Biosensors and Bioelectronics 228 (2023) 115202

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ultra-sensitive label-free SERS biosensor with high-throughput screened DNA aptamer for universal detection of SARS-CoV-2 variants from clinical samples

Ki Sung Park [a], Anna Choi [a], Hyun Jung Kim [a,b], Insu Park [a,c], Mi-Suk Eom [d], Sang-Gu Yeo [d], Ryeo Gang Son [a], Tae-In Park [a], Gyudo Lee [a], Hyongsok Tom Soh [e,f], Yoochan Hong [b,**], Seung Pil Pack [a,*]

[a] Department of Biotechnology and Bioinformatics, Korea University, Sejong, 30019, Republic of Korea
[b] Department of Medical Device, Korea Institute of Machinery and Materials (KIMM), Daegu, 42994, Republic of Korea
[c] Department of Biomedical Engineering, Konyang University, Daejeon, 35365, Republic of Korea
[d] Division of Infectious Diseases, Sejong Institute of Health & Environment, Sejong, 30015, Republic of Korea
[e] Department of Electrical Engineering, Stanford University, Stanford, CA, 94305, USA
[f] Department of Radiology, Stanford University, Stanford, CA, 94305, USA

ARTICLE INFO

Keywords:
SARS-CoV-2 spike binding aptamer
Particle display
Silver nanoforest
SERS-based aptasensor
Label-free SERS biosensor

ABSTRACT

COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused an ongoing global pandemic with economic and social disruption. Moreover, the virus has persistently and rapidly evolved into novel lineages with mutations. The most effective strategy to control the pandemic is suppressing virus spread through early detection of infections. Therefore, developing a rapid, accurate, easy-to-use diagnostic platform against SARS-CoV-2 variants of concern remains necessary. Here, we developed an ultra-sensitive label-free surface-enhanced Raman scattering-based aptasensor as a countermeasure for the universal detection of SARS-CoV-2 variants of concern. In this aptasensor platform, we discovered two DNA aptamers that enable binding to SARS-CoV-2 spike protein via the Particle Display, a high-throughput screening approach. These showed high affinity that exhibited dissociation constants of 1.47 ± 0.30 nM and 1.81 ± 0.39 nM. We designed a combination with the aptamers and silver nanoforest for developing an ultra-sensitive SERS platform and achieved an atto-molar (10^{-18} M) level detection limit with a recombinant trimeric spike protein. Furthermore, using the intrinsic properties of the aptamer signal, we demonstrated a label-free aptasensor approach, enabling use without the Raman tag. Finally, our label-free SERS-combined aptasensor succeeded in detecting SARS-CoV-2 with excellent accuracy, even in clinical samples with variants of concern, including the wild-type, delta, and omicron variants.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogen responsible for COVID-19 (Wu et al., 2020), has caused an ongoing global pandemic with high morbidity and mortality, severely threatening public health and the global economy (Podder et al., 2021; Yang et al., 2021). Effective control of pandemics like COVID-19 should focus on the early detection of infections (Spinelli and Pellino, 2020). Vaccines from Moderna, Pfizer-BioNTech, Johnson & Johnson, Novavax and others are now widely available (Banerji et al., 2021), but early diagnosis of SARS-CoV-2 continues to play an essential role in reducing virus spread. Further development of advanced rapid diagnostic tools will be valuable both here and as a countermeasure for preventing and controlling future pandemics (Simpson et al., 2020).

Currently, early detection of SARS-CoV-2 is primarily achieved by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based molecular diagnostics (Emery et al., 2004; Corman et al., 2020). However, these assays entail a turnaround time of >24 h to screen and diagnose patients with suspected SARS-CoV-2 infection. Lateral-flow-based immunoassays (Li et al., 2020) and CRISPR-based

* Corresponding author.
** Corresponding author.
E-mail addresses: ychong1983@kimm.re.kr (Y. Hong), spack@korea.ac.kr (S.P. Pack).

https://doi.org/10.1016/j.bios.2023.115202
Received 5 December 2022; Received in revised form 28 February 2023; Accepted 4 March 2023
Available online 8 March 2023

tests (e.g., SHERLOCK (Kellner et al., 2019) and DETECTR (Broughton et al., 2020)) can shorten the time required for diagnosis, but typically require high production costs owing to their reliance on antibodies or enzymes. Rapid antigen tests (RATs) such as the lateral-flow immunoassay can be effective for generating quick results at the point of care, but also face hurdles including a high false-positive rate (Gans et al., 2022). As such, there remains an unmet need for rapid, low-cost diagnostics that can achieve high sensitivity and selectivity for target analytes.

