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# Evaluation of mucoadhesiveness of polymers by BIACORE method and mucin-particle method

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## Abstract

To evaluate the reliability of the BIACORE method as a useful method for measuring the mucoadhesive interaction between chitosan and mucin, the mucin-particle method was used for comparison. In this study, the adhesivities of different-molecular-weight chitosans (chitosan Mw. 150,000, CS; low-molecular-weight chitosan, LCS) and hydrophobically modified chitosans (dodecylated CS, d-CS; dodecylated LCS, d-LCS) to mucin were determined. The BIACORE method showed that CS, LCS and d-CS could interact with mucin based on the increased resonance unit (RU) response after mucin was passed over the chitosans-immobilized sensor chip surface. Sensorgrams obtained from the interaction between these polymers and mucin also indicated the rate and strength of binding reaction. The rate and strength were higher for unmodified chitosans than hydrophobically modified chitosans. The simple *in vitro* mucoadhesive test or mucin-particle method revealed that the turbidity of unmodified chitosan/mucin mixtures was higher than that of dodecylated chitosans for all concentration of chitosans and mucin. The results from both BIACORE and the mucin-particle method implied that hydrophobic modification of chitosan reduced its adhesivity to mucin. The results from these two methods corresponded well. Therefore, the BIACORE method has promised as an alternative method for evaluating the adhesivity of adhesive polymers to mucin.

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**Keywords:** BIACORE method; Mucin-particle method; Mucoadhesive; Chitosan; Mucin

## 1. Introduction

Mucoadhesion is defined as the adhesive phenomenon occurring between polymeric materials and the mucus gel layer covering mucosal membranes. As mucoadhesive polymers have the potential to prolong residence time and improve drug bioavailability in drug delivery systems, they have been of interest for use as excipients in various pharmaceutical dosage forms (Imam et al., 2003).

Interactions between such polymers and mucin have been investigated and reported extensively in the literature. Recently, various methods for measuring and detecting the interaction between adhesive polymers and mucin have been reported. These include rheological synergism (Rossi et al., 2001), viscosimetric and turbidimetric measurements (Rossi et al., 2000), sedimentation fingerprinting, which is the analytical ultracentrifuge method (Deacon et al., 1999), mucous glycoprotein assay (Filipovic-Grcic et al., 2001; He et al., 1998), the fluorescence polarization method (Qaqish and Amiji, 1999) and so on. These methods have been used to detect the adhesivity of chitosan or adhesive polymers to mucin under different conditions, and vary according to the main objectives of the authors or the available instruments in their laboratories. Previously, we have developed a simple *in vitro* test, the mucin-particle method, for measuring the adhesivity of polymers to mucin (Takeuchi et al., 1999).

In this method, the adhesive properties between polymers and mucin were determined by using the change in particle size and zeta potential of the original mucin particles after mixing with polymer solutions. Moreover, we have recently developed a novel mucoadhesive test, the BIACORE method (Takeuchi et al., 2005). It was found that the BIACORE method could be used to detect and evaluate the adhesive interaction between polymers and mucin.

Therefore, in this study, to determine the adhesive properties between polymers and mucin and validate the reliability of the BIACORE method as a useful method for measuring

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mucoadhesive interaction, the mucin-particle method, which detects the mucoadhesive strength tendency of adhesive polymers and mucin by means of turbidity, was used as a reference method. Different-molecular-weight chitosans and hydrophobically modified chitosans were used as adhesive polymers to interact with mucin. The correlation between these two methods is described subsequently.

## 2. Materials and methods

### 2.1. Materials

Chitosan Mw. 150,000 (CS) (deacetylation >85%, Seelab, Germany), low-molecular-weight chitosan (LCS) (chitosan 10, deacetylation 90.2%, Wako Pure Chemical, Japan), PVA205 (Kuraray, Japan), Carbopol 971 PNF (BFGoodrich Co., England), and mucin (from porcine stomach, type II crude, bound sialic acid 1%, Sigma, USA) were obtained from the indicated sources. All other reagents used were of analytical grade.

