

Supporting Information

Heparan sulfate proteoglycans as attachment factor for SARS-CoV-2

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Materials

The SARS-CoV-2-spike (S1+S2) was obtained from Sino Biological (40589-V08B1). The Alexa Fluor 647 anti-His tag antibody was obtained from BioLegend (#652513). High grade heparin (HG-Hep, Mw 15,700) for SPR was obtained from Iduron Ltd, UK (#HEP-HG 100). Furin was obtained from New England Biolabs (P8077). Furin inhibitor II (hexa-D-Arginine) was obtained from Sigma-Aldrich (SCP0148). The SARS-CoV-2 (COVID-19) inhibitor screening kit (EP-105), the SARS-CoV-2 inhibitor (Human ACE2; AC2-NA005), and biotinylated ACE2 (AC2-H82F9) were from ACRO Biosystems.

Methods

Expression of SARS-CoV-2 RBD and spike proteins

The RBD modified by an His6-tag and trimerized, soluble spike with His6-tag were expressed in HEK cells and purified as previously described.^{1,2} The RBD trimer used in the tissue binding experiments was expressed as previously described.³

Microarray printing and screening

Aminopentyl modified HS oligosaccharides were printed on NHS-ester activated glass slides (NEXTERION® Slide H, Schott Inc.) using a Scienion sciFLEXARRAYER S3 non-contact microarray equipped with a Scienion PDC80 nozzle (Scienion Inc.). Individual samples were dissolved in sodium phosphate buffer (50 µL, 0.225 M, pH 8.5) at a concentration of 100 µM and were printed in replicates of 10 with a spot volume of ~ 400 pL at 20 °C and 50% humidity. Each slide has 24 subarrays in a 3x8 layout. After printing, slides were incubated in a humidity chamber for 8 h and then blocked for 30 min in a Tris buffer (pH 9.0, 50 mM) containing 5 mM ethanolamine at 40 °C. Blocked slides were rinsed with DI water, spun dry, and kept in a desiccator at room temperature for future use.

Screening was performed by incubating the slides with a protein solution at the indicated time, followed by washing and drying. A typical washing procedure includes sequentially dipping the glass slide in TSM wash buffer (2 min, containing 0.05 % Tween 20), TSM buffer (2 min) and, water (2 x 2 min), followed by centrifugation. For SARS-CoV-2-RBD and SARS-CoV-2-spike protein screening, the slides were incubated with SARS-CoV-2-RBD (10, 30, and 100 µg/mL in TSMBB) or SARS-CoV-2-spike (3, 10, and 30 µg/mL in TSMBB) for 1 h, followed by washing and incubation with suitable detection reagent. For His tagged proteins, a solution of AlexaFluor® 647 conjugated anti-His antibody (10 µg/mL) was used. For Fc-tagged proteins, a solution of AlexaFluor® 647 conjugated goat-anti-human antibody (10 µg/mL) was used. For biotinylated proteins, a solution of Streptavidin- AlexaFluor® 647 or Streptavidin-Cy3 conjugates (10 µg/mL) was used. After washing and drying, the slides were scanned using a GenePix 4000B microarray scanner (Molecular Devices) at the appropriate excitation wavelength with a resolution of 5 µM. Optimum gains and PMT values were employed for the scanning ensuring that all signals were within the linear range of the scanner's detector and there was no saturation of signals. The images were analyzed using GenePix Pro 7 software (version 7.2.29.2, Molecular Devices). The data was analyzed with a home written Excel macro. The highest and the lowest value of the

total fluorescence intensity of the replicate spots were removed, and the remaining values were used to provide the mean value and standard deviation.

Preparation of biotinylated heparin

High grade heparin (HG-Hep, Mw 15,700, Iduron, UK) was biotinylated following a literature procedure.⁴ Briefly, HG-Hep (0.5 mg), biotin-(PEG)₃-NH₂ (0.5 mg, Thermo Scientific, USA) and sodium cyanoborohydride (2.5 mg, NaBH₃CN, AminoLink™ reductant, Thermo Scientific, USA) were combined, dissolved in water (100 µL) and stirred at 70 °C for 24 h. Another portion of NaBH₃CN (2.5 mg) was added and stirring continued for additional 24 h. To remove excess of biotin linker and reductant, the reaction mixture was centrifuged over a spin filter (Mw cutoff 3000) and exchanged with water (2x1 mL, Milli-Q®), concentrated (200 µL) and lyophilized to obtain the desired Biotin-HG-Hep as white fluffy powder. The product was aliquoted and stored at -80 °C.

Surface plasmon resonances experiments

A streptavidin coated sensor chip was prepared from a CM5 chip by standard amine coupling using an amine coupling kit (Biacore Inc. - GE Healthcare). Briefly, the surface was activated using freshly mixed *N*-hydroxysuccinimide (NHS; 100 mM) and 1-(3-dimethylaminopropyl)-ethylcarbodiimide (EDC; 391 mM) (1/1, v/v) in water. The remaining active esters were quenched by aqueous ethanolamine (1.0 M; pH 8.5). Next, streptavidin (50 µg/mL) in aqueous NaOAc (10 mM, pH 4.5) was passed over the chip surface until a ligand density of approximately 3,000 RU was achieved. Next, biotin-HG-Hep (1 µM) was passed over one of the flow channels at a flow rate of 5 µL/min for 2 min resulting in a response of 56 RU. Next, the reference and modified flow cells were washed with three consecutive injections of 1 min with 1 M NaCl in 50 mM NaOH. HBS-EP (0.01 M HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20; pH 7.4) was used as the running buffer for the immobilization, kinetic studies of the interaction of HG-Hep with SARS-CoV-2-RBD and spike protein (S1+S2), and inhibition studies of immobilized HG-Hep with synthetic GAGs oligosaccharides. Analytes were dissolved in running buffer and a flow rate of 30 µL/min was employed for association and dissociation at a constant temperature of 25 °C. A 60 s injection of 0.25% sodium dodecyl sulfonate (SDS) at a flow rate of 30 µL/min was used for regeneration and to achieve prior baseline status. Using Biacore T100 evaluation software, the response curves of various analyte concentrations were globally fitted to the 1:1 binding model.

