

Elucidating the Mechanism of Recognition and Binding of Heparin to Amyloid Fibrils of Serum Amyloid A

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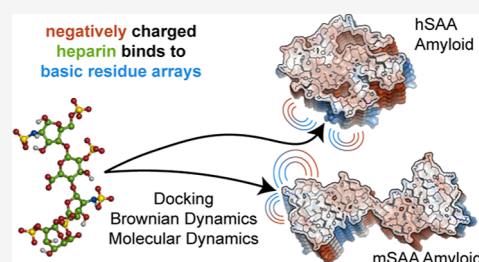
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ABSTRACT: Amyloid diseases feature pathologic deposition of normally soluble proteins and peptides as insoluble fibrils in vital organs. Amyloid fibrils co-deposit with various nonfibrillar components including heparan sulfate (HS), a glycosaminoglycan that promotes amyloid formation in vitro for many unrelated proteins. HS–amyloid interactions have been proposed as a therapeutic target for inflammation-linked amyloidosis wherein N-terminal fragments of serum amyloid A (SAA) protein deposit in the kidney and liver. The structural basis for these interactions is unclear. Here, we exploit the high-resolution cryoelectron microscopy (cryo-EM) structures of ex vivo murine and human SAA fibrils in a computational study employing molecular docking, Brownian dynamics simulations, and molecular dynamics simulations to elucidate how heparin, a highly sulfated HS mimetic, recognizes and binds to amyloid protein fibrils. Our results demonstrate that negatively charged heparin chains bind to linear arrays of uncompensated positively charged basic residues along the spines of amyloid fibrils facilitated by electrostatic steering. The predicted heparin binding sites match the location of unidentified densities observed in cryo-EM maps of SAA amyloids, suggesting that these extra densities represent bound HS. Since HS is constitutively found in various amyloid deposits, our results suggest a common mechanism for HS–amyloid recognition and binding.



INTRODUCTION

Amyloid diseases, including Alzheimer's, Parkinson's, and other diseases such as inflammation-linked amyloid-A (AA) amyloidosis, afflict hundreds of millions of patients worldwide, yet they currently have limited treatment options and no cure.¹ In these diseases, normally soluble proteins or protein fragments misfold, aggregate, and deposit extracellularly as insoluble amyloid fibrils.^{1,2} Amyloid deposition in vital organs (e.g., brain, heart, kidney, liver) can lead to organ damage and, if untreated, death.^{1,3} Over 40 different proteins have been identified that form pathologic amyloid fibrils in humans.² In addition to the fibrillar protein, all amyloid deposits also contain glycosaminoglycans (GAGs), lipids, apolipoproteins, and other nonfibrillar components, which can influence amyloid deposition and biological properties in ways that are just beginning to be understood.^{2,4–8} Identifying the structural basis for the interactions of various ligands with amyloid fibrils may help better understand the biological properties of amyloids, the mechanisms of amyloid formation, factors that promote or prevent this process, and, ultimately, help guide therapeutic targeting of amyloid diseases.

Since 2017, hundreds of atomic structures of amyloid fibrils have been determined by cryogenic electron microscopy (cryo-EM), including patient-derived amyloids. The “resolution revolution” in cryo-EM⁹ facilitates this endeavor. These structures often contain extra densities,^{7,10–12} some of which

may represent tightly bound ligands not removed during fibril isolation from tissues. A striking example is collagen-VI fiber bound alongside patient-derived light chain amyloid fibril in a superhelical assembly.¹³ Furthermore, α -synuclein fibrils grown in vitro in the presence of lipids or heparin clearly show these bound ligands in the cryo-EM maps.^{12,14} Heparan sulfate (HS), a highly sulfated GAG that codeposits with SAA and many other protein amyloids, accelerates amyloid formation by many unrelated proteins^{7,14–21} including SAA,^{22–24} suggesting that it stabilizes common structural motives in amyloids.^{7,14,21,25,26} In this study, we harness cryo-EM structures of ex vivo fibrils of serum amyloid A (SAA), the protein precursor of AA amyloidosis, to explore how HS recognizes and binds to amyloid fibrils.

All amyloid fibrils adopt a common architecture featuring flattened protein molecules assembled into stacks of parallel in-register β -sheets running perpendicular to the fibril axis and stabilized by intermolecular hydrogen bonds.^{27,28} In this

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repeating fibril topology, arrays of identical side chains spaced at approximately 4.8 Å run along the spine of the fibril. For charged residues, the arrays colocalize uncompensated charge that may slow amyloid formation and destabilize amyloid fibrils. Consistent with this view, binding of counterions is expected and observed to accelerate amyloid formation for many unrelated proteins,^{7,14,21,29} perhaps via scaffolding.^{7,26,27,30} We proposed that periodic polyanions with charge and geometry complementary to that of amyloids can bind to basic residue arrays along the fibril spines, thereby promoting amyloid nucleation and stabilizing the growing fibril via scaffolding.⁷ This conjecture resulted from molecular docking of heparin to more than 50 different fibril structures determined by cryo-EM for various tissue-extracted amyloids; all these structures contained unidentified extra densities, and the predicted HS binding sites invariably overlapped some of these extra densities.⁷ Our prediction was later verified experimentally for α -synuclein amyloid fibrils grown in vitro in the presence of heparin; cryo-EM structure of these fibrils showed extra density corresponding to heparin bound along the amyloid fibril spine and coordinated by ladders of basic residues.¹⁴

HS is a key component of the extracellular matrix that is found in most patient-derived amyloid deposits.^{31,32} HS is a biopolymer containing linear chains of glucosamine and uronic acid disaccharide repeats differentially modified by deacetylation, sulfation, and epimerization.³³ These flexible chains contain segments of high sulfation spanning 14–16 monosaccharide units, linked by segments of low sulfation.³⁴ HS chains form helices in solution and have negative charges spaced approximately 5 Å apart, which is compatible with the 4.8 Å spacing in amyloid fibrils.³⁵ Heparin, whose molecular structure represents highly sulfated HS regions,³³ is often used as a shorter mimetic of highly sulfated HS regions in experimental studies in vitro. These studies have established HS/heparin as a potent amyloid agonist,^{18,20,26,30,36,37} even for peptides that have a low intrinsic amyloidogenicity.³⁸ Thus, HS–fibril interactions may be a potential therapeutic target for amyloid diseases.

