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A colorimetric sandwich-type bioassay for SARS-CoV-2 using a hACE2-based affinity peptide pair

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A B S T R A C T

The metallopeptidase angiotensin-converting enzyme 2 (ACE2) is the SARS-CoV-2 receptor required for viral entry based on its specific recognition of the spike protein receptor binding domain (S_RBD) on SARS-CoV-2. We constructed a human ACE2 (hACE2)-based peptide pair by ligating discontinuous key residues involved in the hACE2–S_RBD interaction. We firstly performed in silico simulations to identify a 12-mer and 15-mer peptide pair with capability to bind to the SARS-CoV-2 S_RBD via different binding sites. Then, the bio-layer interferometry validated the specific interactions between the peptides and S_RBD, with affinities at the nanomolar level. Lastly, we developed a colorimetric sandwich-type bioassay based on S_RBD-specific peptide-modified gold nanoparticles and found the colorimetric bioassay offered fast (~30 min), simple, and sensitive detection of S_RBD protein at levels as low as 0.01 nM (0.26 ng mL\(^{-1}\)) in SARS-CoV-2. The linear signals ranging from 10^5 to 10^7 virus copies mL\(^{-1}\) were achieved in typical types of environmental waters spiked with lysed SARS-CoV-2 pseudovirus. The technology can serve as a beneficial supplement to the routine virus nucleic acid detection in environment media and wastewater treatment.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which causes coronavirus disease 2019 (COVID-19), has spread globally since its report in Wuhan, China, in December 2019. As of Sept 4, 2021, the disease had spread to at least 204 countries, infected approximately 200 million people, and resulted in at least 4.5 million deaths worldwide (Anon, 2021). In general, such enveloped viruses are not considered a major threat for the wastewater and water industries due to their assumed low concentrations in municipal wastewater and high susceptibilities to degradation in aqueous environments (Wiggin-ton et al., 2015). However, some patients with SARS-CoV-2 infection have viral RNA or live infectious virus present in faeces, which suggests that the possible route might be faecal–oral transmission (WHO, 2020b). Furthermore, survivability studies show that SARS-CoV-2 is capable of retaining infectivity for days to months in aqueous environments (Larsen and Wigginton, 2020). Therefore, there is rapid ongoing research into cost-effective tools for detecting SARS-CoV-2 in water environment for better understanding of the full extent of the COVID-19 pandemic.

Apart from the routine nucleic acid detection of SARS-CoV-2, detection of the surface protein of virus via bioaffinity materials, also known as virus antigen detection, can provide more information of virus status in water system, even indicating live viruses. The receptor-binding domain in the S1 subunit (S_RBD) of SARS-CoV-2 binds to host-cell receptors and then triggers the SARS-CoV-2 infection process, and atomic-level images and structural modelling studies of S_RBD suggest that SARS-CoV-2 shares stronger affinity for the host cell receptor human angiotensin-converting enzyme 2 (hACE2) with previously discovered coronaviruses, such as SARS-CoV (Daniel Wrapp et al., 2020; Hofmann et al., 2005; Li et al., 2003). Recently, in silico simulations enabled the design of a series of rigid peptides generated by extracting partial helices and folds from hACE2 for binding the SARS-CoV-2 S_RBD (Bressler et al., 2018; Han and Kral, 2020; Ling et al., 2020; Zhang et al., 2020a); however, only discontinuous α-helices or β-sheets with certain key amino acid residues were considered in the design, with neither of these designs capable of sufficiently covering the binding sites. Consequently, the peptides reportedly working as viral inhibitors shared the similar binding sites of SARS-CoV-2 S_RBD and with relatively long residues, none of them have proven to be suitable biorecognition materials for developing the sandwich-type sensing mode. Recently, \textit{de novo} methods have been reported to design hACE2-based peptides as S_RBD...
binders. However, the affinity to S\textsubscript{RBD} of short peptides is still far less than that of ACE2 (Pomplun et al., 2021), while larger peptides (>50-mer) increase the difficulty and cost of chemical synthesis and modification (Cao et al., 2020; Linsky et al., 2020). Although peptide is an attractive affinity probe in many applications, the peptide design methods for SARS-CoV-2 is still under development.

