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Structural Basis of Single Molecular Heparin-FX06 Interaction Revealed by SPM Measurements and Molecular Simulations

Cunlan Guo^a, Bin Wang^a, Lianchun Wang^b, and Bingqian Xu^{*,a}

^aSingle Molecule Study Laboratory, College of Engineering and Nanoscale Science and Engineering Center, University of Georgia, Athens, GA 30602, USA

^bComplex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA

Abstract

Heparin, a functionalized polysaccharide, is observed under scanning tunneling microscope, which shows atomic scale conformation details. The peptide FX06 is found to bind with five consecutive sugar units of heparin and this interaction is directly revealed by atomic force microscopy dynamic force spectroscopy measurements. The determined free energy change agrees well with the dynamic calculation result.

Polysaccharide-protein interactions on cell surface play key roles in a wide range of crucial biological process¹. Heparin as well as heparan sulfate (HS) has been known to play critical roles in many cellular processes ranging from embryonic development, angiogenesis, and blood coagulation to viral infection, inflammation and tumorigenesis by interacting with a remarkable number of biologically important molecules including coagulation factors, proteinase, proteinase inhibitors, adhesion molecules, growth factors, chemokines and morphogens². FX06, the peptide B 15-42 from fibrin fragments, has shown to protect from ischemia/reperfusion-elicited tissue injuries in animal models, such as myocardial infarction, and acute injuries of lung and kidney³. Very recently, a multi-centered phase II clinical trial study concluded that FX06 effectively protects patients with acute myocardial infarction⁴, exhibiting a promising potential to develop FX06 as a novel drug for the treatment of ischemic/reperfusion-related human diseases. FX06 has been observed to bind to heparin⁵, and the interaction has been implicated in endothelial cell adhesion, spreading and proliferation⁶. However, the mechanisms of biophysical interaction of FX06 with heparin and the heparin structure involved, which are crucial to understand the therapeutic effects of FX06 and to develop novel drugs targeting ischemia/reperfusion-elicited human diseases, have yet to be elucidated.

To measure the molecular level interaction of heparin-FX06, as shown in Fig. 1a, heparin was immobilized on 11-mercapto-1-undecanol (MUO) modified Au(111) surface through epoxy group activated hydroxyl groups reaction (ESI[†] SI-3), and FX06 was tethered onto gold coated AFM tip via bifunctionalized polyethylene glycol (PEG linker) mediated conjugation (ESI[†] SI-4). Our simulation results show that the heparin filament adopts a linear conformation in aqueous solution (PDB entry 3IRJ model 1)⁷, and FX06 has a folded tertiary structure with a length of ~2.8 nm in the long axis (ESI[†] Fig. S2a-b)⁸. Because FX06 is small compared to the PEG linker (~15 nm in length with molecular weight (Mw) of

2000), it can move freely without spatial restriction after being linked to the PEG modified tip and maintain its activity during the AFM scanning.

We first used AFM recognition imaging⁹ to locate the heparin molecules as highlighted by dashed circles in Fig. 1b. The bright dots in the topographic image exhibited different shapes with a measured height of 0.19 ± 0.03 nm. We hypothesize that these dots which have corresponding dark signal in recognition image may be individual heparin molecules or heparin aggregates. STM images (Fig. 1c) clearly showed that the linear conformations of heparin agreed well with the simulated results (ESI[†] Fig. S2a). The measured height of heparin molecules from the STM image was 0.23 ± 0.03 nm, which was close to the height of the dots in the AFM topographic image. Further comparison of the AFM and STM topographic images were in ESI[†] Fig S3. It is indicated that the small dots in the AFM images consisted of several heparin molecules lying side by side on the MUO monolayer, or randomly without overlapping. The topography image in Fig. 1d presented the atomic scale details of a part heparin chain from one single heparin filament, including some discrete spherical cores flanked by short “sulfate arms”. The spherical cores are sugar units of heparin according to the simulated structure. The distance between the “sulfate arms” in the STM image (green line) was around 5.7 Å, which agreed well with the simulated 5.73 Å distance from one oxygen atom in the O-sulfate group to another oxygen atom in the sulfamate group. More comparisons of the distances from the STM image and the simulated structure were given in ESI[†] Fig. S4, which showed excellent agreement. Therefore, the sulfate groups on the heparin filament were distinguished on the STM images. Furthermore, the measured most probable length of linear heparin filament was 16.50 ± 1.80 nm (Fig. 1e), which is consistent with the length estimated from the constrained X-ray scattering data (~ 17 nm for a heparin molecule with Mw of 13500)⁷. Besides, the measured width of single heparin molecules at half height was 1.07 ± 0.14 nm, similar to the width of the sugar unit (0.9 nm) in the simulated heparin structure. Meanwhile, the sizes of sulfamate and O-sulfate groups on the sugar units were around 0.3 nm in the simulated heparin conformation, which were also similar to the heparin height measured by STM. Therefore, once the heparin molecules attached to MUO monolayer, most heparin filaments were in the relaxed state, with their sugar units lying flat on the MUO monolayer.

