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A new approach to study mucoadhesion: colloidal gold staining

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Summary

A new in vitro method called "mucin–gold staining" was developed for the quantitative comparison of mucoadhesive properties of various hydrogels. The technique employs red colloidal gold particles which are stabilized by the adsorbed mucin molecules (mucin–gold conjugates). Upon interaction with mucin–gold conjugates, mucoadhesive hydrogels develop a red color on the surface. Thus, the mucoadhesive properties of hydrogels can be compared quantitatively by measuring the intensity of the red color. The pH-dependent stability of mucin–gold conjugates were examined and optimum conditions for mucin–gold staining were determined. The validity of the mucin–gold staining as a method to determine mucoadhesive property was tested using acrylic hydrogels of which mucoadhesive properties were well known. The potential applications and the advantages of this new in vitro technique are discussed.

Introduction

Mucoadhesives have been used to increase gastric residence time of oral dosage forms and thereby enhance the bioavailability of some drugs (Longer et al., 1985; Harris et al., 1987). The mucoadhesive properties of various polymers have been tested by several techniques, such as tensile testing (Park and Robinson, 1985), adhesion weight method (Smart and Kellaway, 1982), flow channel method (Mikos and Peppas, 1986), and falling liquid film method (Teng and Ho, 1987). These methods generally require isolated tissues of the gastrointestinal tract or a large quantity of mucus. Since the research on mucoadhesives is still in its early stage and a large number of polymers need to be examined, development of simpler methods is desired. A method should be able to screen a large number of candidate polymers under the same condition in a quantitative manner. There are a few parameters that can be used for the quantitation of mucoadhesion. They are adhesion strength, adhesion number, and duration of adhesion. While it is not clear which of the above parameters represents mucoadhesion better than the others, each can be used as a reasonable parameter, at least in a comparative sense.

Here we present a new approach that examines mucoadhesive properties of various hydrogels quantitatively by measuring the adhesion number. The technique employs colloidal gold particles which are conjugated with mucin molecules (mucin–gold conjugates). Mucin molecules of the

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conjugates interact with mucoadhesive polymers and the interaction is quantified by measuring the density of the colloidal gold particles adsorbed on polymer surfaces. The technique is simple and requires only a small amount of mucin. A large quantity of mucin–gold conjugates can be made easily in a reproducible manner. Thus, the mucoadhesive property of various candidate materials can be examined and compared under the same condition. The validity of the technique was tested using acrylic hydrogels whose mucoadhesive properties are well characterized (Park and Robinson, 1987).

Background

Colloidal gold particles are most frequently prepared by reducing $HAuCl_4$ with various reducing agents (Mysels, 1959). The size and stability of the formed particles depend on the type and amount of added reducing agents (Frens, 1973; Horisberger and Rosset, 1977; Roth, 1983). Monodisperse gold sols are red and absorb visible light with a single peak of absorption between 520 nm and 550 nm (Jonker, 1952). The position of the peak absorption moves to a longer wavelength as the size of gold particles increases (Horisberger and Rosset, 1977).

The stability of colloidal gold particles in the absence of added salts is maintained by electrostatic repulsion. The negative charge of colloidal gold particles arises from chloride ions (Dean, 1948) which are strongly adsorbed to the surface of gold particles during the process of preparing the sol (Vold and Vold, 1964). The addition of electrolytes to gold sols results in the reduction of charge repulsion and as a consequence gold particles flocculate. At a certain degree of flocculation, gold sols change color from red to blue. Gold sols can be stabilized even at high salt concentrations by adsorbing hydrophilic polymers (protecting agents) onto the surface of the particles. The protected gold particles acquire the properties of the protecting agents. Thus, protected gold particles behave towards salts just like the protecting agents (Von Buzagh, 1937). The effectiveness of various polymers as stabilizers is determined by Zsigmondy's gold number (Jirgensons and Straumanis, 1962; Napper, 1983).

