantages for drug delivery vehicles, for example, by increasing the localization and residence time at the site of drug absorption, and in providing an intensified contact with the mucosa and, subsequently, increasing the drug concentration gradient at the required site (Hejazi & Amiji, 2003). Increasing the molecular mass of the polymer leads to a higher internal cohesion of the molecule that consequently typically increases the mucoadhesion (Tobyn, Johnson, & Dettmar, 1996). Therefore, in this work, the mucoadhesion of the 4-CBS-chitosan, derived from the different (w/w) ratios of 4-CBS:chitosan, with mucin were investigated in SGF (pH 1.2) and PB (pH 5.5) because chitosan is only readily soluble in dilute acid solutions below pH 6. At a pH of above 6, chitosan becomes deprotonated and losses its charge and so becomes insoluble (Pillai, Paul, & Sharma, 2009). The mucoadhesive property is largely derived from the ionic interactions between the polymer and mucin. As the ionic interactions increase, the mucoadhesive properties and viscosity of the mixture increases, and so the mucoadhesion is typically related to the mucoadhesive force between the interacting polymer and the mucin.

The mucoadhesion of chitosan and the four different 4-CBS-chitosan in SGF (pH 1.2) are summarized in Table 1 with a representative example shown in Fig. 5. In SGF, chitosan showed a component of mucoadhesion of 14.6 cps, while the 0.2:1 (w/w) ratio 4-CBS:chitosan showed the highest component of mucoadhesion, almost 20-fold higher than that for chitosan. The trend of mucoadhesion of the 4-CBS-chitosan was not directly related to the increasing (w/w) ratios of 4-CBS:chitosan, but rather was maximal at the 0.2:1 (w/w) ratio. When the 4-CBS:chitosan (w/w) ratio was higher than 0.2:1, the mucoadhesive component of the 4-CBS:chitosan decreased dramatically reaching a 6.3-fold lower level than that of chitosan at a 1:1 (w/w) ratio 4-CBS:chitosan, due to the high steric hindrance effect of the 4-CBS side chain which makes it difficult for the interaction between the COO^- groups of chitosan and the SO^{3-} groups of mucin. Thus, the mucoadhesive forces between the 4-CBS-chitosan and mucin are likely to be dominated by electronic interactions with, to a lesser extent, hydrophobic effects of the $-CH_3$ and aromatic part of 4-CBS groups on the polymer residue that interact with the $-CH_3$ groups on the mucin side chains.

In PB (pH 5.5) (Table 1 and Fig. 5), the 4-CBS-chitosan at a 4-CBS:chitosan (w/w) ratio of 0.05:1–1:1 revealed significantly lower components of mucoadhesiveness, in the range of about 6.2–32.8, which are lower than that for chitosan (36.3). However, the mucoadhesive property of chitosan in PB at pH 5.5 was higher than that in SGF at pH 1.2 because the sialic acid of mucin was in the anionic form and so ionic attraction with chitosan resulted in a higher mucoadhesive force. The 4-CBS-chitosan exhibited significantly lower mucoadhesive properties at pH 5.5 than that of the native chitosan except for the 0.2:1 (w/w) ratio 4-CBS:chitosan which was only slightly lower. However, considering the greatly enhanced mucoadhesion seen at pH 1.2, the 0.2:1 (w/w) ratio 4-CBS:chitosan is the most suitable of these chitosan polymers for further study as a potential mucoadhesive drug delivery system in the stomach (low pH).

3.4. Swelling test

The swelling behavior indicates the relative ease and speed of liquid penetration into a polymer matrix, which is an essential step and important influence on the kinetics of the drug release

