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Research Paper

Effect of a Hydrophobic Phospholipid Lining of the Gastric Mucosa in Bioadhesion

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Purpose. The role of a model hydrophobic phospholipid simulating lining of the gastric mucosa, as to adhesion of polymers with different surface functional groups and surface hydrophobicities, was evaluated using an *in vitro* gastric mucus model.
Materials and Method. Front-faced fluorescence measurement was used to determine adhesion of fluorescent polystyrene microspheres with different surface functional groups. Contact angle measurements and sticking bubble technique were used to measure relative surface hydrophobicity of the polymers.
Results. Adhesion of fluorescent polystyrene microspheres using front-faced fluorescence measurement revealed the hydrophobic phospholipid lining of the *in vitro* gastric mucus model did not allow adhesion of microspheres with –COOH and $–NH_2$ functional groups, whereas it did allow adhesion of microspheres with hydrophobic attributes. In addition, *in vitro* adhesive force studies using diblock copolymers of polystyrene and polyacrylate showed that the *in vitro* adhesive force between the hydrophobic phospholipid lining of the *in vitro* gastric mucus model and the polymer increased when the surface hydrophobicity of the polymer increased.
Conclusion. The hydrophobic phospholipid acts as an adhesion barrier to hydrophilic bioadhesive polymers and polymers with surface functional groups of carboxylic acid and amine. The hydrophobic phospholipid lining of the gastric mucosa should be taken into considerations for screening and designing of a new gastric bioadhesive polymer.

KEY WORDS: adhesion; gastric mucosa; hydrophobic phospholipid; surface functional group; surface hydrophobicity.

INTRODUCTION

It is generally recognized that a short and variable gastric retention time of a drug imposes a limitation on the use of oral extended release dosage forms (1,2). Therefore, prolonging the residence time of a drug delivery system in the stomach to achieve local treatment for gastric ulcers and cancer, as well as to increase dosing compliance and the relative bioavailability of drugs through an extended release dosage form has posed a challenge. Bioadhesion, which is an interfacial phenomenon between a synthetic or natural macromolecule and a biological surface, is a useful concept to prolong residence time of the drug delivery system (2). It has been successfully applied at many mucosal surfaces using hydrophilic bioadhesive polymers (3). Unfortunately, bioadhesive polymers with hydrophilic functional groups did not work in the human stomach (1,4–6). Despite extensive research, no clear answer to the fundamental issue of "why the bioadhesive approach does not work in the stomach" has been proposed. We propose that two factors, application of the wrong type of polymer and the lack of an *in vitro* gastric mucus model representing the stomach surface, are the primary cause of failure of the bioadhesive approach in humans although these hydrophilic polymers did show a strong interaction with a mucus model and animal tissues *in vitro*. The main reason is that the surface of the mammalian gastric mucosa was thought to be hydrophilic because of a high water and glycoprotein content in gastric mucus (Fig. 1). Therefore, hydrophilic polymers used in the stomach as a bioadhesive was unsuccessful. Surprisingly, the stomach lining is relatively hydrophobic due to the presence of a hydrophobic phospholipid lining on the top of the luminal surfaces of rat and canine gastric mucosa (Fig. 1). Unlike hydrophilic mucosal surfaces, the hydrophobic stomach surface is essential in protecting the mucosal surface against

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ABBREVIATIONS: Amine-MS, fluorescent amine-modified polystyrene microspheres; Carboxylate-MS, fluorescent carboxylate-modified polystyrene microspheres; F.I., fluorescence intensity; IGM, *in vitro* gastric mucus model without LPC; IGM-LPC, *in vitro* gastric mucus model with LPC; LPC, egg yolk L-α-phosphatidylcholine; MTS, material testing workstations; Plain-MS, fluorescent plain polystyrene microspheres.

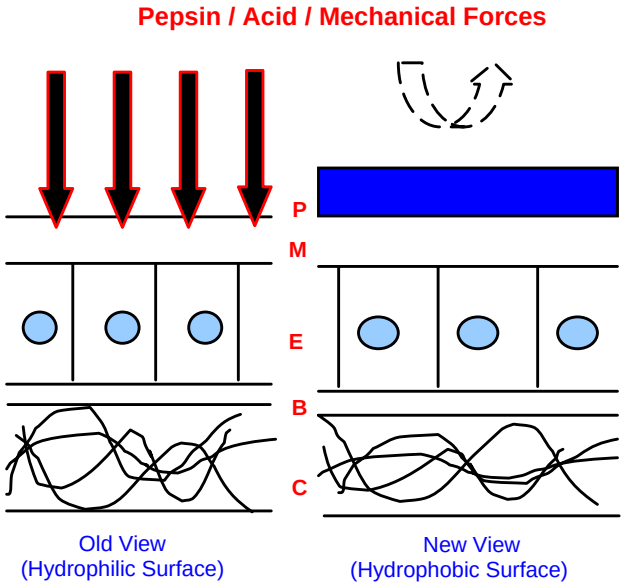

Fig. 1. Schematic illustration of the mammalian gastric mucosa surface. The gastric mucosal surface, on the left hand, is subject to damage by stomach acid, pepsin, and mechanical erosion by food based on an older concept of gastric mucosa. An additional layer contributed by surface-active phospholipids present on the luminal surface of the gastric mucosa may be essential primarily protect the mucosal surface from luminal acid, pepsin, and mechanical erosion as seen in the new concept. *P* Phospholipids layer, *M* mucus layer, *E* epithelial cells, *B* basal membrane, *C* connective tissue

luminal damaging factors such as acids, mechanical erosion, and digestive enzymes (7–13). In addition, alcian blue staining studies in live pigs show that the surface property of the stomach tissue changes from hydrophobic (nonwettable) to hydrophilic (wettable) after the animal is sacrificed (14). The present research is an attempt to develop a simplified *in vitro* gastric mucus model, which can simulate *in vivo* stomach surface properties, and to understand the role of the hydrophobic phospholipid lining of the gastric mucosa in adhesion using an *in vitro* model to provide information for the design of successful gastric bioadhesive polymers. The hypothesis is that the presence of a hydrophobic phospholipid lining of the gastric mucosa acts as an adhesion barrier to hydrophilic polymers, but permits adhesion of polymers with a certain degree of surface hydrophobicity.