Aptamers are single-stranded DNA or RNA reagents with high affinity and specificity to specific target molecules (Ellington and Szostak, 1990). Compared with monoclonal antibodies, DNA aptamers have several benefits, including thermal and pH stability, small size, and straightforward chemical synthesis (Jayasena, 1999), which means that they can be easily and reproducibly produced and modified at a low cost (Zhou and Rossi, 2017). Several DNA aptamer-based analytical approaches have already been developed, such as the aptamer-based lateral-flow assay (Chen and Yang, 2015), the enzyme-linked oligonucleotide assay (ELONA) (Drolet et al., 1996), and aptamer-based field effect transistor (FET) biosensors (Park et al., 2021; Hwang et al., 2021). However, although ultra-sensitive detection methods have been achieved by incorporating aptamers into various sensing platforms (Hu et al., 2012; Taghdisi et al., 2016), these typically require aptamer modification with labels for signal reporting at the 5′ or 3′ end of the aptamer sequence, which increases the production cost in terms of chemical synthesis.

As an alternative, we have developed a label-free surface-enhanced Raman scattering (SERS)-based aptamer sensor. In the last decade, SERS-based aptasensors have been intensively explored for sensitive detection of analytes ranging in size from small-molecule targets to protein targets (Scatena et al., 2019). Although Raman scattering is not inherently ideal for biological applications owing to its inherently weak signal, the use of SERS can provide up to 10^8-fold signal enhancement. This enhancement is derived from the molecular vibrations of analyte molecules on the surface of a nanostructured metal substrate, coupled with locally-amplified electromagnetic fields (Moskovits, 1985; Ambartsumyan et al., 2020). In this study, we isolated two novel DNA aptamers against SARS-CoV-2 spike (S) protein through four rounds of screening using particle display (Wang et al., 2014), a powerful high-throughput aptamer screening approach. The S protein is located on the SARS-CoV-2 surface and is the primary antigen employed in COVID-19 diagnostic tests, allowing aptamer access to the virus surface and rapid detection without virus lysis. Two aptamers were conjugated on a silver nanoforest (SNF) substrate, which was fabricated with a three-dimensional nanostructure in order to achieve effective enhancement of Raman signals using an intrinsic property of the aptamer (Fig. 1). Using this label-free aptasensor approach, we were able to achieve ultra-sensitive detection of the recombinant trimeric S protein, with a detection limit of 100 fg/mL (240 aM). We were able to further validate this assay's diagnosis performance with excellent accuracy in clinical samples, for both the wild-type Wuhan strain as well as the newer delta and omicron variants.

2. Materials and methods

2.1. Materials and reagents

Rhodamine 6G, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), sorbitan monooleate (Span-80), polysorbate-80 (Tween-80), Triton X-100, and mineral oil were purchased from Sigma-Aldrich (St Louis, MO, USA). Carboxylic acid-functionalized MyOne magnetic particles (1 μm size) were purchased from Invitrogen. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Biowest (Nuaillé, France). Silver was purchased from iNexus Inc. (Seongnam, Korea). GoTaq PCR master mix, GoTaq polymerase, and dNTPs were purchased from Promega Corporation (Madison, WI, USA). Recombinant SARS-CoV-2 spike (S) protein extracellular domain (ECD) (ref 40589-V08H4), subunit 1 (ref 40591-V08H), subunit 2 (ref 40590-V08H1),