In addition to hydrophilic polymers, colloidal gold particles have been stabilized by adsorbing various proteins, such as albumin, antibody, protein A, lectin, or enzyme (Albrecht and Hodges, 1988). Protein adsorption on colloidal gold particles is pH-dependent. The protein-adsorbed gold particles (protein–gold conjugates) are known to be most stable when protein is added at a pH close to or slightly basic to the isoelectric pH of the protein (Geoghegan and Ackerman, 1977). The exact condition for the production of the most stable protein–gold conjugates, however, needs to be determined empirically for each protein. It is generally known that proteins adsorb onto gold particles irreversibly, although non-covalently, and still maintain biological activities (Horisberger et al., 1975). The notion of irreversible protein adsorption, however, has been challenged by many investigators (Goodman et al., 1981; Behnke et al., 1986).

We have observed that colloidal gold particles can be stabilized by mucin molecules. The mucin-stabilized colloidal gold particles (mucin–gold conjugates) maintain red color and their solution concentration can be easily measured from the absorbance at a visible wavelength of 525 nm. Upon interaction with mucin–gold conjugates, mucoadhesive polymers develop red color on the surface. Thus, the extent of the mucoadhesive interaction can be quantified by directly measuring the intensity of red color on the polymer surfaces. Since the intensity of red color is essentially the density of colloidal gold particles of the mucin–gold conjugates, the extent of mucoadhesion can be quantified by the "colloidal gold density" or "mucin–gold density". Thus, the whole procedure can be simply called "colloidal gold staining" or "mucin–gold staining".

Materials and Methods

Preparation of colloidal gold particles

Colloidal gold particles with an average diameter of 18 nm were prepared following the procedure described previously (Frens, 1973; Horis-

berger, 1979; Loftus and Albrecht, 1983). 0.5 ml of 4% $HAuCl_4 \cdot 3H_2O$ solution (Fisher) was added to 200 ml of deionized distilled water at room temperature and the solution was brought to a boil. Four ml of freshly prepared 1% trisodium citrate was rapidly mixed into the boiling solution and the mixture refluxed for 30 min. The formation of the monodisperse colloidal gold particles was indicated by the appearance of red color. The concentration of gold particles was calculated from the size of gold particles, the amount of gold added, and the density of gold which is 19.32 (Park et al., 1987). The concentration of 18 nm gold particles was 8.3×10^{11}/ml and the absorbance at 525 nm was 0.975. The colloidal gold solution was cooled and stored at 4° C until use. The pH of the colloidal gold solution at room temperature was 5.7 as measured using a gel-filled combination electrode (Orion).

Preparation of mucin–gold conjugates

Mucin solution of an appropriate concentration was made by weighing powdered mucin (bovine submaxillary mucin, Type I, Sigma) and dissolving it in deionized distilled water. The mucin solution was dialyzed extensively against deionized distilled water to remove salts before preparing mucin–gold conjugates. The minimum amount of mucin necessary to stabilize the colloidal gold particles was determined from adsorption isotherms (Geoghegan and Ackerman, 1977). A series of mucin solutions of increasing concentration was made and 0.1 ml of the mucin solution was added to 1 ml of colloidal gold solution. After 30 min, 1 ml of 10% NaCl was added and rapidly mixed. If the colloidal gold particles were not stabilized due to incomplete surface coverage by mucin molecules, they became aggregated as indicated by a color change from red to light blue. The minimum mucin amount which prevented this color change was determined. The following procedure was used to make large quantities of mucin–gold conjugates.

