Evaluation of gastric mucoadhesive properties of aminated gelatin microspheres

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Abstract

The gastric mucoadhesive properties of aminated gelatin microspheres were evaluated both in vitro and in vivo. The interactions of gelatin, aminated gelatin and microspheres with two kinds of commercial mucin were estimated in aqueous media. At a higher mucin concentration, aminated gelatin demonstrated a stronger interaction with mucin than either kind of the gelatin (isoelectric point (IEP): 5.0 and 9.0) under the same condition, although these interactions varied with varying media. At the same time, a larger amount of mucin was adsorbed to aminated gelatin microspheres than to either of the gelatin microspheres in the same condition. In the in vitro model of isolated and perfused rat stomach, the amount of aminated gelatin microspheres that remained in the stomach after perfusion was significantly larger than that of gelatin microspheres. However, no significant difference was observed whether the test was performed in simulated gastric fluid (SGF) or in phosphate-buffered saline (PBS, pH7.4). In the in vivo experiment, about 47\% of the aminated gelatin microspheres remained in the stomach 2 h after oral administration in a capsule, whereas it was 29 and 34\% for gelatin (IEP=5.0) and gelatin (IEP=9.0) microspheres, respectively. These results indicated that aminated gelatin microspheres demonstrated a higher gastric mucoadhesive ability than gelatin microspheres. The higher amino group content, improved chain flexibility and favorable polymer conformation were suggested to be the main factors that contributed to the stronger mucoadhesive properties of aminated gelatin microspheres than that of gelatin microspheres.

Keywords: Gelatin; Aminated gelatin; Microsphere; Gastric drug delivery; Mucoadhesion

1. Introduction

Mucoadhesive controlled release dosage formulations have gained considerable attention due to their ability to adhere to the mucus layer and release the loaded drug in a sustained manner. The relevant routes of mucoadhesive formulations have involved nasal, gastrointestinal, buccal, ocular, vaginal and rectal ways. By using these dosage forms, the intimate contact time with the mucus surface would increase, thus resulting in an increased drug retention time and drug concentration in the local sites. This would lead to an improved therapeutic effect for the local diseases [1,2]. In addition, mucoadhesive dos-
age forms can also improve the absorption and systemic bioavailability of drugs that are normally poorly absorbed [3].

Mucoadhesion process is rather complex, and several theories have been put forward to explain the mechanisms, including electric theory [4], adsorption theory [5], diffusion theory [6] and wetting theory [7]. Although no theory alone can elucidate this process, it is generally accepted that the first step of mucoadhesion relates to the intimate contact between the mucoadhesive materials and the mucus surface. Subsequently, mucoadhesive molecules would penetrate into the mucus gel network, and entanglements and secondary chemical bonds, such as hydrogen bonding and van der Waals forces, could be formed in this stage [8]. Based on this consideration, the electrostatic attraction was suggested to be able to facilitate the intimate contact, while the presence of hydrophilic groups such as OH, NH, and COOH, sufficient polymer chain flexibility and favorable polymer conformation were considered to be essential for successful mucoadhesion [9].

The widely studied mucoadhesive materials include chitosan, hydroxypropyl cellulose, poly(acrylic acid) and their derivatives. Hydrogen bonding was suggested to be an important factor for the mucoadhesion of acrylic-based polymers [8]. The gastrointestinal mucoadhesive properties of chitosan were in a large part attributed to the electrostatic attraction between its positively charged \(\text{D-}\)glucosamine residues and the negatively charged sialic acid residues of mucin [10]. We have demonstrated in our previous study that aminated gelatin microspheres, with a high primary amino group content, showed a sustained amoxicillin release characteristic and a considerable gastric mucoadhesive property in vitro [11]. The focus of this study was to further investigate the mucoadhesive behaviors of these microspheres in vitro and in vivo in comparison with that of gelatin microspheres.

2. Materials and methods

2.1. Materials

Gelatin (isoelectric point (IEP), 5.0 and 9.0; MW, 100 kDa) was kindly supplied by Nitta Gelatin (Osaka, Japan). Aminated gelatin was synthesized by reaction of gelatin (IEP=9.0) with ethylenediamine in the presence of 1-ethyl-3-(3-diethylamino propyl)-carbodiimide hydrochloride as described previously [11]. Olive oil was obtained from Wako Pure Chemical Industries (Osaka, Japan). Rhodamine B isothiocyanate (RITC), trypsin type II-S (from porcine pancreas), 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 5% (w/v) aqueous solution, mucin type III (from porcine stomach, containing about 1% sialic acid) and mucin type I-S (from porcine pancreas, containing about 12% sialic acid) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade available commercially.

