Evaluation of absorption of heparin-DOCA conjugates on the intestinal wall using a surface plasmon resonance

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Abstract

We validated the application of the surface plasmon resonance (SPR) technique to reliably determine adhesion of drugs to the intestinal wall using heparin-DOCA conjugates, developed to enhance the oral absorption of poorly absorbed heparin. In this study, heparin conjugates, or deoxycholyl-heparin (H-DOCA) and bisdeoxycholyl-heparin (H-bis-DOCA), were synthesized by covalently coupling the synthesized succinimido deoxycholate (DOCA-NHS) or succinimido bis-deoxycholyl-l-lysine (DOCA-bis-NHS) to amine groups of heparin, and their physicochemical and biological properties were evaluated. To mimic the duodenal and ileal surfaces, duodenal and ileal brush border membrane (BBM) vesicles isolated from Sprague–Dawley (SD) rats were immobilized onto a biosensor chip composed of dextran derivatives with modified lipophilic residues. The adhesion of heparin conjugates on the BBM surface was evaluated by measuring the SPR response signal. The adhesion of heparin conjugates was significantly dependent on the conjugated DOCA molecules: that is, they showed higher adhesion signal on the ileal BBM surface than that on the duodenal BBM surface. In particular, the solubilized heparin conjugates in DMSO solution presented significantly increased adhesion affinity on the ileal BBM surface. The adhesion of heparin conjugates on the intestinal surfaces was successfully assayed using the surface plasmon resonance technique with the sensor chip on which BBM vesicles were immobilized.

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1. Introduction

Heparin, one of the most potent anticoagulants, is widely used for the treatment and prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE) [1–3]. The main disadvantage of heparin treatment, however, is that it can be administered only by parenteral administration [4–6]. Heparin is not absorbed in the gastrointestinal (GI) tract because of its large molecular weight and negatively charged structure. The hydrophilic property of heparin also makes it difficult to penetrate through the epithelial cells because of the low permeability and repulsion forces of the polar head groups of the epithelial membrane. To facilitate heparin absorption in the GI tract, researchers have developed various formulations, such as liposome, complexes of heparin with hydrophobic organic bases, enteric coating, and aerosol formulation. There have also been attempts to evaluate the enhancing effect of EDTA, acidic buffer, or sulfated surfactants on heparin absorption in

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the GI tract [7–10]. A recent study has reported sodium N-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) and sodium N-[10-(2-hydroxybenzoyl)amino]decanoate (SNAD) as potent promoters of heparin absorption in the GI tract [11,12].

We have also developed new amphiphilic heparin conjugates for oral formulation by coupling deoxycholic acid (DOCA) molecules to heparin [13–15]. DOCA, as one of the bile acids, is a natural compound consisting of a facially amphiphilic steroid nucleus with a hydrophobic β-side and a hydrophilic α-side [16]. In in vivo animal test, we found that bile acid-modified heparins were highly absorbed in the intestine without damaging the tissue structure of the mucous membrane [15]. Considering the fact that bile acid conjugated prodrugs could preserve some of the properties of bile acids, such as amphiphlicity, capacity of self-assembling, high chemical stability and binding ability to bile acid transporters in the intestine [17,18], our study proposes two possible approaches regarding the enhancement of oral adsorption of heparin conjugates in the GI tract; one is that of imparting lipophilic property by the conjugated DOCA, and the other, utilizing the interaction between the conjugated DOCA and bile acid transporters in the ileum.

We previously studied a new surface plasmon resonance (SPR) system to evaluate drug adhesion on the intestinal surface using biosensor chips on which BBM vesicles were immobilized [20,21]. Surface plasmon resonance technique has been used recently for in an in-real time assay that allows a direct analysis of drug adhesion on the phospholipid surfaces [22–24] and in-real time permeability assays of orally absorbed drugs. Also, this SPR technique has been credited with various advantages as it provides a real-time high throughput drug screening method without labeling requirements, and can be reused for various drugs.

