



Study on the mucoadhesion mechanism of pectin by atomic force microscopy and mucin-particle method

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ABSTRACT

Atomic force microscopy (AFM) has been used to probe the interaction between porcine stomach mucin and a mucoadhesive polymer, pectin, with different chemical characteristics. Images were produced detailing the structures of mucin, pectin and the mixtures of pectin and mucin, in either 0.1 N hydrochloric acid or deionized water. The AFM images of the pectin–mucin mixture in acidic medium showed no association between pectin and mucin. The large aggregates observed after mixing pectin and mucin in deionized water revealed the association between pectin and mucin, probably by the H-bonding. Increasing of pectin in the mixture with mucin resulted in a shift of zeta potential of the mixture to a higher negative value. The electrostatic repulsion with the same charges of pectin and mucin may cause an uncoiling of polymer chains, which facilitated chain entanglement and bond formation. The particle size of the mixtures of pectin and mucin depended on the proportion of either pectin or mucin in the mixture. The results suggested that the mucoadhesion of pectin could be due to the adsorption mechanism on the mucin molecules or electrostatic repulsion between pectin and mucin.

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1. Introduction

The mucoadhesive mechanism was firstly proposed by Duchene, Touchard, and Peppas (1988). The first stage involved an intimate contact between a mucoadhesive and a mucus surface, either from a good wetting of the bioadhesive surface or from the swelling of the mucoadhesive polymer. In the second stage, after contact is established, penetration of the bioadhesive polymer chain into tissue surface or interpenetration of the chains of the bioadhesive polymer and the mucus was occurred. Then, weak chemical bonds could be formed in the final stage. The theories, which were developed to understand and explain the adhesive performance of adhesives, have been adapted to gain an understanding of bio/mucoadhesion. Five theories being proposed to explain the mucoadhesion phenomena are wetting, adsorption, diffusion, electrical and fracture theories.

One of the proposed theories in mucoadhesion is the adsorption theory. After an initial contact between two surfaces, the material adhered because of surface forces acting between the molecules in the two surfaces. Two types of chemical bonds resulting from these forces were established (Ahuja, Khar, & Ali, 1997). The interpreta-

tion in terms of adsorption is generally evaluated by putting particles of polymer in contact with mucosal surfaces and evaluating either the amount of particles attached to the mucosa or the amount of non-adherent particles (Ponchel & Irache, 1998). The adsorption studies of the mucoadhesive potential were examined on a model intestinal absorption of solutes. The adsorption of mucin to aminated gelatin microspheres was examined by determination non-adsorbed mucin after mixing by using colorimetry (Wang, Tabatab, Bi, & Morimoto, 2001). Furthermore, the atomic force microscopy (AFM) has been used to visualize the adsorption of polymer and mucin/mucosal cell surfaces, i.e. the visualization of the mucin–chitosan mixture (Deacon et al., 2000), the visualization of bioadhesive polymer adsorption onto human buccal cells (Patel et al., 2000).

Surface properties of mucoadhesive polymer and mucin in mucus layer can be examined to support the electrical theory. The assumption of this theory is that the mucoadhesive polymer and the mucosal surface have different electrical charges. When the polymers come in contact with the mucosal surface, electron transfer occurs in an attempt to balance Fermi levels, causing the formation of a double layer of electrical charge at the polymer–mucin interface. The mucoadhesive force is believed to be due to attraction forces across this electrical double layer. The system is charged when the polymer and mucosal surface are in contact and discharged when they are separated. The method used for studying electrical structure of polymers is surface charge proper-

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ties measurement using zeta potential meter. Recently, the mucin-particle method measuring the zeta potential and particle size of mucoadhesive materials and mucin particles has been used (Takeuchi et al., 2005).

