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A Universal DNA Aptamer as an Efficient Inhibitor against Spike-Protein/hACE2 interactions

Achut Prasad Silwal, Raunak Jahan, Siddhartha Kalpa Samadhi Thennakoon, Satya Prakash Arya, Rick Mason Postema, Elizabeth Claire VanderArk, and Xiaohong Tan*

Department of Chemistry and Center for Photochemical Sciences, Bowling Green State University

*To whom correspondence should be addressed: tanx@bgsu.edu

Supporting Information

Supporting Figures:

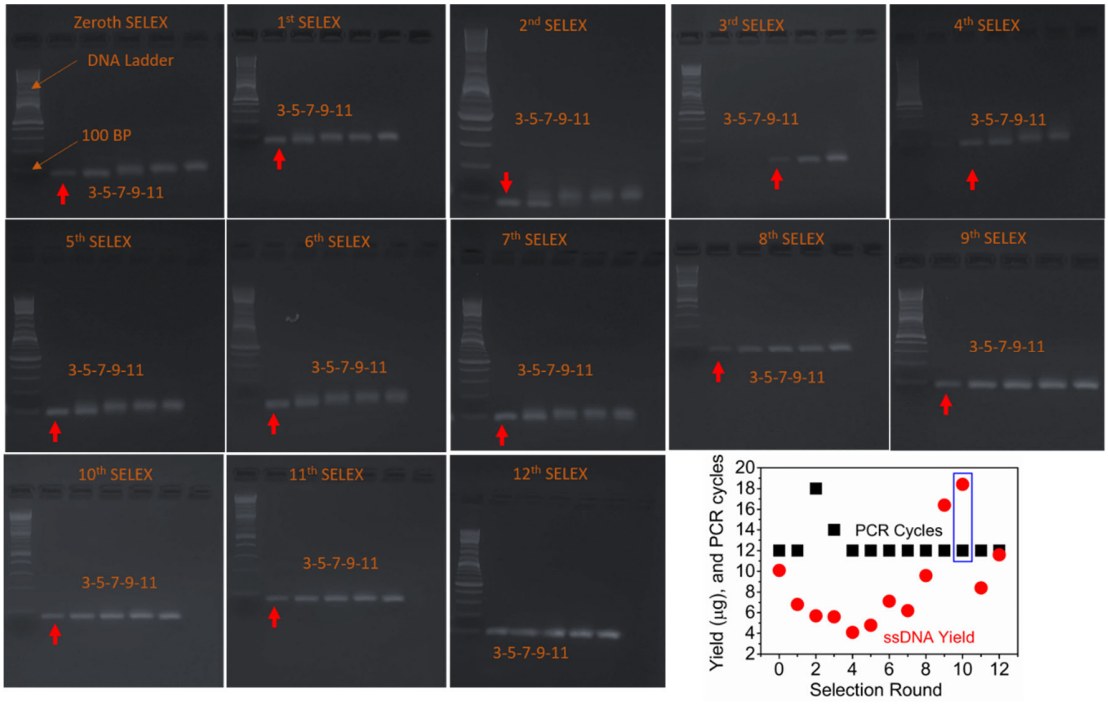

Figure S1. The gel electrophoresis data corresponding to the selection process of the A1C1 aptamer. The gel electrophoresis data assesses the quality of the PCR product to determine the optimized number of PCR cycles (indicated by the red arrow) required for the DNA amplification. The correct size of the PCR product had 73 base pairs of nucleotides. The DNA ladder was used to characterize the size of the PCR products. Prior to the optimization, all selection rounds underwent 9 PCR cycles. The relationship between yield and PCR cycles over the selection rounds (lower right corner) indicates that the 10th selection round has the highest enrichment.