MATERIALS AND METHODS

Materials

Bile (bovine, minimum 50% bile acids), alcian blue 8GX (a cationic dye), paraformaldehyde, glutaraldehyde (grade I, 70% aqueous solution), caodylic acid, sodium cacodylate, Trizma® hydrochloride, Trizma® base, sodium bisulfite, sodium sulfate, ammonium sulfide, crude mucin (type II, from porcine stomach), ethyl alcohol (HPLC grade), and L-α-lysophosphatidylcholine (type I, from egg yolk, >99%) were purchased from Sigma. Noveon ™ AA-1 polycarbophil (polyacrylic acids cross-linked with divinylglycol) was a gift from BF-Goodrich. Fluorescent polystyrene microspheres were obtained from Molecular probes (Eugene, OR) and Bangs Laboratories. Poly(styrene-*b*-acrylic acid) amphiphilic block copolymers were purchased from Polymer Source, Inc. Methanol (HPLC grade), hydrogen hexachloroplatinate (IV) hydrate, and potassium iodide were purchased from Aldrich Chemical Company.

Animals

Healthy domestic farm swines of either sex weighing between 25 to 30 kg were used. Pigs fasted for 24 h were killed by intravenous overdose of 20 mM potassium chloride through the jugular vein. The stomach and proximal duodenum were rapidly removed and used. All experiments using pigs in this study were carried out according to the guidelines provided by the Campus Animal Care and Use Committee (The University of Wisconsin-Madison).

Preparation of an *In Vitro* Gastric Mucus Model

The basic gastric mucus gel was prepared by dispersing 10% (*w*/*w*) polycarbophil and 3% (*w*/*w*) crude pig gastric mucin, followed by incubation of the dispersion at room temperature for 48 h, and mixing the gel to homogeneity on a glass plate. An aqueous egg yolk L-α-phosphatidylcholine (LPC) sample was prepared by shaking 10% (*w*/*w*) LPC in 0.2 M HCl buffer, pH 2.0, until a homogeneous dispersion of liposomes was obtained. An *in vitro* gastric mucus model was constructed by the following sequential processes: (1) The basic gastric mucus gel was coated on parafilm to a thickness of 0.6–0.7 mm, (2) LPC molecules were layered onto the gel surface (IGM-LPC) by dipping in the LPC liposomal dispersion of the IGM and subsequently slow removal, (3) The gel coated with LPC molecules was kept at 4°C overnight prior to use. A gel coated on parafilm without LPC molecules on the surface (IGM) was prepared as a control surface to represent the hydrophilic mucosal surface.

Alcian Blue Staining

An alcian blue staining technique was used to confirm whether there is a hydrophobic layer present on the surfaces of IGM, IGM-LPC, pig gastric and duodenal mucosa. Alcian blue was prepared as a 0.5% (*w*/*v*) solution in 0.2 M HCl buffer, pH 2.0. The stomach was separated into lower (pyloric region) and upper (body and cardiac regions) parts. Some pieces of the gastric and duodenal mucosa were immersed into a 2% bile solution, pH 7.2, for 10 min at room temperature and washed with saline buffer, pH 7.2, as control samples. The *in vitro* gastric mucus models and the pig mucosal tissue pieces were immersed in the alcian blue dye solution for 2 min at room temperature, washed with distilled water, and comparatively observed.

Surface Hydrophobicity Measurements

Contact angles were measured by the "captive bubble technique" (15) in an aqueous environment. Briefly, experimental objects were measured for contact angle using a contact-angle goniometer equipped with an envi-

ronmental chamber to control temperature of the medium. The clean environmental chamber was mounted on the goniometer stage and filled with distilled water. Temperature of the chamber was kept constant at 37±0.5°C. The *in vitro* gastric mucus model or the tissue sample was fixed on a glass slide using superglue and was immersed upside-down in the water chamber. The glass was then suspended in water and equilibrated for about 3 min. Air bubbles and mineral oil droplets of about the same size were placed on the tissue surface. Several bubbles and droplets were measured for each gel surface. If possible, two contact angles (left and right) were measured per bubble. Sometimes, however, the contact angle points were not clearly distinguishable due to roughness of the surface. Contact angles from duodenal, bile treated gastric mucosal, and bile untreated gastric mucosal surfaces were compared for relative surface hydrophobicity using an unpaired Student's *t* test with significance assumed when $p<0.05$. Results are expressed as mean±SD.

Proton Permeability Measurements

The permeability of hydrogen ions through gastric mucosal tissues, IGM, and IGM-LPC was measured in a diffusion chamber. The muscular layer was carefully removed from the gastric mucosa after incision for the proton permeability assay. To preserve the extracellular material over the gastric mucosal surface, caution was taken in removing the muscle layer. Some of the gastric mucosal pieces were immersed into a 2% bile solution for 10 min at room temperature and washed with saline buffer to remove the surface hydrophobic barrier. The gastric mucosal sample or IGM or IGM-LPC was positioned in the center panel separating the two compartments, filled on one side with 0.155 M HCl (pH~0.9) and on the other with 0.155 M NaCl (pH~6.5). The sample was then bathed on both sides with 10 ml of each solution at 37°C in a thermostatically controlled Plexi-glass chamber. The pH in the NaCl compartment was monitored using a pH electrode in 5-min intervals for a total of 2 h. The concentration of hydrogen ions diffusing through the gel was calculated in mmoles/liter. The permeability coefficients of the gastric mucosal samples in cm/sec were calculated by plotting concentrations of hydrogen ion as a function of time using Fick's first law equation. Results are expressed as mean±SD. All experiments were repeated five times for reproducibility.

