Peptide Binder with High-Affinity for the SARS-CoV-2 Spike Receptor-Binding Domain

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ABSTRACT: Rapid antigen detection tests are urgently needed for the early diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The discovery of a binder with high affinity and selectivity for the biomarkers presented by SARS-CoV-2 is crucial to the development of the rapid antigen detection method. We utilized the surface biopanning to identify a peptide binder R1 from a phage-displayed peptide library consisting of $10^9$ independent phage recombinants. The R1 peptide exhibited high-affinity for specific binding with the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein with a dissociation constant $K_D$ of $(7.5 \pm 1.9) \times 10^{-10}$ M, which maintained high binding affinity with the RBD derived from Gamma, Lambda, Delta, and Omicron variants. The composition and sequence dependence of binding characteristics in R1–RBD interactions was revealed by the binding affinity fluctuations between RBD and the scrambled sequences or single-site mutants of R1. The R1-functionalized gold nanoparticles possessed concentration-dependent response to RBD and selectivity over bovine serum albumin and human serum albumin. The peptide binder R1 shows the potential to be used for constructing a rapid detection method for the early-stage diagnostics for SARS-CoV-2.

KEYWORDS: affinity peptide, interface, receptor-binding domain, sensor, assembly

INTRODUCTION

An early diagnosis of SARS-CoV-2 is urgently needed for tracking the SARS-CoV-2 replication dynamics and treating patients with coronavirus disease 2019 (COVID-19). COVID-19 disease progression exhibits three escalating phases, including early infection (phase I), pulmonary involvement (phase II), and systemic hyper inflammation (phase III).1 Viral replication is predominantly pathogenic in the early infection phase, whereas the host inflammatory response overrides the progression of COVID-19 across phase II and phase III.1,2 It is crucial to distinguish the COVID-19 disease phases for each patient and determine the use of either an antiviral agent or an anti-inflammatory agent.3,4 However, the clinical signs associated with phase I present as either asymptomatic infections or mild constitutional symptoms,5 making it difficult for people who are infected to notice their symptoms. The statistics show that the median time for infected patients to seek medical treatment is 5 days after developing symptoms.6 In contrast, phase I subsides quickly (approximately 1 week after the exposure to SARS-CoV-2).7 Consequently, the administration of antivirals, such as remdesivir and the combination of lopinavir and ritonavir, shows reduced or no clinical benefit due to the close of the antiviral window.8 With regards to overcoming this practical challenge, an early diagnosis of SARS-CoV-2 is still needed for taking interventions to interrupt viral replications as early as possible.
Several aptamer-based antigen tests have been reported to detect SARS-CoV-2 with high sensitivity and specificity.\textsuperscript{14–17} For example, a dimeric DNA aptamer-based electrochemical sensor allowed the detection of the SARS-CoV-2 virus in patient saliva samples.\textsuperscript{16} Some affinity peptides and antibodies have been screened to be applied to the detection of SARS-CoV-2 as well.\textsuperscript{18–23} However, current antigen tests for SARS-CoV-2 are generally less sensitive than RT-PCR, and endeavors should still be made toward improving their sensitivity and specificity. SARS-CoV-2 biomarkers, such as the spike protein (S-protein) on the surface of the SARS-CoV-2 envelope,\textsuperscript{24} can be employed as promising targets for screening high-affinity binders. The S-protein is responsible for initiating the invasion of the coronavirus into the host cell.\textsuperscript{25} The extracellular region of the S-protein includes two functional subunits, S1 and S2. The S1 subunit is essential for the virus to recognize angiotensin-converting enzyme 2 (ACE2) receptors in the host cells, whereas the S2 subunit facilitates the fusion of the virus envelope and the human cell membrane.\textsuperscript{26} In the S1 subunit, the key domain mediating the molecular recognition of ACE2 receptors is commonly referred to as the receptor-binding domain (RBD),\textsuperscript{27} making it the promising target for the development of advanced diagnostic or therapeutic modalities. Thus, the high-affinity binders with selectivity for the SARS-CoV-2-spike-RBD are important detection kits for the diagnosis of SARS-CoV-2.