This concept has been particularly well established for AA amyloidosis,^{22–24} a major complication of chronic inflammation in diseases such as tuberculosis, rheumatoid arthritis, certain cancers, or Crohn's disease.^{39–42} AA amyloidosis is caused by the extracellular deposition of N-terminal fragments of SAA (termed AA) mainly in kidney, liver and spleen, leading to organ failure if untreated.^{43,44} SAA (12 kDa) is an evolutionarily conserved lipid-binding protein systemically elevated in inflammation; chronically elevated plasma levels of SAA (3–10 fold)⁴⁵ are associated with AA amyloidosis.⁴⁴ In a mouse model of AA amyloidosis, overexpression of HS accelerated the disease. Conversely, overexpression of heparanase that cuts HS chains into short fragments was protective, leading to the proposal that interactions of HS with SAA in its native conformation or in the form of amyloid provide a therapeutic target for AA amyloidosis.²⁴ However, the HS binding site on SAA was unknown. A small-molecule HS mimetic, Kiacta, was proposed to block SAA–HS interactions in AA amyloidosis⁴⁶ but failed to show efficacy in phase-III clinical trials,⁴⁷ perhaps because this disulfonate molecule was too small to compete with long HS chains for binding to amyloid. This highlights the need for a detailed understanding of amyloid–HS interactions before attempting their therapeutic targeting.

In this study, we used molecular docking, Brownian dynamics simulations, and molecular dynamics (MD) simulations to elucidate how heparin/HS recognizes and binds to SAA amyloid fibrils. To this end, we harnessed cryo-EM structures of ex vivo amyloid fibrils of murine SAA1 (mSAA) and human SAA isoform 1.1 (hSAA), which are the major amyloid-forming protein isoforms in mice and humans. For cryo-EM studies, the fibrils were isolated from murine spleen or human kidney using an extraction procedure that employs collagenase and avoids harsh detergents or high salt concentrations and preserves the fibril morphology.^{48,49} The results of our computational studies are supported by experimental evidence including extra densities observed in the cryo-EM maps. These results demonstrate how electrostatic interactions drive heparin/HS binding to basic residue arrays along the fibril site, and how structural polymorphism of amyloid fibrils influences the binding. While the computational exploration of glycans binding to globular proteins has been covered and reviewed previously,^{50–52} we believe this to be the first study exploring the direct binding of heparin to an amyloid fibril through a multiscale approach.

METHODS

Unless stated otherwise, system setup and analysis was done in Python 3 and made use of the NumPy,⁵³ SciPy,⁵⁴ and MDAnalysis packages.⁵⁵ Figures were created in Matplotlib⁵⁶ and PyMOL.⁵⁷ Molecular mechanics Poisson–Boltzmann surface area (MMPBSA) calculations were performed using gmx_MMPBSA⁵⁸ and are described in Supplemental Methods II.

Molecular Docking. We used the heparin mode of ClusPro to dock a heparin tetrasaccharide structure to the fibril structures of ex vivo mSAA and hSAA amyloids (PDB IDs: 6DSO and 6MST, resolution 3.0 and 2.7 Å, respectively).⁴⁸

ClusPro uses fast Fourier transform algorithms to determine the most likely clusters of binding for two partners in a three-step process.⁵⁹ First, it uses rigid body docking to exhaustively sample billions of conformations for the fixed protein and mobile ligand structures using the PIPER docking program.^{59,60} Next, ClusPro uses root-mean-square deviation (RMSD) values to cluster the 1000 lowest-energy structures into the most likely complexes, returning up to 30 complexes that each represent a cluster.⁶⁰ For the heparin mode, the interaction energy is calculated as $E = w_1 E_{\text{rep}} + w_2 E_{\text{attr}} + w_3 E_{\text{elec}}$. The first two terms are the repulsive and attractive van der Waals contributions describing shape complementarity, and the third is electrostatic contribution. Lastly, these representative structures are refined by energy minimization using a fixed backbone for the protein while the heparin tetrasaccharide is allowed to be fully flexible.⁶⁰ Since the ranking of the results is based on cluster population rather than energy score, the results provide no information on binding free energy.

For heparin docking to the mSAA amyloid, no modifications were made to the fibril structure. For docking to the hSAA fibril, we blocked the central cavity, which was too small to accommodate heparin but was predicted to be a heparin binding site in the initial round of docking because of ClusPro's apparent limited ability to handle steric clashes.

Fibril Models for Brownian Dynamics and Molecular Dynamics. The 6-layer cryo-EM fibril structures of hSAA and mSAA were obtained from the protein databank.⁴⁸ The CHARMM-GUI pdb reader and manipulator was used to

add hydrogens to and to neutralize the C-terminal ends of each peptide in the fibrils' cores (their disordered segments were not included).⁶¹ Additional layers were then added to the fibrils to provide an ample surface for the heparin decasaccharide. The positions of each atom in the existing layers were used to predict the atomic positions in the next layer, according to

$$p_{N_l+1} = \frac{1}{N_l - 2}[(2N_l - 3)p_{N_l} - (N_l - 1)p_{N_l-1} + p_1 - p_2] \quad (1)$$

where p_l is the position of a given atom in layer l and N_l is the total number of existing layers in the fibril. The last existing layer in the fibril was then RMSD-fit to the predicted positions using the Kabsch algorithm.⁶² This process was repeated to extend each fibril to 12 layers.

Each fibril model was solvated using the TIP3P water model and 0.15 mol NaCl and simulated in GROMACS 2020.1 using the CHARMM36m force field at 310.15 K.^{63–65} A dodecahedron box was employed with a minimum of 2 nm on either side of the fibril to prevent interperiodic self-contacts in the case of any delamination of the terminal fibril layers. After energy minimization, the system was equilibrated for 5 ns in the canonical ensemble using the velocity rescaling thermostat and with spherical position restraints applied to the backbone atoms (force constant of 400 kJ mol^{−1} nm^{−2}) and the side chain atoms (force constant of 40 kJ mol^{−1} nm^{−2}).⁶⁶ The system was then equilibrated for 5 ns in the isobaric–isothermal ensemble at 1 bar using the velocity rescaling thermostat and the Berendsen barostat with a compressibility of 4.5×10^{-5} bar^{−1}.^{66,67} The same spherical position restraints were applied to the backbone and side chain atoms. Next, the system was equilibrated in the isobaric–isothermal ensemble at 1 bar with the Nose–Hoover thermostat and the Parrinello–Rahman barostat with a compressibility of 4.5×10^{-5} bar^{−1} with no position restraints for 5 ns.^{68–70} Each equilibration step employed a 1 fs time step. Finally, two replicate simulations of each model were performed for 500 ns in the isobaric–isothermal ensemble at 1 bar using the Nose–Hoover thermostat and the Parrinello–Rahman barostat.^{68–70} For this production simulation, a 2 fs time step was used.