Electron Microscopy

The preparation of the pig gastric mucosa sample for electron microscopic observations was modified somewhat from the method described by Kao and Lichtenberger (8). Pigs fasted for 24 h were sacrificed by intravenous overdose of 20 mM potassium chloride through the jugular vein. The stomach was rapidly removed. Freshly prepared cold fixative (2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 2 mM $CaCl_2$) was then introduced by an intravenous (IV) tubing connected with an IV bag into the gastric lumen through the esophagus. By this means, the gastric mucosa was pre-fixed *in situ* for 30 min. The stomach was incised along the greater curvature and the luminal surface of the stomach exposed. The stomach luminal surface was gently washed with 0.1 M cacodylate buffer, pH 7.4. The specimens were cut into small pieces. Some of the specimens were immersed in 2% bile solution for 10 min at room temperature and washed with saline buffer to remove the hydrophobic layer. Both bile salt-treated and untreated specimens were bathed in cold fixative overnight. The tissue samples were washed thoroughly with cold 0.1 M cacodylate buffer, pH 7.4, before iodoplatinate (IP) reaction. About 1 mm^3 tissue blocks were cut out. The tissue samples were incubated in Tris–IP solution (10% hydrogen hexachloroplatinate IV, distilled water, 0.2 M Tris buffer, pH 6.9, and 6% potassium iodide in 1:16:16:33 proportions) for 3.5 h. These reagents must be prepared freshly and mixed immediately before use. The final pH of Tris–IP solution was adjusted to pH 6.5–6.8. A solution of 0.3% sodium bisulfite was then added dropwise until the color of the reaction medium changed to brownish yellow. The tissue samples were then incubated in this solution for another 1 h. After three 10-min. washings in 0.05 M Tris buffer, pH 6.9, containing 1.0% sodium sulfate, it was finally incubated for 1 h in a solution composed of one part diluted 20% ammonium sulfide (1:500) and three parts of 0.05 M Tris buffer, pH 6.9, washing buffer containing 1% sodium sulfide. The tissue samples were washed in 0.05 M Tris buffer, pH 6.9, with three 5-min washings. The samples were dehydrated in a graded series of ethanol at 22°C (50–100%, *v*/*v*). The dehydrated samples were infiltrated and embedded using Spurr's low viscosity resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Final embedding took place in 100% resin at 65°C for 24 h. Ultrathin sections (70–80 nm) were collected on a Reichert–Jung Ultracut E ultramicrotome, placed on 300 mesh Cu Gilder thin-bar grids. The sections were post-stained in uranyl acetate and Reynold's lead citrate, and viewed at 80 kV on a Philips CM 120 transmission electron microscope (FEI Corp., Eindhoven, Netherlands). Digital micrographs documenting samples were taken using a SIS MegaView III CCD camera (Soft Imaging Systems Corp., Lakewood, Co., USA).

Stability of LPC on the IGM-LPC

For the stability study, the gel was coated on the surface of a 14 ml polystyrene round-bottom tube to a depth of 3 cm from the bottom of the tube. The tube coated with the gel was then immersed in the 10% LPC dispersion and slowly removed. It was then kept at 4°C for 12 h prior to use. Twenty-five microliters (μl) of the 10% dispersion, before and after coating LPC onto the gel surface, was used as a control to calculate how many LPC molecules were coated on the gel surface. Fifty milliliters (ml) of each pH buffer solution (pH 2.0, 4.0, and 6.0) was transferred to a 100 ml beaker in a 37°C water bath to keep a constant temperature. The tube was immersed in a buffer solution and 25 μl of each buffer solution was periodically placed into 25 ml glass tubes for 2 h. The concentrations of the LPC in buffer solutions were assayed by a modified method of phosphorous assay (17). Percent loss of LPC is expressed as the ratio of the total amount of LPC detected from the solution to the total amount of LPC coated on the gel surface multiplied by 100.

Adhesion Assay of Microspheres

All microspheres were washed once with distilled water to remove residual surfactants and antibacterial agents, separated by centrifugation, and dispersed in distilled water. Five milliliters of each fluorescent polystyrene microsphere suspension was centrifuged for 15 min at 4,000 rpm to remove surfactants and ions adsorbed onto the sphere (Beckman CPR). After decanting the supernatant, the pellet was redispersed in 5 ml of distilled water by sonication for 1 min in a water bath-type sonicator (AmericanBrand ™ ultrasonic cleaner, American Scientific products). These cleaning procedures were performed twice. The buffers used for this study were 50 mM phosphate buffers (pH 2.0 and 6.0) for fluorescent carboxylate-modified polystyrene microspheres (carboxylate-MS, an anionic sphere) and fluorescent plain polystyrene microspheres (Plain-MS, a hydrophobic sphere), and 50 mM glycine buffer (pH 2.0 and pH 6.0) for fluorescent amine-modified polystyrene microspheres (amine-MS, a cationic sphere). 10 ml of each pH buffer solution was transferred into a 10 ml beaker and then kept in a 37°C water bath. A binding assay was carried out in two steps, as follows: (1) A gel piece was immersed in an appropriate pH buffer solution for 2 min and then removed. The gel was then placed above the surface of a triangular fluorometer cell using double- sided tape. The fluorescence intensity (F.I) of the gel surface was measured at the given emission wavelength of each microsphere as baseline F.I. Six readings were taken for each sample and averaged. (2) A microsphere suspension prepared as described earlier was diluted in the same buffer as in the baseline test to a final concentration of approximately 1.5×10^8 spheres per ml. To obtain a homogeneous dispersion, the diluted suspension was sonicated for 30 s in the water bath. The gel used in the first step for the baseline test was immersed in the diluted suspension for 3 min and taken out. Free and loosely-bound microspheres were washed away using saline buffer for 1 min at the rate of 30 ml/min using a peristaltic pump (Wason–Marlow, Bacon Technical Industries). The gel was placed above the triangular cell and the gel surface F.I. was measured at the same emission wavelength used in the first step as total fluorescence intensity. Six readings were taken and averaged for each sample. The "real fluorescence intensity", from only microspheres bound on the gel surface, was obtained from a difference between the total F.I. and the baseline F.I. (= total F.I.−baseline F.I.). All adhesion studies were repeated three times. A standard curve of each microsphere suspension on each gel surface was constructed by plotting fluorescence intensity versus the number of microspheres applied to the gel surface. The real fluorescence intensity was correlated to the number of microspheres bound to the gel surface using a standard curve.