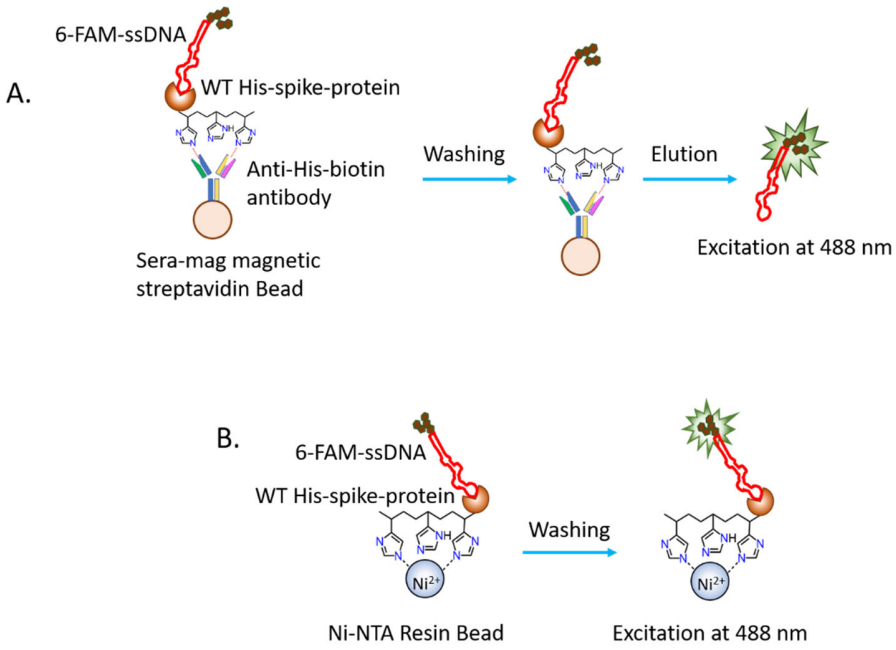

Figure S2. The schematic representation of the fluorescence emission measurement experiments (not to scale). (A) The specific 6-FAM-ssDNA strongly binds to the His-tagged target protein and persists to remain after washing. The bound 6-FAM-ssDNA was eluted by a hot SELEX buffer at 95°C, and the fluorescence emission intensity from the elution was measured by a Clariostar microplate reader at λ_{max} = 520 nm with excitation by 488 nm light. (B) The ssDNA specific to spike-protein has stronger interaction with the His-tagged WT spike-protein; it remains after washing and imparts the brighter fluorescence image to the Ni-NTA resin bead. The 6-FAM-ssDNA bound to Ni-NTA resin bead coupled with WT spike-proteins were collected using a digital inverted fluorescence microscope (Invitrogen EVOS FL).

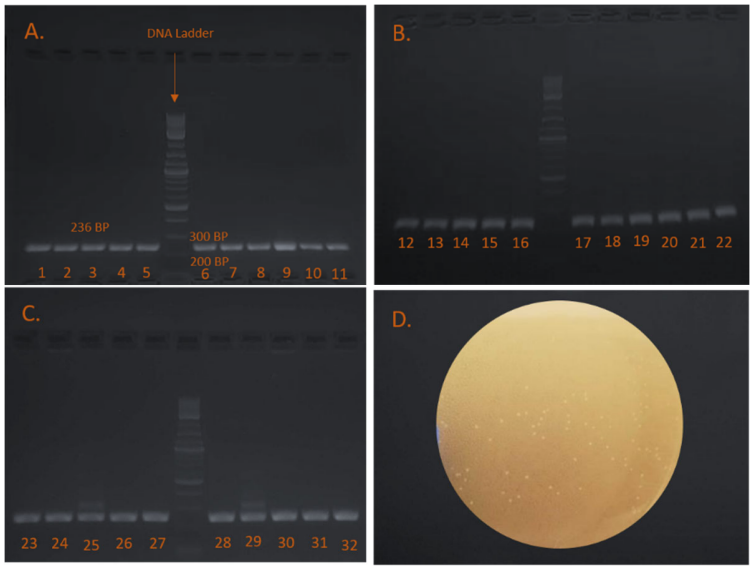

Figure S3. (A-C) The gel electrophoresis data of the bacterial PCR product, the desirable insert has 236 nucleotides. The 1 µL of bacterial culture was used for the PCR, and the remaining was used to extract the purified plasmids for DNA sequencing. We measured the concentrations of the purified plasmids and used 24 plasmid samples with higher concentrations for the DNA sequencing. (D) The ampicillin-resistant bacteria culture in the agar plate. The purified dsDNA received from the 10^{th} round of selection was ligated with the TOPO vector, and the recombinant DNA was used to transform the *E. coli* component cell. Each single colony of the bacteria was transferred to culture the bacteria in Luria broth solution.