Heparin Models for Brownian Dynamics and Molecular Dynamics. The heparin decasaccharide molecule, 2-O-sulfo- α -L-iduronic acid-[1 $\rightarrow$ 4]-2-deoxy-2-sulfamido- α -D-glucopyranosyl-6-O-sulfate (α -L-IdoA(2S)-[1 $\rightarrow$ 4]- α -D-GlcNS(6S)), was constructed using the CHARMM-GUI glycan reader and modeler.^{71,72} The heparin molecule was solvated using the TIP3P water model and 0.15 mol NaCl in a cubic box with a minimal space of 1.5 nm on each side to prevent interperiodic self-contact, and was simulated in GROMACS 2020.2 with the CHARMM36m force field.^{63,65} This force field has been validated for similar glycans in the past.⁷³ After energy minimization, the system was equilibrated in the canonical ensemble using the velocity rescaling thermostat.⁶⁶ During equilibration, the glycan rings were restrained using a spherical potential with a 400 kJ mol^{−1} nm^{−2} force constant, all other atoms were restrained using a spherical potential with a 40 kJ mol^{−1} nm^{−2} force constant, and all glycan ring dihedrals were restrained with a force constant of 4 kJ mol^{−1} nm^{−2}. Next, the system was equilibrated in the isobaric–isothermal ensemble at standard pressure using the velocity rescaling thermostat and the Berendsen barostat with a

compressibility of 4.5×10^{-5} bar^{−1}.^{66,67} Both equilibration steps were performed at 310.15 K for 2 ns with a 1 fs time step. Finally, the system was simulated in the same isobaric–isothermal ensemble at 310.15 K and standard pressure for 1 μ s using a 2 fs time step.

Brownian Dynamics of Heparin–Fibril Binding. The frames from the MD trajectory of the fibrils and heparin were clustered using agglomerative clustering with Ward's minimum variance metric.⁷⁴ All clustering was completed in Julia using the Clustering.jl package.⁷⁵ For hSAA, this was done for two sets of atomic positions for the CZ atom of R39 and for the NZ atom of R46. For mSAA, this was done based on the positions of the CZ atom R38. For heparin, the clustering was done based on the positions of the sulfur atoms. This approach was used to sample a broad selection of conformations across the species' conformational ensembles. Based on the silhouette scores, we ultimately selected 6 clusters for each clustering of hSAA, 16 clusters for mSAA, and 13 clusters for heparin.⁷⁶ The medoid frame in each cluster was used as an input structure for the Brownian dynamics simulation.

For each input structure, electrostatics were computed with the Adaptive Poisson–Boltzmann Solver (APBS).⁷⁷ For this, the radii and charges of each atom were set in accordance to the CHARMM36m force field. The calculations were performed at 310.15 K with a solvent dielectric constant of 78, a solute dielectric constant of 4, a solvent radius of 1.4 Å, and an ion concentration set according to 1.5 mol NaCl (cation: +1 charge, 1.41075 Å radius; anion: −1 charge, 2.27 Å radius). SPL2 point charge mapping was used with 10 quadrature points per Å² and a surface cubic spline window of 0.3 Å. The grid sizes for each species are listed in Tables S1–S3.

The Brownian dynamics simulations were performed in BrownDye2 according to the Northrup–Allison–McCammon (NAM) algorithm.⁷⁸ The solvent was parametrized with a relative viscosity of 1, a dielectric constant of 78, a relative kT of 1 kJ, and a desolvation parameter of 1. Reactions were defined by a minimum number of 3 residue–heparin contacts with a distance threshold of 7.5 Å between reference atoms. For heparin, the reference atoms were S2 and S6 of each α -D-GlcNS(6S) and S2 of each α -L-IdoA(2S). For mSAA, reaction centers were residues K24, K29, and R38. For hSAA, reaction centers were R15, R39, and K46. The reference atoms for all arginine and lysine reaction centers were their CZ and NZ atoms, respectively.

For each fibril, 1000 trajectories were generated for each input structure with each heparin input structure. This led to 156,000 total trajectories for hSAA and to 208,000 total trajectories for mSAA. The maximal number of steps was set to 1,000,000. The time step tolerance was 1.0 when unbound and 0.1 when bound.

Molecular Dynamics of Heparin–Fibril Complexes. To obtain starting configurations of heparin–fibril complexes for further simulations, the final frames from the Brownian dynamics simulations ending with heparin bound to either R38 or K29 of mSAA or to either R39 or K46 of hSAA were considered if the heparin molecule was within the bounds of the fibril along the z-axis and the heparin's orientational angle was $\leq 45^\circ$ with respect to the fibril's principal axis. The fibril's principal axis was defined by the vector passing from the centroid of the fibril's third layer to the centroid of the fibril's third-to-last layer. Heparin's orientational vector was defined to pass from the centroid of the first ring (including atoms C1,