Herein, we report the discovery of peptide binder R1 (12 amino acid residues) with high affinity and selectivity for the SARS-CoV-2-spike-RBD via a biopanning approach with a phage display peptide library. The random phage display library provides a wide range of affinity peptide sources, i.e., 10\textsuperscript{9} peptide sequences, for screening the efficient binders for the target protein. After the surface biopanning experiment, we investigated the intermolecular interactions between the SARS-CoV-2-spike-RBD and R1 via the biolayer interferometry (BLI) assay and determined their binding affinity to be 0.75 nM. We further functionalized the surface of colloidal gold nanoparticles with R1 and developed a surface plasmon resonance (SPR)-based assay. As reflected by the change in the excitation of surface plasmon, R1-functionalized gold nanoparticles specifically and sensitively respond to the presence of SARS-CoV-2-spike-RBD in solution, suggesting the potential to be applied as an optically active sensor in the diagnostic test of SARS-CoV-2.

Figure 1. A peptide binder with a high affinity for SARS-CoV-2-spike-RBD was identified by phage display screening. (a) Phage display screening for SARS-CoV-2-spike-RBD and the potent binding affinity peptide sequences. (b) Representative curve of a BLI sensor with sequential steps: (1) baseline, (2) peptide loading, (3) baseline, (4) association, and (5) dissociation. (c) BLI curves for the association and dissociation of R1–R4 peptides to 300 nM SARS-CoV-2-spike-RBD. (d) BLI responses correspond to the specific binding of 300 nM SARS-CoV-2-spike-RBD on the R1–R4 immobilized BLI sensor surfaces. (e) BLI curves for association/dissociation of R1 peptide to SARS-CoV-2-spike-RBD. (f) BLI curves for association/dissociation of ACE2 to SARS-CoV-2-spike-RBD. (g) BLI curves for association/dissociation of R1 peptide to HSA. (h) Comparison of BLI responses of R1 to 500 nM RBD versus 500 nM HSA.
RESULTS AND DISCUSSION

A peptide phage library was constructed by fusing different dodecapeptide regions to the N-terminus of phage coat protein pIII in an M13 bacteriophage display framework. The peptide library, consisting of approximately 10^9 independent phage recombinants, was screened by the iterative cycles of binding, elution, and amplification against the target SARS-CoV-2-spike-RBD, which was immobilized on a plastic surface. Four peptides with high affinity for the target SARS-CoV-2-spike-RBD were identified from the peptide library, including R1 (DVDVLIKYQFSF-CONH₂), R2 (SSVDQASHVNKK), R3 (RTNLHTLIATFN), and R4 (YGRTHSTFSPSA) (Figure 1a).

To evaluate the binding affinity of peptides R1–R4 for SARS-CoV-2-spike-RBD, we synthesized the peptides R1–R4 with C-terminal amidation and an addition of biotin-GG-motif (G represents glycine) at the N-termini. Peptides were immobilized on the surfaces of streptavidin (SA)-coated BLI optical sensors via the strong interaction between SA and biotin (Figure 1b). The adsorption or desorption of molecules on the sensor surface causes a shift in the interference pattern of light waves that reflects the spectrophotometers from two interfaces between the optical sensor and solution: the internal reference interface and the external interface composing of a layer of immobilized molecules.28 The shifts in optical interference can be quantified to determine the kinetics of association or dissociation in real-time.29 We investigated the

Figure 2. Contributions of amino acid sequence, terminal functionality, and amino acid residues to the affinity of R1 binding to SARS-CoV-2-spike-RBD. (a) Ratios between the binding energy of R1-RBD (ΔGA-R1) and the binding energy of R1 variants-RBD (ΔGA-variant). (b) BLI responses of scrambled R1 peptides and R1 with a different terminal functionality (R1–0) to 500 nM RBD. (c–k) Kinetic binding curves of R1 mutants for RBD protein with the concentration of 1000, 500, 250, 125, and 62.5 nM.
association and dissociation kinetics of SARS-CoV-2-spike-RBD interacting with peptides by monitoring the changes in the BLI signals (Figure 1b).