In this study, we have validated the application of the SPR technique to reliably determine binding patterns of heparin-DOCA conjugates to the intestinal surface. These experiments also examined the absorption of heparin-DOCA conjugates on the BBM surfaces according to varying BBM compositions and DMSO concentrations.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (LMWH, Fraxiparin®), whose average molecular weight is about 10,000 Da, was obtained from Sanofi-Synthelabo Co. (Gentilly, France). Deoxycholic acid (DOCA), N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), tetrahydrofuran (THF), anhydrous N,N-dimethylformamide (DMF), triethylamine (TEA), ethyl acetate (EtOAc), dimethyl sulfoxide (DMSO) and absolute ethanol (EtOH) were purchased from Sigma–Aldrich Co. (St. Louis, MO). The Coatest Factor Xa assay kit was purchased from Chromogenix (Milano, Italy). All reagents and organic solvents used were at least of ACS grade.

2.2. Preparation of DOCA derivatives

Succinimido deoxycholate (DOCA-NHS): DOCA (200 mg, 0.5 mmol) and NHS (76 mg, 0.67 mmol) were dissolved in anhydrous THF (20 mL). DCC (136 mg, 0.67 mmol) was added and stirred at 4 °C for 6 h. After area derivatives produced as a side product were filtered, the filtrate was poured into cold n-hexane (120 mL) and the precipitate was dried in vacuum. The synthesized succinimido deoxycholate was obtained as white powder.1H NMR, 350 MHz, CDCl3: 0.59 (s, 3H, 18-CH3); 0.86 (s, 3H, 19-CH3); 0.91 (d, 3H, 21-CH3); 1.03–2.50 (25H, steroidal H); 2.81 (s, 4H, CH2CH2); 3.78 (m, 1H, 3-H); 4.06 (br, s, 1H, 12-H).

Succinimido bisdeoxycholyl-l-lysine (bis-DOCA-NHS): A suspension of L-lysine ethyl ester HCl (50 mg, 0.24 mmol) in anhydrous DMF (15 mL) containing TEA (140 μL, 1 mmol) was stirred at room temperature for 30 min, followed by adding DOCA (190 mg, 0.48 mmol) and NHS (71 mg, 0.62 mmol). DCC (129 mg, 0.62 mmol), dissolved in anhydrous DMF (5 mL), was added drop-wise to the mixture and then stirred at room temperature for 12 h. The mixture was filtered and the filtrate was diluted with EtOAc (40 mL). The organic mixture was washed with 15 mL of 0.5N HCl, distilled water, 0.5N NaOH and distilled water, in the order just described. The dried products were dissolved in EtOH (10 mL) containing saturated NaOH followed by refluxing at 60 °C for 1 h. After filtering at room temperature, the filtrate was added drop-wise to a cold acid solution (pH 3). The precipitate was filtered, washed and dried in vacuum. Finally, bisdeoxycholyl-l-lysine was obtained as white powder. The carboxylic group of bisdeoxycholyl-l-lysine was activated by NHS as described above to obtain succinimido bisdeoxycholyl-l-lysine.1H NMR, 350 MHz, CDCl3: 0.58 (s, 6H, 2 × 18-CH3); 0.84 (s, 6H, 2 × 19-CH3); 0.92 (d, 6H, 2 × 21-CH3); 1.05–2.40 (56H, steroidal 50H, 3 × CH2); 2.75 (s, 4H, CH2CH2); 2.98 (m, 2H, CH2-NHCO); 3.61 (m, 2H, 2 × 3-H); 4.16 (br s, 1H, 2 × 12-H); 4.47 (s, 1H, CH2-NHCO); 7.73 (br s, 1H, CH2-NHCO); 7.96 (br s, 1H, CH-NHCO).

