A Highly Stable Covalent Conjugated Heparin Biochip for Heparin–Protein Interaction Studies

Fuming Zhang,* Melissa Fath,† Rory Marks,‡ and Robert J. Linhardt*,†,§,[1]

**Department of Chemistry, †Division of Medicinal Chemistry, College of Pharmacy, and §Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52242; and ‡Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109*

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Heparin is a proteoglycan composed of highly sulfated linear polysaccharides of alternating uronic acid and glucosamine that interacts with a wide variety of proteins and peptides (1). Heparin and the structurally related heparan sulfate are the most acidic polysaccarides in the human body and, as a result, interact with many cationic proteins, giving rise to myriad biological activities (2). Some of these interactions have received extensive attention in recent years, including heparin's binding to growth factors (3, 4) influencing angiogenesis and other proliferation-dependent processes, and its binding to the ectodomain proteins of pathogens influencing infection (5).

Surface plasmon resonance (SPR)[2] spectroscopy has become one of several established methods for measuring biomolecular interactions (6). SPR measures binding interactions on the surface of a biosensor chip, and SPR biosensors have been successfully used for quantitative modeling of heparin–protein interactions (3, 7–9). These experiments require the immobilization of either heparin or the heparin-binding protein on the surface of a biosensor chip, over which its binding partner (heparin-binding protein or heparin) is passed. While a number of immobilization chemistries have been developed to immobilize proteins for SPR (6), this may be problematic as proteins differ with respect to ease of covalent attachment to a surface with retention of native conformation, accessibility of active sites, and bioactivity. In natural biological systems, heparan sulfate is found immobilized on the cell surface through its core protein (10), and captures heparin-binding proteins that flow over the cell surface. Modeling this interaction by SPR would best be achieved by immobilizing heparin/heparan sulfate rather than the heparin-binding protein. However, the presence of only a single reducing-end amine group in glycosaminoglycans such as heparin and heparan sulfate makes the usual amine chemistry-based immobilization chemistries problematic, and we have been exploring alternative approaches. In all previous heparin–protein interaction studies using SPR, heparin was immobilized through a bridging avidin–biotin system (3, 7, 8), biotinylated heparin being bound to avidin or streptavidin immobilized on the chip surface. However, studies in our laboratory demonstrated that many heparin-binding proteins interact nonspecifically with avidin and streptavidin, making the use of such heparin-containing biochips problematic (9). Furthermore, we also found that the streptavidin–biotin heparin chip was not stable, especially when the heparin-binding protein is tightly bound and harsh regeneration conditions (e.g., washing the surface with NaOH) are required, leading to damage to the linkage between the ligand and surface.

Our laboratory (9) and others (11) have previously immobilized albumin–heparin conjugates on functionalized polystyrene surfaces to study heparin–protein interactions. In the present study, we describe a new method for the covalent immobilization of heparin on a biochip using a preformed albumin–heparin conjugate and its utility for studying interactions with heparin–binding proteins.

Materials and Methods

Acidic fibroblast growth factor (aFGF) was a gift from Amgen (Thousands Oaks, CA). Recombinant dengue virus envelope protein was prepared as previously described (9). Pioneer Sensor C1 Chip, *N*-hydroxysuccinimide (NHS), and *N*-ethyl-*N*-(dimethyaminopropyl)-carbodiimide (EDC) were from BIAcore (Biosensor AB, Uppsala, Sweden). Bovine serum albumin was from Amresco (Solon, OH). Albumin–heparin and other chemicals were obtained from Sigma–Aldrich Company (St. Louis, MO) and were of the highest purity commercially available. SPR measurements were performed on a BIAcore 3000 operated using BIAcore 3000 version software. Buffers were filtered and degassed.

Immobilization of albumin–heparin. Albumin–heparin was covalently immobilized to the biosensor surface through its primary amino groups in the following manner. Carboxymethyl groups on the C1 Chip surface was first activated using an injection pulse of 50 μl (flow rate, 5 μl/min) of an equimolar mix of NHS/EDC (final concentration 0.05 M, mixed immediately prior to injection).