Human angiotensin-converting enzyme 2 (ACE2) with Fc tag (Sino biology 10108-H02H) (5 µg/mL in HBS-P buffer) was captured to a human IgG Fc antibody chip at a flow rate of 5 µL/min for 300 s before the injection of analytes. SARSCov2RBD and spike protein were diluted in running buffer and a flow rate of 30 µL/min was employed for association and dissociation at a constant temperature of 25 °C. A 30 s injection of 10 mM glycine (pH 1.5) at a flow rate of 50 µL/min was used for regeneration and achieved prior baseline status. Using Biacore T100 evaluation software, the response curves of various analyte concentrations were globally fitted to the 1:1 binding model.

Competition experiments using immobilized ACE2 on streptavidin coated slides

Biotinylated ACE2 (ACRO Biosystems, AC2-H82F9) was printed on streptavidin coated glass slides (SuperStreptavidin microarray substrate slides, ArrayIt Inc) at 12.5, 25, and 50 µg/mL using a Scienion sciFLEXARRAYER S3 non-contact microarray equipped with a Scienion PDC80 nozzle (Scienion Inc) in replicates of six. The average drop volume was 400 pL. The slides were stored at 4 °C after printing and were blocked with TSM binding buffer (20 mM Tris·Cl, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 2 mM MgCl₂, 0.05% Tween-20, 1% BSA) for 1 h at 4 °C prior to use.

The protein (SARS-CoV-2 RBD-His or spike monomer-His 2.5 µg/mL) was premixed with AlexaFluor 647 conjugated anti-His antibody (2.5 µg/mL), followed by addition of human ACE2 as SARS-CoV-2 inhibitor (ACRO Biosystems, AC2-NA005) at various concentrations. The mixture was incubated at 4°C for 15 min before added to the microarray surface. The microarray slide was incubated at room temperature for 45 min, washed with TSM washing buffer, TSM buffer, water, and spun dried. The washing steps was carried out with the cassette attached to protect the unused blocks. After staining, the slides were scanned using a GenePix 4000B microarray scanner (Molecular Devices) at the appropriate excitation wavelength with a resolution of 10 µm. Optimum gains and PMT values were employed in the scanning to ensure that all the signals were within the linear range of the scanner's detector and there was no saturation of signals. The image was analyzed using GenePix Pro 7 software (version 7.2.29.2, Molecular Devices). The data was analyzed using a home written Excel macro. The highest and the lowest value of the total fluorescence intensity of the six replicates spots were removed, and the remaining four values were used to provide the mean value and standard deviation.

The ability of heparin to inhibit spike protein RBD or spike protein monomer by ELISA

ACE2 binding was determined by a colorimetric ELISA assay (SARS-CoV-2 inhibitor screening kit, ACROBiosystems). The assay was performed according to the manufacturer's instruction. Briefly, desired spike protein component was immobilized (2.5 µg/mL, 100 µL/well) at a high binding surface microplate (clear bottom) at 4 °C for 16 h. The microwells were then incubated with biotinylated human ACE2 (1 µg/mL, 50 µL/well) either in the presence of heparin (HG-heparin, 200 µg/mL, 50 µL/well) or in the absence of heparin (dilution buffer, 50 µL/well) at 37 °C for 1 h. Next, streptavidin-HRP (0.1 µg/mL, 100 µL/well) was added to each microwell and incubated at 37 °C for 1 h, followed by treatment with colorimetric HRP substrate (TMB One Component, SouthernBiotech, 200 µL/well) at 37 °C for 20 min. The reaction was terminated by the addition of a stop solution (1 M sulfuric acid, 50 µL/well) and absorbance was measured at 450 nm (POLARstar Optima, BMG labtech). The absorbance values were corrected for measured blank value and plotted using Prism 9 software (GraphPad Software, Inc.), bars represent the mean ± SD for each treatment. The assay was performed (in triplicate) at least two times.

Furin cleavage assay

Furin cleavage reactions were performed with 2 µg of SARS-CoV-2 spike protein, 1 µL furin and different concentrations of heparin or furin inhibitor II in 25 mM Tris-HCl

buffer (pH 7.0) with 2 mM of CaCl₂ in a total volume of 10 µL. The reactions were incubated at 37 °C for 1 h before the reaction was loaded on an SDS-gel for analysis.

Tissue binding experiments

Serial sections of formalin-fixed, paraffin-embedded ferret lungs were obtained from the Department of Veterinary Pathobiology, Faculty of Veterinary Medicine, Utrecht University and the Department of Viroscience, Erasmus University, The Netherlands, respectively. Tissue sections were rehydrated in a series of alcohol from 100%, 96% to 70%, and lastly in distilled water. Tissues slides were boiled in citrate buffer pH 6.0 for 10 min at 900 kW in a microwave for antigen retrieval and washed in PBS-T three times. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 30 min. Tissues were subsequently incubated with 3% BSA in PBS-T overnight at 4 °C. The next day, the purified viral spike proteins (50 µg/mL), a rabbit antibody against ACE2 (5 µg/mL; Abcam 272690) and a mouse antibody against heparan sulphate (10 µg/mL; Abcam 370255-S) were added to the tissues for 1 h at RT. With rigorous washing steps in between, the proteins were detected using goat anti mouse (AlexaFluor 555) and anti-rabbit (AlexaFluor 488) antibodies at 400 ng/mL (Life Biosciences). Where indicated, tissues were incubated overnight at 37 °C with 0.2 µg/mL heparanase (HPSE). HPSE was expressed in HEK 293T cells using Addgene plasmid #53407.⁵

Safety statement

No unexpected or unusually high safety hazards were encountered.