2.3. Preparation of heparin-DOCA conjugates

Heparin–deoxycholate (H-DOCA) and heparin-bis-deoxycholate (H-bis-DOCA) were synthesized by chemical coupling of heparin with DOCA-NHS and bis-DOCA-NHS, respectively, as shown in Fig. 1. Heparin (100 mg, 2.2 μM) was dissolved in DMF/H2O (5 mL, 1/1, v/v, pH 9.5) at room
Fig. 1. Synthetic scheme for bile acid-modified heparin conjugates.

temperature. DOCA-NHS or bis-DOCA-NHS (440 μM) was dissolved in anhydrous DMF (1 mL) and added to the heparin solution. After 5 h, the solution was quenched by adding distilled water and pH was adjusted to 2 with 1N HCl. After mixtures were precipitated in an excess of cold acetone, the precipitate was washed three times with acetone to remove excess DOCA and bis-DOCA, followed by drying in vacuum. The dried heparin conjugate was dissolved in water and it was lyophilized to give white powder. The presence of H-DOCA and bis-DOCA in heparin conjugate was confirmed by the characteristic peaks of bile acid appearing at 0.65–2.3 ppm. The new amide linkages between heparin and DOCA in heparin appeared at 8.04, in the $^1$H NMR spectra.

2.4. Characterizations of heparin-DOCA conjugates

The degree of substitution (DS), defined as the number of conjugated DOCA or bis-DOCA per one heparin molecule, was determined by bile acid assay, as reported in a previous study [27,28]. 3-α-Hydroxyl group of DOCA molecule is specifically converted to the 3-keto derivatives in the presence of NAD$^+$ and 3-α-hydroxysteroid dehydrogenase, and its product, NADH, reacts with nitrotetrazolium blue under the catalytic influence of diaphorase to give blue formazan derivative. The absorbance of the blue formazan derivative formed by DOCA was measured at 550 nm. Thus, DS of the conjugated DOCA or bis-DOCA per heparin molecule was determined from the standard curve of each DOCA and bis-DOCA, respectively. The chemical structures of heparin conjugates were analyzed using FT-IR spectrophotometer (1725 v, Perkin-Elmer) and $^1$H NMR (JEOL JNM-LA 300 WB FT-NMR, Tokyo, Japan).

Anti-coagulant activities of heparin conjugates were determined with a kinetic method using an anti-FXa chromogenic assay kit with a chromogenic substrate sensitive to factor Xa (S 2222). The mean diameter and the zeta potential of aggregates formed by heparin conjugates in buffer were determined...
using dynamic light scattering (Lexel Laser Model 95) and an ELS-8000\(^\circ\) electrophoretic light scattering spectrophotometer (Otsuka, Electronics Co. Ltd., Japan), respectively.

### 2.5. Immobilization BBM vesicles on the biosensor chip

Duodenum and ileal BBM vesicles were prepared by the Ca\(^{2+}\) precipitation method, as described previously [22,23]. Duodenum and ileum segments, obtained from the small intestines of SD rats (200–220 g, fasted overnight), were used for the preparation of small intestinal BBM vesicles. The purity of BBM vesicles was determined by the established procedure of an activity assay for alkaline phosphatase [29] and Na\(^+\)/K\(^+\) ATPase [30]. The prepared BBM vesicles were used for the SPR experiments within 6 h without any loss of their carrier proteins and enzymatic activity. After all the SPR experiments, we evaluated the bioactivity of BBM vesicles to confirm whether BBM vesicles maintained the same property during the SPR experiments.

Prior to use, the biosensor chip surface was washed with an injection of 40 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate at a flow rate of 5 L/min for 4 min, followed by washing with a running buffer at a flow rate of 5 \(\mu\)L/min for 4 min. Then, each BBM vesicle solution (2.5 mg/mL) was injected at the flow rate of 2 \(\mu\)L/min and the running buffer was allowed to elute continuously to remove any slightly absorbed BBM vesicles. Finally, the response level according to the immobilized BBM vesicles was equilibrated within 10 min.

