Probing the Interaction of Dengue Virus Envelope Protein with Heparin: Assessment of Glycosaminoglycan-Derived Inhibitors

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A structure−activity relationship study was carried out to facilitate development of inhibitors of dengue virus infectivity. Previous studies demonstrated that a highly charged heparan sulfate, a heparin-like glycosaminoglycan found on the cell surface, serves as a receptor for dengue virus by binding to its envelope protein. Interventions that disrupt this binding effectively inhibit infectivity. A competitive binding assay was developed to screen polyanionic compounds for activity in preventing binding of dengue virus envelope protein to immobilized heparin; compounds tested included drugs, excipients, and larger glycosaminoglycans and their semisynthetic derivatives. Results of this competitive binding assay were used to select agents for detailed evaluation of interactions by surface plasmon resonance spectroscopy, which afforded binding on-rates, off-rates, and dissociation constants. From these data, an understanding of the structural requirements for polyanion binding to dengue virus envelope protein has been established.

Introduction

Dengue virus is a mosquito-transmitted flavivirus that causes a febrile disease in humans.[1] More than 2.5 billion people in over 100 countries are at risk of infection, and there are at least 20 million infections per year.[2] There is no treatment for dengue infection, and no vaccine is available. New approaches for the control of dengue infection are urgently needed. Studies of dengue virus pathogenesis have been hampered by the lack of suitable animal models, and there is little understanding of the pathophysiology of infection.[1] Studies of other pathogenic viruses have revealed that the binding of viral ectodomain molecules to specific receptors on target cells is a critical factor in pathogenicity since it determines cell and tissue tropism.[3] We previously showed that dengue virus envelope protein utilizes a highly sulfated form of the glycosaminoglycan (GAG) heparan sulfate as a receptor.[4] Five distinct cell types, derived from five different organs and five different species[4] (and unpublished), utilize heparan sulfate as a receptor; thus, it is likely that a single cell-surface GAG represents a conserved physiologically relevant receptor for dengue virus. Although many microorganisms interact with glycosaminoglycans, the dengue virus interaction is unusual for its high affinity and its specificity for an highly sulfated form of heparan sulfate.[4]

Since a heparan sulfate GAG is a putative receptor for dengue virus envelope protein, soluble GAGs and other highly charged polyanions could be effective inhibitors of viral infectivity. In support of this hypothesis, we found that heparin, heparin-derived oligosaccharides, and the polysulfonated urea Suramin, inhibited dengue virus envelope protein binding and viral infectivity in vitro.[4] We are building on these data to systematically generate and test related chemical structures for antiviral activity and potential use as therapeutics. We developed a 96-well-based competition assay for testing the ability of soluble compounds to inhibit the binding of dengue virus envelope protein to immobilized heparin. We used this assay, in conjunction with surface plasmon resonance spectroscopy (SPR), to investigate the interaction of heparin-like molecules with dengue virus envelope protein and report the relationship of molecular size, level of sulfation, and other structural properties to inhibitory activity.

Materials and Methods

Chemicals. Chondroitin sulfate (molecular weight average (MW_{av}) 15 000), from bovine tracheal cartilage, and dermatan sulfate (MW_{av}, 30 000) from porcine skin were a kind gift from Shin-Nippon Yakugyo Co. (Tokyo, Japan). Hyaluronan (MW_{av}, 100 000) from *Streptococcus zooepidemicus* was purchased from Kibun Food Chemipha Co. (Tokyo, Japan). Heparin (MW_{av}, 16 000) and heparan sulfate (MW_{av}, 14 800) from porcine intestinal mucosa were purchased from Celsus (Cincinnati, OH). Suramin was from Sigma (St. Louis, MO). Sulfated lactobionic acid was a generous gift from Professor Jawed Fareed (Loyola University Medical Center, Maywood, IL). Sulfated β-cyclodextrin was from American Maize (Hammond, IN). Sucrose octasulfate was from Toronto Research Chemicals (Toronto, Canada). Hyaluronidase from *Streptomyces hyalurolyticus* (lyase, E. C.4.2.2.1) was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Sephadex G-50 (superfine) and Hi-Trap desalting columns were purchased from Pharmacia Biotech. Dialysis tubing (MWCO 500) was pur-

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chased from Wako, Japan. *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide (EDC), and CM5 chips were from Biacore (Uppsala, Sweden). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Instruments. The gradient HPLC system used to demonstrate the purity of hyaluronic acid (HA) oligosaccharides was assembled with gradient pumps (Jasco 980-PU, intelligent HPLC pumps), an eluent mixer (Jasco HG-980-3, solvent mixing module), and a fluorescence-detector (Jasco FP-920S intelligent fluorescence detector) from Nihon Bunko Co., Japan. A variable sample injector (VMD-350) was from Shimamura Instrument Co., Japan. A UV-detector (D-2500) was from Hitachi Seisakusho Co., Japan. A conductivity detector (CM-8) for ion chromatography was purchased from TOSOH Co., Japan. The capillary electrophoresis system was assembled with a Beckman capillary electrophoresis system (P/ACE 5010) equipped with an UV detector and an operating system using version 0.4P/ACE station on an IBM-compatible PC, from Beckman, U.S.A. JEOL ECP400 and 600 NMR instruments, equipped with a 5 mm field gradient tuneable probe with standard JEOL software, were used for 1- and 2D ^{1}H NMR experiments at 303 K on 500 μL each sample. A BIAcore 2000 (Biacore, Uppsala, Sweden) was employed for surface plasmon resonance (SPR) spectrometry.

Preparation of Chemically Persulfated Glycosaminoglycans. Chemical O-sulfation to obtain persulfated glycosaminoglycans was carried out under mild conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents.[5] Fully O-sulfated glycosaminoglycans were prepared according to the method described previously.[6] Briefly, the GAG tributylamine (TBA) salt was obtained from 100 mg of each sodium salt by strong cation-exchange chromatography and lyophilized. The resulting dry TBA salt was dissolved in 0.8 mL of *N*,*N*-dimethylformamide (DMF) to which a required excess (15 mol/equiv of available hydroxy group) of pyridine-sulfur trioxide complex had been added. After 1 h at 40 °C, the reaction was interrupted by addition of 1.6 mL of water, and the raw product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate and then collected by centrifugation. The resulting fully O-sulfated GAGs were dissolved in water, dialyzed to remove salts, and lyophilized. In the cases of O-sulfation of heparin and heparan sulfate, partial hydrolysis of the sulfate group from the *N*-sulfoglucosamine residues occurred. These derivatives were re-N-sulfated according to the method described previously.[7]

Analysis of Glycosaminoglycans. The average molecular weight of each sample was estimated using gradient polyacrylamide gel electrophoresis (PAGE) analysis in a 12−22% gradient mini-gel, visualized with Alcian Blue.[8] The relative molecular weights of each GAG were confirmed by their elution position from a gel permeation chromatography−HPLC column eluted with 50 mM sodium acetate, pH 7.4, at a flow rate of 1 mL/min with detection at 206 nm.[9]

GAG samples were prepared for determination of sulfate and hexosamines by exhaustive dialysis against distilled water using MWCO 3500 tubing, lyophilization, and drying for 2 days in a desiccator over P_2O_5. The determination of sulfate groups was performed following combustion and after acid hydrolysis of the sample in 6 M HCl at 100 °C for 2.5 h by HPLC using a TSKgel IC-Anion-PW (4.6 mm × 50 mm) column from Tosoh Co. (Tokyo, Japan) and a suppressor column of Dowex 50W-X8 (H+ form) (50 mm × 200 mm) from The Dow Chemical Co. The mobile phase was a mixture of 1.42 mM sodium bicarbonate and 1.5 mM sodium carbonate, with flow rate 1.0 mL/min. Detection was based on conductivity (Tosoh model CM-8). Hexosamine was analyzed by the postcolumn HPLC method after acid hydrolysis under the same conditions as for sulfate analysis.[10]

Preparation of Hyaluronic Acid Oligosaccharides. The large-scale, partial depolymerization of HA by bacterial hyaluronidase was carried out on 1.0 g of HA. To a solution containing 1.0 g of HA in 200 mL buffer was added 2 mL of hyaluronidase solution (400 TRU/mL), and the enzymatic digestion was performed in a glass flask at 60 °C. When the reaction reached 40% digestion based on the absorbance at 232 nm, the reaction was stopped by boiling for 3 min. The sample was cooled in ice and dialyzed for several days at 4 °C in 500 MWCO dialysis tubing against deionized and distilled water and then freeze-dried. The resulting white powder was dissolved in 20 mL water and was directly applied onto a low-pressure gel permeation chromatography column.