### 2.2. Synthesis of dodecylated chitosan (d-CS) and dodecylated low-molecular-weight chitosan (d-LCS)

Hydrophobically modified chitosans (d-CS and d-LCS) were synthesized by following the method of Desbrieres et al. (1996) with some modifications. Four grams of CS or LCS were dissolved and stirred in 220 mL of 0.2 M acetic acid overnight for complete dissolution. Then, 150 mL of ethanol was slowly added into the solution to allow the aldehyde used for alkylation to remain in the solvation medium. Afterwards, the pH of the solution was adjusted to 5.1 using 0.2 M sodium hydroxide in order to prevent precipitation of the macromolecules (the optimal pH for this reaction is between 4 and 8). Subsequently, a solution of dodecyl aldehyde (92%, Mw. 184.32, Aldrich) in ethanol was added so that the ratio of the number of amino groups of chitosan to aldehyde was 1–0.05. Afterwards, an excess amount of sodium cyanoborohydride (NaCNBH<sub>3</sub>) was added to the solution as a catalyst. The mixture was stirred at room temperature for 24 h and then the by-product was removed by filtration. d-CS or d-LCS was precipitated with ethanol (gel-like form) and separated by centrifugation at 20,000 rpm for 5 min. The precipitate was washed with ethanol/water mixtures with increasing ethanol content from 70 to 100% (v/v). Then, the d-CS or d-LCS was dried in a vacuum at room temperature and stored at room tem-

perature. The chemical reaction used for the synthesis of d-CS and d-LCS is shown in Fig. 1.

### 2.3. Determination of substitution degree (SD) of dodecylated chitosan (d-CS) and dodecylated low-molecular-weight chitosan (d-LCS)

Two milligrams of dried d-CS or d-LCS was dissolved in 0.75 mL of 2% (v/v) of acetic acid-D<sub>4</sub> (CD<sub>3</sub>COOD, 99.94%D, Cambridge Isotope laboratories, Inc., USA) in deuterium oxide (D<sub>2</sub>O, 99.97%D, Euriso-top, France). Then the solution was transferred into an NMR tube and subjected to a 400 MHz <sup>1</sup>H NMR spectrometer (FT-NMR; AL-400; JEOL, Japan). The structures of d-CS and d-LCS were analyzed by <sup>1</sup>H NMR spectra, and the substitution degrees were calculated by the following equation:

$$SD = \left( \frac{\delta_{1.1\text{ppm}}}{3} \right) \times \left( \frac{100}{\delta_{3.4\text{ppm}}} \right) \quad (1)$$

where chemical shifts ( $\delta$ ) were expressed in ppm with respect to the CH<sub>3</sub> triplet at 1.1 ppm and the proton at the amino group at 3.4 ppm (Liu et al., 2003). It was found that the percentage of alkyl substitution of d-CS and d-LCS was around 3%.

### 2.4. Measurement of mucoadhesive interaction of polymers on mucin by the BIACORE method

#### 2.4.1. Preparation of mucin suspension

A mucin suspension was prepared at a concentration of 1.0% (w/v) by suspending and continuously stirring in 10 mM acetate buffer solution (ABS), pH 4.5, overnight. Then it was incubated at 37 °C for one night. After that it was ultra-sonicated with a probe sonicator (Branson Sonifier 250) until the particle size was smaller than 1 μm. Afterwards, it was centrifuged at 4000 rpm for 20 min to extract the supernatant portion. The particle size of mucin in the supernatant portion was less than 200 nm (Zetasizer 3000HSA; Malvern Instrument Ltd., UK). The supernatant portion was diluted to a concentration of 0.01% (w/v) with 10 mM ABS, pH 4.5, before use.

#### 2.4.2. Interactions between adhesive polymers and mucin

The interaction between adhesive polymers and mucin was observed under following conditions: running buffer, 10 mM

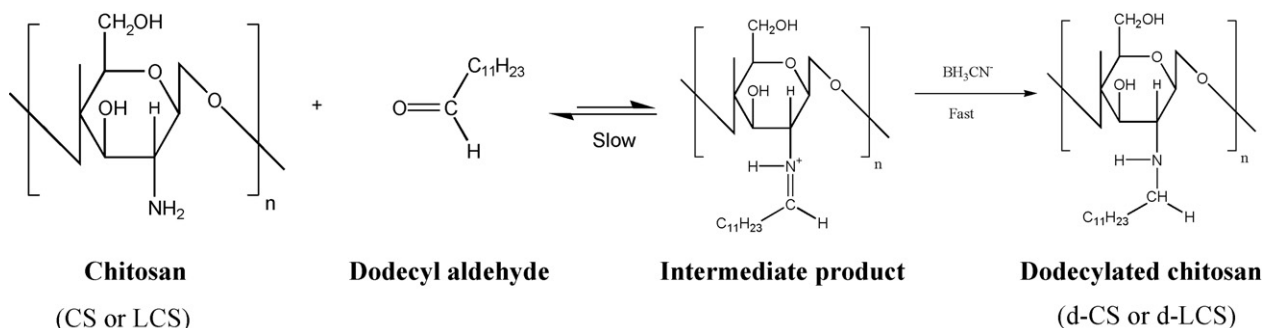


Fig. 1. Synthesis of hydrophobically modified chitosan.

