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Synthesis of a fluorescent chitosan derivative and its application for the study of chitosan–mucin interactions

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Abstract

Fluorescein isothiocyanate-labeled chitosan was synthesized as a macromolecular fluorophore to investigate the molecular mechanism of interactions between chitosan and mucin by the fluorescence polarization method. The percent change in polarization upon chitosan–mucin association was measured as a function of the molecular weight of chitosan, and the pH and ionic strength of the medium. Dextran sulfate and albumin were used as positive and negative controls of polyelectrolyte complexation, respectively. The degree of polarization increased significantly with increasing mucin/chitosan (molecular weight, 750 000 daltons) molar ratio in 0.10 M acetic acid to suggest that there was multivalent binding between the two polymers. In contrast, only one dextran sulfate molecule was able to bind with the chitosan molecule. Albumin, having a net positive charge at a pH of 2.9, did not bind with chitosan. There was a possibility of multivalent association between high molecular weight chitosan and mucin as compared to univalent association with low molecular weight chitosan. The results of pH and ionic strength studies suggest that, in addition to electrostatic interactions between D-glucosamine residue of chitosan and the sialic acid residue of mucin, there could be other attractive forces involved in chitosan–mucin binding such as hydrogen bonding and/or hydrophobic interaction.

Keywords: Chitosan; Mucin; Mucoadhesive drug delivery; Fluorescence polarization; Fluorescein isothiocyanate

1. Introduction

Since the introduction of the concept by Park and Robinson (1984), mucoadhesive polymeric systems have generated significant interest over the years as a method to improve the performance of controlled-release drug delivery systems. Mucoadhesive polymers can localize the formulation at the site of interest for local or systemic therapy (Park et al., 1985; Kamath and Park, 1988), optimize contact with the gastro-intestinal tissue surface to improve drug permeability (Veillard et al., 1987), and in some instances even inhibit degradative enzymatic activity (Leußen et al., 1985). Mucoadhesive formulations have been developed for ocular, nasal, buccal, gingival, gastro-intestinal, rectal, and vaginal drug administration (Leung and Robinson, 1992). The basic component of mucus at all of these sites is the mucin glycoproteins which forms an unstirred gel layer over the epithelial cells of the mucosa (Silberberg, 1988). Mucin glycoproteins are made up of subunits with a molecular weight ranging from 380 000 to 720 000 daltons (Marriott and Gregory, 1990). The subunits assemble through disulfide bonds to create the mucin glycoproteins with terminal oligosaccharide side chains. D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (sialic acid) are the predominant terminal carbohydrate residues of mucin glycoproteins (Mantle et al., 1978).

For optimum mucoadhesion, there has to be an intimate contact between the adhesive and the substrate and interpenetration of the polymer chains with the mucin glycoprotein network. Peppas and Buri (1985) have described the mucoadhesive processes in the context of electronic, adsorption, wetting, diffusion, and fracture theories. The initial attraction between the adhesive and mucus layer can be through van der Waals forces, electrostatic interactions, hydrogen bonding, or hydrophobic interactions (Mikos and Peppas, 1990). Interpenetration of the polymer with mucin occurs through diffusion and entanglements of the chains. For mucoadhesion with poly(acrylic acid) hydrogels, for instance, interpenetration of the polymer chains leading to improved bonding was enhanced with lower degree of crosslinking (Ch'ng et al., 1985).

A number of studies have shown that chitosan adheres to

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mucosal surfaces and can enhance the absorption of drugs, including protein and peptide drugs (Artursson et al., 1994; Illum et al., 1994; Leußen et al., 1994; Schipper et al., 1997). Chitosan, a natural cationic polymer, is obtained from *N*-deacetylation of chitin. Chitin, the second most abundant natural polymer, is harvested mainly from the exoskeleton of marine crustaceans such as crabs and shrimps (Li et al., 1992). Chitosan has been proposed for a number of medical and pharmaceutical applications (Chandy and Sharma, 1990). We have examined the possibility of using chitosan hydrogels as pH-sensitive swelling systems for localized drug release in the stomach (Patel and Amiji, 1996). The degree of deacetylation and molecular weight of chitosan is determined by the temperature and duration of the reaction and the concentration of the base (NaOH or KOH) used for deacetylation. Commercial chitosan typically has a wide molecular weight range and degree of deacetylation of $> 75\%$. The pK_a of the primary amine group D-glucosamine residue of chitosan is about 6.5 (Claesson and Ninham, 1992). As such, the polymer is positively charged when dissolved in dilute acidic solutions or in salt forms. The mucoadhesive properties of chitosan is, therefore, thought to be mediated by electrostatic interactions between the positively-charged D-glucosamine residue and the negatively-charged sialic acid residue of mucin (Artursson et al., 1994; Illum et al., 1994).

