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Bioadhesive interaction and hypoglycemic effect of insulin-loaded lectin–microparticle conjugates in oral insulin delivery system

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Abstract

Biodegradable microparticles were prepared with alginate by the piezoelectric ejection process, and lectin (wheat germ agglutinin, WGA) was conjugated to alginate microparticles to take advantage of the protective effects of alginate microparticles and the mucoadhesive properties of WGA for improved oral delivery of insulin. Their specific interaction with model mucin was determined by pig mucin (PM) immobilized surface plasmon resonance (SPR) biosensor and in vitro adsorption studies. The hypoglycemic effects of alginate and WGA-conjugated alginate microparticles were examined after oral administration in streptozotocin-induced diabetic rats. The alginate microparticles were fabricated by ejecting alginate/insulin solution into 0.1 M $CaCl_2$ solution through a nozzle actuated by the piezoelectric transducer. The WGA was conjugated to alginate microparticles by activating hydroxyl groups with carbonyldiimidazole (CDI). The affinity constant (K_A) of alginate–WGA microparticles from the SPR data (K_A=5.455 g^{-1} L) was about nine times greater than alginate microparticles (K_A=0.628 g^{-1} L). In vitro experiments in the mucin solution showed that the conjugated WGA enhanced the interaction about three times. In vivo studies with diabetic rats showed that the blood glucose level of SPF rats was lowest when alginate–WGA microparticles were orally administered. Larger K_A of alginate–WGA microparticles resulted in larger glucose change (%) from base level. Still, it is not clear whether the transport of insulin through the intestinal mucous membrane was influenced by the increase of residence time at intestinal membrane through the specific adsorption of WGA-conjugated microparticles. However, it is concluded that alginate–WGA microparticles enhance the intestinal absorption of insulin sufficient to drop the glucose level of blood.
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1. Introduction

Oral delivery is the most convenient and desired way for drug delivery, especially when repeated or routine administration is necessary. In spite of these

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advantages, most peptide and protein drugs are not administered orally due to their gastrointestinal degradation by digestive enzymes [1]. A number of different strategies, such as use of permeation enhancers, enteric coatings, protease inhibitors and microsphere encapsulation, have been explored to improve the oral bioavailability of insulin [2]. In addition, extensive research to overcome drawbacks has been devoted to finding specific carriers for oral delivery [3] and their bioadhesive interactions with mucous membrane [4]. Bioadhesion can be obtained by either nonspecific or specific interactions with surface ligands at a mucosal surface. It prolongs the residence time at the site of drug absorption and reduce the dosing frequency [5]. Specific bioadhesive devices may be formed by small colloidal particles (drug carriers), coupled with lectins that are able to interact with receptors at the surface of gastrointestinal mucosa [6]. Lectins are proteins or glycoproteins of nonimmunological origin which specifically recognize sugar molecules and therefore are capable of binding to glycosylated membrane components [7]. Sugars are all present in glycoproteins and glycolipids of mammalian mucosa, either at the surface of epithelial cells or in mucous layers [8]. For pharmaceutical purposes, especially nontoxic lectins of plant origin being part of the regular diet of man seem to be suited, such as tomato lectin and wheat germ agglutinin (WGA) [9]. WGA binds to *N*-acetylglucosamine and sialic acid residues exhibiting a molecular weight of 36 kDa. As compared with plant lectins with different carbohydrate specificity, it was known that the WGA-binding rate to intestinal cell lines of human origin, human colonocytes and prostate cancer cells was highest [10]. Most lectin–particle conjugates have been reported with polystyrene particles, which are unacceptable as a drug carrier because of its nondegradability and there are only few studies concerning biodegradable particles [5,9].

Alginate is a naturally occurring biodegradable polymer and has several unique properties that have enabled it to be used as a matrix for the entrapment and delivery of proteins, drugs and cells [11,12]. Alginate microparticles were prepared with various methods [13–16]. In this study, alginate microparticles were prepared by the piezoelectric ejection process [17] that did not require use of toxic organic solvents like conventional methods, that did not denature proteins. In addition, in situ formation of alginate microparticles could be achieved by ionic gelation. The piezoelectric ejection process is spraying a polymer solution though a small orifice which is vibrated by a piezoelectric transducer. This method combined with carrier stream can achieve precise control of sphere size and monodisperse size distribution. It is a highly cost-effective, easy for scale-up, environmentally friendly method [18].

In this work, biodegradable microparticles were prepared with alginate by the piezoelectric ejection process, and WGA was conjugated to alginate microparticles to take advantage of the protective effects of alginate microparticles on the one hand and the mucoadhesive properties of WGA on the other hand. The interaction between WGA-conjugated alginate microparticles and model pig mucin (PM) was characterized by use of surface plasmon resonance (SPR) biosensor. Recently, the biosensor based on SPR has been used to analyze interactions of glycoproteins, glycopeptides and oligosaccharides with lectins in real time without fluorescence or radioisotope labeling. The method is rapid and sensitive, and yields rich and useful results, including kinetic parameters in studies of glycoprotein–lectin interactions [19]. In vitro adsorption studies were conducted to evaluate the activities of alginate and WGA-conjugated alginate microparticles with PM. The hypoglycemic effects of alginate and WGA-conjugated alginate microparticles were examined after oral administration in streptozotocin-induced diabetic rats. In addition, the affinity constant (K_A) from SPR data was compared with in vitro adsorption studies and in vivo studies to study the relation between the affinity constant and in vitro, in vivo studies.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt with medium viscosity, lectin from Triticum vulgaris [wheat germ agglutinin (WGA)], 1,1′ -Carbonyldiimidazole (CDI), glycine, sodium citrate, insulin from bovine pancreas, phosphate-buffered saline (PBS), pH 7.4, containing 0.138 M NaCl and 0.0027 M KCl (PBS 7.4) and crude

mucin from pig (PM) were purchased from Sigma (St. Louis, MO, USA). Calcium chloride dehydrate (99+%), sodium tetraborate (99.998%), 11-Mercaptoundecanoic acid (MUA), 1-[3-(Dimethylamino)-propyl]-3-ethyl carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride and Triton X-100 were purchased from Aldrich (Milwaukee, WI, USA). Acetone, ethyl acetate and ethanol were purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Junsei (Tokyo, Japan). Streptozotocin was purchased from Wako (Osaka, Japan). All chemicals were used as received.