Pectin is regarded as safe for human consumption and has been used successfully for many years in food and pharmaceutical industries. As it is rich of carboxylic groups and possible to interact with functional groups in mucus layer, it has been used as a mucoadhesive polymer for controlled drug delivery (e.g. Liu, Fishman, Hicks, & Kende, 2005; Schmidgall & Hensel, 2002; Thirawong, Kennedy, & Sriamornsak, 2008; Thirawong, Nunthanid, Puttipipatkachorn, & Sriamornsak, 2007). Liu et al. (2005) reported that pectin with higher net electrical charges showed a higher mucoadhesion with porcine colonic tissues than the less charged ones. Recently, Thirawong et al. (2007) reported the mucoadhesive performance of various pectins onto the gastrointestinal tract, investigating by texture analysis. The results demonstrated the mucoadhesive properties of pectin against GI mucosa with the strongest mucoadhesion in large intestine. Moreover, mucoadhesive performance of pectins largely depended on their characteristics, i.e. degree of esterification and molecular weight. The wetting behavior of pectin surfaces increased with the decreased degree of esterification, indicating hydrophilic nature of the molecules (Sriamornsak, Wattanakorn, Nunthanid, & Puttipipatkachorn, 2008). The rheological parameters, which investigated by a viscometer (Thirawong et al., 2008) or a dynamic oscillatory rheometer (Sriamornsak & Wattanakorn, 2008), increased after mixing of pectin and mucin indicating the interaction between pectin and mucin due to physical entanglement.

Pectin is a cell wall structural carbohydrate present in all higher plants. Commercially available pectin is obtained from edible plants. Though it is a heterogeneous polysaccharide, pectin contains linear chains of (1–4)-linked α -D-galacturonic acid residues. The linear structure of pectin is partly interrupted by (1,2)-linked side-chains consisting of L-rhamnose residues and some others neutral sugars (Rolin, 1993). The galacturonic acids have carboxyl groups, some of which are naturally presented as methyl esters and others which are reacted with ammonia to produce carboxamide groups. The degree of esterification (DE) and degree of amidation (DA), which are both expressed as a percentage of carboxyl groups (esterified or amidated), are an important means to classify pectin. The DE less than 50% is so-called low methoxy pectin while DE more than 50% is so-called high methoxy pectin (Rolin, 1993).

Our previous studies have shown the mucoadhesive properties of various pectins by wetting (Sriamornsak et al., 2008), diffusion (Sriamornsak & Wattanakorn, 2008; Thirawong et al., 2008) and fracture (Thirawong et al., 2007) theories. The objective of this study was, therefore, to reveal further the mucoadhesive properties of pectin according to the adsorption and electrical theories. As per adsorption theory, the interaction between pectin and mucin was visualized by using AFM. The mucin-particle method, which measures zeta potential and particle size, was used to explain the mucoadhesive strength tendency of pectin and mucin according to the electrical theory.

2. Materials and methods

2.1. Materials

Three commercial pectins with different DEs and molecular weights (MWs), namely CU201, CU701 and CU020 (see Table 1), were kindly provided by Herbstreith & Fox KG (Germany). Porcine stomach mucin, type II, with bound sialic acid 9–17% was purchased from Sigma Chemical Co., Ltd. (USA). All other chemicals were analytical grade and used as received without further purifi-

cation. Deionized (DI) water was prepared by reverse osmosis throughout all experiments.

2.2. Atomic force microscopy (AFM) observation

Pectin, mucin and a mixture of pectin and mucin (ratio 1:1) were diluted with filtered DI water to 2–4 $\mu\text{g/mL}$. An aliquot (2 μL) of the diluted sample solutions were immediately spread on freshly cleaved mica surfaces. The sample was then allowed to dry at ambient temperature (20 °C) for 20 min. Samples in 0.1 N hydrochloric acid (HCl) were also prepared in the same manner as those in DI water. Samples in other media, e.g. in phosphate buffer saline (PBS, pH 6.8), could not be observed because of the interference of phosphate ions. Tapping mode was carried out using a multimode NanoScope IIIa AFM (Digital Instruments, USA) equipped with Phosphorus (n) doped Si (Veeco, model RTESP) cantilever with a quoted spring constant of 20–80 N/m. The scan speed of 2 Hz was used for imaging. Several images of different zones were examined since AFM images are generally limited to small scanned areas. Height mode was used for image analysis. The correction of the images by commercial image processing software (Adobe Photoshop, Version 6.0.1, Adobe Systems Inc., USA) with glowing edges in grayscale mode was performed to enable a reduction of the noise.