### 2.6. SPR experiments

Each sample, whose concentration ranged from 12.5 to 200 \(\mu\)M, was dissolved in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 0, 0.5 and 1% (v/v) DMSO, respectively. A BIAcore 3000 (Biacore AB, Uppsala, Sweden) was used with a biosensor chip, which is composed of dextran derivatives with modified lipophilic groups of heparin, as active sites of heparin as an anticoagulant drug [32]. The synthesized heparin conjugates presented the amide linkages between heparin and DOCA derivatives at the wavelength of 1665 cm\(^{-1}\) in the FT-IR spectra. The presence of DOCA derivatives in heparin was also confirmed by the characteristic peaks of DOCA appearing at 0.65–2.3 ppm [33] and the new amide linkages between heparin and DOCA derivatives appeared at 8.04 ppm in the \(^{1}H\) NMR spectra (data not shown) [34].

The degree of substitution of DOCA derivatives to heparin was calculated by measuring the remaining carboxylic groups in heparin conjugates using the bile acid assay kit (Table 1). The results indicated that the DS of H-DOCA and H-bis-DOCA was 1.3 and 1.5, respectively. But the weight fraction of DOCA molecules in H-bis-DOCA was about two times higher than that of H-DOCA, because the bis-DOCA molecule contained two DOCA molecules. These heparin conjugates still presented a high anticoagulant activity of about 86.6–91.7%.

The mean size of self-aggregates (1 mg/mL in HEPES-buffered saline), determined by dynamic light scattering, was in the range of 600–820 nm, as shown in Table 1. Since the conjugated hydrophobic DOCA promoted intramolecular or intermolecular association in the aqueous solution, the heparin conjugates formed self-assembled nano-aggregates. Moreover, the zeta-potential of the aggregates was found to be very negative, about \(-51\) to \(-54\) mV (Table 1), which

### Table 1

**Characterizations of heparin-DOCA conjugates**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular weight</th>
<th>DS(^a)</th>
<th>WF (%)(^b)</th>
<th>BA (%)(^c)</th>
<th>(D) (nm)(^d)</th>
<th>(\zeta) (mV)(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-DOCA</td>
<td>4950</td>
<td>1.33</td>
<td>9.09</td>
<td>91.7</td>
<td>820</td>
<td>(-54)</td>
</tr>
<tr>
<td>H-bis-DOCA</td>
<td>5508</td>
<td>1.50</td>
<td>18.3</td>
<td>66.7</td>
<td>600</td>
<td>(-51)</td>
</tr>
</tbody>
</table>

\(^a\) DS, degree of substitution of H-DOCA and H-bis-DOCA.

\(^b\) WF, weight fraction of heparin conjugates.

\(^c\) BA, bioactivity of heparin conjugates.

\(^d\) \(D\), diameter of heparin conjugates which was measured using dynamic light scattering.

\(^e\) The \(\zeta\) potential of the heparin conjugates.
indicates that negatively charged heparin molecules covered the surface of the aggregates. These results imply that, in the aqueous phase, their hydrophobic cores of DOCA derivatives are surrounded by a hydrophilic and negative charged heparin shell.

The solubility of heparin-DOCA conjugates differed according to the concentration of DMSO in buffer (Fig. 2). When the DMSO concentration increased to 1% (v/v) in buffer, the light scattering intensity was decreased about 50%, compared to the heparin conjugates in buffer; this is because DMSO is a good solvent for DOCA derivatives. On the other hand, the light scattering intensity was nearly zero in 5% DMSO solution, indicating that the heparin-DOCA conjugates were completely solubilized without forming any aggregates.

3.2. Immobilization of BBM vesicles on the LI chip®

BBM surface was prepared by immobilizing BBM vesicles on the LI chip®, as previously described [23]. Briefly, BBM vesicles, isolated from duodenum or ileum segment, were immobilized on a single flow cell of LI chip® by injecting 5 μL of BBM vesicle solution at the elution rate of 2 μL/min. The resonance response of BBM surfaces become stabilized to 4260 resonance unit (RU) within 5 min. We already confirmed in our previous studies that the BBM surface fully covers the LI chip® surface at this optimized condition and that this BBM surface prevents non-specific binding of samples to the bare LI chip® surface [22–23]. Since H-DOCA or H-bis-DOCA was randomly adsorbed on the BBM surface immobilized onto the biosensor chip by

Fig. 2. Effect of DMSO on scattering intensity of heparin conjugates.

