Characterization of Interactions between Heparin/ Glycosaminoglycan and Adeno-Associated Virus

Fuming Zhang,† Javier Aguilera,‡ Julie M. Beaudet,¶ Qing Xie,‖ Thomas F. Lerch,‖ Omar Davulcu,‖ Wilfredo Colón,‡ Michael S. Chapman,*,‖ and Robert J. Linhardt*,†‡§

ABSTRACT: Adeno-associated virus (AAV) is a key candidate in the development of gene therapy. In this work, we used surface plasmon resonance spectroscopy to study the interaction between AAV and heparin and other glycosaminoglycans (GAGs). Surface plasmon resonance results revealed that heparin binds to AAV with an extremely high affinity. Solution competition studies showed that binding of AAV to heparin is chain length-dependent. AAV prefers to bind full chain heparin. All sulfo groups (especially N-sulfo and 6-O-sulfo groups) on heparin are important for the AAV–heparin interaction. Higher levels of sulfo group substitution in GAGs enhance their binding affinities. Atomic force microscopy was also performed to image AAV-2 in a complex with heparin.

Adeno-associated virus (AAV) is a small DNA-containing human parvovirus that is a leading candidate vector for in vivo human gene therapy.¹ Gene therapy involves the delivery of genes into cells for the treatment or prevention of a disease, supplying a replacement for a faulty or missing gene, or sensitizing cells to (cancer) prodrugs.² The development of gene therapy currently centers mostly on basic science, but early clinical successes include demonstrated long-term efficacy in experimental treatments for retinopathies and hemophilia B.³ Viruses are the usual choice for gene delivery as they have naturally evolved for this purpose. The examples mentioned above show that among several viruses that have been engineered for gene therapy, AAV is increasingly prominent, considered a relatively safe vector for moderately sized genes of <5 kb. AAV is much less immunogenic than adenoviruses (no relation), and less oncogenic than retroviruses, because they transduce cells episomally or integrate with partial site specificity.¹⁶⁻⁹ A lack of understanding of the tropism and cell specificity of AAV has impeded its application in gene therapy.

In vivo, multiple factors determine AAV tissue tropism, including its traversal of the endothelium, immune neutralization, and blood clearance.¹⁰⁻¹³ Interactions with host cell molecules, starting with the primary receptor, are important both in vivo and in vitro. Cell entry for AAV starts with attachment to extracellular glycoconjugates followed by binding to one or more membrane-anchored coreceptors (or secondary receptors) prior to endosomal entry.¹⁴⁻²⁰ Virus–glycan interactions, involving glycoproteins, glycolipids, or proteoglycans (PGs), figure prominently in the cell entry of many viruses.²¹ However, the importance of these virus–glycan interactions varies, particularly with respect to their role in conferring cell and tissue specificity.²²⁻²⁶ Multiple lines of evidence implicate an important role of glycans in AAV attachment, including the effect of mutations at the glycan binding site on both in vitro cell specificity and in vivo tissue tropism.¹¹,²⁷⁻³² AAV serotypes have different preferences for glycans in their attachment. AAV-2 and AAV-3 are thought to use heparan sulfate (HS) PGs as the primary receptor in cell attachment.³³,³⁴ These serotypes, as well as AAV-6 to lesser extent, bind to heparin, a commonly used HSPG analogue.³⁵ In vivo, AAV-6 has a preference for glycans terminated with sialic acid, as do AAV-1, -4, and -5,³⁷⁻³⁹ whereas AAV-9 binds to a galactose-terminated glycans.³⁵,³⁶ Heparin/HS belongs to the glycosaminoglycan (GAG) family of anionic, and often highly sulfated, complex polydisperse linear polysaccharides. An increasing number of GAG-binding proteins have been reported in the literature.⁴¹ A number of diverse pathophysiological processes are mediated through the interaction between heparin/HS and proteins, including blood coagulation, cell growth and differentiation, host defense and viral infection, lipid transport and metabolism.
cell-to-cell and cell-to-matrix signaling, inflammation, and cancer.\textsuperscript{41–44} Generally, an understanding of heparin/HS–virus particle interactions at the molecular level is of fundamental importance to biology and will aid in the development of highly specific glycan-based therapeutic agents.\textsuperscript{41,45} For AAV, it is hoped that an improved understanding of GAG interactions will eventually allow more exquisite and rational modulation of cell attachment, transduction, and tissue tropism, beyond the gross changes demonstrated with chimeric transducing vectors.\textsuperscript{30,35,36}

Heparin has been used as a model GAG in the study of HS–protein interaction, because it mimics the interactions of proteins with the HS present on cell surfaces and in the extracellular matrix.\textsuperscript{21} The goal of this study is to analyze molecular interactions of heparin/GAGs with adeno-associated virus (AAV). Three different variants were used: natural human serotypes AAV-2 and AAV-6 and a chimeric gene-shuffled recombinant, AAV-DJ, which has been developed as a hepatotropic gene therapy vector. A BIAcore 3000 system was used to measure the strength of AAV–GAG interactions by surface plasmon resonance (SPR). Atomic force microscopy was also performed to image the complexes from AAV-2–heparin interaction.