2.3. Zeta potential measurement

Pectin and mucin were separately dispersed in DI water, 0.1 N HCl and PBS (pH 6.8) to make stock solutions of 1% w/w. Consequently, the pectin and mucin stock solutions were diluted with corresponding media to make the final concentration of 0.5–0.01% w/w. The pH of solutions was also determined. The zeta potential measurement for all formulations was conducted using a zetasizer (Model 3000HSA, Malvern Instrument Ltd., UK). All samples were controlled at 25 ± 0.1 °C during the test. These experiments were repeated 10 times and the mean values and standard deviations were calculated.

2.4. Mucin-particle method

Pectin and mucin were separately suspended in PBS (pH 6.8) with a concentration of 1% w/w. The sample solutions were then centrifuged at a speed of 50,000 rpm ($27,950 \times g$) for 15 min. An appropriate amount of supernatant of pectin and mucin was mixed together in various ratios (i.e. 100:1, 10:1, 1:1, 0.1:1 and 0.01:1). The mixtures were incubated at 37 °C for 1 h prior to the test. The zeta potential and particle size of each sample were detected with a particle sizer (Zetasizer 3000HSA, Malvern Instrument Ltd., UK).

2.5. Statistical analysis

Analysis of variance (ANOVA) and Levene's test for homogeneity of variance were performed using SPSS version 10.0 for Windows (SPSS Inc., USA). *Post hoc* testing ($p < 0.05$) of the multiple comparisons was performed by either the Scheffé or Games-Howell test depending on whether Levene's test was insignificant or significant, respectively.

3. Results and discussion

3.1. AFM images

Mucin, a key component in mucus layer, is a block copolymer with branched and un-branched blocks. Both blocks contain

Table 1

Designation and properties of pectin examined in the study.

Pectin type and designation	Degree of esterification (DE, %)	Degree of amidation (DA, %)	Molecular weight (Da)
High methoxy pectin CU201	70	0	200,000
Low methoxy pectin CU701	38	0	80,000
Amidated low methoxy pectin CU020	29	20	150,000

Note: The DE, DA and molecular weight are specified and reported by the manufacturer.

protein backbone chains and the branched blocks contain many highly branched oligosaccharide chains. The oligosaccharide side chains have sugar residues such as galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid. Figs. 1 and 2 show the AFM images of porcine gastric mucin in 0.1 N HCl and DI water, respectively. Mucin particles in acidic medium showed aggregated chains with a large size, whereas those in DI water showed a particle-like structure with the aggregation of small particles which were smaller than in 0.1 N HCl. This is probably due to the porcine stomach mucin formed gel at low pH but can disperse at higher pH environment. As the pH was lowered in dilute solutions, porcine stomach mucin underwent a conformational change from an isotropic random coil at pH about 7 to an anisotropic extended random coil at pH 1.2. This agreed with Bansil and Turner (2006) in which the conformation of commercial porcine gastric mucin in dilute solution examined by viscosity and circular dichroism measurements revealed similar changes, as a function of pH, which were attributed to the unfolding of hydrophobic domains at low pH. Marriott and Gregory (1990) revealed that, at pH > 3, sialic acid