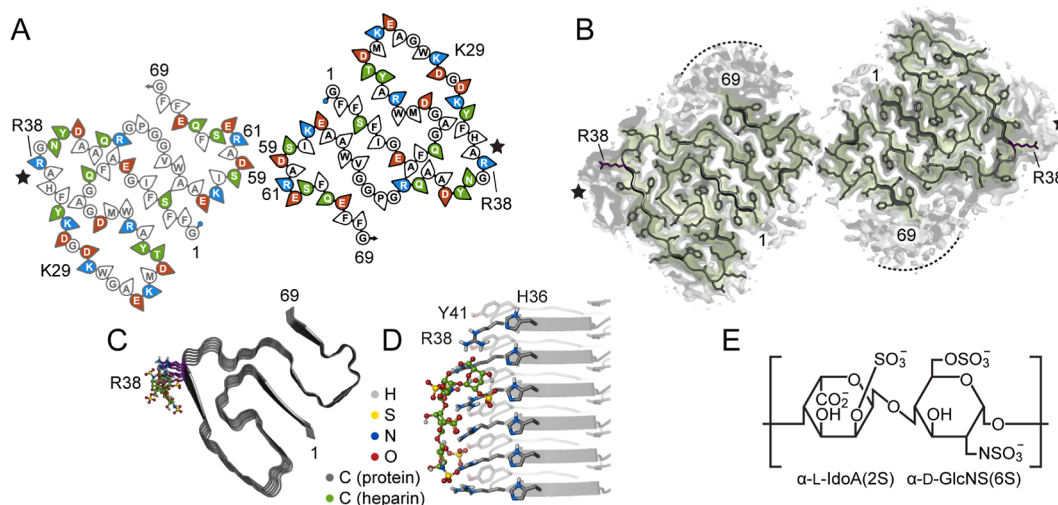


Figure 1. Summary of molecular docking of heparin to the mSAA amyloid fibril. Panels A–C show top-down views of the fibril. (A) Single layer of mSAA fibril labeled by amino acid. Residues 1–69 form the fibril core, residues 70–83 are unstructured, and the remainder of the C-terminal segment is truncated. Stars indicate location of predicted heparin binding site on each protofilament at R38/H36. (B) Cryo-EM map of ex vivo mSAA fibrils. Ordered fibril core is shown in stick model and green volume at 4.5σ level, and unidentified extra density shown in gray volume at 3σ level. Stars highlight gray extra densities that match heparin docking results. Dashed lines indicate densities from the disordered C-terminal segments. (C) ClusPro docking results predict one major heparin binding site at R38/H36 of each protofilament. Representative docking result is shown. Six layers of mSAA fibril are shown in dark gray, with R38 residue array in purple. (D) Side view of heparin docking pose at residues R38/H36, showing the coordination of heparin's sulfate and carboxylate groups to the arginine and histidine side chains of mSAA. (E) Molecular structure of heparin disaccharide unit; molecules containing 2 to 5 such units were used in this study. Cryo-EM map created using data from Libertà et al., 2019.⁴⁸

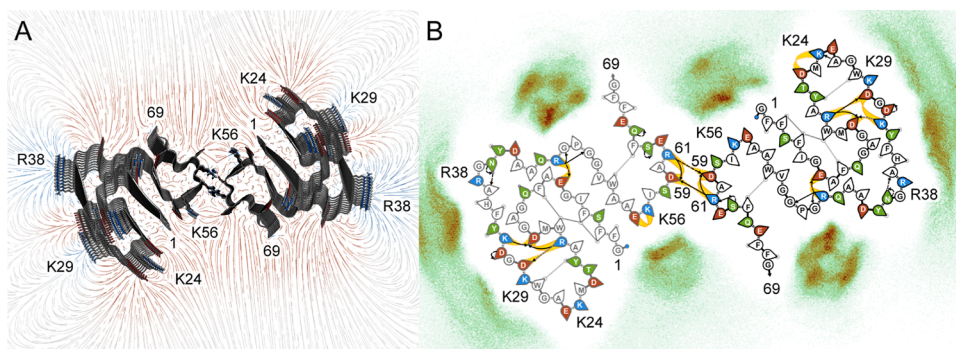


Figure 2. Summary of Brownian dynamics of heparin-mSAA fibril. (A) Brownian dynamics simulations are guided by the electric field of the fibril, which is computed by APBS and shown in cyan and red on top-down view of fibril. Side chains of selected basic and acidic residues are shown. (B) Lateral density of the final positions of the heparin sulfur atoms from Brownian dynamics trajectories. Hydrogen bonds (black arrows), salt bridges (yellow swashes), and pi-stacking interactions (gray lines) computed from MD simulation of mSAA in solution are shown. Interactions that have a probability greater than 0.5 are shown. A complete legend is provided in Figure S3.

C2, C3, C4, C5, and O5) to the centroid of the last ring. For each binding site, the configurations were then clustered with agglomerative clustering and Ward's minimum variance metric by the pairframe dRMSDs of the distance between each heparin sulfur atom and the nearest atom of interest in the binding residue.^{74,75} For K29 of mSAA and K46 of hSAA, the atom of interest was NZ. For R38 of mSAA and R39 of hSAA, the atom of interest was CZ. The medoid configuration of 10 clusters for each binding site was selected for further simulation.

Each starting complex was solvated with the TIP3P water model and 0.15 mol NaCl in a dodecahedron box with 2 nm of space on each side. This resulted in systems containing ~400,000 atoms for the mSAA fibril and ~200,000 atoms for the hSAA fibril. After a brief energy minimization, the complex was run through a series of three 1 ns equilibrations, each with a 1 fs time step: (1) An NVT equilibration at 310.15 K using

the velocity rescaling thermostat with backbone ($400 \text{ kJ mol}^{-1} \text{ nm}^{-1}$) and side chain ($40 \text{ kJ mol}^{-1} \text{ nm}^{-1}$) position restraints,⁶⁶ (2) an NPT equilibration at 310.15 K and 1 bar using the velocity-rescaling thermostat and the Berendsen barostat with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ and with the same position restraints,⁶⁷ and (3) an unrestrained NPT equilibration at 310.15 K and 1 bar using the Nose–Hoover thermostat and the Parrinello–Rahman barostat with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$.^{68–70} Finally, each system was simulated for 20 ns in the same NPT ensemble as equilibration 3 using a 2 fs time step. Simulations were performed in GROMACS 2021.5 using the CHARMM36m force field.⁶³

RESULTS AND DISCUSSION

Molecular Docking of Heparin Tetrasaccharide to mSAA Fibril. Figure 1 summarizes the heparin docking results

for the mSAA fibril structure. The fibril is comprised of two protofilaments related by an approximate 2-fold symmetry and packed via a narrow interface stabilized by intermolecular salt bridges between the juxtaposed pairs of D59 and R61. Each protofilament contains a well-ordered polypeptide segment G1–G69 that forms the fibril core and is folded in an all- β Greek key motif (Figure 1A). The disordered C-terminal segment 70–103 is truncated near residue 83.⁴⁸ Similar C-terminal truncations are characteristic of AA deposits in humans and other species.^{79,80}

For heparin docking to this mSAA fibril structure, ClusPro returned 19 docking poses, all of which predicted the R38/H36 residue array as the binding site. This result is not surprising, since the R38/H36 array is the major area of uncompensated positive charge on the fibril surface (Figure 1A). This docking result is consistent with the cryo-EM map that shows an additional density outside the amyloid core near residues R38/H36 (Figure 1B). We propose that this extra density represents bound polyanions such as HS.