2.2. Microparticle fabrication by the piezoelectric ejection process

The schematic diagram of the piezoelectric ejection process is shown in Fig. 1. Solution containing the polymer and any active agent (protein drug) to be encapsulated pass through a small nozzle (30 μm in diameter) to form a smooth, cylindrical jet. The nozzle is vibrated by a piezoelectric transducer driven by a function generator. A wave of acoustic energy generates periodic instabilities that break the stream into a train of uniform droplets.

Alginate (0.5% w/v) and insulin (0.25% w/v) was dissolved in 0.01 N NaOH solution. The sample solution was filtered with syringe filter (0.45 μm) and pumped through an orifice (30 μm diameter) at a flow rate of 0.4 ml/min. The optimum frequency for microparticle fabrication was 24.2 kHz. Droplets went into in 0.1 M $CaCl_2$ solution to form alginate microparticles containing insulin by ionic gelation. Ionic gelation was conducted for 15 min with vigorous stirring at room temperature. The microparticles in the solution were examined by an optical microscope and the dried microparticles were characterized by scanning electron microscopy (Philips 535 M, the Netherlands).

2.3. Encapsulation efficiency (EE%)

Fifteen milligrams of alginate microparticles were dissolved in 3% w/v sodium citrate solution (10 ml) for 24 h and centrifuged at 8000 rpm for 30 min. The insulin concentration was measured at 244 nm by UV spectrophotometer (Jasco V-530, Japan) with standard

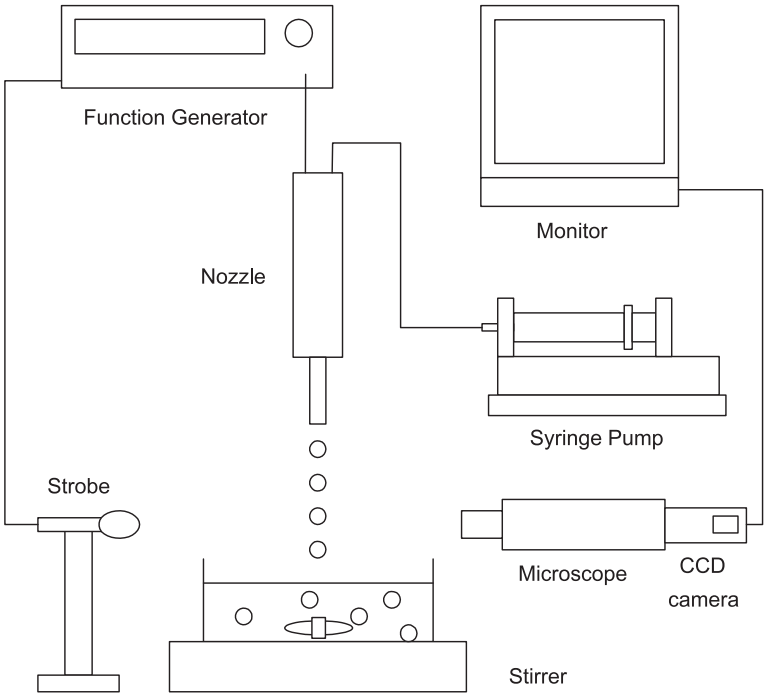


Fig. 1. The schematic diagram of the piezoelectric ejection process.

curve. The encapsulation efficiency (EE%) was calculated with following equation: EE%=actual drug content/theoretical drug content. The EE% was 61.02%.

2.4. Conjugation of WGA to microparticles

WGA was conjugated to alginate microparticles by activating hydroxyl groups with carbonyldiimidazole (CDI) in aprotic solvent, such as acetone [20]. Briefly, the microparticles were incubated in 10 ml of anhydrous acetone containing 0.4 g of CDI (4.0% w/v) for 12 h at room temperature with shaking. The CDI solution was discarded and the microparticles were washed several times with anhydrous acetone to remove unbound CDI. WGA was coupled to the activated alginate microparticles. The activated microparticles were resuspended in 2 ml of 0.2 M sodium borate buffer, pH 9.5, to which 0.4 mg of WGA was added. The microparticles were washed several times in the borate buffer to remove unbound lectin and incubated for 1 h in 0.5% glycine (w/v) in borate buffer to quench activated sites to which lectin was not bound.

2.5. SPR measurements

2.5.1. Attachment of model mucin (PM) on sensor surface

The interaction of alginate–WGA microparticles and model mucin from pig (PM) was examined with the PM immobilized SPR biosensor. The schematic diagram of a typical SPR instrument is depicted in Fig. 2 [21]. In this study, SpreetaTM Evaluation