Surface Free Energy Measurements

Surface free energy of the materials used for adhesion studies was determined by the "sticking bubble technique" described by Keurentjes *et al.* (18), in which the surface hydrophobicity is expressed as surface tension (mN/m). IGM and IGM-LPC were prepared as described earlier. 0.45 g of poly(styrene-b-acrylic acid) copolymers was compressed by PARK Instrument Press into tablets with a diameter of 12 mm. Mixtures of water and methanol were prepared to generate an appropriate surface tension ranging from 73 to 22 mN/m, and the surface tensions of the mixtures were measured by the Wilhelmy plate method using a platinum plate at 37°C. Three measurements were taken for each liquid mixture and averaged as a mean±SD. A polymer tablet or IGM or IGM-LPC was placed horizontally at the bottom of a 20 ml beaker containing a 15 ml water–methanol mixture with a surface tension, γ_L, using superglue (DURO®). Forty air bubbles were brought into contact with each surface of the tablet or IGM or IGM-LPC using a 25 μl syringe (Hamilton, Co.) with a flat ended needle (horizontal). The number of air bubbles sticking to the surface was counted from each sample in a specific surface tension of a water–methanol mixture. The surface tension at detachment (γ_d) was determined by plotting the percentage of bubbles, which sticks, versus the surface tension of the water-methanol mixture (γ_L). A sudden transition was observed at the region of γ_d. Therefore, from the plot the surface hydrophobicity of the polymers was expressed in terms of γ_d, the surface tension at which an air bubble brought into contact with the top surface of the tablet has a 50% chance of detaching from the surface. Results were expressed as a mean±SD of n=3.

In Vitro Adhesive Force Measurements

The bioadhesive force between the polymer and the *in vitro* gastric mucus model was measured using material testing workstations (MTS) synergie 200 as determination of the force required to separate a polymer sample from the *in vitro* gastric mucus model gel surface (IGM or IGM-LPC). A tablet of the polymer was attached to the movable probe and the *in vitro* gastric mucus gel was placed on a 250 ml beaker, supported with test weights, using superglue. The 250 ml beaker was filled with 100 ml of pH 2.0 HCl buffer and equilibrated at a temperature of 37°C. The tablet and the *in vitro* gel were then pre-incubated for 1 min and 30 s. The steel probe with the tablet was lowered at a rate of 1.0 mm/s until contact was made between the tablet and the *in vitro* gel. The tablet was left in contact with the *in vitro* gel for 40 s with an applied force of 5 g and was raised to separate it from the *in vitro* gel to determine the required force at detachment. All experiments were repeated three times at the same condition for reproducibility. Results were expressed as mean±SD of n=3. The force of detachment was calculated in N/m^2 using contact surface area of the tablet as the adhesive force between the polymer and the *in vitro* gel.

RESULTS

Development and Validation of an *In vitro* Gastric Mucus Model

To determine whether the *in vitro* gastric mucus model (IGM-LPC) is functionally and structurally similar to that of animals, wettability to alcian blue, contact angle, and proton permeability of the IGM-LPC were examined as compared to those of the control surface (IGM, representing hydrophilic mucosal surfaces). In alcian blue staining, the IGM-LPC was not stained with alcian blue, whereas IGM without

LPC was strongly stained, indicating that LPC molecules were nicely deposited on the IGM surface. Contact angles formed by both the air bubble and mineral oil droplets on the IGM-LPC (63.5°±3.0° and 87.3°±1.3° as mean±SD, respectively, n=6) were higher than those on IGM without LPC (37.3°±1.5° and 44.8°±1.7°, respectively, n=6, p<0.05), showing that the surface of IGM-LPC is relatively hydrophobic due to LPC molecules present on the IGM surface. Proton permeability coefficients through the IGM and IGM-LPC were $10.6\pm2.3\times10^{-6}$ and $4.25\pm0.45\times10^{-6}$ cm/s, respectively. This explains that the absence of LPC on the IGM surface was accompanied by about a 149% reduction in the ability of IGM to retard the passage of hydrogen ion compared with that of IGM-LPC. Finally, we determined the stability of LPC molecules deposited on the IGM gel surface. The stability of the L-α-phosphatidylcholine molecules deposited on the IGM surface was tested in three different pH buffers. The result from Fig. 2 showed that LPC was not detected significantly after 2 h in pH 2.0 buffer solution. In pH 6.0 buffer solution, however, a detectable amount of LPC was observed in the buffer after 90 min. The main reason for this is due to the swelling property of this system at a higher pH, resulting in surface erosion. The swelling characteristic of Polycarbophil (PC) in water was well known to be pH-dependent, with increased swelling as pH increases. At low pH (pH 1–3), PC absorbs~15–35 ml of water per gram, whereas in neutral or basic media it can absorb ~100 ml/gram. In contrast, LPC molecules were relatively stable in a pH 4.0 buffer solution. We can maintain this system for at least 1 h in a broad range of pH buffer solutions, which is enough time for all *in vitro* tests.