Two ml of the dialyzed mucin solution (0.3 mg/ml) was added to 20 ml of 18 nm gold particles (pH 5.7) to make the final mucin concentration of 27 μg/ml. The absorbance of colloidal gold particles at 525 nm was 0.97. The volume of 20 ml was chosen, since the whole procedure can be done using a 50 ml centrifuge tube (Beckman). Thus, larger quantity of mucin–gold conjugates can be made by increasing the number of centrifuge tubes. The solution was gently mixed for a few seconds and left at room temperature for 30 min without any further disturbance, since continuous agitation resulted in precipitation or aggregation of gold particles. To the mucin–gold solution was added 100 μl of albumin solution (0.2 mg/ml) to further stabilize the conjugates. The addition of albumin alone in that amount in the absence of mucin could not stabilize the colloidal gold particles. Albumin molecules are believed to adsorb onto small bare spots on the colloidal gold particle where mucin molecules do not cover. The whole solution was gently mixed a few times and left for 10 more min. The mucin–gold conjugates were separated from the bulk mucin and albumin molecules by centrifuging in an angle head rotor (Beckman, Model J2-21) at 12,000 rpm (maximum centrifugal force of 17,400 *g*) for 30 min. The supernatant was discarded and the sedimented mucin–gold conjugates were resuspended in 1 ml of deionized distilled water. The concentrated mucin–gold conjugates were diluted with desired solution, such as pH 1.3 solution (0.05 N HCl solution) and other buffer solutions. The concentration of the diluted mucin–gold conjugates was calculated by measuring the absorbance at 525 nm as described above in '*Preparation of colloidal gold particles*'. For most studies, absorbance at 525 nm (Abs_{525}) was used to compare the concentration of colloidal gold particles instead of the exact number of particles.

Synthesis of acrylic hydrogels

Acrylic acid (Aldrich) was mixed with deionized distilled water to make the final concentration of 30 w/w%. Acrylamide (Bio-Rad) was dissolved in deionized distilled water to make the same final concentration. In the synthesis of copolymers of acrylic acid and acrylamide [P(AA-co-AM)], comonomers were mixed in the desired initial feed composition while maintaining the total concentration of 30 w/w%. *N,N'*-methylene-bis-acrylamide (cross-linking agent, Bio-Rad) and ammonium persulfate (initiator, Bio-Rad) were ad-

ded to make the final concentrations of 0.05 w/w% and 1.0 w/w% of the monomers, respectively. This concentration of cross-linking agent was used throughout the experiments, except for the study on the effect of cross-linking density on mucin–gold staining. The mixture was degassed by vacuum aspiration and poured into two glass plates which were separated by a U-shaped mylar film (0.72 mm thickness, Polysciences). The mixture was polymerized upon incubation at 60 °C for 2 h. The synthesized hydrogels were washed extensively in deionized distilled water and finally in the desired solution. The cleaned, transparent hydrogels were cut into either rectangular shape (7 mm x 15 mm) or discs (diameter of 10 mm) using a cutting mold. The thickness was varied from 1.5 mm to 3 mm depending on the type of hydrogel and pH of the solution.

Staining of hydrogels with mucin–gold conjugates

A cross-linked polymer strip or disc was placed vertically into the center of a polyacrylate cuvette (Sarstedt) and 2.5 ml of mucin–gold solution was slowly added to the cuvette. The concentration of the conjugate solution was varied (see Results). At predetermined time points, the polymer strip was taken out, gently rinsed in a buffer solution, and carefully placed on a wall of a new polyacrylate cuvette. Three ml of fresh buffer solution was added to the new cuvette. The intensity of red color (colloidal gold density) on the polymer strip was quantified by measuring absorbance at 525 nm (Abs_{525}), using a transparent control polymer strip as a blank. An attempt to calculate the number of gold particles on a unit surface area was not made, since the exact surface area of hydrogels was not known. The direct measurement of Abs_{525} of the polymer strip gave precise and reproducible readings, although the absolute value was relatively small. More than one polymer strip can be placed in the same cuvette to increase the absolute absorbance value. Alternatively, the concentration of mucin–gold conjugates in the bulk solution after removing the polymer strip was also measured. In this case, the magnitude of the decrease in the absorbance value from the initial value (ΔAbs) was used as a quantitative parameter indicating the interaction between protein–gold conjugates and hydrogels. In addition to these two methods, we have used an image analyzer (PC Consulting) to quantify the intensity of red color on the polymer surfaces. The images of colloidal gold-stained mucoadhesive hydrogels were transferred to a computer using a video camera (model 65, Dage-MTI) and the staining intensities of the polymers were compared using 256 gray levels (Imaging Technology). This method will be particularly useful for the mucoadhesive polymers which are not transparent.