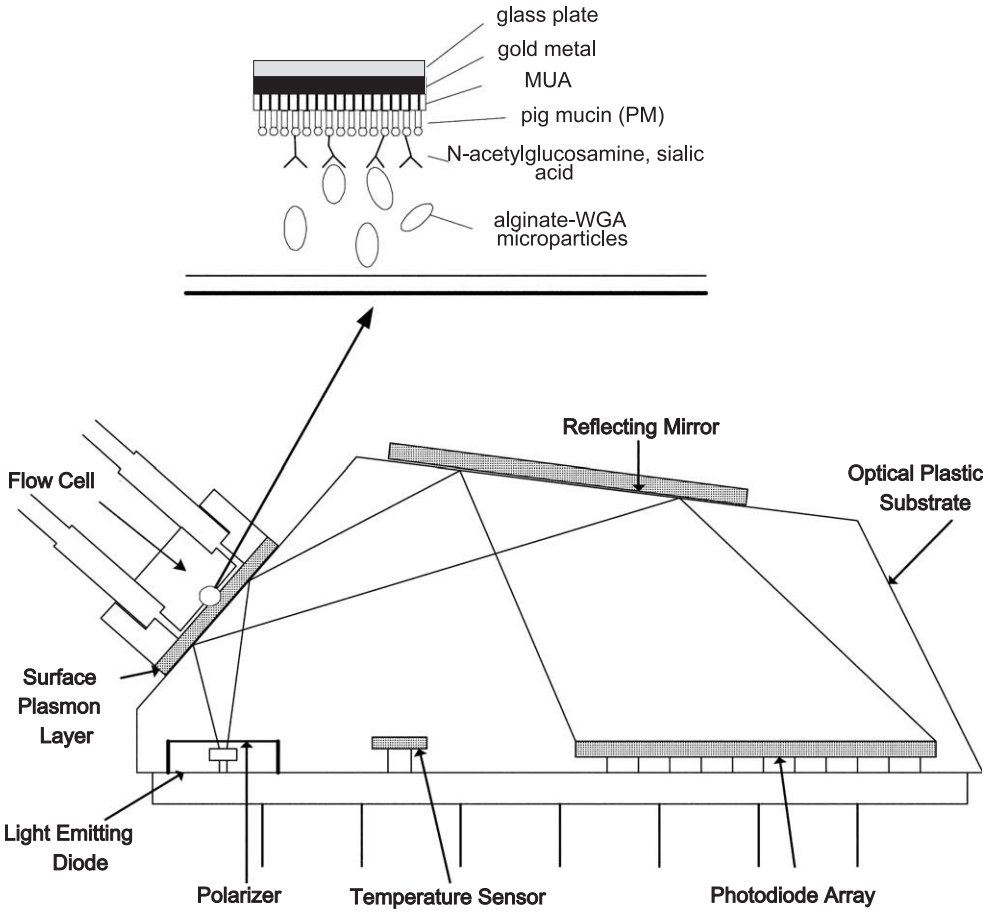


Fig. 2. The schematic diagram of a typical SPR instrument.

Module (SPR-EVM-BT) Surface Plasmon Resonance Biosensor (Nomadics OK, USA) was used. The sensor gold surface was cleaned with a cleaning solution, and the sensor was equilibrated in PBS 7.4. MUA monolayers were formed on a gold film by flowing a MUA solution through the flow cell of the SPR sensor for 45 min. The concentration of the MUA solution was 1 mM in PBS 7.4 and the flow rate was 40 μl/min at room temperature [22]. The carboxylic groups of MUA monolayers were activated by flowing 0.2 M EDC and 0.05 M NHS solution in PBS 7.4 for 45 min. After activation, PM was attached to the sensor surface by flowing the 100 μg/ml solution of PM in PBS 7.4 for 1 h at a flow rate of 40 μl/min at room temperature. The remaining surface NHS-esters were then blocked by flowing 1 M ethanolamine hydrochloride solution [23–25].

2.5.2. Evaluation of activity between alginate–WGA microparticles and PM

The interaction of alginate–WGA microparticles and PM was examined by flowing the 1 mg/ml dispersion of alginate–WGA microparticles over the PM immobilized SPR sensor in PBS 7.4 containing 0.1% of Triton X-100. Triton X-100 was used to reduce the aggregation of alginate–WGA microparticles. The interaction was measured for 1 h at a flow rate of 40 μl/min at room temperature [21]. The interaction of alginate microparticles with the PM immobilized SPR sensor was also examined as control.

2.6. In vitro adsorption studies with PM

The activities of alginate–WGA microparticles were determined by mixing 1 ml of the PM suspension at PBS 7.4 with the equal volume of alginate–WGA microparticles suspension for an incubation period of 60 min [8]. The samples were centrifuged (10 min at 4000 rpm) and the remaining free PM in the supernatants was measured at 251 nm by UV spectrophotometer, since interacted PM was sedimented together with alginate–WGA microparticles. A centrifuged solution of PM in PBS 7.4 was used as reference. The activities of alginate microparticles with PM were measured as control.

2.7. In vivo studies

2.7.1. Experiment animals

Sprague–Dawley (SPF) with 210±10 g of weight which was supplied by Bio-Genomics (Seoul, Korea) was used as an experiment animal. They were adjusted in breeding room which was kept in constant temperature and humidity for 7 days and investigated to discriminate healthy ones for experiment. Five animals were bred in every stainless steel breed box (420 W×500 L×300 H mm) under following conditions: 12 h interval of artificial illumination (from 8 a.m. to 8 p.m.), the intensity of illumination was 300–500 lx, temperature 22±1 °C, humidity 55±5%, evacuation was 10–18 times/h. They were allowed to take Purina and water freely.

2.7.2. Induction of diabetes

Diabetes was induced for 1 week by an intravenous injection of 45 mg/kg of streptozotocin (Wako) dissolved in 0.1 M citrate buffer (pH 4.0) for two times with the interval of 24 h, in the tail vein of SPF fasted for 12 h. The glucose level was measured 2 h before experiment and rats which had glucose level of only 280–380 mg/dl were used for experiment. The 24 diabetic rats were randomly divided into four groups, and each group (n=6) was housed in one cage. Formulations following were administered to them orally: (1) control insulin solution (insulin dose 50 I.U./kg); (2) alginate microparticles (insulin dose 50 I.U./kg); (3) alginate–WGA microparticles (insulin dose 50 I.U./kg). The microparticles were suspended in the distilled water before oral administration [26].

2.7.3. Measurement of glucose level

Blood samples were collected from the tail vein of the rats to measure the glucose levels by Glucotrend II (Roche, Germany). Glucose levels were measured at −2 (2 h before oral administration), 0, 1, 2, 4, 6, 8, 10, 12 and 24 h after oral administration. Results were shown as the mean values of plasma glucose levels (±standard deviation) of 6 animals. The mean blood glucose levels determined in samples collected before insulin administration were taken as the baseline levels. Using these values, the percentage of glucose reduction at each time after dosing was calculated and plotted against time. Data from different experimental

groups were compared with the corresponding control groups (control insulin solution) by the *U*-test of Mann and Whitney with significance level of $p<0.05$ [27].