Twenty microliters of a solution of albumin–heparin (200 μg/ml in sodium acetate buffer (10 mM) with 2 M guanidine, pH 4.0) was then applied. Excess unreacted sites on the sensor surface were blocked with a 50-μl injection of 1 M ethanolamine. Successful immobiliza-

[1] To whom correspondence should be addressed. Fax: (319) 335-8766. E-mail: Robert-linhardt@uiowa.edu.

[2] Abbreviations used: SPR, surface plasmon resonance; aFGF, acidic fibroblast growth factor; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide.

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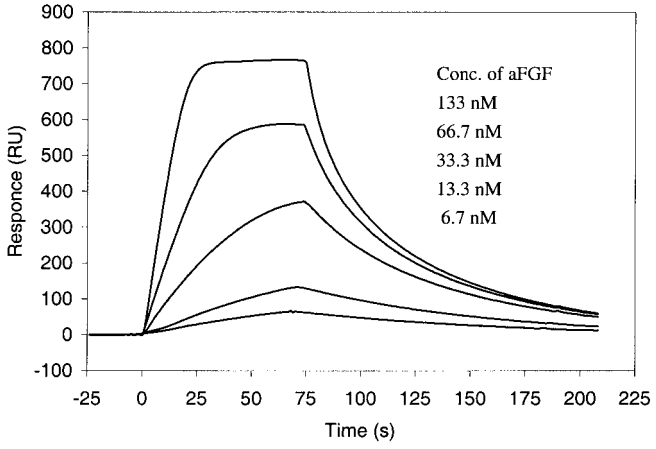

FIG. 1. SPR sensograms of aFGF and heparin interaction.

tion of albumin–heparin was confirmed by the observation of a ~300-RU response.

To prepare the control flow cell, bovine serum albumin was immobilized on the surface using a similar coupling procedure. After the surface was activated with NHS/EDS, 5 μl of albumin (20 mg/ml in sodium acetate buffer, pH 4.0) solution was injected and a ~300-RU response observed.

Kinetic measurement of protein interactions with immobilized heparin. aFGF in HBS-EP buffer (0.01 M Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) was injected over the albumin–heparin and control albumin surfaces at different concentrations at a flow rate of 20 μl/min. At the end of the sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation studies. After a suitable dissociation time (4 min), the sensor surface was regenerated by injecting with 20 μl of 2 M NaCl. Dengue virus envelope protein was similarly assessed except that full regeneration of the sensor surface required serial injections of 20 μl of 2 M NaCl, 20 μl of 10 mM glycine–HCl (pH 2), and 20 μl of 50 mM NaOH.

Responses were monitored as a function of time (sensogram) at 25°C. Kinetic parameters were evaluated using the BIA Evaluation software (Version 3.1, 1999).

Results and Discussion

Immobilization of albumin–heparin on Pioneer Sensor C1 Chip. Amine coupling is the most common choice for immobilization of proteins onto biosensor chips. *N*-Hydroxysuccinimide esters are formed by treating the carboxymethyl groups on the biosensor surface with a mixture of NHS and EDC. These active esters then react spontaneously with amines on the protein ligand to form covalent links. After coupling, ethanolamine is added to deactivate remaining active esters. A Pioneer Sensor C1 Chip with a flat carboxymethylated surface was used in this study to minimize nonspecific binding. The buffer system selected was a very important facet in achieving successful immobilization of albumin–heparin. Initial experiments in which albumin–heparin in sodium acetate buffer, pH 4, was passed across the activated surface failed to result in any immobilization. This was likely the result of unfavorable interactions between the negative charges of albumin–heparin and those on the carboxymethyl groups of the biosensor surface. The addition of 2 M guanidine to the sodium acetate buffer (pH 4) resulted in sufficient charge shielding for the successful covalent immobilization of 300 RU of albumin–heparin conjugate on the biosensor surface. Next, the utility of this heparinized surface for binding studies was examined.

