Why is Chitosan Mucoadhesive?

Ioannis A. Sogias, Adrian C. Williams, and Vitaliy V. Khutoryanskiy*

School of Pharmacy, University of Reading, Whiteknights, Post Office Box 224, Reading RG6 6AD, United Kingdom

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Chitosan is a biocompatible and biodegradable amino polysaccharide, which is soluble in aqueous solutions at pH < 6.5. It has been widely used for developing drug delivery systems because of its excellent mucoadhesive properties. Although many studies report on chitosan being mucoadhesive, the nature of interactions between chitosan and mucin remains poorly defined. Here, we have examined the role of primary amino groups and the role of electrostatic attraction, hydrogen bonding, and hydrophobic effects on aggregation of gastric mucin in the presence of chitosan. Reducing the number of amino groups through their half acetylation results in expansion of chitosan's pH-solubility window up to pH 7.4 but also reduces its capacity to aggregate mucin. We demonstrated that electrostatic attraction forces between chitosan and gastric mucin can be suppressed in the presence of 0.2 mol/L sodium chloride; however, this does not prevent the aggregation of mucin particles in the presence of this biopolymer. The presence of 8 mol/L urea or 10% v/v ethanol in solutions also affects mucin aggregation in the presence of chitosan, demonstrating the role of hydrogen bonding and hydrophobic effects, respectively, in mucoadhesion.

Introduction

Mucosal membranes are the moist surfaces lining the walls of various body cavities such as the gastrointestinal, respiratory and reproductive tracts. They consist of connective tissue overlaid with an epithelial layer, the surface of which is covered by mucus. The epithelium may be either single-layered, as in the stomach, small and large intestine, or multilayered/stratified as found in the esophagus, vagina, and cornea. The single-layered membranes contain goblet cells that secrete mucus directly onto the epithelial surfaces, whereas the multilayered membranes contain or are adjacent to tissues containing specialized glands (e.g., salivary glands) that secrete mucus onto the epithelial surface. Mucus is present as either a gel layer adhered to the mucosal surface or as a luminal soluble or suspended form, where it protects epithelial cells from physical and chemical damage, provides lubrication, acts as a wetting agent, and modulates water content in the underlying tissue.[1] The major components of all mucus gels are mucin glycoproteins (usually termed mucins), lipids, inorganic salts, and water, the latter accounting for more than 95% of mucus weight, providing a highly hydrated system.[1,2] The molecular weight of mucins varies from 500 kDa to 20 MDa, but they tend to form larger aggregates through hydrophobic interactions between nonpolar groups, hydrogen bonding between sugar units, and disulphide linkages between cysteine residues. Most mucins are negatively charged due to the presence of sialic acids and ester sulfates, which are fully ionized at pH > 2.6.[3,4]

Numerous polymers adhere to mucosal tissues, that is, they are mucoadhesive. These include poly(acrylic acid) (PAA), the sodium salt of carboxymethyl cellulose (NaCMC), and chitosan.[5,6] Several theories have been reported to explain the mucoadhesive properties of polymers,[7–11] some of which stress the importance of specific interactions between macromolecules and mucins. Thus, for PAA, hydrogen bonding to mucin is likely to be the main reason for the strong adhesion to the mucosal membranes.[12,13]

Chitosan is a natural cationic polysaccharide derived from chitin by partially deacetylating its acetamido groups with strong alkaline solutions.[14] Over the last two decades, chitosan has been used for various biomedical and drug delivery applications due to its low toxicity and good biocompatibility and antimicrobial and mucoadhesive properties.[15–17]

Although chitosan mucoadhesion has been widely studied, the basis for these properties remains unclear. Electrostatic interactions of cationic chitosan with the negatively charged mucin have been reported as the main driving force for its strong mucosal adhesion.[18] However, Snyman et al.[19] examined mucoadhesive interactions of trimethylated chitosan (TMC) with different levels of quaternization and demonstrated that the presence of quaternary ammonium groups is detrimental to mucoadhesion. The authors related this adverse effect to conformation changes in TMC. Alternatively, we believe that this effect could also be due to the nature of the quaternized amino groups, which could still interact electrostatically with mucins but would no longer form hydrogen bonds.