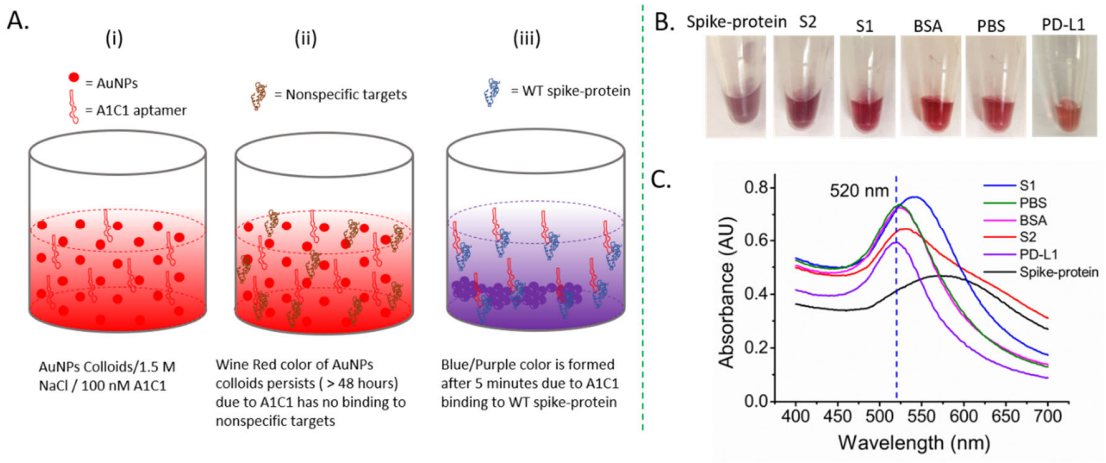

Figure S4. The Schematic representation of gold nanoparticle-based colorimetric assay to measure the specificity of the A1C1 aptamer against WT spike-protein. The addition of non-specific targets (PBS, BSA) to AuNPs/NaCl/A1C1 did not affect the AuNPs/aptamer interaction; hence, the red color was intact for more than 48 h (ii). The addition of 250 nM WT spike-protein dramatically changed AuNPs color to purple or blue within 5 minutes, suggesting A1C1 is preferably bound to WT spike-protein (iii). (B) Specificity test for the A1C1 aptamer using an AuNPs-based colorimetric assay. The addition of nonspecific targets, such as phosphate buffer saline (PBS), bovine serum albumin (BSA) to the colloids of AuNPs, A1C1 aptamers and NaCl, they did not affect the aptamer; hence, red-wine color persisted. When spike-protein was added, it removed the A1C1 from the vicinity of AuNPs, and blue or purple color was observed. (C) UV-Vis absorption spectra from the colloids of AuNP, A1C1, and NaCl after addition of WT spike-protein, WT S1-protein, WT S2-protein, BSA, or PBS. The redshift from 520 nm indicates the aggregation of the AuNPs colloids.

