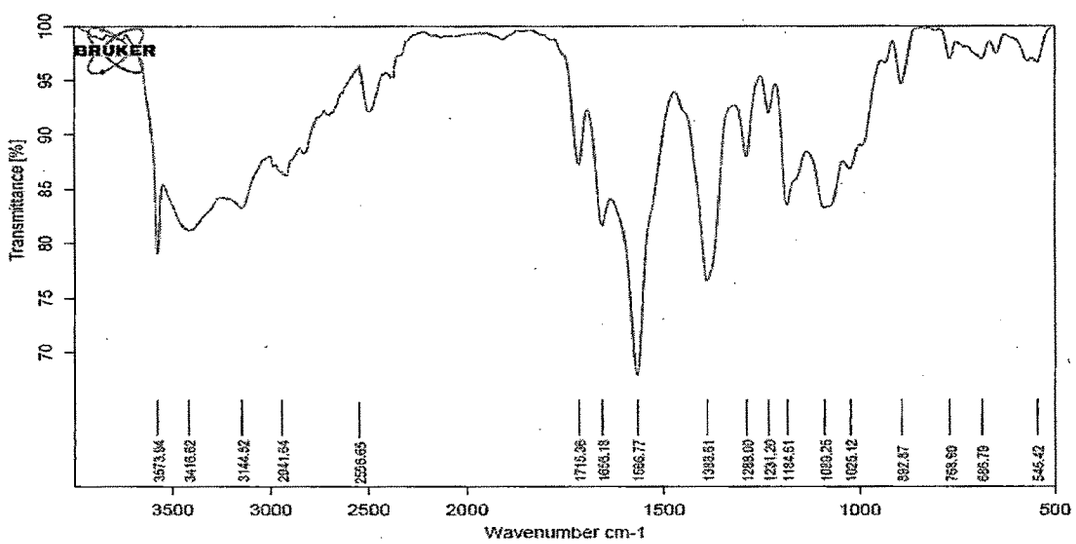
Chapter 4 Modification of chitosan



4.1. Preparation of thiolated chitosan

Thiolated chitosan was synthesized by previously reported method (Hornof et al., 2003). Each type chitosan (low and medium molecular weight) was dissolved at 500 mg of concentration in 50 ml of 1.0% acetic acid. In order to facilitate reaction with thioglycolic acid (TGA), 100 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added to the chitosan solution. After EDC was dissolved, 30 ml of TGA was added and the pH was adjusted to 5.0 with 3 N NaOH. In order to evaluate the influence of the pH-value on the coupling reaction, the pH was adjusted using 3 N NaOH to pH 3, 4, and 5 respectively. The reaction mixture was stirred and left for 3 h at room temperature. To eliminate the unbound TGA and to isolate the polymer conjugates, the reaction mixture was dialyzed against 5 mM HCl five times (molecular weight cut-off 10 k*Da*) over a period of 3 days in the dark, then two times against 5 mM HCl containing 1.0% NaCl to reduce ionic interactions between the cationic polymer and the anionic sulfhydryl compound (Hornof et al., 2003). Thiol groups in thiolated chitosan were analysed quantitatively by 'Ellman's method' and qualitatively confirmed by the FTIR. The influence of the chitosan to thioglycolic acids as well as the pH (Constantia et al., 2001) on the share of immobilized thiol group was shown in Table 4.1.

Table 4.1: Amount of reagents used for the reaction mixtures in order to evaluate the influence of the chitosan to thioglycolic acids as well as the pH on the share of immobilized thiol group in mmol.

Polymer	pH	Chitosan 500mg/50 ml	EDC (mg)	TGA (ml)	Thiol group/gm of polymer (mmol)
LMC	3	500	100	15	10.67±0.67
	3	500	100	30	16.90±0.98
	4	500	100	15	21.87±1.9
	4	500	100	30	29.8±2.2
	5	500	100	15	38.67±2.4
	5	**500**	**100**	**30**	**42.33±2.7**
MMC	3	500	100	15	55.89±3.9
	3	500	100	30	75.95±4.9
	4	500	100	15	79.45±5.1
	4	500	100	30	98.43±4.6
	5	500	100	15	109.78±6.9
	5	**500**	**100**	**30**	**125.50±9.8**

(Mean ± S.D., $n = 3$)

LMC- Low molecular weight chitosan

MMC- Medium molecular weight chitosan

4.2. Characterization of thiolated chitosan

4.2.1. Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity (Ellman's method)

Thiol groups in thiolated chitosan were analysed quantitatively by 'Ellman's method' (Snyder et al., 1983, Sreenivas et al., 2008, Andreas et al., 2003) and Orientating studies with these thiolated chitosans showed that a degree of modification of 25–250 mmol thiol groups per gram chitosan leads to the highest improvement in the mucoadhesive and permeation enhancing properties. Reaction Buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA) was used for the preparation of thiolated chitosan solution and Ellman's reagent. For each polymer sample to be tested, prepare a tube containing 50 µl of Ellman's Reagent Solution (4 mg Ellman's Reagent in 1 ml of reaction buffer) and 2.5 ml of reaction buffer and add 250 µl of each unknown polymer solution to the separate test tubes. As a blank, add 250 µl of reaction buffer to a separate test tube. For the unknown sample, make dilutions so that the 250 µl sample applied to the assay reaction has a sulfhydryl concentration less than 1.0 mM. Concentrations exceeding 1 mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient. Finally mix the content of above prepared test tubes and incubate at room temperature for 15 minutes and measure the absorbance at 412 nm using spectrophotometer. Calculate the amount and concentration of sulfhydryl in the sample from the molar extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB) (14,150 $M^{-1}cm^{-1}$). Figure 4.1 shows the Structure of Ellman's Reagent and Reduction of Ellman's Reagent.

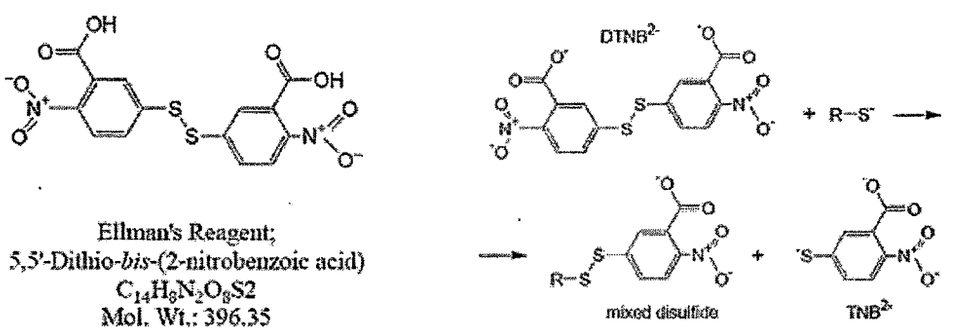


Figure 4.1: (a) Structure of Ellman's Reagent (b) Reduction of Ellman's Reagent

4.2.2. Infrared spectra of NPs

Infrared spectra of different molecular weight chitosan and thiolated chitosan were obtained and analyzed for confirmation of the thiol group. The IR spectrum of chitosan and thiolated chitosan was obtained by dispersing the sample in KBr disc.