While there is considerable interest in elucidating interactions between polymers and mucins,[11] few such studies have been described in the literature.[20–24] Here, we have probed the mechanisms of chitosan−mucin interactions in aqueous solutions by determining the contribution of different factors, namely, the role of primary amino groups and the effects of electrostatic attraction, hydrogen bonding and hydrophobic effects to chitosan mucoadhesion.

Experimental Section

Materials and Methods. *Materials.* Lyophilized porcine mucin (type III, bound sialic acid 0.5−1.5%, stored at −4 °C) and medium viscosity chitosan were purchased from Sigma-Aldrich (U.K.). Acetic acid and other chemicals and solvents, such as acetic anhydride, ethanol, acetone, NaOH, NaCl, and urea, were purchased from Fisher Chemicals (U.K.)

* To whom correspondence should be addressed. E-mail: v.khutoryanskiy@reading.ac.uk.

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and were used as received. Deinonized water was used for all solutions. Dialysis membranes (molecular weight cutoff 12−14 kDa) were purchased from Medicell International Ltd. (U.K.).

Synthesis of Half-Acetylated Chitosan (HACHI). HACHI was produced by acetylation of chitosan with acetic anhydride, using a method adapted from Qin et al.[25] Briefly, 1.5 g of chitosan was dissolved in 50 mL of dilute acetic acid (4% v/v), and 0.56 g of acetic anhydride was dissolved in 50 mL of ethanol. Solutions were then mixed and placed in a water bath at 40 °C, shaking for 12 h. The final product was obtained by precipitation with NaOH at pH 12 and was washed twice with ethanol. To remove impurities and solvents, the polymer was redissolved in water and then dialyzed against 5 L of deionized water (five changes over 72 h). The final product was recovered by freeze-drying in a Heto PowerDry LL3000 Freeze-Dryer (Thermo Scientific).

Characterization. The molecular weight of chitosan was determined by gel permeation chromatography (GPC); the solvent was 500 mM acetate buffer pH 4.6 at 1 mL/min at 25 °C. The molecular weight of chitosan was 163 kDa and the polydispersity index (M_w/M_n) was 1.13. The degree of acetylation (DA) of chitosan and of HACHI was determined by ^{1}H NMR spectroscopy, according to the integration pattern of the respective protons. ^{1}H NMR spectra were obtained on a Bruker DPX 250 MHz spectrometer by dissolving chitosan or HACHI in D_2O, acidified with trichloroacetic acid. The degree of acetylation DA was calculated from

$$\mathrm{DA} = (I_{\mathrm{CH_3}}/3)/(I_{\mathrm{H2-H6}}/6) \times 100\% \quad (1)$$

where $I_{\mathrm{CH_3}}$ is the integral intensity of *N*-acetyl protons and $I_{\mathrm{H2-H6}}$ is the sum of the integral intensities of H-2, -3, -4, -5, and H-6 of the acetylated rings. ^{1}H NMR spectroscopy was also used to confirm the purity of HACHI.

Preparation of Samples. Mucin samples were prepared by adding mucin to deionized water to give stable colloidal dispersions of 1 mg/mL. The pH was adjusted by adding NaOH or HCl. The samples were sonicated for 15 min then centrifuged for 5 min at 1000 rpm. The supernatant was recovered and used in experiments. All mucin dispersions were freshly prepared before each experiment. Polymer solutions were prepared by adding chitosan or HACHI to deionized water, and pH was adjusted with HCl or NaOH.

Size and ζ-Potential Measurements. Particle *z*-average diameter of mucin and mucin−polymer mixtures were determined by dynamic light scattering using a red laser (633nm) and detection of scattered light at 173° (Malvern Zetasizer Nano-S, Malvern Instruments). The ζ-potential was determined using a Malvern Zetasiser 3000 HS (Malvern Instruments). All measurements, performed at 25 °C, were repeated in triplicate and the values are reported as mean ± standard deviation.

Turbidimetric Titrations. Mixtures of mucin dispersions with polymer solutions were prepared at various ratios, by adding polymer solution to mucin dispersions. The turbidity of these mixtures was measured at 400 nm using a V-530PC spectrophotometer (Jasco, U.K.). All measurements were performed at 25 °C and each sample was analyzed three times. All experiments were repeated in triplicate and the turbidity values are reported as mean ± standard deviation of the experimental triplicates.