\section*{EXPERIMENTAL PROCEDURES}

\textbf{Materials.} The GAGs used were porcine intestinal heparin (16 kDa), low-molecular weight (LMW) heparin (4.8 kDa), and porcine intestinal heparan sulfate (12 kDa) (Celsius Laboratories, Cincinnati, OH), chondroitin sulfate A (20 kDa) from porcine rib cartilage (Sigma, St. Louis, MO), dermatan sulfate (also known as chondroitin sulfate B, 30 kDa, from porcine intestine, Sigma), dermatan disulfate (4,6-disulfo DS, 33 kDa, Celsius) prepared through the regioselective chemical 6-O-sulfonation of dermatan sulfate,\textsuperscript{16} chondroitin sulfate C (20 kDa, from shark cartilage, Sigma), chondroitin sulfate D (20 kDa, from whale cartilage, Seikagaku, Tokyo, Japan), chondroitin sulfate E (20 kDa from squid cartilage, Seikagaku), and hyaluronic acid sodium salt (100 kDa, from Streptococcus zooepidemicus, Sigma). Fully desulfated heparin (14 kDa), N-desulfated heparin (14 kDa), and 2-O-desulfated IdoA heparin (13 kDa) were all prepared using the method of Yates et al.\textsuperscript{37} 6-O-Desulfated heparin (13 kDa) was a generous gift from L. Wang (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Heparin oligosaccharides included disaccharide [degree of polymerization of 2 (dp2)], tetrasaccharide (dp4), hexasaccharide (dp6), octasaccharide (dp8), decasaccharide (dp10), tetradecasaccharide (dp14), hexadecasaccharide (dp16), and octadecasaccharide (dp18) and were prepared from the controlled partial heparin lyase 1 treatment of bovine lung heparin (Sigma) followed by size fractionation. Chemical structures of these GAGs and heparin oligosaccharides are shown in Figure 1. Sucrose octasulfate (SOS) was from Toronto Biochemicals (Toronto, ON). Sensor SA chips were from GE Healthcare (Uppsala, Sweden). SPR measurements were performed on a BIAcore 3000 system operated using BIAcore 3000 control and BIAnalysis software (version 4.0.1). Atomic force microscopy was conducted on an MFP-3D atomic force microscope (Asylum Research, Santa Barbara CA) using Asylum Research version 6.22. AFM scans were performed with 2 nm (±1 nm) Super Sharp Silicon-NCLR (Non-Contact Long-cantilever Reflex-Coating) AFM cantilevers (Nano and More, Soquel, CA) on highest-grade (V1) mica sheets (Ted Pella, Redding, CA). Milli-Q water was degassed and refiltered using 0.22 μm cellulose acetate membranes (Millipore, Billerica, MA).

\textbf{AAV Expression and Purification.} The production of AAV-2 and AAV-DJ virus-like particles (VLPs) in SF9 cells

Figure 1. Chemical structures of heparin, heparin-derived oligosaccharides, and GAGs.
followed methods developed for AAV-2 that used the Bac-to-Bac Baculovirus Expression Vector System (Invitrogen) and were adapted for AAV-DJ as previously described. VLPs are devoid of infectious viral DNA, but the outer protein capsids appear to be structurally and functionally identical to the wild-type virus. These empty capsids were purified by three rounds.

Figure 2. SPR sensorgrams of the AAV-2—heparin interaction (top). Concentrations of AAV were 5, 2.5, 1.25, 0.63, and 0.32 nM (from top to bottom, respectively). SPR sensorgrams of the AAV-6—heparin interaction (middle). Concentrations of AAV were 10, 5, 2.5, 1.25, and 0.63 nM (from top to bottom, respectively). SPR sensorgrams of the AAV-DJ—heparin interaction (bottom). Concentrations of AAV were 1.25, 0.63, 0.32, 0.16, and 0.08 nM (from top to bottom, respectively).
of CsCl density gradient ultracentrifugation, followed by heparin affinity chromatography, eluting with a NaCl gradient. AAV-6 was prepared in HeLa cells as infectious virions and purified by three rounds of CsCl gradient ultracentrifugation, all using protocols that have been described previously. All samples were dialyzed into phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$ (pH 7.4)].