Figure 1D shows a representative docking pose for the heparin tetrasaccharide at this site. The sulfate and carboxylate groups of heparin bind via electrostatic interactions to R38 and H36 side chains arrays along the length of the fibril. No close contacts involving sugar moieties of heparin were seen in this or other docking poses, suggesting that the binding is driven mainly by electrostatic interactions. To test this conjecture and to obtain a more complete picture of heparin–mSAA amyloid interactions, we performed MD simulations using a longer molecule of heparin decasaccharide as described below.

Brownian Dynamics and Molecular Dynamics of mSAA and Heparin. Initial simulations of mSAA in solution were performed to determine key native contacts within the fibril structure (including hydrogen bonds, pi-stacking interactions,⁸¹ and salt bridges), which are shown in Figure 2B. Further details about the calculation of these contacts are provided in Supplemental Methods IV. The key intermolecular interactions identified on the exterior of the fibril are R61–D59 salt bridges and hydrogen bonds between the two protofilaments and K56–E55/K24–D22 salt bridges.

The initial simulations of mSAA and heparin in solution were clustered by agglomerative clustering leading to 16 distinct clusters for mSAA and 13 clusters for heparin. Analysis of the conformations of heparin in solution are provided in Supporting Information Results I. Brownian dynamics simulations were performed using the medoids of these clusters to identify key locations of bound heparin on the fibril. All these locations involve outward-facing basic residues (K24, K29, R38, and K56) that correspond to positive regions of the fibril's electric field (Figure 2A). Of the 208,000 trajectories, heparin binds to K24 in 6313 (3.0%), K29 in 34,504 (16.6%), R38 in 27,326 (13.1%), and K56 in 54,709 (26.3%); heparin escapes binding in 52,977 (25.5%); and in 32,171 (15.5%) heparin binds elsewhere, predominantly near the capped C-terminus of the ordered structure. Since the K24 site has very low occupancy while the sites at the capped C-terminus and K56 are occluded by the disordered C-terminal tail (which appears as diffuse density in the cryo-EM map, Figure 1B), these sites were not considered for further analysis. Therefore, our all-atom MD simulations used binding conformations for sites K29 and R38 only.

These locations, which correspond to the largest regions of positive charge in the fibril's electric field, are flanked by negatively charged residues. Even in the case of our finite fibril

and heparin models, this guides heparin to take an upright orientation along the array of K29 and R38 residues. The negative regions in the electric field from D32 between K29 and R38, from E25 on the far side of K29, and from D42 on the far side of R38 guide heparin toward the K29 and R38 binding sites. This suggests a heparin–fibril interaction driven largely by electrostatic steering.

Final frames from the Brownian dynamics trajectories with heparin bound to K29 or R38 within the bounds of the fibril z -axis and with an orientational angle of less than 45° were clustered with agglomerative clustering to identify 10 distinct binding modes for each site.^{74,75} Each of these configurations was then individually simulated with all-atom MD for 20 ns. In the analysis of these simulations, a fibril side chain or heparin charged group (HCG) was considered bound if its cationic nitrogen or anionic oxygen was within 4.5 Å of its oppositely charged counterpart. Here, HCGs include sulfate, sulfamido, and carboxylate groups.

Of the 10 trajectories with heparin initially bound at the K29 site, only 6 end with heparin still bound to K29. In the remaining 4 trajectories, heparin dissociates from K29 and binds to R38. Largely due to these dissociation events, a median of only 1 K29 residue is bound to a HCG throughout the trajectories, and a median of only 2 HCGs are bound to a K29 residue (Figure 3A). Nonetheless, for the trajectories where heparin remains bound to the K29, HCGs are able to bind to a maximum of 10 K29 (representative frame shown in Figure 3B). When considering only the trajectories in which heparin does not dissociate from the K29 site, a median of 4 K29 residues are bound to a HCG (Figure 3A). The binding energies were calculated by MMPBSA using the gmx_MMPBSA package (as described in Supporting Information Methods II).⁵⁸ When fully bound, heparin has a binding energy of roughly -30 kcal mol⁻¹ with K29 (Figure 3E). This indicates that K29 is a possible heparin binding site on the mSAA fibril.

Unlike K29, in all 10 simulations with heparin initially bound at the R38 site, the heparin remains bound for the entire trajectory. In these trajectories, a median of 9 R38 residues are bound to a HCG, and a median of 8 HCGs are bound to a R38 residue (Figure 3C). Additionally, a maximum of 11 HCGs are able to bind to R38 groups and vice versa (representative frame shown in Figure 3D). When fully bound, heparin has a binding energy of roughly -40 kcal mol⁻¹ with R38 (Figure 3E). This suggests that the R38 residue array is the primary heparin binding site for this mSAA amyloid structure, and that K29 is a possible secondary heparin binding site. This result is consistent with the excess densities seen in the cryo-EM map near R38 of mSAA (Figure 1B).

Molecular Docking of Heparin Tetrasaccharide to hSAA Fibril. Despite high amino acid sequence similarity of mSAA and hSAA (75% identity; 84% similarity; Figure S1), the fibril structures are different and the two sequences are incompatible for adopting the other protein's protofilament fold.⁴⁸ Ex vivo fibrils of hSAA contain two protofilaments that are related by an approximate 2-fold symmetry and are packed together via a large hydrophobic interface (Figure 4A). Each protofilament is comprised of residues 2–67. (Residue numbering in hSAA and mSAA differs by 1 since hSAA has an additional N-terminal Arg, which is truncated in amyloid). Well-ordered residues 2–55 of hSAA fold into an S-shaped motif in the fibril core (Figure 4A,B), while C-terminal segment 56–67 is disordered. Each protofilament contains a