Transmission Electron Microscopy (TEM). TEM images of mucin and mucin with chitosan mixtures were acquired using a Philips CM20 analytical TEM at 80 kV. For sample preparation, the copper grids were brought into contact with aqueous dispersions of the samples for 30 s, before the grids were treated with 2% w/v phosphotungstic acid for 10 s before drying.

Results and Discussion

Gastric Mucin and Its Properties. Gastric mucin has recently attracted considerable attention from researchers due to its unique structure and biophysical properties.[26–31] Solutions of porcine gastric mucin undergo a marked increase in viscosity at low pH, which regulates diffusion through the mucus gel and protects the stomach against digestion by secreted gastric acid.[32]

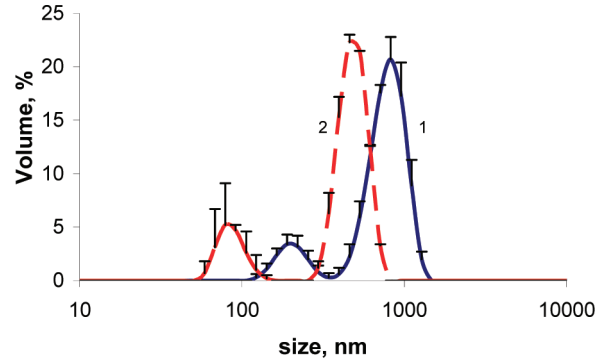

Figure 1. Dynamic light scattering measurements for pig gastric mucin particles *z*-average diameter at pH 2.0 (1) and pH 7.0 (2). Mean *z*-average size ± SD, $n = 3$.

Several authors show that native mucin freshly isolated from pig stomach exhibits some biophysical properties, which are partially lost upon its purification and storage.[2,4] In our study, we have chosen a commercial sample of lyophilised porcine mucin (type III). This product may differ slightly from the native porcine mucin because purification and storage may result in a partial degradation of glycoproteins and also in the formation of disulphide bridges due to oxidation of thiol groups in cysteine-rich subdomains. Nevertheless, commercial mucin is often used in studies of mucoadhesion because it shows less batch-to-batch variability and gives more reproducible results.[22,23]

The structure and behavior of mucin in aqueous solutions was studied at both pH 2.0 and 7.0 to correspond with the environment in the fasting stomach and when neutralized by the presence of food or antacids, respectively.[33,34] At low concentrations (1 mg/mL), mucin forms colloidally stable dispersions, which allows their assessment by dynamic light scattering (Figure 1).

The size distributions of mucin are bimodal at both pHs, with a population of smaller particles of around 91 ± 30 nm and larger aggregates of 531 ± 30 nm at pH 7.0. Under acidic conditions (pH 2.0), both particle populations are larger (220 ± 30 nm and 825 ± 30 nm), probably caused by further aggregation resulting from suppression of sialic acid group ionization, which would facilitate hydrogen bonding and hydrophobic interactions. Similar results showing bimodal distributions of mucin particles and their aggregation at different pHs were reported in our previous publication.[35]

The charge of mucin particles as a function of pH was assessed by measuring ζ-potential (Figure 2).

At pH 7.0, the mean ζ-potential of mucin aggregates is −19.4 mV. However, acidification reduces the particles negative charge, which approaches electroneutrality at around pH 2.0. These results are in good agreement with our previous findings[35] and data reported by Takeuchi et al.[24] Recently, Maleki and co-workers[36] have studied the effects of pH on the association behavior of pig gastric mucin in aqueous solutions by dynamic light scattering (DLS), turbidity, and rheo-small angle light scattering methods. They also reported that mucin is uncharged at pH 2.0, but because it is amphoteric, it acquires a negative charge at pH > 2.0 and positive charge at pH < 2.0. Our measurements performed at pH 1.0 did not confirm the existence of positively charged mucin particles and zeta potential was found to remain close to electroneutrality at −3.0 mV. However, our results showed considerable variability at pH < 2; repetitive