3. Results and discussion

3.1. Preparation of alginate microparticles

The alginate microparticles were obtained by the piezoelectric ejection process. The uniform droplets ejected from the dispenser are shown in Fig. 3A. The droplets of an alginate/insulin solution entered into a 0.1 M $CaCl_2$ solution, and gelated to form a layer near the interface encapsulating insulin and finally turned into microparticles by ionic gelation. The piezoelectric ejection process did not include emulsification step, thereby minimizing inactivation of encapsulated drugs. The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure. The divalent cations bind to the –L-guluronic acid blocks in a highly cooperative manner and the size of the cooperative unit is more than 20 monomers. Each alginate chain can dimerize to form junctions with many other chains, and as a result, gel networks are formed rather than insoluble precipitates [11]. The obtained microparticles in the solution had average diameter of 60–80 μm which is the slightly larger value than the theoretical diameter (Fig. 3B) [18]. It seems that the droplets coalesced before ionic gelation to form larger microparticles than theoretical microparticles diameter. After drying, the microparticles deswelled and had average diameter of 1–20 μm (Fig. 3C).

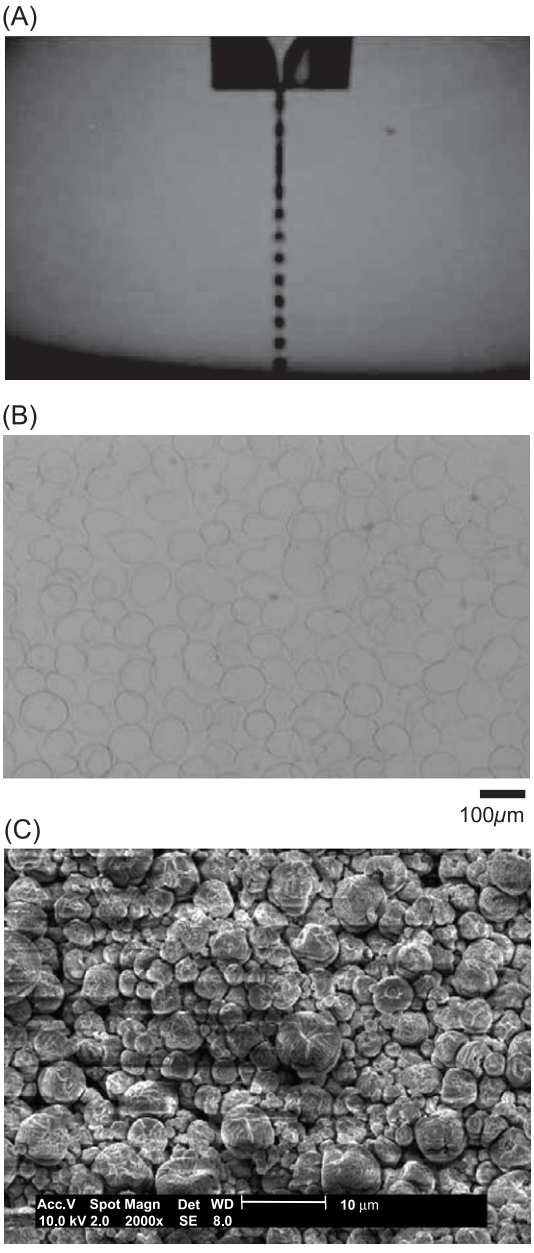


Fig. 3. The fabrication of alginate microparticles by the piezoelectric ejection process. (A) The uniform droplets ejected from the dispenser. (B) The optical microscope of alginate microparticles. (C) The SEM photograph of alginate microparticles after drying.

3.2. Conjugation of WGA to alginate microparticles

The procedure of WGA conjugation to alginate microparticles is depicted in Fig. 4 [28]. CDI forms an imidazolyl carbamate complex with the hydroxyl group of alginate microparticles which may be displaced by binding the free amino group of a ligand, such as a protein. The reaction is an *N*-nucleophilic substitution and results in a stable *N*-alkylcarbamate linkage of the ligand to the polymer. The resulting ligand–polymer complex is stable and resists hydrolysis for extended periods of time [20].

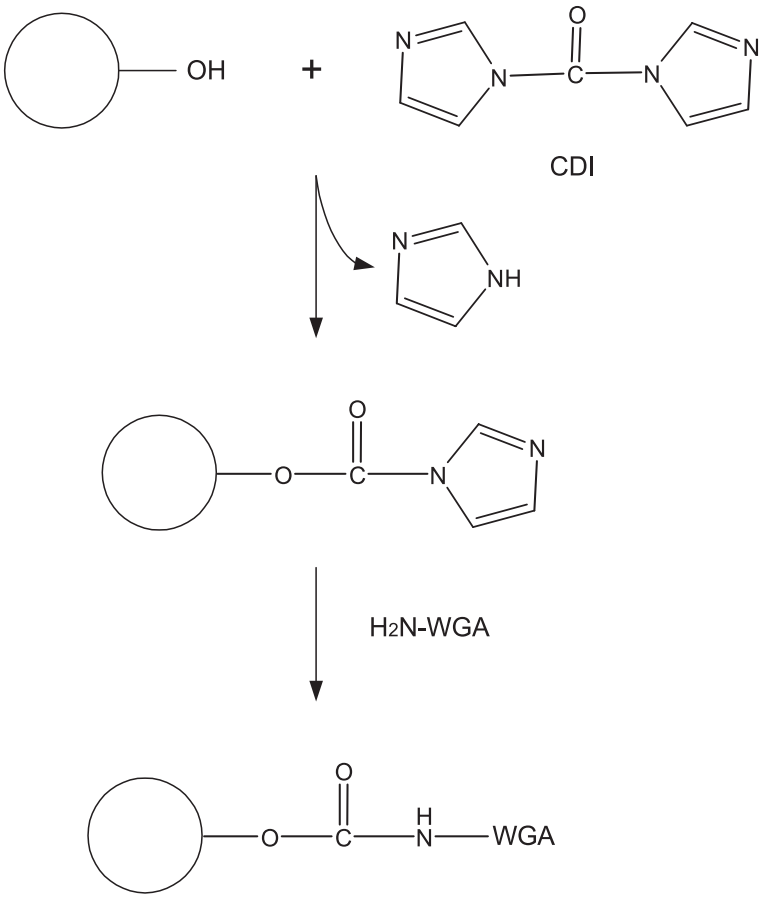


Fig. 4. The reaction scheme of WGA conjugation to alginate microparticles.