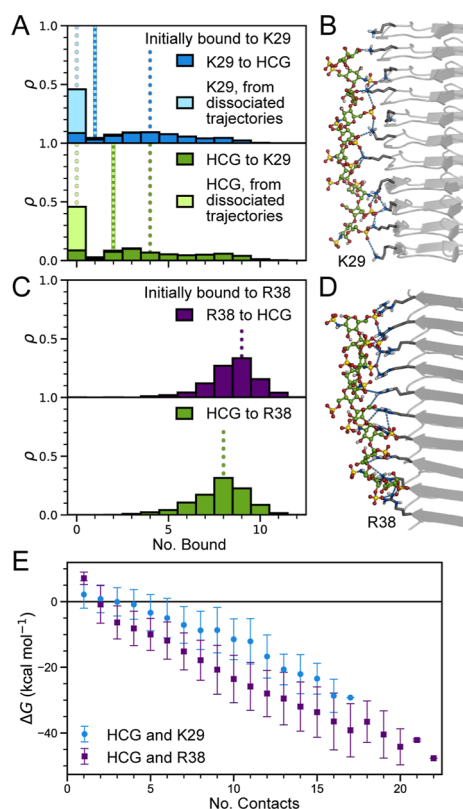


Figure 3. Summary of results for heparin-mSAA MD simulations. (A,B) For the trajectories starting with heparin bound to K29, (A) shows the probability of numbers of K29 bound to a HCG and HCGs bound to K29. Lighter bars show contributions from the dissociated trajectories. Vertical lines indicate medians. (B) A frame of the trajectory with heparin bound to K29. (C,D) For the trajectories starting with heparin bound to R38, (C) shows the probability of different numbers of R38 residues bound to HCGs and HCGs bound to R38. (D) A frame of the trajectory with heparin bound to R38. In (B,D) cyan dashed lines indicate R38/K29–HCG distances ≤ 4.5 Å. (E) The binding energy calculated by MMPBSA as a function of the total number of R38/K29–HCG distances ≤ 4.5 Å in frames with heparin bound to that residue.

water channel lined by polar residues; since these channels are too narrow to accommodate heparin, they were blocked during docking as described in Methods.

For heparin docking to the hSAA fibril structure, ClusPro predicted 20 docking poses and identified R15/R19 as the strongest heparin binding site (12 of 20 results), followed by K46 (7 results) and R39/H37 (1 result). However, the R15/R19 site is probably occluded by the disordered C-terminal tail (which appears as diffuse density near A55 in Figure 4B). Therefore, residues R15 and R19 were blocked in a subsequent round of docking using the Repulsion feature in ClusPro. This round returned 16 binding poses; heparin was predicted to bind to arrays of K46 (9 poses) and R39/H37 (7 poses) (Figure 4C). As for the mSAA fibril, these sites constitute the major arrays of uncompensated positive charge on the hSAA fibril surface. The heparin binding site at K46 of hSAA was not predicted for mSAA which has Gln45 in the corresponding position. As for mSAA, at each predicted binding site of hSAA the docking results showed heparin binding along the fibril length to the basic arrays of Lys or Arg side chains. Representative binding poses of heparin bound at the R39/H37 and K46 sites are shown in Figure 4D,E, respectively.

We conclude that the docking results for hSAA and mSAA fibrils are qualitatively similar. Heparin is strongly predicted to bind via electrostatic interactions to arrays of uncompensated basic residues along the fibril sides. Importantly, as for mSAA, the docking results for hSAA match the location of extra densities seen in the cryo-EM map (Figure 4B). Moreover, in the cryo-EM map, the extra density at K46 is stronger than that at R39/H37, suggesting that the ligand bound at K46 may have higher occupancy than that at R39/H37. This is consistent with the distribution of ClusPro docking poses.

Brownian Dynamics and Molecular Dynamics of hSAA and Heparin. Similar to mSAA, initial simulations of hSAA in solution were used to identify key contacts (including hydrogen bonds, salt bridges, and pi stacking interactions) within the fibril (Figure 5B). The sole interaction on the exterior of the fibril potentially relevant to heparin binding is an E9–R39 salt bridge spanning the two protofilaments.

Frames from the simulations of hSAA in solution were clustered by agglomerative clustering leading to a total of 12 distinct structures.^{74,75} Brownian dynamics simulations were performed using each of these structures and each of the 13 heparin structures to identify key locations of the bound heparin on the fibril. These locations are near the exposed basic residues (R15, R39, and K46) in regions of the positive electric field of the fibril (Figure 5A). Of the 156,000 total trajectories, heparin binds to R15 in 26,220 (16.8%), to R39 in 28,950 (18.6%), and to K46 in 33,947 (21.8%); in 22,993 (14.7%) it escapes the electric field of the fibril, and in 43,890 (28.1%) it binds elsewhere on the fibril. R15 is probably occluded by the disordered C-terminal tail. Therefore, we proceeded with all-atom MD simulations of heparin binding conformations only to R39 and K46.

Similar to mSAA, these sites correspond to large positive regions in the electric field. The K46 site is flanked by the C-terminal intrinsically disordered domain on one side, and a small negative region of the electric field on the other side close to R39. Continuing around the fibril, there is a large negative region in the electric field on the far side of the R39 side stemming from the E9 and D12 residues. This negative region can direct heparin back toward the K46 and R39 binding sites. Consistent with our results for mSAA, we propose that interactions between heparin and the fibril at these sites are driven by electrostatic steering. The arrays of positive K46 and R39 residues running the length of the fibril favors an upright orientation of bound heparin. Regions of negative charge surrounding the ladders of positively charged residues creates a funneling effect that steers the heparin toward the favored binding sites. This electrostatic steering effect should enhance the rate of heparin binding overestimates based on spatially isotropic diffusion limited reaction rates.

Final frames from the trajectories with heparin bound to K46 or R39 were clustered with agglomerative clustering to identify 10 distinct binding modes for each binding site. These configurations were then simulated for 20 ns.