We then measured the single molecular heparin-FX06 interaction by monitoring the rupture force between the heparin (gold surface) and the FX06 (AFM tip) (Fig. 2). More than 300 force-distance curves at each force loading rate were collected to construct the force histograms (Fig. 2b)¹⁰. Rupture forces were determined to be from 11.77 ± 6.72 pN to 59.79 ± 13.39 pN with the loading rate increasing from 10 to 403 nN/s (ESI[†] Table S1). These force values were smaller than the unbinding force of antibody-antigen¹¹, indicating a moderate interaction between heparin and FX06. To understand the interaction, a long linear heparin molecule model (ESI[†] Fig. S2a) was used to simulate the binding with FX06. From the docking prediction¹², a shallow groove on FX06 surface can only be closely embedded any five consecutive sugar units of heparin (HEP) in the most stable complex conformation (COM) (ESI[†] Fig. S2c), while the other sugar units remained in the extended conformations far away for efficient interactions (Fig. 3a). The heparin used in our experiments is a much longer (~ 46 sugar units) but still linear filament (Fig. 1c), so it will bind FX06 with HEP when FX06 is driven close to them. The docking results also indicate that the relatively weak heparin-FX06 interaction is mainly due to the FX06 small size that has limited contact surface to the heparin.

Moreover, by linear fitting the rupture force and natural logarithmic loading rates (Fig. 2c), we showed that the dissociation of the heparin-FX06 complex followed Bell's single-barrier model¹³, which is also consistent with the simulation of COM structure. Neither FX06 nor HEP has complicated structure to generate more than one energy barrier during the

interaction. The values of kinetic off-rate k_{off} and energy barrier width x for heparin-FX06 reaction were derived to be 0.27 s^{-1} and 0.31 nm , respectively, from Bell's model¹³. The k_{off} of heparin-FX06 interaction was bigger than that of antibody-antigen complex¹⁴, while the x was similar to the reported barrier width of $0.12 \sim 0.3 \text{ nm}$ ¹⁵. These results showed a moderate binding affinity, which was further verified by the simulated results for COM. Fig. 3b (built by molecular modeling software VMD¹⁶) showed that the six hydrogen bonds spread on separate residues along HEP (ESI[†] SI-8). Fig. 3c shows that in COM the positively charged FX06 residues form a localized highly charged domain near the negatively charged HEP groups ($-\text{CO}^{2-}$ and $-\text{SO}^{3-}$). However, FX06 only has an overall charge of +3 to neutralize the HEP (overall charge = -10) (ESI[†] Fig. S8 and Table S2)¹⁷, thus the binding affinity of FX06 to heparin is only moderate, which is confirmed by the experimental data.

The Jarzynski equality with the exponential weighting of integrated work was further applied to reconstruct the Gibbs free energy change of heparin-FX06 interaction at equilibrium state¹⁸. Through reconstructing the force trajectory by exponential weighting averaging force-distance curves (Figs. 2d and ESI[†] S5), the Gibbs free energy profile was rebuilt (Fig. 2e). The related change of Gibbs free energy for heparin-FX06 interaction was calculated to be 16.92 kcal/mol . Moreover, the measured force-distance curves had different stretch length, which might come from the pulling of PEG linker or part of heparin filament before the breakage of bonds formed in heparin-FX06 interaction. To eliminate this entropy part effect, the beginning parts of the force-distance curves without apparent force change were cut away (ESI[†] Fig. S6). The obtained change of Gibbs free energy after removing the entropy effect was 14.24 kcal/mol . The further simulated Gibbs free energy change of HEP-FX06 interaction was -14.37 kcal/mole at the equilibrium state in aqueous solvent (by molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) method⁸). It agrees well with the experimental result when the entropy effect was removed. The electrostatic interaction from heparin and the positively charged FX06 residues was the main contributor for lowering the Gibbs free energy and stabilizing the complex (ESI[†] SI-10 and SI-11).