ABS (pH 4.5); sensor chip, CM5 (carboxymethylated dextran); flow rate, 15  $\mu\text{L}/\text{min}$ ; temperature, 25  $^{\circ}\text{C}$ .

Prior to starting each experiment, an immobilization process was required. 0.02% (w/v) of polymer solution (CS, LCS and d-CS), prepared in 10 mM ABS, pH 4.5 (filtered through 0.2  $\mu\text{m}$  cellulose acetate membrane filter before use), was passed over the surface of a CM5 sensor chip for 10 min and baseline data was collected for 1 h. Then 0.01% (w/v) of mucin suspension was injected for 10 min and the sensorgram was collected until equilibrium. Afterwards, 0.02% (w/v) of polymer solution was injected again for 10 min, ensuring a complete equilibrium.

### 2.5. Measurement of mucoadhesive tendency of adhesive polymers on mucin by the mucin-particle method

#### 2.5.1. Preparation of mucin suspension

Mucin powder was suspended and stirred in 100 mM ABS, pH 4.4, overnight for complete dispersion. Then the mucin suspension was ultra-centrifuged (Hitachi, Japan) at 50,000 rpm at 4  $^{\circ}\text{C}$  for 15 min; the supernatant portion was collected and used in the experiment. The particle size of mucin in the supernatant portion was measured by Zetasizer 3000HSA; Malvern Instrument Ltd., UK.

#### 2.5.2. Preparation of polymer solutions

The polymers used in this experiment were CS, LCS, d-CS, and d-LCS, with PVA205 and carbopol 971 PNF as negative and positive control polymers, respectively. Polymer solutions were prepared at concentrations of 0.1, 0.3 and 0.6% (w/v) in 100 mM ABS, pH 4.4. Each polymer solution was filtered through 0.20  $\mu\text{m}$  of cellulose acetate membrane filter before use.

#### 2.5.3. Evaluation procedure of turbidimetric measurement

Equal volumes of each polymer solution and the mucin suspension were mixed by vortexing for 1 min. The mixtures were incubated at 37  $^{\circ}\text{C}$  for 1 h; then, UV spectrophotometry was performed to measure the turbidity of the mixtures at 500 nm. Each test was measured in triplicate. [www.biacore.com](http://www.biacore.com)

## 3. Results and discussion

### 3.1. Mucoadhesive interaction by BIACORE method

According to the principle of the BIACORE system, if binding occurs, the response on a prepared sensor surface will increase as the sample passes over it. If equilibrium is reached, a constant signal will be seen. The rate of reaction and the strength of the binding can be determined from the slope and the value of the RU response from the sensorgram, respectively ([www.biacore.com](http://www.biacore.com), view in 2007). Here, the adhesivity of chitosan to mucin was detected and the results of the sensorgrams are shown in Fig. 2. This figure shows that the RU response increased after mucin was passed over the CS- or LCS-immobilized sensor chip surface. The increase in RU response was the result of the change of the refractive index on the sensor chip surface when the binding molecules interacted with the immobilized molecules. Thus, these results indicate that mucin

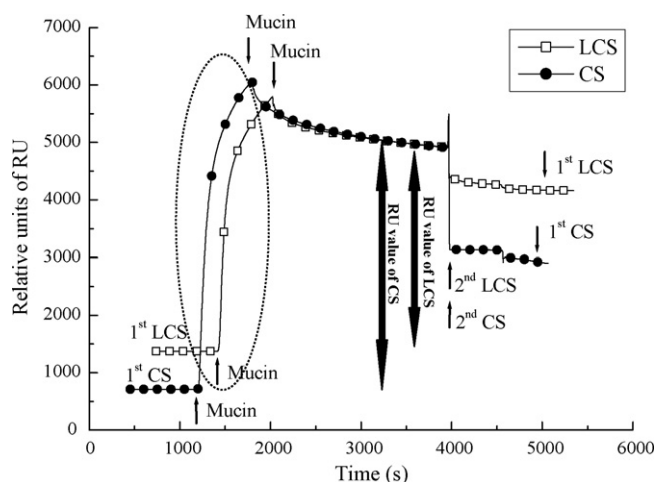


Fig. 2. Overlay sensorgrams of CS–mucin–CS and LCS–mucin–LCS interactions on CM5 sensor chip surfaces: ( $\uparrow$ ) start injection and ( $\downarrow$ ) stop injection.