Surface Hydrophobicity of the Pig Gastric Mucosa

In pig stomach surface characterization, the stomach was separated into lower (pyloric region) and upper (body and cardiac regions) parts because some food particles remained only on the upper part of the pig stomach after fasted for 24 h. There is no regional variation in surface hydrophobicity, wettability to alcian blue dye, and proton permeability within the pig stomach. It is explained that the turnover rate of either the phospholipid layer or mucus layer on the upper part of the pig stomach may be slower than that of the lower part. Fig. 3 shows that the pig gastric mucosa did not stain with alcian blue dye molecules, whereas the pig gastric mucosa treated with bile salts, which remove a hydrophobic molecule from the surface, and the duodenal mucosa did stain well with alcian blue dye molecules. It indicates that there are hydrophobic molecules covering the gastric mucosal surface. The contact angles formed by air bubbles on the intact pig stomach in both the upper an lower parts (60.4°±3.0° and 59.4°±1.1° as mean±SD, respectively, n=6) were comparable to those on pig gastric mucosa in both the upper and lower parts after treatment with bile salts, and pig duodenum mucosal surface (33.2°±1.3°, 34.2°±1.5°, and 29.7°±1.8, respectively, n=6, p<0.05). It demonstrates that the pig gastric mucosal surface is relatively hydrophobic in nature. Proton permeability studies (Table I) revealed that an additional hydrophobic lining present on the luminal surface of the pig gastric mucosa retarded permeation of hydrogen ions through the mucus layer. An electron micrograph of the pig gastric mucosa untreated with bile salts (Fig. 4b) showed a continuous iodoplatinate-reactive filamentous phospholipid layer on the top of the mucus layer. However, after luminal exposure to a 2.0% (w/v) bile salts known to be a hydrophobic barrier breaking agent for 10 min, an electron micrograph (Fig. 4a) of the pig gastric mucosa showed loss of the integrity of the apical plasma membrane and damage of the extracellular mucus layer as well as the continuous phospholipid layer. A continuous phospholipid layer on the top of the luminal gastric mucosa surface contributes to surface hydrophobicity, which provides a hydrophobic (non-wettable) barrier to hydrogen ion permeability and is removed by surface-active materials and alcohol, thereby becoming a wettable surface.

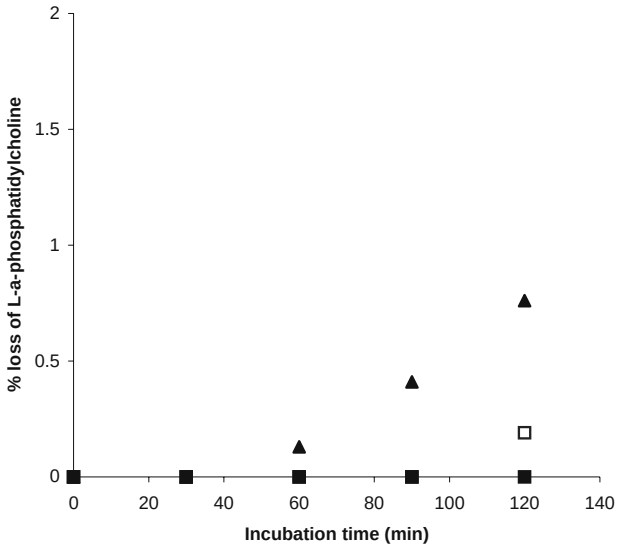

Fig. 2. The stability of LPC molecules deposited on the IGM surface in different pH buffer solutions (37°C). *Triangles*, *closed* and *open squares* represent the stability of LPC molecules in pH 6.0, 4.0, and 2.0 buffer solution, respectively

Effect of the Surface Functional Groups of Polymers on Adhesion

Adhesion of three different fluorescent polystyrene microspheres on the hydrophilic IGM gel surface (Fig. 5a) revealed that a number of both fluorescent polystyrene microspheres with carboxylic acid functional groups (carboxylate-MS) and with amine functional groups (amine-MS) adhered to the negatively charged hydrophilic IGM surface, whereas negligible number of fluorescent polystyrene microspheres with no additional functional groups (plain-MS) adhered to the same gel surface in pH 2.0 buffer solution. On the other hand, many amine-MS particles adhered to the same gel surface, however; negligible number of both carboxylate-MS and plain-MS adhered to the same gel surface in pH 6.0 solution. Conversely, adhesion of three different microspheres on the hydrophobic IGM-LPC (Fig. 5b) revealed that none of two microspheres with hydrophilic functional groups (Carboxylate-MS and Amine-MS) adhered to the hydrophobic phospholipid layer, whereas a significant number of Plain-MS adhered to the same surface in both pH buffer solutions.

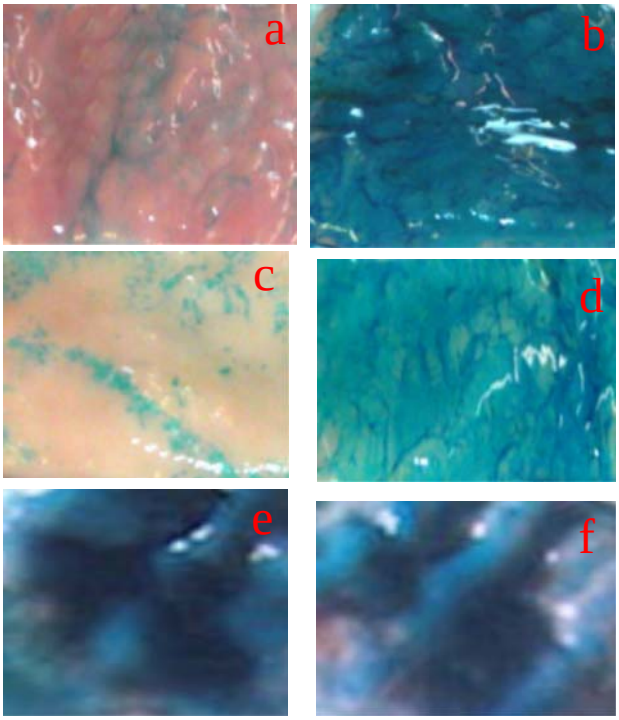

Fig. 3. The pig gastric and duodenal mucosal tissues before and after treatment with bile salts. **a** A picture of upper part of the pig gastric mucosal surface before treatment with bile salts. **b** A picture of upper part of pig gastric mucosal surface after treatment with bile salts. **c** A picture of lower part of the pig gastric mucosal surface before treatment with bile salts. **d** A picture of lower part of pig gastric mucosal surface after treatment with bile salts. **e** A picture of the pig duodenum mucosal tissue before treatment with bile salts. **f** A picture of the pig duodenum mucosal tissue after treatment with bile salts

