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RESEARCH PAPER

# Evaluation of the Mucoadhesive Properties of *N*-Trimethyl Chitosan Chloride

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## ABSTRACT

Previous studies have established that *N*-trimethyl chitosan chloride (TMC) is a potent absorption enhancer for peptides and large hydrophilic compounds across mucosal surfaces, especially in neutral and basic environments where chitosan is ineffective as an absorption enhancer. The degree of quaternization of TMC plays an important role on its absorption-enhancing properties. Several TMC polymers with different degrees of quaternization were synthesized and the molecular mass of the polymers was determined by SEC/MALLS. The mucoadhesive properties of the TMC polymers were measured with a modified tensiometer based on the Willhelmy plate method. The effect of the TMC polymers on the surface tension of a mixture of polymer and mucus was measured with a Du Noüy tensiometer. The degrees of quaternization of the synthesized TMC polymers were between 22.1% and 48.8% and the molecular mass was above 100,000 g/mole for all the polymers. A decrease in mucoadhesivity with an increase in the degree of quaternization of the TMC polymers was found. Surface-tension analysis of a mixture of polymer and mucus showed the effect of excessive polymer hydration on mucoadhesion. The results show that the degree of quaternization of TMC had a pronounced effect on the mucoadhesive properties of this polymer. Although the mucoadhesive profiles for the TMC polymers were lower than the original chitosan, they still retained sufficient mucoadhesive properties for successful inclusion into mucoadhesive dosage forms.

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*Key Words:* Mucoadhesion; Chitosan; *N*-Trimethyl chitosan chloride (TMC); Size-exclusion chromatography (SEC); Multiangle laser light scattering (MALLS); Surface-tension analysis; Tensile separation testing.

## INTRODUCTION

Chitosan is a mucoadhesive polysaccharide that is obtained through the deacetylation of the natural polymer chitin.[1,2] It has been shown that chitosan enhances the absorption of peptide and protein drugs across nasal[3] and intestinal epithelia[4,5] in acidic environments. Chitosan has an apparent $pK_a$ value of 5.60–6.00 (as measured by potentiometric titration) and is only soluble in acidic solutions with pH values lower than 6.[6] This interferes with the biomedical application of chitosan, especially at the physiological pH value ($pH = 7.40$), where chitosan is insoluble and ineffective as an absorption enhancer.[7]

*N*-Trimethyl chitosan chloride (TMC) is a partially quaternized derivative of chitosan with improved solubility and easy preparation.[6,8] This polymer has excellent absorption enhancing effects, especially in neutral environments where chitosan is ineffective as an absorption enhancer.[9,10] It has been shown that the degree of quaternization has an effect on the absorption-enhancing properties of TMC[10] and it is expected that the same could be true for the mucoadhesive properties of this polymer. During the synthesis of TMC, the amount of fixed positive charges on the polymer chain is increased, probably causing expansion of the polymer in solution. As the mucoadhesive properties of a polymer is a function of the chain flexibility, it is expected that the degree of quaternization will have an effect on its mucoadhesive properties as an increase in the degree of quaternization decreases this chain flexibility. Furthermore, during synthesis, degradation of the polymer chain occurs due to factors such as the strong alkaline environment and elevated temperatures.[2,6,11] According to Gurny and coworkers,[12] the molecular weight of a polymer has an effect on its mucoadhesive properties up to a value of 100,000 g/mole, thereafter any further increases had no noticeable effect.

In the development of mucoadhesive drug-delivery systems, the contact time at the site of absorption of the intended drug molecule is important for selective and effective drug absorption. The mucoadhesive effect of an excipient in a controlled-release mucoadhesive dosage form should decrease the rate of clearance and movement of the drug from the mucosal epithelia, such as in the nasal cavity and gastrointestinal tract, to allow for longer contact time with the absorptive epithelium. This extension of contact with the absorbing epithelia may be beneficial, especially for hydrophilic drugs or for drugs with a high molecular weight such as peptides and proteins. This study evaluates the effect of the degree of quaternization of TMC on the mucoadhesive properties of this polymer for future dosage form development.