Results

Effect of pH on the stability of mucin–gold conjugates

The mucin–gold conjugates were very stable at pH's 1.3, 6.0, and 7.4 (Fig. 1). The conjugates were less stable at pH's 2.0 and 3.0. The conjugates slowly aggregated during a period of days at pH 5. At pH 4, the mucin–gold conjugates were very unstable and became aggregated after a few hours. It appeared that mucin molecule itself was not stable at pH 4 and could not protect colloidal gold particles. Since the conjugates were stable at pH 1.3, it was possible to use them to test mucoadhesive properties of hydrogels under the gastric condition. The fact that mucin–gold conjugates are

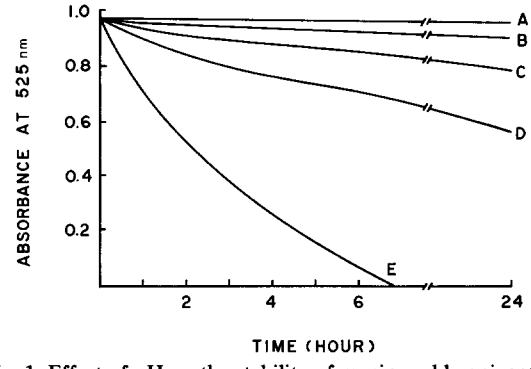

Fig. 1. Effect of pH on the stability of mucin–gold conjugates. Absorbance of mucin–gold solution was measured at 525 nm as a function of time. The decrease in absorbance is due to the aggregation of the unstable conjugates. The mucin-gold conjugates were stable at pH's 7.4 (A), 6.0 (A), and 1.3 (B). The conjugates were less stable at pH's 2.0 (C), 3.0 (C), and 5.0 (D). The conjugates were very unstable at pH 4.0 (E).

very stable at the neutral pH suggests that they can also be used to examine mucoadhesive properties of various hydrogels under conditions found in the intestine.

Factors affecting colloidal gold staining

The interaction between mucin–gold conjugates and the hydrogel surface (colloidal gold staining) occurs as a result of diffusion of the conjugates from the bulk solution to the surface. The diffusion process from an unstirred solution to a planar adsorbing surface can be described by the following equation.

$$q = cn(kTt/yr)^{1/2} \quad (1)$$

where q is the number of mucin-gold conjugates reaching the surface up to time t, c is a constant, n is the bulk concentration of the conjugates, k is Boltzmann's constant, T is temperature in degrees Kelvin, y is the viscosity of the dispersed medium, and r is the radius of the mucin–gold conjugates. The value of c varies depending on the assumptions used in the derivation of the equation. The values used are 0.163 (Mysels, 1959), 0.172 (Miller and Bach, 1973), and 0.260 (Ward and Tordai, 1946). We have found that the colloidal gold staining can be best described by using the c value of 0.163 (Park et al., 1987). The above equation clearly shows that a few experimental parameters, such as n, T, y, r, and t, should be controlled for the reproducible results. At a given experimental condition, the temperature (T) and the radius of the mucin-gold conjugates (r) are assumed to be constant. In addition, the viscosity of the solution (y) remains essentially constant, since the volume fraction of the colloidal gold particles is negligible. Thus, the most important parameters which affect the colloidal gold staining are the concentration of the mucin–gold conjugates (n) and the time for the staining (t). The effects of these two parameters were examined according to Eqn. 1.