For better understanding of the mechanism of interactions leading to enhanced mucoadhesion, in the present study, we have used the fluorescence polarization technique to characterize chitosan–mucin binding. Fluorescein isothiocyanate (FITC)-labeled chitosan was synthesized and used as a macromolecular fluorophore. Fluorescence polarization measurements of association between chitosan and mucin were carried out with different molecular weights of chitosan, and at different pH and ionic strengths of the solution. Dextran sulfate and albumin were used as positive and negative controls of polyelectrolyte complexation with FITC-labeled chitosan.

2. Theory of polarization

Fluorescence polarization was first described by Perrin (1926) and was later reviewed for biological applications by Weber (1953). When intrinsic or extrinsic fluorescent molecules are excited with plane polarized incident light, the emitted signal remains in the same polarized plane, provided that the molecule remains stationary throughout the lifetime of the excited state. If the fluorescent molecule, however, rotates or tumbles out of the plane of polarized light during the excited state, the emitted signal then will be in a different plane from that of the excitation plane. When a vertically polarized incident light (I_V) is used to excite the fluorescent molecule, the emission light intensity can be monitored in both vertical (I_V) and horizontal planes (I_H). The degree to which the emission signal moves from the vertical to the horizontal plane is related to the mobility of the fluorescent molecule. Fluorescent macromolecules will not be very mobile during the lifetime of the exited state and, as such, the emitted light remains highly polarized with respect to the incident light. For small fluorophores, on the other hand, the mobility is very high and the resulting emission light is highly depolarized with respect to the incident light (Bolger and Checovich, 1994).

The degree of steady-state polarization (P) is defined as follows:

$$P = (I_V - I_H G)/(I_V + I_H G) \quad (1)$$

where I_V and I_H are the intensities of vertically and horizontally polarized emissions, respectively, with vertically polarized incident light. 'G', the grating factor, defined as I_V/I_H with horizontally polarized incident light, is a wavelength-dependent polarization response due to the components of the emission detection system.

Perrin (1926) related the degree of polarization to the rotational relaxation time (ρ) of a fluorophore as:

$$(1/P - 1/3) = (1/P_o - 1/3)(1 + 3\tau/\rho) \quad (2)$$

where P_o is the limiting polarization observed when depolarization due to the Brownian motion is absent and τ is the excited state lifetime of the fluorophore. The rotational relaxation time (ρ) is defined as the time required for a molecule to rotate through an angle of approximately 68.5°. The rotational relaxation time for a spherical fluorophore is related to the molecular volume (V) as follows:

$$\rho = 3\eta V/RT \quad (3)$$

where η is the viscosity of the medium, R is the universal gas constant, and T is the absolute temperature. Under these conditions, the Perrin equation ((Eq. (2)), can be re-written as follows:

$$(1/P - 1/3) = (1/P_o - 1/3)[1 + (RT\tau/\eta V)] \quad (4)$$

As can be seen from Eq. (4), the degree of polarization is directly proportional to the molecular volume of the fluorophore when the excited state lifetime, viscosity, and temperature are maintained constant. It is important to note here that neither the chitosan nor mucin molecule in solution in our study would be represented by the perfect spherical shape. Under these conditions, the rotational relaxation time should be thought of in terms of the effective rotational diffusion time.

Based on the above relationship, steady-state fluorescence polarization is a powerful tool to study macromolecular interactions. It is widely used to study antigen–antibody binding in fluorescent immunoassay (Dandliker and De Saussure, 1970), membrane fluidity (Sorensen, 1988), protein–protein interactions (Jiskoot et al., 1995; Lundblad et al., 1996), protein–DNA interactions (Lundblad et al., 1996), and polymer–polymer interactions (Morawetz, 1979; Heyward and Ghiggino, 1989).

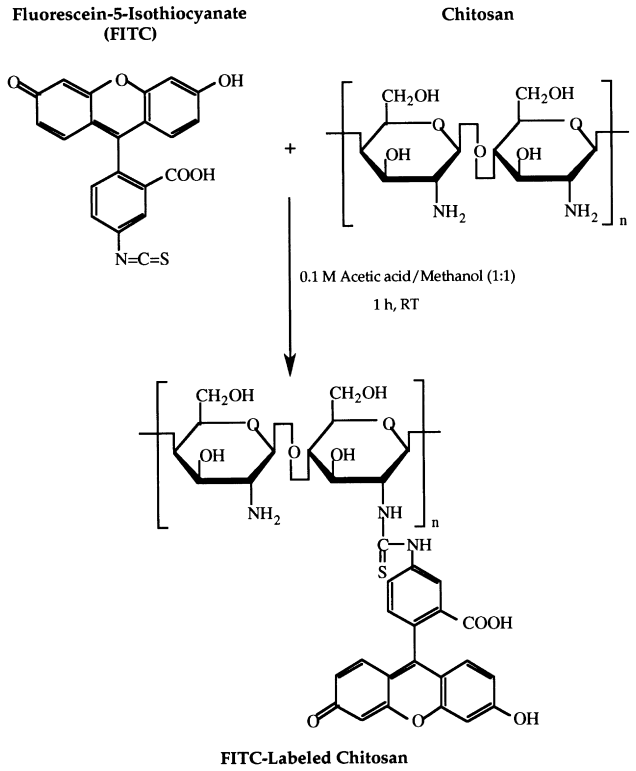

Fig. 1. Schematic illustration of the chemical synthesis of FITC-labeled chitosan. FITC was reacted with chitosan, dissolved in 0.10 M acetic acid–methanol (1:1) mixture, for 1 h at room temperature.