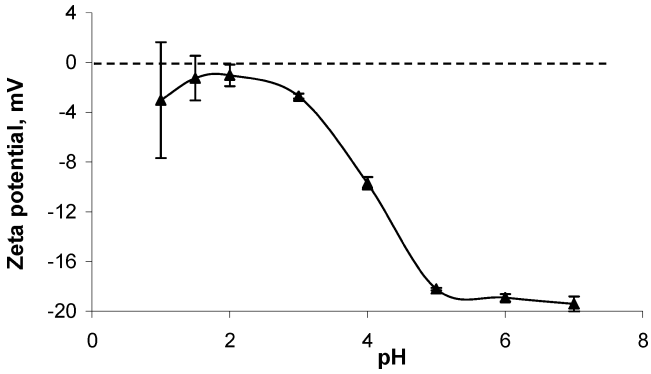

Figure 2. ζ-Potential of 0.1% w/v pig gastric mucin dispersion as a function of pH. Mean ζ-potential ± SD, $n = 3$ for pH 3.0−7.0 and $n = 5$ for pH 1.0−2.0.

experiments (SD = 4 mV, $n = 5$) indicated some colloidal instability of this disperse system under acidic conditions.

Aggregation of Mucin in the Presence of Chitosan and Its Half-Acetylated Derivative. Previously, we reported on interactions between gastric mucin and a series of synthetic cationic copolymers with varied hydrophilic−hydrophobic balance.[35] These copolymers were synthesized by copolymerizing cationic [2-(methacryloyloxy)ethyl]trimethylammonium chloride with a number of nonionic (meth)acrylates. Hydrogen bonding between these copolymers and mucin was prevented by the absence of proton-donating and proton-accepting groups in their structures, so interactions were mainly driven by electrostatic and hydrophobic effects. Chitosan is a cationic polysaccharide bearing primary amino and hydroxyl groups in each repeating unit, except for the acetylated units, which are without primary amines. When protonated, primary amino groups carry a positive charge, which may facilitate electrostatic interactions with negatively charged mucin macromolecules. When these amino groups are deprotonated, they can participate in hydrogen bonding with mucin along with the nonionic hydroxyl groups. However, the ability to manipulate protonation−deprotonation of chitosan amino groups is limited due to its insolubility at pH > 6.5, resulting from the semicrystalline nature of this polymer.

The solubility window of chitosan may be extended by chemical modifications. A half-acetylated derivative can be prepared by reaction with acetic anhydride with the resulting product containing approximately 50 mol % of acetylated amino groups.[25] Through this modification, the crystallinity of chitosan is partially disrupted, which increases its solubility window, elevating the pH at which it precipitates. In the present study, we have synthesized half-acetylated chitosan (HACHI) to clarify the role of amino groups in mucoadhesion. The degree of acetylation of the parent chitosan determined by ^{1}H NMR spectroscopy was found to be 14 ± 2 mol %, whereas for HACHI it was 52 ± 4 mol %. HACHI was found to be soluble over a wider range, up to pH 7.4 (see NMR spectra and pH-solubility profiles of chitosan and HACHI in Supporting Information).

Dynamic light scattering measurements of mucin in the presence of chitosan and its half-acetylated derivative have revealed that both polymers cause further aggregation of particles leading to agglomerates, whose final size exceeds 1000 nm (Figure 3). The biggest particles are observed for the mixture of mucin with HACHI at pH 2.0. Aggregation has also been demonstrated by transmission electron microscopy; Figure 3 (insets) shows aggregation of mucin particles taken from the smaller size population (separation of smaller and larger particle populations was possible due to the tendency of larger particles to concentrate at the edges of the grid). In the presence of chitosan, individual mucin particles (<100 nm) tend to form larger aggregates whose size exceeds 200 nm.