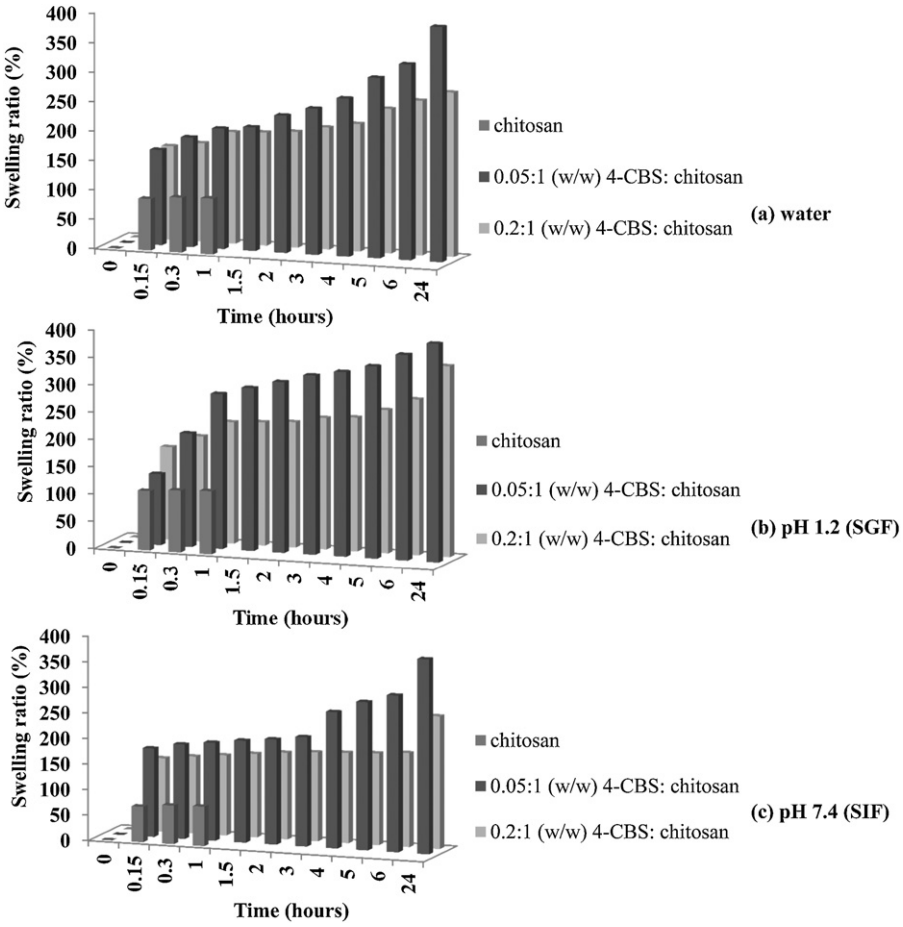

Fig. 6. Swelling behaviors of the 4-CBS-chitosan derived from the 0.05:1 and 0.2:1 (w/w) ratio of 4-CBS:chitosan in (a) water, (b) SGF (pH 1.2) and (c) SIF (pH 7.4). Data are shown as the mean ± 1 SD and are derived from three independent repeats.

process. In addition, the swelling behavior of mucoadhesive polymers has a strong influence on their adhesive properties, water-uptake and stability (Hornof, Weyenberg, Ludwig, & Bernkop-Schnurch, 2003). A rapid swelling behavior may improve the inter-diffusion process between the polymer and the mucus layer, providing a strong adhesion and then leading to an enhanced drug delivery rate (Mathiowitz, Chickering, & Lehr, 1999). In order to investigate the possibility of using 4-CBS-chitosan as a drug delivery system in general and more specifically in the gastrointestinal tract, the swelling behavior was observed in water, SGF and SIF. That for the 0.05:1 and 0.2:1 (w/w) ratio 4-CBS:chitosan as examples compared to chitosan are shown in Fig. 6.

The swelling behavior of chitosan in water (Fig. 6a) mainly depends on the difference in the osmotic pressure inside and outside of the chitosan specimen (Sarkyt & Vladimir, 1999). Taking the 0.05:1 and 0.2:1 (w/w) 4-CBS:chitosan as examples, they swelled rapidly within 1 h in water, and then continued to swell gradually thereafter to a final swelling ratio of over 418.9% and 280.0% more than the original starting size, respectively. Within 24 h, they had swollen to almost 4.4-fold and 2.9-fold more than that seen with chitosan at 1 h. This was probably because the 4-CBS prevented the intermolecular interactions between the $-NH_2$ groups of chitosan, so more water could penetrate into the polymeric networks to increase the degree of swelling. The dissolution mechanism may be related to the content of the aromatic sulfonamide groups coupling onto the chitosan, leading to increased steric effects that prevent the intermolecular interactions of the $-NH_2$ groups of chitosan. This notion would then explain why the 0.05:1 (w/w) ratio 4-CBS:chitosan swelled more than that for the 0.2:1 (w/w) ratio 4-CBS:chitosan.

In the acidic SGF medium (pH 1.2) (Fig. 6b) within 1 h of immersion, the 0.2:1 (w/w) ratio 4-CBS:chitosan swelled about 2.4-fold and 1.9-fold more than that of the chitosan and continued to swell to reach a swelling ratio of 456.1% and 349.4% more than its initial dried size after 24 h. The degree of swelling of all 4-CBS:chitosan (w/w) ratio were larger in SGF than in water, due to the $-NH_2$ groups being protonated ($-NH_3^+$) at pH < 6.0 and so the hydrogen bonds are dissociated, inducing the network to become loose and leading to an increased degree of swelling (Hejazi & Amiji, 2003; Kim, Park, Kim, & Cho, 2003). This causes repulsion among the polymer chains allowing more water to enter the polymer film than that which occurs in water or SIF (pH 7.4).