Supplementary Figures

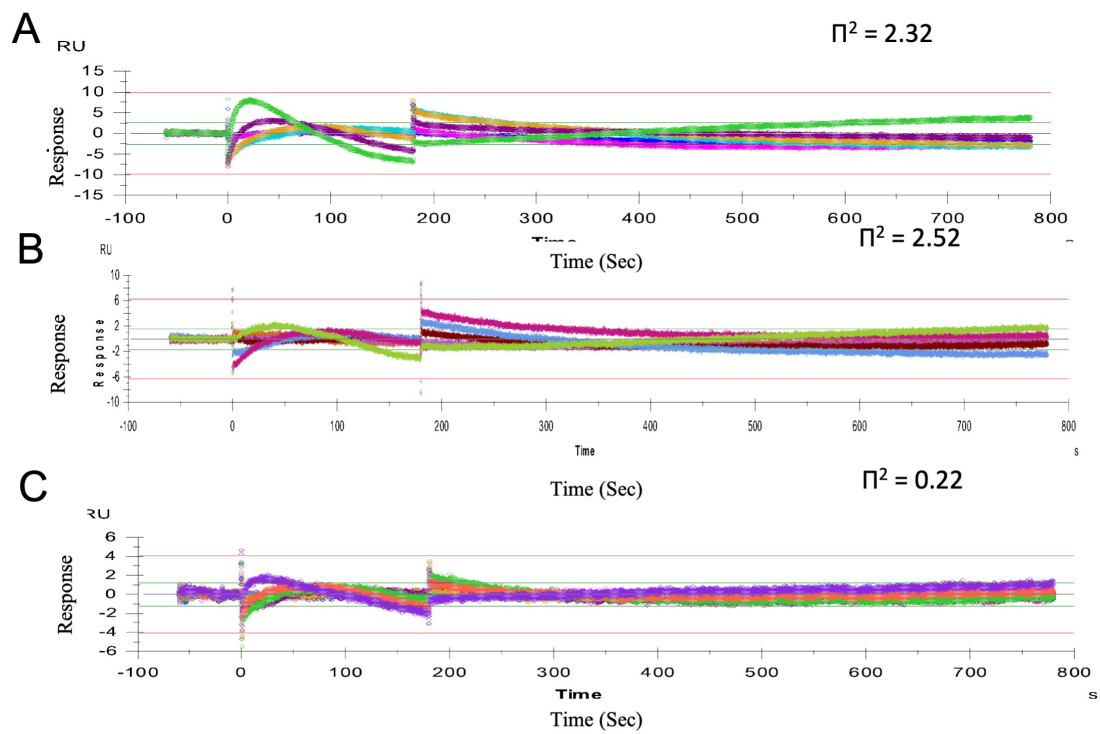
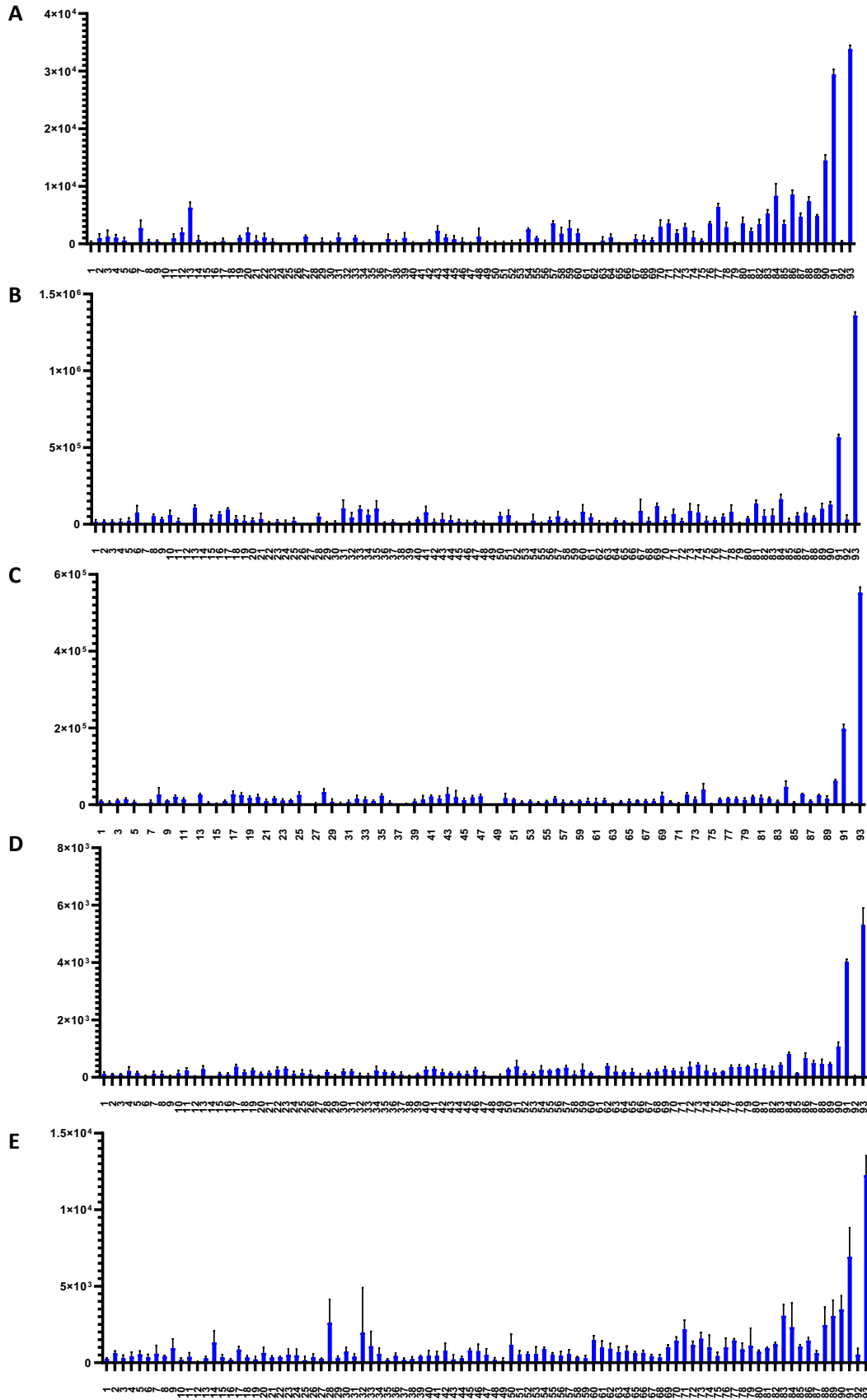