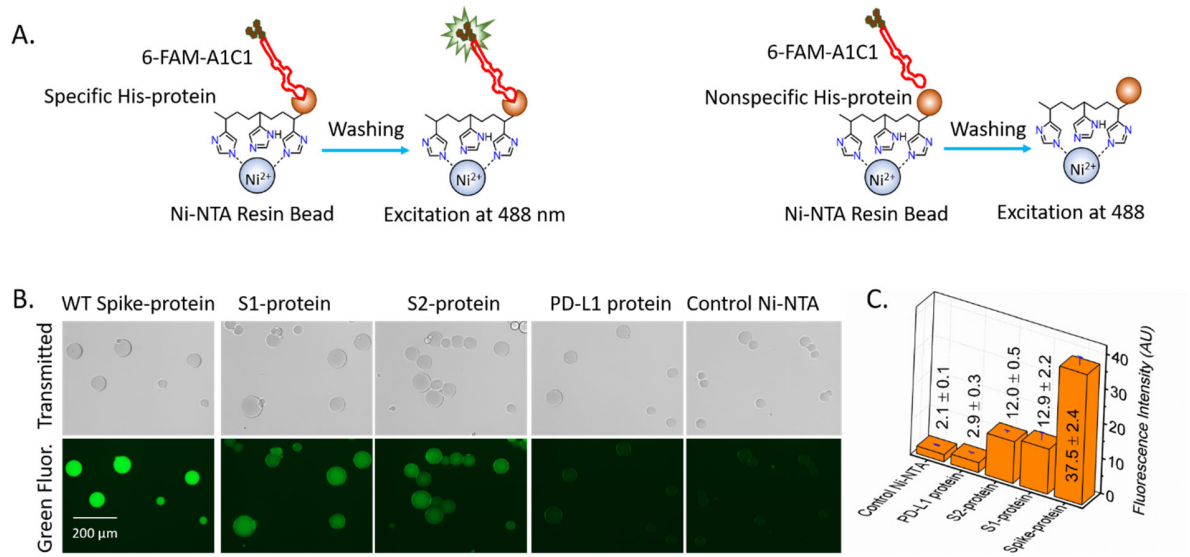

Figure S5. The fluorescence microscopy imaging to measure the binding potential of A1C1 aptamer to various targets. (A) The schematic representation of the A1C1 interaction with the His-tagged WT spike-protein and non-specific protein in a course of fluorescence imaging experiment. The 6-FAM-A1C1 aptamer persists to remain on Nickel-nitrilotriacetic acid (Ni-NTA) resin bead after washing only in the presence of specific His-tag protein. Therefore, fluorescence images of the 6-FAM-A1C1 and Ni-NTA resin bead has a brighter fluorescence emission in the presence of WT spike-proteins in comparison to nonspecific His-tag proteins. The fluorescence images were collected using a digital inverted fluorescence microscope (Invitrogen EVOS FL) with the excitation by 488 nm light. (B) The fluorescence images of the 6-FAM-A1C1 aptamer and Ni-NTA resin beads in the presence of various His-tag proteins to determine the binding potential of the A1C1 aptamer against isolated S1- and S2-, WT spike-protein, and controlled protein like PD-L1 proteins. (C) The average fluorescence intensities of the 6-FAM-A1C1 aptamers bound to the Ni-NTA beads coupled with WT spike-protein, S1-protein, S2-protein, PD-L1 protein, and control Ni-NTA beads alone. For binding to the FAM-A1C1 aptamer, S1-protein, S2-protein, PD-L1 protein, and control Ni-NTA shows 34.4, 31.9, 7.6, and 5.5 % fluorescence intensity, respectively, in comparison to the normalized 100% fluorescence intensity produced by the intact WT spike-protein. The ImageJ software was used for the quantification of the fluorescence intensity of the fluorescence microscopy images. The A1C1 aptamer preferably binds to the intact spike-protein over S1 or S2-protein alone. The binding potential of the A1C1 aptamer to various proteins in the trend of WT spike-protein > S1-protein ≈ S2-protein >> PD-L1 protein.

