Structural basis of single molecular heparin–FX06 interaction revealed by SPM measurements and molecular simulations†

Cunlan Guo,a Bin Wang,a Lianchun Wangb and Bingqian Xua*

Received 8th September 2012, Accepted 1st November 2012
DOI: 10.1039/c2cc36546d

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

Polysaccharide–protein interactions on the cell surface play key roles in a wide range of crucial biological processes. 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 morphogens. FX06, the peptide B15–42 derived from fibrin fragments, has been shown to protect against ischemia/reperfusion-elicted tissue injuries in animal models, such as myocardial infarction, and acute injuries of lung and kidney. Very recently, a multi-centered phase II clinical trial study concluded that FX06 effectively protects patients with acute myocardial infarction, exhibiting promising potential for the development of FX06 as a novel drug for the treatment of ischemic/reperfusion-related human diseases. FX06 has been observed to bind to heparin, and the interaction has been implicated in endothelial cell adhesion, spreading and proliferation. 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-elicted 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 the 11-mercaptop-1-undecanol (MUO) modified Au(111) surface through epoxy group activated hydroxyl groups reaction (ESI† S3), and FX06 was tethered onto a gold coated AFM tip via bifunctionalized polyethylene glycol (PEG linker) mediated conjugation (ESI† S4). Our simulation results show that the heparin filament adopts a linear conformation in aqueous solution (PDB entry 3IR1 model 1), and FX06 has a folded tertiary structure with a length of ~2.8 nm in the long axis (ESI† Fig. S2a and b). Because FX06 is small compared to the PEG linker (~15 nm in length with a 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 a corresponding dark signal in the recognition image may be individual heparin molecules or heparin aggregates. STM images (Fig. 1c) clearly showed that the linear conformation 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 is shown in Fig S3 (ESI†). 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 presents the atomic scale details of a part of the 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. Moreover, the measured most probable length of the 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 a $M_w$ of 13 500).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 attach to the 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$^{-1}$ (ESI,† Table S1). These force values were smaller than the unbinding force of heparin–FX06 interaction. The values of kinetic off-rate $k_{off}$ and energy barrier width $\Delta x$ for heparin–FX06 reaction were derived to be 0.27 s$^{-1}$ and 0.31 nm, respectively, from Bell’s model.12 $k_{off}$ of heparin–FX06 interaction was larger than that of the antibody–antigen complex,14 while $\Delta x$ was similar to the reported barrier width of 0.12–0.3 nm.15 These results showed a moderate binding affinity, which was confirmed by the experimental data. 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 model,13 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 $\Delta x$ for heparin–FX06 reaction were derived to be 0.27 s$^{-1}$ and 0.31 nm, respectively, from Bell’s model.12 $k_{off}$ of heparin–FX06 interaction was larger than that of the antibody–antigen complex,14 while $\Delta x$ was similar to the reported barrier width of 0.12–0.3 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) shows the six hydrogen bonds spread on separate residues along HEP (ESI,† S8). 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 $-\text{SO}_3^-\text{H}$). However, FX06 has an overall charge of only +3 to neutralize the HEP (overall charge = −10) (ESI,† Fig. S8 and Table S2),12 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 the equilibrium state.18 Through reconstructing the force trajectory...
Moreover, the measured force–distance curves had different beginning parts of the force–distance linker or part of the 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 an apparent force change were cut away (ESI,† Fig. S6). The obtained change in Gibbs free energy after removing the entropy effect was 14.24 kcal mol$^{-1}$. Moreover, the measured force–distance curves had different stretch lengths, which might come from the pulling of the PEG linker or part of the 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 an apparent force change were cut away (ESI,† Fig. S6). The obtained change in Gibbs free energy after removing the entropy effect was 14.24 kcal mol$^{-1}$. The further simulated Gibbs free energy change of HEP–FX06 interaction was $-14.37$ kcal mol$^{-1}$ at the equilibrium state in aqueous solvent (by the molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method). It agrees well with the experimental result when the entropy effect was removed. The electrostatic interaction between heparin and the positively charged FX06 residues was the main contributor for lowering the Gibbs free energy and stabilizing the complex (ESI,† S10 and S11).

To better understand the measured force–distance curves, we constructed the 2D event distribution contour map of both the rupture force and stretch distance (Fig. 4a). With the loading rates increasing from 10 nN s$^{-1}$ to 403 nN s$^{-1}$, more stretch distances shifted to small values with increasing force (ESI,† S12). However, the stretch distances showed no obvious correlation with the loading rates (Fig. 4b), which indicates that the stretch distance may correspond to the stretching of only the PEG linker (lower panel), or the stretching sum of PEG and portion of the heparin chain (upper panel). This further implies that different values of the stretch distance came from various ways of heparin immobilization on the MUO monolayer as well as diverse sites of heparin binding with FX06. The initial stretching portion in the entropic regime, pulling of the unimmobilized heparin filament, needs small force (5–10 pN, Fig. S6a, ESI†) and does not contribute to the dissociation of the heparin–FX06 complex, which rationalizes the free energy calculation obtained by different stretch distances 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 for cardiovascular diseases.

We thank J. H. Prestegard for the fruitful discussions, R. J. Woods and R. L. Foley for their help in AMBER, Dr F. Y. Dupradeau for his instructions on R.E.D. webserver, and Dr A. M. J. J. Bonvin for the help on HADDOCK. This work is supported by the U. S. National Science Foundation: ECCS 0823849, CBET 1139057 (B.X.) and National Institute of Health: NIH R01HL093339, RR005351,GM103390 (L.W.)

Notes and references