3. Materials and methods

3.1. Materials

Chitosan of molecular weights 70 000 (70K), 750 000 (750K), and 2 000 000 (2.0M) were puchased from Fluka Chemika/Biochemika (Ronkonkoma, NY) (Table 1). Fluorescein isothiocyanate (FITC, Type I) was purchased from Molecular Probes (Eugene, OR). Bovine submaxillary mucin (Type I-S), with a molecular weight of 400 000 daltons and containing 12% sialic acid residues, was purchased from Sigma Chemical Company (St. Louis, MO). Bovine serum albumin (Fraction V) was also purchased from Sigma. Mucin and albumin were purified by extensive equilibrium dialysis against deionized distilled water (NANOpure II, Barnsted/Thermolyne, Dubuque, IO) and then freeze-dried. Dextran sulfate (molecular weight, 500 000 daltons) was purchased from Polysciences (Warrington, PA). All other chemicals were of reagent grade and were used as received.

3.2. Synthesis of FITC-labeled chitosan

Chitosan was purified from proteins and inorganic impurities by dissolving in 0.10 M acetic acid, followed by precipitating in 0.10 M sodium hydroxide and extensive washing of the precipitate with deionized distilled water. The dissolution–precipitation–washing process was repeated twice and the polymer was freeze-dried. The exact degree of deacetylation of chitosan, ranging from 82 to 85%, was determined by ultra-violet analysis, as described by Muzzarelli and Rocchetti (1985), using a Shimadzu UV-160U spectrophotometer (Columbia, MD). One gram of freeze-dried chitosan was dissolved in 100 ml of 0.10 M acetic acid. To the chitosan solution, 100 ml of dehydrated methanol was slowly added with continuous stirring. FITC, dissolved in methanol at 1.0 mg ml^{-1} concentration, was slowly added to the chitosan solution. The final concentration of FITC in the reaction medium was controlled to give the label to D-glucosamine residue ratio of 1:50. The reaction between the isothiocyanate group of FITC and the primary amine group of the D-glucosamine residue, as shown in Fig. 1, was allowed to proceed for 1 h in the dark at room temperature. FITC-labeled chitosan was precipitated in 0.1 M sodium hydroxide solution. The precipitate was washed extensively with deionized distilled water until there was complete absence of free FITC fluorescence signal in the washing medium. The labeled polymer was then freeze dried. The labelling efficiency was found to be approximately one FITC molecule per 70 D-glucosamine residues of chitosan. Typical reaction yield was 85%.

3.3. Fluorescence polarization experiments

FITC-labeled chitosan of molecular weights 70K, 750K, and 2.0M, each dissolved in 0.10 M acetic acid (pH 2.9), was mixed with mucin, albumin, and dextran sulfate at the different chitosan to biopolymer molar ratios. The chitosan to mucin, albumin, or dextran sulfate molar ratio was calculated by keeping the chitosan concentration in solution constant, and progressively increasing the other biopolymer

Table 1
Properties of the chitosans used in the study [a]

Type	Average molecular weight (daltons)	Degree of deacetylation (%)
Chitosan 70K	70 000 [b]	83.2 [c]
Chitosan 750K	750 000	85.7
Chitosan 2.0M	2 000 000	82.7

[a] Chitosans of different molecular weights were purchased from Fluka Chemika/Biokemica (Ronkonkoma, NY).
[b] The average molecular weight was determined from the viscosity of a 1.0% (w/v) chitosan solution in acetic acid by the supplier.
[c] The degree of deacetylation was measured by ultra-violet analysis as described by Muzzarelli and Rocchetti (1985).

concentrations. Albumin and dextran sulfate were used as negative and positive controls of polyelectrolyte complexation, respectively. In some instances, the pH and ionic strength of the final mixture was varied. The pH of solution was varied from 2.9 to 5.0 using 0.10 M acetic acid/sodium acetate buffer. The pH values ranging from 2.9 to 5.0 were optimized based on the FITC-labeled chitosan fluorescence signal to noise ratio and the pH-dependent solubility of chitosan. For mucoadhesion, the pH values in the gastrointestinal tract can range from 1.5 in the fasted stomach to 7.0 in the small intestine. Additionally, the pH of vaginal mucosa is around 4.5 due to the secretion of lactic acid. The ionic strength of the mixture was varied by addition of sodium chloride to the solution in 0.10 M acetic acid at a final concentration ranging from 0.01 M to 1.0 M. Fluorescence polarization experiments were performed with a Perkin–Elmer LS 50B fluorescence spectrophotometer (Norwalk, CT) equipped with Glans–Thompson excitation and emission polarizers. The Perkin–Elmer Fluorescence Data Manager software that runs on an IBM 433DX/Si (International Bussiness Machines, White Plains, NY) automatically calculates the '*G*' factor, polarization, and anisotropy using built-in functions. FITC-labeled chitosan was excited at 470 nm and the emission maximum was observed at 525 nm (Fig. 2). The excitation and emission slit width were 10 nm and 2.5 nm, respectively, and the signal was collected at 10 s intervals. The percentage change in polarization was calculated according to the following expression:

$$\text{Percent change} = [(P_2 - P_1)/P_1] \times 100 \quad (5)$$

where P_1 and P_2 are the degrees of steady-state polarization in the absence and presence of the associating agent, respectively.