Targets that pose a significant public health threat (such as a deadly virus) are of particular importance for developing rapid, low-cost, simple-to-use, and instrument-free in-field assays that impart minimal scientific burden on the user (Wang et al., 2017). Gold nanoparticles (AuNPs) have emerged as excellent nanomaterials due to their unique physical and chemical properties (Link and El-Sayed, 2003; Saha et al., 2012). The distinctive color change arising from interparticle plasmon coupling during AuNP aggregation or redispersion has attracted considerable attention for applications as a simple and rapid detection assay with great potential for in-field analysis (Elgammal et al., 1997; Ghosh and Pal, 2007). Compared with unmodified AuNPs (in the general sense, citrate-capped AuNPs), AuNPs functionalized using thiol-Au chemistry can offer resistance to harsh environments and higher reliability. Integration with different biorecognition materials, such as DNA, RNA, peptides, or antibodies, has allowed the development and utilization of functionalized AuNP-based colorimetric assays for detecting various viral targets (Draz and Shaﬁee, 2018). Inspired by the high affinity of hACE2 to the S protein on the SARS-CoV-2 surface, we attempted to develop a rapid, simple, and colorimetric assay for detecting SARS-CoV-2 using AuNPs as indicators. However, AuNPs-aggregation-based bioassays rely heavily on the surface chemistry of biorecognition elements in the correct orientation relative to their targets. The complexity and diversity of protein compounds make the synthesis of controllable and stoichiometrically deﬁned and oriented NP–biomolecule complexes a great challenge (Liu et al., 2009). Peptides are considered ideal candidates for proteins as biological-recognition elements, such as their small size, low cost, ease of synthesis and modiﬁcation, high surface density, and fast binding kinetics, compared with proteins (Cui et al., 2012; Pappas et al., 2016). Speciﬁcally, a common approach involves the use of thiol-Au chemistry to achieve a self-assembled peptide monolayer exhibiting a high degree of orientation on the AuNP surface. Furthermore, short peptides can minimize the aggregation-based interparticle distance, thereby improving assay sensitivity.

To address these challenges, in the present study, we report the development and validation of a matched hACE2-based S\textsubscript{RBD}-specific peptide pair and their application in an AuNPs-based colorimetric bioassay for SARS-CoV-2 detection in water environment. Based on the identification and analysis of key residues from the binding site in hACE2 that interacts with S\textsubscript{RBD}, we generated a peptide pair specific for SARS-CoV-2 rather than extracting individual hACE2 residues as described previously (Han and Kral, 2020; Zhang et al., 2020a). Through in silico simulations, we analyzed the conformations and binding energies of the peptide pair–S\textsubscript{RBD} complexes. The results identiﬁed the new S\textsubscript{RBD}-speciﬁc peptide pair targeting S\textsubscript{RBD} with suitable length, and we veriﬁed the binding afﬁnity of each with S\textsubscript{RBD} by bio-layer interferometry (BLI). Lastly, we exploited the interparticle plasmon coupling of AuNPs and the binding capabilities of the matched peptide pair to develop a simple and fast colorimetric detection platform targeting the S\textsubscript{RBD} of SARS-CoV-2, as well as other viruses sharing a similar cell-entry method. Considering the diverse applications of AuNPs with peptides, this study can also serve as a good reference in providing important implications in the sensing and peptide-directed nanoparticle assembly for coronavirus tracing and COVID-19 control.

2. Material And Methods

2.1. The hACE2-based peptide pair design and in silico simulation

The parent peptide was designed by recognizing and reorganizing key residues in hACE2 involved with binding the SARS-CoV-2 S\textsubscript{RBD} (Fig. 1a), and -GGG- ammonia acids were used to link residues 24–41, 349–360 and 82–83 of hACE2, in order to avoid or limit interference between the original residue interactions. Subsequently, the peptide pair (Pep15 and Pep12) with non-overlapping sequences was generated from the parent peptide. The sequences are shown in Fig. 1b.