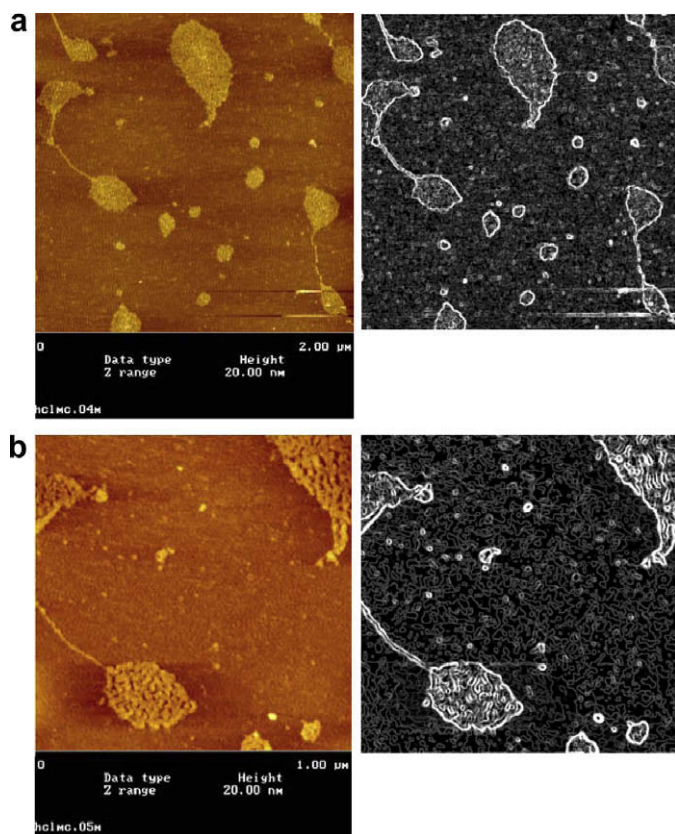


Fig. 1. Topographical (left) and equivalent processed (right) images from AFM of mucin in 0.1 N HCl; (a) image size 2.0 × 2.0 μm² and (b) image size 1.0 × 1.0 μm².

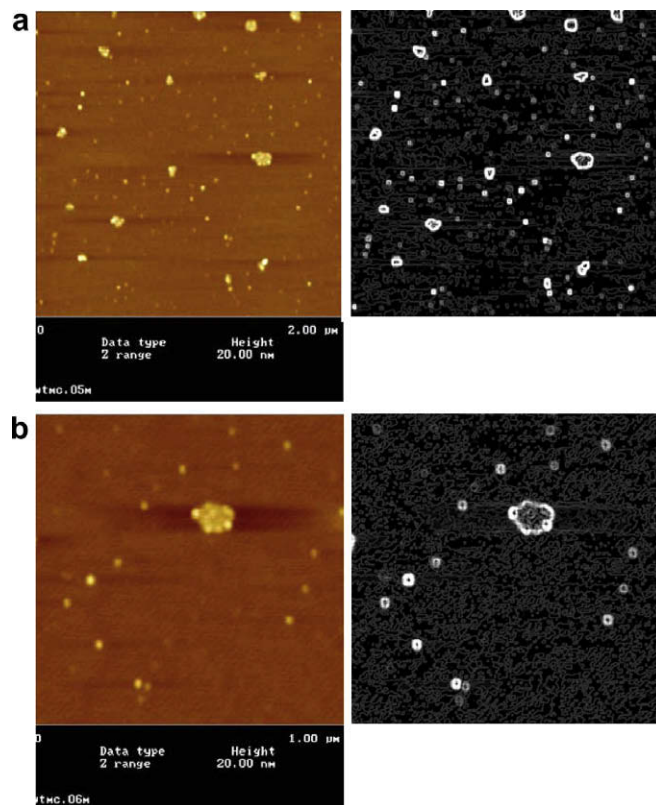


Fig. 2. Topographical (left) and equivalent processed (right) images from AFM of mucin in DI water; (a) image size 2.0 × 2.0 μm² and (b) image size 1.0 × 1.0 μm².

and sulfated sugars were fully ionized and this conferred a net negative charge to the molecule. The dried mucin could be dispersed in aqueous medium because it contains numerous H-bonding groups, e.g. the hydroxyl groups in the branched sugar chains, the amide groups in the backbone chains, and some carboxylic or sulfate groups in the terminal segments of branch chains (Peppas & Huang, 2004).

Hong et al. (2005) demonstrated that mucin was extended fiber-like molecule at pH 6, aggregated at pH 4 and formed well-defined clusters at pH 2. However, the long filamentous strands (about 2 μm length) with diameter of 16 nm of purified porcine gastric mucus glycoprotein in 0.1 M sodium acetate buffer have been reported by Deacon et al. (2000). The difference in the observed structure may be due to the different test conditions and dispersion media. Brayshaw, Berry, and McMaster (2003) suggested that the most favorable conditions for imaging ocular mucin in air under AFM are in 10 mM buffered solution with 2 mM NiCl₂. The NiCl₂ could increase the amount of mucin bound to the mica since both mica and mucin are negatively charged (Brayshaw et al., 2003; Hansma & Laney, 1996).

All pectins in DI water showed a chain-like structure with a small number of branches (Fig. 3a). It is likely that CU201 in 0.1 N HCl showed more chain aggregation than in DI water (Fig. 3b). The possible explanation is that pectin was unionized in acidic environment, resulting in unexpanded chains. In other words, gelation or aggregation of pectin was induced by the decreased pH of medium. The morphology of a mixture of CU201 and mucin in 0.1 N HCl is shown in Fig. 4. The images displayed some large particles and small chains spreading all over the image, without cross-linking. This suggested that pectin and mucin molecules were separated from each others in acidic environment. This is probably due to both pectin and mucin were in unionized form, leading to coil formation.