## EXPERIMENTAL SECTION

### Materials

Seacure 244 (93% deacetylated chitosan) was a gift from Pronova Biopolymer (Drammen, Norway). The chitosan was milled in a Retsch-mill (Retsch K G, Haan, Germany) to obtain a powder to improve the solubility rate of the polymer. Iodomethane, ammonium acetate, acetic acid, sodium hydroxide pellets, sodium iodide, sodium chloride, absolute ethanol, and diethylether (Merck, South Africa) and *N*-methyl-2-pyrrolidone (Riedel-de Haën, South Africa) were also used during the synthesis of TMC. Carbopol 934P (BF Goodrich), pectin USP (Sigma-Aldrich, Germany), chitosan hydrochloride, chitosan glutamate (Pronova Biopolymer, Norway), and mucus (Sigma-Aldrich, Germany) (partially purified porcine gastric mucin type III) were used in the mucoadhesion experiments. All chemicals and materials used in this study were of analytical grade.

### Synthesis of TMC Polymers

TMC polymers were synthesized by reductive methylation of chitosan that was accomplished by a chemical reaction between chitosan and iodomethane in the presence of sodium hydroxide based on the method previously described.[6,11] The reaction step was repeated several times with the product obtained from each step to increase the degree of quaternization of TMC. The reaction steps used to synthesize the different TMC polymers are described below and

***Table 1.*** Number of reaction steps for the synthesis of TMC polymers.

| Polymer | Number and sequence of reaction steps |
|---|---|
| TMC 1 | Step 1 |
| TMC 2 | Step 1 and 2 |
| TMC 3 | Steps 1, 2, and 3 |
| TMC 4 | Steps 1, 2, 3, and 4 |

the compilation of reaction steps for each of the polymers (TMC 1–4) is shown in Table 1.

Reaction Step 1. A mixture of 2 g chitosan, 4.8 g of sodium iodide, 11 mL of a 15% w/v aqueous sodium hydroxide solution and 11.5 mL of iodomethane in 80 mL of *N*-methylpyrrolidone was stirred on a waterbath at a temperature of 60°C for 45 min. The iodomethane was kept in the reaction mixture with a Liebig's condenser. The product was precipitated from solution with ethanol and isolated by centrifugation.

Reaction Step 2. The product obtained from the previous reaction step was dissolved in 80 mL *N*-methylpyrrolidone and 4.8 g of sodium iodide after which 11 mL of a 15% w/v aqueous NaOH solution and 11 mL of iodomethane were added. This mixture was stirred on a waterbath at a temperature of 60°C for 30 min. The product was precipitated from solution with ethanol and isolated by centrifugation.

Reaction Step 3. Before precipitation of the product in Step 2, an additional 2 mL iodomethane and 0.6 g NaOH pellets were added to the mixture, while stirring was continued for another 45 min at a temperature of 60°C. The product was precipitated from solution with ethanol and isolated by centrifugation.

Reaction Step 4. The product obtained from the previous reaction step was dissolved in 80 mL *N*-methylpyrrolidone and 4.8 g of sodium iodide, after which 11 mL of a 15% w/v aqueous NaOH solution and 11.5 mL of iodomethane were added. The mixture was stirred on a waterbath at a temperature of 60°C for 30 min. The product was precipitated from solution with ethanol and isolated by centrifugation.

Ion Exchange. After washing with ethanol and diethylether, the final products were dissolved in 40 mL of a 5% w/v aqueous NaCl solution to exchange the iodide-ion with a chloride-ion. The polymer was precipitated from solution with ethanol and was isolated by centrifugation.

The product was finally dissolved in 40 mL water to remove the remaining NaCl from the material and precipitated from solution with ethanol. This product was dried for 12 hr in a vacuum oven at 40°C.

## Characterization of TMC Polymers

### Degree of Quaternization

The degree of quaternization of the products were determined from $^1$H-NMR spectra (600 MHz) obtained with a BRUKER DNX-600 spectrometer (Karlsruhe, 76189 Germany) in $D_2O$ at 80°C with suppression of the water peak. The degree of quaternization was calculated from $^1$H-NMR spectra as described in previous studies[6,11] with the following equation:

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

where $\%\, DQ$ is the degree of quaternization as a percentage, $[(CH_3)_3]$ is the integral of the trimethyl amino group peak at 3.1 ppm, and $[H]$ is the integral of the $^1$H peaks between 4.7 and 5.7 ppm.