4.2.3. Mucoadhesion by mucin particle method

Mucoadhesive properties of chitosan and thiolated chitosan were evaluated using the mucin particle method. Submicronsized mucin (ss-mucin) suspension (1% w/v) was prepared by suspending and continuously stirring mucin type III powder in 10 mM Tris buffer, pH 6.8, for 10 h. Mucin suspension was then incubated at 37°C overnight. The size of mucin was reduced by ultrasonication until particle size was found around 300–400 nm and then centrifuged at 4000 rpm for 20 minutes to extract submicron-sized mucin particles in the supernatant. The particle size and zeta potential of the precisely size-controlled ss-mucin were 400 ± 12 nm and -16.1 ± 1.8 mV, respectively. Around one milliliter of 1% w/v ss-mucin suspension was mixed with different volumes of 1 mg/ml each polymer solutions under mild stirring and the particle size and zeta potential values were measured using a dynamic light scattering with a Malvern Zetasizer (NanoZS, Malvern Instruments, UK) (Anchalee et al., 2008).

4.3. Results and discussion

4.3.1. Preparation of thiolated chitosan

Thiolation of chitosan was carried out by carbodiimide method. Thiol groups in thiolated chitosan were analyzed quantitatively by 'Ellman's method'. TGA was attached covalently to the primary amino groups on the surface of chitosan. The carboxylic acid moieties of TGA were activated by EDC that forms an intermediates *O*-acylurea derivative, which reacts with the primary amino groups of chitosan. In order to optimize the synthesis of thiolated chitosan, the influence of the pH (pH 3 to 5) and the amount of chitosan to TGA during the coupling reaction on the amount of polymer-immobilized thiol groups was evaluated (Table 4.1). Results of optimization studies were demonstrated that the pH-value at the coupling reaction has a great impact on the amount of TGA bound to chitosan. At pH 3 the amount of covalently attached thiol groups was comparatively low, as EDC could not gain its full reactive potential at this pH. Hence, at pH 4 the amount of attached thiol groups was

significantly higher than the pH 3 and lower that the pH 5. Thiolation was maximum at pH 5 and above pH 5 the yield of polymer-bound thiol groups decreased again because the oxidation of the sulfhydryl groups during the coupling reaction, which is favored at higher pH (Bernkop-SchnuK et al., 2000, Constantia et al., 2001). In order to achieve a further improvement in the thiol groups attachment to the polymer, the influence of the polymer to TGA amount during the coupling reaction was evaluated at pH 5. Results of this study demonstrated the highest coupling rate was found at 500 mg of polymer and 30 ml of TGA. At less or more than 30 ml of TGA with 500 mg of polymer led to lower yields of immobilized thiol group on the polymer. The results indicate that the amount of covalently bound thiol groups cannot be increased by raising the share of TGA during the coupling reaction.

The lyophilized thiolated chitosan appeared as white, odorless powder of fibrous structure. They were easily swellable in aqueous solutions at a pH below 5 and formed transparent gels of high viscosity. The lyophilized thiolated chitosan stored at 4°C were stable towards air oxidation which could be proved in a study that has been carried out for six months.

4.3.2. Characterization of thiolated chitosan

4.3.2.1. Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity (Ellman's method)

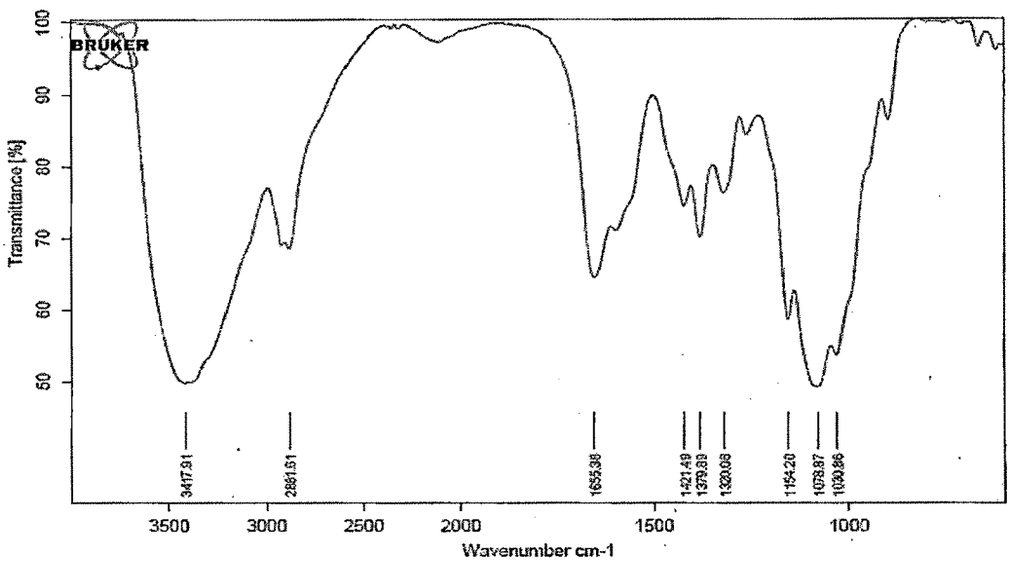
The thiolated chitosan produced by carbodiimide exhibited 42.33 mmol and 125.5 mmol immobilized free thiol groups per gram of low and medium molecular weight polymer respectively. The presence of thiol groups on the thiolated chitosan surface at high concentration increased the mucoadhesion capacity of NPs by forming covalent bonds with the cysteine residues of the mucus glycoproteins. The results indicate that the amount of covalently bound thiol groups can be increased approximately twice as much as by allowing the reaction to go in a way that prevents the oxidation of thiol groups (Snyder et al., 1983, Sreenivas et al., 2008, Andreas et al., 2003).

4.3.2.2. Infrared spectra

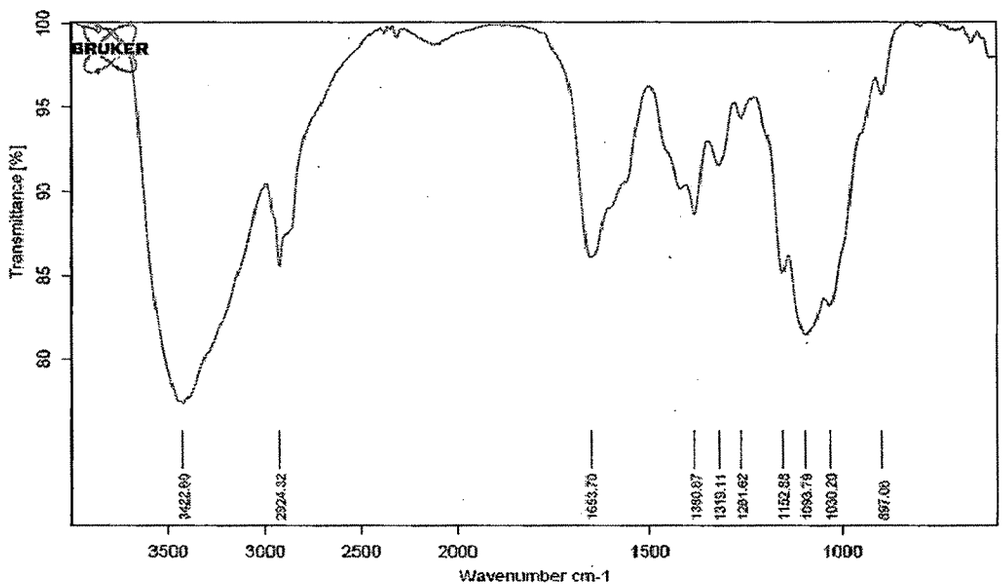
The coupling of TGA to chitosan was confirmed by the presence of S-H group. The spectra from thiolated samples contained a signal peak within the thiol group range at 2550-2600 cm^{-1}, which indicated the thiol group attached to the surface of chitosan, a

peak that is not found in the spectrum of the unmodified chitosan. Figure 4.2 shows the IR spectrum of chitosan and thiolated chitosan.