particles could interact with chitosans (CS and LCS) immobilized on the sensor chip surface. The value of the RU response for the CS-immobilized sensor chip surface was slightly higher than that for the LCS-immobilized sensor chip surface (Fig. 2; two-way arrows). These findings implied that CS showed a slightly stronger binding property to mucin than LCS. Additionally, the slopes of the sensorgrams after mucin passage were the same for both the CS- and LCS-immobilized sensor chip surface (Fig. 2; dot-circle). This means that CS and LCS interacted with mucin particles at the same rate of reaction.

When CS and LCS solutions were passed over (second time) the mucin-immobilized sensor chip surface, the RU responses of both CS and LCS decreased. One possible way to explain these results is that CS might catch or hold the mucin particles on the sensor chip surface, leading to dissociation of the mucin particles from the sensor chip surface.

Fig. 3 shows the overlay sensorgrams of CS–mucin–CS and d-CS–mucin–d-CS and CS–mucin–PVA205 interactions on CM5 sensor chip surfaces. The kinetic interactions of CS–mucin and d-CS–mucin were different. At the initial step,

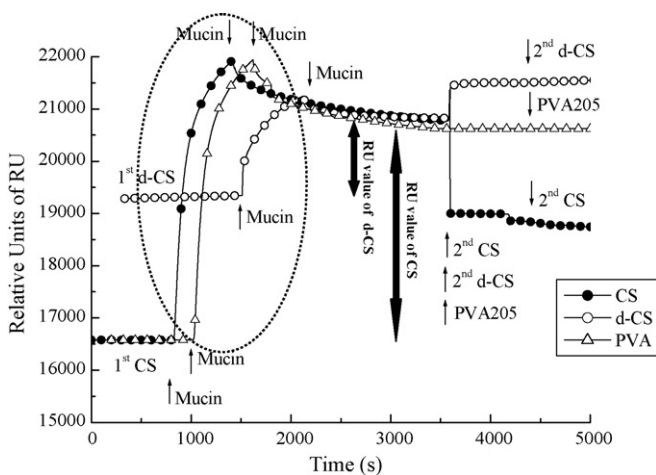


Fig. 3. Overlay sensorgrams of CS–mucin–CS, d-CS–mucin–d-CS and CS–mucin–PVA205 interactions on CM5 sensor chip surfaces: ( $\uparrow$ ) start injection and ( $\downarrow$ ) stop injection.

Table 1

Percentage of RU response decrease after injection of various adhesive polymer solutions on mucin-immobilized sensor chip (Takeuchi et al., 1999)

Polymers	Decrement of RU (%)
LCS	22.01
CS	46.34
Carbopol 971 PNF	29.58
Carbopol 974 PNF	27.57
PVA 205	3.26
PVP 25 (Kollidon 25)	7.92
TC-5 S (HPMC 2910)	6.78
90SH-400 (HPMC 2208)	8.58
65SH-50 (HPMC 2906)	9.60

the RU response after mucin was passed over the CS or d-CS immobilized sensor chip surface increased in both cases. However, after CS or d-CS was passed over (second time) the mucin-immobilized sensor chip surface, the RU response of CS decreased whereas that of d-CS increased. In addition, the slope of the RU response after mucin was passed over the sensor chip was lower for the d-CS immobilized surface than the CS immobilized surface (Fig. 3; dot-circle). However, the RU response after CS/d-CS solution was passed over mucin on the chitosan-immobilized sensor chip surface was much higher for CS than for d-CS (Fig. 3; two-way arrows). The differences in slope and RU values show that d-CS interacted with mucin at a slower rate of reaction and with a weaker mucoadhesive force than CS. This means that hydrophobic modification of chitosan might reduce its mucoadhesive properties. Since PVA205 is non-mucoadhesive (Davies et al., 1991), there was no much change in the RU response when it was passed over the mucin-immobilized-over-CS sensor chip (Fig. 3 and Table 1).