Preparation of the Heparin Biochip. The biotinylated heparin was prepared by reaction of sulfo-N-hydroxysuccinimide long chain biotin (Piez, Rockford, IL) with the free amino groups of unsubstituted glucosamine residues in the polysaccharide chain, following a published procedure. The biotinylated heparin was immobilized to a streptavidin (SA) chip using the manufacturer's protocol. In brief, a 20 μL solution of the heparin–biotin conjugate (0.1 mg/mL) in HBS-EP running buffer was injected over flow cell 2 (FC2) of the SA chip, at a flow rate of 10 μL/min. The successful immobilization of heparin was confirmed by the observation of an ∼50 resonance unit (RU) increase in the sensor chip. The control flow cell (FC1) was prepared by a 1 min injection with saturated biotin.

Measurement of the Interaction between Heparin and AAV Using BIAcore SPR. The protein samples were diluted in HBS-EP buffer [0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (pH 7.4)]. Different dilutions of protein samples were injected at a flow rate of 30 μL/min. At the end of the sample injection, the same buffer was passed over the sensor surface to facilitate dissociation. After dissociation for 3 min, the sensor surface was regenerated with 30 μL of 0.5% SDS and then with 2 M NaCl to yield a fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C.

Inhibition of Heparin Binding by Oligosaccharides. AAV-2 at 8.3 pM (equivalent to capsid protein at 0.5 nM) was mixed with 1000 nM heparin oligosaccharides, including disaccharide (dp2), tetrasaccharide (dp4), hexasaccharide (dp6), octasaccharide (dp8), decasaccharide (dp10), tetradecasaccharide (dp14), hexadecasaccharide (dp16), and octadecasaccharide (dp18), all in HBS-EP buffer. Samples were injected over the heparin chip at a flow rate of 30 μL/min. After each run, dissociation and regeneration were performed as described above. For each set of competition experiments on SPR, a control experiment (target without ligand) was performed to make sure the surface was completely regenerated and that the results obtained between runs were comparable.

Inhibition of Heparin Binding by GAGs and Chemically Modified Heparin. AAV-2 at 8.3 pM was premixed with 100 nM GAG or chemically modified heparin. Otherwise, SPR was performed as described above.

Atomic Force Microscopy. AAV-2 was combined with 100 nM GAG and incubated at 22 °C for 10 min. A 20 μL aliquot was spotted on a sheet of immobilized mica and allowed to set for 1 h before being washed with Milli-Q water. The sample was dried for 6 h. Raster images were obtained using 10 μm squares, and a representative area was rescaned at a higher resolution with 2 μm squares. Using Asylum Research version 6.22, particle heights were obtained for the 10 μm squares in representative sections.

### RESULTS

**Kinetics of the AAV–Heparin Interactions.** The binding to immobilized heparin was measured for the three virions (AAV-2, AAV-6, and AAV-DJ) using SPR. The SPR binding sensorgrams of the AAV-2–heparin, AAV-6–heparin, and AAV-DJ–heparin interactions are displayed in Figure 2 showing different binding profiles. The sensorgrams were fit globally to obtain apparent on ($k_a$) and off ($k_d$) rates for the binding equilibrium (Table 1), using the BiaEvaluation software and assuming a 1:1 Langmuir model.

<table>
<thead>
<tr>
<th>interaction</th>
<th>$k_a$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-2–heparin</td>
<td>1.2 × 10$^7$</td>
<td>1.2 × 10$^{-3}$</td>
<td>1.0 × 10$^{-10}$</td>
</tr>
<tr>
<td>AAV-6–heparin</td>
<td>1.61 × 10$^4$</td>
<td>0.0197</td>
<td>1.22 × 10$^{-8}$</td>
</tr>
<tr>
<td>AAV-DJ–heparin</td>
<td>4.3 × 10$^7$</td>
<td>4.6 × 10$^{-3}$</td>
<td>1.1 × 10$^{-10}$</td>
</tr>
</tbody>
</table>

Polymer Length. Relative avidity, determined through SPR measurements of AAV-2–heparin binding inhibited by different oligosaccharides, was used to establish the dependence of binding on saccharide chain length. These heparin-derived oligosaccharides ranged from disaccharides to octadecasaccharides (dp2 to dp18). The extent of competitive inhibition increases in two steps, once near a chain length of four saccharide units, and the other at 16 saccharide units (Figure 3). This can be rationalized if the binding sites on the side of each spike are approximately five saccharides in length, and if a chain length of 16 saccharide units is the minimum for a polysaccharide to bind to two neighboring sites on spikes related by one of the viral 3-fold axes of symmetry.