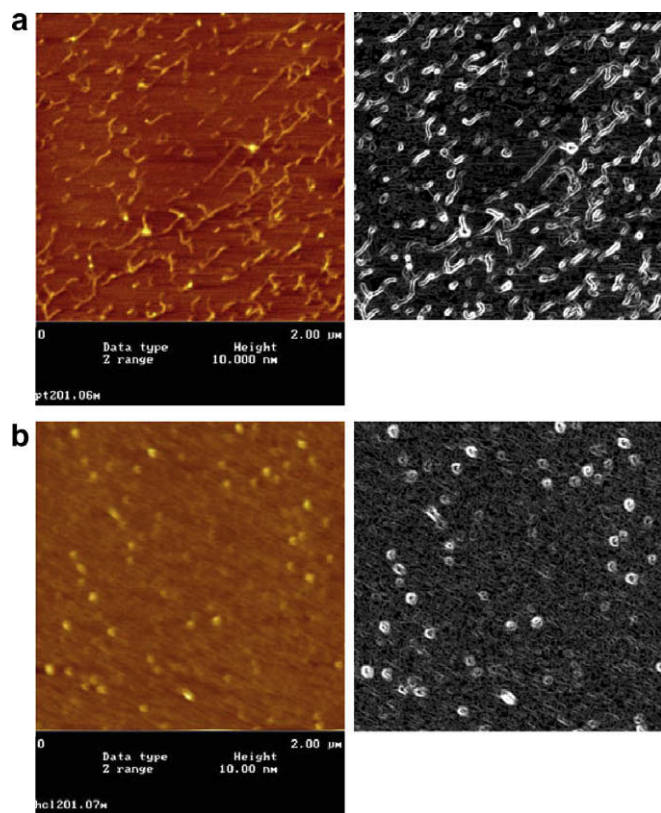


Fig. 3. Topographical (left) and equivalent processed (right) images from AFM of CU201 in (a) DI water and (b) 0.1 N HCl. Image size is $2.0 \times 2.0 \mu\text{m}^2$.

In contrast, the mixture of pectin and mucin in DI water showed the association of the chains (Fig. 5). It is thought that the structure of mucin was changed from the agglomerated state to the expanded chain and facilitated the interaction with pectin chain. Deacon et al. (2000) reported that the large clump-like aggregates with surrounding of a tangled arrangement of filaments were observed after mixing of porcine gastric glycoprotein and chitosan in 0.1 M sodium acetate buffer, due to the electrostatic interaction. In the case of pectin, the electrostatic attraction might not be occurred as both pectin and mucin showed negative charge in DI water (see Section 3.2). The possible explanation for these interactions is that both pectin and mucin in water were partially ionized, leading to coil expansion which facilitated chain entanglement. The hydrophobic interaction between pectin and mucin may also establish for bond formation. In addition, the uncharged segments in pectin molecules could interact with mucin via H-bond formation. Due to the presence of amide groups, together with carboxyl groups, in the structure of CU020, the H-bond formation may be stronger than that of CU701 which has only carboxyl groups (Srimornsak et al., 2008).

3.2. Surface charges of pectin and mucin

Pectin showed negative charge in DI water and pH 6.8 PBS (Table 2). Mucin also showed negative charge in DI water and pH 6.8 PBS. The negative charges of pectin and mucin were due to the ionization of carboxyl groups in pectin and sialic acid in mucin since the environmental pH was higher than their pK_a ; pK_a of pectin and mucin were about 3–4 and 2.6, respectively. Pectin in DI water showed more negative charge than in PBS. It is thought that the ions in the medium would suppress the expression of negative charge. Zhu, Fan, Li, Xiao, and Zhang (2007) found that the zeta