Fig. 2 describes the result of the staining of cross-linked poly(acrylic acid) strips with 3 different concentrations of mucin–gold conjugates. The mucin–gold density, or colloidal gold density, on the polymer strips as measured by the absorbance

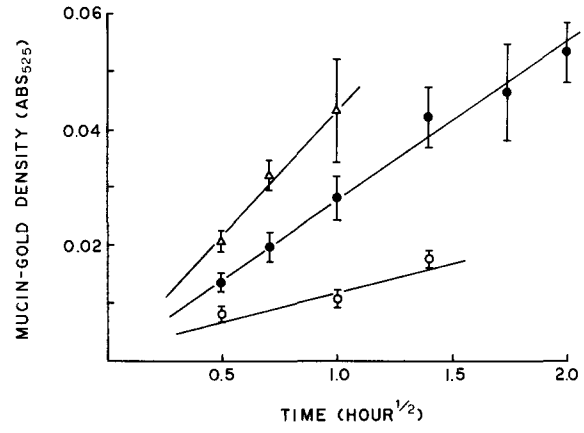

Fig. 2. The density of mucin–gold conjugates on cross-linked poly(acrylic acid) as a function of square root of the staining time (average ± S.E.M., $n = 4$). Staining was carried out at pH 1.3 using mucin–gold concentrations of $Abs_{525} = 0.47$ (○), $Abs_{525} = 0.98$ (●), and $Abs_{525} = 1.45$ (Δ).

at 525 nm represents the extent of mucoadhesion. As Eqn. 1 predicts, the mucin–gold density on the polymer surface is a linear function of the square root of time (Fig. 2). If Eqn. 1 is correct, we should also observe a linear increase in the mucin–gold density on the polymer surface as the bulk concentration of mucin–gold conjugates is increased. Indeed, the colloidal gold staining was proportional to the bulk concentration of mucin–gold conjugates (Fig. 3). Figs. 2 and 3 clearly show that the concentration of mucin–gold conjugates and the staining time have to be carefully controlled for reproducible results. Thus, quantitative comparison of mucoadhesive properties of various hydrogels is possible, if the same concentration of the mucin–gold conjugates and the staining time are used. If the concentration of mucin–gold conjugates is changed, the staining time has to be adjusted according to Eqn. 1 for reproducible results.

Effect of acrylic acid composition on mucoadhesion

Under the same staining condition as described above, the intensity of the red color (colloidal gold density) can be considered as a parameter indicating the extent of interaction between mucin and polymer chains of the hydrogels. Fig. 4 shows the staining of P(AA-co-AM) when the content of

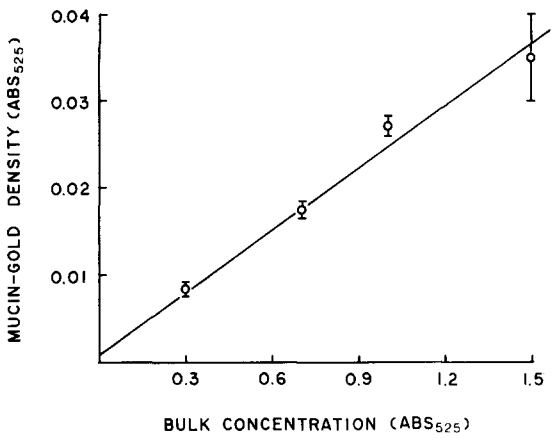

Fig. 3. The density of mucin–gold conjugates on cross-linked poly(acrylic acid) as a function of bulk mucin–gold concentration (average ± S.E.M., $n = 4$). The density and bulk concentration of mucin–gold conjugates are described in units of absorbance at 525 nm. Staining was carried out at pH 1.3 for 1 h.

acrylic acid is varied from 0 to 100%. The mucin-gold densities on different hydrogels can be determined visually due to the appearance of red