2.2. Preparation and characterization of microspheres

Gelatin microspheres (GMS, IEP=5.0 and 9.0) were prepared according to the modified method of Tabata and Ikada [12]. Aminated gelatin microspheres (AGMS) were prepared using essentially the same method as described previously [11], except that a relatively lower glutaraldehyde concentration (0.06%, w/v) was adopted for the cross-linking of the microspheres and the cross-linking time was extended to 48 h. The size of the microspheres was determined by observation under a light microscope. The available amino group content of microspheres was determined using a TNBS method [13].

2.3. Interactions between mucin and gelatin and aminated gelatin in aqueous solution

Mucin solutions were prepared with relevant media and filtered through a 0.22-\(\mu\)m filter before use. Gelatin and aminated gelatin were dissolved in corresponding media at 37°C for 6 h before use. The concentrations of mucin and polymer solutions were 0.5 and 1 mg/ml, respectively. For evaluation of the interactions, mucin solution and gelatin solution were mixed at different volume ratios (from 1:3 to 9:1), and 30 min later the absorbances of these mixtures were measured at 500 nm using an UV spectrophotometer (U-1100 Spectrophotometer, Hitachi, Japan). The absorbances of the individual mucin and polymer solutions were also measured in the same way, from which the theoretical absorbance
values of the mixed solutions were calculated. The difference between the experimental and theoretical values ($\Delta A$) was taken as an index of the strength of the interaction between mucin and polymers.

2.4. Adsorption of mucin to microspheres

Five mg of microspheres were suspended in 5 ml of mucin solution (0.5 mg/ml), vortexed, and then incubated at 37°C in a shaking water bath for 1 h. After centrifugation at 3600 rpm for 4 min, the concentration of mucin in the supernatant was determined using a colorimetry [14]. Briefly, to 2 ml of the supernatant, 0.2 ml of periodic acid reagent was added. The mixture was incubated at 37°C for 2 h, then 0.2 ml of Schiff reagent was added at room temperature. Thirty minutes later, the absorbance of the solution was measured at 555 nm using an UV spectrophotometer (U-1100 Spectrophotometer, Hitachi). The mucin concentration was calculated from a calibration curve, and the amount of mucin adsorbed to the microspheres was calculated as the difference between the total amount of mucin added and the free mucin in the supernatant. Periodic acid reagent was freshly prepared by mixing 0.1 ml of 5% (w/v) periodic acid and 7 ml of 7% (w/v) acetic acid. The Schiff reagent stock solution was prepared by mixing 20 ml of 1 M HCl and 100 ml of 1% (w/v) basic fuchsin. Just before use, 0.1 g of metabisulfite was added to 6 ml of Schiff reagent stock solution, and the resultant solution was incubated at 37°C for 1.5 h until it became colorless.

2.5. In vitro evaluation of gastric mucoadhesion of microspheres

The in vitro gastric mucoadhesive properties of microspheres were evaluated using RITC-labeled microspheres in an isolated rat stomach as described previously [11]. In brief, male Wistar rats, weighting 200–250 g, were fasted overnight before the experiment, but were allowed free access to water ad libitum. The stomach was excised under anesthesia and perfused with physiological saline to remove the content of the stomach. The cleaned stomach was used immediately after preparation. Three milligram of RITC-labeled microspheres that were suspended in simulated gastric fluid (SGF) or phosphate-buffered saline (PBS, pH7.4) was filled into the cleaned stomach, ligated, and then incubated in physiological saline at 37°C for 30 min. The liquid content of the stomach was then removed by injection of air, and the stomach was perfused with SGF or PBS (pH 7.4) for 30 min at a flow rate of 1 ml/min. The stomach was cut open, and the microspheres that remained in the stomach were recovered by washing with PBS (pH 7.4). The final volume of the washing solution was adjusted to 50 ml with PBS (pH 7.4), and then 0.125 g of trypsin II-S was added and dissolved, followed by incubation at 37°C for 2 h for complete degradation of the microspheres. After filtration through a 0.22-μm filter, the fluorescence intensity of the filtrate was determined by a fluorescence spectrophotometer (FP-770, Japan Spectroscopic) at an emission wavelength of 578 nm (EX 554 nm). The amount of RITC-labeled microspheres that remained in the stomach was calculated from a calibration curve. The gastric mucoadhesion was expressed as the percent of microspheres remaining in the stomach after perfusion.