Figure S1. The residue plot and χ^2 of curve fitting for binding kinetic experiments of heparin interaction with SARS-CoV-2 related proteins (A) RBD, (B) spike monomer, and (C) spike trimer.



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1	IdoA-GlcNAc6S	32	GlcA-GlcNAc-IdoA2S-GlcNS6S	63	GlcA-GlcNS6S-GlcA-GlcNS6S-IdoA-GlcNS6S
2	GlcA-GlcNAc6S	33	GlcA-GlcNS-IdoA-GlcNS6S	64	GlcA-GlcNS6S-IdoA-GlcNS6S-IdoA-GlcNS6S
3	IdoA2S-GlcNAc6S	34	IdoA-GlcNS6S-GlcA-GlcNS	65	GlcA-GlcNS6S-GlcA-GlcNS3S-GlcA-GlcNS6S
4	IdoA2S-GlcNS6S	35	IdoA2S-GlcNAc6S-GlcA-GlcNAc6S	66	GlcA-GlcNS6S-IdoA-GlcNS3S-GlcA-GlcNS6S
5	GlcA-GlcNAc-GlcA-GlcNAc	36	IdoA2S-GlcNS-GlcA-GlcNS	67	GlcA-GlcNS6S-GlcA-GlcNS3S-IdoA-GlcNS6S
6	GlcA-GlcNAc-IdoA-GlcNAc	37	GlcA-GlcNS6S-IdoA-GlcNS	68	GlcA-GlcNS6S-IdoA-GlcNS3S-IdoA-GlcNS6S
7	GlcA-GlcNAc-IdoA2S-GlcNAc	38	IdoA-GlcNS-IdoA-GlcNS6S	69	GlcA-GlcNS6S-GlcA-GlcNS3S-IdoA2S-GlcNS6S
8	GlcA-GlcNAc-GlcA2S-GlcNAc	39	GlcA-GlcNAc6S-GlcA2S-GlcNAc6S	70	GlcA-GlcNS-IdoA2S-GlcNS6S-IdoA2S-GlcNS
9	GlcA-GlcNAc-IdoA-GlcNAc6S	40	IdoA-GlcNS6S-GlcA-GlcNS6S	71	GlcA-GlcNAc6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS
10	IdoA-GlcNAc6S-GlcA-GlcNAc	41	GlcA-GlcNS6S-IdoA-GlcNS6S	72	GlcA-GlcNS6S-GlcA-GlcNS3S6S-GlcA-GlcNS6S
11	IdoA2S-GlcNAc-GlcA-GlcNAc	42	GlcA-GlcNS6S-GlcA-GlcNS6S	73	GlcA-GlcNS6S-IdoA-GlcNS3S6S-GlcA-GlcNS6S
12	IdoA-GlcNAc-IdoA-GlcNAc6S	43	IdoA-GlcNS6S-IdoA-GlcNS6S	74	GlcA-GlcNS6S-IdoA2S-GlcNS6S-GlcA-GlcNS6S
13	IdoA-GlcNAc6S-IdoA-GlcNAc	44	GlcA-GlcNS-GlcA2S-GlcNS6S	75	GlcA-GlcNS6S-GlcA-GlcNS3S6S-IdoA-GlcNS6S
14	GlcA-GalNAc-GlcA-GalNAc4S	45	GlcA-GlcNS-IdoA2S-GlcNS6S	76	GlcA-GlcNS6S-IdoA-GlcNS3S6S-IdoA-GlcNS6S
15	IdoA-GlcNS-IdoA-GlcNAc	46	IdoA2S-GlcNS6S-GlcA-GlcNS	77	GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA-GlcNS6S
16	IdoA-GlcNAc-IdoA-GlcNS	47	IdoA2S-GlcNAc6S-IdoA2S-GlcNAc6S	78	GlcA-GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S
17	IdoA-GlcNAc6S-IdoA-GlcNAc6S	48	IdoA-GlcNS-IdoA2S-GlcNS6S	79	GlcA-GlcNS6S-IdoA-GlcNS6S-IdoA2S-GlcNS6S
18	IdoA-GlcNAc6S-GlcA-GlcNAc6S	49	IdoA2S-GlcNS6S-IdoA-GlcNAc6S	80	GlcA-GlcNS6S-IdoA2S-GlcNS3S-GlcA-GlcNS6S
19	GlcA-GlcNAc6S-IdoA-GlcNAc6S	50	GlcA-GlcNS6S-IdoA2S-GlcNS6S	81	GlcA-GlcNS6S-IdoA2S-GlcNS3S-IdoA-GlcNS6S
20	GlcA-GlcNAc6S-GlcA-GlcNAc6S	51	IdoA2S-GlcNS6S-GlcA-GlcNS6S	82	GlcA-GlcNS6S-IdoA-GlcNS3S-IdoA2S-GlcNS6S
21	GlcA-GlcNAc-GlcA2S-GlcNAc6S	52	IdoA-GlcNS6S-IdoA2S-GlcNS6S	83	GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS
22	GlcA-GlcNAc-IdoA-GlcNS6S	53	GlcA-GlcNS3S-IdoA2S-GlcNS6S	84	GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-GlcA-GlcNS6S
23	GlcA-GlcNAc-IdoA2S-GlcNAc6S	54	IdoA2S-GlcNS6S-IdoA2S-GlcNS	85	GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA-GlcNS6S
24	IdoA2S-GlcNAc6S-GlcA-GlcNAc	55	GlcA-GlcNS6S-GlcA2S-GlcNS6S	86	GlcA-GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S
25	GlcA-GalNAc4S-GlcA-GalNAc4S	56	IdoA2S-GlcNS6S-IdoA2S-GlcNS6S	87	GlcA-GlcNS6S-IdoA-GlcNS3S6S-IdoA2S-GlcNS6S
26	GlcA-GlcNS6S-GlcA-GlcNAc	57	GlcA-GlcNS3S6S-IdoA2S-GlcNS6S	88	GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S
27	IdoA-GlcNS-IdoA-GlcNS	58	GlcA-GlcNAc-IdoA2S-GlcNAc6S-GlcA-GlcNAc	89	GlcA-GlcNS6S-IdoA2S-GlcNS3S-IdoA2S-GlcNS6S
28	GlcA-GlcNAc6S-IdoA2S-GlcNAc6S	59	GlcA-GlcNS-IdoA2S-GlcNS6S-GlcA-GlcNS	90	GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S
29	IdoA-GlcNAc6S-IdoA2S-GlcNAc6S	60	GlcA-GlcNAc-IdoA2S-GlcNS6S-IdoA2S-GlcNS	91	IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S
30	GlcA-GlcNS-IdoA2S-GlcNS	61	GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S	92	GlcA-GlcNS6S-IdoA-GlcNS-IdoA2S-GlcNS6S-IdoA-GlcNAc6S
31	GlcA-GlcNS-GlcA2S-GlcNS	62	GlcA-GlcNS6S-IdoA-GlcNS6S-GlcA-GlcNS6S	93	IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S