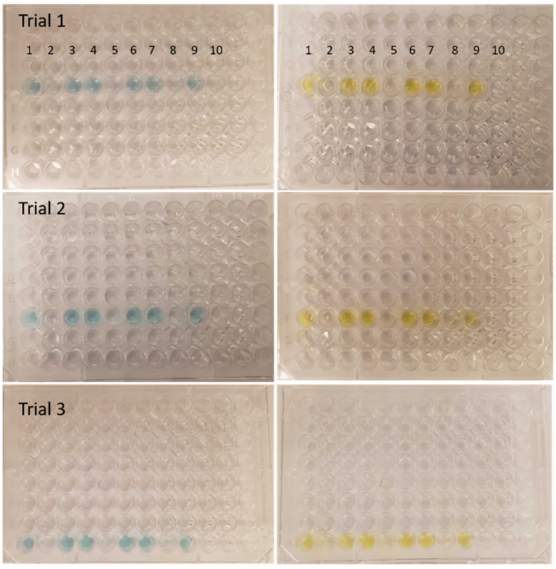

Figure S6. The ELISA data determined the neutralization efficacy of the aptamer against the Omicron Variant spike-protein. (A) The well plate shows the intensity of the color product formed due to HRP mediated oxidation of TMB. The left and right panels were obtained from the same sample before and after the use of 2 µL of concentrated H_2SO_4, respectively. The sample description is as follows: 1 = WT spike-protein w/o aptamer, 2 = WT spike-protein with the A1C1 aptamer, 3 = WT spike-protein with the random sequence, 4 = Delta spike-protein w/o aptamer, 5 = Delta spike-protein with the A1C1 aptamer, 6 = Delta spike-protein with the random sequence, 7 = Omicron spike-protein w/o aptamer, 8 = Omicron spike-protein with the A1C1 aptamer, 9 = Omicron spike-protein with the random sequence, 10 = TMB buffer. The absorbance from the yellow color was measured at λ_{max} = 450 nm. The neutralization efficacy of the aptamer was analyzed as an inversely related property of the absorbance. The absorbance measurement showed that A1C1 aptamer reduces the absorbance by approximately 89.1, 87.3, and 85 %, which was contributed by the WT, Delta, and Omicron spike-protein/hACE2 interactions, respectively.

Table S1: The details of the selection.

Selection Round	WT spike-protein (pmol)	Counter Selection (Ni-NTA bead/ incubation time)	Binding Time (min)	Washing repetition	Total PCR Cycles	Yield (µg)
0	100				9+3=12	10.1
1	100		60	2	9+3= 12	6.8
2	100		60	2	9+3=12	5.7
3	100	2 µL, 5 min	60	3	9+9=18	5.6
4	50		50	3	9+5=14	4.1
5	50	2 µL, 10 min	50	4	9+3=12	4.8
6	50	2 µL, 10 min	50	4	9+3=12	7.1
7	50		50	5	9+3=12	6.2
8	25	2.5 µL, 15 min	40	5	9+3=12	9.6
9	25	2.5 µL, 15 min	40	6	9+3=12	16.4
10	25		30	6	9+3=12	18.4
11	25	2.5 µL, 20 min	30	7	9+3=12	8.4
12	25	3 µL, 20 min	20	7	9+3=12	11.6

Table S2: The fluorescence emission from the bound 6-FAM-ssDNA over various selection rounds.

Selection Round	Emission at $\lambda_{max} = 520$ nm							
	Before Background Correction			After Background Correction				
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Average	Standard Error
Buffer	558	590	551	0	0	0	0	0
0	3570	3837	3329	3570	3937	3229	3578.7	354.1
7	8725	9594	8822	8725	9594	8822	9047	476.2
8	17680	19006	17476	18680	19006	17476	18387.3	805.9
9	20818	22379	20578	21818	22379	20578	21591.7	921.6
10	22580	24273	22320	22580	24273	22320	23057.7	1060.5

Table S3: The Sequence obtained from purified plasmid samples. The randomized sequences of the aptamers are flanked by reverse and forward primers on their 5' and 3' ends, respectively, as represented by 5'-GGAGGCTCTCGGGACGAC-N40-GTCGTCCCGCCTTTAGGATTTACAG-3'.

Aptamer	Sequences	Percentage (%)
SA1	5'-GGAGGCTCTCGGGACGACGACGGACATCGTGAGAAATGGTCGACCTTGTGTCTGTCGTCCCGCCTTTAGGATTTACAG-3'	50
SA2	5'-GGAGGCTCTCGGGACGACTCTCGTAGGGATGGGTTGAGGGATAGGGTTCGCTGTGTCGTCCCGCCTTTAGGATTTACAG-3'	16.7
SA3	5'-GGAGGCTCTCGGGACGACGTGCAGGCACAACTAATGCTAATGATTGTCTGTCATGTCGTCCCGCCTTTAGGATTTACAG-3'	8.4
SA4	5'-GGAGGCTCTCGGGACGACTAGGCACTGCAAGGGATTACTATTCCGACAGTC5'-TACGTCGTCCCGCCTTTAGGATTTACAG-3'	8.4
SA5	5'-GGAGGCTCTCGGGACGACCGCCGGTACGGACATCGAGTACTTTGTGTATCCGCAGTCGTCCCGCCTTTAGGATTTACAG-3'	8.4
SA6	5'-GGAGGCTCTCGGGACGACGTTCTTCTTGGAGGGAGGGGATGTGGGGTGGGATGGGTCGTCCCGCCTTTAGGATTTACAG-3'	8.4

Table S4: The measurement of the absorbance (λ_{450} nm) in ELISA competition Assay to test the inhibition efficacy of A1C1 aptamer against the spike-protein/hACE2 interaction.