Effect of the Surface Hydrophobicity of Polymers on Adhesion

The adhesive force between the polymer and IGM decreased as the surface free energy of the polymers decreased, whereas the adhesive force between the polymer and IGM-LPC increased as the surface free energy of the polymers decreased (Fig. 6a). Furthermore, the adhesive force between the polymer and IGM decreased as the contact angle increased, however; the adhesive force between the polymer and IGM-LPC increased as the contact angle increased (Fig. 6b). There is the relationship between the surface hydrophobicity of the polymers and the adhesive force, showing that the *in vitro* adhesive force between the polymer and IGM-LPC increased when the surface hydrophobicity of the polymers increased.

DISCUSSION

Development and Validation of an *In Vitro* Gastric Mucus Model

Development of a reliable *in vitro* gastric mucus model is essential because the surface property of the stomach tissue changes from hydrophobic to hydrophilic after the animal is sacrificed (14). The *in vitro* model should basically include the structural characteristic of the gastric mucosal surface as seen in Fig. 1 and the functional property of the hydrophobic barrier to proton permeation in mimicking the *in vivo* pig stomach surface properties. With these pieces of information from the surface characterization study of the pig stomach, a simplified *in vitro* gastric mucus model consisting of crude pig gastric mucin, polycarbophil, and egg yolk phosphatidylcholine was developed. Polycarbophil was specifically introduced into this gel system to control swelling and prevent erosion of the system during experimentation. It forms negative charges in a pH buffer solution of greater than its p*K*a, around 4.5, which represents a negatively charged biological cell surface. Egg yolk LPC was selected as a LPC source because the composition of egg yolk LPC containing 34% palmitic acid, 32% oleic acid, and 18% linoleic acid is similar to that of the most abundant phosphatidylcholine species (21–23) in rat, dog, pig, and human gastric mucus (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine. The effect of LPC present on the IGM surface may be primarily protection of epithelial cells from acid back-diffusion. Several studies (24–26) have shown that animal gastric mucus retarded the permeability of small molecules. It is most likely that the *in vitro* model is functionally similar to the property of animal gastric mucosal surface based on the retardation of hydrogen ion permeability. Alcian blue staining, contact angle measurement, and proton permeability studies confirmed that that the *in vitro* gastric mucus model is structurally and functionally similar to the property of the animal gastric mucosa surface. The *in vitro* gastric mucus model was specifically designed to understand the role of the phospholipid lining of gastric mucus on bioadhesion of bioadhesive polymers as well as factors affecting bioadhesion. Although this *in vitro* gastric mucus model cannot reproduce some properties of the *in vivo* gastric mucus such as a pH gradient in gastric mucus gel, it is valuable enough to understand the role of the phospholipid lining in bioadhesion of polymers, the mechanism of bioadhesion and *in vitro* screening of bioadhesive polymers, which stick to the hydrophobic stomach surface.

Surface Hydrophobicity of the Pig Gastric Mucosa

Characterization of the surface property of the pig gastric mucosa is an essential component in our study because the physiology and anatomy of the pig stomach are

Table I. Effect of the Surface Hydrophobic Material Depletion on the Permeability of Pig Gastric Mucosa to Hydrogen Ion

Material	Permeability Coefficient (cm/s)	
	A	B
Upper part of pig stomach (1)	$(1.017\pm0.094)\times10^{-6}$	$(3.12\pm0.12)\times10^{-6}$
Lower part of pig stomach (2)	$(1.104\pm0.034)\times10^{-6}$	$(3.34\pm0.090)\times10^{-6}$

No statistically significant difference between (1)-A and (2)-A; (1)-B and (2)-B. Statistically significant difference between A and B. Unpaired *t* test, $p<0.05$. Data reported as mean±SD ($n=5$)
A Before treatment with bile, *B* after treatment with bile

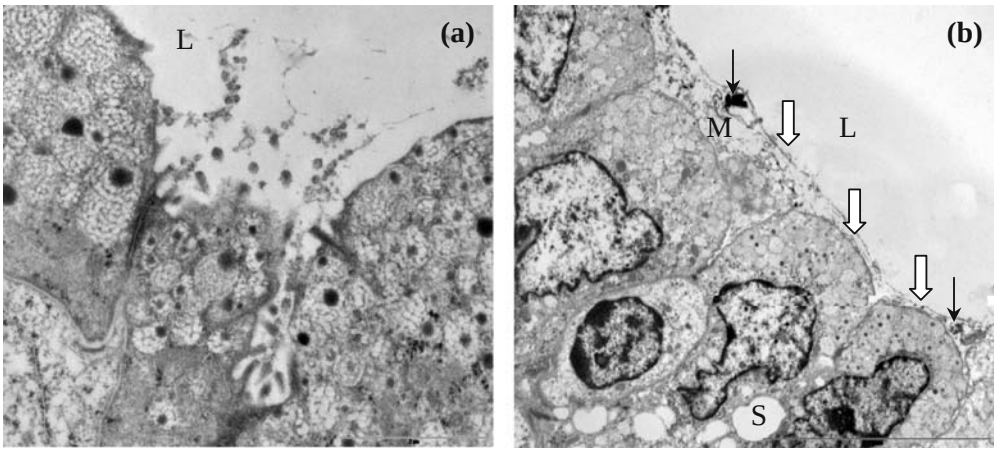

Fig. 4. Electron micrographs of surface mucous cells (SMCs) of pig gastric mucosa treated with iodoplatinate. **a** Bile salt-treated pig gastric mucosa shows loss of apical cell membrane integrity as well as extracellular mucus layer of the SMCs. **b** Intact pig gastric mucosa (bile salt-untreated) shows a continuous iodoplatinate-reactive filamentous fine phospholipid layer deposited on the luminal surface of the mucus gel (*open arrows*) and IP-reactive materials in the form of lamellated vesicles appear in close association with the phospholipid layer and within the mucus layer (*solid arrows*). *L* Lumen, *S* secretory mucus granules, *M* extracellular mucus gel layer