### Molecular Weight

The molecular weight of the TMC polymers was measured by using a size-exclusion chromatograph (SEC) (Hewlett Packard, 1100, Geneva, 1217, Switzerland) connected to a multiangle laser light scattering detector (MALLS) that consisted of a laser photometer (Dawn DSP, Wyatt Technology Corporation, Santa Barbara, 93117, CA, USA) coupled to a refracting index detector (ERC 7515A, Tokyo, 101-032, Japan). The TMC polymers were dried at 40°C in a vacuum oven for 24 hr and dissolved in distilled water at concentrations of 5 mg/mL, from which 0.8 mL samples were filtered through 0.2 μm membrane filters and collected in chromatographic sample vials. The mobile phase consisted of 0.2 M ammonium acetate and the pH was adjusted to 4.50 with acetic acid. The experimental setup consisted of a HP 1100 vacuum degasser, isocratic pump, and auto sampler connected to a TSK-guard PWH (Toso Haas, Gruenstrasse, D-79232, Germany) in-line column. The size-exclusion columns included a TSK G6000 PW (Toso Haas, Gruenstrasse, D-79232, Germany, inside diameter = 7.5 mm, length = 30 cm, particle size > 17 μm, pore size > 1000 Å) column connected in series with a TSK G5000 PW (Toso Haas,

Gruenstrasse, D-79232, Germany, inside diameter = 7.5 mm, length = 30 cm, particle size = 17 μm, pore size = 1000 Å) column. Samples of 100 μL were injected at a flow rate of 0.8 mL/min and were analyzed with the laser photometer (He/Ne laser, $\lambda = 633$ nm) and the refracting index detector. The data from the detector was interpreted with a computer using Astra® for Windows (Wyatt Technology Corporation, Santa Barbara, 93117, CA, USA).

### Mucoadhesive Evaluation of the TMC Polymers

#### Tensile Separation Testing

The mucoadhesive properties of the TMC polymers were measured with the use of a modified tensiometer based on the Willhelmy plate-separation method.[13] Round aluminium plates were prepared by adding 0.5 g of the respective TMC solutions (1% w/v in distilled water) on each plate to produce ±0.05 g of polymer film after drying for 12 hr at room temperature. Mucus samples were prepared by adding 1.5 g mucin to 5 mL of distilled water. This solution was stirred for 2 min until the consistency of the mucus was uniform. The beaker with mucus was placed in a waterbath at 25°C to decrease the effect of dehydration on the mucus layer and left to reach equilibrium. The aluminium plate was suspended from a microbalance (Hugo Sachs Elektronik, Gruenstrasse, D-79232, Germany, Force Transducer F30, Type 372) using a fine metallic thread that was free from elasticity. The plate was lowered until contact with the mucus was achieved and the tension between the plate and the microbalance declined, ensuring a 2-g downward pressure on the mucus. The plate was left in this position for different time intervals for hydration of the polymer to occur after which it was again lifted at a rate of 0.25 mm/s. The separation was registered by software (Chart for Windows v3.4, Powerlab System, OX44 7RW, UK) and the maximum detachment force was noted. The detection system was calibrated with standard calibration weights (Hugo Sachs Elektronik, 1 g). The experiments were done in triplicate at hydration time intervals of 20, 40, 60, 80, 100, and 120 sec. Similar experiments were performed with Carbopol 934 P, chitosan hydrochloride, chitosan glutamate, and pectin.

A straight line for each of the mucoadhesion profiles was fitted through the datapoints at each hydration interval with the use of straight-line regression and the $y$-intercept and $r^2$ values for each of these lines was determined. The intrinsic mucoadhesivity (IM) for each of the polymers were determined by calculating the projected maximum detachment force (MDF) from these straight lines at a time of 120 sec. These IM values were used to rank the polymers according to their mucoadhesive strength. Pectin was chosen as a 100% reference to which the other polymers were related.[13,14] The baseline (clean-plate reference standard) was subtracted from the IM value of each of the polymers to decrease the effect of dehydration on the system and the percentage mucoadhesivity of each polymer relating to pectin was calculated.