2.6. In vivo evaluation of gastric mucoadhesion of microspheres

Male Wistar rats, 200–250 g, were fasted for 24 h before the experiments, but were allowed free access to water ad libitum. RITC-labeled microspheres (2 mg) that were filled in capsules (PCcaps™-kit, CAPSUGEL) were administered to rats using a gastric sonde. Two hours after administration, the rats were sacrificed, and the stomach was removed and washed with phosphate-buffered saline (pH 7.4) to recover the remaining microspheres. The amount of RITC-labeled microspheres that remained in the stomach was determined using the same method as described in the above in vitro perfusion experiment.

3. Results

3.1. Characterization of microspheres

Aminated gelatin microspheres were prepared by emulsification of aminated gelatin solution in olive oil followed by cross-linking with glutaraldehyde. In this study, a relatively low cross-linking agent con-
centration (0.06%) was adopted, but the cross-linking reaction time was extended to 48 h in order to obtain microspheres with a possibly higher amino group content as well as a slow drug release rate. Amoxicillin release from the microspheres prepared in this study was similar to that from microspheres prepared with 0.12% glutaraldehyde and 20 h cross-linking (data not shown). The mean size and available amino group content of the microspheres were shown in Table 1. All the microspheres presented nearly the same size, but the amino group content of aminated gelatin microspheres was significantly higher than that of the gelatin microspheres.

3.2. Interaction between mucin and gelatins

The interactions of gelatin and aminated gelatin with mucin in aqueous solutions were estimated using a turbidimetric method and the results were shown in Fig. 1. In the case of gelatin (IEP=5.0), nearly no interaction with mucin was observed under all conditions investigated. The interactions of gelatin (IEP=9.0) and aminated gelatin with mucin followed different patterns in different media. However, at a higher mucin concentration, aminated gelatin invariably demonstrated a stronger interaction with mucin than gelatin in the same condition.

In purified water (Fig. 1A), a strong interaction was observed in the case of gelatin (IEP=9.0) and aminated gelatin. For gelatin (IEP=9.0), there existed a maximum interaction at a mucin concentration of about 0.35 mg/ml (weight ratio of mucin to gelatin of about 1:1). At either a lower mucin concentration or a lower gelatin concentration (higher mucin concentration), the interaction decreased. For aminated gelatin, the interaction appeared to be very weak at a low mucin concentration, but increased quickly when the mucin concentration increased.

![Fig. 1. Turbidimetric measurement of the interactions of gelatin and aminated gelatin with mucin (type III) in (A) purified water, (B) phosphate-buffered saline (PBS) and (C) simulated gastric fluid (SGF). Mucin solution (0.5 mg/ml) and gelatin solution (1 mg/ml) were mixed at volume ratios between 1:3 to 9:1. ΔA represents the difference between the experimental and theoretical absorbances of the mixed solution. Each point represents the mean of three experiments. Key: (△) gelatin (IEP=5.0); (□) gelatin (IEP=9.0); (◇) aminated gelatin.](image)

Table 1

<table>
<thead>
<tr>
<th>Characterization of microspheresa</th>
<th>GMS (IEP=5.0)</th>
<th>GMS (IEP=9.0)</th>
<th>AGMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean size (μm)</td>
<td>52</td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td>Amino group content (μmol/mg)</td>
<td>0.301±0.072</td>
<td>0.369±0.076</td>
<td>0.861±0.108</td>
</tr>
</tbody>
</table>

a GMS and AGMS represent gelatin and aminated gelatin microsphere, respectively. Data of amino group contents are expressed as mean±S.D. (n=3).
reached 0.4 mg/ml, and showed a strong interaction even at a relatively very low concentration of aminated gelatin.