Estimating ζ-potential of mucin particles in the presence of polymers was found to be a good tool to study mucoadhesive interactions.[24,35] Figure 4 shows the changes in mucin particles' ζ-potential upon addition of increasing amounts of chitosan (pH 2.0) and HACHI (pH 2.0 and 7.0). In all three experiments, the polymers recharge the mucin particles, confirming adsorption of cationic macromolecules onto their surfaces. At pH 2.0, the recharging of mucin particles caused by addition of chitosan happens at lower weight ratios compared to the effect of its half-acetylated derivative. Isoelectric point (ζ-potential = 0 mV) in this case is achieved at a lower ratio and the maximal value of ζ-potential, reached after saturation, is 10 mV higher. This effect is expected because at pH 2.0 the amino groups of chitosan are protonated and so bear a positive change. The number of these groups in HACHI is almost halved because of acetylation and, therefore, more polymer is needed to interact with the negatively charged mucins. In both cases, the maximal effect was achieved at a [polymer]/[mucin] weight ratio approximately equal to 0.5 g/g.

At pH 7.0 the addition of HACHI still recharges mucin particles, but the isoelectric point is observed at a [HACHI]/[mucin] weight ratio ~0.8 and saturation at a ratio >2.0. Under these pH conditions, most HACHI amino groups are deprotonated (noncharged) and interaction with mucin is likely to be driven primarily by hydrogen bonding and hydrophobic effects.

Effect of Different Additives on Mucoadhesive Interactions. To clarify the role of electrostatic attractive forces, hydrogen bonding and hydrophobic effects on the mucoadhesive interactions, we have used turbidimetric titration as a simple tool to monitor mucin particle aggregation in the presence of polymers. This technique has been shown to be useful both for the study of mucin transformations at different pHs[35,36] and for polymer−mucin interactions.[18,35] Addition of chitosan to mucin at pH 2.0 is accompanied by a marked increase in solution turbidity until the [chitosan]/[mucin] weight ratio reaches 0.05; turbidity then decreases with further polymer addition (Figure 5).

The position of maximum turbidity coincides with the [chitosan]/[mucin] weight ratio at which inversion of charges was observed during ζ-potential measurements (ζ-potential = 0 mV). These dramatic changes in solution turbidity are related to the aggregation of mucin particles in the presence of small portions of chitosan and subsequent disaggregation caused by excess of the cationic polymer in the solution (Figure 6). Once disaggregation is complete, further addition of chitosan is accompanied by a slow decrease in turbidity due to dilution.

To estimate the role of electrostatic attraction between oppositely charged mucin and chitosan, we added 0.2 mol/L of NaCl in each solution and examined its effects on aggregation. It is well-known that this concentration of NaCl is able to disrupt interaction between oppositely charged synthetic polyelectrolytes.[37] Indeed, the turbidimetric titration profile in the presence of NaCl differs from the results obtained in salt-free solutions (Figure 5). Turbidity of mucin in the presence of salt is reduced, which may relate to a reduction of its particle size caused either by compaction or by further disaggregation. The turbidity maximum in the titration curve was displaced to a higher [chitosan]/[mucin] weight ratio, close to 0.1. Adding NaCl affects the interaction between chitosan and mucin due to screening of electrostatic charges; however, it does not com-