#### Surface Tension Analysis

The effect of the different TMC polymers on the surface tension of a physical mixture of polymer and mucus was measured with a Du Noüy tensiometer (Cambridge Instrument Co., MA, 02492, USA) to give an indication of the effect of hydration on the polymer film. A 2.5% w/v polymer solution was prepared in distilled water and stirred for 4 hr at a temperature of 20°C. The surface tensions of the polymer solutions were measured as a control value at 20°C ($t = 0$ min) as the behavior of surface tension varies significantly with the concentration of the solution that may increase with dehydration of the mixture. The surface tension of a mixture of polymer and mucus was measured by adding 0.5 g of mucus to 5 mL of the respective polymer solutions to provide a 10% w/v mucus and 2.5% w/v polymer mixture. This mixture was stirred for short intervals during which hydration of the matrix occurred. The surface tension was measured after time intervals of 10, 20, and 30 min, respectively. The mixtures were stirred every 2 to 3 min to ensure that consistency of the mixture was uniform. Surface tension measurements were expressed in dyn/cm. Each experiment was done in triplicate.

## RESULTS

### Synthesis and Characterization of TMC Polymers

The results of the $^1$H-NMR and molecular weight characterization of the TMC polymers are

shown in Table 2. The $^1$H-NMR spectra of TMC 1 is shown in Fig. 1 with an indication of the quaternary and $^1$H peaks used to calculate the degree of quaternization of the polymers. The degrees of quaternization of the synthesized TMC polymers were found to be between 22.1% and 48.8%. The effect of repeated reductive methylation can be seen by the significant increase in the degree of quaternization of the polymers. A one-step reaction gave a polymer with a degree of quaternization of 22.1%. A maximum degree of quaternization of 48.8% was reached for the polymer produced by the four-step synthesis. Two- and three-step reactions gave polymers with intermediate degrees of quaternization. This range of quaternized chitosan polymers was considered to be sufficient for effective mucoadhesive testing. The SEC/MALLS experiments showed the combined effect of degradation of the polymer chain and addition of methyl groups to the amino groups on the C-2 position of the chitosan chain during synthesis. The molecular weight of all the TMC polymers stayed above 100,000 g/mole and, therefore, it was assumed that the small differences in molecular weight of the respective polymers will be negligible on the mucoadhesive properties of these polymers.[12]

***Table 2.*** The degree of quaternization and molecular weight of the TMC polymers.

| Polymers | Degree of quaternization (%) | [a]Molecular weight (g/mole × $10^5$) |
|---|---|---|
| Chitosan | — | $1.48 \pm 0.074$ |
| TMC 1 | 22.1 | $2.47 \pm 0.099$ |
| TMC 2 | 38.1 | $1.70 \pm 0.068$ |
| TMC 3 | 42.8 | $2.11 \pm 0.148$ |
| TMC 4 | 48.8 | $1.94 \pm 0.116$ |

[a]$n = 3$.

## Mucoadhesive Properties of TMC

Figure 2a shows the regression line fitted through the mucoadhesive data points of TMC 1, including the standard deviation. The mucoadhesive bond increases with time from $t = 0$ to $t = 120$ sec as interpenetration of the polymer chains into the mucus layer increases with hydration of the polymer film, resulting in an increased mucoadhesive bond with the mucus. Figure 2b shows the straight lines fitted