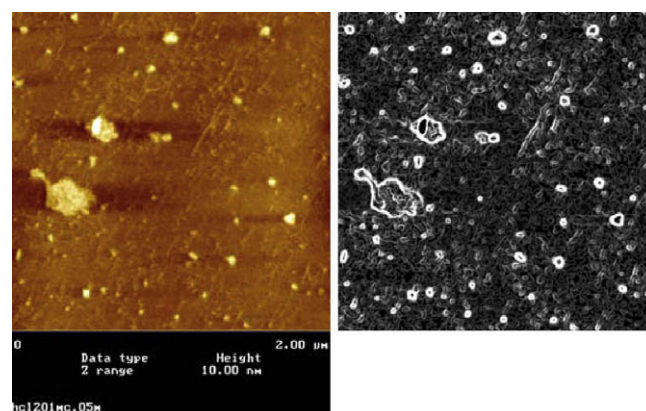


Fig. 4. Topographical (left) and equivalent processed (right) images from AFM of the mixture between CU201 and mucin in 0.1 N HCl. Image size is $2.0 \times 2.0 \mu\text{m}^2$.

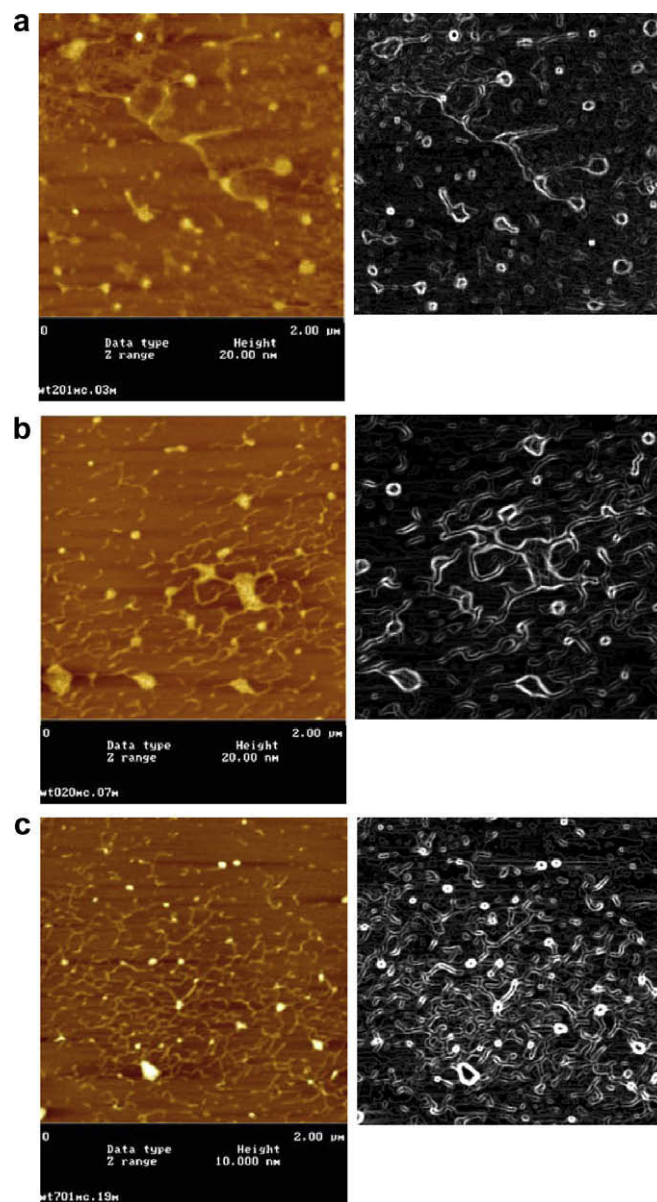


Fig. 5. Topographical (left) and equivalent processed (right) images from AFM of the mixture between pectin and mucin in DI water, (a) CU201, (b) CU020 and (c) CU701. Image size is $2.0 \times 2.0 \mu\text{m}^2$.

Table 2Zeta potential and pH of pectin and mucin in different media ($n = 10$).

Medium	Polymer	Zeta potential (mV)	pH
DI water	0.05% w/w Mucin	-23.94 ± 1.67	6.53
	0.01% w/w CU201	-48.93 ± 2.46	4.51
	0.01% w/w CU020	-73.28 ± 1.61	5.53
	0.01% w/w CU701	N/A ^a	4.41
Phosphate buffer saline (pH 6.8)	0.05% w/w Mucin	-19.08 ± 0.94	6.95
	0.01% w/w CU201	-32.80 ± 0.60	6.93
	0.01% w/w CU020	-43.57 ± 1.02	6.95
	0.01% w/w CU701	-42.99 ± 1.31	6.88
0.1 N Hydrochloric acid	0.05% w/w Mucin	$+1.38 \pm 0.11$	2.12
	0.01% w/w CU201	-2.42 ± 0.33	2.12

Note: N/A = not applicable.