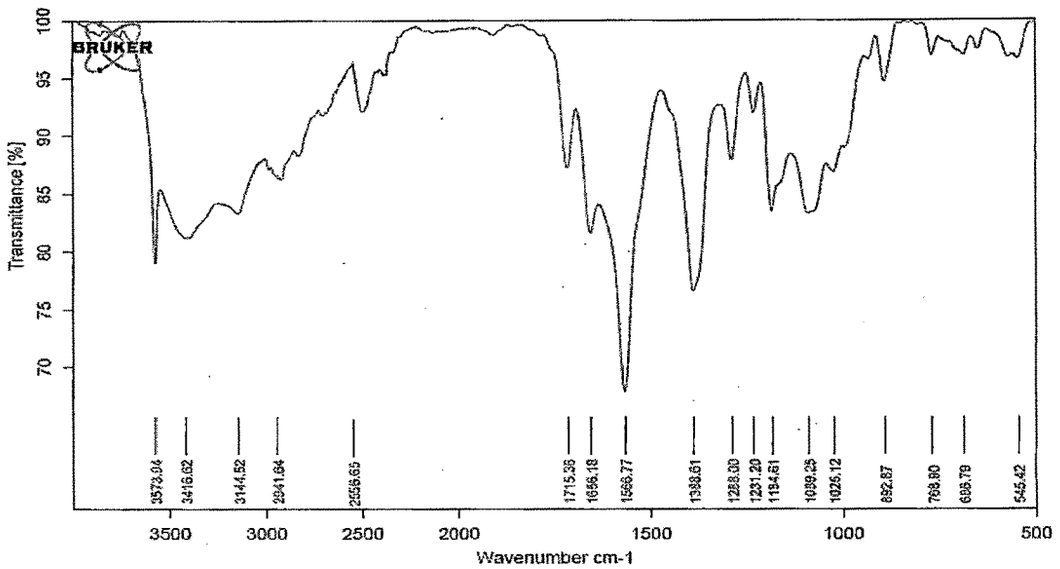
[a] Low molecular weight chitosan



[b] Medium molecular weight chitosan



[c] Low molecular weight thiolated chitosan



[d] Medium molecular weight thiolated chitosan

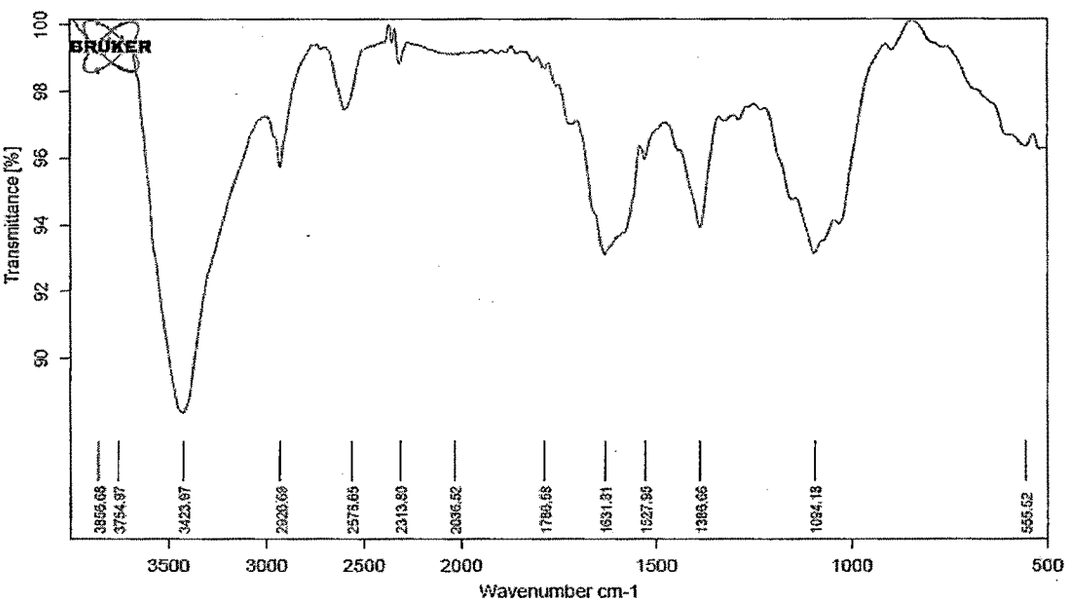


Figure 4.2: Infrared spectra: (a) Low molecular weight chitosan (b) Medium Molecular weight chitosan (c) Low molecular weight thiolated chitosan (d) Medium Molecular weight thiolated chitosan

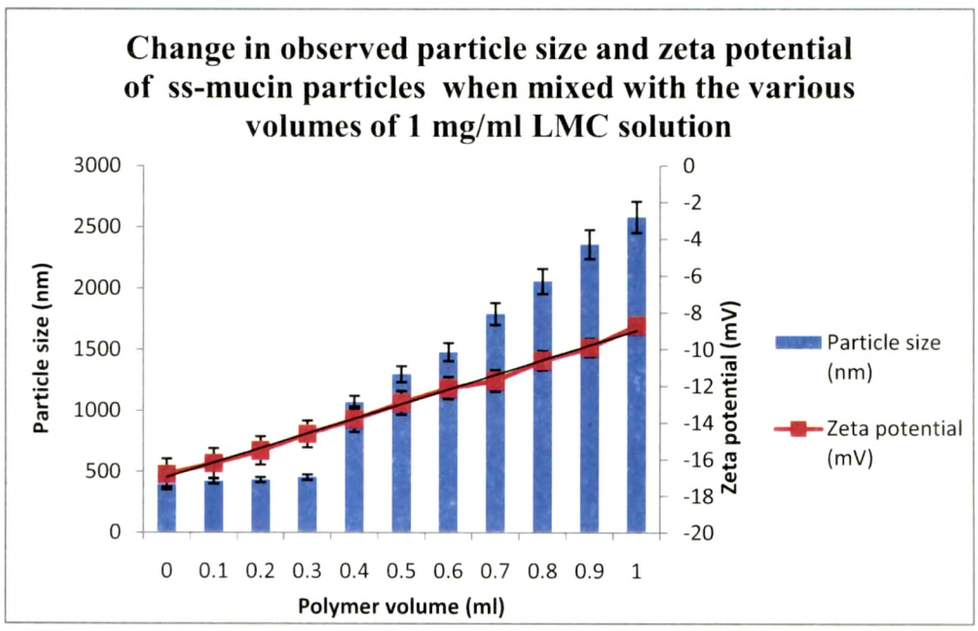
4.3.2.3. Mucoadhesion by mucin particle method

Mucoadhesion of modified chitosan (thiolated chitosan) were evaluated using the mucin particle method based on the change in surface properties of mucin particle, such as particle size and zeta potential, due to mucoadhesion of the thiolated chitosan