The 4∼20-mer HA oligosaccharides were fractionated on a Sephadex G-50 (superfine) column (4.4 cm × 1 m) and eluted with 0.2 M sodium chloride at an optimum flow rate of 2 mL/min. The freeze-dried HA oligosaccharide mixture (∼100 mg) was dissolved in 20 mL water and applied to the column, 300 fractions were collected (5 mL/tube), and absorbance at 232 nm of each fraction was measured. Each unified HA oligosaccharide fraction was collected and concentrated by evaporation. This chromatographic separation was repeated as necessary.

Each size-uniformed HA oligosaccharide fraction, obtained from a second round of low-pressure gel permeation chromatography, was desalted on a Hi-Trap desalting column eluted with water at 1.0 mL/min. The eluent was collected, and the fractions containing HA oligosaccharides were combined. The HA oligosaccharide samples were freeze-dried.

Preparation of fully O-sulfated HA oligosaccharides was based on the method described previously,[6] with some modifications. To obtain the tributylamine (TBA) salt of HA oligosaccharides, 50 μL of TBA was added to size-uniform HA oligosaccharides (1.0 mg) in 1.0 mL of distilled water adjusted to pH 2.80 with 0.1 M hydrochloride, mixed vigorously, and freeze-dried. The resulting salt was dissolved in 0.2 mL of *N*,*N*-dimethylformamide (DMF) to which a required excess (15 mol/equiv of available hydroxy group in HA oligosaccharides) of pyridine-sulfur trioxide complex had been added. After 3 h at 40 °C, the reaction was interrupted by addition of 0.5 mL of water, and the raw product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate and then collected by centrifugation. The resulting fully O-sulfated HA oligosaccharides were dissolved in water and centrifuged, and the supernatant was repeatedly concentrated by pressure filtration using a MW 3000 cutoff filter device (Microcon YM-3) and finally freeze-dried.

Characterization of Hyaluronic Acid Oligosaccharides. Purified HA oligosaccharides were analyzed by a new separation method, based on normal-phase HPLC using an Amide-80 column. Conditions for gradient elution were as follows: The stepwise gradient elution was started at 78% buffer A (a mixture of acetonitrile/distilled water/0.2 M sodium phosphate buffer (pH 7.0)/3.0 M ammonium chloride = 32/11/1/1, by volume) and 22% buffer B (a mixture of acetonitrile/distilled water/0.2 M sodium phosphate buffer (pH 7.0)/3.0 M ammonium chloride = 16/21/1/1, by volume) and was subsequently changed to 65% buffer A and 35% buffer B for 15 min, 50% buffer A and 50% buffer B for 35 min, 25% buffer A and 75% buffer B for 75 min, and finally maintained in 100% buffer B for 10 min, and then it was returned to the initial condition: flow rate, 1.0 mL/min; column temperature, 50 °C; UV detection at 232 nm.

The purity of HA oligosaccharides was confirmed by capillary electrophoresis in the normal polarity mode, using a mixture of 40 mM disodium phosphate/40 mM sodium dodecyl sulfate/10 mM sodium tetraborate adjusted to pH 9.0 with 1.0 M hydrochloric acid as previously described.[11] The fused silica capillary (75 μm i.d. × 375 μm o.d., 67 cm long) was automatically washed before use with 0.1 M sodium hydroxide, followed by nitrogen gas pressure injection (5 s) at a constant current 15 kV. The samples (0.1 mg/mL) were dissolved in water and loaded (7 nL) with nitrogen gas pressure injection.

Gradient PAGE was used to monitor the preparation and purification of O-sulfated 10∼20-mer HA oligosaccharides as well as to confirm depolymerization of HA oligosaccharides by the O-sulfation reaction. Polyacrylamide linear gradient resolving gels (14 × 28 cm, 10−20% acrylamide gel) were purchased and processed as previously described.[8] O-Sulfated HA oligosaccharides were visualized by Alcian Blue staining.

Determination of sulfate groups was performed by anion exchange HPLC using a conductivity detector, after acid hydrolysis of the sample in 6 M hydrochloric acid at 100 °C for 2.5 h. Hexosamine was analyzed by the postcolumn HPLC derivatization method[10] after acid hydrolysis under the same conditions as described for sulfate analysis.

Spectral Characterization. One- and two-dimesional ^{1}H NMR spectroscopy were performed using conditions described previously.[6] Briefly, each sample (approximately 2.0 mg) was dissolved in 0.5 mL of D_2O (99.9%) and repeatedly freeze-dried to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. The thoroughly dried sample was then dissolved in 500 μL of D_2O (99.96%) and passed through a 0.45 μm syringe filter and transferred to an NMR tube (5.0 mm o.d. × 25 cm). The HOD signal was suppressed by presaturation during 3 or 1.5 s for 1D or 2D NMR spectra, respectively. To obtain 2D spectra, a 1024 × 512 data matrix for spectral width of 2000 Hz was measured, and the time domain data were multiplied after zero-filling (data matrix size 1K × 1K) with a shifted sine-bell window functions for 2D double quantum filtered correlation spectroscopy (DQF-COSY).

For infrared (IR) spectroscopy of solid samples, a Jasco model FTIR 230 (Tokyo, Japan) was used. A 100 μg portion of glycosaminoglycan was mixed with 500 μg of dried KBr, and a salt disk (3 mm diameter) was placed in the spectrometer.

The same dried samples used for sulfate analysis were used to measure optical rotation. Samples were weighed and dissolved in distilled water at a concentration of 5 mg/mL, and their optical rotation was determined. Measurements were made at the sodium D-line on a Jasco model DIP-140 spectropolarimeter (Tokyo, Japan).

Envelope Protein Construct. Two forms of recombinant dengue virus envelope protein were used for experiments. For surface plasmon resonance studies, we utilized a fusion protein that was based on nucleotides 1−1272 of the dengue 2 virus envelope protein cDNA (Tonga 1974 strain, Genbank accession X54319), representing the entire envelope protein ectodomain region. This protein was expressed as an amino-terminal fusion with the Fc region of human IgG (env-IgG). The cDNA was incorporated into an eukaryotic expression plasmid, and protein expressed after transfection of COS cells. Details of the expression system have been published.[12] For the competitive binding microplate assay, a larger amount of recombinant protein was required, and a second form of the envelope protein was developed for expression in bacteria; this incorporated the same envelope protein ectodomain region included in the original eukaryotic expression construct. Details of the bacterial expression system are given below.