3.3. SPR measurements

3.3.1. Attachment of model mucin (PM) on sensor surface

A pig mucin (PM) as a model mucin has been immobilized on the gold surface of an SPR sensor and the attachment chemistry of surface layers was characterized with SPR measurements. The steps employed in the surface modification are shown in Fig. 5. Monolayers of MUA were deposited on a gold surface with carboxylic group outwards. The carboxylic groups of MUA were reacted with a carbodiimide and stabilized with *N*-hydroxysuccinimide, to form a succinimidyl ester. The resulting ester reacted with amine residues on the PM. In this way, it was possible to bind PM to the modified gold surface [22]. Each binding step on the sensor surface was monitored in real time as an increase in the relative index of refraction at the sensor surface over time. When the gold surface has been cleaned, and a steady PBS 7.4 baseline has been established, the gold surface was first coated with a self-assembled monolayer (SAM) of MUA. The refractive index increases, as MUA binds to the gold surface. Small amount of the bound MUA was rinsed off after switching back to the PBS 7.4 buffer (Fig. 6A). The carboxylic groups of MUA were activated by EDC and NHS to form activated ester. Increase in the initial index of refraction of the EDC/NHS solution is due to its higher refractive index compared to that of PBS 7.4 buffer. The formation of activated ester was confirmed by the increased refractive index after rinsing the sensor with PBS buffer (Fig. 6B). The activated ester groups were reacted with amine groups in PM to form a peptide bond which is resistant to hydrolysis [20]. The refractive index increased with PM binding and very

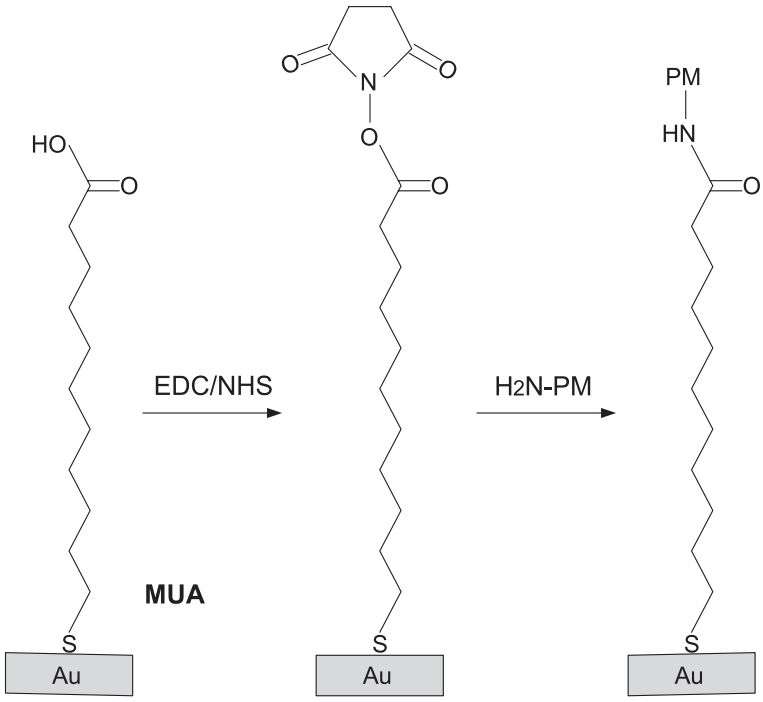


Fig. 5. The surface modification for attaching PM onto a gold surface.

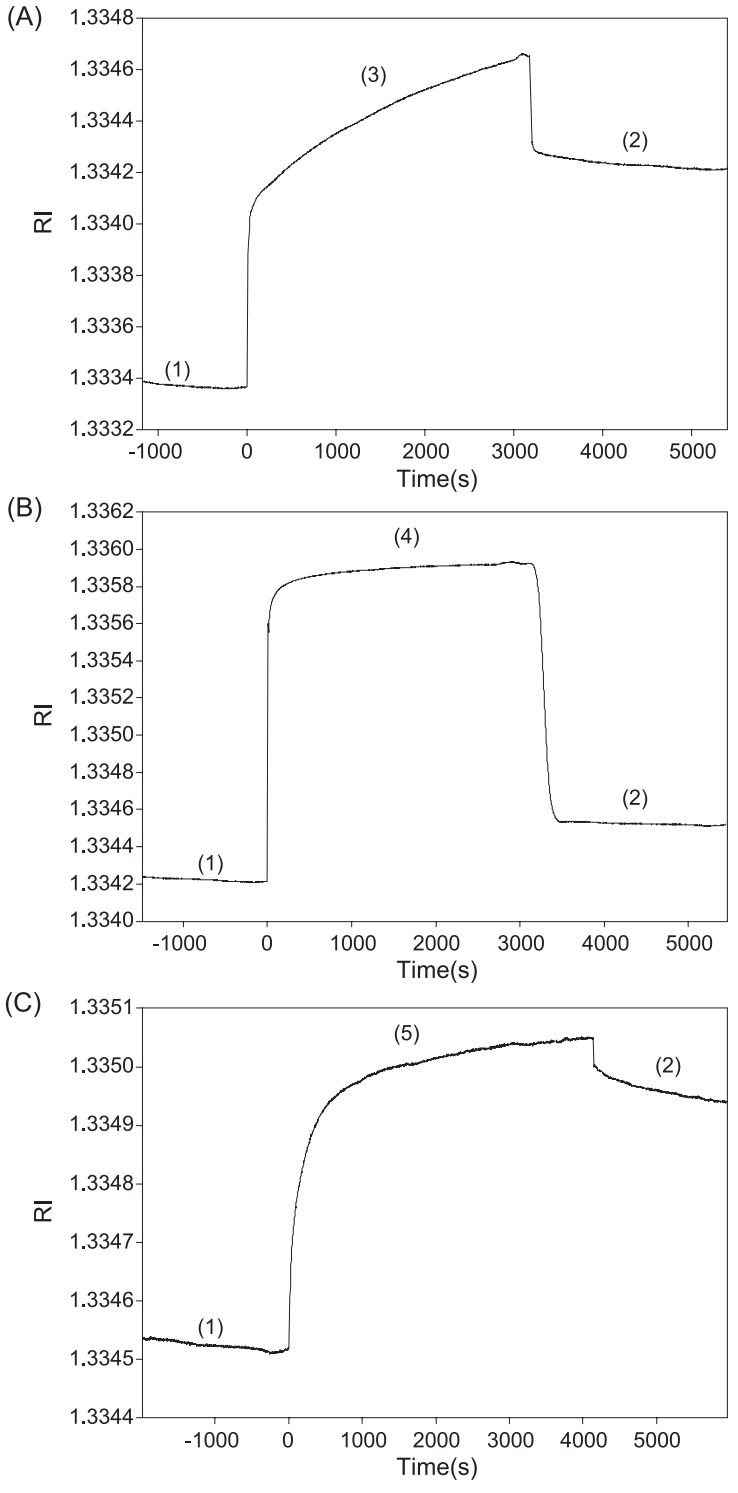
little of PM was rinsed off after switching back to the PBS buffer (Fig. 6C). Consequently, the model mucin (PM) was attached to the gold surface of SPR sensor and the biosensor for measuring the interaction of alginate–WGA microparticles with the PM was constructed.