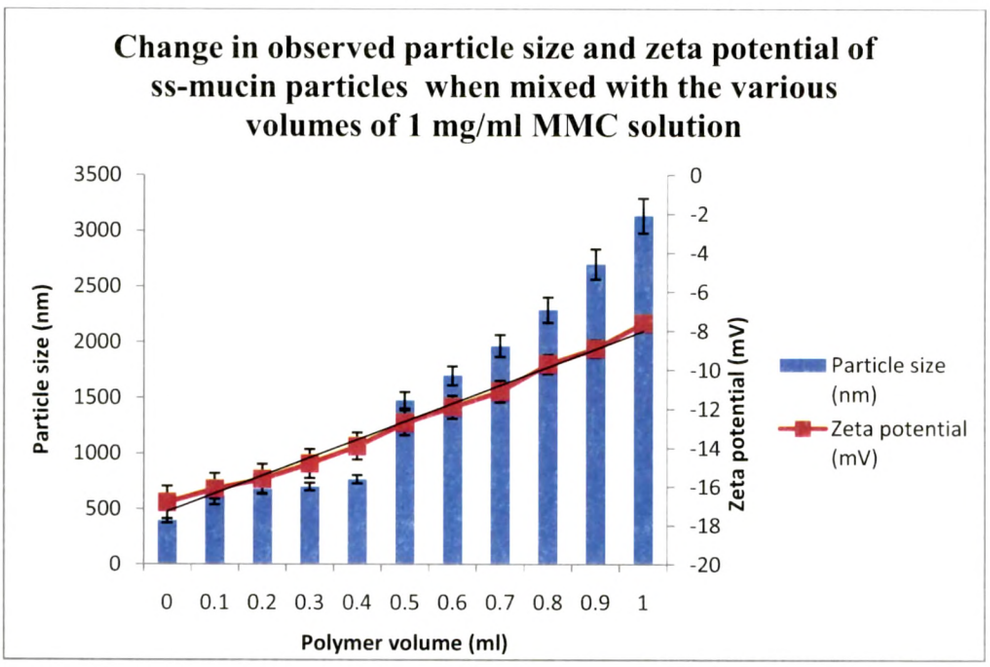
with the ss-mucin. It was found that the suspension of ss-mucin particles when mixed with a different volume of polymer solution, would induce the ss-mucin particles to aggregate, if the polymer had a strong affinity to them. Commercially available procine gastric mucin type III mucin was used for the study and the interaction was determined at pH 6.8 in Tris buffer where chitosan was insoluble and lost mucoadhesive properties. Figure 4.3 shows particle size and zeta potential of ss-mucin particles versus added volume of 1 mg/ml polymer solution (LMC, MMC, LMTC and MMTC). Two regions were found in all above graph. In region I all polymer did not affect the size and zeta potential of ss-mucin particles. Increases in the size and zeta potential were observed in region II where the aggregation occurred after the zeta potential of ss-mucin exceeded the critical zeta potential (-7 mV) of ss-mucin. These findings may be explained by DLVO theory (Anchalee et al., 2008). The slope of zeta potential profiles in region II and an extrapolated critical volume (Vc) of polymer used to neutralize negative charge of ss-mucin to zero could be used as indices of mucin–polymer adhesive bond strength of polymers. The stronger the mucoadhesive bond strength, the higher the value of slope as well as the lower the Vc value was observed.

By referring to the results of ss-mucin–polymer interaction studies (Table 4.2), it can be deduced that exhibited mucoadhesive characteristic and the rank order of mucoadhesive bond strength of polymers was MMTC > LMTC > MMC > LMC. Mucin particle method suggests the thiolated chitosan having higher mucoadhesive strength than the chitosan. The findings may be explained by the formation of covalent bonds between the thiol groups of thiolated chitosan and cysteine residue. Furthermore, it was observed that the interaction between ss-mucin particles and polymer was molecular weight-dependent. The interaction was decreased with decreased molecular weight.

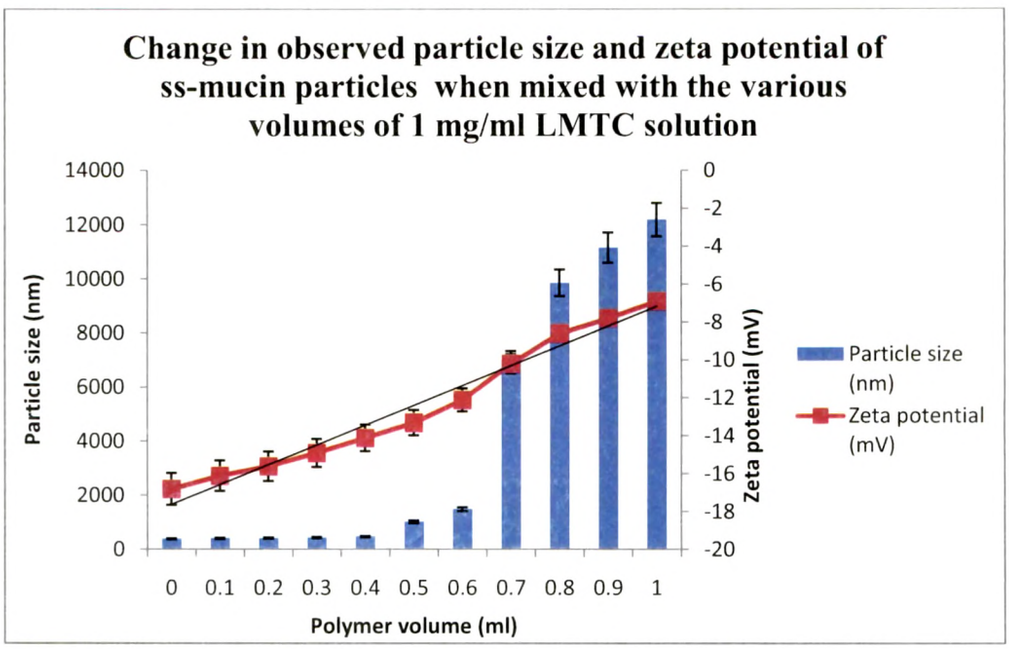
(a)


Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml LMC solution
Particle size (nm)
Zeta potential (mV)
Polymer volume (ml)
Particle size (nm)
Zeta potential (mV)

(b)


Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml MMC solution
Particle size (nm)
Zeta potential (mV)
Polymer volume (ml)
Particle size (nm)
Zeta potential (mV)

(c)



(d)

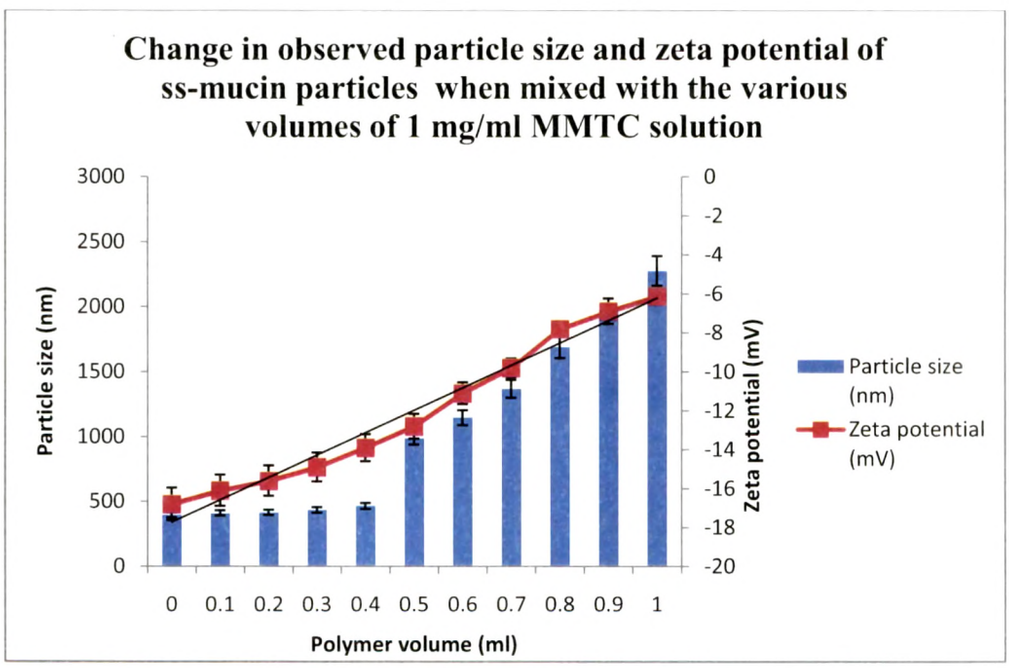


Figure 4.3 : Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml polymer solution (a) LMC (b)MMC(c)LMTC (d) MMTC