R1−R4 modified sensors showed different BLI responses to 300 nM SARS-CoV-2-spike-RBD (Figure 1c), where the BLI responses were ranked as R1 (0.50 nm) > R3 (0.42 nm) > R2 (0.36 nm) > R4 (0.13 nm). R1 peptide exhibited a higher BLI response relative to R2−R4, indicating the stronger potency of R1 for inducing RBD adsorption (Figure 1d). The binding affinity of R1 peptide with SARS-CoV-2-spike-RBD was exploited by using RBD solutions with a varying concentration range from 62.5 to 4000 nM (Figure 1e). The apparent dissociation constant $K_D$ was determined to be $(7.5 \pm 1.9) \times 10^{-10}$ M for R1 binding with RBD (the detailed calculation method is provided in the Materials and Methods section, eqs 3−7). The binding affinity $K_D$ is related to the free energy of association ($\Delta G_A$), as:

$$\Delta G_A = -RT \ln K_A = RT \ln K_D$$

where $K_A$ is the equilibrium constant for the association, $R$ is the gas constant, and $T$ is the temperature of the reaction in Kelvin. Thus, the free energy of association ($\Delta G_A$) for the interaction between R1 and RBD can be calculated to be $-12.6 \pm 0.2$ kcal/mol accordingly. In contrast, R2−R4 showed weaker binding affinity toward RBD, in which the magnitudes of $K_D$ for R2−R4 interacting with RBD were measured to be on the order of $10^{-9}$ or $10^{-8}$ M (Figure S1). The comparisons of R1−R4 performance in terms of specific adsorption and apparent dissociation constant lead to a preliminary statement that, compared with R2−R4, R1 performed better as an affinity peptide for binding with SARS-CoV-2-spike-RBD.

To provide insights into the interactions between R1 and SARS-CoV-2-spike-RBD, two sets of additional measurements were performed. First, biotinylated ACE2 was immobilized on the SA-coated BLI sensor surfaces to interact with SARS-CoV-2-spike-RBD in a concentration range from 62.5 to 4000 nM (Figure 1f). The magnitude of $K_D$ for the interaction between ACE2 and RBD was determined to be $(5.8 \pm 0.2) \times 10^{-9}$ M, approximately 8 times greater than that for the interaction between R1 and RBD. It demonstrates that R1 is a stronger binder for SARS-CoV-2-spike-RBD than ACE2, the mammalian receptor responding for RBD. Second, the responses of R1-coated sensor surfaces upon the adsorption of human serum albumin (HSA) were recorded by BLI experiments (Figure 1g). HSA, constituting about 60% of total plasma protein, is predominant in the intravascular and extracellular space within a human body. Within the range of experimental concentrations, the BLI responses of HSA binding to R1 peptide were below 0.05 nm (Figure 1g). The interaction of the R1-immobilized sensor surface with 500 nM SARS-CoV-2-spike-RBD was monitored by BLI signals (Figure 1b).
RBD versus 500 nM HSA resulted in distinguishable BLI responses of 0.50 nm versus 0.04 nm, respectively (Figure 1h). Thus, R1 possesses a promising selectivity toward the recognition of RBD.

To elucidate the molecular mechanism for the interaction between SARS-CoV-2-spike-RBD and R1, the contributions of the R1 sequence and composition were evaluated. The conformation of R1 was examined using circular dichroism (CD) spectroscopy. At the concentration of 50 μM in sodium phosphate buffer (pH = 7.3, the ionic strength was 0.17 M), R1 displayed a CD minimum at 203 nm, which indicates the adoption of a random coil secondary structure (Figure S2). To validate the dependence of R1 binding affinity to RBD on the amino acid sequence, two scrambled forms of R1 peptide (sc1-R1 and sc2-R1) were synthesized and analyzed (Figure 2a). The disruption of the R1 sequence decreased the BLI response to 1 μM RBD from 0.54 (R1) to 0.27 (sc1-R1) and 0.32 nm (sc2-R1) (Figure 2b). To reflect the influence of sequence mutation on the free energy of association, the ratios of ΔGA for the peptide-RBD association between peptide mutants and R1 (ΔGAvariant/ΔGAR1) were calculated. Figure 2a reveals that the disruption of the R1 sequence reduced the ΔGA for peptide binding RBD and the ΔGAvariant/ΔGAR1 value becomes less than 1 for sc1-R1 and sc2-R1.