^a The values could not be detected due to the sensitivity limit of the instrument.

potential of calcium phosphate particles in medium was influenced by pH, ionic strength, concentration of calcium and phosphate ions. High DE pectin in 0.1 N HCl showed very low zeta potential values because the pH of 0.1 N HCl was lower than its pK_a , resulting in unionization of molecules. In 0.1 N HCl, the mucin showed a positive charge. This is because the isoelectric point (pI) of mucin, a glycoprotein, is about three. At a pH below its pI, it shows a net positive charge; above its pI it shows a net negative charge.

3.3. Interaction between mucin particles and pectin

Fig. 6 shows the zeta potential and mean particle size of mucin and the mixture, after mixing of pectin in various ratios. The results showed that the zeta potential of mucin was negative charge and it was shifted to a higher negative value after mixing with pectin. This strongly supported what happened in the mixture and the possibility of the mucin-particle method for evaluating the mucoadhesive properties of pectin (Takeuchi et al., 2005).

A higher pectin to mucin (P:M) ratio showed a higher negative zeta potential value. This might be owing to the increased number of negatively charged pectin in the mixtures. Similar results (Gu, Robinson, & Leung, 1988) were also observed for carbomer934P having negative charge similar to pectin. It has been suggested that the interaction of carbomer with mucin was due to the dissociation of carboxyl groups and electrostatic repulsion between the negative charges of carboxyl groups and sialic acid in mucin, causing the uncoiling and expansion of the molecules which facilitated for molecular entanglement (Liu et al., 2005).

It is likely that the interaction between pectin and mucin is similar to that of carbomer and mucin because of the same negative charges. Our previous study (Sriamornsak et al., 2008) also showed no ionic interaction between pectin and mucin when investigated by FTIR. However, other bond formations such as H-bond and hydrophobic interaction may be established. High DE pectin showed a lower negative value than low DE pectin after mixing with mucin (Fig. 6). This is probably due to a smaller number of free carboxyl groups in the structure of high DE pectin. It is suggested that an increase in the content of hydrophobic groups (e.g. methoxy group) in polymer chain led to a more efficient adsorption of pectin on the surface of mucin particles, which shows the importance of hydrophobic effects in mucoadhesion (Fefelova, Nurkeeva, Mun, & Khutoryanskiy, 2007). The results are also in agreement with the mucoadhesive properties of pectins investigated by texture analyzer (i.e. fracture test) in which a higher DE and molecular weight of pectin (i.e. CU201) demonstrated a stronger mucoadhesion (Thirawong et al., 2007).

The mean particle size of mucin was around 120 nm and that of pectin was around 1 μm for CU201, 2.5 μm for CU020 and 0.5 μm for CU701. However, the polydispersity indices of both mucin and