To better understand the measured force-distance curves, we constructed the 2D event distribution contour map of both the rupture force and stretch distance (Figure 4a). With the loading rates increasing from 10 nN/s to 403 nN/s , more stretch distances shifted to small values with increasing force (ESI[†] SI-12). However, the stretch distances showed no obvious correlation with the loading rates (Figure 4b), which indicates that the stretch distance may correspond to the stretching of only PEG linker (lower panel), or the stretching sum for PEG and portion of the heparin chain (upper panel). This further implies that different values of stretch distance came from various ways of heparin immobilization on MUO monolayer as well as diverse sites of heparin binding with FX06. The initial stretching portion in the entropic regime, pulling of unimmobilized heparin filament, needs small force ($5\text{--}10 \text{ pN}$, Fig. S6a) and does not contribute to the dissociation of heparin-FX06 complex, which rationalizes the free energy calculation obtained by different stretch distance of force-distance curves.

In conclusion, we have successfully imaged heparin at subnanometer resolution and elucidated the molecular basis of its interaction with FX06 including the key structural and dynamic features. This work may provide a new approach to probe and understand the mechanisms of the interactions between heparin and proteins, which are very critical for applications in biophysics and biochemistry as well as the development of specific therapeutic agents in cardiovascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Imaging heparin on MUO modified gold substrate. (a) Schematic of experiment setup for AFM imaging and recognizing of heparin with a FX06 modified tip. (b) Topographic (left) and corresponding recognition (right) images of heparin. (c) STM topography images of heparin molecules on MUO monolayer. (d) One single heparin molecule marked with circle in (c) (left), and part of the heparin molecule compared with the simulated structure (right); (e) Histogram of heparin length (100 heparin molecules from 5 STM images).

Fig. 2.

Heparin-FX06 interaction measured by force spectroscopy. (a) Typical rupture force-distance curves of heparin-FX06 interactions under eight different loading rates. (b) Corresponding distributions of rupture force at eight different loading rates. (c) Most probable rupture force at different loading rate. (d) Force-distance curve obtained by averaging 20 curves using weighted work integral averaging. (e) The reconstructed free energy for heparin-FX06 interaction.

Fig. 3.

Simulation of heparin-FX06 binding mode. (a) A heparin filament (24 sugar units, stick) interacts with a FX06 peptide (translucent surface). HEP (colorized) is used for docking simulation. (b) The hydrogen bonds between FX06 and HEP are highlighted in black dotted lines (enlarged in the frame). The FX06 residues involved in hydrogen bonds are labeled in stick. (c) The electrostatic interactions involved in HEP-FX06 binding (enlarged in the frame). FX06 and HEP are shown in stick. The positive and negative charged atoms of FX06 are highlighted in dotted spheres (blue for positive and red for negative). The neutral polar residues are in green and non-polar residues in gray. In HEP, oxygen atoms are in red, sulfur atoms in yellow, nitrogen atoms in blue, carbon atoms in cyan, and hydrogen atoms in white. The structure in c was 90° counterclockwise rotated b along the axis between (b) and (c).

Fig. 4.

Typical 2D event distribution plots associated with both rupture force and stretch distance. The loading rate of (a) is 10 nN/s. Inset in (a) shows the typical rupture force-distance curves corresponding to the most probable distribution of rupture force and distance, and the relevant schematics for potential binding modes of FX06 with heparin on the MUO monolayer. The schematics only show three of the potential binding modes. However, FX06 could bind to any HEP. (b) Two of the most probable stretch distances at different loading rates (Inset: fitted peak 1 and peak 3 at 10 nN/s).