Samples		Absorbance (λ_{450} nm)							
		Before Background correction			After Background correction				
Protein	Aptamer	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Average	Standard Error
Omicron spike-protein	Random Sequence	0.472	0.495	0.495	0.432	0.455	0.455	0.447333	0.013279
	A1C1 Aptamer	0.105	0.112	0.121	0.065	0.072	0.081	0.072667	0.008021
	W/o Aptamer	0.522	0.53	0.534	0.482	0.49	0.494	0.488667	0.00611
Delta spike-protein	Random Sequence	0.508	0.527	0.506	0.468	0.487	0.466	0.473667	0.01159
	A1C1 Aptamer	0.106	0.113	0.112	0.066	0.073	0.072	0.070333	0.003786
	W/o Aptamer	0.578	0.598	0.597	0.538	0.558	0.557	0.551	0.011269
WT spike-protein	Random Sequence	0.51	0.523	0.461	0.47	0.483	0.421	0.458	0.032696
	A1C1 Aptamer	0.098	0.103	0.093	0.058	0.063	0.053	0.058	0.005
	W/o Aptamer	0.573	0.572	0.576	0.533	0.532	0.536	0.533667	0.002082
NA	TMB	0.04	0.04	0.04	0	0	0	0	0

Materials and Methods

Chemicals and reagents. All proteins used in this work, including hACE2 and the WT and variant SARS-CoV-2 spike-proteins, were purchased from Sino Biological and used without further purification. All aptamers and other nucleic acids were obtained from Integrated DNA Technologies, Inc. (www.idtdna.com) as lyophilized powders and were dissolved in nanopure water upon receipt. All chemicals were purchased from Sigma unless mentioned otherwise.

SELEX procedure. We performed the DNA-SELEX using the WT spike-protein as a target. We used an oligonucleotide library obtained from IDT which is composed of 40 random nucleotides flanked by constant primer sequences (Table S3). For the first round of the selection, 100 pmol of the spike-protein and 1 µL of nickel nitrilotriacetic acid (Ni-NTA) beads (Ref# 062N-A; G-bioscience) were diluted into 100 µL of SELEX buffer (PBST-Mg buffer, PBS with 1mM $MgCl_2$, pH 7.4, 0.01% tween) and incubated at room temperature (RT), rotating for 1 h. Meanwhile, 3 nmol of DNA library was diluted into 100 µL of PBST-Mg and treated at 95 °C for 5 min, on ice (or 4 °C) for 5 min, RT for 5 min, and placed in ice. When 1 h of incubation was completed, the protein-bead (P-B) complex was washed two times by 200 µL SELEX buffer and combined with a heat-treated DNA library, with 1 µL of 100 times concentrated tRNA, and incubated for 1 h at RT with rotation. After incubation, the protein-bead-library (PBL) complex was washed two times with 200 µL SELEX buffer to remove the unspecific library. After washing, the bound library was eluted 2 times by 30 µL of hot water at 95°C. The selected library was amplified by polymerase chain reaction (PCR). For the first round of selection, the PCR mixture contained 60 µL of the library, 39 µL of nuclease-free water, 100 µL of 2 × PCR solution, and 1 µL of Easy Taq polymerase. The 50 µL of the PCR mixture was loaded into each PCR tube and amplified in the conditions of 2 min at 95°C; 9 cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C, 2 min at 72°C. After completing PCR, all PCR product was collected in a tube. To optimize the PCR cycle number for bulk amplification, 5 µL of the PCR product, 119 µL water, 125 µL of 2 × PCR solution, and 1.25 µL Easy Taq polymerase were mixed in a tube and then distributed equally (50 µL) into 5 PCR tubes. Amplification conditions were 2 min at 95°C; 3-11 cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C, 2 min at 72°C. The PCR tubes were taken out in 3, 5, 7, 9, and 11 cycles, respectively, and kept in ice. Then, PCR products were assessed with 2 % agarose gel electrophoresis to determine the suitable number of PCR cycles (X). The suitable number of PCR cycles would provide the right PCR product and was confirmed by a brighter and smear-free band at 73 base pairs (Figure S1). Once the number of suitable PCR cycles (X) was determined, the bulk PCR reaction was run to generate 1 (or 2) mL of PCR mixture (20 µL of the 1st round PCR solution, 475 µL water, 500 µL of 2 × PCR solution, and 5 µL of Easy Taq Polymerase). The PCR amplification conditions were set to 2 min at 95°C; X cycles of 45 s at 95°C; 30 s at 54°C; 30 s at 72°C; 2 min at 72°C.