Figure S2. Binding of synthetic heparan sulfate oligosaccharides to SARS-CoV-2-related proteins, including (A) RBD-His (100 µg/mL), (B) RBD-Fc (10 µg/mL), (C) RBD trimer-streptavidin (3 µg/mL), (D) Spike monomer-His (3 µg/mL), and (E) Spike trimer-streptavidin (10 µg/mL). (F) Compound numbering and structures.

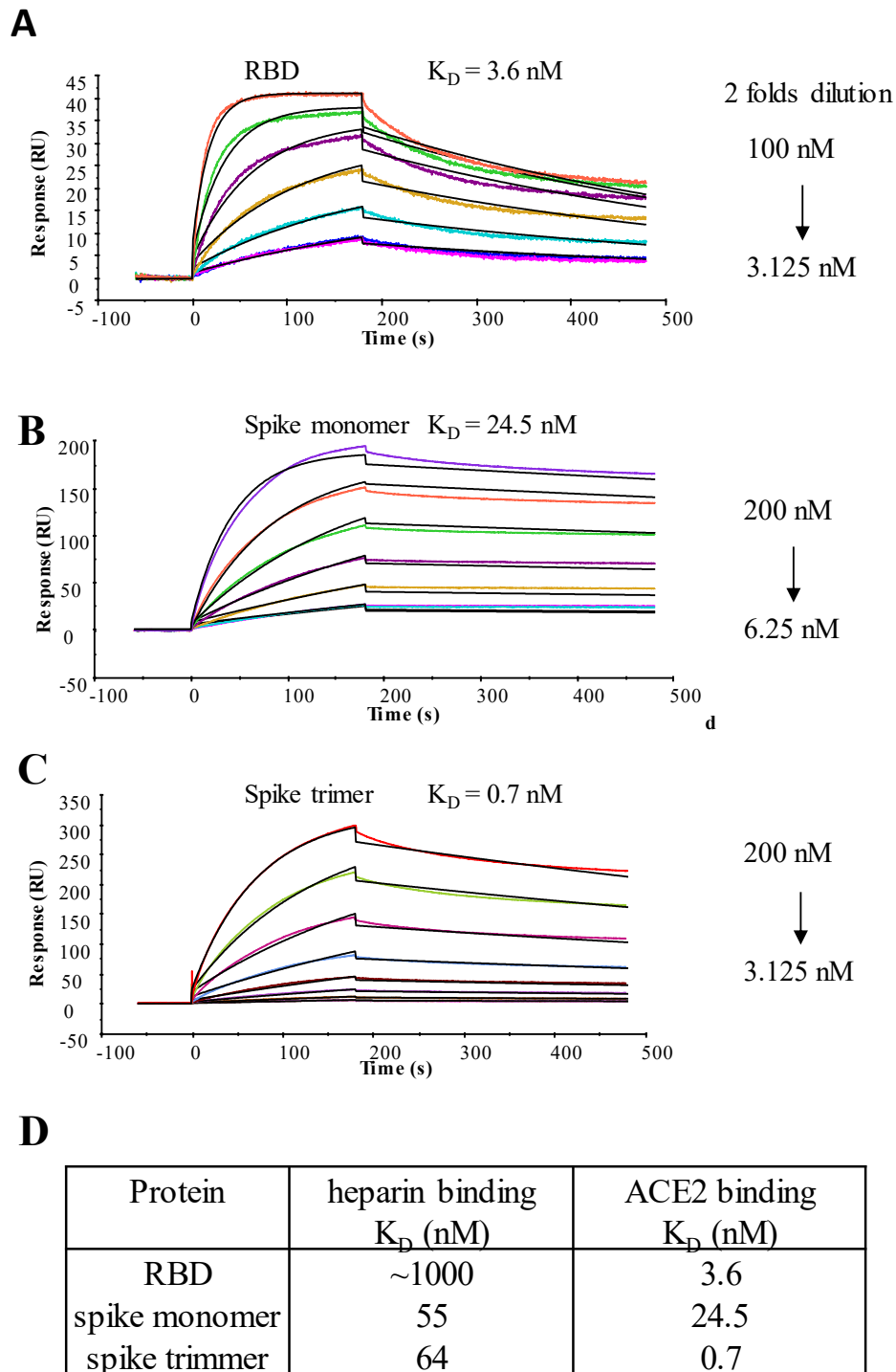


Figure S3. Sensorgrams representing the concentration-dependent kinetic analysis of the binding of immobilized ACE2 with SARS-CoV-2 derived proteins (A) RBD, (B) spike monomer, and (C) spike trimer. (D) Comparison of the K_D values of heparin binding and ACE2 binding to SARS-CoV-2 related proteins.