The S\textsubscript{RBD} structure used for in silico simulations was extracted from the complex structure [PDB ID: 6M17 (chain E)], and a de novo approach (PEP-FOLD3) was applied for structural modelling of the peptides. Initial structures were generated by aligning the hACE2-based peptides (Pep15, Pep12) to the position of hACE2 in the complex crystal structure. Additionally, we used Pep24–42 (generated from residues 24–42 of hACE2) as a positive control and two random peptides [a 12-mer (Ran12) and 15-mer (Ran15)] as negative controls. MD simulations of 1:1 peptide-S\textsubscript{RBD} complexes were conducted in Gromacs (v.2016.4; http://www.gromacs.org/) with a CHARMM27 force field (Berendsen et al., 1995). To explore the interaction among the peptide pair and S\textsubscript{RBD}, the 1:1:1 peptide-S\textsubscript{RBD}-peptide ternary complexes were also simulated. Simulations were performed in the presence of solvent, following energy minimization, and under constant-volume ensemble (NVT), isobaric–isothermal ensemble (NPT), and periodic boundary conditions. Single-point-charge water molecules were used to solvate the complex in a dodecahedral box, and the minimum distance between the box and the complex was 1.5 nm. Na\textsuperscript{+} and Cl\textsuperscript{-} ions were added to achieve charge balance. A 50,000-step energy minimization was performed based on the steepest-descent method, followed by 2-ns NVT and 2-ns NPT using the Parrinello–Rahman method at 300 K. The particle grid Ewald method and the modified Berendsen thermostat method were used for long-distance electrostatic and temperature coupling, respectively. The electrostatic cut-off and van der Waals cut-off were both set to 1.4 nm. The final MD simulation step was set to 2 fs, with the total time of 50 ns and output data saved every 1 ps. The root mean square fluctuation of the S\textsubscript{RBD} main chain, the RMSD of the S\textsubscript{RBD} backbone, and the secondary structure and trajectories were extracted from Gromacs.

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**Fig. 1.** Construction of an hACE2-based affinity peptide pair for SARS-CoV-2. a. The binding site of the hACE2 and S\textsubscript{RBD} is analyzed to extract important key ammonia acids from hACE2 (in wheat shadow). b. Sequences of parent peptide, S\textsubscript{RBD}-specific peptide pair and random peptides for comparison. Parent peptide represented with green background is followed with the truncated peptides (Pep15, Pep12) with blue background. Random peptides are represented with black font.
binding energy was calculated using the g_mmpbsa tool based on the MM/PBSA method (Kumari et al., 2014). The conformation files were visually displayed in PyMol (v.2.0.4; https://pymol.org/2/) and data were analyzed using Origin (v.9.1; OriginLab, USA).

2.2. Reagents

The SARS-CoV-2 S_RBD-His recombinant protein (S_RBD), SARS-CoV S_RBD-His recombinant protein, MERS-CoV S_RBD-His recombinant protein, HCoV-NL63 S1-His recombinant protein and SARS-CoV-2 spike pseudovirus were obtained from Sino Biological, China. LY-13 lysis buffer purchased from AcroBioSystem, China. The modified peptides [3-(CH₂)$_n$-CONH-Cys] were purchased from Gibcobio, China and the purities (95%) were measured by LCMS (Fig. S1). AuNPs in diameter of 10–20 nm were purchased from XFNANO, China. Phosphate-buffered saline (PBS; 10 mM sodium phosphate and 25 mM NaCl (pH 7.2)) was prepared using BupH Packs and NaCl from Thermo Fisher Scientific. Other chemicals were obtained from Sigma-Aldrich. All solutions were prepared using molecular-grade, ultrapure, sterilized water. Before use, stock solutions of 0.25 mg mL$^{-1}$ recombinant proteins and 1 mg mL$^{-1}$ affinity peptides were prepared in ultrapure water and stored at 4°C until dilution to the required concentrations with PBS.

2.3. BLI assays

Assays were performed on an Octet Red96 instrument (ForteBio, USA). Affinity peptides were biotinylated and immobilized on a streptavidin-modified BLI chip, and 1 × kinetics buffer (10 mM PBS, 0.1% BSA, and 0.05% Tween-20) was used for the assays. Before the measurements, the kinetics buffer was passed over the chip for 300 s to block possible nonspecific binding sites and establish a stable baseline. Association of various concentrations of SARS-CoV-2 S_RBD in 1 × kinetics buffer following a two-fold dilution series from 200 nM to 6.25 nM was performed for 600 s prior to dissociation for 600 s. Data were aligned using a 1:1 binding model with ForteBio data analysis software and $k_{on}$, $k_{off}$, and Kd values were recorded.