All above results implied that the BIACORE method could be used for detection and prediction of the binding properties of chitosan to mucin. Not only could chitosan's mucoadhesive binding be detected by BIACORE, but that of other adhesive polymers could also be determined, as shown in Fig. 4. In addition,

BIACORE could be used for characterization of polymer properties. In Table 1, the difference of RU decrease of these polymers could be used to determine the difference in adhesive binding strength of these polymers to mucin as well. The results show that LCS, CS and carbopol possess higher adhesivity to mucin than PVA and HPMC because of higher values of RU decrease.

There are several theories to explain mucoadhesion of polymeric materials. A diffusion theory is one of the main theories proposed to describe mucoadhesion which includes the action of the polymer-chain entanglement (Chickering and Mathiowitz, 1999). Diffusion theory states that interpenetration of the chains of polymer and mucus may lead to sustained mucoadhesion and by mechanical interlocking between mucin and mucoadhesives. The fundamental concept of the diffusion theory is that adhesion arises through the inter-diffusion of molecules in the adhesive and adherent. The diffusion theory is primarily applicable when both the adhesive and adherent are polymeric, having compatible long-chain molecules capable of movement. Parameters affecting the diffusion process are: contact time, temperature, molecular weight of polymers and physical form (liquid, solid). In these experimental conditions, we may conclude that contribution of physical entanglement to adhesion phenomenon between PVA (and HPMC) and the surface of mucin particles is not so strong in this measurement with BIACORE.

### 3.2. Mucoadhesive tendency by mucin-particle method

As in our previous studies, the mucin-particle method was used for screening and detecting the mucoadhesive properties of polymers by measuring the change in particle size and zeta potential of mucin original particles. In this study, based on the mucin-particle method with some modifications, the turbidity value after mixing of the polymer and mucin is another factor that could be used to roughly determine the adhesivity between polymers and mucin. If the initial turbidity of mucin increased in the presence of the adhesive polymers, the interaction between polymer and mucin probably occurred because of the aggregation of mucin particles in a system having adhesive polymers as a linkage.

In this study, turbidities as a function of relative adhesive strength between polymers and mucin under various concentrations of mucin (2, 4 and 12 mg/mL) and polymer solution at 0.1, 0.3 and 0.6% (w/v) were determined. PVA205, a non-adhesive polymer, and carbopol 971 PNF, which possesses adhesivity to mucin (Takeuchi et al., 2003), were used as a negative and positive control in this experiment, respectively. In Figs. 5–7, when the polymer concentrations were retained at constant, the turbidity of the carbopol/mucin, CS/mucin, LCS/mucin, d-CS/mucin and d-LCS/mucin increased with increasing concentrations of mucin. The turbidity of the LCS/mucin mixture at any concentration was higher than that of CS/mucin. Moreover, the turbidity of the d-CS/mucin mixture at any concentration was higher than that of the d-LCS/mucin mixture. Thus, the turbidity of the mixtures could be ordered from high to low as LCS, CS, d-CS and d-LCS. On the other hand, when mucin concentrations were kept constant, the turbidity of carbopol/mucin, CS/mucin,

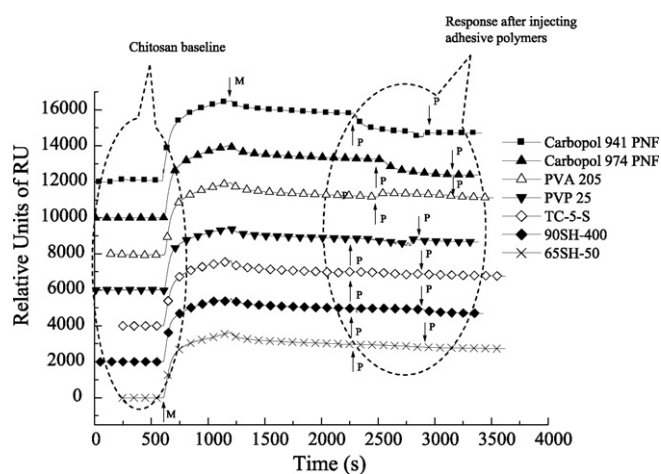


Fig. 4. Overlay sensorgrams of mucin-immobilized sensor chip after injection of the various adhesive polymer solutions: (↑M and ↓M) start and stop mucin injection, respectively and (↑P and ↓P) start and stop polymer injection, respectively (Takeuchi et al., 1999).