![Image](image1.png)

Fig. 3. The complete cycle of a sensorgram of the assay of BBM surfaces/heparin conjugates containing (a) BBM capturing, (b) drug binding, and (c) regeneration, respectively.
both non-specific and specific binding, the binding affinity of the heparin conjugates were evaluated by the amount of bound heparin conjugates rather than association rate constant [22,24]. In order to remove any free BBM vesicles and slightly absorbed BBM vesicles on the L1 chip®, the chip was washed with the running buffer for 30 min. Then, the resonance signal of BBM surfaces were stabilized at 4235 RU with a drift of 0.5 RU/min. Each sample was injected into the BBM surface at the rate of 20 μL/min for 2 min. After the sample screening, the BBM surface was completely stripped off with a 40 μL injection of a 50/50 (v/v, %) mixture of 100 mM HCl/isopropanol. The entire assay procedure was automated to repeat the immobilization of BBM vesicles, the sample assay, and the BBM surface regeneration over 50 times for each flow cell of the L1® chip. The complete sensorgram obtained from a typical binding cycle is shown in Fig. 3.

As shown in Fig. 4, the efficiency and reproducibility of BBM vesicle immobilization were not altered in the range of 0-1% DMSO in buffer, and a stable BBM surface was produced. However, when the DMSO concentration was increased above 5% in a running buffer, BBM vesicles were dissociated and the RU value was significantly decreased (data not shown). Thus, the maximum DMSO concentration in buffer was set up at 1% for the SPR assay of heparin conjugates.

3.3. Absorption of DOCA salt to BBM surfaces

To evaluate the role of DOCA as a promoter for oral absorption, we examined the absorption of DOCA to the BBM surface, in which duodenal and ileal BBM vesicles were each loaded onto different sensor channels. The results indicated that SPR signals of DOCA salt rapidly increased in both ileal and duodenal surfaces due to the presence of bound DOCA, which were then rapidly dissociated when the solution was replaced with the running buffer (Fig. 5). The sensorgram in Fig. 5 is quite different from a typical SPR sensorgram, which is shown in Fig. 4. The sensorgram was obtained at a high flow rate of 20 μL/min, which is 10 times higher than that used in BBM vesicle immobilization in Fig. 4. It is known that this sensorgram with a fast steady-state binding level is useful for assaying the binding level of orally absorbed drugs on the lipid-based SPR system [19–23]. In the case of duodenal BBM surface, the SPR signals might have resulted from non-specific physical interactions, such as lipophilicity, hydrogen bonding and ionic strength, because the bile acid transporters are absent in the duodenum. It is important to note that DOCA presented increased SPR signal (about 65%) on the ileal BBM surface, compared to that of duodenal surfaces. This result explicitly suggests that DOCA molecules specifically interact with bile acid transporters in the ileal BBM vesicles.

3.4. Absorption of heparin-DOCA conjugates to BBM surfaces

Heparin showed low absorption rate (less than 100 RU) on the duodenal and ileal BBM surfaces due to the high molecular weight and strong negative charges of heparin molecules.
It was also found that the binding response of heparin did not depend on the DMSO concentration. However, heparin-DOCA conjugates exhibited different absorption patterns according to varying BBM compositions and DMSO concentrations. On the duodenal BBM surface, the SPR signal was dependent on the lipophilicity of the heparin conjugates and the DMSO concentration (Fig. 6a).

The binding reaction of heparin-DOCA conjugates increased with the increase in the number of conjugated DOCA molecules; that is, H-bis-DOCA > H-DOCA > heparin (Fig. 6b and c). As the number of conjugated DOCA molecules increased, the lipophilicity of heparin-DOCA conjugate was increased, thereby increasing its take-up in the intestinal tissues. When 1% DMSO solution was used as the running buffer, the binding responses of H-DOCA and H-bis-DOCA on the duodenal BBM surface slightly increased. On the other hand, the binding reaction of heparin-DOCA conjugates in the ileal BBM surface was higher than that in the duodenal BBM surface (Fig. 7b). Furthermore, the number of the conjugated DOCA molecules and DMSO had more significant effects on the ileal BBM surface than on the duodenal BBM surface. Therefore, the highest binding response was obtained from H-bis-DOCA in 1% DMSO solution, and this SPR signal was about 8 times higher than that of heparin in the running buffer (Fig. 7c). Even though we could not obtain the SPR sensorgram in 5% DMSO/buffer solution.