close to that of humans (19). The results from the surface property characterization confirm that the luminal surface of pig gastric mucosa is relatively hydrophobic in nature due to the presence of a hydrophobic phospholipid lining on the mucus layer. Contact angles of air bubbles on the luminal surface after treated with bile salts become smaller than their on the luminal surface before treated with bile salts. Moreover, the luminal surface did stain well with alcian blue dye after bile salt treatment, which originally did not stain with alcian blue before treatment. It is concluded that bile salts solubilize hydrophobic molecules from the luminal surface of the gastric mucosa and then make the luminal surface more hydrophilic and wettable. This is supported by Goggin *et al.* (27) that the contact angles for gastric ulcer human subjects with relatively a high bile salt concentration in gastric juice had significantly lower contact angles than healthy subjects, indicating the surface hydrophobicity is reduced by refluxed bile. Spychal *et al.* (28) measured the contact angles of saline drops applied to endoscopic biopsy specimens of human gastrointestinal mucosa, showing that a contact angle on the gastric mucosa surface is greater than its on duodenal mucosa surface. Our results for the hydrophobic profile of the pig gastric mucosa entirely agree with the published data (10,27) on the dog and human that the mammalian gastric mucosa surface is relatively hydrophobic and non-wettable. Several studies (24–26) demonstrated that the hydrophobic stomach surface functions as a hydrophobic barrier to protect epithelial cells from luminal acid-back diffusion. A study by Slomiany *et al.* (29) demonstrated that preincubation of pig gastric mucin with 10 mM lysolecith, which is a highly surface active material, produced a marked decrease in retardation ability of the glycoprotein to hydrogen ion, whereas preincubation with 10 mM lecithin increased retardation ability to hydrogen ion. These results concluded that an additional hydrophobic phospholipid lining of the mucus gel layer plays a significant role in retardation of hydrogen ion diffusion. The results of our proton permeability using pig gastric mucosal samples before and after treatment with bile salts showed that removal of a surface hydrophobic phospholipid by bile salts caused an increase in the permeability of hydrogen ion through pig gastric mucosa compared to the intact pig gastric mucosa. This suggests that the hydrophobic phospholipid present on the gastric mucosal surface is essential to provide hydrophobic barrier to acid-back diffusion. There were morphological studies (8,20) using selective histochemical iodoplatinate staining and electron microscopic techniques, showing that a continuous filamentous phospholipid layer is present at the luminal interface of rat and dog gastric mucus gel surface. It was confirmed that the surface hydrophobicity of small animal gastric mucosa is attributable to an adsorbed layer of surface-active phospholipids. Our morphological studies of pig gastric mucosa revealed that a continuous iodoplatinate-reactive filamentous

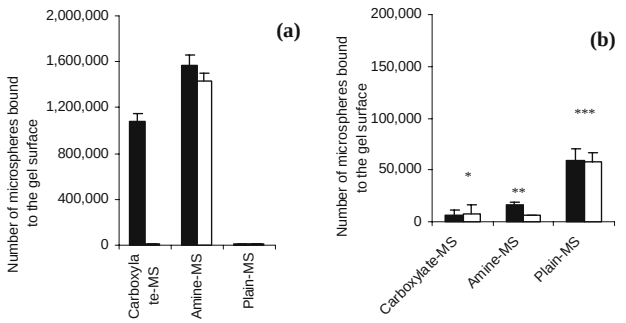

Fig. 5. Adhesion of microspheres on IGM-LPC and IGM surfaces in different pH buffer solutions (*solid bars*; pH 2.0, *open bars*; pH 6.0). **a** Adhesion of microspheres on the hydrophilic IGM surface. **b** Adhesion of microspheres on the hydrophobic IGM-LPC. No statistically significant different between * and **. Statistically different between * or ** and ***. Unpaired *t* test $P<0.05$. Data reported as mean±SD ($n=3$)

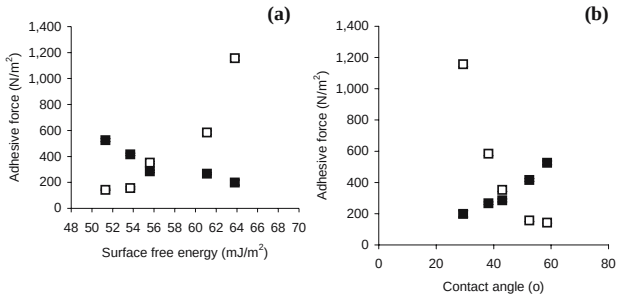

Fig. 6. Correlations between the surface hydrophobicity of the polymers and adhesive force between the polymer and IGM-LPC or IGM in pH 2.0 buffer at 37°C. **a** Relationship between surface free energies of the polymers and the adhesive force between the polymer and IGM or IGM-LPC. The adhesive forces of the polymers on the IGM surface with a surface free energy of 62.7±0.2 mJ/m^2 (*open squares*) and on the IGM-LPC surface with a surface free energy of 48.1±0.5 mJ/m^2 (*solid squares*). **b** Relationship between contact angles of the polymers and the adhesive force between the polymer and IGM or IGM-LPC. The adhesive forces of the polymers on the IGM surface with a contact angle of 37.3±1.5° (*open squares*) and on the IGM-LPC surface with a contact angle of 63.5±3.0° (*solid squares*). Data reported as mean±SD (n=3)

phospholipid layer is deposited on the luminal surface of the mucus layer. However, after luminal exposure to 2.0% bile salt for 10 min, electron micrographs of the pig gastric mucosa treated with bile salt showed loss of the integrity of the apical plasma membrane integrity and damage of the extracellular mucus layer as well as the continuous phospholipid layer. This strongly indicates that the surface hydrophobicity of human stomach may be contributed by an adsorbed phospholipid layer, which primarily functions as proton diffusion barrier.