To study the composition effect underlying the R1 binding affinity for SARS-CoV-2-spike-RBD, the contributions of the terminal identity and single-site residue to the interaction between R1 variants and RBD were assessed. R1-0 was synthesized to share the same sequence as R1 but alter the C-terminus from amide to carboxylic acid (Figure 2a). Relative to R1, R1-0 mediated a weakened BLI response to the adsorption of 500 nM RBD and a smaller ΔGAvariant/ΔGAR1 for binding RBD, suggesting that the binding of R1 with RBD is vulnerable to the chemical functional groups located at the end of a polypeptide (Figure 2b). To assess the contribution of single residues in the R1 recognition of RBD, alanine scanning mutagenesis of the R1 sequence was performed. Twelve peptide variants with single-site substitutions by alanine (A) at different positions were synthesized. V4A, K7A, and Y8A mutants were insoluble in an aqueous solution, and thus we performed BLI experiments with the nine soluble peptides and determined their binding affinities for RBD (Figure 2c–k). As summarized in Figure 2a, D1, V2, and L5 residues ranked in the top 3 based on the change of ΔGA, and thus played a relatively important role in the binding affinity between the R1 peptide and RBD protein. In contrast, R1–RBD interaction was inert to the mutation of Q9A. Other residues, including D3, I6, F10, S11, and F12, occupied the middle ground for mediating R1–RBD interaction. This figure highlighted the contributions of sequence, terminal amidation, and single-site identity to the binding affinity between R1 and RBD.

The high-affinity peptide binder R1 can be utilized in the construction of a colloidal gold-based detection kit for the diagnosis of SARS-CoV-2. We synthesized gold nanoparticles (AuNP) via citrate reduction and immobilized R1 on the AuNP surfaces by incorporating a cysteine (C) at the C-terminus of R1 to form an Au−S bond (Figure 3a). Transmission electron microscopy (TEM) images of R1-
immobilized AuNPs revealed the spherical morphology of AuNPs with diameters ranging from 18 to 32 nm (Figure 3b,c). The immobilization of R1 lowered the zeta potential of pristine AuNPs from $-24.2 \pm 0.6$ to $-26.2 \pm 1.0$ mV (Figure 3d). The performance of R1-functionalized AuNPs in the detection of SARS-CoV-2 was examined by monitoring the UV–vis absorption for R1-functionalized AuNPs in the presence of RBD at different concentrations (Figure 3e). The collective oscillations of conduction band electrons in AuNPs excited by the electromagnetic of incident light are known as localized surface plasmon resonance (SPR), which can be modulated by the local refractive index near the AuNP surface.33 Thus, the adsorption of RBD on the R1-functionalized AuNP surfaces can be reflected by a change in the SPR peak of AuNPs in the UV–vis absorption spectra. As shown in Figure 3e, the titration of RBD into the solution containing 0.17 nM (i.e., $1.0 \times 10^{11}$ particles/mL) R1-functionalized AuNPs resulted in a redshift for the SPR peak in a concentration-dependent manner. Specifically, the SPR peak position moved from 523.2 to 551.5 nm as the RBD concentration increased from 0 to 6.5 μg/mL. We performed parallel measurements by titrating bovine serum albumin (BSA) or HSA into the R1-functionalized AuNP solution. The SPR peak was almost unaffected by the addition of BSA and HSA (Figure 3f–h). These results demonstrated the specificity of interaction between R1-functionalized AuNPs and RBD.

To determine the minimum detectability of R1-functionalized AuNPs for SARS-CoV-2-spike-RBD detection, we plotted the SPR peak position versus the increased RBD concentration from 6 to 30 nM (Figure 3i). A linear regression curve with the concentrations of RBD was generated according to the method of Funk et al., and thus the limit of detection (LOD) for the R1-functionalized AuNPs can be deduced as follows:34

$$\text{LOD} = \frac{3S_r}{b}$$

where $S_r$ is the standard error of the linear regression curve and $b$ is the slope. Consequently, the LOD for R1-functionalized AuNPs detecting RBD was quantified to be 9.9 nM. The BLI characterization of the R1 peptide binding with SARS-CoV-2-Spike pseudovirus was also performed, and the BLI response was increased with the increased titer of pseudovirus in a titer-dependent manner (Figure S3a,b). The response to 110 and 333 TU/mL of pseudovirus was significantly stronger than the control (Figure S3c), and the LOD for detecting pseudovirus was calculated to be 73 TU/mL (Figure S3d). The SPR peaks of R1-immobilized AuNPs also exhibited a titer-dependent response to the addition of SARS-CoV-2-Spike pseudovirus in the UV–vis absorption spectra (Figure S4).