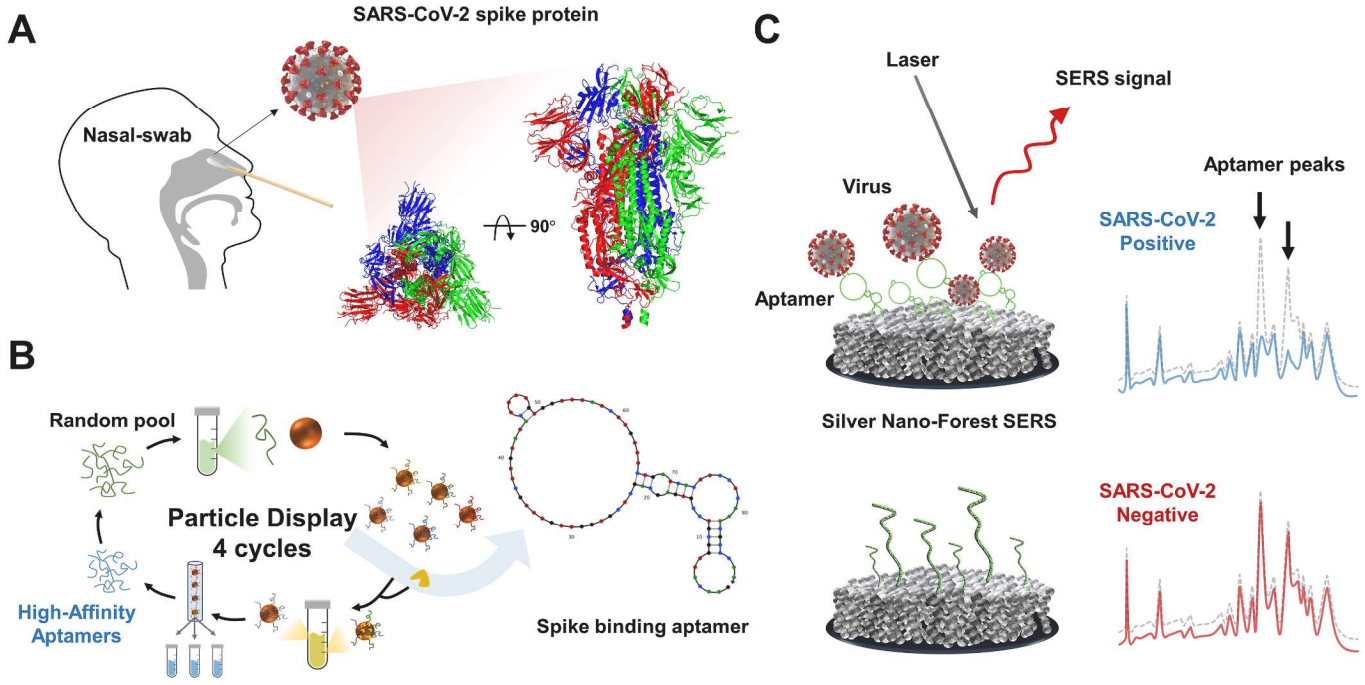

Fig. 1. Design strategy for our label-free SERS-based aptasensor platform for SARS-CoV-2. (A) Targeted aptamer screening against spike (S) protein for detecting SARS-CoV-2 from clinical samples. Side and top view of the trimeric S protein on the surface of SARS-CoV-2 (PDB: 6VXX). (B) The particle display aptamer discovery process, in which solution-phase aptamer library molecules are converted to monoclonal aptamer particles, incubated with fluorescently-labeled S protein, and then subjected to fluorescence-activated cell sorting (FACS) to enrich library molecules with strong affinity for this target. (C) The aptamer is then conjugated onto a silver nanoforest (SNF) substrate for the detection of SARS-CoV-2. The intrinsic aptamer peaks shift in response to the conformational changes triggered by S protein binding to the aptamer.

and receptor binding domain (RBD) (ref 40592-V08H) were purchased from Sino Biological (Beijing, China).

2.2. Library and primer construction

The single-stranded DNA (ssDNA) library, modified aptamer, and primers were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). The 90-mer Shao library (Shao et al., 2011) comprised 52-nt randomized sequences flanked by 19-nt PCR primer sites: 5′-GAACATTGGCGTCCGTGAG-[N52]-CACTTCCTCAAACGCCCAA-3′. The randomized sequences were synthesized with an equal probability of A, T, G, or C. All sequences described in this work are listed in Table S1.