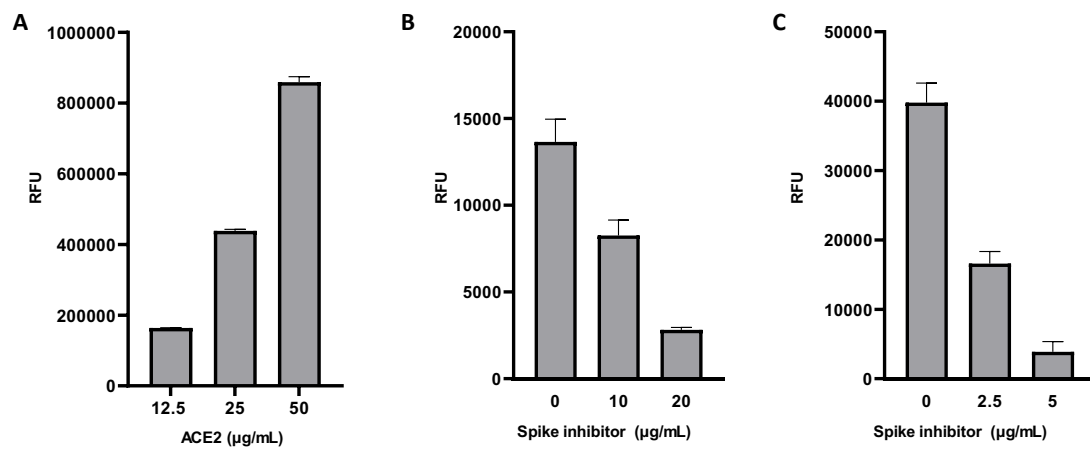


Figure S4. (A) Dose response of ACE2-Fc-biotin printed on streptavidin-coated slides at the indicated concentrations and detected by a goat-anti-human Fc antibody conjugated with AlexaFluor 647. (B) Humans ACE2 efficiently inhibits RBD-ACE2 and (C) spike-ACE2 binding. In (B) and (C) ACE2-Fc-biotin was printed at 50 µg/mL.

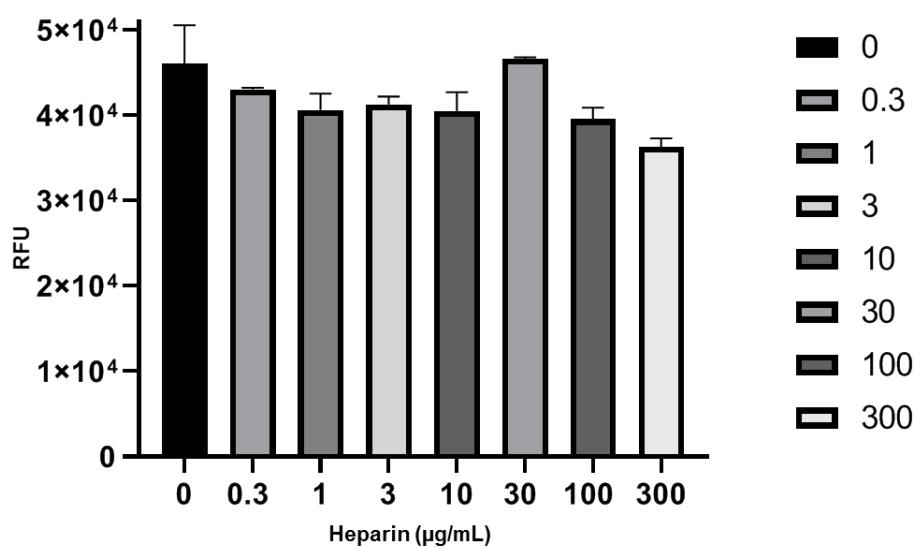


Figure S5. Extended dataset on the effect of heparin on binding of spike monomer to ACE2 immobilized on microarray slides. The concentration of heparin is from 0 to 300 $\mu\text{g/mL}$.