Finally, we made two additional comments for the performance of R1. First, the utilization of R1 is not only limited to the detection of RBD derived from pristine SARS-CoV-2 but also the RBD derived from SARS-CoV-2 variants. The dynamic evolution of SARS-CoV-2 has achieved significant genetic diversity in the past two years. The rapid mutation rate of SARS-CoV-2 has posed an increased risk to global public health, challenging the administration of vaccines and drugs.35 The RBD derived from the SARS-CoV-2 Lambda variant (C.37) has three mutated residue sites, including K417T, E484K, and N501Y (Figure 4a).36 Whereas, the RBD derived from Gamma variant (P.1) contains two mutated sites, including L452Q and F490S (Figure 4a).37 Similarly, the RBD derived from the Delta variant (B.1.617.2) contains two mutated sites, including L452R and T478K (Figure 4a).36 The RBD derived from the SARS-CoV-2 Omicron variant (B.1.1.529) has 15 mutated residue sites, including G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, and Y505H (Figure 4a).36 Structure comparison of the RBD derived from wild type virus (PDB ID: 6M0J) with its variants (Gamma, Lambda, Delta, and Omicron) revealed that the RBD core region, a twisted five-stranded antiparallel β sheet surrounded by short helices, was conserved throughout viral evolution (Figure 4b–e). In contrast, the structure of the RBD accessory subdomain was prone to substantial fluctuations due to the structural flexibility (Figure 4b–e). We evaluated the binding affinity of R1 to the Gamma, Lambda, Delta, and Omicron RBDS. The binding affinity of R1 for Gamma, Lambda, Delta, and Omicron RBDS was determined to be (2.6 ± 0.1) × 10^{-9}, (3.6 ± 0.4) × 10^{-10}, (9.0 ± 0.2) × 10^{-9}, and (4.5 ± 0.1) × 10^{-9} M, respectively (Figure 4f–i). This result suggested that the high affinity of R1 binding to wild-type RBD was preserved in the interaction between R1 and the RBD derived from Gamma, Lambda, Delta, and Omicron variants. The binding affinity of the R1 peptide for MERS-CoV-Spike-RBD and SARS-CoV-1-Spike-RBD proteins was also investigated by BLI. The R1 peptide maintained the relatively high binding affinity with SARS-CoV-1-Spike-RBD in comparison with that of MERS-CoV-Spike-RBD (Figure S5). The spike protein of the other human beta coronavirus HKU1 was investigated by BLI, and the binding affinity of R1 for HCoV-HKU1-S1 protein was measured to be (3.0 ± 0.1) × 10^{-8} M (Figure S6). The binding affinity of R1 for HCoV-HKU1-S1 protein was significantly weaker than that for the SARS-CoV-2-Spike-RBD
protein by two orders in terms of the $K_d$ value, which demonstrated the binding selectivity of the R1 peptide for SARS-CoV-2-Spike-RBD.

Second, R1 showed promising biocompatibility to mammalian cells. We examined the cytotoxicity of R1 by incubation with cell lines for 48 h, including the green monkey kidney cells (Vero E6), human embryonic kidney cells with ACE2-overexpression (HEK293T(ACE2+)), human non-tumorigenic lung epithelial cells (Beas), and adenocarcinomic human alveolar basal epithelial cells (A549). Compared to the control group, no measurable cytotoxicity was observed in the R1-treated groups below the dosing level of 100 $\mu$M (Figure 5).

### CONCLUSIONS

In conclusion, we have discovered a peptide R1 that was able to bind with SARS-CoV-2 Spike-RBD by using the screening method of phage-display, with a combinatorial peptide library that has a library size of $10^9$ peptides. R1 exhibited a high affinity for binding RBD with an apparent dissociation constant of $(7.5 \pm 1.9) \times 10^{-10}$ M. Compared with the other reported affinity peptides, the R1 peptide had a stronger binding affinity for its target RBD protein. R1 can recognize the RBDs derived from Gamma, Lambda, Delta, and Omicron variants with promising binding affinity, whereas the recognition of RBD mutations has not been reported by previously reported polypeptides.  