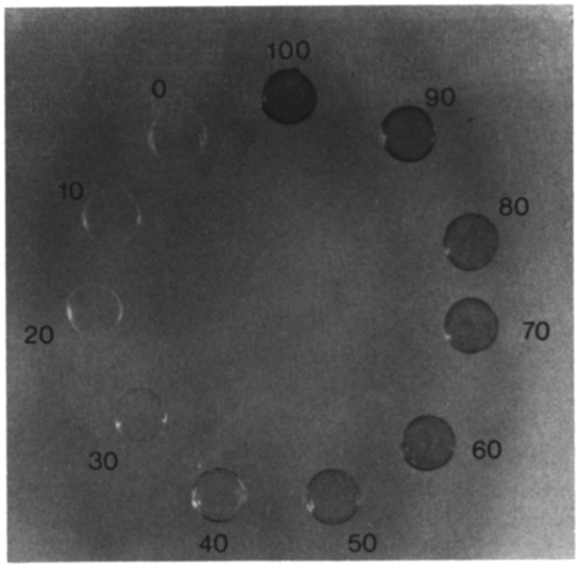

Fig. 4. Interaction of mucin–gold conjugates with cross-linked P(AA-co-AM) varying the content of acrylic acid. The interaction between the conjugates and hydrogels is indicated by the appearance of red color. The numbers describe percentages of acrylic acid used to make copolymers. The total concentration of acrylic acid and acrylamide was kept at 30 w/w%. Staining was carried out for 1 h at pH 1.3 using a mucin–gold concentration of $Abs_{525} = 1.45$.

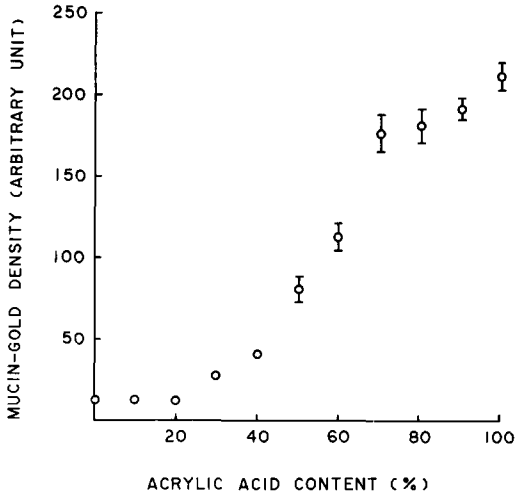

Fig. 5. The density of mucin-gold conjugates on cross-linked P(AA-co-AM) as a function of the acrylic acid content. The mucin–gold density of the hydrogels in Fig. 4, was quantified using 256 gray levels of an image analyzer. The mucin-gold density was an average (± S.D.) of 64 pixels.

color. No significant interaction was observed until the content of acrylic acid was increased up to 50%. Above this acrylic acid content the intensity of red color was further increased.

For quantitative comparison we used an image analyzer. The intensity of red color was quantitated using 256 grey scales of an image analyzer. The result of the quantitative analysis is shown in Fig. 5. The mucoadhesive property of P(AA-co-AM) increased sharply when the acrylic acid content reached 70%. Above 70% acrylic acid content, the intensity of red color of the copolymers did not increase significantly. The same data were obtained when the hydrogels were quantified by ΔAbs or Abs_{525}. The result obtained using mucin-gold conjugates is very similar to that of the mucoadhesion tests using gastric tissues (Park and Robinson, 1987). Thus, it appears that there is a critical acrylic acid content above which the mucoadhesive property remains about the same. As shown in Fig. 5, small differences in the colloidal gold density are easily identified using an image analyzer. The image analyzer will be particularly useful if mucoadhesive polymers are not transparent or the geometry is such that the direct measurement of absorbance at 525 nm is not possible.