3.3.2. Evaluation of the interaction between microparticles and PM

The schematic diagram of the interaction between alginate–WGA microparticles and the PM immobilized SPR sensor is depicted in Fig. 7. As alginate–WGA microparticles interacted with the PM immobilized SPR sensor, the refractive index increased and small amount of alginate–WGA microparticles were rinsed off after switching back to the PBS buffer (Fig. 8A). The interaction of alginate microparticles (control) with the PM immobilized SPR sensor is shown in Fig. 8B. A large signal in the initial index of refraction of alginate microparticles dispersion is due to its higher refractive index compared to that of the PBS buffer. This is indicated by their inability to maintain altered refractive index after rinsing the sensor with buffer. There were little interactions between alginate microparticles and PM. These results indicated that model mucin (PM) was attached on the gold surface of SPR sensor successfully and the interactions between lectin (WGA) and protein (PM) occurred at the SPR sensor surface.

The dissociation constant (k_d), association constant (k_a) and affinity constant (K_A) of alginate–WGA and alginate microparticles were calculated according to Refs. [29,30]. k_d, k_a and K_A of alginate–WGA

Fig. 6. The change in refractive index over time during immobilization of PM on SPR sensor. (A) Binding of MUA on a gold surface of SPR sensor. (B) Activation of carboxylic groups with EDC and NHS. (C) Attachment of model mucin (PM) on activated ester groups. (1) The baseline with PBS 7.4. (2) The flow of PBS 7.4 for washing. (3) The application of 1 mM MUA solution in PBS 7.4. (4) The application of EDC/NHS in PBS 7.4. (5) The application of 100 μg/ml PM in PBS 7.4.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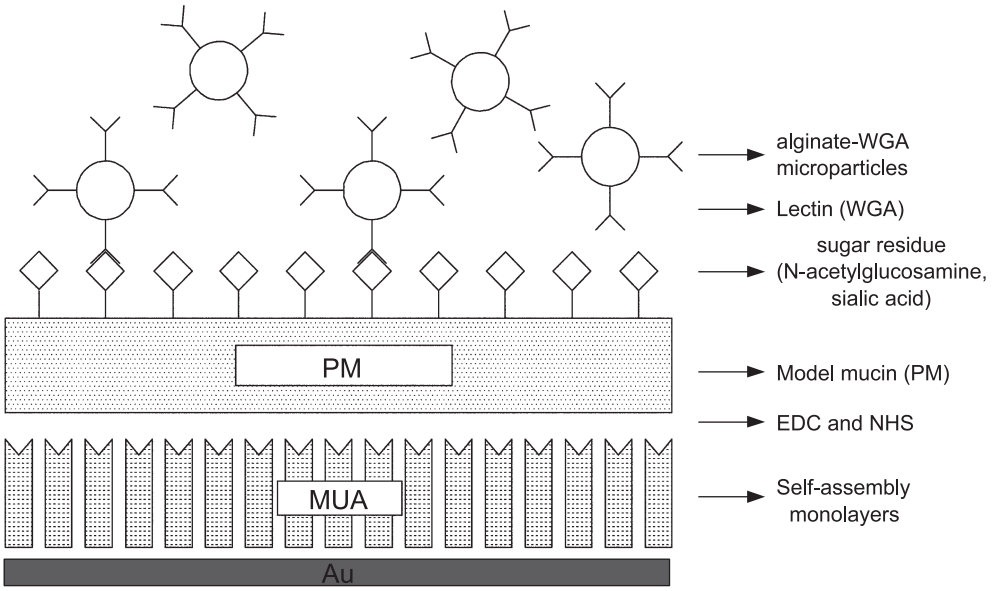

Fig. 7. The schematic diagram of the interaction of alginate–WGA microparticles with the PM-immobilized SPR sensor.

microparticles were 4.852×10^{-8} s^{-1}, 2.647×10^{-7} g^{-1} L s^{-1} and 5.455 g^{-1} L, respectively. In addition, k_d, k_a and K_A of alginate microparticles were 5.748×10^{-8} s^{-1}, 3.609×10^{-8} g^{-1} L s^{-1} and 0.628 g^{-1} L, respectively. K_A of alginate–WGA microparticles was about nine times greater than alginate microparticles. The larger k_a resulted in larger K_A of alginate–WGA microparticles, which is due to the greater interaction of WGA in alginate–WGA microparticles with mucin layer of SPR sensor than that of alginate microparticles.