The dengue 2 virus envelope protein cDNA was obtained as a plasmid clone in the vector pcDNA3.[12] The region encoding the envelope protein, extending from the start codon to the beginning of the transmembrane region (nucleotides 1−1272), was subcloned using PCR amplification. Additional nucleotides were included in the sense and antisense strand oligonucleotides to generate upstream Xho I and downstream Not I restriction sites and a translation-termination codon. The sense strand oligonucleotide primer sequence was (Xho I site underlined) 5′-CTC GAG ATG CGC TGC ATA GGA ATA TCA AAT AGG G. The antisense strand oligonucleotide primer was (Not I site underlined, termination codon in italics) 5′-GCG GCC GC*T TA*A GAT CCA AAA TCC CAG GC. PCR amplification was performed using Platinum Taq DNA polymerase and buffer solutions (Life Technologies, Rockville, MD). One nanogram of the original plasmid clone was used as template, and PCR conditions were as follows: denaturation 94 °C 2 min, followed by 25 cycles of (i) denaturation (94 °C 45 s), (ii) annealing (58 °C 30 s), and (iii) elongation (72 °C 90 s), followed by a final 10 min elongation period at 72 °C. The PCR product was ligated into the plasmid pCR2.1 and transformed into InvαF′ strain *Escherichia coli* (TA cloning kit, Invitrogen, Carlsbad, CA). Correct transformants were identified by restriction digest analysis and digested with Xho I and Not I, and the released cDNA insert was recovered from an agarose gel after electrophoresis.

The plasmid utilized for expression was pThioHisA (Invitrogen), a vector designed for prokaryotic expression of heterologous proteins fused to the carboxy terminus of the *E. coli* protein thioredoxin. Expression is driven by the *trc* promoter and is regulated by the *lac*I^q repressor. pThioHisA was digested with Xho I and Not I and then treated with calf intestinal alkaline phosphatase (Life Technologies). Digested plasmid was separated from the liberated stuffer fragment and recovered after agarose gel electrophoresis. The envelope protein cDNA insert was then ligated, in frame, into the prepared pThioHisA plasmid and transformed into InvαF′ strain *E. coli*, correct transformants were identified by restriction digestion, and large-scale plasmid preparations were prepared. The construct is named "TR-env". Standardized protocols were used for molecular biology procedures.[13]

Expression and Recovery of Envelope Protein. pTR-env plasmid was transformed into DH5α strain *E. coli* (Life Technologies), and colonies were selected on ampicillin containing media and confirmed as containing the correct insert by restriction mapping, as well as by PCR amplification of appropriately sized inserts using series of flanking and internal oligonucleotide primers. A single colony was expanded in LB broth containing ampicillin 100 μg/mL, used to inoculate a 250 mL flask of the same media, and grown in a shaking incubator to mid-log phase at 37 °C (OD_{600nm} = 0.5). At this point, 1 mM isopropylthio-β-galactosidase (Life Technologies) was added to remove the *lac* repressor and permit expression of the TR-env protein. The culture was harvested after a further 3 h period (time of peak expression).

Initial experiments indicated that all the recombinant protein was expressed as intracellular insoluble inclusion bodies. Cultures were centrifuged at 3000*g* for 10 min at 4 °C, the supernatant was discarded, and the bacterial pellet was stored at −80 °C. For processing, the pellet was resuspended in 15 mL of bacterial protein extraction reagent (BPER, Pierce, Rockford, IL), containing 5 μg/mL DNAase (Boehringer Mannheim, Indianapolis, IN), and incubated with gentle mixing for 10 min at room temperature. The preparation was centrifuged at 27000*g* for 15 min at 4 °C, and the supernatant was discarded. Another 15 mL of BPER, containing 200 μg/mL lysozyme (Boehringer Mannheim), was used to resuspend the residual insoluble material and was incubated with gentle mixing for 5 min at room temperature. A total of 100 mL of a 1/10 dilution of BPER in water was added and mixed by extensive vortexing. Inclusion bodies were collected by centrifuging at 27000*g* for 15 min at 4 °C and washed twice more with 100 mL of 1/10 diluted BPER. Inclusion body preparations were stored at −80 °C until used.

Solubilization and Refolding of Envelope Protein. Insoluble inclusion body preparations were washed twice by resuspension in 20 mM Tris pH 8.5, 2.5 mM EDTA, 5 mM imidazole, 1% Triton X-100, and collected by centrifugation at 27000*g* for 10 min at 4 °C. Inclusion bodies were then solubilized in 15 mL of 0.3% w/v *N*-lauryl sarcosine (ICN, Aurora, OH), 50 mM CAPS (ICN) pH 11, and 1 mM dithiothreitol (DTT), and they were incubated for 30 min at room temperature. The preparation was centrifuged at 10000*g* for 15 min at 4 °C, the supernatant recovered, and remaining insoluble material discarded. Detergent was removed, and the protein was refolded by dialysis (12−14 kD exclusion cellulose tubing, Life Technologies) without stirring, two exchanges, 4 h per exchange, 4 °C, sequentially against (i) 20 mM Tris pH 8.5, 150 mM NaCl, 0.1 mM DTT; (ii) 20 mM Tris pH 8.5, 150 mM NaCl; (iii) 20 mM Tris pH 8.5, 150 mM NaCl, 0.2 mM oxidized glutathione (Calbiochem, San Diego CA), 1 mM reduced glutathione (Calbiochem).

Evaluation of Envelope Protein. Protein expression was confirmed by denaturing SDS−PAGE using a Laemmli buffer system,[14] followed by Coomassie blue and silver staining. A predominant band migrating at the expected 61 kD molecular weight was observed and constituted >90% of protein found in inclusion body preparations and in the solubilized and

refolded preparations. Electrophoresis in nonreducing gels revealed that the protein predominantly migrated as a monomer. pTR-env encodes an enterokinase cleavage site at the junction between the amino-terminal thioredoxin and the carboxy-terminal envelope protein, and digestion of 61 kD TR-env with enterokinase generated the expected 13 kD and 48 kD bands. Both forms of recombinant envelope protein bound to Vero and HepG2 cells. Binding was prevented by pretreating cells with with heparin lyase I (degrades heparin and highly sulfated domains in heparan sulfate) and with heparin lyase III (degrades only heparan sulfate), but binding was not affected by chondroitin ABC lyase. These data confirm that the envelope proteins bind to cell-surface heparan sulfate.[4] Both forms of envelope protein bound to heparin with similar high affinity (elution from heparin-Sepharose requiring 0.6−0.8 M NaCl), compatible with previous studies using intact native dengue virus.[4]

Envelope Protein Binding Assay. The binding assay was based on using an antibody to bovine albumin as a capture reagent to coat plastic micro-wells, followed by addition of heparin-conjugated albumin, which was immobilized by the albumin-antibody. Recombinant TR-env envelope protein was then added, in the presence or absence of potential inhibitors, and bound envelope protein was quantitated with secondary immunoreagents. Immulon-4 96-well flat-bottom microtiter plates (Dynex, Chantilly, VA) were coated with 50 μL of 4 μg/mL affinity purified rabbit antibody to bovine serum albumin (ICN) diluted in cation-free phosphate buffered saline pH 7.4 (PBS, Life Technologies) and incubated overnight at 4 °C. Wells were washed three times with 200 μL of PBS containing 0.05% v/v Tween-20 (PBST), and residual nonspecific binding sites were blocked by incubating with 200 μL of 0.2% Tween-20 in PBS for 1 h at room temperature. Wells were washed three times with PBST, 35 μL of 10 μg/mL heparin-albumin (Sigma H-0403) diluted in PBS was added, and the mixture was incubated for 1 h at room temperature. Unbound material was removed by washing three times, and the following reagents, diluted in PBS, were sequentially added for 1 h at room temperature, with incubations separated by three washes with PBST: (i) 35 μL of 3 μg/mL envelope protein in the presence or absence of test inhibitor compounds; (ii) 35 μL of 0.5 μg/mL mouse anti-thioredoxin antibody (Invitrogen); (iii) 35 μL of B-Phycoerythrin conjugated F(ab′)$_2$ fragment of goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA). After final washing, fluorescence/well was quantitated using a Cytofluor 2300 fluorescence plate reader (Applied Biosystems, Foster City, CA) using 530 nm excitation and 595 nm emission filters. Three wells were used for each test condition, and the results were expressed as the mean and standard deviation for each replicate.