4. Results and discussion

4.1. Fluorescence polarization to study macromolecular association

We have synthesized FITC-labeled chitosan as a water-soluble macromolecular fluorophore to investigate the interactions with mucin by fluorescence polarization method. The reaction for conjugation of the fluorescein moiety to the primary amine group of D-glucosamine residues in chitosan is illustrated in Fig. 1. The fluorescence excitation and emission spectra of FITC-labeled chitosan are shown in Fig. 2. For polarization studies, the excitation wavelength was set at 470 nm and the emission wavelength was set at

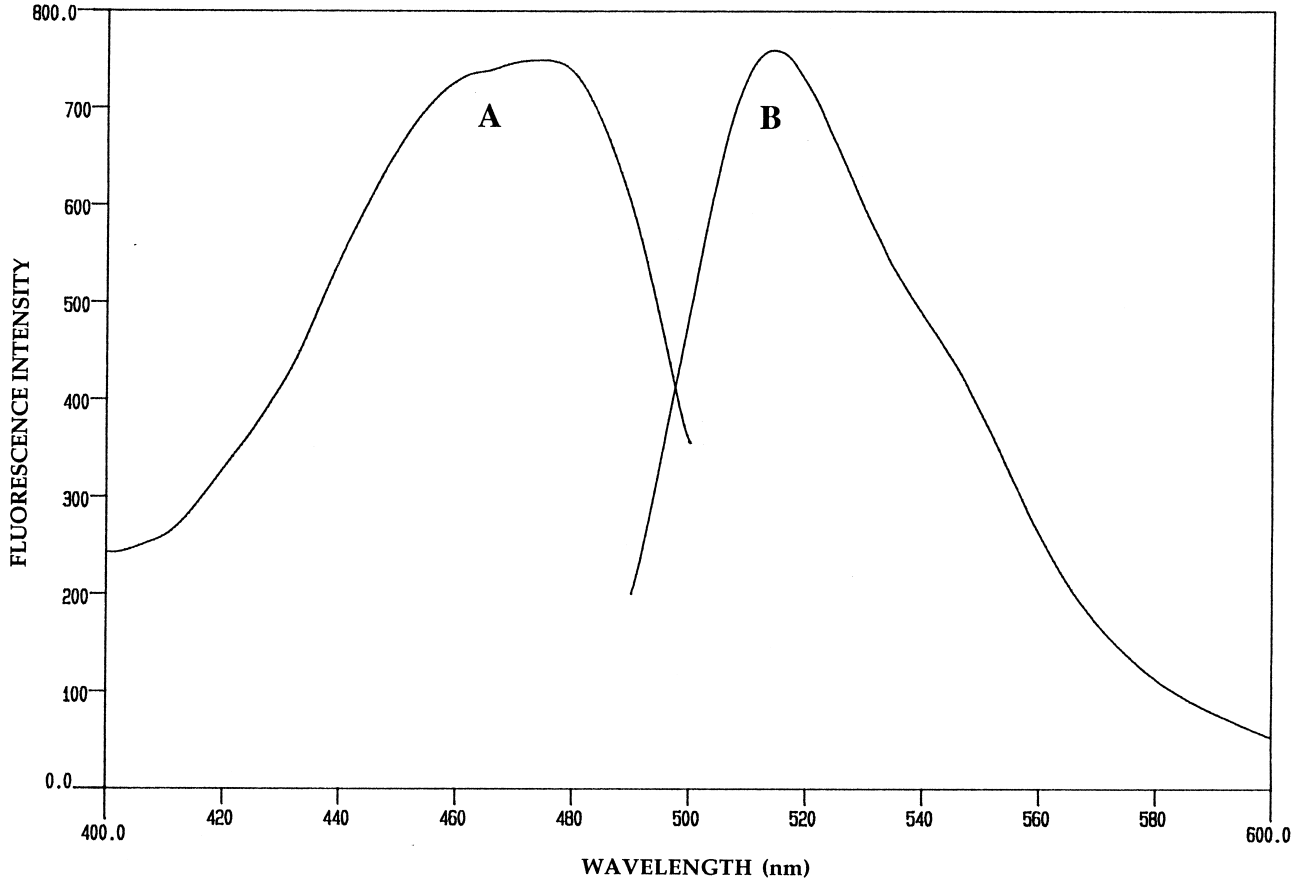

Fig. 2. Excitation (A) and emission (B) spectra of FITC-labeled chitosan in 0.10 M acetic acid (pH 2.9). FITC-labeled chitosan (molecular weight, 750 000 daltons) was excited at 470 nm and the emission maximum was at 525 nm. The concentration of fluorescent conjugate was 1.33 μM.