After a bulk PCR, 20 µL of neutravidin beads were washed two times by 400 µL of SELEX buffer and incubated with 1 mL of PCR products for 15 min rotating at RT. Then, the beads were washed two times with 400 µL of the SELEX buffer. The sense strand was separated from the beads by denaturing in 200 µL of 100 mM NaOH solution for 1 min; the solution was immediately neutralized by 0.2 M HCl. Then, the beads were again eluted by 212 µL of SELEX buffer, combined with the previous solution, and centrifuged using a desalting column (3K) at 12000 g for 10 min. The remaining solution in the desalting column was washed two times using 400 µL of the SELEX buffer. The eluted library was quantified by nanodrop. Then, it was treated at 95 °C for 5 min, ice for 5 min, and RT for 5 min and stored at -20°C.

For subsequent rounds of selection, the 100 pmol DNA library (~2 µg) was incubated with 100 pmol protein (bead complex). The amount of protein and incubation time were consistently decreased for the following selection rounds to increase the selection pressure (Table S1), while the number of washes to the PBL complex was consistently increased to ensure the removal of the unspecific libraries. The bound libraries were eluted two times by 30 µL of hot water at 95°C. Then, 20 µL from the total 60 µL elution was used for the PCR amplification, and the remaining 40 µL was stored at -20° C. The PCR amplification, purification, desalting, and quantification were similarly followed for the subsequent rounds as they were in the first SELEX. However, after the second SELEX, we introduced the counter selection (CS) in every other round. For that, we incubated the ssDNA with the unembellished Ni-NTA magnetic beads. The unspecific library bound to the magnetic beads was discarded, while the specific library present in the supernatant was used to start the next round of selection.

Plasmid preparation for DNA sequencing. The label-free dsDNA obtained from the 10^{th} selection round was purified using NucleoSpin Gel and PCR Clean-up kit (Ref# 740609-250; Macherey-Nagel) and used as an insert. We used the TOPO TA cloning kit (Ref# 45-0071; invitrogen) for ligation and transformed the *E. coli* component cells using recombinant DNA. The ampicillin-resistant bacterial colonies were cultured on Luria broth (LB) agar plates containing ampicillin (100 µg/mL), following the standard protocol. The bacterial culture was subjected to PCR to assess the correct insert using gel electrophoresis (Figure S3). The plasmid containing the desirable insert was extracted from the bacterial solution using E.Z.N.A.® Plasmid DNA Mini Kit (Ref# D6942-01; Omega Bio-Tek) and sequenced by the Human Genetics Comprehensive Cancer Center DNA Sequencing Facility at the University of Chicago.

ELISA assay with HRP anti-His tag antibody. The 0.5 µg hACE2 in 50 µL 0.1 M $NaHCO_3$ (pH 8.6) was added to the high-binding 96 well plates (Ref# 12565501; Fisher brand) and incubated overnight. The solution was removed and incubated with 100 µL of 5 mg/mL BSA in 0.1 M $NaHCO_3$ for 1 h at RT and washed 3 times with 200 µL SELEX buffer containing Tween 20. Then, a 50 µL solution of 100 nM His-tagged spike-protein and the aptamer in SELEX buffer was incubated at RT for 1 h. The plate was then

washed six times with 200 µL SELEX buffer to remove the unbound spike-protein. Then, 50 µL of 2000 times diluted anti-His-tagged HRP antibody (catalog# AE028; ABclonal) in SELEX buffer was added, which bound to the remaining His-tagged spike-protein. The wells were incubated at RT for 30 min and washed six times. When the aptamer shows the capacity to block spike-protein/hACE2 interaction, HRP will not persist in the well plate after washing. Finally, 50 µL of TMB substrate solution was added to the well and incubated for 30 minutes at RT. The intense blue color produced in this step was caused by the strong spike-protein/hACE2 interaction. When 2 µL of concentrated sulfuric acid was added to the blue product, the yellow color was formed. The absorbance of the yellow product was measured at λ_{max} = 450 nm using the Clariostar microplate reader (BMG LABTECH).