In some initial studies, unconjugated bovine albumin was used as a control in place of heparin-albumin. In these experiments, binding of albumin or heparin-albumin to the capture anti-albumin antibody was assessed by adding 35 μL of 100 μg/mL rhodamine conjugated IgG antibody to bovine albumin (ICN) to the wells for 1 h at room temperature, followed by washing and quantitating fluorescence with 530 nm excitation and 620 nm emission filters.

Immobilization of Envelope Protein for Surface Plasmon Resonance. Envelope protein (env-IgG) was covalently bound to the sensor surface through its primary amino groups. The carboxymethylated dextran surface of the CM-5 sensor chip was first activated using an injection pulse of an equimolar mix of NHS and EDC (35 μL, 5 μL/min, final concentration 0.05 M, mixed immediately prior to injection). Prior to injection over the sensor chip, envelope protein was mixed with a 10-fold molar excess of the polyanion Suramin, for 30 min at 4 °C, to protect the positively charged basic amino acids that define the glycosaminoglycan-binding domains of the envelope protein. Citrate buffer (200 μL) pH 5.5 containing envelope protein (30 μg/mL) and Suramin (1.7 μg/mL) was injected into the first flow cell. The second flow cell was injected with human IgG to serve as a control for the envelope protein. The third flow cell was activated with NHS and EDC, but no protein was immobilized on its surface. Unoccupied active sites on the sensor surfaces were then blocked with a 35 μL injection of 1 M ethanolamine. Successful immobilization of envelope protein and IgG were indicated by 4045 and 11 000 increases in resonance unit (RU) signal, respectively.

Kinetic Measurement of Envelope Protein−Polyanion Interaction by Surface Plasmon Resonance. Samples to be tested for interaction with immobilized envelope protein were prepared in phosphate buffered saline (0.01 M sodium phosphate, 2.7 mM KCl, 0.137 M NaCl, pH 7.4). Experiments were carried out at 25 °C. Fifteen microliters of sample was injected into each flow cell at a rate of 5 μL/min. At the end of each sample injection, the same buffer was continuously flowed over the sensor surface to monitor dissociation. At the end of each run, the sensor surface was regenerated by injecting 10 μL of 2 M NaCl; this was followed by reinstituting flow with PBS and the next test compound. Sensorgrams were generated that related signal to time, and kinetic parameters were determined using Biacore BIA Evaluation software (ver. 3.0.2, 1999). Signals from control cells were subtracted from the signal generated by the flow cell containing envelope protein. However, in the experiments shown here, none of the compounds interacted significantly with either control IgG protein or the dextran surface of the CM5 chip.

Results

Preparation and Characterization of Polyanions. The polyanions examined in this study include several that are currently used as drugs and excipients. Small synthetic polyanions (Figure 1) including Suramin (an antineoplastic and antihelminthic agent),[15] sucrose octasulfate (the active component in Sucralfate),[16] sulfated lactobionic acid (an antithrombotic in clinical evaluation),[17] and sulfated β-cyclodextrin (an excipient)[18] were evaluated. We also evaluated the natural polyanionic polysaccharides known as glycosaminoglycans (GAGs): heparin (an anticoagulant/antithrombotic), heparan sulfate, chondroitin sulfate, and dermatan sulfate (all components of the antithrombotic ORG10172)[19] and hyaluronic acid (an ophthalmic and antiarthritic agent). With the exception of hyaluronic acid, all these GAGs have substantial sequence heterogeneity (Figure 1). In addition, all GAGs are highly polydisperse mixtures having a range of polysaccharide chain lengths, characterized by an average molecular weight (MW_{av}). The GAGs were subjected to per-O-sulfation with sulfur trioxide-pyridine to both increase their negative charge and to decrease (or eliminate in the case of chondroitin and dermatan sulfate) their sequence heterogeneity (Figure 1). Since we had previously described a highly sulfated heparin decasaccharide that effectively blocked the binding of dengue virus envelope protein to target cells,[4] we decided to prepare a collection of highly sulfated GAG-derived oligosaccharides ranging in size from a decasaccharide (degree of polymerization (dp) 10) to an ecosisaccharide (dp 20) for evaluation. Hyaluronic acid was used as the starting material as it has no sequence heterogeneity. Partial enzymatic depolymerization of hyaluronic acid with bacterial hyaluronidase (an eliminase), followed by fractionation, afforded a collection of HA oligosaccharides of dp 10, 12, 14, 16, 18, and 20. These were determined to be analytically pure by capillary electrophoresis and had the structures shown in Figure 1. Exhaustive treatment of these HA oligos with sufur trioxide-pyridine complex afforded the persulfated HA oligosaccharides. The purity of these oligosaccharides was confirmed by HPLC, capillary electrophoresis, and