2.4. Preparation and characterization of peptide-functionalized AuNPs

Unmodified AuNPs dispersed in water were purchased from XFNANO, and the diameters (d) were calculated by UV-spectra according to a previously described method (Haiss et al., 2007) and confirmed by TEM analysis. AuNP concentration (~10 nM) was evaluated based on analytical relationships between the extinction efficiency and d (Haiss et al., 2007). Peptides (sequence-(CH₂)$_n$-CONH-Cys) were conjugated to the AuNPs by mixing 3.5 mL of 10 nM AuNPs with 30 μL of 350 μM peptides and incubation for 24 h with gentle rotation. The solution was then washed twice with ultrapure water by centrifugation at 16,000 × g for 20 min and then resuspended in 4 mL PBS. The UV-spectrum of the supernatant was measured to determine the absence of unconjugated peptide. 5 μL of the peptide–AuNP suspension in PBS was deposited on a 300-nm carbon-coated copper grid and dried in a 70 °C oven for 30 min. TEM images were obtained using a H-7650SB microscope (Hitachi, Japan) to evaluate the peptide–AuNP conjugates and also after S_RBD and Mg$^{2+}$ addition. Particle-size analysis was performed for the conjugates by counting ~100 particles from TEM images acquired at 200 kV. The DLS measurements were conducted by using the Zetasizer Nano ZS90 (Malvern PANalytical, UK) with a 633 nm wavelength laser light. The viscosity of the solution for DLS was adjusted to 0.887 cP with the selected dispersant refractive index of 1.330. Triplicate measurements were conducted for each sample. The size distributions of AuNP solutions were characterized by the number percent (in diameter, nm).

2.5. Sensitivity analysis

All absorbance spectra were recorded using a Synergy HTX multimode reader (BioTek, USA) and 384-well plates. A series of S_RBD standard solutions (0.1, 1, 10, 20, 50, 80, 100, and 200 nM) was prepared by diluting the stock solution with PBS, followed by addition of 12.5 μL of each respective S_RBD solution to 25 μL of the peptide–AuNP suspension (a 1:1 mixture of modified AuNPs functionalized with different peptides) for incubation at 37 °C for 10 min. Subsequently, 1.2 μL of 125 mM Mg$^{2+}$ solution was added for to the mixture for incubation at 37 °C for another 10 min, followed by transfer of 35 μL of the samples to a 384-well plate for absorbance measurements at 400–700 nm at room temperature (~25 °C) and the absorbance at 532 nm ($A_{532}$) was used as an index to indicate sensitivity. In order to visually display the color changes, the detection volume was scaled up six times and photographed within a transparent 96-well plate.

2.6. Selectivity analysis

To investigate assay selectivity, 12.5 μL of the SARS-CoV S_RBD and MERS-CoV S_RBD were added to 25 μL of the peptide–AuNP solution, respectively, and differences in the absorbance were analyzed. Three different concentrations for each spike protein variant (10, 50, and 80 nM) were used, and normalized signals ($[(A_{532}–A_{532=0})/A_{532=0}]$) were used as an index to indicate selectivity.

2.7. Detection of lysed pseudovirus in typical water environmental samples

Three sources of real-water samples, including surface water (Hetang Lake, Tsinghua University, China), wastewater influent and effluent from Daoxianghu Lake Water Recycling Plant which uses MBR biological treatment and ozone disinfection process (Beijing, China), were selected to investigate the practicability of the developed biosensor in urban water samples. Before analysis, water samples were filtered using a 0.22 μm filter. All water samples were centrifuged at 4000 rpm for 5 min to remove precipitates. Lysed pseudovirus stock solution was prepared by mixing pseudovirus (10$^{10}$ virus copies mL$^{-1}$) with LY-13 lysis buffer (1:9), followed by 10 min incubation. In bioassay detection, 15μL spiked sample prepared by mixing lysed pseudovirus solution with environmental samples and 10 × PBS (1:8:1) were added to 25 μL of the peptide–AuNP solution, respectively, for incubation at 37 °C for 10 min. Subsequently, 0.9 μL of 125 mM Mg$^{2+}$ solution was added for to the mixture for incubation at 37 °C for another 10 min. Other experimental conditions followed those in the sensitivity assay and all measurements were performed in triplicate.

2.8. Statistical analysis

In this work, error bars in all figures represent the standard deviations from three individual experiments.