Fluorescence microscope-based binding assay. The 20 µL of 10 times diluted Hispur Ni-NTA resin bead (Ref# 88221; Thermo scientific) was washed two times with 500 µL of SELEX buffer, resuspended in 50 µL of SELEX buffer, and incubated with 5 pmol of His-tagged target protein for 30 minutes at RT with rotation. The resin-protein complex was washed two times with 500 µL of SELEX buffer to remove unbound protein and resuspended with 50 µL of SELEX buffer. The 10 pmol 6-FAM-labeled ssDNA or aptamer was incubated in this resin-protein complex at RT for 30 min. After washing two times with 500 µL of SELEX buffer, the complex was finally resuspended in 50 µL of SELEX buffer and transferred on a glass slide for the fluorescence measurement. The fluorescence images were collected using both the green fluorescence and transmitted light channels by the digital inverted fluorescence microscope (Invitrogen EVOS FL). The fluorescence microscopy images were analyzed by the software Image J.

Determination of binding capability. The 100 nM His-tagged protein and anti-His-tagged biotinylated antibody were incubated at RT for 2 h in 50 µL of SELEX buffer. The protein-antibody complex was then incubated with 2 µL of sera-mag magnetic streptavidin-coated particles (Ref# 30152103010150; Cytiva) at RT for 2 h and stabilized overnight at 4°C. After washing two times with 200 µL of SELEX buffer, the protein-bead complex was incubated with the 6-FAM-labeled aptamer of 100 nM concentration in 1.5 mL Eppendorf tubes for 2 h. The unbound aptamer was removed by washing three times with 200 µL of SELEX buffer; the bound aptamer was eluted using 30 µL of hot SELEX buffer at 95°C. The fluorescence intensity from the sample at 520 nm was collected using the Clariostar microplate reader (BMG LABTECH).

Synthesis of gold nanoparticles. A three-necked round-bottom flask was cleaned with freshly prepared aqua regia (concentrated HNO_3 and HCl in 1:3 molar ratio), rinsed with nuclease-free water, and perfectly dried before use. The AuNPs colloid was synthesized from $KAuCl_4$ (Ref# 334545-1G; Sigma Aldrich) precursor using the classical citrate reduction method. Briefly, 100 mL of 1 mM $KAuCl_4$ solution was heated to boiling. Then, 2 mL of 194 mM sodium citrate solution (CAS# 1545801, Sigma Aldrich) was added, and the mixture was boiled for an additional 15 min with good stirring. The color of the solution changed from

yellow, clear/gray, and finally to dark wine-red. After 15 min of boiling the reaction, the flask was taken out and cooled slowly to room temperature.

Flow cytometry experiment to measure the binding affinity of the aptamers. The 100 µL of 200 nM His-tagged spike-protein was prepared in SELEX buffer and incubated with 1 µL of Ni-NTA magnetic beads (Ref# 062N-A; G-Biosciences,), rotating for 1 h at RT. The Ni-NTA bead/spike-protein complex was washed twice with 200 µL of SELEX buffer and incubated with 100 µL of 3, 10, 30, 100, 300, and 1000 nM of 6-FAM-labeled aptamer prepared in SELEX buffer for 1h at RT with rotation. After incubation, the beads were washed two times with 200 µL of SELEX buffer and finally resuspended with 100 µL of SELEX buffer. The 6-FAM-labeled aptamers bound to the protein/bead complex were analyzed by Flow cytometry (Catalog # 0500-4005; Guava easyCyte 5HT), counting approximately 5000 events. Each experiment was run for three trials to calculate the mean fluorescence intensity (X_C) and standard error. The binding affinity (K_d) of the 6-FAM-A1C1 aptamer against spike-protein was determined by an intensity vs. concentration plot using the Origin software.