520 nm. Since it is speculated that chitosan may bind with mucin primarily by electrostatic interactions, we have used dextran sulfate and albumin as positive and negative controls of polyelectrolyte complexation, respectively. Being a strong polyanion, dextran sulfate would completely ionize in solution, independent of the pH. The net charge of albumin with an iso-electric point of 4.7 (Peters, 1975), on the other hand, is dependent on the pH of the solution. In 0.10 M acetic acid (pH 2.9), albumin would have a net positive charge and, as such, would not interact well with chitosan.

As shown in Fig. 3, the percentage change in the degree of polarization of FITC-labeled chitosan 750K in 0.10 M acetic acid (pH 2.9) was significantly increased upon association with mucin. When the mucin/chitosan molar ratio was 19:1, for instance, there was a 61% increase in the degree of polarization from baseline. The degree of polarization continued to increase with increasing mucin/chitosan molar ratio. This suggests that there are multivalent interactions between mucin and chitosan. For dextran sulfate, on the other hand, the degree of polarization increases with lower dextran sulfate/chitosan molar ratio and remains constant after a 1:1 molar ratio. The percentage change in polarization remains at around 60% when the dextran sulfate/chitosan molar ratio exceeds 1:1. Based on the electrostatic interactions, it is expected that one molecule of dextran sulfate would bind with only one molecule of chitosan. Albumin did not bind at all to chitosan, as seen from the polarization data. The percentage change in the degree of polarization increased to only 3.0% even when the albumin/chitosan molar ratio was 55:1.

The above results show that the fluorescence polarization method can be used to examine macromolecular interactions where an increase in polarization is indicative of an increase in the molecular volume of the fluorophore. The multivalent binding of mucin with chitosan was observed as the degree of polarization increased with increasing mucin/chitosan molar ratio. This is in marked contrast with dextran sulfate–chitosan interactions where one-to-one binding was observed.

4.2. Effect of chitosan molecular weight on association with mucin

FITC-labeled chitosan, of molecular weight 70K, 750K, and 2.0M, was mixed with mucin in 0.10 M acetic acid (pH 2.9) at different molar concentrations. The percentage change in degree of polarization of FITC-labeled chitosan upon mixing with mucin was measured and is shown in Fig. 4. The increase in the degree of polarization of 60–65% occurred when the mucin/chitosan molar ratios were 1.75:1 for chitosan 70K, 19:1 for chitosan 750K, and 50:1 for chitosan 2.0M. Based on these data, it is clear that high molecular weight chitosan (i.e. 2.0M) offers multiple sites

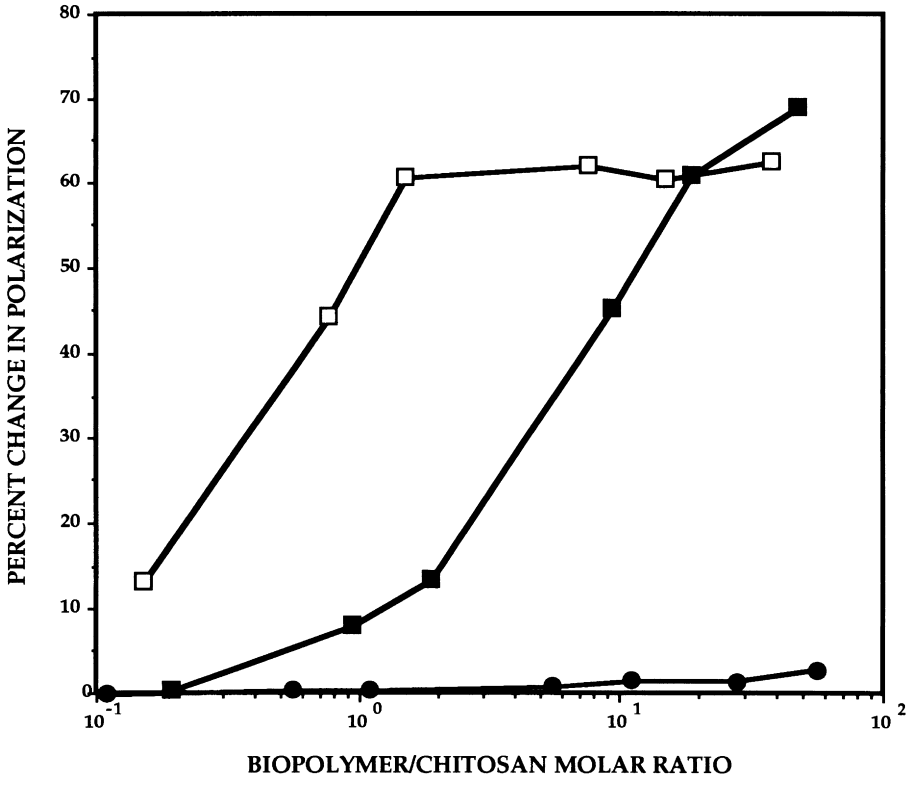

Fig. 3. Percentage change in polarization of FITC-labeled chitosan upon association with mucin (■), dextran sulfate (□), and albumin (●). Dextran sulfate and albumin were used as positive and negative controls of polyelectrolyte complexation, respectively. FITC-labeled chitosan (molecular weight, 750 000 daltons) was mixed with mucin, dextran sulfate, and albumin in 0.10 M acetic acid (pH 2.9).