2.3. Forward primer (FP) conjugation to magnetic particles

Carboxylic acid-functionalized MyOne magnetic particles (1 μm size, 500 μL with 10^7/μL) were washed using 500 μL of 0.01 N sodium hydroxide (NaOH) and washed thrice with an equal volume of ultra-pure water. After washing, the beads were re-suspended in a 150 μL reaction mixture containing 30 nmol of 5′-C12-amino-modified FP, 250 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1 mM imidazole chloride, and 200 mM NaCl. The mixture was vortexed and sonicated, and then incubated for 16 h at 25°C with gentle rotation. After complete covalent coupling, the remaining functional groups on the particle surfaces were blocked with 20 mM amino-PEG-methyl. Finally, the FP-coated beads were washed twice with quenching buffer (50 mM Tris-HCl buffer, pH 8.0, with 0.05% Tween-20) and stored in 500 μL of 0.01% Tween-20 in 10 mM Tris-HCl buffer, pH 8.0. To characterize the FP conjugation efficiency, the FP-coated beads were hybridized with 1 μM FAM-modified FP-complementary oligonucleotides in PBST (0.025% Tween-20 in DPBS) buffer, followed by fluorescence analysis using flow cytometry (Fig. S1).

2.4. Aptamer particle generation using emulsion PCR (ePCR)

The oil phase comprised 0.05% Triton X-100, 0.45% Tween-80, and 4.5% Span-80 in mineral oil. The aqueous phase comprised 1x PCR master mix, 40 nM FP, 1 μM reverse primer (RP), 62.5 U/mL GoTaq polymerase, 3.5 mM of each dNTP, 25 mM $MgCl_2$, 3 pM library pool, and 3×10^8 FP-coated beads in a final volume of 1 mL. The emulsion was generated using an Ultra-Turrax Device (IKA) with a DT-20 tube (IKA) at 500 rpm for 5 min. The ePCR was carried out as the following cycling conditions: 95°C for 3 min, followed by 50 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 75 s. Then, the emulsion was broken, and the aptamer particles were cleaned up. Based on the Poisson distribution, we estimated that 20 ± 15% of the synthesized aptamer particles should include beads displaying the PCR product, favoring the generation of monoclonal aptamer particles (Dressman et al., 2003; Diehl et al., 2006; Wang et al., 2014). To characterize these particles, the synthesized beads were hybridized with 1 μM FAM-modified reverse primer in PBST buffer, followed by fluorescence analysis using flow cytometry (Fig. S2).

2.5. Preparation of monoclonal aptamer particles

After cloning and sequencing, the monoclonal aptamer particles displaying individual aptamer sequences were generated by particle PCR. Each 100 μL particle PCR comprised 1 × PCR Master Mix, 25 mM $MgCl_2$, 1 μM FAM-modified RP, 10 nM aptamer template, and 3×10^7 FP-coated particles. PCR was carried out under the following conditions: 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. To avoid particle aggregation and increase particle PCR efficiency, it was vortexed and sonicated the reaction every five cycles at the 72°C elongation step. After PCR amplification, the fluorescence intensities were measured using flow cytometry to confirm the particle PCR efficiency.

2.6. Binding assay and specificity test

The relative binding abilities of aptamer candidates in each consensus sequence were confirmed through a bead-based fluorescence binding assay (Ahmad et al., 2011). Each of the monoclonal aptamer particle preparations was incubated with 100 nM biotinylated S protein trimer, receptor-binding domain (RBD), subunit 1 (S1), subunit 2 (S2) and BSA, respectively, for 1 h in PBSMCT (1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.025% Tween-20 in DPBS) binding buffer. After binding, the particles were washed thrice, followed by labeling with 50 nM Alexa Fluor 647-conjugated streptavidin (Invitrogen, CA, USA) in a binding buffer for 10 min. It is crucial to limit the labeling time to avoid non-specific binding during fluorescence labeling. We then washed the beads thrice with a binding buffer and measured the fluorescence using flow cytometry.