3. Results And Discussion

3.1. Construction of an hACE2-based peptide pair

Based on the recently solved crystal structures of S_RBD–hACE2 complexes (PDB IDs: 6M17, 6M0J, and 6W11) and identification of the key amino acids in the binding sites (Lan et al., 2020; Shang et al., 2020; Yan et al., 2020), we chose the 2.9-A cryo-electron microscopy structure (PDB ID: 6M17) given a full-length hACE2 three-dimensional conformation contained. As shown in Fig. 1a, three peptide fragments (residues 24–42, 82–83 and 353–357, shown in wheat shadow) from hACE2 play important roles in the binding interaction of hACE2 and S_RBD. Among these, Gln24, His34, Tyr41, Gln42, Met82, Lys353, and Arg357 of hACE2 form a hydrogen bond network with S_RBD (Yan et al., 2020).
whereas other hACE2 residues within 5 Å of bound $S_{RBD}$ also represent potential key amino acids. To cover the binding site to the greatest degree possible, we constructed the parent peptide by adding linkers to the discontinuous fragments at specific positions during the design of the peptide pair. Given that the distances (Cα to Cα) between Gln24 and Tyr83 and Tyr41 and Lys353 were only 8.1 Å and 7.1 Å, we used three residues -GGG- as linkers. Based on the construction of the parent peptide, two peptides (Pep15 and Pep12) having non-overlapping sequences were selected in order to obtain the $S_{RBD}$ specific peptide pair for sandwich-type bioassay.

3.2. MD simulation

To investigate the binding affinities and binding mechanism to $S_{RBD}$, we conducted all-atom MD simulations to acquire a dynamic view of peptide binding to $S_{RBD}$ at the molecular level. MD simulation of the $S_{RBD}$ protein with the peptide pair (Pep15, Pep12), Pep24–42 as a positive control, and two random peptides (Ran12, Ran15) as negative controls, was performed for 50 ns, and for each simulation, two independent cycles were performed with different initial velocities. We used root mean-square deviation (RMSD) as the index to measure the conformational changes in the simulated structures. Other information with respect to structural changes of peptide–protein complexes during MD simulation, including the root mean square fluctuation (RMSF),

Fig. 2. MD simulations of peptide–$S_{RBD}$ complexes. a. RMSDs of the peptide–$S_{RBD}$ complexes following a 50-ns simulation. b. The average binding energy obtained during the final 1 ns was calculated using the MM/PBSA method. c–e. Conformations of the binding sites in each complex of Pep15–$S_{RBD}$-Pep12, Ran15–$S_{RBD}$, and Ran12–$S_{RBD}$, respectively. Hydrogen bonds are shown in yellow, peptides (except Pep15) in cyan, Pep15 in magenta, and $S_{RBD}$ in green.
secondary structure changes, and Ramachandran plots, is supplemented in Fig. S2.

Fig. 2a and Fig. S3 shows the time evolution of RMSDs for peptides. A plateau was reached with fluctuations of < 0.1 nm in the last 1 ns in each trajectory, indicating acquisition of equilibrium. We evaluated the binding energy of the peptide–protein complexes using the Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method (Srinivasan et al., 1998; Wang et al., 2019). We found that the truncated peptides (Pep15, Pep12) obtained binding energies < −80 kJ/mol, which were significantly lower than the negative controls (42.54 kJ/mol for Ran15, and −43.82 kJ/mol for Ran12) (Fig. 2b). The positive control, Pep24–42, generated through residues 24–42 of hACE2, showed a binding energy as low as −144.00 kJ/mol, similar to results from a previous report demonstrating high-affinity binding to $S_{RBD}$ (Zhang et al., 2020a, 2020b).

We then analyzed conformational stability in the peptide–protein complexes (Fig. 2c–f and S3a-b). All peptides expect Ran15 maintained their positions within the hACE2-$S_{RBD}$ binding site along with the hydrogen bonds formed with $S_{RBD}$ residues. Residues Gln476, Gln493 and Gln498 of $S_{RBD}$ were identified as key residues involved in the peptide–protein interactions, generally consistent with results observed in the recently solved crystal structure (PDB ID: 6M17). Notably, in the Pep15–$S_{RBD}$-Pep12 complex, Pep15 and Pep12 maintained the stable binding conformation with $S_{RBD}$ by forming three hydrogen bonds via completely different binding sites (Fig. 2c). By contrast, we observed the formation of only one hydrogen bond in the Ran12–$S_{RBD}$ complexes (Fig. 2e) and even a 5 nm long-distance between Ran15 and $S_{RBD}$ (Fig. 2d). Results indicated that negative controls might exhibit weak or no binding affinity with $S_{RBD}$, which was consistent with the predicted binding energies > −80 kJ/mol. The structural stability of Pep15–$S_{RBD}$-Pep12 complex provides the basis for its application as a peptide pair specific to $S_{RBD}$.

The above work provides insights into the mechanism of protein–protein interaction-based affinity peptides reconstruction guided by in silico simulations. Notably, the simulation results and followed BLI experiments were verified to be in good agreement. Overall, our work highlights the important guiding role of in silico simulations in unveiling the binding activity of $S_{RBD}$-specific peptide pairs, which can be generalized to the simulation of other peptide–protein structures, dynamics and binding potencies to gain insights at the molecular level.