3.4. In vitro adsorption studies with PM

In the GI tract, mucus gel layers protect the mucous membrane against the harsh conditions of gastric surroundings. Its principal components are mucus glycoproteins (mucins) with a carbohydrate content of 70% to 80%. The mucins are formed by numerous oligosaccharide side chains with *O*-glycosidic linkage to serine and threonine residues in the polypeptide backbone. These carbohydrate chains are built by five different sugars: *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose and sialic acid. These sugar moieties may constitute possible sites of lectin (WGA) attachment [8]. A crude mucin from pig (PM) was chosen as a model mucin to determine the in vitro activity of WGA–microparticle conjugates towards the sugar residues of a glycoprotein. The activities of microparticles were determined by mixing the PM suspension in PBS 7.4 with the same volume of the WGA–microparticle conjugate suspensions. Fig. 9 shows the amounts of PM% interacted with microparticles and the relation between K_A and the amount of interacted PM%. The alginate microparticles had little interaction with the PM. On the contrary, the alginate–WGA microparticles showed about three times larger interactions with the PM than alginate microparticles, since the interacted PM precipitated together with the alginate–WGA microparticles. It is believed that the WGA is covalently bound to alginate–WGA microparticles and interacted with PM. Therefore, it can be said that the bioadhesive alginate–WGA microparticles can bind with mucin in the intestinal mucous membrane. As shown in the figure, the larger K_A of alginate–WGA microparticles resulted in the larger interaction with PM.

3.5. In vivo studies

Fig. 10 showed the behavior of different formulations administered orally to diabetic rats. The

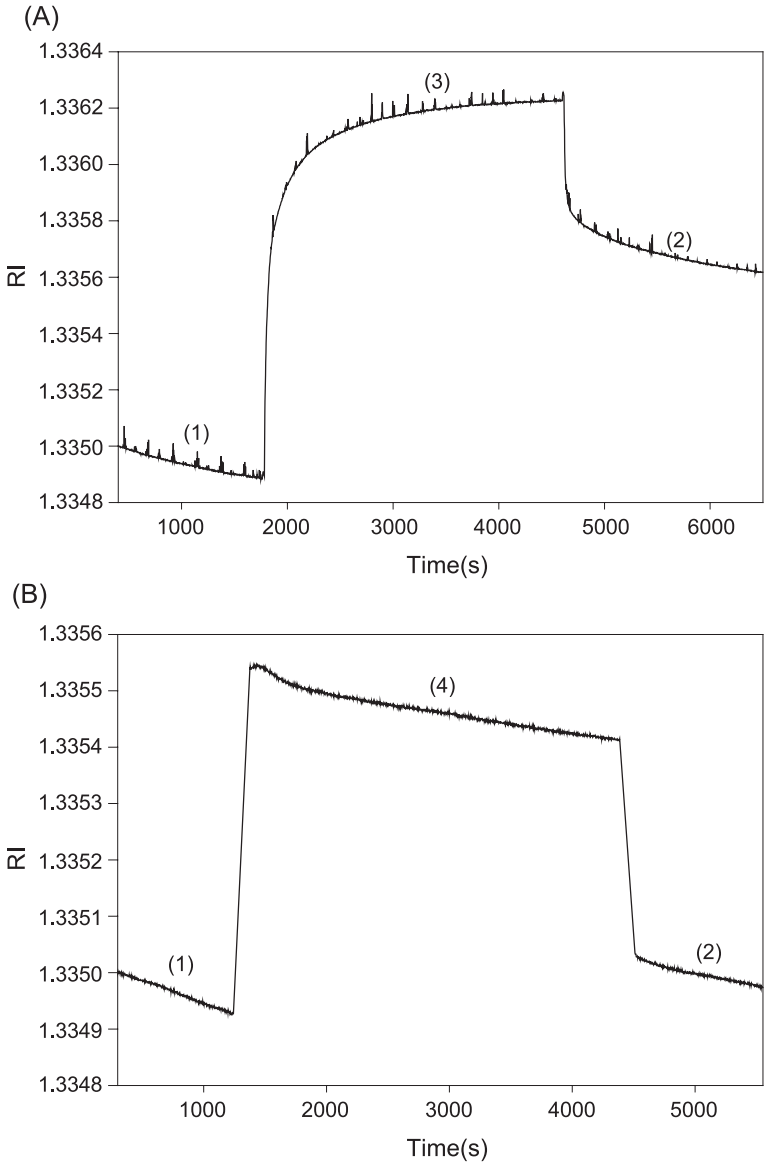

Fig. 8. The interaction of microparticles with the PM immobilized SPR sensor. (A) The interaction of alginate–WGA microparticles with the PM immobilized SPR sensor. (B) The interaction of alginate microparticles with the PM-immobilized SPR sensor. (1) The new baseline with PBS 7.4 containing 0.1% Triton X-100. (2) The flow of PBS 7.4 containing 0.1% Triton X-100 for washing. (3) The application of alginate–WGA microparticles in PBS 7.4 containing 0.1% Triton X-100. (4) The application of alginate microparticles in PBS 7.4 containing 0.1% Triton X-100.

efficacy of the formulations was estimated by measuring the plasma glucose concentration. When the formulations were orally administered to the diabetic rats at a dosage of 50 I.U./kg, no significant decrease of the plasma glucose was found during the first 2 h. However, after 4 h, the decrease in plasma glucose levels of alginate and alginate–WGA microparticles was significantly different from that induced by the insulin control solution ($p<0.05$) and this hypoglycemic effect