The high affinity between R1 and RBD made R1 a potential candidate in the development of affinity-based diagnostic tools. The surface functionalization of gold nanoparticles with R1 led to a colloidal gold-based detection method with minimum detectability of nanomolar concentration for RBD and selectivity over BSA and HSA. Rapid diagnostics of COVID-19 can offer us the opportunities to track the emergence of SARS-CoV-2, interrupt the spread of the virus, and maximize the efficacy of antivirals. Although the antigen-based detection tests suffer from low sensitivity when compared with PCR, there is still a need in clinical practice to improve the performance of such detection tests due to its advantage as a rapid diagnostic tool. The high-affinity peptide identified in our study might provide a feasible solution to improve the sensitivity of antigen-based detection methods, facilitating the achievement of early-stage diagnostics.

### MATERIALS AND METHODS

**Materials.** Lyophilized powders of R1-related affinity peptides were synthesized and purified by Banpeptide Biological Technology Co., Ltd. The quality of R1-related peptide (purity: above 98%) was verified by high-performance liquid chromatography and mass spectrometry. SARS-CoV-2-Spike-RBD protein and variant-derived RBD were purchased from Sino Biological (40592-V088 WT), 40592-V08H113 (Lambda), 40592-V08H86 (Gamma), 40592-V08H90 (Delta), 40592-V08H121 (Omicron)). Biotinylated ACE2 protein was purchased from Sino Biological (10108-H27B-8), Vero E6, Beas, and A549 cells were purchased from the Peking Union Medical College Cell Culture Center. ACE2-overexpression HEK293T cells (HEK293T(ACE2+)) were purchased from Sino Biological (Beijing, China). MERS-CoV-Spike-RBD protein (40071-V08B1), SARS-CoV-1 Spike-RBD protein (40150-V08B2), and hCoV-HKU1-S1-Protein (40602-V08H) were purchased from Sino Biological. SARS-CoV-2 Spike (GFP-T2A-Luciferase) pseudovirus was purchased from Genomeditech (Shanghai, China). Guanidinium chloride (GdmCl), Trizol reagent, and bovine serum albumin were purchased from Sigma-Aldrich and used without any further purification. Related reagents in the experiments were purchased from commercial vendors as follows: 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Innochem), polyethylene glycol-8000 (PEG-8000, Macklin), sodium iodide (NaI, Macklin), ethylenediaminetetraacetic acid (EDTA, Solarbio), ethanol (DAMAo), human serum albumin (HSA, Solarbio). Assay kits and commercial vendors are listed as follows: Cell Counting Kit-8 (CCK8) assay (Cat.#ck04–10, DOJINDO), DMEM basic media (Gibco), and F12 basic media (Gibco).

**Screening of Affinity Peptides by Phage Display.** Recombinant RBD was used as the target protein in a surface panning experiment with M13 phage vector-based Ph.D.-12 phage display libraries (New England Biolabs Inc.). Linear dedcapeptides are displayed as N-terminal fusions to the minor coat protein pIII in this phage display system. The library includes approximately $10^9$ independent dedcapeptide sequences. The phage panning experiment was performed following the protocol of Ph.D.-12 display phage library surface panning. In brief, RBD was directly immobilized on a plastic plate surface by nonspecific hydrophobic and electrostatic interactions. The panning was carried out by binding the phage display libraries with the RDB-immobilized plate, washing away the unbound phage, and eluting the specifically bound phage. We amplified the eluted bound phage and performed 3–4 iterative cycles of binding, elution, and amplification to isolate the specifically bound phage. Eventually, individual clones were collected for DNA sequencing.

**Phage DNA Purification and Sequencing.** Single-stranded phage DNA was extracted and purified for sequencing with the sodium iodide method of Wilson. A total of 500 $\mu$L of phage-containing supernatant was transferred to a fresh microfuge tube and added into 200 $\mu$L of 20% (w/v) PEG-8000/2.5 M NaCl solution. The solution was mixed by inverting several times and incubated for 15 min at room temperature. Subsequently, the solution was centrifuged at 12,000 rpm for 10 min at 4 °C and then the supernatant was discarded. The resulting pellet was suspended with 100 $\mu$L of iodide buffer (10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 4 M NaI, stored at room temperature in the dark) by vigorously taping the tube. A total of 250 $\mu$L of ethanol was added into the solution and incubated for 15 min at room temperature to precipitate single-stranded phage DNA. The solution was centrifuged again at 14,000 rpm for 10 min at 4 °C. The pellet was washed with 500 $\mu$L of ice-cold 70% ethanol and centrifuged at 14,000 rpm for 10 min at 4 °C. The single-stranded DNA pellet was dried at room temperature until all remaining ethanol was evaporated. Single-stranded phage DNA was resuspended in 20 $\mu$L of PCR H2O and sequenced by the Tsingke Biotechnology Co., Ltd.