Specificity for Different GAGs. The SPR competition assay was also utilized to determine the preferences of AAV-2 in binding to various GAGs (Figure 1). SPR competition sensorgrams and inhibition levels are displayed in Figure 4. The results showed that CSE provided the strongest inhibitory effect (70–80%) in terms of binding of AAV-2 to immobilized heparin. DiS-DS also inhibited binding by ∼40%. Other GAGs and SOS (as a non-GAG polyanion control) had little effect. The data suggest that the binding interactions are dependent on GAG structure and strongly influenced by the level of GAG sulfation.

Chemically Modified Heparins. SPR competition sensorgrams and inhibition profiles with chemically modified heparins are displayed in Figure 5 for AAV-2. Inhibitory activity is lost upon all types of desulfation (fully desulfated heparin, N-desulfated heparin, 2-O-desulfated heparin, and 6-O-desulfated heparin). However, with 2-O-desulfated heparin, the extent of the loss of inhibition is much smaller with AAV-2. This implies that N-sulfo groups and 6-O-sulfo groups are more critical than 2-O-sulfo groups to the AAV-2–heparin binding interaction.

**AFM Analysis.** In the absence of heparin oligosaccharides, AFM shows a field of approximately round particles of AAV-2 with heights in the range of 15–30 nm (Figure 6). This is consistent with particle heights reported earlier by Chen and the observation that empty particles were measured with somewhat reduced diameters because of deformation under the ATM tip. In the presence of heparin oligosaccharides, the average particle size increases with chain length, indicating aggregation (Table 2 and Figure 6). Even at dp6, some larger particles are observed, suggesting a limited ability to cross-link. There is a marked increase in both the average size and the frequency of larger aggregates in dp14, dp16, and full-length heparin. The large particles 60–90 nm in height seen with dp16 oligosaccharides and dominant with heparin correspond to
several particle diameters and indicate the improving ability of multiple virions to be bound along the GAG chain and of each particle to host interactions with multiple GAGs, presumably at binding sites related by icosahedral symmetry.

**Molecular Modeling.** An extended chain model of \( \beta-D\text{-GlcA-(1,4')}\)-\(\alpha-D\text{-GlcNAc-(1,4')}\)_8 was generated using the GLYCAM Web tool (http://www.glycam.com). This was fit into the difference density obtained by subtracting the cryo-electron microscopy (cryo-EM) reconstruction of AAV-2 from that of the AAV-2–heparin complex at 8.3 Å resolution. Coot was used for computer-aided manual fitting of the model into the difference density contoured at 6σ. Stereochemical distortions, introduced during modeling, were corrected through molecular mechanics energy minimization using NAMD. Before final energy minimization with NAMD and checking of stereochemical parameters, close contacts with the virus were resolved and the fit to density was improved using Coot. Figure 7 was generated using PyMol.

**DISCUSSION**

Through SPR, we now have fully quantitative measures of the avidity of AAV for heparin. Prior quantitation used the proxy of
NaCl concentration needed to elute AAV from a heparin affinity column for rank ordering. Heparin affinity elution had shown that, of natural serotypes, AAV-2 binds strongest and AAV-6 weakest of those measurable. SPR is consistent with this and now shows a 100-fold difference in avidity (Table 1). AAV-DJ is a chimeric combination of AAV-2, -8, and -9 created by random gene shuffling, followed by selection for liver cell transduction and escape from human polyclonal neutralizing sera. There are several potential causes for the improved in vivo (mouse) hepatotropism, of which altered attachment has been considered a possibility. SPR, like heparin affinity and cell binding, indicates weakened binding relative to that of AAV-2. Quantitatively, the SPR-derived 10% difference in the apparent $K_D$ (Table 1) is consistent with the structures of AAV-2 and AAV-DJ being very similar in the heparin-binding region, and differing in calculated electrostatic potential only on the periphery of the binding site. A 10% difference in binding avidity is unlikely to account, through cell attachment alone, for >10-fold differences in in vitro transduction efficiencies and in vivo tissue tropism. Suspicion then naturally falls on the region of the structure where there are the greatest differences, the external loop that constitutes variable region 1. Given that this is the center of the AAV-2 epitope for neutralizing monoclonal antibody A20, it is likely that structural differences resulted here from AAV-DJ’s stringent selection by escape from neutralizing sera. It is possible that changed tissue tropism in vivo results from altered immune clearance, sequestration, or other effects. As for changes to transduction in