Table 4.2: Characteristic of the interaction between ss-mucin particles with the modified and unmodified chitosan

Polymer	Slope (mV/ml)	*Vc (ml)
LMC	8.2±0.5	2.08±0.5
MMC	10.25±0.6	1.75±0.1
LMTC	13.28±0.7	1.5±0.1
MMTC	13.70±0.9	1.4±0.1

*The extrapolated volume of 1mg/ml polymer solution used to neutralized negative charge of 1 % w/v SS-mucin to zero

4.4. Preparation of Trimethyl chitosan

Trimethyl chitosan (TMC) with a different degree of quaternization was synthesized by methylation of different molecular weight chitosan using methyl iodide (CH_3I) in the presence of a strong base (NaOH). 1 gm of chitosan, 2.4 gm of sodium iodide, and 10 ml of 20 % w/v aqueous sodium hydroxide solution were dissolved in 60 ml of N-methyl-2- pyrrolidinone (NMP) and the mixture was kept in a water bath at 60°C for 20 minutes. Subsequently, 10 ml of methyl iodide was added to the above mixture and the reaction was carried out in a Liebig condenser for 30 minutes. Afterwards, again 10 ml of methyl iodide and 10 ml of 20 %w/v aqueous sodium hydroxide were added in second step and the reaction was further continued at same temperature for 30 minutes in a Liebig condenser. The product was precipitated using ethanol three times. The products obtained were pooled and dissolved in 10%w/v sodium chloride solution to exchange the iodide ion with chloride. The final product was freeze-dried after dialysis and kept in light-protected desiccators. The purified TMC was analyzed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. The NMR spectrum of the TMC in D_2O at 80°C was recorded with a NMR spectrometer for determination of the degree of quaternization (DQ) (Worawan et al., 2008). Molecular weight of TMC was measure using Gel permeation chromatography method.

4.5. Characterization of trimethyl chitosan

4.5.1. Infrared spectrum

Infrared spectra of different molecular weight chitosan and trimethyl chitosan were obtained and analyzed for confirmation of the trimethylation.

4.5.2. ^{1}H NMR

The NMR spectrum of the TMC in D_2O at 80°C was recorded with a NMR spectrometer for determination of the degree of quaternization (DQ).

4.5.3. Mucoadhesion by mucin particle method

Mucoadhesive properties of chitosan and trimethyl chitosan were evaluated using the mucin particle method (same as described in section 4.2.3).

4.6. Results and discussion

4.6.1. Preparation of trimethyl chitosan

Methylation of amino groups in chitosan can be achieved using methyl iodide at elevated temperature in presence of strong alkaline environment to bind the acid being generated during the reaction taking place and to avoid protonation of the unreacted primary amino groups (Figure 4.4). The degree of quaternization (DQ) can be altered by increasing the number of reaction steps.

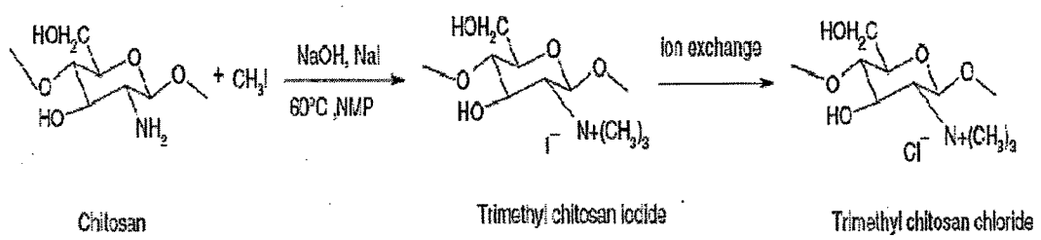


Figure 4.4: Synthesis of trimethyl chitosan

TMC with a different degree of quaternization (DQ) was synthesized by methylation of chitosan using CH_3I in the presence of a strong base (NaOH). The purified TMC was analyzed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. The NMR spectrum of the TMC in D_2O at 80 °C was recorded with a NMR spectrometer for determination of the degree of quaternization (DQ). This quaternized derivative of chitosan possesses a positive charge and is soluble over a wide range of pH. TMC was enriched with positive charge shows better mucoadhesive and permeation enhancement properties. The absolute molecular weight of the TMC polymers decreased with an increase in the DQ. Table 4.3 shows the characteristics of chitosan and synthesized TMC.

Table 4.3: The characteristics of chitosan and trimethyl chitosan

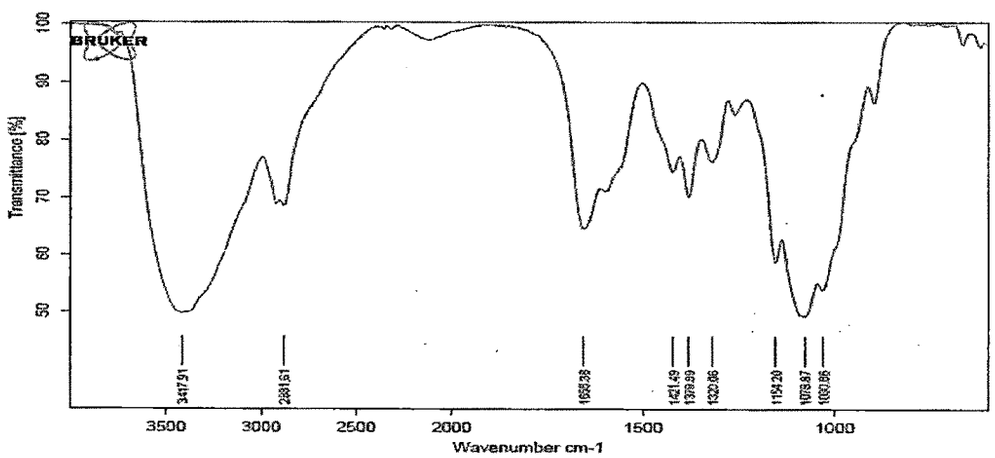
Type of chitosan	Number of reaction step	DQ (%)	Molecular weight (kDa)
Low molecular weight trimethyl chitosan (60 kDA)	2	38.78	39 kDA
Medium molecular weight trimethyl chitosan (450 kDA)	2	44.43	242 kDA