### 3.3. Verification of binding affinities

We then performed BLI to verify the binding affinity of each of the peptides with $S_{RBD}$. Interactions between $S_{RBD}$ and the hACE2-based peptides, a positive control (Pep24–42), and two negative controls (Ran15, Ran12) were measured by immobilizing the biotinylated peptides on a streptavidin (SA)-functionalized solid surface, and the surface of the SA sensor was blocked with 1 mg mL$^{-1}$ bovine serum albumin (BSA) prior to measurement to eliminate nonspecific interactions. Data collected after background subtraction of nonspecific adsorption signals were recorded and are shown in Fig. 3 and S3d-e. Table 1 and Table S1

![Fig. 3. Determination of peptide binding affinities. BLI measurements for a. Pep15–$S_{RBD}$, b. Pep12–$S_{RBD}$, c. Ran15–$S_{RBD}$, and d. Ran12–$S_{RBD}$. Alignment curves are indicated by dashed lines.](image-url)
show that all association–dissociation processes fit the binding model well ($R^2 > 0.99$). Pep15, Pep12 and their parent peptide showed affinities in the nanomolar range toward $S_{\text{RBD}}$, with Pep15 exhibiting the lowest $K_d$ in agreement with MD simulation results (Fig. 3a-b), whereas Ran15 and Ran12 showed no binding affinity with $S_{\text{RBD}}$ (Fig. 3c-d). Among them, Pep24–42, which combines two hACE2 peptide sequences sharing 82.6% amino acid homology and previously reported as a first-in-class peptide binder to the SARS-CoV-2 spike protein (Zhang et al., 2020a), exhibited a comparable affinity for $S_{\text{RBD}}$ with that reported previously (Fig. S4e and Table S1) (Zhang et al., 2020a). Notably, we found that the parent peptide constructed by extracting key binding residues with glycine linker to the discontinuous fragments showed a higher binding affinity for $S_{\text{RBD}}$ than Pep24–42 ($K_d$: 9.44 nM vs 23.35 nM), which only considered certain key amino acid residues from hACE2. Results suggested the efficacy of the peptide-construction method employed in the present study, and the binding affinity of peptides improves ($K_d = 14.50–31.91$ nM) even compared with the very recent affinity peptide study (peptides with $K_d = 80–970$ nM) (Pomplun et al., 2021). Moreover, Pep15 and Pep12 with completely different sequences, as the shortest affinity peptides to $S_{\text{RBD}}$ than previously reported, providing possibilities to work as an affinity peptide pair to design more flexible downstream biosensing methods for the detection of SARS-CoV-2. And it is suggested that these two peptides may bind to $S_{\text{RBD}}$ by different sites, because the association-dissociation process between $S_{\text{RBD}}$ and Pep15 is rarely affected by the binding of $S_{\text{RBD}}$ and Pep12 (Fig. S5).

### 3.4. Peptide-functionalized AuNP colorimetric bioassay for $S_{\text{RBD}}$ detection

We then used the truncated peptides to develop a sandwich-type colorimetric bioassay targeting $S_{\text{RBD}}$ using peptide-modified AuNPs for detecting of SARS-CoV-2. A schematic illustration of the sensing process, b. DLS measurement of the size distribution showing the unmodified AuNPs in the red line and the peptide-modified AuNPs in the black line, separately. c. Visible spectra of the bioassay following addition of various concentrations of $S_{\text{RBD}}$. d. Linear fit of the calibration curve for $S_{\text{RBD}}$ ($R^2 = 0.99$) with a 95% confidence band (shown in red shadow). Each data point represents the average $A_{532}$ with standard deviation for triplicate experiments. e. Selectivity of the bioassay toward $S_{\text{RBD}}$ or the S1 subunit of SARS-CoV-2, SARS-CoV, and MERS-CoV.

| Table 1 | Equilibrium dissociation constants ($K_d$) for peptide binding to SARS-CoV-2 S$_{\text{RBD}}$. |
|----------|--------------------------------------------------|
| Pep15 | Pep12 |
| $k_{\text{on}}$ ($M^{-1} \cdot S^{-1}$) | $1.76 \times 10^5$ | $0.79 \times 10^4$ |
| $k_{\text{off}}$ ($S^{-1}$) | $2.55 \times 10^4$ | $2.53 \times 10^4$ |
| $K_d$ (nM) | $14.50 \pm 0.41$ | $31.91 \pm 0.31$ |
| $R^2$ | 0.9941 | 0.9922 |