![Figure 4](https://dx.doi.org/10.1021/bi4008676 | Biochemistry 2013, 52, 6275–6285)
vitro, quantitatively similar dissociation constants draw attention away from initial attachment to speculation that selection of neutralization escape variants may have serendipitously altered coreceptor binding, if neutralizing epitopes are well-represented at currently uncharacterized coreceptor binding sites.

The dependence of AAV-2−GAG interactions on chain length can be interpreted in light of the cryo-EM structure of AAV-2 complexed with a 17 kDa fragment of heparin (Figure 7). The heparin in the cryo-EM visualization lacks molecular detail, because of the modest 8 Å experimental resolution, and because we see the average of heterogeneous sequences that are bound to the thousands of particles imaged. The virus structure in the complex is better defined, because the 3 Å resolution crystal structure can be docked as a rigid body by superimposing the icosahedral symmetry. The maximal heparin density, implying the tightest binding, is adjacent to Arg and Arg. Strong density extends about the length of a pentasaccharide to cover other positively charged residues implicated by mutagenesis in heparin binding: Arg, Arg, and Lys as well as Lys. With short oligosaccharides, Figure 5B shows progressively stronger binding, until

Figure 5. Sensorgrams for the AAV-2−heparin interaction inhibited by heparins that have been chemically desulfonated in various ways. The AAV-2 concentration was 0.5 nM, and concentrations of chemically modified heparins were 100 nM. Bar graphs (errors from triplicate experiments) showing relative levels of AAV-2 binding in the presence of GAGs with different sulfonation patterns.
presumably all the main interactions of a single site on AAV are contributing. The next big change comes between dp14 and dp16, which might correspond to the chain length required for a change in avidity with the contribution of two symmetry-related sites on the virus. Density bridging between adjacent sites in the EM reconstruction is approximately half the maximal height, implying looser association, but still experimentally significant at 6σ. Modeling a canonical glycosaminoglycan through the path of this density shows that a minimal chain length of 13 is required to make contacts with two symmetry-related copies of Lys\textsubscript{532} (Figure 7). Intriguingly, density continues away from the 3-fold axis in each direction such that a 16-mer can be fit into the EM density. This indicates continuing GAG–virus interactions beyond Lys\textsubscript{532}. It may be that the stronger binding of dp16 evident in Figure 5B and the length of the density seen in the EM reconstruction are both consequences of two-site attachment becoming optimal with a chain length of at least 15. Some caution is warranted,

### Table 2. Description of Particle Heights Observed via AFM on AAV-2–Heparin Oligosaccharide Complexes

<table>
<thead>
<tr>
<th></th>
<th>predominant particle size range</th>
<th>minor particle morphologies</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-2 only</td>
<td>15–30 nm</td>
<td></td>
</tr>
<tr>
<td>AAV-2–dp6</td>
<td>15–30 nm</td>
<td>30–80 nm</td>
</tr>
<tr>
<td>AAV-2–dp14</td>
<td>15–30 nm</td>
<td>30–80 nm</td>
</tr>
<tr>
<td>AAV-2–dp16</td>
<td>15–60 nm</td>
<td>60–90 nm</td>
</tr>
<tr>
<td>AAV-2–heparin</td>
<td>60–90 nm</td>
<td>15–30 nm</td>
</tr>
</tbody>
</table>

![Figure 6. AFM images of AAV-2 and complexes with different heparin oligosaccharides.](image)
of any of the serotypes, the inhibition patterns for heparins desulfated at specific locations begin to offer guiding principles. The presence of sulfo groups at the N- and 6-O-positions is more critical than at the 2-O-position. Such information might support the first step in an interactive process through which one might hone in on heparin oligomers with stronger binding and inhibitory activity, and thereby advance our understanding of AAV attachment by combining structural and virological studies.

**REFERENCES**


Biochemistry

Article

6284 dx.doi.org/10.1021/bi4008676 | Biochemistry 2013, 52, 6275–6285
biochemistry interactions.

Identification of the galactose binding domain of the AAV9 capsid. J. Virol. 86, 7326−7333.