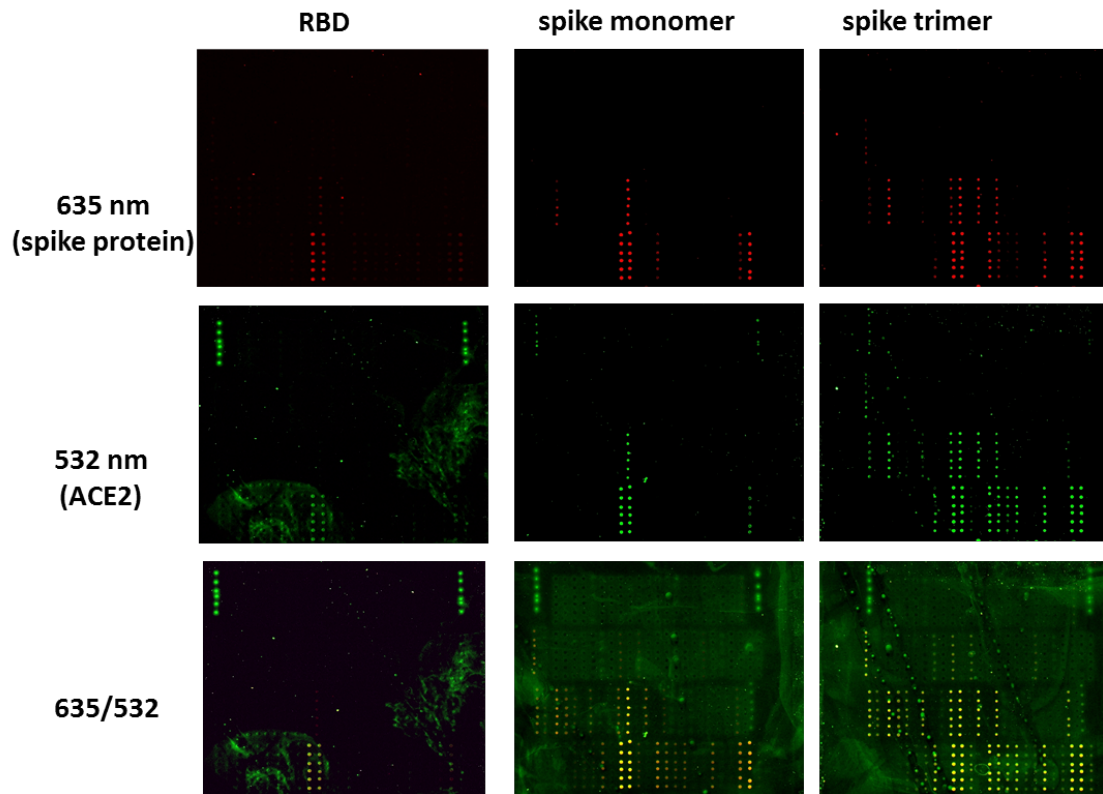


Figure S6. Microarray scanning images of the spike protein-ACE2 complex binding on the heparan sulfate array. Spike proteins were detected at 635 nm, while ACE2 was detected at 532 nm.