Data are derived from that provided in Fig. 3. *Values represent the average ± standard derivation.*
colorimetric bioassay for rapid detection of S\textsubscript{RBD}. In the proposed bioassay (Fig. 4a), we used the identified peptide pair consisting of Pep15 and Pep12 that specifically bound to the SARS-CoV-2 S\textsubscript{RBD} via different binding sites (Fig. 2c). A sulphydryl group was functionalized to each peptide by introducing an (CH\textsubscript{2})\textsubscript{2}–CONH–Cys flexible linker, and the sulphydryl-functionalized peptides (Sulfhydryl-Pep15 and Sulfhydryl-Pep12) were conjugated to AuNPs, respectively. Transmission electron microscopy (TEM) revealed the diameters of peptide-functionalized AuNPs as ~18 nm to ~20 nm, which was larger than the unmodified AuNPs (~17–18 nm) (Fig. S6a–b). The dynamic light scattering (DLS) measurement revealed that the mean hydrodynamic size of AuNPs changing from 21 nm to 25 nm after the functionalization of peptides (Fig. 4b). The target-triggered variations in the absorption spectra of AuNPs provide a qualitative basis for colorimetric sensing. In the presence of S\textsubscript{RBD}, the functionalized peptides captured S\textsubscript{RBD} and formed a sandwich assembly that promoted AuNP aggregation (Fig. S6c), which resulted in a distinctive color change arising from the interparticle plasmon coupling and absorption at 532 nm (A\textsubscript{532}). By contrast, in the absence of S\textsubscript{RBD}, we observed no color development. It is noted that AuNPs aggregation does not occur when a single type of peptide-modified AuNPs bind to S\textsubscript{RBD} (Fig. S7). Under optimal assay conditions (Fig. S8 and the Supplementary Note), we investigated the visible spectra associated with the peptide-functionalized AuNPs in the presence of various concentrations of S\textsubscript{RBD} (up to 200 nM) (Fig. 4c). A standard curve obtained by plotting the A\textsubscript{532} value (Fig. 4d) demonstrated a linear working range of 0.01–80 nM (0.26–2080 ng/mL), with a limit of detection (LOD) for S\textsubscript{RBD} < 0.01 nM (0.26 ng/mL), which showed a better working range compared with a logistic function fitting curve (Fig. S9). Notably, this facile and time-saving one-step assay was comparable with currently available S\textsubscript{RBD} ELISA assay using an immobilized hACE2, with linear range of 8–125 ng/mL using an immobilized hACE2 (acrobiosystems, 2020a), and a naked-eye LOD against S\textsubscript{RBD} was achieved down to 50 nM (130 ng/mL). Inset of Fig. 4c, suggesting the utility of the assay for in-field applications without the aid of instrumentation.

Coronaviruses use the spike glycoprotein to bind host receptors and facilitate viral entry. Therefore, it is possible that a colorimetric bioassay developed using hACE2-based affinity peptides might exhibit cross-reactivity with other coronaviruses sharing similar entry mechanisms. SARS-CoV S\textsubscript{RBD} reportedly shares ~74% sequence homology that of SARS-CoV-2 (Fig. S10) while showing a slightly lower binding affinity for hACE2 (On et al., 2020). Furthermore, MERS-CoV, which belongs to the same family as SARS-CoV and SARS-CoV-2, recognizes human dipeptidyl peptidase (Rabaan et al., 2020; Wang et al., 2013), suggesting the same family as SARS-CoV and SARS-CoV-2, recognizes human for hACE2 (Ou et al., 2020). Furthermore, MERS-CoV, which belongs to the same family as SARS-CoV and SARS-CoV-2 (Fig. S10) while showing a slightly lower binding affinity for hACE2 (acrobiosystems, 2020a), and a naked-eye LOD against S\textsubscript{RBD} as reported in previous studies (Rabaan et al., 2010; Surette et al., 2021). Therefore, it is valuable to systematically investigate the effect of water matrix constituents before moving to the practical applications.