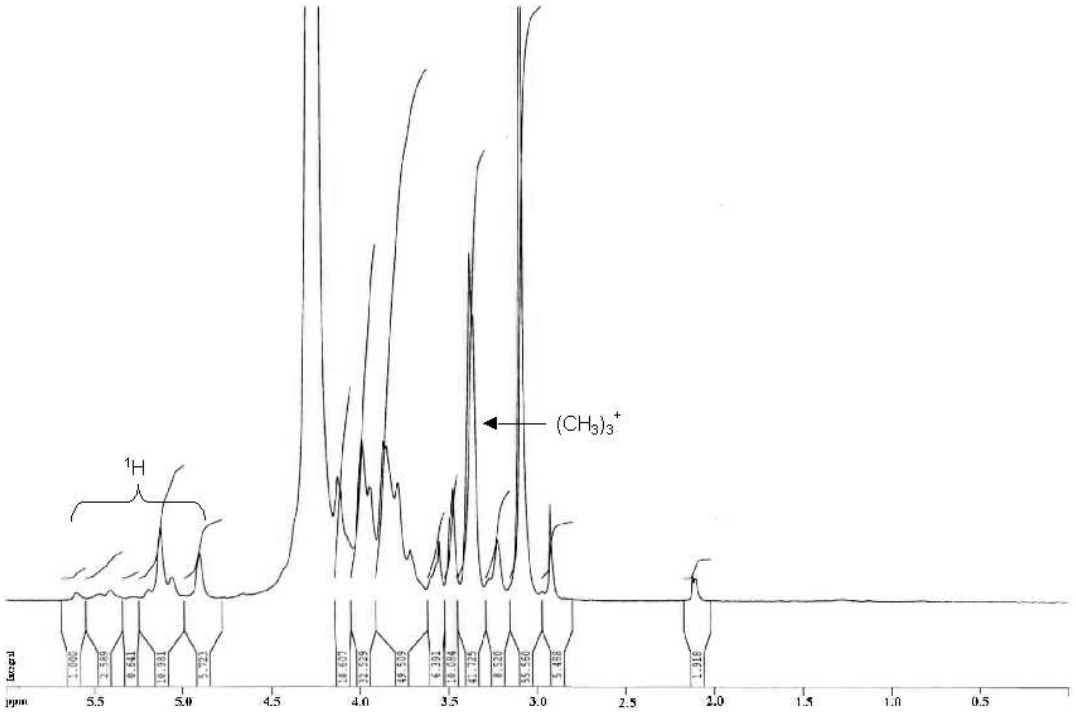


***Figure 1.*** $^1$H-NMR spectra of TMC 1.

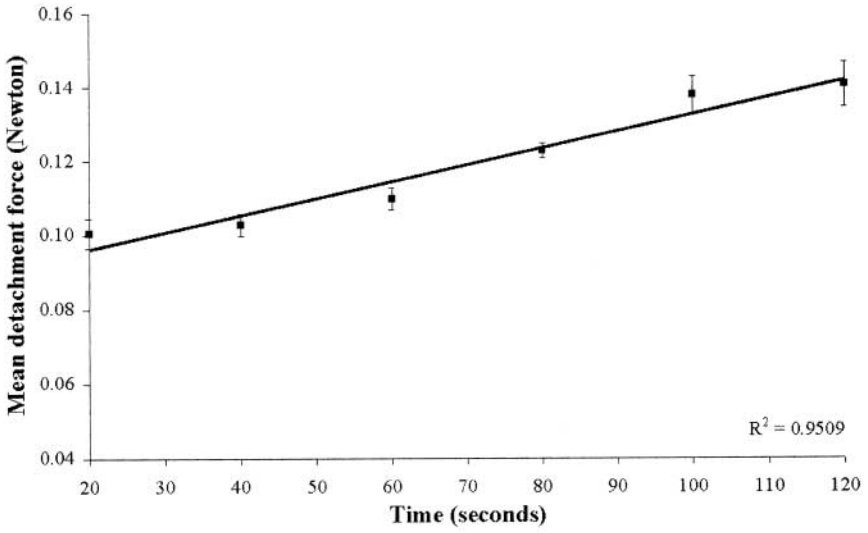


***Figure 2a.*** Regression line fitted through the mucoadhesive profile of TMC 1.

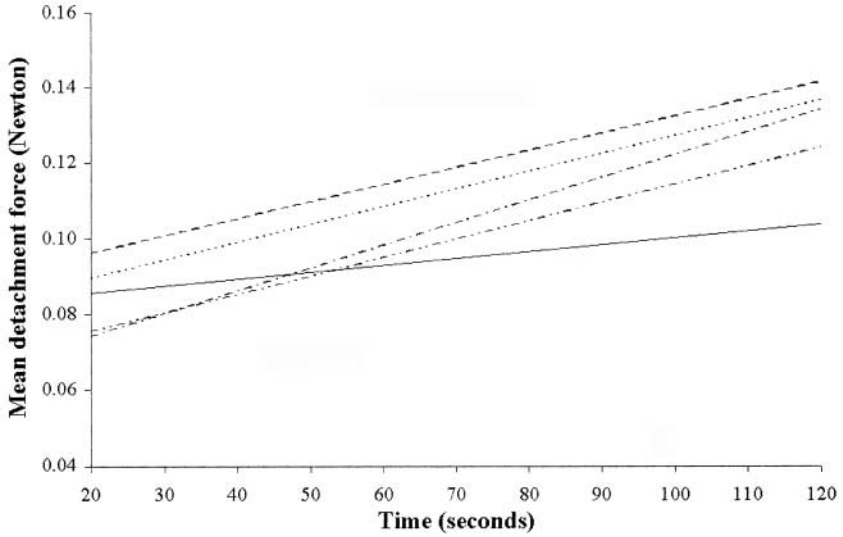


***Figure 2b.*** Straight lines fitted through the mucoadhesive profiles of the TMC polymers. Key: TMC 1 (- - - - -), TMC 2 (. . . . . .), TMC 3 (-·-·-·-·), and TMC 4 (-···-···-···) with the clean plate (———) as control.

through the mucoadhesive profiles (MDF with time) of the TMC polymers. The mucoadhesive profiles of TMC 3 and TMC 4 begin at lower mucoadhesive strengths as the clean-plate reference. This may indicate the formation of a layer (polymer, mucus, and water) not adhering to either the mucus or polymer layers that inhibits the penetration of the polymer chains into the mucus. The straight lines fitted through the mucoadhesive data of the TMC polymers clearly show that the mucoadhesion decreases as the degree of quaternization increases.

The intrinsic mucoadhesivity of the TMC polymers, Carbopol 934P, chitosan hydrochloride, chitosan glutamate, and pectin are presented in Table 3. Carbopol 934P was found to be the best mucoadhesive polymer. The calculated value of 193% was found to be close to the known value of 185% (related to pectin).[13,14] Chitosan hydrochloride (181%) and chitosan glutamate (167%) possess stronger mucoadhesive properties than the TMC polymers (105% to 169%). This decrease in mucoadhesivity could be explained by changes to the original

polymer chain structure during synthesis of TMC. However, even the TMC polymer with the highest degree of quaternization (48.8%) was still sufficiently mucoadhesive to be included into a mucoadhesive dosage form.