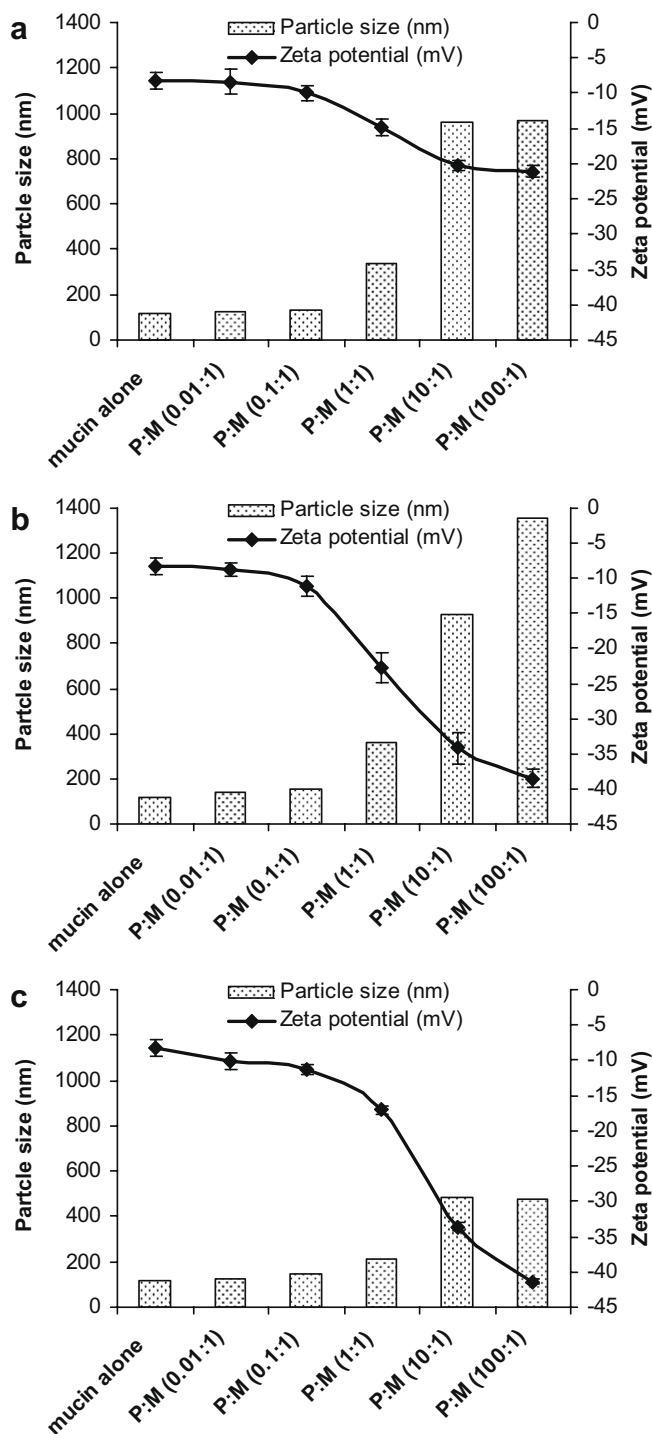


Fig. 6. Zeta potential (mV) and mean particle size (nm) of mucin (type II) and the mixtures between pectin (P) and mucin (M) in various ratios; (a) CU201, (b) CU020 and (c) CU701. The samples were dispersed in phosphate buffer saline solution (pH 6.8).

pectin particles were very high (around 0.8–1.0), indicating the particle size distribution was in a wide range. After mixing pectin with mucin, the particle size of the mixture was changed according to proportion of either pectin or mucin in the mixture. Once P:M ratio was low, the particle size of the mixture decreased due to a higher amount of mucin-particle. The polydispersity indices of all the mixtures showed the number close to 1, as their particle size distribution was still in a wide range. Because a large particle size with wide distribution of pectin was mixed with a small size of

mucin, the particle size of the mixture would be large. So the aggregation of particles could not be observed by particle size measurement. It was thought that small particles (e.g. submicron-sized particles) with a narrow distribution of both mucin and polymer particles would be necessary for measuring the mucoadhesive interaction by mucin-particle method. Therefore, the mucin-particle method may not be suitable for studying pectin and mucin interaction because of a very large size and a wide size distribution of pectin. However, in this study, the relationship between the particle size and zeta potential value was observed. The results revealed that the increase of particle size in the mixture related to a higher negative value of zeta potential since the amount of pectin, which was large in size and contained a large number of carboxyl groups in the mixture, were increased.

4. Conclusions

The visualization of pectin adsorption on mucin molecules was revealed by the AFM. The AFM images of the pectin–mucin mixture in acidic medium showed no association between pectin and mucin. On the contrary, the association of the pectin and mucin molecules in water was observed. The association might be due to the H-bond formation. The H-bonding may be stronger with CU020 than other types of pectin as the association was clearly seen in the AFM images. These results suggested that the mucoadhesive properties of pectin could be due to the adsorption mechanism on the mucin molecules. Increasing of pectin in the mixture with mucin, the resultant zeta potential of the mixture shifted to a higher negative value because of the negative charge of pectin. The electrostatic attraction between negative charges would not occur in the mixture of pectin and mucin. However, the electrostatic repulsion with the same charges might result in an uncoiling of polymer chains, which facilitated chain entanglement and bond formation. The study of interaction between pectin and mucin by mucin-particle method was limited due to a wide size distribution of pectin samples.

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