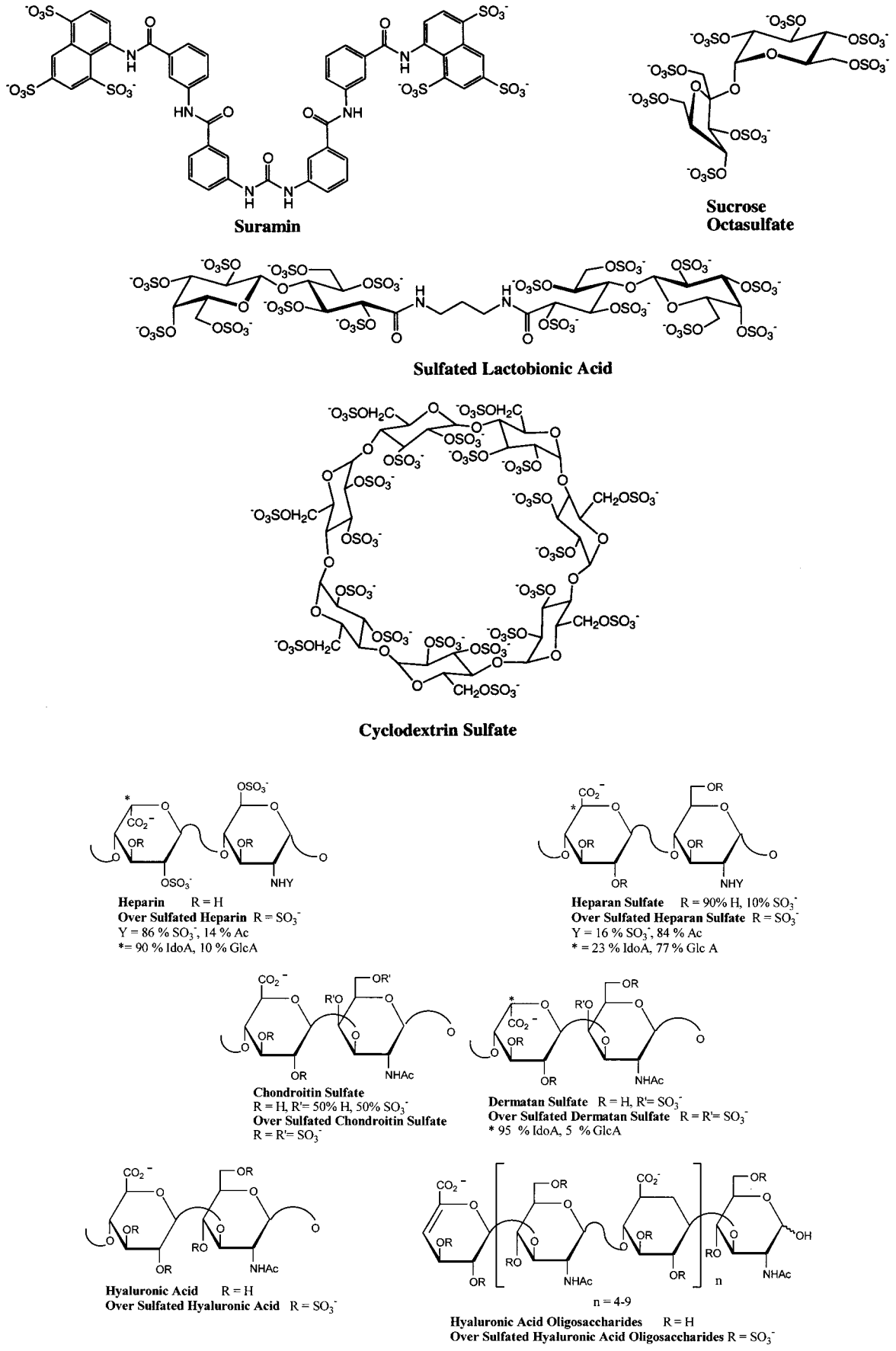

Figure 1. Structures of small polyanions and structures of GAGs, oversulfated GAGs, and HA oligosaccharides.

gradient PAGE, and their structure was confirmed (Figure 1) by NMR spectroscopy and quantitative sulfate analysis. All of the polyanions investigated here for their ability to bind to dengue virus envelope pro-

tein are considered to be heparinoids or heparin-like agents.

Envelope Protein−Heparin Microtiter Plate Binding Assay. In initial experiments we attempted to take advantage of the nonspecific binding interaction between proteins and protein-binding microtiter plates to directly immobilize heparin-conjugated albumin, with binding detected using a fluorochrome-labeled anti-albumin antibody; as expected, unconjugated albumin was readily immobilized. However, no signal was detected after similarly attempting to immobilize heparin-albumin, possibly due to electrostatic repulsion between the highly negatively charged heparin and the plastic surface. To overcome this effect, we first immobilized a saturating amount of antibody to albumin in wells, for use as the initial capture reagent, and this was followed by addition of albumin or heparin-albumin. Figure 2a demonstrates that this approach did lead to successful immobilization of both albumin and heparin-albumin, and this method was adopted for all later studies. Figure 2b demonstrates that the envelope protein bound to immobilized heparin-albumin, but there was no binding to an equivalent amount of nonconjugated albumin. Figure 2c demonstrates that co-incubation of heparin with envelope protein led to a concentration-dependent reduction of binding, consistent with competition for the envelope protein between immobilized heparin-albumin and soluble heparin.

Competitive Binding Studies. Competitive binding studies between the soluble heparinoids shown in Figure 1 and immobilized heparin for dengue virus envelope protein was investigated, since heparin had previously been demonstrated to elicit the strongest interaction with envelope protein and intact virus.[4] Initial evaluation focused on the low molecular weight structurally defined heparinoids, Suramin, sucrose octasulfate, sulfated lactobionic acid, and sulfated β-cyclodextrin. Displacement of envelope protein was measured as a function of polyanion concentration (Figure 3). Sucrose octasulfate showed no inhibition of envelope protein binding to heparin even at concentrations of up to 100 μg/mL, while sulfated lactobionic acid and sulfated β-cyclodextrin showed low but comparable activities at concentrations above 10 μg/mL. Suramin showed potent activity, exceeding that of soluble heparin (Table 1).

Next we examined the GAGs for activity in competitively inhibiting binding of envelope protein to heparin. None of the GAGs examined, with the exception of heparin, showed inhibitory activity (Figure 4A). Previous studies demonstrated avid binding of highly sulfated heparan sulfate to envelope protein; however, the heparan sulfate used in this experiment was a commercial low-sulfate preparation and as expected did not bind envelope protein.[4] However, persulfation of these GAGs afforded O-sulfated GAGs with very potent and nearly equal inhibitory activity, demonstrating approximately 100-fold stronger binding than heparin (Figure 4B, Table 1).

The size dependence of oligosaccharide binding to envelope protein was examined using HA-derived oligosaccharides. In the absence of sulfate groups, HA oligosaccharides of degree of polymerization (dp) 10−20 showed no activity (not shown). However, persulfonated HA oligosaccharides (dp 10−20) showed activity that generally increased with oligosaccharide dp (Figure 5). The O-sulfated HA ecosisaccharide (dp 20) showed activity comparable to that of heparin (on a weight basis) but significantly lower than that of full-length O-sulfated-HA.

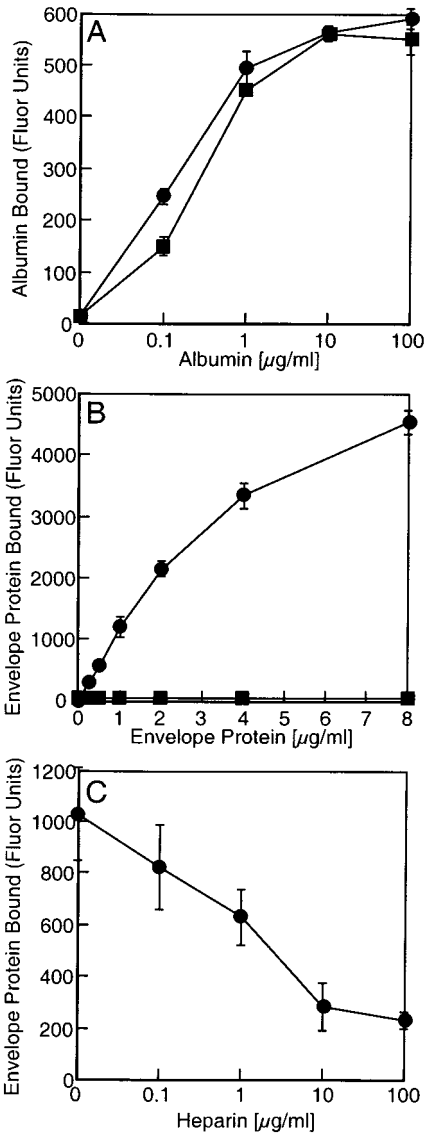

Figure 2. Microtiter plate assay. (A) Immobilization of heparin-albumin and albumin. Wells were first treated with antibody to albumin, followed by addition of heparin-albumin or unconjugated albumin. Albumin bound was detected with fluorochrome-labeled anti-albumin antibody. Abscissa: (●) heparin-albumin or (■) albumin added 0−100 μg/mL. Ordinate: albumin bound (fluorescence units per well). (B) Envelope protein binds to immobilized heparin-albumin. Wells were treated with antibody to albumin, followed by (●) heparin-albumin or (■) unconjugated albumin. Envelope protein was then added, followed by detection of bound envelope protein with secondary immunoreagents. Abscissa: envelope protein added 0−8 μg/mL. Ordinate: envelope protein bound (fluorescence units per well). (C) Soluble heparin blocks binding of envelope protein to immobilized heparin-albumin. Wells were treated with antibody to albumin, followed by heparin-albumin, and then by envelope protein in the presence or absence of heparin. Bound envelope protein was then detected with secondary immunoreagents. Abscissa: envelope protein 3 μg/mL with heparin 0−100 μg/mL. Ordinate: envelope protein bound (fluorescence units per well). Results are expressed as mean ± standard deviation of replicates.