Pronounced differences between the mucoadhesive properties of the TMC polymers and the mixing effects on the surface tension of the polymer–mucus mixture were observed, as evident in Fig. 3. The surface tension values for clean water and for clean mucus were 72.8 dyn/cm and 64.0 dyn/cm, respectively. The mucoadhesion profiles as depicted by the surface-tension analysis show the rate of hydration for each of the TMC polymers. The surface tension of the clean polymer is given by the values at the $x$-axis as determined at $t = 0$. Directly after addition of the mucus, a decrease in the surface tension was observed. As hydration of the mixture, interpenetration, and subsequent complexation between the polymer and mucus increased, the surface tension increased. A maximum occurred at different times for each of the polymers, possibly indicating differences in hydration rate or complexation rate between the mucus and polymer. In general, this maximum surface tension was upheld for a period of time, after which excessive hydration of the complex caused the mucoadhesion to decrease until no effective mucoadhesion could be seen. These results highlight the suitability of TMC polymers as mucoadhesive excipients for inclusion into mucoadhesion dosage forms.

***Table 3.*** Intrinsic mucoadhesivity of the different polymers.

| Polymer | Intrinsic mucoadhesivity ($\times 10^2$) | Related to pectin (%) |
|---|---|---|
| Carbopol 934P | $15.1 \pm 0.05$ | 192.93 |
| Chitosan hydrochloride | $14.7 \pm 0.02$ | 181.27 |
| TMC 1 | $14.4 \pm 0.03$ | 169.96 |
| Chitosan glutamate | $14.3 \pm 0.02$ | 167.14 |
| TMC 2 | $14.0 \pm 0.01$ | 156.18 |
| TMC 3 | $13.4 \pm 0.01$ | 134.98 |
| TMC 4 | $12.6 \pm 0.03$ | 105.30 |
| Pectin | $12.4 \pm 0.02$ | 100.00 |

## DISCUSSION

Mucoadhesion, among other factors, such as opening of the tight junctions, is a key element of TMC polymers for being effective as absorption enhancers at mucosal surfaces. The degree of quaternization of TMC had a pronounced effect on the mucoadhesive properties of this polymer and this may be due to the presence of fixed positive charges and their interaction with the negative sialic groups