4.6.2. Characterization of trimethyl chitosan

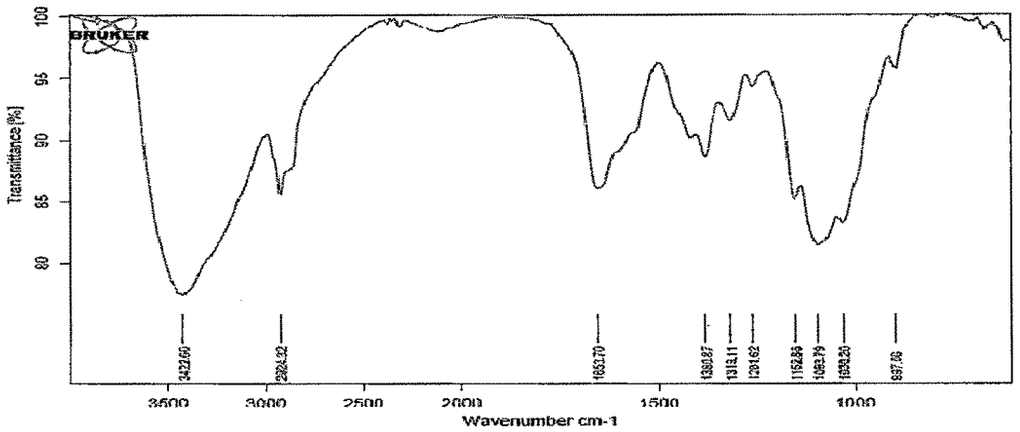
4.6.2.1. Infrared spectrum

The IR spectrum of chitosan and TMC was obtained by dispersing the sample in KBr disc. The IR spectrum of TMC was provided the confirmation for the occurrence of methylation in the region 1,700–1,200 cm^{-1}. The confirmation was found at (a) the band centered at 1,475 cm^{-1} in the spectrum of TMC due to the asymmetric angular deformation of C–H bonds of methyl groups, this band was absent in the spectrum of chitosan (de Britto et al., 2007) (b) the band due to the angular deformation of N–H bond of amino groups occurs in both spectra, at 1,577 cm^{-1} (1,500–1,620 $cm^{-1)}$ for chitosan and at 1,559 cm-1 for TMC, but it was weaker or disappeared due to the occurrence of N-methylation (Domard et al., 1986). A new peak was seen at a high wave number 1,630–1,660 cm^{-1} which indicates the quaternary ammonium salt (de Britto et al., 2007). The peak at 1,415–1,430 cm^{-1} was due to the characteristic absorption of N–CH_3 (Mourya et al., 2009). Figure 4.5 shows the IR spectrum of chitosan and trimethyl chitosan.

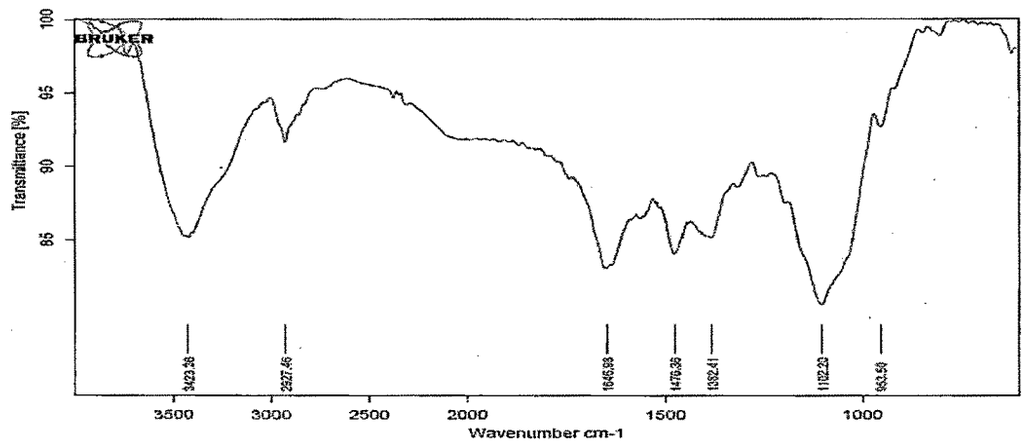
(a) Low molecular weight chitosan



(b) Medium molecular weight chitosan



(c) Low molecular weight trimethyl chitosan



(d) Medium molecular weight trimethyl chitosan

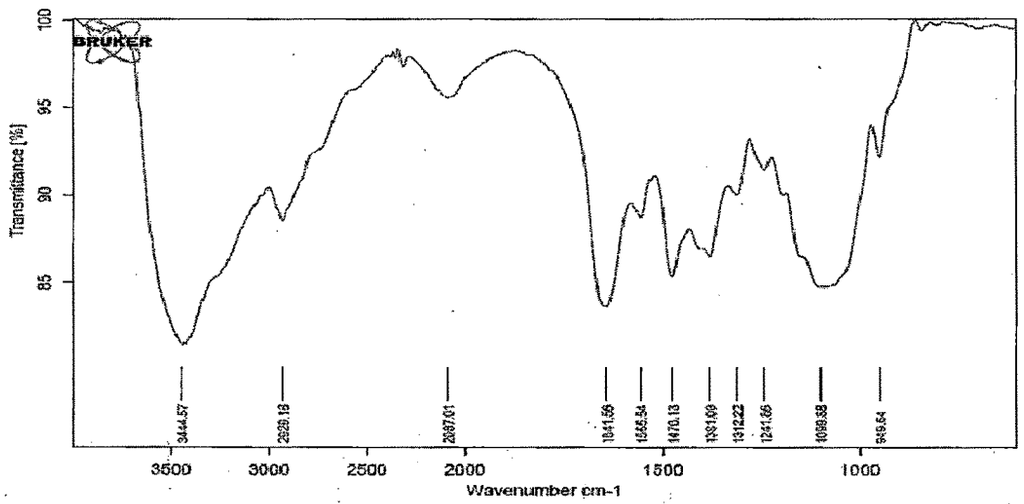


Figure 4.5: Infrared spectrum: (a) Low molecular weight chitosan (b) Medium molecular weight chitosan (c) Low molecular weight trimethyl chitosan (d) Medium molecular weight trimethyl chitosan

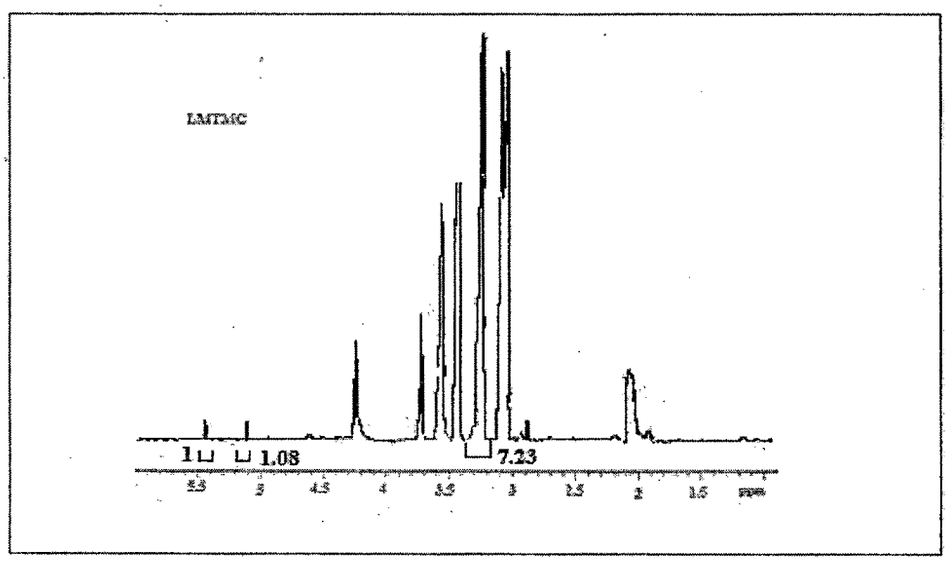
4.6.2.2. ^{1}H NMR

^{1}H NMR spectra were measured with a 300 or 600 MHz spectrometer by dissolving chitosan and TMC samples in D_20 at 80°C. The signals include a peak at 3.4 ppm for quaternized amino group, a peak at 2.5 ppm for dimethyl amino group and a peak at 3.36–3.56 ppm for O-methylated group. The signal of native monomers were confirmed as peaks at, 4.5–5.0 ppm for hydrogen bonded to the anomeric carbon 1; at 3.4–4.0 due to hydrogen bonded to the carbon atoms 3, 4, 5 and 6 of the glycopyranose unit; 3.18 attributed to the hydrogen atom bonded to the carbon 2 of the glycopyranose ring and ~2 corresponding to the hydrogen atoms of the methyl moieties of the acetamido groups. Table 4.3 shows the characteristics of chitosan and trimethyl chitosan. Degree of quaternization was calculated using equation:

$$\mathbf{DQ\ (\%) = \{[(CH3)3]/[H]*1/9\}*100}$$

where DQ (%) is the degree of quaternization as percentage, $[(CH3)_3]$ is the integral of the chemical shift of the N-trimethyl amino group at 3.3 ppm attributed to the nine hydrogen atoms of the methyl groups pertaining to trimethylated amino groups. [H] is the integral of the ^{1}H peaks between 4.7 – 5.7 ppm (reference signals) representing the protons attached to the carbon of the glucosamine unit of the glucopyranose ring. Figure 4.6 shows the ^{1}H NMR spectra of chitosan and trimethyl chitosan.

(a) Low molecular weight trimethyl chitosan



(b) Medium molecular weight trimethyl chitosan

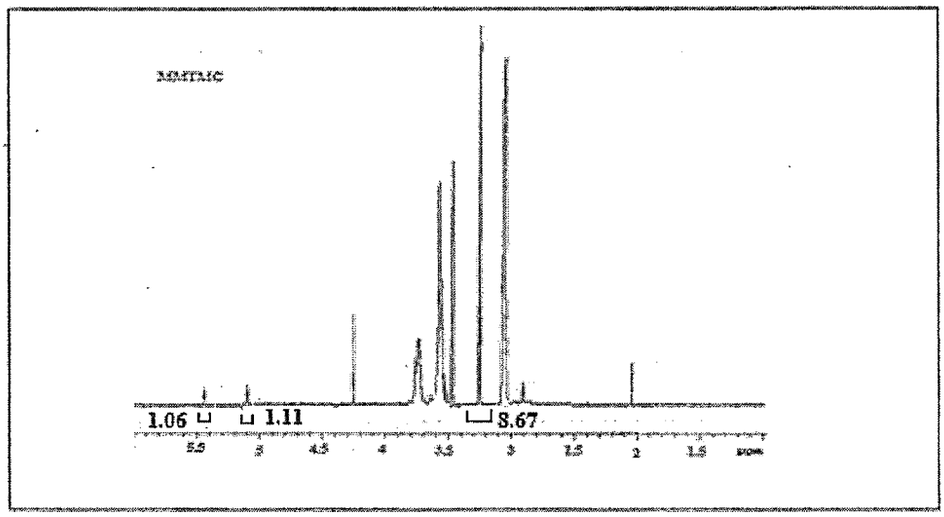
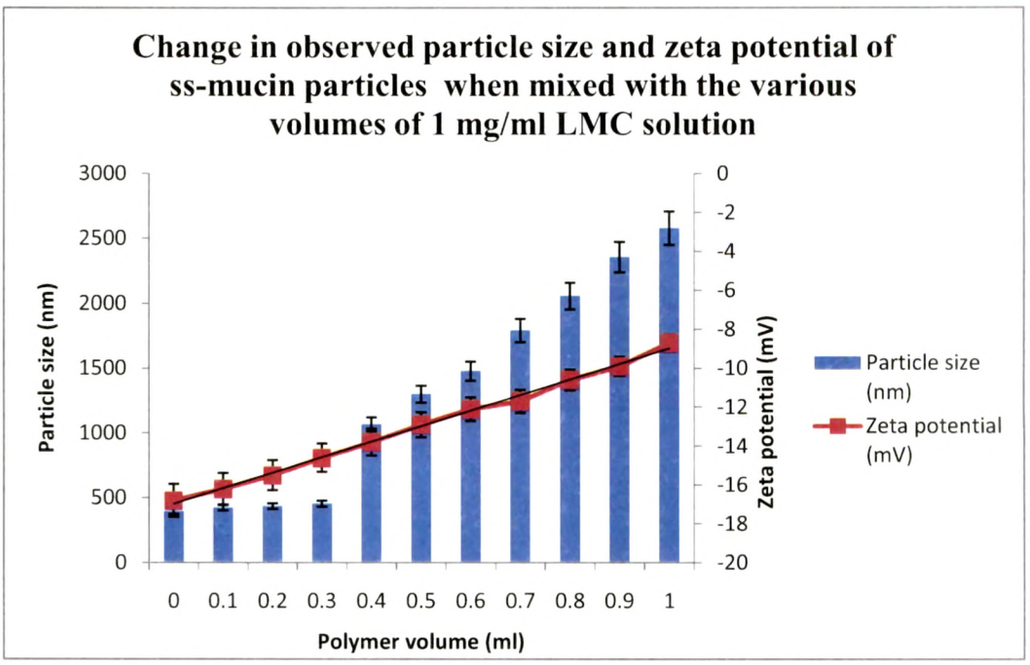


Figure 4.6: ^{1}H MNR spectra of: (a) Low molecular weight trimethyl chitosan (b) Medium molecular weight trimethyl chitosan

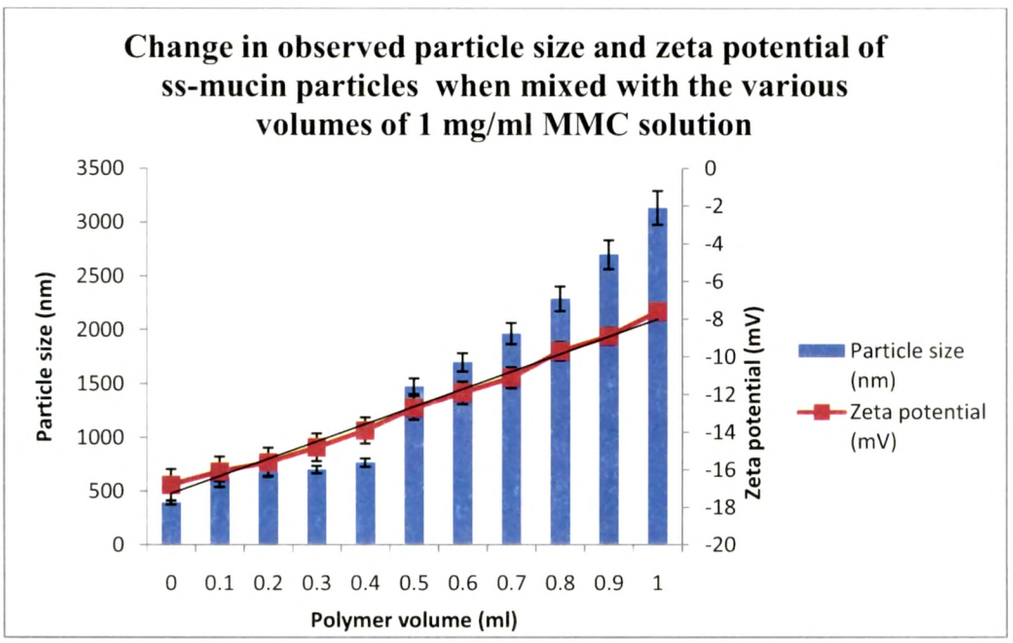
4.6.2.3. Mucoadhesion by mucin particle method