Fig. 6. Relative SPR responses for heparin and heparin conjugates to duodenal BBM surfaces: (a) heparin, (b) H-DOCA, and (c) H-bis-DOCA. Each sample was injected at 12.5, 25, 50, 100, and 200 μM in a HEPES-buffered solution or in a 1% (v/v) DMSO mixed HEPES-buffered solution, respectively.
in which the BBM surface are dissolved, the effect of DMSO on the binding level of heparin-DOCA conjugates to the BBM surfaces was evaluated by increasing the DMSO concentration from 0.5 to 1% DMSO in the buffer solution. Based on the solubility data (Fig. 2), the binding level of heparin-DOCA conjugates in 5% DMSO/buffer solution may be expected to increase further, because the heparin-DOCA nanoparticles are completely dissolved in the 5% DMSO/buffer solution.

The effect of DMSO concentration on the binding level of heparin conjugates to BBM surfaces is summarized in Fig. 8. With the increase of the DMSO concentration from 0.5 to 1% in the running buffer, the binding responses of heparin-DOCA conjugates on duodenal BBM surfaces increased about 10–24% (Fig. 8a). In particular, on the ileal BBM surfaces, heparin-DOCA conjugates showed a highly increased binding response by about 26 and 42% by adding 0.5 and 1% DMSO into the running buffer, respectively (Fig. 8b).

The conjugated DOCA molecules could form hydrophobic cores by hydrophobic interactions, and since they were covered with hydrophilic heparin shells, heparin-DOCA conjugates could form self-assembled aggregates in the aqueous condition. Hence, the conjugated DOCA molecules could not be fully exposed to BBM surfaces, thereby reducing their roles as a promoter for enhancing drug absorption on the intestinal surface. When the aggregates of heparin-DOCA conjugates were solubilized in DMSO solution, the conjugated DOCA molecules could more freely bind to the BBM surface.

Heparin-DOCA conjugates could be absorbed on the intestinal surfaces both by non-specific absorption and spe-
Fig. 8. Binding patterns of heparin conjugates to BBM surfaces as a function of BBM composition and buffer DMSO concentration. Heparin and heparin conjugates were injected over each BBM surface at a concentration of 200 HEPES-buffered solution containing 0, 0.5, 1% (v/v) DMSO, respectively. (a) Duodenal BBM surface and (b) ileal BBM surface.

Specific binding with the bile acid transporters, which are located in the ileal surface. Therefore, the SPR response of heparin-DOCA conjugate in the ileal BBM surface was found to be higher than that in the duodenal BBM surface. In particular, since heparin-DOCA could be dissolved in DMSO solution, the absorption of heparin-DOCA on the ileal BBM surface in DMSO solution was higher than that in the running buffer.

4. Conclusions

The current study demonstrates that immobilized duodenal and ileal BBM surfaces on a biosensor chip closely mimic the intact small intestine surface and that this can be used to predict the interaction between prodrugs and BBM surfaces. The binding patterns of heparin-DOCA conjugates were found to be closely related to the physicochemical properties of each BBM surface and the drug solubility. Heparin-DOCA conjugates showed an increased level of binding on the ileal BBM surface due to the presence of bile acid transporters. In addition, the solubilized heparin-DOCA conjugate in DMSO solution further increased the SPR response because the conjugated DOCA molecules of solubilized heparin-DOCA conjugate interacted more effectively with bile acid transporters on the ileal BBM surface. The binding between heparin-DOCA conjugates and small intestinal surfaces was successfully assayed using the SPR technique.

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