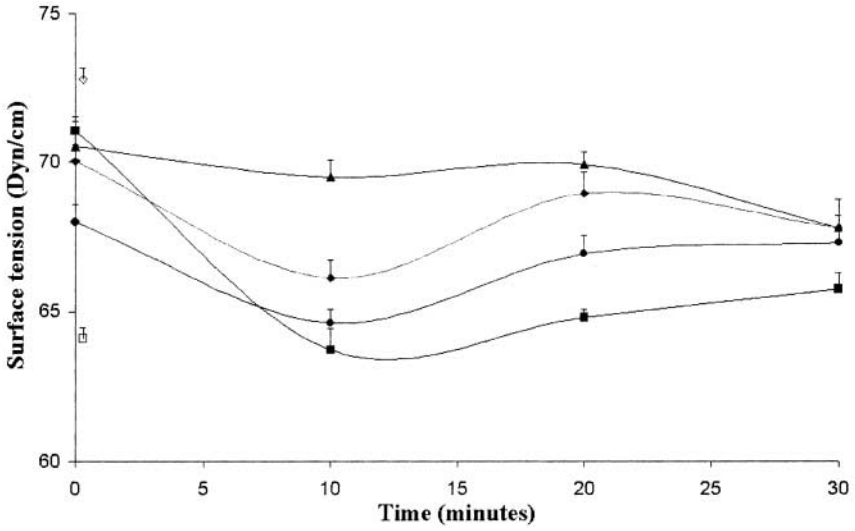


***Figure 3.*** Surface-tension analysis of a mixture of mucus and TMC polymers. Key: TMC 1 (▲), TMC 2 (■), TMC 3 (●), TMC 4 (◆), clean water (◇), and clean mucus (□).

on the mucus protein structure. The mucoadhesivity decreased when the degree of quaternization of TMC increased.

The decrease in mucoadhesion with an increase in the degree of quaternization may be explained by changes in the conformation of the respective TMC polymers due to interactions between the fixed positive charges on the C-2 position of each polymer. These interactions may force the polymer to change its conformation with a decrease in polymer-chain flexibility. This decrease in flexibility influences both the rate and amount of charge exchange between the negatively charged sialic groups of the mucus and the fixed positive charge of the TMC polymers and the interpenetration into the mucus layer with a subsequent lower mucoadhesivity. The rate of penetration of TMC 3 and TMC 4 may also be slower than the rate of penetration of TMC 1 and TMC 2 into the mucus layer because of sterical hindrance or intermolecular associations, and this may be related to the higher degree of quaternization of TMC 3 and TMC 4 compared to TMC 1 and TMC 2. This decrease in interpenetration into the mucus is proposed as an explanation for the behavior of the higher quaternized polymers (TMC 3 and TMC 4), both of which showed initial mucoadhesion below that of the clean-plate reference (tensile separation testing, Fig. 2b), indicating the formation of an interfacial layer between the mucus and the mucoadhesive-film surface. Further experiments investigating the molecular interactions between charges on the polymer chain and the resulting conformational changes of the polymer are, however, needed to fully explain the mucoadhesive behavior of these TMC polymers.

The results of the surface-tension experiments show that no direct relationship between the degree of quaternization of the TMC polymers and the effect on the surface tension of a physical mixture of polymer and mucus could be found. However, clear differences in the rate of mucoadhesive bonding as well as the effect of excessive hydration on the matrix were observed.

Previous studies have established that a high degree of quaternization of TMC (approximately 60%) was the most effective in opening the intercellular spaces of epithelial cells to allow for the paracellular transport of hydrophilic macromolecules.[10] These studies attribute the absorption-enhancing properties of TMC to a combination of its transient opening of tight junctions and its mucoadhesive properties. In this study, the mucoadhesive properties of TMC polymers clearly show that mucoadhesion decreases with an increase in quaternization, but TMC polymers with degrees of quaternization between 22% and 48% still retained sufficient mucoadhesive properties for inclusion into mucoadhesive drug-delivery systems. These factors and their respective contribution to absorption enhancement should be considered carefully in the selection of a specific TMC polymer for development of a mucoadhesive dosage form.

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