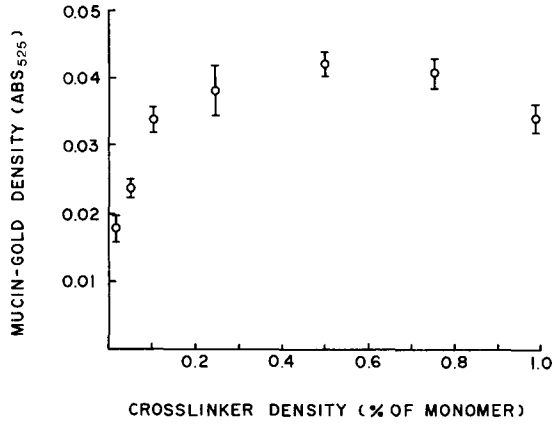

Fig. 6. The density of mucin-gold conjugates on cross-linked poly(acrylic acid) as a function of the nominal cross-linker density (average ± S.E.M., $n = 6$). The nominal cross-linker density is presented as the weight percent of monomer in the feed. Staining was carried out at pH 1.3 using a mucin-gold concentration of $Abs_{525} = 0.92$ for 1 h.

Effect of cross-linker density

The effect of the density of cross-linking agent on mucoadhesion was examined using cross-linked poly(acrylic acid) at pH 1.3. Since the final cross-linking density was not known, the initial density of cross-linking agent was used for convenience. The density of cross-linking agent was varied from 0.02 to 1.0 w/w% of monomer. As shown in Fig. 6, the extent of mucoadhesion was dependent on the cross-linker density of the poly(acrylic acid) hydrogels. Hydrogels of different cross-linker density were swollen to equilibrium at room temperature and the same size of gels were prepared. Thus, the total number of poly(acrylic acid) chains available for the interaction with mucin–gold conjugates was increased as the cross-linker density was increased. This explained the gradual increase in mucoadhesion as the cross-linker density was increased from 0.02 w/w% to 0.5 w/w%. Above the cross-linker density of 0.5 w/w%, the mucoadhesion started decreasing. This is most likely due to the reduced chain flexibility of poly(acrylic acid) at high cross-linker density.

The effect of cross-linker density on mucoadhesion observed in this study was different from that of Park and Robinson (1987) who used gastric tissue. They observed higher mucoadhesion with lower cross-linker density. Mucoadhesion was continuously decreased as the cross-linker density was increased from 0.1 w/w% up to 2 w/w%. It should be remembered that tension tests using gastric tissues involves three-dimensional interactions, while colloidal gold staining involves two-dimensional interactions. In three-dimensional interaction, entanglement between polymer chains and the mucus layer is important and such entanglement is enhanced by the low cross-linker density. In colloidal gold staining, however, individual mucin-gold conjugates interact with only surface polymer chains of hydrogels. Thus, the concentration of poly(acrylic acid) chains available for interaction is more important than the easy entanglement in the mucin–gold staining.

Stable complex formation between mucin and poly(acrylic acid)

Since one of the reasons for studying mucoadhesion is to apply the mucoadhesives to oral controlled-release dosage forms, it is important to know the effect of pH changes from 1 to 7. Poly(acrylic acid) hydrogels were allowed to interact with mucin–gold conjugates at pH 1.3 (A in Fig. 7) and then transferred to a pH 7 phosphate buffered saline solution (B in Fig. 7). The hydro-

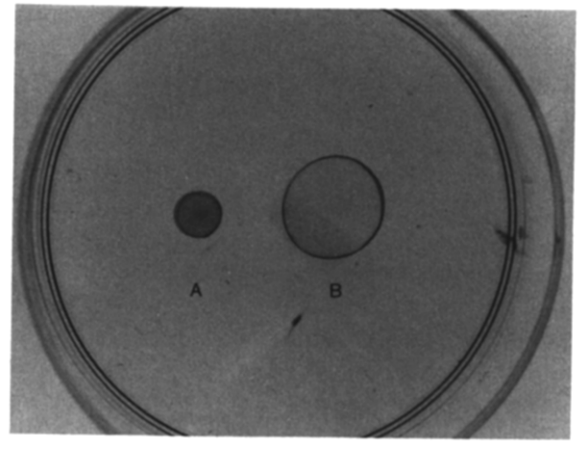

Fig. 7. Very stable complex formation between mucin–gold conjugates and cross-linked poly(acrylic acid). Cross-linked poly(acrylic acid) hydrogels were stained with mucin–gold conjugates at pH 1.3 for 1 h (A) and then transferred to a pH 7 buffer solution (B). It is noted that the red mucin–gold conjugates remain attached to the hydrogel even after swelling at pH 7 (B).