In the case of PBS (Fig. 1B), neither gelatin (IEP=5.0) nor gelatin (IEP=9.0) showed any interaction with mucin. On the contrary, aminated gelatin still revealed a relatively strong interaction with mucin at a wide range of mucin concentrations. However, the interaction at a higher mucin concentration decreased in comparison with that in purified water. In simulated gastric fluid (Fig. 1C), a relatively weak interaction was observed for both kinds of gelatin and aminated gelatin. With the increase of mucin concentration, the interaction increased slightly until a platform was arrived. The difference in interaction between gelatin and aminated gelatin appeared at a higher mucin concentration.

3.3. Mucin adsorption to microspheres

Figs. 2–4 showed the results of mucin adsorption to microspheres. A relatively larger amount of mucin was adsorbed to aminated gelatin microspheres than to either of the gelatin microspheres although the results differed in varying conditions. In purified water, both types of mucin demonstrated a relatively strong adsorption to gelatin (IEP=9.0) and aminated gelatin microspheres. However, little mucin was adsorbed to gelatin (IEP=5.0) microspheres. In PBS and SGF, the amount of mucin adsorbed to all microspheres decreased markedly (Fig. 2).

The effect of pH on mucin adsorption to microspheres is shown in Fig. 3. For mucin type III (Fig. 3A), pH did not significantly affect the adsorption of mucin to microspheres except for gelatin (IEP=5.0) microspheres. On the other hand, with decrease of the pH from 7.0 to 3.5, the amount of mucin type I-S adsorbed to all the three kinds of microspheres increased subsequently (Fig. 3B).

Ionic strength of the media was found to have a significant effect on mucin (type III) adsorption to microspheres (Fig. 4). With the increase of ionic strength, the amount of mucin adsorbed to all microspheres decreased accordingly.

3.4. In vitro evaluation of mucoadhesion

The in vitro gastric mucoadhesion of microspheres were evaluated by using fluorescent-labeled microspheres in an isolated and perfused rat stomach. After 30-min perfusion, about 68 and 74% of the aminated gelatin microspheres remained in the stomach when the examinations were performed in SGF and PBS, respectively. These results were significantly different than that of gelatin (IEP=5.0) microspheres (Fig. 5). However, there was not a significant difference between the two gelatin microspheres, nor was a significant difference observed whether the test was performed in PBS or in SGF for the same microspheres.

3.5. In vivo gastric mucoadhesion

The in vivo evaluation of gastric mucoadhesion was performed using RITC-labeled microspheres in rats. The percentage of aminated gelatin microspheres remained in the stomach 2 h after the administration in a capsule was increased by about
Fig. 4. Effect of ionic strength on mucin (type III) adsorption to microspheres in acetate buffer (pH 5.0). Ionic strength was adjusted with sodium chloride. Error bars indicate standard deviation (n = 3).

Fig. 5. In vitro gastric mucoadhesion of microspheres in isolated and perfused rat stomach. Error bars indicate standard deviation (n = 3–5).

4. Discussion

Mucoadhesion involves different kinds of interaction forces between mucoadhesive materials and mucus surface, such as electrostatic attraction, hydrogen bonding, van der Waals forces and mechanical interpenetration and entanglement. Many methods have been employed to evaluate these interactions in vitro and in vivo. Adhesive strength measurement [15], perfusion-washing technique [16], rheological test [17] and surface energy analysis [18] have been commonly used for the in vitro evaluation. Gamma scintigraphy [19], isolated loop techniques [20] and transit studies with radio-labeled or fluorescent-labeled dosage formulations [21] have been reported for in vivo estimation. Of the in vitro test, commercial mucin is frequently used as a substitute for fresh mucin because of its reproducible quality and easy...
availability, although it does not possess the same viscoelastic properties as freshly isolated mucus gel [22]. Nevertheless, it is still proved to be effective to provide a preliminary information about the interactions with mucoadhesive materials [10,23]. In this study, two kinds of commonly used commercial mucin with different contents of sialic acid were utilized to evaluate their interaction with gelatins and microspheres. In addition, the mucoadhesive properties of aminated gelatin microspheres were estimated using fluorescent-labeled microspheres both in vitro and in vivo in rats.