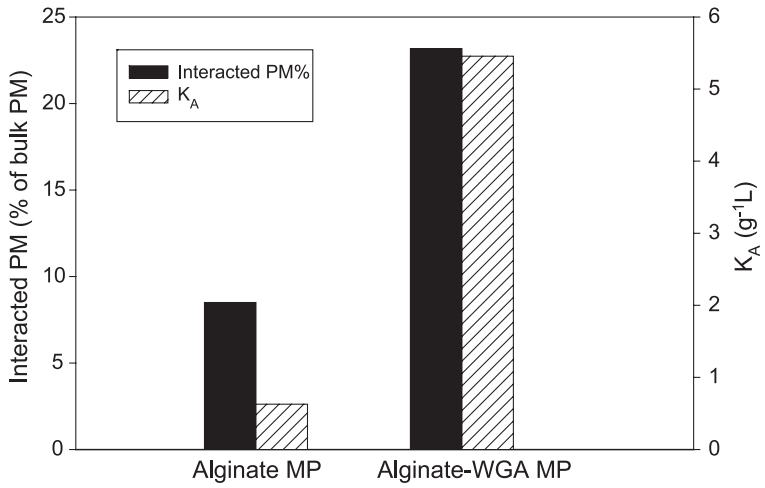

Fig. 9. The affinity constant and the amount of interacted PM with microparticles.

lasted about 8 h. It is believed that alginate and alginate–WGA microparticles made the entrapped insulin more stable and protect it from degradation in harsh conditions of gastrointestinal tract [31]. The hypoglycemic effect observed with alginate–WGA microparticles was greater than that observed with alginate microparticles. It is believed that conjugated WGA induced strong mucoadhesion and deep penetration to the mucous layer, which increased the residence time and close contact of the insulin at the absorption site to enhance the insulin absorption [32]. The results clearly evidenced the ability of alginate–WGA microparticles to enhance the intestinal absorption of insulin. Fig. 11 shows the relation between K_A from SPR data and the glucose change (%) from base level. As shown in

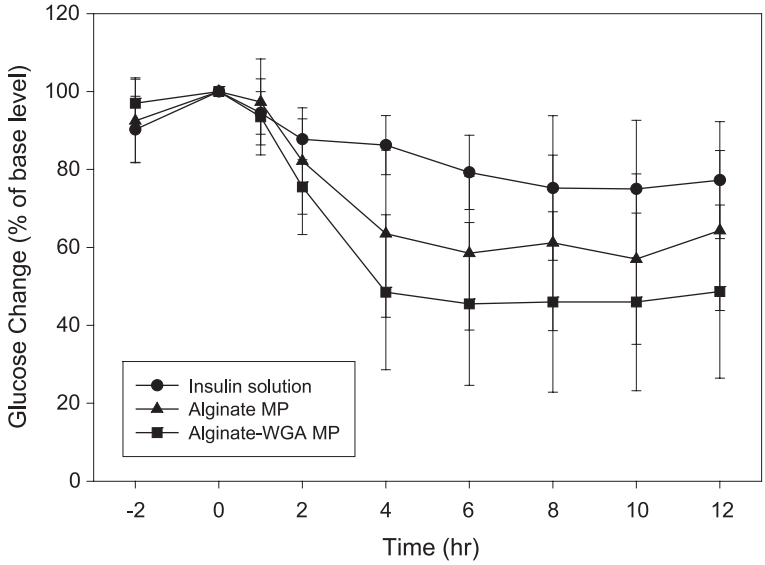

Fig. 10. Hypoglycemic effect of oral administration of: control insulin solution (●); alginate microparticles (▲); alginate–WGA microparticles (■). Data represent the mean±S.D., n=6 per group.

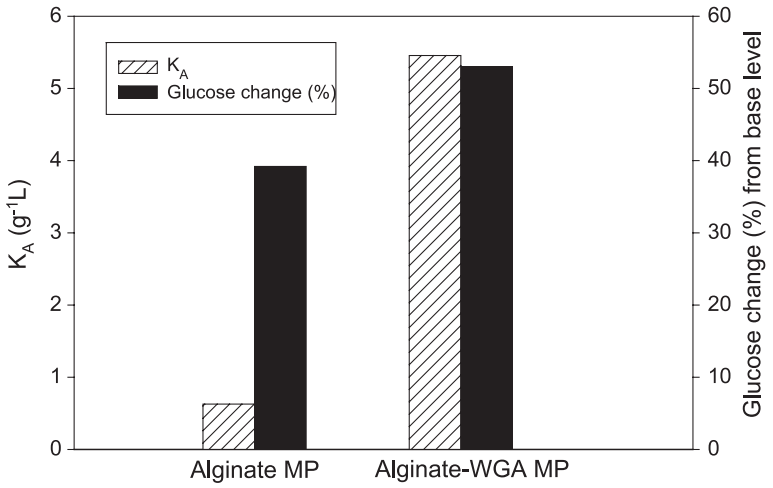

Fig. 11. The relation between affinity constant (K_A) from SPR data and the glucose change (%) from base level.

the figure, the larger K_A of alginate–WGA microparticles resulted in larger glucose change (%) from base level. The precise and adequate correlation between K_A from SPR data and the hypoglycemic effect needs more experiments to be clarified.

4. Conclusion

The work described in this paper shows that alginate–WGA microparticles enhanced the intestinal absorption of insulin. It seemed that the protective effects of alginate microparticles and the mucoadhesive properties of WGA improved oral delivery of insulin. Furthermore, the alginate microparticles were fabricated by the piezoelectric ejection process, which ejected alginate/insulin solution into a 0.1 M $CaCl_2$ solution through a nozzle actuated by the piezoelectric transducer. The model mucin (PM) was attached on the gold surface of SPR biosensor successfully and the interaction of alginate–WGA microparticles with PM was examined. The K_A of alginate–WGA microparticles (K_A=5.455 g^{-1} L) was about nine times greater than alginate microparticles (K_A=0.628 g^{-1} L). The larger K_A of alginate–WGA microparticles resulted in larger glucose change (%) from base level. A further study will examine the precise correlation between K_A from SPR data and the hypoglycemic effect of WGA-conjugated microparticles.

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