In 2 of 10 trajectories starting with heparin bound to R39, heparin completely dissociates from the fibril, and in 1 trajectory heparin turns laterally to bind at one end to R39 and at the other end to K46. In these simulations, the median number of R39 residues bound to an HCG is 3, and the median number of HCGs bound to an R39 residue is 2 (Figure 6A). Like with mSAA, binding energies were computed using MMPBSA in the gmx_MMPBSA package (described in Supporting Information II).⁵⁸ When fully bound, heparin

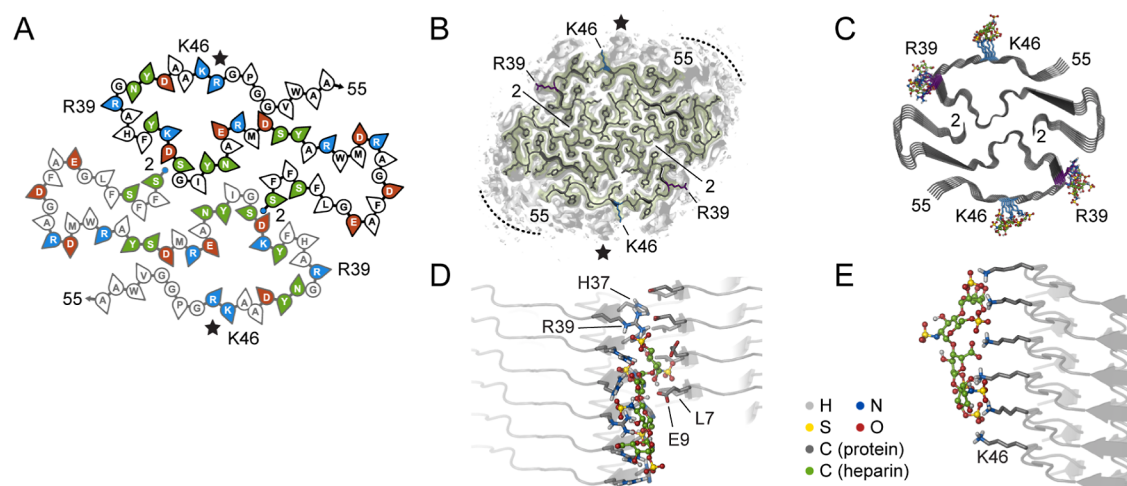


Figure 4. Summary of molecular docking of heparin to the hSAA amyloid fibril. Panels (A–C) show top-down views of the fibril. (A) Single fibril layer labeled by amino acid. Residues 1–55 form the ordered fibril core, while residues 56–67 are unstructured and the remainder of the C-terminal segment is truncated. Stars indicate the location of predicted heparin binding sites. (B) Cryo-EM map of ex vivo hSAA fibrils. Ordered peptide core is shown in stick model and green volume at 4.5σ level, and unidentified extra density is in gray volume at 4σ level. Stars highlight extra densities that match the location of heparin docking results and gray lines indicate densities from the disordered C-terminal segments. (C) ClusPro docking results using the hSAA fibril structure predict two major heparin binding sites on each protofilament at K46 and R39. Representative docking results at each site are shown. Six layers of hSAA fibril are shown with main chain in gray, K46 in blue, and R39 in purple. (D,E) Side views of representative heparin docking poses at R39 (D) and K46 (E) show the coordination of heparin's sulfate and carboxylate groups to the arginine and lysine side chains. Cryo-EM map created using data from Liberta et al., 2019.⁴⁸

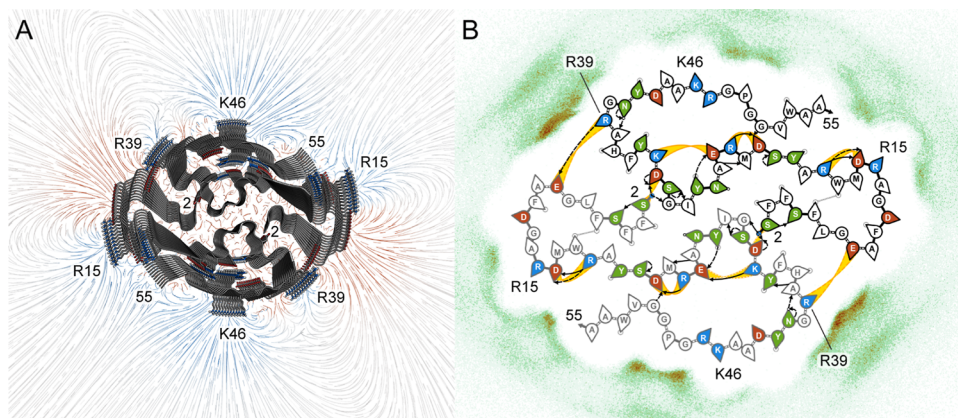


Figure 5. Summary of Brownian dynamics of heparin-hSAA fibril. (A) Brownian dynamics simulations are guided by the electric field of hSAA, which is computed by APBS and shown in green and red on top-down view of fibril. Basic and acidic residues are shown. (B) Lateral density map shows the final positions of the heparin sulfur atoms from Brownian dynamics trajectories. Hydrogen bonds (arrows), salt bridges (yellow swashes), and pi-stacking interactions (gray lines) computed from MD simulation of hSAA in solution are shown. A complete legend is provided in Figure S3.

does not have a significant binding energy with R38 (Figure 6E). While this result indicates relatively weak binding, the maximal number of bound R39 residues is 10, showing that extensive heparin–fibril contacts are possible at this site (Figure 6C).

Unlike heparin dissociation from R39, heparin that was initially bound to K46 remains bound in all 10 simulations. A median of 7 K46 residues and 8 HCGs are bound to one another (Figure 6C). When fully bound, heparin has a binding energy of roughly $-45 \text{ kcal mol}^{-1}$ with K46 (Figure 6E). These results suggest that K46 forms the primary heparin binding site in this hSAA fibril structure. A representative trajectory frame is shown in Figure 6D. Notably, the primary binding site for mSAA, R38, corresponds to the lesser binding site on hSAA, R39, reflecting structural differences between these fibrils (Figure S1). Weaker heparin binding at R39 in hSAA reflects weaker negative electric field at this site due to the proximity to

E9 from the adjacent protofilament, which can form a salt bridge with R39 to balance its charge.