Mucoadhesive properties of modified chitosan (trimethyl chitosan) were evaluated using the mucin particle method based on the change in surface properties of mucin particle, particle size and zeta potential, by the mucoadhesion of the polymer with the mucin particle. Figure 4.7 shows particle size and zeta potential of ss-mucin particles versus added volume of 1 mg/ml polymer solution (LMC, MMC, LMTMC and MMTMC solution. The results were found to be similar as thiolated chitosan (section 4.3.2.3). By referring to the results of ss-mucin–polymer interaction studies (Table 4.4), it can be deduced that exhibited mucoadhesive characteristic and the rank order of mucoadhesive bond strength of polymers was MMTMC> LMTMC> MMC> LMC. Mucin particle method suggests the trimethyl chitosan having higher mucoadhesive strength than the chitosan. The findings may be explained by the formation of ionic bond between positive charges of TMC and negatively charged sialic groups on the mucus protein structure. Furthermore, it was observed that the interaction between ssmucin particles and polymer was molecular weight-dependent. The interaction decreased with decreased molecular weight.

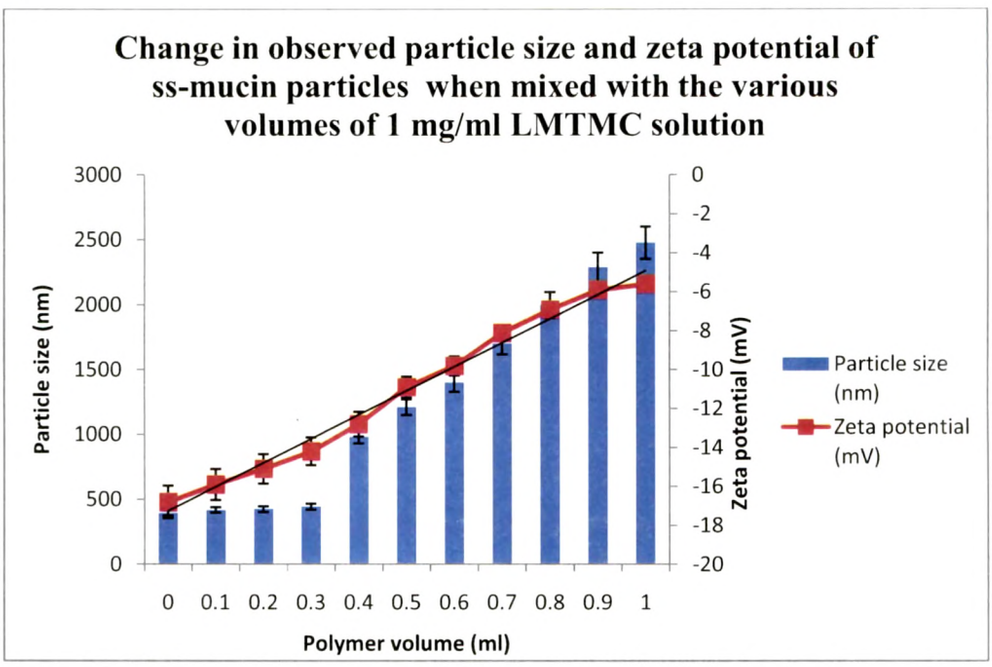
(a)


Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml LMC solution
Particle size (nm)
Zeta potential (mV)
Polymer volume (ml)
Particle size (nm)
Zeta potential (mV)

(b)


Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml MMC solution
Particle size (nm)
Zeta potential (mV)
Polymer volume (ml)
Particle size (nm)
Zeta potential (mV)

(c)



(d)

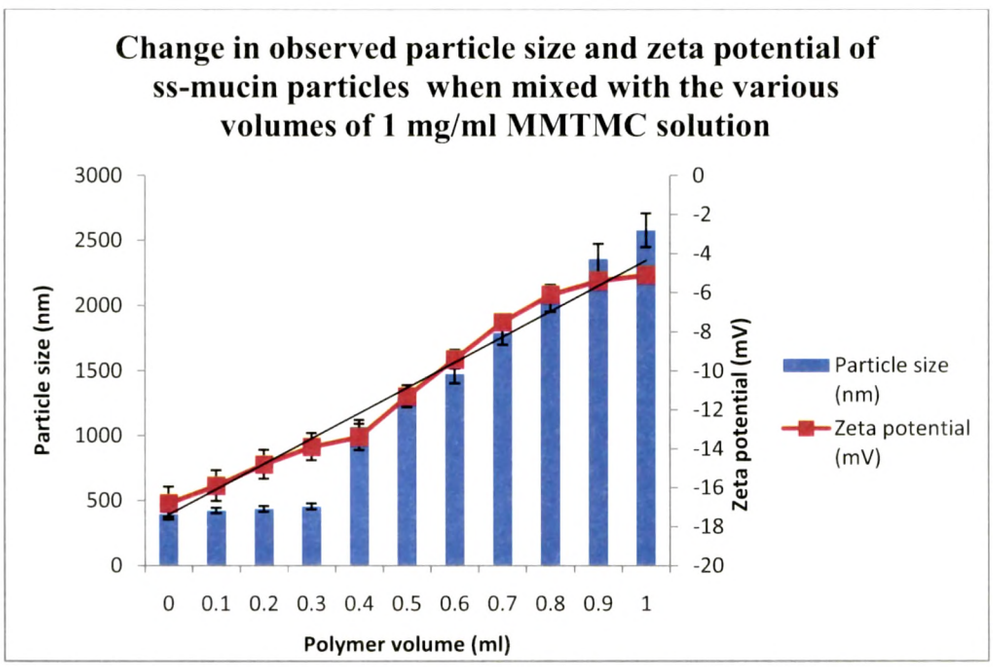


Figure 4.7 : Change in observed particle size and zeta potential of ss-mucin particles when mixed with the various volumes of 1 mg/ml polymer solution (a) LMC (b)MMC(c)LMTMC (d) MMTMC

Table 4.4: Characteristic of the interaction between ss-mucin particles with the modified and unmodified chitosan

Polymer	Slope (mV/ml)	*Vc (ml)
LMC	8.2±0.5	2.08±0.5
MMC	10.25±0.6	1.75±0.1
LMTMC	11.25±1.1	1.5±0.2
MMTMC	12.68±0.4	1.3±0.2

*The extrapolated volume of 1mg/ml polymer solution used to neutralized negative charge of 1 % w/v SS-mucin to zero

4.7. Conclusions

Thiolated chitosan was successfully prepared using carbodiimide method and quantitative and qualitative evaluate for thiol group using Ellman's method and IR respectively. Trimethyl chitosan was successfully prepared using methyl iodine in presence of strong alkali (NaOH). Degree of quarterization was calculated by ^{1}H NMR and qualitatively confirm by IR. Both the modified polymer was sucessfuly evaluated for mucoadhesive strength using mucin particle method. Mucin particle method suggests the thiolated chitosan and trimethyl chitosan having higher mucoadhesive strength than the chitosan. The findings may be explained by the formation of covalent bonds between the thiol groups of thiolated chitosan and cysteine residue and the formation of ionic bond between positive charges of TMC and negatively charged sialic groups on the mucus protein structure.

4.8. References

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