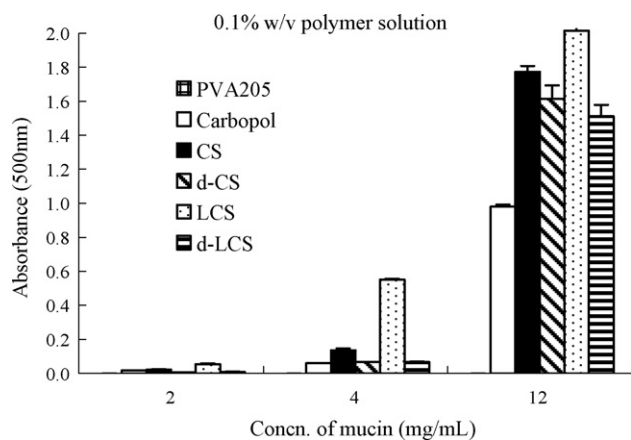


Fig. 5. Turbidity as a function of adhesive strength between 0.1% (w/v) of polymer solution and mucin ( $n=3$ ).

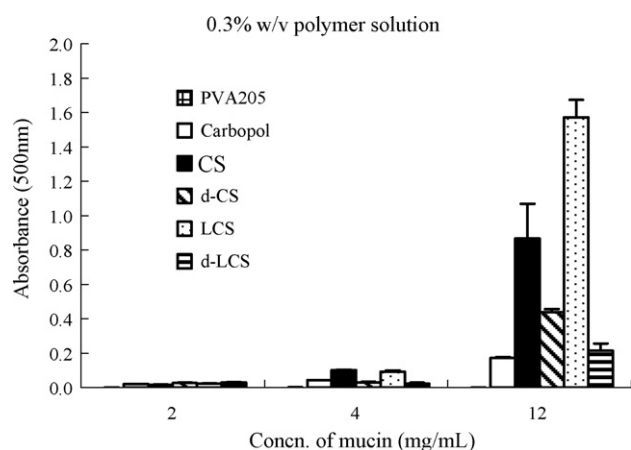


Fig. 6. Turbidity as a function of adhesive strength between 0.3% (w/v) of polymer solution and mucin ( $n=3$ ).

LCS/mucin, d-CS/mucin and d-LCS/mucin mixtures decreased with increasing concentrations of polymer. These results can be explained by considering the interaction between these polymers and mucin. Mucins are elongated rod-shaped molecules

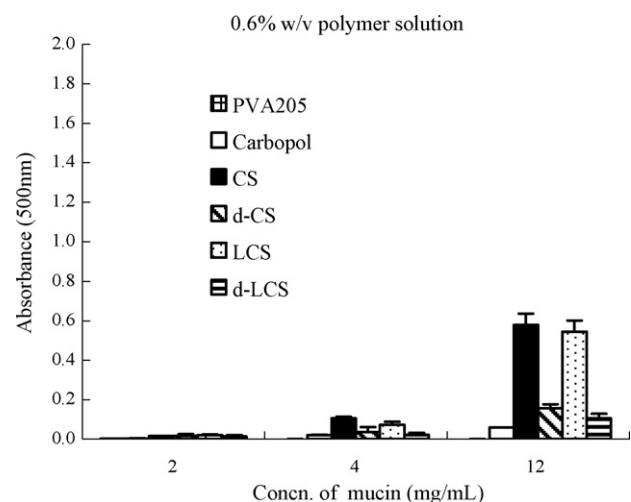


Fig. 7. Turbidity as a function of adhesive strength between 0.6% (w/v) of polymer solution and mucin ( $n=3$ ).

composed of (1) branching sugar chains enriched with serine, threonine and proline, which possess hydroxyl, carboxylic and sulfate groups resulting in highly hydrophilic properties and (2) a backbone chain enriched with an amide group resulting in hydrophobic properties. In addition, mucin has an amino terminus and a carboxyl terminus at either end of its molecule (Bansil et al., 1995; Peppas and Huang, 2004). Therefore, the structural characteristics of mucin can be used to explain the interaction between these polymers and mucin. The explanations will be presented in the following order: first, for the results at a constant concentration of polymer, and then for the constant mucin concentration.