SUMMARY AND CONCLUSIONS

The results of our molecular docking, supported by the Brownian dynamics and MD simulations, indicate that heparin/HS binds along the uncompensated arrays of basic residues on the exterior of mSAA and hSAA fibrils. The longitudinal binding mode (shown in Figures 1D,E and 4D,E for heparin tetrasaccharides and in Figures 3B,D and 6B,D for heparin decasaccharides) can be readily extrapolated to longer regions of high sulfation, including the 14–16 monosaccharide domains of high sulfation present in HS.³⁴ These results suggest that the excess densities seen in the cryo-EM maps near residues R39 and K46 of hSAA and near R38 of mSAA likely represent bound chains of HS, which codeposit with AA fibrils and other amyloids.⁸² While attempts to build ligand

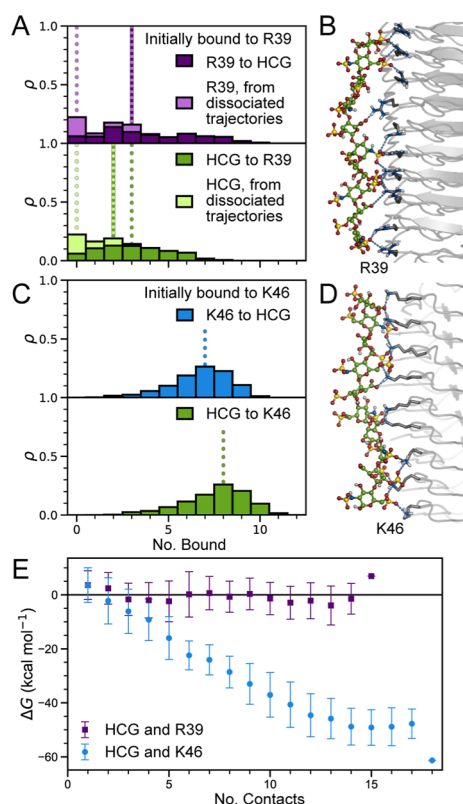


Figure 6. Summary of results for heparin-hSAA MD simulations. (A,B) For the trajectories starting with heparin bound to R39, (A) shows the probability of numbers of R39 bound to a HCG and HCGs bound to an R39 residue. Lighter shaded bars show contributions from the dissociated trajectories. Vertical lines indicate medians. (B) A frame of the trajectory with heparin bound to R39. (C,D) For the trajectories starting with heparin bound to K46, (C) shows the probability of different numbers of K46 residues bound to sulfates and sulfates bound to K46. (D) A frame of the trajectory with heparin bound to K46. In (B,D), cyan dashed lines indicate K46–HCG distances ≤ 4.5 Å. (E) The binding energy calculated by MMPBSA as a function of the total number of R39/K46–HCG distances ≤ 4.5 Å in frames with heparin bound to that residue.

molecules into these extra densities were inconclusive, this likely resulted from the mismatch between the helical symmetry of the fibril and the ligand, compounded by the partial occupancy of HS and its variable conformation in regions of high and low sulfation.

Several lines of additional experimental evidence support our conjecture. (1) mSAA fibrils grown in vitro from an ex vivo fibril seed in the absence of heparin/HS adopt the same protofilament fold as the seed. Despite the improved resolution for in vitro vs ex vivo fibrils (2.7 Å vs 3.0 Å), the strong extra density near R38/H36 seen in the cryo-EM map of the ex vivo fibril was replaced by a very weak diffuse density around the fibril core.⁸³ This observation supports the conjecture that the extra density seen in the ex vivo fibrils represents a bound ligand such as HS, which was present in vivo but was missing from the in vitro preparations. (2) mSAA amyloid fibrils grown in vitro without seeding had a Greek key fold similar to that of the ex vivo or seeded mSAA fibrils, but with a much smaller ordered core only containing residues 1–37.⁸⁴ In contrast, the polypeptide chain starting from R38 was disordered, probably due to uncompensated charge at R38. This suggests that either a polyanion such as HS or a seed is needed to stabilize the

positively charged R38 array in a well-ordered amyloid fibril structure. (3) Experimental binding studies using mSAA fragments have strongly implicated R38/H36 as the heparin/HS binding site, demonstrating that H36 protonation is important for binding and acts as a molecular switch for amyloid formation.²² Furthermore, the heparin-amyloid binding mode described in the current work is supported by in vitro studies of α -synuclein amyloid fibrils grown in the presence of heparin.¹⁴ In this study, heparin chains were unambiguously fit into the 3D cryo-EM density maps of these fibrils, showing that heparin binds to basic residue arrays along the spines of amyloid fibrils, similar to the binding modes shown in Figures 3 and 6 of this study. A similar binding mode has also been observed in the cryo-EM density maps of tau fibrils grown in vitro in the presence of heparin, which display strong extra densities near basic residue arrays.⁸⁵ Taken together, existing evidence suggests strongly that R38/H36 array forms the major heparin/HS binding site in mSAA amyloid, while the K46 array is the major binding site in hSAA amyloid. Our results further substantiate these arrays as heparin's primary binding sites, with the R38 site of mSAA and the K46 site of hSAA having binding energies of roughly -40 kcal mol⁻¹ and roughly -45 kcal mol⁻¹, respectively (Figures 3E and 6E).

Our Brownian dynamics and MD results reveal that heparin binding is driven by long-range interactions. (Figures 2, 5). Arrays of positively charged Lys and Arg residues surrounded by regions of negative charge create electrostatic funnels that steer the polyanionic heparin toward the positively charged residues. Once bound, heparin adopts conformations stabilized by salt bridges, mainly between the HCGs and arrays of Arg or Lys side chains in amyloid (Figures 3 and 6). At the primary heparin binding sites, approximately 40% of all HCGs in heparin decasaccharide are involved in binding mSAA or hSAA amyloid (Figures 3C and 6C), suggesting that amyloid binding is critically influenced by the degree of sulfation in HS. These findings are consistent with experimental data showing that the degree of GAG sulfation strongly correlates with the GAG's ability to promote SAA amyloid formation, and that the presence of sulfate groups is important for facilitating fibrillogenesis regardless of the GAG backbone structure.^{86,87}

Our studies provide the structural basis necessary for designing HS mimetics with optimal molecular size, shape, and sulfation which do not promote amyloid formation, yet compete with long HS chains for binding to amyloid. Additionally, our work provides the basis for future computational studies investigating the binding of heparin/HS and related molecules to SAA and other amyloid fibrils.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.4c00529>.

Contains Supplemental Methods and Results, including sequences of mSAA and hSAA, MMPBSA methods, APBS grid sizes, native contact calculation procedure and legend, and heparin conformation results (PDF)

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Notes

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