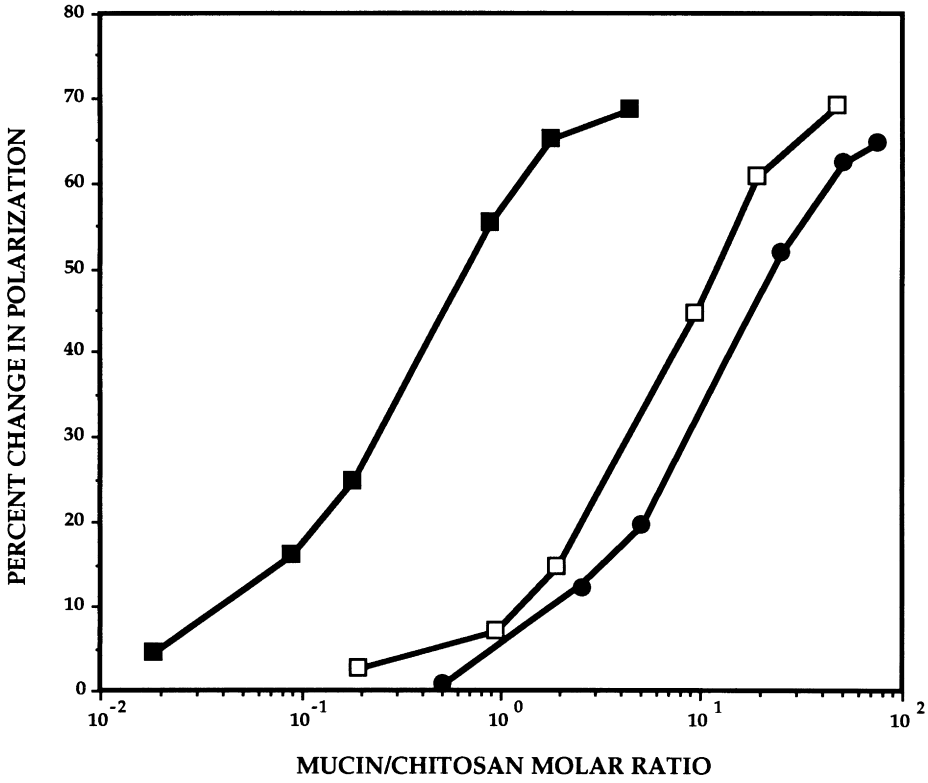

Fig. 4. Percentage change in polarization of FITC-labeled chitosan upon association with mucin as a function of the molecular weight of chitosan. FITC-labeled chitosan of molecular weight 70 000 (■), 750 000 (□), and 2 000 000 (●) were mixed with mucin in 0.10 M acetic acid (pH 2.9).

for mucin attachment than the low molecular weight chitosan (i.e. 70K). Increasing the number of contact points is important for the initial attachment of the two polymers and interpenetration between the chitosan and mucin chains for optimum mucoadhesion. For chitosan hydrogels, the crosslinking density would be an important determinant of the mucoadhesive strength. Hydrogels with lower crosslinking density (or higher swelling) tend to have higher mucoadhesive strength than those with higher crosslinking density (Ch'ng et al., 1985).

4.3. Effect of solution pH on chitosan–mucin association

The pH effect is important for mucoadhesion since the delivery systems are intended for administration to regions of the body with significant pH variation. The percent ionization of D-gluosamine residue ($pK_a = 6.5$) of chitosan and sialic acid residue ($pK_a = 2.6$) of mucin at different pH values according to the Henderson–Hasselbach equation are listed in Table 2. At a pH value of 4.0, for instance, more than 99% of the D-gluosamine residues and 96% of the sialic acid residues would be ionized. If electrostatic interactions were the primary mode of chitosan–mucin association, the maximum strength of interaction would be at a pH of around 4.0. The fluorescence polarization data, as shown in Fig. 5, however, shows that the highest increase in polarization occurs at more acidic pH. The percentage changes in polarization were 61, 55.8, 47.6 and 44.2%, when the pH values of the mixture were 2.9, 3.4, 4.0, and 5.0, respectively, at the mucin/chitosan (molecular weight, 750K) molar ratio of 19:1. At a pH of 2.9, even though almost 100% of the D-glucosamine would be ionized, only 20% of the sialic acid residues are ionized. As such, the complete ionization of D-glucosamine seems to be an

Table 2
Percentage ionization of D-glucosamine residues of chitosan and sialic acid of mucin in different pH environments [a]

pH	Ionization of D-glucosamine (%)	Ionization of sialic acid (%)
2.9	99.97	20.08
3.4	99.92	66.61
4.0	99.68	96.17
5.0	96.93	99.75

[a] Percentage ionization was calculated using the Henderson–Hasselback equation assuming the pK_a of D-glucosamine and sialic acid to be 6.50 and 2.60, respectively.