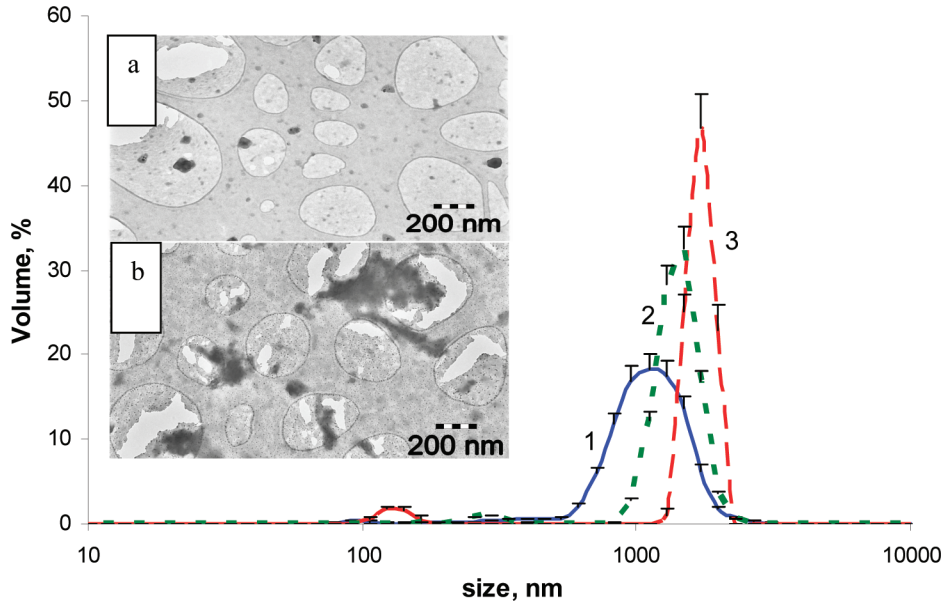

Figure 3. Dynamic light scattering size measurements of pig gastric mucin mixed with chitosan at pH 2.0 (1), HACHI at pH 7.0 (2), and HACHI at pH 2.0 (3) at [polymer]/[mucin] weight ratio = 0.05. Insets: pig gastric mucin at pH 2.0 before (a) and after (b) addition of chitosan.

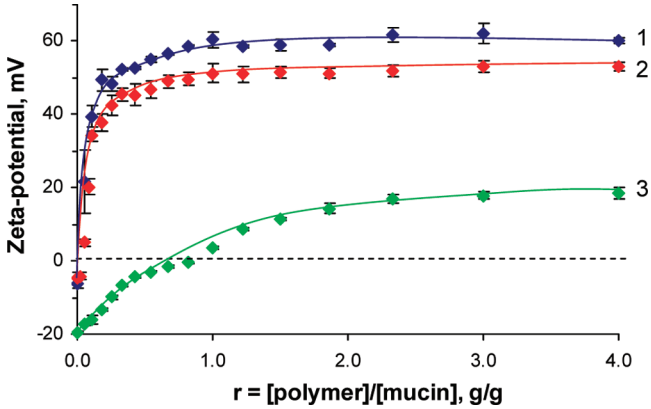

Figure 4. ζ-Potential measurements of 1 mg/mL pig gastric mucin solution, with 1 mg/mL chitosan at pH 2.0 (1), HACHI at pH 2.0 (2), and HACHI at pH 7.0 (3). Mean ζ-potential $\pm$ SD, $n = 3$. Error bars within the size of symbol are not shown.

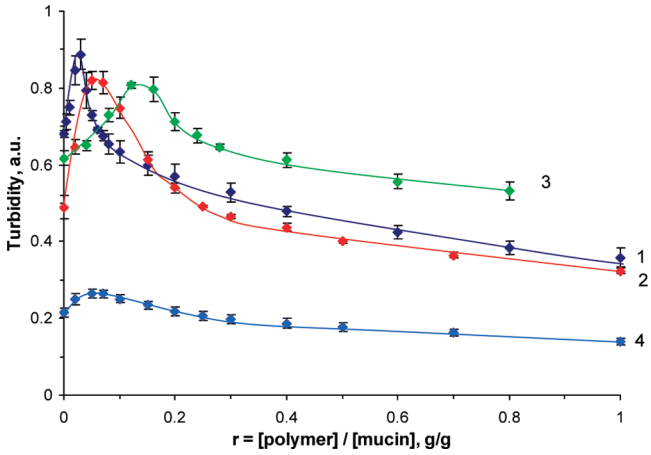

Figure 5. Turbidimetric titration of 1 mg/mL pig gastric mucin solution by 1 mg/mL chitosan at pH 2.0 in the absence (1) and presence of different additives, 0.2 mol/L NaCl (2), 10% v/v ethanol (3), and 8 mol/L urea (4). Mean turbidity $\pm$ SD, $n = 3$.

pletely prevent association. This observation shows that chitosan mucoadhesion is not solely driven by electrostatic attractions.

To probe these interactions further, we performed turbidimetric titrations in the presence of 10 v/v % ethanol. Lower alcohols like methanol, ethanol, and isopropanol are known to disrupt hydrophobic effects and also may affect hydrogen bonding through competition between biopolymer−polymer and biopolymer−ethanol interactions.[38–41] Although the presence of ethanol in solution reduced the initial turbidity of mucin, the position of the turbidity maximum shifted dramatically to a [chitosan]/[mucin] weight ratio ~0.15. This result confirms the existence of nonelectrostatic interactions between chitosan and mucin that are likely to be a combination of hydrogen bonding and hydrophobic effects.