Effect of the Surface Functional Groups of Polymers on Adhesion

Using the validated IGM-LPC and IGM surfaces, we investigated the effect of functional groups of polymers on adhesion to the hydrophobic phospholipid lining of the IGM-LPC using fluorescent polystyrene microspheres with three different surface functional groups (COOH, NH_2, aromatic ring; Table II). Bioadhesive polymers, which work well at the hydrophilic mucosal surfaces such as the vagina, eye, nose, and buccal and do not work at the hydrophobic stomach surface, are generally hydrophilic and contain COOH, NH_2, OH functional groups in their molecules. These fluorescent polystyrene microspheres are a useful polymer model for the purpose of examining the effect of surface functional groups on adhesion with the additional advantage of no swelling characteristic and a constant particle size. In this study, a new fluorescence technique named front-faced fluorescence measurement was used, in which the fluorescence intensity of the gel surfaces of IGM-LPC and IGM was measured before and after they were soaked in the fluorescent polystyrene microsphere suspensions as to degree of adhesion of the microspheres on to the specific gel surface. The results demonstrated that adhesion of the microspheres to negatively charged gel surface is mainly through both electrostatic interaction and hydrogen bonding. For Carboxylate-MS, a number of the microspheres adhered to the hydrophilic gel surface at pH 2.0, but did not adhere at pH 6.0. This is explained in that Carboxylate-MS is predominantly in the unionized form at pH 2.0, which is less than its pKa of about 2.5, thereby increasing hydrogen bonding between the microsphere and the IGM gel, and is predominantly in the negatively charged form at pH 6.0, resulting in an increase in charge repulsion. For amine-MS, a number of Amine-MS adhered to the IGM gel surface at both pH 2.0 and 6.0. This is explained in that the Amine-MS is predominantly in the positively charged form at pH 2.0, which is less that its p*K*a of about 6.0, resulting in an increase in electrostatic interaction between the positively charged amine-MS and the IGM gel, and is in about 50% negatively charged and 50% un-ionized forms at pH 6.0 indicating that both electrostatic interaction and hydrogen bonding contribute to adhesion of the microspheres. For plain-MS, it is explained that adhesion of plain-MS to the hydrophobic phospholipid lining of the IGM-LPC gel is facilitated by hydrophobic interaction. This could be a main reason that hydrophilic bioadhesive polymers do not work in the stomach.

Effect of the Surface Hydrophobicity of Polymers on Adhesion

Lastly, we investigated how the surface hydrophobicity of polymers influences adhesion to a hydrophobic phospholipid lining of IGM-LPC, relative to the hydrophilic IGM surface, using diblock-copolymers of poly(styrene) and poly(acrylic acid) with various chain lengths to give different surface hydrophobicity. We correlated the surface hydrophobicity,

Table II. Characteristics of Fluorescent Polystyrene Microspheres Used for Adhesion Studies

Microspheres	p*K*a[a]	Mean Diameter (um)[b]	Surface Charge Density[c] (meq./g)	Number[d] of Microspheres/ml Suspension	Ex/Em Wavelength
Carboxylate-Modified	2.5 (4.0)	1.0	0.179	3.64×10^{10}	365/415 (green)
Amine-Modified	6.0 (9.0)	0.93	0.335	4.5×10^{10}	505/515 (yellow-green)
Plain	–	2.03	–	2.28×10^{9}	480/520 (yellow-green)

[a] p*K*a values from a reference (22)
[b] As determined by TEM, SEM, and DLS
[c] As measured by conductiometric titration
[d] As calculated by an equation of number of microspheres/ml of suspension = $6C\times10^{12}/(\rho\times\pi\times\varphi^3)$, where C = concentration of suspended spheres in g/ml, φ = diameter of microspheres, and ρ = density of polymer in g/ml

either in terms of contact angle or surface free energy, of the polymers with the adhesive force (N/m^2) between the polymer and IGM-LPC or IGM. The results indicate that a hydrophilic polymer will adhere to a hydrophilic mucosal surface, but will not adhere to a hydrophobic biological surface such as the stomach surface. In the case of a hydrophobic polymer, however, it will adhere to the hydrophobic stomach surface, but will not adhere to hydrophilic mucosal surfaces. It is concluded that the hydrophobic phospholipids lining of the IGM-LPC acts as an adhesion barrier to hydrophilic bioadhesive polymers, which have shown a strong adhesion at hydrophilic mucosal surfaces.

CONCLUSIONS

The present research primarily focused on understanding the role of a biochemical factor, the presence of the hydrophobic phospholipids lining of the gastric mucosa, in adhesion of polymers. It is the first *in vitro* attempt to show that the presence of a hydrophobic phospholipid layer on the surface of gastric mucus gel was thoroughly explored as a major factor leading to poor bioadhesion of hydrophilic bioadhesive polymers in the stomach. It clearly demonstrates that the hydrophobic phospholipid lining of the *in vitro* gastric mucus model (or of the mammalian gastric mucosa) acts as an adhesion barrier to hydrophilic bioadhesive polymers, however; adhesion is improved when the surface hydrophobicity of polymers increases. It is concluded that this biochemical factor might be the key factor affecting poor bioadhesion in the stomach. Therefore, the hydrophobic phospholipid lining of the gastric mucosa must be taken into consideration as a potential target in development of bioadhesive controlled or sustained drug delivery systems.

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