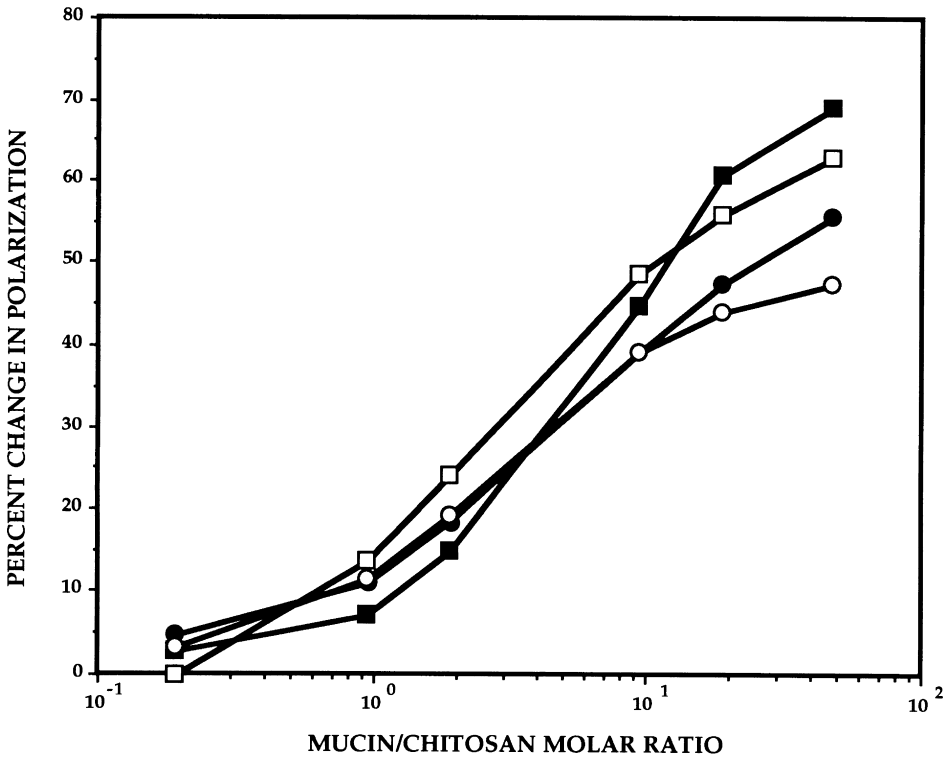

Fig. 5. Percentage change in polarization of FITC-labeled chitosan upon association with mucin as a function of the pH of solution. FITC-labeled chitosan (molecular weight, 750 000 daltons) was mized with mucin and the final pH of solution was adjusted to 2.9 (■), 3.4 (□), 4.0 (●), and 5.0 (○).

important factor in the interactions between mucin and chitosan. The mucin used in this study had a sialic acid content of only 12%. In other sources of mucin, the sialic acid content can be as high as 60% (Wolf et al., 1980). As such, the effect of pH on mucin–chitosan interactions would be dependent on the sialic acid content of the mucin.

4.4. Effect of solution ionic strength on chitosan–mucin association

We varied the ionic strength of FITC-labeled chitosan mixture with mucin in 0.10 M acetic acid (pH 2.9) by addition of sodium chloride. The final sodium chloride concentration ranged from 0.01 to 1.0 M. Dextran sulfate and albumin were again used as positive and negative controls of polyelectrolyte complexation. As shown in Fig. 6, the degree of polarization of FITC-labeled chitosan upon association with mucin increases with increasing sodium chloride concentration. The percentage change in polarization of 30% from baseline was observed with 0.50 M sodium chloride. On increasing sodium chloride concentration to 1.0 M, the degree of polarization increased to 36%. In the FITC-labeled chitosan–albumin mixture, the degree of polarization also increased with increasing sodium chloride concentration. In contrast, when FITC-labeled chitosan was mixed with dextran sulfate, the degree of polarization increased to 32% with 0.50 M sodium chloride and then decreased to 13% when the sodium chloride concentration was increased to 1.0 M.

The increase in polarization of chitosan–albumin and decrease in polarization of chitosan–dextran sulfate complex in the presence of 1.0 M sodium hydroxide was due to charge neutralization as a result of counterion binding. In mucin, however, there was actually an increase in the polarization with increasing ionic strength. Unlike dextran sulfate, which associates with chitosan by electrostatic interactions only, it seems that the mucin–chitosan complex, at least at low pH and high ionic strength, may involve other attractive forces such as hydrogen bonding and/or hydrophobic interactions, in addition to electrostatic interactions. Previous studies have shown that poly(acrylic acid) hydrogels interact with mucin at low pH values, predominantly by hydrogen bonding (Park and Robinson, 1985; Park and Robinson, 1987).