The current state-of-the-art analytical method for SARS-CoV-2 still heavily depends on RT-qPCR, which needs well-equipped laboratory and well-trained personnel (Rabaan et al., 2020; Zhu et al., 2020). As a supplement to the laboratory method, oligonucleotide-capped AuNPs have been constructed for the colorimetric detection of the conserved gene fragment of MERS-CoV (Liu et al., 2009) and SARS-CoV-2 (Cui et al., 2012). However, due to the unstable nature of viral RNA molecules, nucleic acid-based testing requires professional isolation and extraction of viral RNA and is easy to result in unavoidable RNA loss (Pappas et al., 2016). Moreover, nucleic acid-based testing does not provide information concerning viral particles that are intact and infectious. In contrast, virus antigen detections are valuable in providing immediate results for large-scale screening testing and environmental monitoring in order to track the diffusion of the disease and to get an early warning of future outbreaks (Olearo et al., 2021). Recently, several hACE2-based SARS-CoV-2 antigen tests have been reported with LoDs at the nanomolar scale. However, hACE2 protein cannot be chemically synthesized, and the storage and transportation conditions are strict, which limit the usage in environmental monitoring (D’Agostino et al., 2021; Peng et al., 2021). Evolving from in silico simulations based on hACE2 protein, new SARS-CoV-2 S\textsubscript{RBD}–specific peptide developed in this work can serve as a class of promising biorecognition material. The constructed surface-protein-based tools have unique advantages such as simple pretreatment procedures, easy capture of infectious viruses, and

\[
y = -2.56 \times 10^{-8} x + 0.27 \\
R^2 = 0.99
\]

\[
y = -4.08 \times 10^{-8} x + 0.29 \\
R^2 = 0.99
\]

\[
y = -3.08 \times 10^{-8} x + 0.28 \\
R^2 = 0.99
\]

Fig. 5. Bioassay performance in a. surface water, b. wastewater influent, and wastewater effluent, respectively. Linear fit of the calibration curves for lysed pseudovirus (R\textsuperscript{2} = 0.99) in red line, a 95% confidence band shown in red shadow. 15 μl spiked sample and 25 μl peptide–AuNP solution (1:1 mixture of modified AuNPs functionalized with Pep15 and Pep12 peptide) were used in each test, and each data point represents the average A\textsubscript{532} with standard deviation for triplicate experiments.
being closest to a one-step assay. A previous study showed that even the shortest peptides (comprising two or three amino acids) can act as powerful self-assembly motifs (Pappas et al., 2016). For example, using the identified affinity peptide pair sharing non-overlapping sequences and independent binding sequences specific for S$_{RBD}$, a sandwich-type AuNPs-based colorimetric bioassay for rapid detection of S$_{RBD}$ exhibited a LOD down to 0.01 nM (0.26 mg mL$^{-1}$), and obtained results were comparable to ELISA conventional method using hACE2 (acrobiomery-systems, 2020a) and antibody-based biosensors (Fabiani et al., 2021; Kim et al., 2021). The bioassay also offered a linear detection range for lysed SARS-CoV-2 pseudovirus from $10^5$ to $10^7$ virus copies mL$^{-1}$ in various environmental samples, close to the recently reported method using a matched pair consisting of ACE2 and an S1-mAb antibody (Lee et al., 2021). Besides, compared with the reported method, this bioassay relying on a matched peptide pair is significantly cheap and facile, thereby demonstrating new possibilities for instrument-free, in-field detection of SARS-CoV-2.

4. Conclusion

There is an urgent need for low-cost and efficient detection methods for highly pathogenic viruses in water environment. Here, we constructed parent peptides from hACE2 based on the co-crystal structure of hACE2 and SARS-CoV-2 S$_{RBD}$, and identified a peptide pair consisting of a 15-mer peptide and a 12-mer peptide which specifically associated with the SARS-CoV-2 S$_{RBD}$ via different binding sites and exhibited nanomolar levels of affinity to S$_{RBD}$. As a proof-of-concept, we developed a colorimetric sandwich-type bioassay using the S$_{RBD}$-specific peptide pair and AuNPs that demonstrated fast, simple, and sensitive detection of the SARS-CoV-2 S$_{RBD}$ protein at levels as low as 0.01 nM (0.26 mg mL$^{-1}$). Additionally, we observed discriminable signals from the peptide-AuNP bioassay in real-water samples spiked with lysed SARS-CoV-2 pseudovirus, demonstrating new possibilities for its instrument-free in-field detection of the SARS-CoV-2 virus. Furthermore, the high-affinity peptides and peptide pair targeting S$_{RBD}$ showed potential utilization in future COVID-19 treatment and diagnostic methods.

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CRediT authorship contribution statement

Qian Zhu: Methodology, Investigation, Visualization, Writing – original draft. Xiaohong Zhou: Methodology, Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of Interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127923.

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