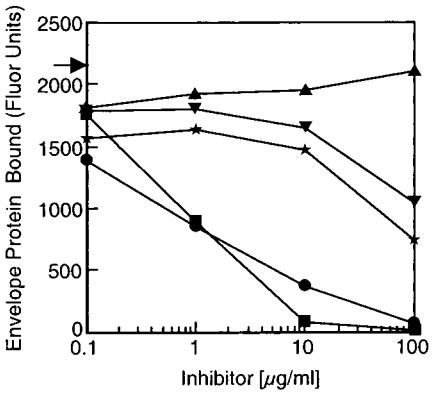

Figure 3. Envelope protein competitive binding studies with heparin and small heparinoid polyanions. Wells were treated with antibody to albumin, followed by heparin-albumin, and then by envelope protein in the presence or absence of heparin or heparinoid inhibitors. Bound envelope protein was then detected with secondary immunoreagents. Abscissa: inhibitor doses. Ordinate: envelope protein bound (fluorescence units per well). Results are expressed as mean ± standard deviation of replicates. Key: (●) heparin, (■) Suramin, (▲) sucrose octasulfate, (▼) sulfated lactobionic acid, (★) sulfated β-cyclodextrin. Arrow at ordinate represents binding of envelope protein in the absence of any inhibitor.

Table 1. Comparison of the Binding Avidity of Suramin, Heparin, and O-Sulfated Heparin[a]

drug	ID_{50} [M]	ID_{100} [M]
Suramin	7.0×10^{-7}	7.0×10^{-6}
heparin	7.6×10^{-7}	2.5×10^{-4}
O-sulfated heparin	7.6×10^{-9}	2.5×10^{-7}

[a] The molar concentration of each compound required to inhibit binding of envelope protein to immobilized heparin in the competition plate assay by 50% (ID_{50}) and 100% (ID_{100}) was determined from several experiments.

Surface Plasmon Resonance Analysis of Envelope Protein−Polyanion Interactions. The interaction of heparin with the envelope protein was first analyzed at different heparin concentrations to permit calculation of rate constants and affinity parameters. Sensograms for the binding of envelope protein to heparin are shown in Figure 6. The association phase of the binding between envelope protein and heparin was fast, and the mean association rate constant was 4.6×10^5 M^{-1} s^{-1}. The dissociation phase was slow, and the mean dissociation rate constant was 2.6×10^{-2} s^{-1}, corresponding to a K_d of 56 nM. Both the association and dissociation kinetics fit a one-site model.

Similar SPR measurements on sulfated lactobionic acid, sulfated β-cyclodextrin, sucrose octasulfate, and the unmodified GAGs (chondroitin, dermatan, and heparan sulfates, and hyaluronic acid) showed no measurable interaction (not shown). In contrast, the O-sulfated GAGs showed very strong interactions with envelope protein (Table 2). The sensograms for the binding of Suramin to envelope protein (Figure 7) showed a fast (as compared to heparin) association rate constant of 1.0×10^6 M^{-1} s^{-1}. The dissociation rate constant 4×10^{-2} s^{-1}, however, was faster than that observed for heparin. Both the association and dissociation kinetics fit to a one-site model and afforded a K_D of 40 nM, comparable with heparin.

The kinetic analysis of the interaction of HA oligosaccharides with envelope protein are summarized in Table

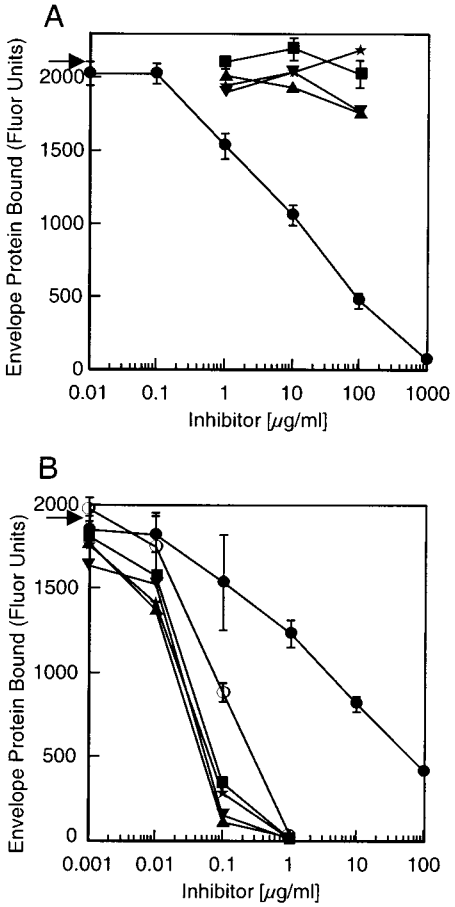

Figure 4. Envelope protein competitive binding studies with GAGs and persulfated GAGs. Wells were treated with antibody to albumin, followed by heparin-albumin, and then by envelope protein in the presence or absence of inhibitors. Bound envelope protein was then detected with secondary immunoreagents. Abscissa: inhibitor doses. Ordinate: envelope protein bound (fluorescence units per well). Results are expressed as mean ± standard deviation of replicates. (A) Native GAGs. Key: (●) heparin, (■) heparan sulfate, (★) chondroitin sulfate, (▲) dermatan sulfate, (▼) hyaluronic acid. (B) Persulfated GAGs. Key: (●) heparin, (○) O-sulfated (*O*S-) heparin, (■) *O*S-heparan sulfate, (★) *O*S-chondroitin sulfate, (▲) *O*S-dermatan sulfate, (▼) *O*S-hyaluronic acid. Arrow at ordinate represents binding of envelope protein in the absence of any inhibitor.

3. The unmodified HA oligosaccharides failed to interact, while the persulfated HA oligosaccharides interacted with increasing strength as a function of increasing oligosaccharide size.

Discussion

Previous studies in our laboratory identified a heparin-binding motif in the dengue virus envelope protein, and experiments using isothermal titration calorimetry demonstrated that heparin bound to envelope protein with a K_d of 15 nM.[4] The binding site(s) in the envelope protein required a decasaccharide for strong interaction.[4] While typical heparan sulfate failed to interact with envelope protein, a highly sulfated heparan sulfate, isolated from bovine liver, bound nearly as tightly as heparin,[4] suggesting that cell surface associated heparan sulfate was a putative receptor for dengue virus.

The current study focused on examining small polyanionic drugs and excipients as well as glycosaminogly-

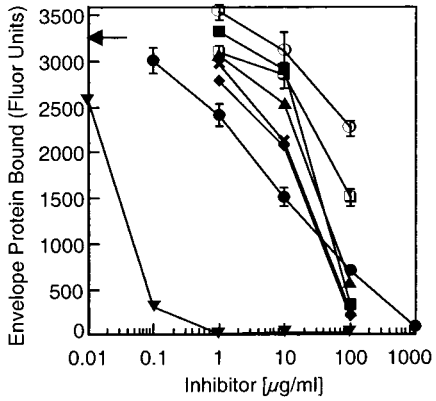

Figure 5. Envelope protein competitive binding studies with persulfated HA oligosaccharides, persulfated HA, and heparin. Wells were treated with antibody to albumin, followed by heparin-albumin, and then by envelope protein in the presence or absence of inhibitors. Bound envelope protein was then detected with secondary immunoreagents. Abscissa: inhibitor doses. Ordinate: envelope protein bound (fluorescence units per well). Results are expressed as mean ± standard deviation of replicates. Key: (●) heparin, (○) persulfated hyaluronic acid (HA) dp 10, (■) persulfated HA dp 12, (□) persulfated HA dp 14, (▲) persulfated HA dp 16, (×) persulfated HA dp 18, (◆) persulfated HA dp 20, (▼) persulfated full-length HA. Arrow at ordinate represents binding of envelope protein in the absence of any inhibitor.

cans and their derivatives, as potential inhibitors of dengue virus envelope protein binding to its receptor,

Table 2. Kinetic Measurement of O-Sulfated GAGs Binding to Immobilized Envelope Protein, Determined by Surface Plasmon Resonance

GAGs	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)	R_{max}
O-sulfated heparin	2.0×10^3	1.1×10^{-5}	5	550
O-sulfated heparan sulfate	2.5×10^3	1.0×10^{-5}	4	523
O-sulfated chondroitin sulfate	1.7×10^3	1.6×10^{-5}	9.5	563
O-sulfated dermatan sulfate	1.2×10^3	1.8×10^{-5}	15	497
O-sulfated hyaluronic acid	1.8×10^3	1.0×10^{-5}	6	400
N- and O-sulfated heparin	2.3×10^3	1.1×10^{-5}	5	650

highly sulfated heparan sulfate. The goal of this study was to develop a structure−activity relationship that could assist in the design of potential therapeutic agents for treatment of dengue virus infection. Two related forms of recombinant dengue virus envelope protein were used for these studies, and both bound to target cell heparan sulfate and to heparin with similar binding and infectivity profiles to that of intact dengue virus, indicating that both expressed critical conformational and functional elements of the native envelope protein.