When immersed within SIF (Fig. 6c), the 0.05:1 and 0.2:1 (w/w) 4-CBS:chitosan gave a 1.9-fold and 1.6-fold greater swelling ratio, respectively, than that of chitosan at 1 h, and they still continued to swell over the 24 h period attaining a 381.1% and 259.4%, or some 4.9-fold and 3.4-fold greater level than the maximum swelling level of chitosan (at 1 h).The lower swelling ratios of chitosan and the modified 4-CBS-chitosan when immersed in the weak alkaline SIF (pH 7.4) than that seen in the acidic SGF (pH 1.2) media can be explained simply as that in alkali medium, the $-NH_3^+$ groups of chitosan are deprotonated and uncharged leading to a re-association of the hydrogen bonds and, consequently, weaker interactions between the polymer chains and the water. This leads to a decreased level of water entry and, subsequently, a

reduced degree of swelling. The observed trend is consistent with the reported behavior of chitosan (Mitsumata et al., 2003).

In conclusion, the swelling behavior of the 4-CBS-chitosan depended on the pH of the medium and the composition of the polymeric matrix. Moreover, the pH-dependent variations in the degree of swelling for chitosan and the 4-CBS-chitosan were potentially related to the association and dissociation of hydrogen bonds. In a low pH medium, the degree of swelling was high (maximal) because of the protonation of the $-NH_2$ moieties in the chitosan polymeric chains.

In all three media (water, SGF and SIF), 4-CBS-chitosan did not dissolve within 1 h and all swelled more than chitosan, although the 0.05:1 (w/w) ratio 4-CBS:chitosan swelled more than the 0.2:1 (w/w) ratio 4-CBS:chitosan in all three media. This is consistent with the formation of slippery mucilage (Smart, 2005). Therefore, the 0.2:1 (w/w) ratio 4-CBS:chitosan appeared to be the best drug delivery system under acidic (SGF; pH 1.2) conditions, because the polymer can gradually and suitably swell within 24 h without dissolution.

3.5. *In vitro cell line cytotoxic activity of the different 4-CBS:chitosan*

The potential cytotoxic activity of the chitosan and the 4-CBS-chitosan with a 0.2:1 (w/w) ratio of 4-CBS:chitosan were evaluated *in vitro* against the primate Vero cell line and the KB, MCF-7 and NCL-H187 transformed (cancer-derived) human cell lines (data not shown). The 4-CBS-chitosan, like chitosan, was found be display no detectable cytotoxicity (over the 0.015–0.035 μg/μL tested range) to all four tested cell lines.

3.6. *Inhibition of 4-CBS-chitosan against E. coli and S. aureus bacteria*

A numbers of reports have indicated that chitosan exhibits antibacterial activities, such as against the growth of *E. coli* and *S. aureus* bacteria (Gerasimenko et al., 2004). In this study, the 0.2:1 (w/w) ratio 4-CBS:chitosan was found to inhibit the growth of *E. coli* and *S. aureus* bacteria using the agar diffusion method (Table 1), with a 1.5- and 1.3-fold wider diameter of inhibition zone against *E. coli* and *S. aureus*, respectively, than that of the chitosan. Taking into account all the results, the 0.2:1 (w/w) ratio 4-CBS:chitosan is a potentially good system for further study as a polymer for a gastric drug delivery system because it has antibacterial activity, is non-toxic, and has mucoadhesive properties in SGF, good swelling properties and resistance in an acidic environment.

4. Conclusions

The mucoadhesive 4-CBS-chitosan can be successfully synthesized *via* a coupling reaction using various (w/w) ratios of 4-CBS to chitosan (0.05:1–1:1) in the presence of EDAC as a coupling reagent. The 4-CBS-chitosan was confirmed by ^{1}H NMR, FT-IR and TGA analysis and the % DS of sulfonamide groups onto chitosan were in the range of 4.1–13.3% for 4-CBS:chitosan (w/w) ratios of 0.05:1–1:1, respectively. The 4-CBS-chitosan gave a higher component of mucoadhesion and swelling ratio than the pure chitosan in the acidic SGF (pH 1.2), suggesting that they can tolerate the acidic stomach condition for at least 24 h. In addition, the 0.2:1 (w/w) ratio 4-CBS:chitosan displayed antibacterial activity against *E. coli* and *S. aureus*, and appeared non-toxic to Vero, KB, MCF-7 and NCI-H187 cell lines in tissue culture.

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