2.7. Measurement of binding affinity

To measure the binding affinities of the consensus sequences, an individual monoclonal aptamer particle was generated by particle PCR following the protocol above. The aptamer displayed particle was incubated with various concentrations of biotinylated S protein (serially diluted from 150 nM to 0.07 nM). All samples were washed thrice and labeled with 50 nM Alexa Fluor 647-conjugated streptavidin under equal conditions. The aptamer-target complex on the bead was washed thrice with binding buffer, and the fluorescence intensities were measured using flow cytometry. The dissociation constant was calculated using GraphPad software with one site–specific binding equation:

$$Y = Bmax \times X / (Kd + X)$$

2.8. Fabrication of the silver nanoforest (SNF) substrate

In previous work, our group established the fabrication of the SNF substrate using the nano-sputtering method (Kim et al., 2021). SERSpace substrates without surface modifications were provided by Kwang-Lim Precise Manufacturing. Briefly, the system conducted for nano-sputtering-based fabrication for nano-structure was applied to fabricate the nano-porous silver structures, and the SNF substrates were fabricated on six-inch silicon wafers. The basic conditions for fabrication of silver nanoporous structured substrate were as follows. The aperture diameter and length of the nano-sputtering system were 5 mm and 45 mm, respectively. The additional conditions were considered to fabricate SNF substrate: 400 mTorr of sputtering pressure in clustering chamber; 300 W of DC power for cluster source; and 20°C of temperature for cluster source. 85 and 15 sccm of the gas flow rates were used for argon and helium, respectively. Additionally, the finished substrate on six-inch wafer was cut to a size of 4×4 mm^2.

2.9. Surface modification of the SNF substrate

To activate the –SH groups at the 5′ end of the aptamers, the aptamers were treated with 100x TCEP for 4 h at 25°C. The remaining reactant was removed by centrifugation using Centricon (Amicon, Beverly, MA, USA) with a 1.5 mL microcentrifuge tube at 13,500 rpm for 30 min. DPBS buffer was added to the samples to adjust the pH to 7. 10 μL of the resulting aptamer solution (1 μM) was then dropped onto the SNF substrate and dried at 25°C for 1 h. The SNF substrates treated with aptamer solution were washed using DPBS for 4 h with gentle stirring and dried at 25°C.

2.10. SERS signal measurements

A 785 nm excitation laser in an upright microscope (Olympus, Japan) was used to acquire SERS signals from samples. It applied 80 mW of laser power, with 1 s of integration time. The Raman signal of the

aptamer was measured at a specific position before sample treatment, and again at the same position after sample treatment, with locations confirmed using a microscope. The sample volume used in this study was fixed at 10 μL. The incubation time between aptasensor and samples was also set for 5 min. All experiments with SERS signal measurements were carried out under identical conditions.

2.11. Clinical samples and RT-qPCR

Nasopharyngeal swab samples from patients were collected in viral transport media (VTM) on different dates. These samples were obtained from the Sejong Institute of Health & Environment. SARS-CoV-2 was determined by RT-qPCR using a Standard M nCoV Real-Time Detection Kit according to the manufacturer's protocol (SD Biosensor, Republic of Korea). SARS-CoV-2 delta (B1.617.2) and omicron (B.1.1.529) variants were detected using a PowerChek SARS-CoV-2 S-gene Mutation Detection Kit (Kogene Biotech, Republic of Korea). The Raman signals from clinical samples were measured under the following conditions. The 10 μL of the sample was incubated with SpS1-C4 aptamer-conjugated silver nanoforest for 5 min, the washing was conducted using DW, and the average spectrum was obtained by measuring ten spectra per sample.

3. Results and discussion

3.1. Particle display selection of novel DNA aptamers against SARS-CoV-2 S protein

The S protein is located on the SARS-CoV-2 surface and facilitates entry into host cells by interacting with human angiotensin-converting enzyme 2 (ACE2), and this protein is the primary antigen employed in COVID-19 diagnostics, as it is accessible on the virus surface and thus allows rapid detection without virus lysis. As the target antigen for aptamer screening in this study, we used the recombinant SARS-CoV-2 S protein extracellular domain (ECD), which is optimized for pre-formation of a homotrimer. Several proline mutations (F817P, A892P, A899P, A942P, K986P, V987P) and alanine mutations (R683A and R685A) were introduced to stabilize the pre-fusion state of the homotrimer and abolished the furin cleavage site, respectively (Hsieh et al., 2020).