Two approaches were used to assess the interaction of envelope protein with polyanions. The first relied on a newly developed competitive binding microtiter plate assay to compare the potency of each polyanion at inhibiting envelope protein binding to heparin, which serves as a model for the highly sulfated heparan sulfate receptor. The second assay relied on direct measurement of the kinetics of polyanion interaction with envelope

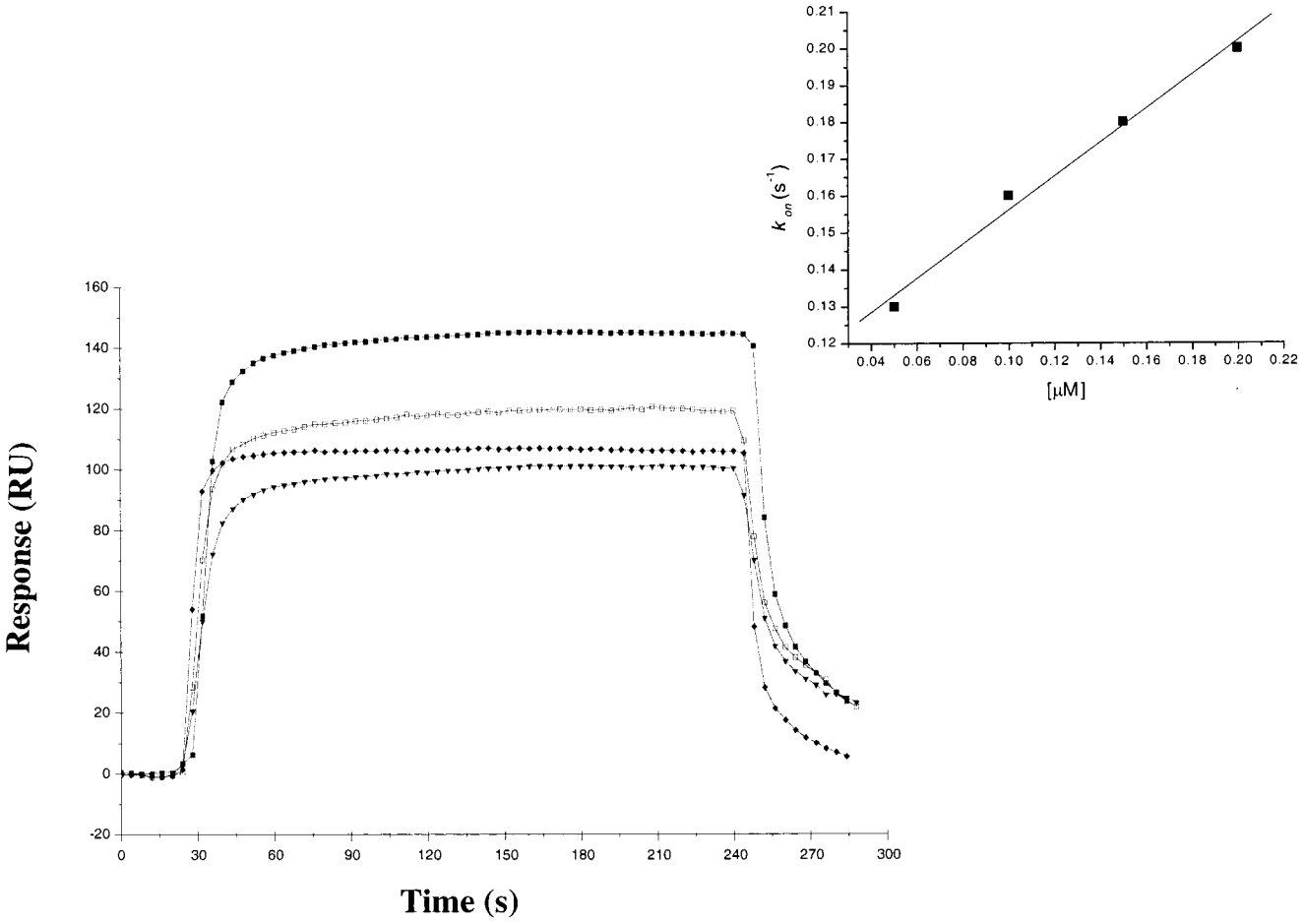

Figure 6. Surface plasmon resonance sensograms of immobilized dengue virus envelope protein interacting with soluble heparin 20 μM (■), 15 μM (□), 10 μM (◆), and 5 μM (▼). Abscissa: time (s). Ordinate: response (resonance units). The RU maximum increases with increasing concentrations. Inset shows a plot of k_s as a function of heparin concentration used to calculate k_{on}.