The following discussion treats changes in turbidity when the polymer concentrations were kept constant. As expected, no turbidity of the mixture of PVA205 and mucin was observed at any concentration because the molecular structure of PVA205 does not provide any active charges or active group with adhesivity to mucin. This result corresponded to the BIACORE method results (Figs. 3 and 4) showing no change of RU response after PVA205 was passed over mucin-immobilized-over-CS sensor chip surfaces. In the case of carbopol 971 PNF, the turbidity of its mixture showed the same trend as that of chitosan and hydrophobically modified chitosan, but the turbidity of the mixture of carbopol 971 PNF and mucin was lower. This result indicated that even carbopol 971 PNF could interact with mucin but the strength of its mucoadhesivity was less than that of the chitosans. These results also corresponded to the BIACORE method results.

When unmodified chitosans (CS and LCS) interacted with mucin, the turbidity of the LCS/mucin mixture at any concentration was higher than that of CS/mucin mixture. These results can be explained by considering molecular weight and size. The molecular weight of LCS (about 22,000 Da (Yamada et al., 2003)) was about seven times lower than that of CS; it is considerably smaller. Therefore, the number of LCS molecules interacting with mucin was greater than that of CS when compared at the same concentration. Given the small molecular size of LCS, it may insert itself into the branching sugar chains of mucin, allowing interactions between amino groups on LCS molecules and active groups on the sugar side-chains of mucin, and thereby resulting in stronger mucoadhesivity. At this point, it is important to note that the mucoadhesive strength results of CS/mucin and LCS/mucin from the BIACORE method were different from those of the turbidimetric method. This difference might be the result of the interaction process. In the BIACORE method, the interaction process between chitosan and mucin is a flowing movement (flow rate: 15  $\mu\text{L}/\text{min}$ ) while in the turbidimetric method, the interaction process is a vortex movement.

In the case of hydrophobically modified chitosans, the turbidity results of d-CS/mucin and d-LCS/mucin mixtures were lower than that of unmodified chitosan/mucin mixtures. These results indicated that the hydrophobic modification of chitosan (d-CS and d-LCS) led to a decrease in the strength of adhesivity between the polymers and mucin. This phenomenon might be due to the structure of mucin. The hydrophobic region of mucin is covered with the hydrophilic region of branching sugar chains (Bansil et al., 1995; Harginc, 2003). The carbon side chain of

Table 2

Changes in particle size of the various concentrations of polymer/mucin mixture dispersed in DI water

Mucin concentration* (mg/mL)	Particle size (nm)											
	0.1% (w/v)				0.3% (w/v)				0.6% (w/v)			
	CS	d-CS	LCS	d-LCS	CS	d-CS	LCS	d-LCS	CS	d-CS	LCS	d-LCS
2	195.5	174.3	159.1	173.7	238.5	183.2	169.4	184.6	410.7	523.5	200.7	349.0
4	230.6	224.8	216.9	202.6	335.7	256.9	228.1	196.9	435.5	329.9	236.8	336.4
12	509.0	477.4	314.9	302.0	557.5	430.1	352.9	379.8	790.4	618.1	431.4	480.1

\* Initial particle size of mucin: 90–100 nm.

d-CS and d-LCS could not interact with the hydrophilic region of mucin leading to lower turbidity and less adhesion on the sensor chip in the BIACORE method. Protonated amino groups on the d-CS and d-LCS molecule could potentially interact with the sialic group on mucin resulting in mucoadhesivity, but this process may be impeded by the long hydrocarbon side chain on d-CS and d-LCS molecules, leading to lower mucoadhesivity. These results also corresponded to the BIACORE results.

Next, we discuss the change in turbidity when mucin concentrations were kept constant. The turbidity of all of the polymers decreased with increasing concentrations of polymer. One possible explanation is that the particle size of the polymer–mucin complex increased, as shown in Table 2, leading to a decrease in turbidity. In this case, the decrease of turbidity when the polymer concentration increased does not indicate lower mucoadhesivity. Consequently, the optimum concentration of a polymer is a factor that should be concerned in the mucoadhesivity determination process. These false negative results are a weakness of the mucin-particle method (turbidity method) not shared by the BIACORE method.

#### 4. Conclusion

The mucoadhesive properties between polymers and mucin could be detected by the BIACORE method and the mucin-particle method. Consequently, the BIACORE method should be considered a useful alternative to the mucin-particle method for determining the adhesivity between polymers and mucin.

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