To effectively and economically discover high-affinity aptamers, we employed the particle display (Wang et al., 2014) screening platform, which quantitatively measures the binding ability of monoclonal aptamer particles derived from every aptamer candidate in a random pool and then individually sorts them using FACS in a high-throughput manner. In this approach, the aptamer particles are synthesized using an emulsion polymerase chain reaction (ePCR) (Shao et al., 2011). The

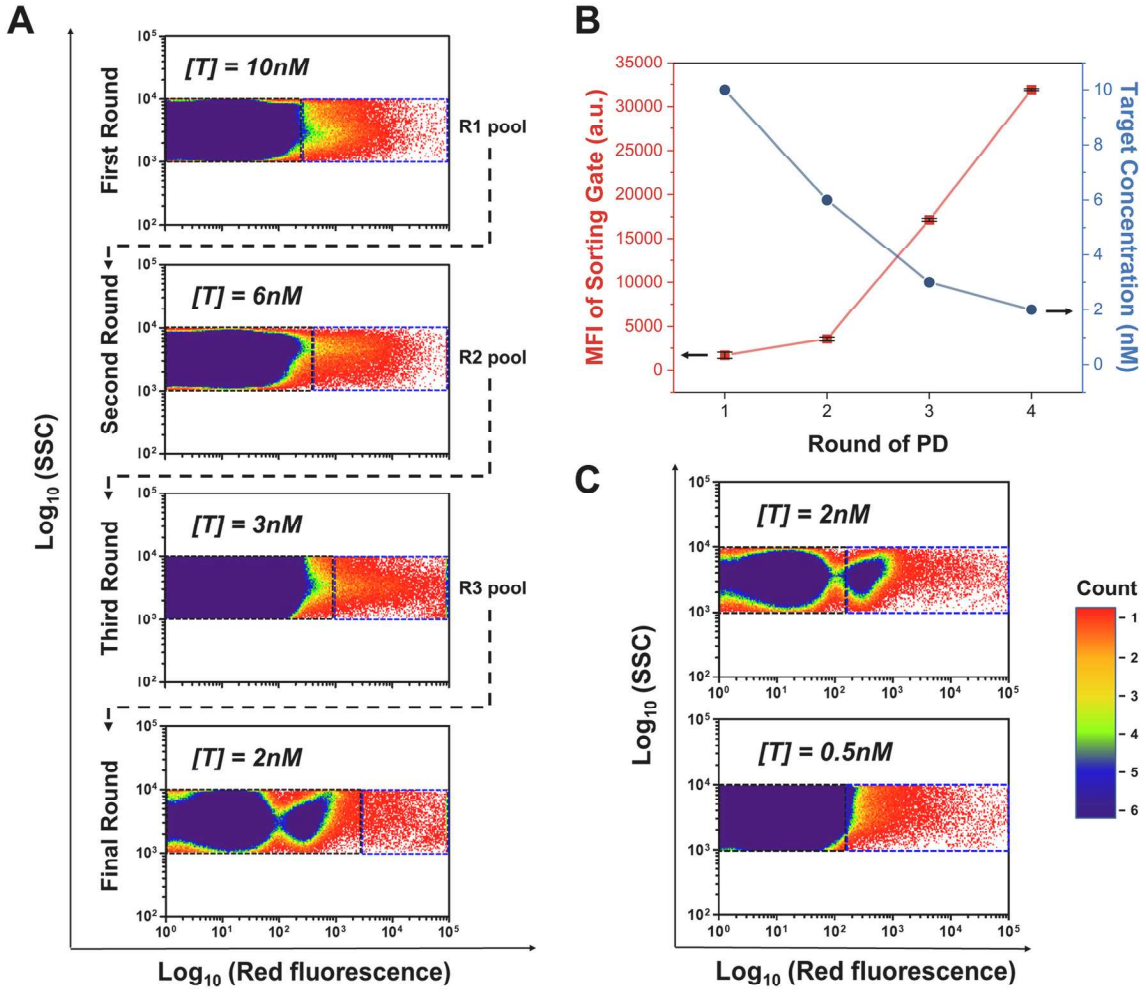

Fig. 2. Round by round assessment of the particle display screening process. (A) FACS dot plots from all four rounds of screening at the stated concentrations (*[T]*) of recombinant trimeric S protein. (B) Mean fluorescence intensity (MFI) within the sort gate at different target concentrations in each of the four rounds. (C) Additional sorting experiments in the final round of screening with another sort gate at different target concentrations. Overall, 5% of the top binders at 2 nM, 0.15% of the top binders at 2 nM, and 0.1% of the top binders at 0.5 nM of S protein were sorted using FACS.