gel swelled at pH 7 due to the ionization of poly(acrylic acid). The swollen hydrogel still possessed red color, i.e., the mucin–gold conjugates still remained on the hydrogel surface. Vigorous washing of the swollen gel did not change the intensity of red color. When the swollen gel was transferred back to the pH 1.3 solution, the gel shrank and the intensity of the original red color was restored. Thus, the reduced intensity of red color at pH 7 (B in Fig. 7) was simply due to the increase in size of the gel. It has been proposed that the interaction between poly(acrylic acid) and mucin molecules occurs through hydrogen bonding (Park and Robinson, 1987). It is known that a stable complex can be formed through hydrogen bonding, if a certain number of undissociated carboxyl groups exist (Bekturov and Bakauova, 1986). Thus, very stable complex formation at pH 1.3 is understandable.

Discussion

In the pharmaceutics field, the ultimate application of mucoadhesives is in the design of platforms for oral controlled release dosage forms. Thus, it is desirable for the accurate assessment of candidate mucoadhesives to use test methods that simulate the in vivo gastrointestinal state most closely. In vivo animal tests or tests using isolated gastric tissues satisfy such a condition. Those test methods, however, are not suitable for the testing of numerous polymers to find new mucoadhesives. The test methods using animal tissues are expensive and time-consuming. Thus, there is a need to use a simpler technique that can provide information on the mucoadhesive properties of various polymers. The results obtained using mucin–gold conjugates are similar to those of tensile tests using gastric tissues (Park and Robinson, 1987), at least for the acrylic hydrogels. This implies that the mucoadhesive properties can be tested by colloidal gold staining instead of using animal tissues. We have used bovine submaxillary mucin as a representative mucin in this report. Certainly, it is possible to use other mucins prepared from specific tissues in each laboratory. Since mucin molecules obtained from commercial sources or prepared in laboratories may not represent intact mucin molecules in the mucous layer in vivo, caution is necessary in the extrapolation of the result of mucin–gold staining to the in vivo mucoadhesion behavior.

There are some advantages of using mucin–gold conjugates over using animal tissues. First, the colloidal gold staining technique is simple to perform. No special instrument other than a spectrophotometer is necessary for the technique. The use of an image analyzer is optional. Second, the experimental cost is much lower than using tissues obtained from animals. Compared to animal studies, the cost of colloidal gold staining is negligible. Third, the colloidal gold staining technique allows study of interaction between mucin molecules and polymer chains at the molecular level. Unlike other tests using the whole mucus layer of gastric tissues or mucin solution, the colloidal gold staining technique measures the interaction between individual mucin molecules and polymer chains. Thus, mucoadhesion results obtained using mucin–gold conjugates tend to be less influenced by other factors, such as the density and thickness of the mucus layer or tissue viability. Fourth, the same experimental condition can be maintained and the results are highly reproducible. Thus, data obtained at different times can be easily compared. Finally, it is possible to make mucin–gold conjugates in large quantity, so that mucoadhesive properties of a large number of different polymers can be compared at the same time under the same condition. Therefore, this technique will be very useful in screening a large number of candidate mucoadhesives.

A variation of the mucin–gold staining can be developed for the study of mucoadhesion in general. Instead of mucin molecules, various mucoadhesive polymers may be used to stabilize colloidal gold particles. The polymer-protected colloidal gold particles can be directly applied to the surface of target tissues. In this way, site-specific interactions between a certain polymer and a particular mucin can be examined. These improvements will be useful in the design of multiple unit dosage forms in which individual units are coated with different mucoadhesive polymers.

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