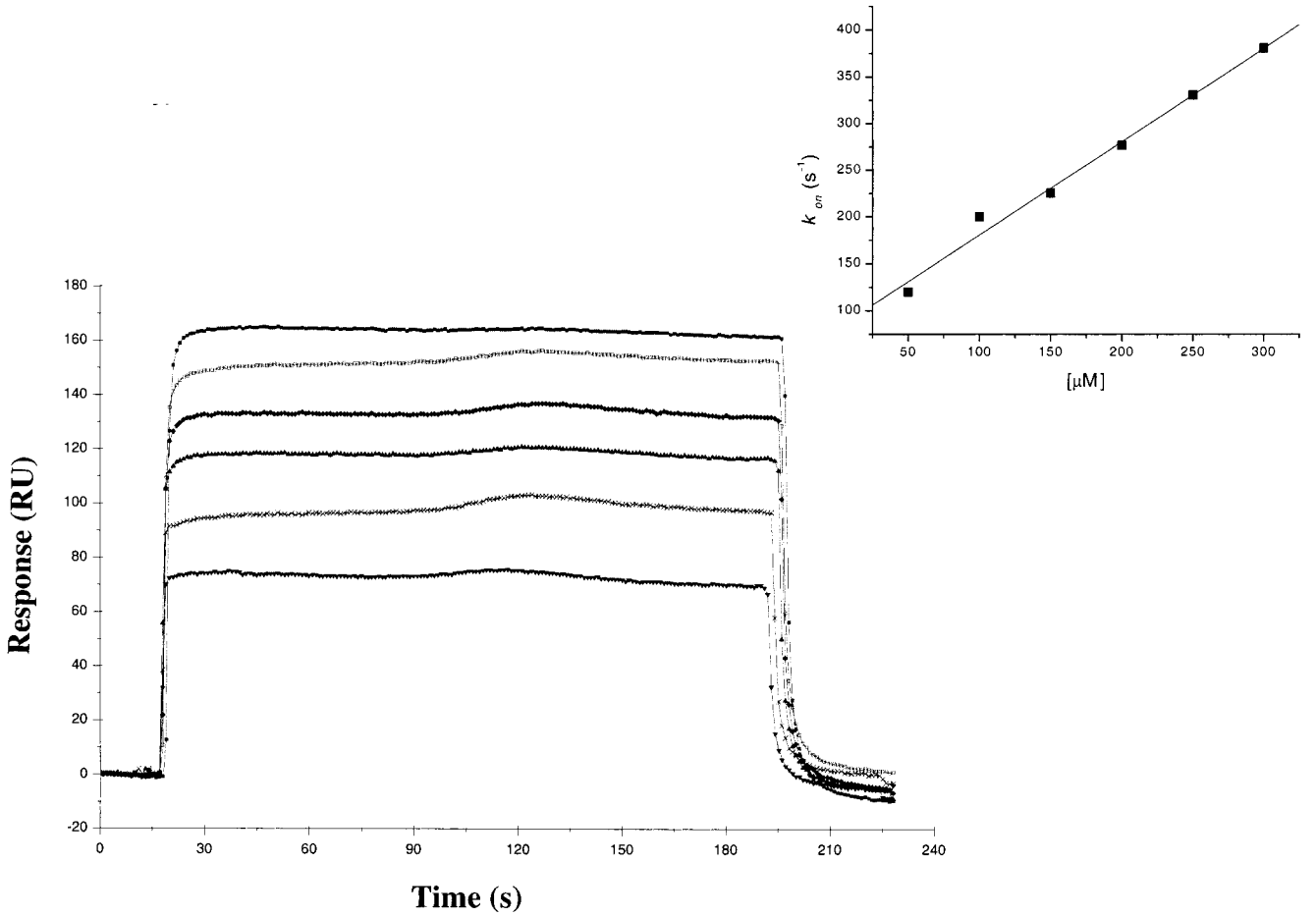

Figure 7. Surface plasmon resonance sensograms of immobilized dengue virus envelope protein interacting with soluble Suramin 300 μM (■), 250 μM (□), 200 μM (◆), 150 μM (▲), 100 μM (×), and 50 μM (▼). Abscissa: time (s). Ordinate: response (resonance units). The RU maximum increases with increasing concentrations. Inset shows a plot of k_s as a function of heparin concentration used to calculate k_{on}.

Table 3. Kinetic Measurement of O-Sulfated HA Oligosaccharides Binding to Immobilized Envelope Protein Determined by Surface Plasmon Resonance

sulfated HA oligos	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)	R_{max}
10-mer	1.0×10^2	1.1×10^{-5}	100	80
12-mer	1.0×10^2	1.0×10^{-5}	100	85
14-mer	1.3×10^2	1.0×10^{-5}	76	80
16-mer	1.3×10^2	1.2×10^{-5}	76	86
18-mer	1.5×10^2	1.0×10^{-5}	66	79
20-mer	1.7×10^2	1.0×10^{-5}	57	90

protein immobilized on a biosensor chip using surface plasmon resonance spectroscopy.

Both assays afforded comparable results for each polyanion examined in the current study and were similar to those previously published for heparin using isothermal titration calorimetry,[4] validating the newly developed, high-throughput, microtiter plate assay to screen for agents that could inhibit dengue virus infectivity. The small polyanions examined showed substantial differences in binding to dengue virus envelope protein. Sucrose octasulfate (the active component of Sucralfate, a drug used to treat peptic ulcers), while having the highest charge density, failed to show any interaction with envelope protein. Sulfated lactobionic acid (an antithrombotic agent) and sulfated β-cyclodextrin showed low but significant interactions with envelope protein. These results suggest that a disaccharide, no matter how highly sulfated, is not able to bind to the relatively elongated polyanion-binding site found in dengue virus envelope protein.[4] The sulfated lactobionic acid tetrasaccharide and sulfated β-cyclodextrin hexasaccharide, while sufficiently large to interact with the envelope protein, are still insufficiently large to bind strongly to its polyanion-binding site. Indeed, previous studies using heparin oligosaccharides demonstrated that a decasaccharide showed interaction with this site comparable to that of heparin.[4] A model of Suramin shows that it is comparable in size with the heparin decasacharide.[4] Suramin binds envelope protein with the identical avidity as heparin, consistent with its occupying the entire polyanion-binding site in dengue virus envelope protein.

Next, our attention turned to the examination of GAGs and modified GAGs. Of the GAGs examined, only heparin bound to envelope protein. The interaction of heparin measured by SPR afforded a K_d of 56 nM, consistent with the value of 15 nM previously reported using isothermal titration calorimetry.[4] Using sulfation chemistry recently developed in our laboratory,[6] we prepared persulfated GAGs, where every hydroxyl group had been converted to an O-sulfo group. Analysis of sulfate content of these GAGs by hydrolysis and ion chromatography, as well as by ^{1}H NMR spectroscopy, was used to confirm the structure of these derivatized GAGs.[20] All of the persulfated GAGs showed similar and

at least 10-fold stronger binding to the envelope protein than heparin, as measured by both the competition assay and by direct assay using SPR. Oligosaccharides were prepared from the structurally simplest GAG, hyaluronic acid (Figure 1), using a bacterial hyaluronidase. These HA oligosaccharides were fractionated on the basis of size to obtain a series of oligosaccharides ranging from decasaccharide to ecosisaccharide, and were demonstrated to be pure by HPLC, capillary electrophoresis, and ^{1}H NMR.[21] Each HA oligosaccharide was persulfated using the same method used for the intact GAGs, and the products were analyzed by capillary electrophoresis and gradient PAGE for purity, following hydrolysis for sulfate content and by ^{1}H NMR.[15,16] While the persulfated HA oligosaccharides bound significantly more weakly than did the intact persulfated HA, the larger persulfated HA oligosaccharides bound nearly as tightly as heparin, assessed by both competitive and direct binding assays. The strength of this interaction increased with oligosaccharide size from dp 10 through dp 20. Persulfated HA, while polydisperse, has an average molecular weight of 184 kD corresponding to a dp of 500. Thus, it is clear from these data that much larger persulfated HA oligos than those currently accessible will be required to obtain binding affinity comparable to that observed for the persulfated GAGs.

SPR provides information not only about the overall strength of binding but also about the binding kinetics, the on-rate (k_{assoc}) and off-rate (k_{dissoc}). Thus, it is most informative to compare the kinetics of binding for heparin (the model for the natural ligand, highly sulfated heparan sulfate), the most tightly interacting polysaccharides (persulfated GAGs), the most tightly interacting oligosaccharide (persulfated HA ecosisaccharide), and the most tightly interacting small polyanion (Suramin). The persulfated GAGs and persulfated HA oligosaccharides both had relatively slow on-rates of from 10^2 to 10^3 M^{-1} s^{-1} but had extremely slow off-rates 10^{-5} s^{-1}. In contrast, both heparin and Suramin had fast on-rates 10^5 M^{-1} s^{-1} but had slow off-rates 10^{-2} s^{-1}. The conformational flexibility of the heparin and the Suramin might permit these molecules to more easily adopt a productive conformation for interaction with the envelope protein than the more conformationally constrained persulfated GAGs and oligosaccharides.[21] Similarly, the more highly sulfated persulfated GAGs and oligosaccharides (4−5 sulfates/disaccharide repeat) once bound may be released much more slowly than the less highly sulfated heparin (2.7 sulfates/disaccharide repeat) and Suramin, due to additional ion-pairing or hydrogen bonding contacts with envelope protein.[4] It is important to note that sulfation density and flexibility are not sufficient for tight interaction, as the highly flexible[16] sucrose octasulfate, which contains 8 sulfate groups/disaccharide, does not interact at all with envelope protein. These data suggest a structure−activity relationship in which three factors control the strength of polyanion interaction: (1) a minimum size of approximately 39 Å; (2) a high charge density; and (3) a high level of structural flexibility. Design of new agents for testing as inhibitors of dengue virus binding and infectivity will need to focus on optimization of these three structural features.

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