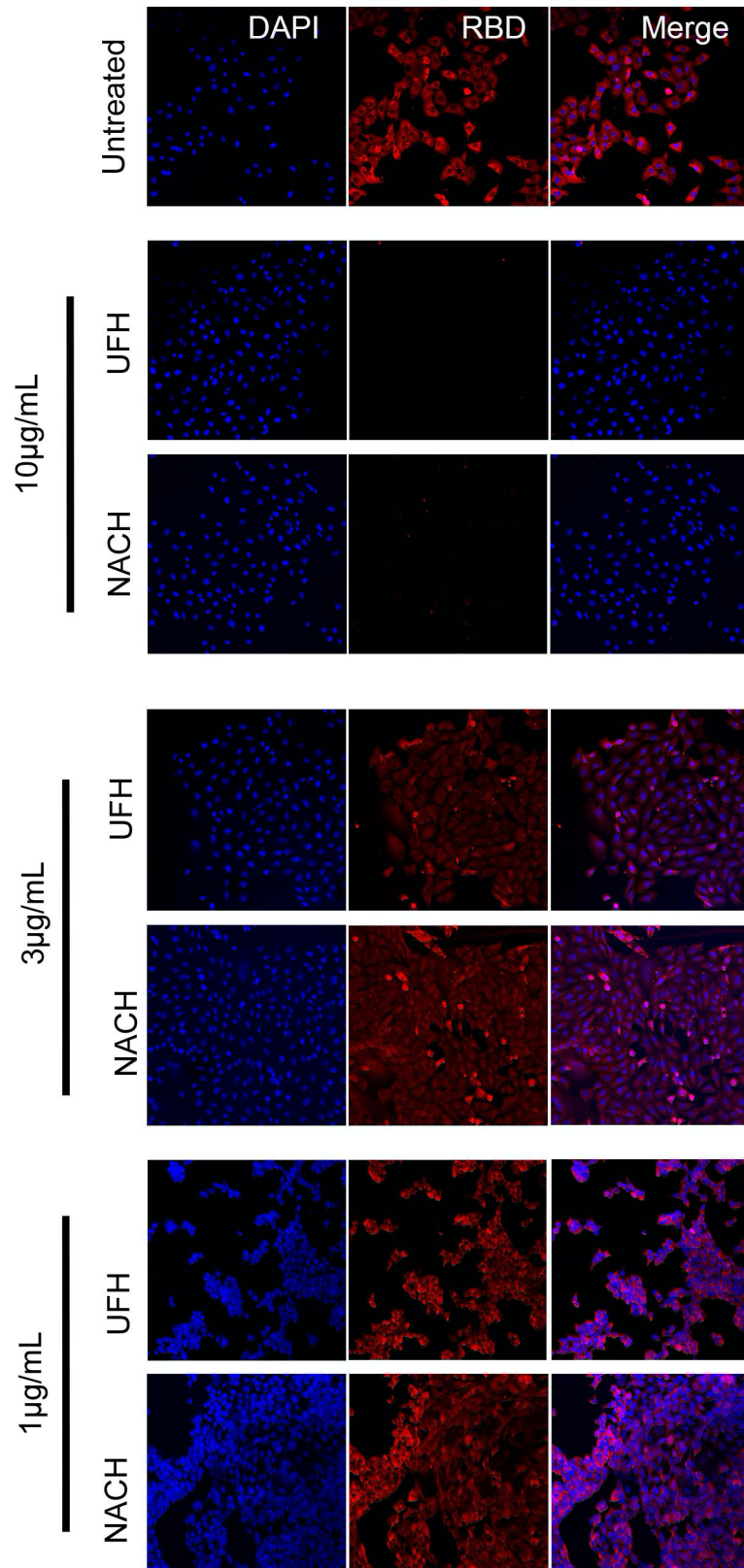


Figure S7. Binding of SARS-CoV-2 RBD pretreated with GAGs to Vero-E6 cells. (A) top-to-bottom: Untreated RBD, Unfractionated heparin (UFH) and Non-anticoagulant heparin (NACH) in a dilution series.

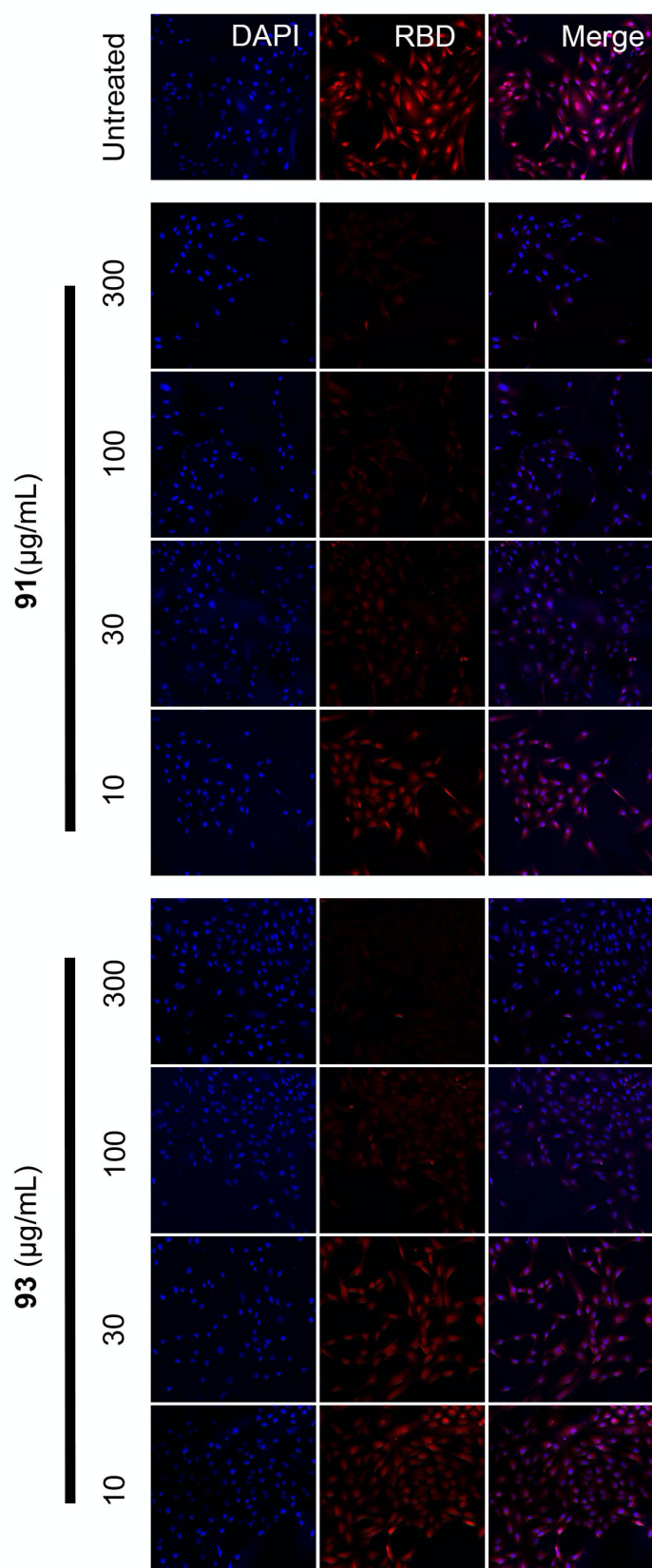


Figure S8. Binding of SARS-CoV-2 RBD pretreated with compounds 91 and 93 in a dilution series to Vero-E6 cells.

References

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