**Biolayer Interferometry.** Two glycine residues were added to the peptide N-termini as a linker. The peptide N-terminus was biotinylated for the immobilization onto the sensor surface. Powders of biotinylated peptides were dissolved to 1 mM and diluted by 2000-fold into 1× PBS for the immobilization onto streptavidin (SA) Octet biosensors (ForteBio). Proteins were prepared as analytes at an appropriate concentration (or nothing for baseline steps) in 1× PBS buffer. A total of 220 $\mu$L of 1× PBS buffer, peptide solution, or target protein solution was filled into the corresponding wells in 96 well plates (ForteBio). Before each run for binding measurements, SA biosensors were hydrated by soaking the tips in 1× PBS for at least 30 min. Affinity measurements were all carried out at 30 °C as follows: (1) obtaining baseline in 1× PBS buffer for 120 s; (2) loading peptide in 500 nM biotinylated peptide solution for 90 s; (3) obtaining baseline in 1× PBS buffer for 120 s; (4) associating with target protein for 180 s; (5) dissociating with target protein in 1× PBS buffer for 600 s. Data analysis was performed with the ForteBio Data Analysis software (ForteBio). BLI signals were processed by subtracting signals of the reference well (with no analytes in buffer) and aligning the curves to the beginning of the association. The kinetics and $K_d$ values of peptide–protein interactions were calculated by the global kinetic fitting of BLI curves with a 1:1 model. Herein, the analysis of kinetics and the $K_d$ value were briefly introduced. The association and dissociation reactions can be depicted as follows:

$$A + B \overset{k_1}{\rightleftharpoons} AB \overset{k_2}{\rightarrow}$$ (3)
where the $A$ is the target protein, $B$ is the peptide. For kinetics measurement,\textsuperscript{39,40}

\[
R = R_0 + R_0 \exp (-k_{a} t)
\]

(4)

where the $t$ is the elapsed time for association, $R$ is the measured BLI signal, $R_0$ is the BLI signal at the beginning of association ($t = 0$), and the $R_0$ is the calculated response value at equilibrium for the given concentration of A. $k_{a}$, the observed binding rate, reflects the overall rate of the combined association and dissociation of A and B. For the dissociation rate constant $k_d$ measurement,

\[
R = R_0 + R_0 \exp (-k_{d} t)
\]

(5)

The association rate constant $k_a$ can be calculated as follows:

\[
k_a = \frac{k_{obs} - k_d}{[A]}
\]

(6)

Finally, the $K_D$ value is calculated as:

\[
K_D = \frac{[A] \times [B]}{[AB]} = \frac{k_j}{k_a}
\]

(7)

Circular Dichroism (CD) Spectroscopy. R1 peptide was dissolved in the sodium phosphate buffer (pH = 7.3, the ionic strength is 0.17 M) with the final concentration of 50 μM. The CD measurements were performed at room temperature with a circular dichroism spectropolarimeter system (Jasco J-1500, Tokyo, Japan), using a quartz cuvette with a 1 mm path length. The CD spectra were recorded by using the following parameters: a scan speed of 100 nm/min, a digital integration time of 1 s, a bandwidth of 2 nm, and step increments of 1 nm in wavelength. At least two scans were accumulated from 260 to 190 nm for far-UV scans. The phosphate buffer was detected as the background, and its signal was subtracted from the sample spectra. CD signals were converted to molar ellipticity ($\theta$, deg cm$^2$ dmol$^{-1}$) according to the equation\textsuperscript{41,42}

\[
[\theta] = 1000 \times \Psi/[n][c]
\]

(8)

where $\Psi$ is the CD signal in mdeg, $n$ is the number of amino acid residues, $l$ is the path length in mm, and $c$ is the concentration in mM.