Heparin interaction with aFGF. Fibroblast growth factor mediates a variety of critical biological processes, such as cell proliferation, differentiation, migration, morphogenesis, and angiogenesis (12), and the interaction of aFGF with polyanionic glycosaminoglycans is of functional significance (3). Sensorgrams (Fig. 1) for the binding of aFGF to the albumin–heparin C1 Chip were identical to previous sensorgrams modeling the same interaction with biotinylated heparin immobilized on a streptavidin chip (3). Binding observed was specific for heparin as no interaction was observed with the control flow cell containing only immobilized unsubstituted albumin. Each set of sensorgrams fits well the Langmuir 1:1 binding model. The kinetic parameters calculated from our binding data (Table 1) were very close to the heparin–aFGF interaction kinetic data reported by Kamei *et al.* (14): $k_{on} = 8.6 \times 10^4$ (M^{-1} s^{-1}), $k_{off} = 0.016$ (s^{-1}), and $K_D = 180$ nM, where heparin was coupled to carboxymethyl dextran using glycolchitosan as a multivalent linker. The calculated $K_D = 160$ nM is also comparable to the K_D (50–140 nM) we previously obtained with biotinylated heparin (3). In these experiments residual aFGF was easily removed and the biosensor surface regenerated with 2 M NaCl.

Heparin interaction with dengue virus envelope protein. Dengue virus is a mosquito-transmitted flavivirus that causes a febrile disease in humans (13). Our previous studies showed that dengue virus envelope protein uses a highly sulfated form of heparan sulfate as a receptor (13). Initial SPR studies on the interac-

TABLE 1

Kinetic Parameters for Interaction of Proteins with Heparin

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
aFGF	9.2 (±4.3) × 10^4	0.015 (±0.004)	160
Dengue virus envelope protein	5.5 (±3.8) × 10^3	1.7 (±0.7) × 10^{-4}	31

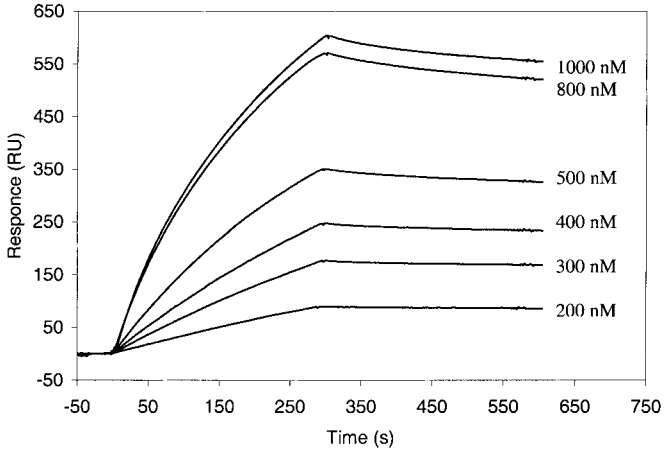

FIG. 2. SPR sensograms of dengue virus envelope protein and heparin interaction.

tion between biotinylated heparin and dengue virus envelope protein were complicated by both nonspecific interactions between streptavidin and the envelope protein and the instability of the biosensor surface under the harsh conditions required for regeneration (9). Sensorgrams for the binding of dengue virus envelope protein to the albumin–heparin conjugate surface are shown in Fig. 2. Binding between heparin and dengue virus envelope protein was very tight, with only 35% of the protein being removed with 2 M NaCl in the regeneration procedure. The surface can, however, be fully regenerated with 50 mM NaOH and the albumin–heparin conjugate was fully stable under these harsh regeneration conditions. The kinetic parameters were calculated (Table 1) with a Langmuir 1:1 binding model. The K_D value of 31 nM for heparin binding to dengue virus envelope protein calculated in these experiments is comparable to the value of 15 nM obtained using isothermal titration calorimetry (13).

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