As shown in this study, the interactions between gelatin and mucin changed dramatically in varying environmental conditions. However, under the same condition, aminated gelatin invariably demonstrated a stronger interaction with mucin than both kinds of the native gelatins at a high mucin concentration. This result might be mainly explained by the different physicochemical properties between the native gelatins and aminated gelatin. In purified water, gelatin (IEP=9.0) is positively charged, a strong interaction with mucin is expected as the result of the electrostatic attraction with mucin that is negatively charged under this condition. However, aminated gelatin, with a higher positive charge density (higher amino group content) than gelatin, apparently showed a lower interaction with mucin at a low mucin concentration (Fig. 1A). This phenomenon could be understood by the fact that aminated gelatin presented a higher level of solubility in water than gelatin. For example, it can dissolve in water to give a 5% (w/v) solution at room temperature (24°C), whereas gelatin was in a gelation state under that condition. Therefore, the turbidity could not be detected probably due to the solubility of the interaction complex at a lower mucin concentration. However, owing to its high positive charge density, a strong interaction was observed at a higher mucin concentration even when the aminated gelatin concentration was relatively very low. In contrast, gelatin (IEP=5.0) did not show any interaction with mucin in purified water because it carried a net negative charge as mucin did.

In the case of PBS (Fig. 1B), gelatin (IEP=9.0), as well as gelatin (IEP=5.0), showed no interaction with mucin, although gelatin (IEP=9.0) was expected to reveal an electrostatic attraction with mucin as in the case of purified water. This result might be ascribed to the unfavorable polymer conformation in the presence of high electrolyte concentration. Under that condition, gelatin was dehydrated, thus shielding the free amino group content and reducing the available positive charge. For aminated gelatin, however, a relatively strong interaction could still be observed in virtue of its higher amino group content and probably higher hydration degree in the presence of electrolyte. In simulated gastric fluid, all the three kinds of gelatins are positively charged. However, due to the existence of electrolyte, furthermore, the ionization of sialic acid residues of mucin was greatly reduced, the electrostatic attraction between mucin and gelatins deceased, therefore resulting in a weakened interaction for all kinds of gelatins.

The interaction between mucin and microspheres was consistent with that between mucin and gelatin in aqueous solutions. A larger amount of mucin was adsorbed to aminated gelatin microspheres than to gelatin microspheres under the same condition. This result suggested that the interactions between mucin and microspheres were in a large part resulted from the electrostatic attraction and repulsion. Mucin type I-S presented a much higher sialic acid content than mucin type III, therefore possibly showed a stronger interaction with positively charged microspheres under the condition of purified water. The pH change from 7.0 to 3.5 did not significantly affect the ionization degree of the amino group of gelatin (IEP=9.0) and aminated gelatin, therefore the
amount of mucin (type III) adsorbed to these microspheres did not change significantly. On the contrary, gelatin (IEP=5.0) changed from a net negative to a net positive charge, thus resulting in an evident increase in electrostatic attraction with mucin. However, the amount of mucin type I-S adsorbed to all microspheres increased as the pH decreased from 7.0 to 3.5. Especially, at pH 3.5 nearly no free mucin could be detected in the case of gelatin (IEP=9.0) and aminated gelatin microspheres. This result cannot be fully explained by the different electrostatic interactions between mucin and microspheres, but probably came from the specific physicochemical properties of mucin type I-S, such as a high sialic acid content.

The effect of ionic strength on mucin adsorption to microspheres was nearly in the same way as in the case of the interactions in aqueous solutions. In the presence of high concentration of electrolyte, the amount of mucin adsorbed to all microspheres decreased dramatically (Fig. 4). However, in the in vitro model of isolated rat stomach, a relatively strong interaction was observed between microspheres and mucus surface either in PBS or in SGF. This difference could be understood by the different characteristics between commercial mucin and fresh mucus surface. In the test of mucin adsorption to microspheres, the mucin concentration was relatively very low (0.5 mg/ml), thus the main force involved was probably electrostatic interaction, which was significantly affected by ionic strength as well as pH. In the case of isolated rat stomach, the fresh mucosal surface was a viscous gel network. The stronger interaction with the microspheres, especially aminated gelatin microspheres, most probably resulted from the interpenetration and entanglement of the polymer chains with the mucus gel. The probably improved chain flexibility of aminated gelatin might have contributed to the stronger interaction of aminated gelatin microspheres with mucosal surface than that of gelatin microspheres. This was further supported by the result of the in vivo experiment.

5. Conclusion

In this study, aminated gelatin microspheres demonstrated a considerable gastric mucoadhesion both in vitro and in vivo in rats. The high amino group content, improved chain flexibility and favorable polymer conformation might work together to explain the improved mucoadhesive properties in comparison with that of gelatin microspheres.

References