Gold Nanoparticle Synthesis. A total of 150 mL of sodium citrate was added into a 250 mL flask and stirred using a magnetic stirrer. This solution was heated at 90 °C. A total of 1 mL of 25 mM tetrachloroauric(III) acid was added and reduced by sodium citrate. After 30 min, a mixture of 60 mM sodium citrate (1 mL) and 25 mM tetrachloroauric acid (1 mL) was added into the flask and stirred for 30 min. This procedure was repeated for another 30 min. Finally, the AuNPs were synthesized after cooling.\textsuperscript{43}

Transmission Electron Microscopy. A total of 10 μL of sample solution was deposited onto the 200 mesh Formvar carbon-coated copper TEM grids for 2 min at room temperature. The excess solution was removed by filter paper and allowed to dry for 2 h at room temperature. Topography images were recorded on a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

The Response of Peptide Modified AuNPs to Target Protein. To load R1 peptide onto the AuNP surfaces, 20 μL of R1 peptide solution (0.5 mg/mL) was mixed with 200 μL of AuNPs suspension solution and incubated for 30 min at 30 °C in a water bath. This solution was centrifuged at 12,000 rpm for 5 min at room temperature, and the supernatant was discarded. The pellet was resuspended in 200 μL of ddH$_2$O, and the peptide-modified AuNPs were collected. The response of peptide-modified AuNPs to the solutions of RB, HSA, or BSA was determined by monitoring the shift of UV absorbance spectra, which were measured by a UV–vis spectrophotometer (PerkinElmer, United States).

Zeta-Potential Measurement. A zetasizer Nano ZS (Malvern, UK) system was used for zeta-potential measurement. A total of 750 μL of the sample was added into the zeta cell. We gently tapped the zeta cell to dislodge any air bubbles and visually checked that both electrodes had been submerged. The zeta cell was placed into the instrument and the zeta-potential of samples was collected. Three runs per sample were performed for the reproducibility of data.

Cell Viability Assay. The cell cytotoxicity of the R1 peptide was evaluated by the CCK8 assay. Cells were pre-seeded into a transparent 96-well plate at the density of around 5000 cells in each well for 12 h and then incubated with 3-fold serial dilutions of R1 peptide in a fresh culture medium for 48 h. The culture medium was added with 10 μL of CCK8 solution for each well and incubated for an additional 3 h. The optical density at a wavelength of 450 nm (OD$_{450}$) was recorded by using the Synergy H4 microplate reader (BioTek, USA). The viability of the cell incubated with a fresh culture medium defines 100%.

Statistical Analysis. Replicate measurements were carried out for all experiments, and the number of replicates was provided in the result section of the experiment. Student’s $t$-test was performed to assess the statistical significance of the results ($^*P < 0.1$ and $^{**}P < 0.01$).

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c03707.

(Figure S1) BLI curves for the association and dissociation of R2 peptide, R3 peptide, and R4 peptide; (Figure S2) CD measurements with 50 μM R1 in sodium phosphate buffer; (Figure S3) the response of R1 peptide to SARS-CoV-2-Spike pseudovirus by BLI; (Figure S4) the response of R1 peptide-modified AuNPs to SARS-CoV-2-Spike pseudovirus; (Figure S5) kinetic binding curves of R1 peptide to MERS-CoV-Spike-RBD and SARS-CoV-1-Spike-RBD with he concentrations of 1000, 500, 250, 125, and 62.5 nM; and (Figure S6) kinetic binding curves of R1 peptide to 15.6, 31.3, 62.5, 125,250, and 500 nM HCoV-HKU1-S1 protein (PDF)

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L.Y., R.W., and T.W. contributed equally. L.Y. did the conceptualization, methodology, experiments, writing, and funding acquisition. R.W. did the experiments and validation. T.W. did the experiments and validation. L.L. did the writing of the review & editing. T.W. did the experiments. S.L. did the conceptualization, methodology, writing, supervision, resource gathering, and funding acquisition. C.W. did the conceptualization, methodology, writing, supervision, resource gathering, and funding acquisition. All authors have given approval to the final version of the manuscript.

Notes
The authors declare the following competing financial interest(s): C.W., L.Y., R.W., H.X. and T.W. are inventors on a pending Chinese patent related to this work filed by Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College (application no. 202110178816.3). The authors declare no other competing interests.

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