5. Conclusions

We have synthesized FITC-labeled chitosan to examine the mechanism of interactions between chitosan and mucin by the fluorescence polarization method. An increase in the degree of polarization is an indicator of the increase in the molecular volume of the fluorophore upon association. As such, fluorescence polarization is used widely to study macromolecular association. Upon association with mucin, the degree of polarization increased significantly with increasing mucin/chitosan 750K molar ratio. At the mucin/chitosan molar ratio of 19:1, for instance, the

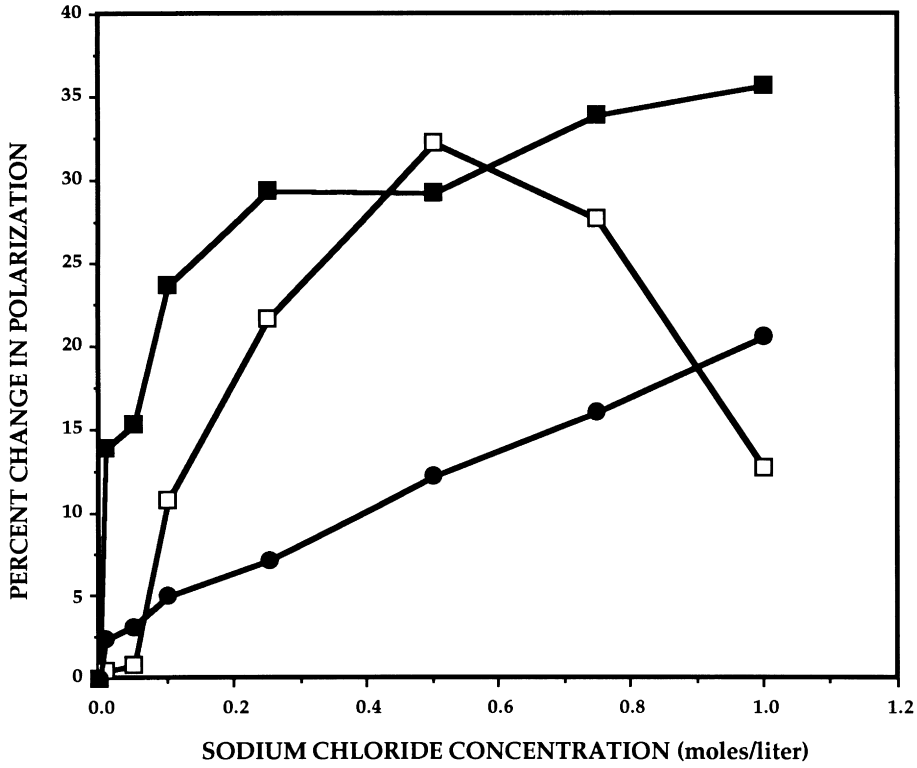

Fig. 6. Percentage change in polarization of FITC-labeled chitosan upon association with mucin (■), dextran sulfate (□), and albumin (●) as a function of the ionic strength of solution. The molar ratios of mucin, dextran sulfate, and albumin to FITC-labeled chitosan (molecular weight, 750 000 daltons) in the mixture was 19:1, 15:1, and 28:1, respectively. The ionic strength of the solution in 0.10 M acetic acid (pH 2.9) was adjusted with sodium chloride.

increase in polarization was 61%. This suggests that more than one mucin molecule can associate with each chitosan molecule. In contrast, the interactions between dextran sulfate, a strong polyanion, and chitosan occurred by one-to-one binding. Positively-charged albumin at pH 2.9 did not bind with chitosan at all. High molecular weight chitosan offers multiple attachment sites for mucin binding than low molecular weight chitosan. The pH and ionic strength of solution also had a profound effect on the interactions between chitosan and mucin. The maximum change in polarization of FITC-labeled chitosan–mucin occurred at a pH of 2.9. Although nearly 100% of the D-glucosamine residues ($pK_a = 6.5$) of chitosan would be ionized at the pH of 2.9, only 20% of the sialic acid ($pK_a = 2.6$) of mucin would be ionized. In addition, increasing the ionic strength of the medium with sodium chloride increased the degree of polarization of the FITC-labeled chitosan–mucin mixture. The degree of polarization of the FITC-labeled chitosan–dextran sulfate mixture decreased when the sodium chloride concentration exceeded 0.50 M. As such, the pH and ionic strength results suggest that there may be multiple modes of interactions between chitosan and mucin involving other forces such as hydrogen bonding and/or hydrophobic interactions, in addition to electrostatic interactions.

The results of this study show that fluorescence polarization is a useful method to examine the association behavior of water-soluble fluorescent macromolecules like chitosan and mucin. For mucoadhesion, more than one mucin molecule can bind with a chitosan molecule. In addition, the pH and ionic strength data suggest that the binding may involve other forces such as hydrogen bonding and/or hydrophobic interactions, in addition to electrostatic interactions, between D-glucosamine and sialic acid residues.

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