Adding 7−8 mol/L urea to aqueous solutions of polymers and biopolymers is known to break hydrogen bonding and to weaken hydrophobic effects.[40,42] We thus prepared solutions of chitosan and mucin in 8 mol/L urea at pH 2.0 and studied the interactions (Figure 5). The initial turbidity of these mucin solutions is approximately 3 times lower than the value observed without any additive. This notable difference is possibly related to the ability of urea to denature or disaggregate mucins, resulting in significantly smaller particles. It can be assumed that this denaturation/disaggregation of mucin in the presence of urea will only affect the particle size and supramolecular structure and will not alter surface functionality that drives mucoadhesive interactions. Indeed, the addition of chitosan to mucin in the presence of urea is accompanied by some increase in turbidity; the turbidity maximum is broader and slightly shifted to a higher [chitosan]/[mucin] weight ratio compared to the titration curve obtained without additive. Thus, in the system where hydrogen bonding is fully prevented and hydrophobic effects are weakened, chitosan still interacts with mucin presumably via electrostatic forces only.

Interestingly, addition of NaCl, ethanol, or urea affects the interactions between mucin and HACHI more dramatically (Figure 7). The presence of NaCl reduces the turbidity maximum to a greater extent than the titrations with unmodified chitosan. This screening effect is probably related to the lower number of amino groups in HACHI that bind with mucin electrostatically. The maximum turbidity observed for the titration in the presence of ethanol is not significantly different for HACHI compared with unmodified chitosan, but its position is further

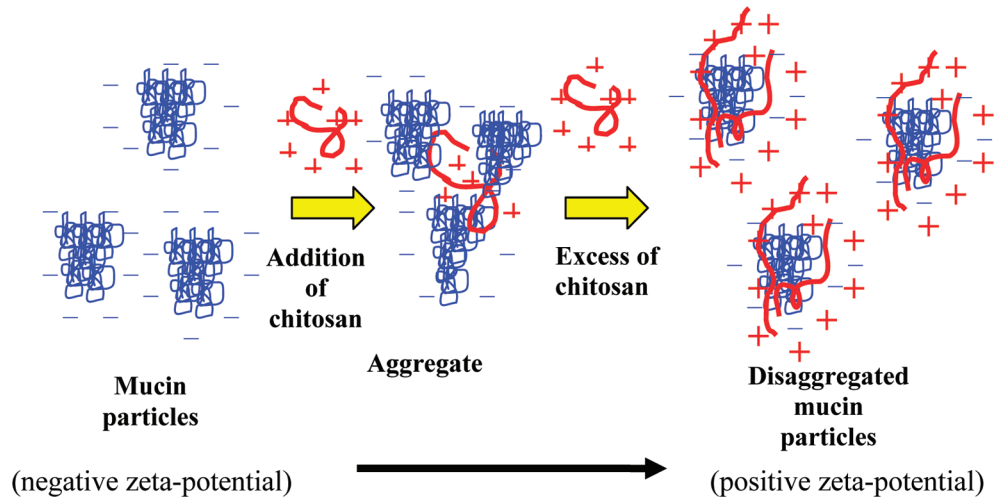

Figure 6. Diagram depicting aggregation/disaggregation of pig gastric mucin in the presence of chitosan.

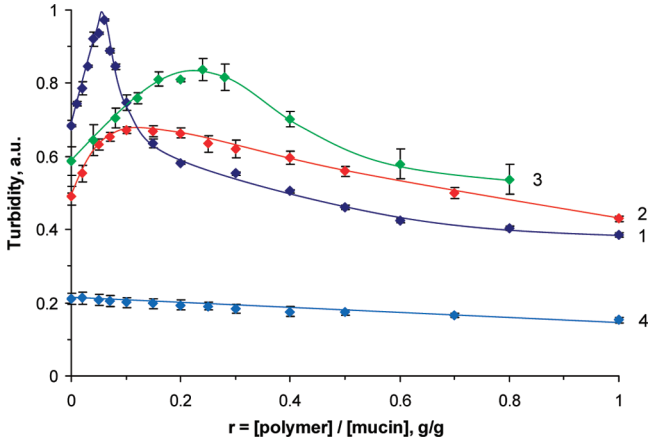

Figure 7. Turbidimetric titration of 1 mg/mL pig gastric mucin by 1 mg/mL half-acetylated chitosan solution at pH 2.0 in the absence (1) and presence of different additives, 0.2 mol/L NaCl (2), 10% v/v ethanol (3), and 8 mol/L urea (4). Mean turbidity ± SD, $n = 3$.

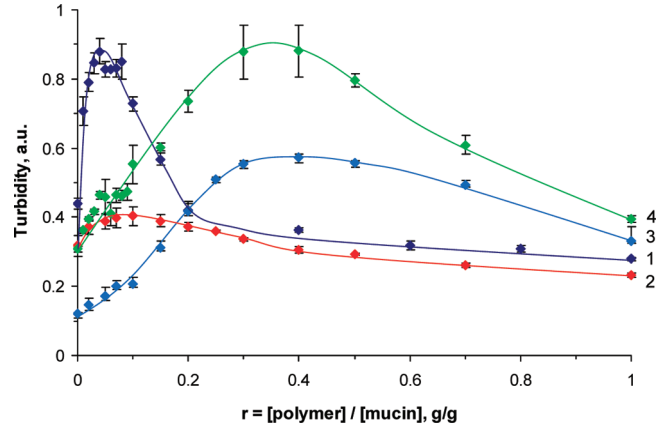

Figure 8. Turbidimetric titration of 1 mg/mL pig gastric mucin by 1 mg/mL half-acetylated chitosan solution at pH 7.0 in the absence (1) and presence of different additives, 0.2 mol/L NaCl (2), 8 mol/L urea (3), and 10% v/v ethanol (4). Mean turbidity ± SD, $n = 3$.

shifted to a [HACHI]/[mucin] weight ratio ~0.25, indicating that partial elimination of amino groups may also affect hydrogen bonding. The titration in the presence of urea provided a near flat curve showing no interaction between the components. Perhaps, in this case, a reduction in the contribution of electrostatic contacts as well as hydrogen bonds via partial elimination of primary amino groups, coupled with disruption of hydrogen bonding and weakening of hydrophobic contacts, completely prevents mucoadhesive interactions.

When similar experiments were performed with HACHI and mucin at pH 7.0 (Figure 8), we observed that NaCl markedly reduces the interactions: the maximum turbidity is approximately halved in the presence of NaCl compared to salt free solutions. With ethanol, the position of the maximum shifted from a [HACHI]/[mucin] weight ratio of 0.05 to 0.35, but the absolute value of turbidity remained unchanged. The titration profile in the presence of urea is very similar to that with ethanol but the turbidity values are greatly reduced. It is likely that under these pH conditions, the major contribution to mucoadhesive interactions results from electrostatic attraction because of the high negative charge on the mucin particles (see zeta potential results above). Consequently, the strongest disruption was observed in the presence of NaCl due to effective screening of electrostatic charges. Ethanol and urea still affect the interaction by disrupting/weakening hydrogen bonding/hydrophobic effects, but these additives do not affect the main driving force (electrostatic components) in this case.

Conclusions

It is widely recognized that specific interactions between polymers and mucins play an important role in mucosal adhesion at the molecular level. In this study we have isolated and estimated the contribution of different physical interactions through manipulating chitosan structure via partial acetylation and adding NaCl, ethanol or urea. Although it was not possible to completely "switch off" selected interactions, we have demonstrated that mucoadhesive interactions between chitosan and mucin are complex with contributions from electrostatic attraction, hydrogen bonding, and hydrophobic effects. Electrostatic attraction appears to be the major mechanism for chitosan mucoadhesion but is also accompanied by contributions from hydrogen bonding and hydrophobic effects. Solution pH as well as the presence of other chemicals in the solutions can change the relative contributions of each physical interaction. These findings should support the development of chitosan-based mucoadhesive drug delivery systems.

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Supporting Information Available. Solubility profiles and ^{1}H NMR spectra of chitosan and half-acetylated chitosan. This information is available free of charge via the Internet at http://pubs.acs.org.

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