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Electronic Supporting Information (ESI)

Effective ACE2 peptide-nanoparticle conjugation and its binding with the SARS-CoV-2 RBD quantified by dynamic light scattering

Vince St. Dollente Mesias,[a] Hongni Zhu,[a,b] Xiao Tang,[c] Xin Dai,[a] Yusong Guo,[c] Wei Liu,[d] and Jinqing Huang [a,b,]*

[a]Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China

[b]Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China

[c]Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, China

*Corresponding Author: Jinqing Huang

Email: jghuang@ust.hk

Contents

Preparation and purification of SARS-CoV-2 spike protein RBD

The glutathione transferase tagged (GST) SARS-CoV-2-Spike (RBD) purification was performed as described previously.[1] Briefly, SARS-CoV-2-Spike (RBD) was cloned in a pGEX-2T vector (GE Healthcare Biosciences, NJ). The constructs were transformed in BL21 cells and individual colonies were grown to O.D. 0.6 in 500 ml of Luria broth (LB) at 37°C. Protein expression was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) for 16 h at 25°C. Cells were centrifuged, washed with PBS and lysed in lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol, 0.5 mg/mL lysosome, proteinase inhibitor cocktail, complete, EDTA free, one tablet for 50 ml solution, Roche, Mannheim, Germany). After 30 min on ice, the cell lysates were adjusted to contain 0.5% TX-100 and sonicated four times for 30 s each time and centrifuged at 55k rpm for 20 min in a Beckman TLA-110 rotor for the ultracentrifuge. The supernatant fraction was incubated with 200 µl glutathione-agarose beads at 4°C overnight. After incubation, the beads were washed four times with PBS containing 1 mM DTT and 0.1% Tween 20 then two times with PBS. The bound GST tagged proteins were then eluted using elution buffer (50 mM Tris, pH 8.0, 250 mM KCl, 1 mM DTT, 25 mM glutathione, pH 8.0, proteinase inhibitor cocktail).

Sequence of the modified tACE2 and SARS-CoV-2 spike protein RBD

The following are the peptide and protein sequence used in this study:

tACE2:
CAAAEEQAKRFLDKFNHEAEDLFYQSSLASWYNA

SARS-CoV-2 RBD:
FTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISN
CVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQ
TGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDI
STEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAP
ATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDA

Preparation of tACE2-AuNP

Gold nanoparticles (O.D 1, Sigma Aldrich) were conjugated with the tACE2 peptide (GL Biochem Ltd, Shanghai, China) according to the method of thiol-gold functionalization described by Gao et. al with some modifications.[2] Thus, 1 mL of gold nanoparticles were mixed with 100 µL of tACE2 peptide in various concentrations (0.1, 0.5, 1, 5, 10, 20, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 80, 100 µg/ mL) and the pH was adjusted to 8.0 by 0.1 M sodium hydroxide solution (Sigma Aldrich). The mixture was stirred (200 rpm) for 10 mins and incubated for 12 h at around 4-8ºC, then washed twice to remove the excess peptides through centrifugation (3000 rcf, 15 min). Finally, the conjugated nanoparticles were resuspended using a buffer solution, 1X PBS before subjecting to UV-Vis spectroscopy, the resulting spectra for all tACE2 concentrations are presented in figure S1. Hydrodynamic diameters and zeta potential were measured using the ZetaPLUS Zeta Potential and Particle size Analyzer (Brookhaven Instruments, USA). Particle size

detection mode was set to 90º scattering with an active dust filter, while the zeta potential measurements were derived from electrophoretic mobility using the Smoluchowski model. Each reported value is an average of at least five measurements.

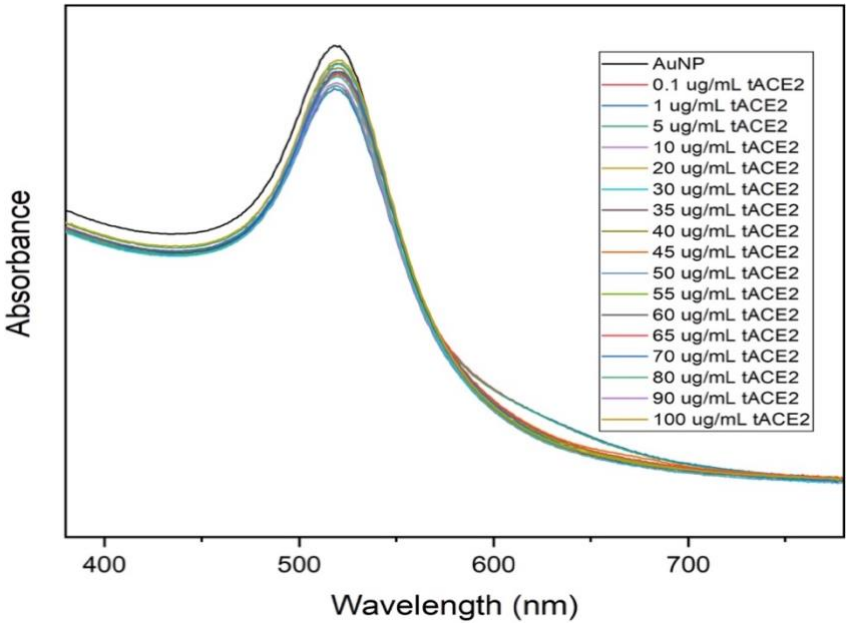

Figure S1. UV-Vis spectra of gold nanoparticles and its tACE2 peptide conjugates of varying concentration

Binding experiments of tACE2-AuNP with SARS-CoV-2 spike protein RBD

The tACE2-AuNP (700 µL) were added to 50 µL of varying concentrations (0.01, 0.025, 0.1, 0.25, 0.5, 0.75, 1, 5 µM) of SARS-CoV-2 RBD to yield the following RBD/ACE2-AuNP ratio: 0.43, 1.7, 4.28, 10.7, 21.4, 32.1, 42.8, 214, 652. Note that mole ratio can be calculated the variables of RBD (concentration and volume) and AuNP (volume and number of particles per mL). The mixtures were stirred for 60 s and incubated for 12 h. They were centrifuged (3000 rcf, 15 min) and washed twice with 1X PBS then resuspended using the same buffer. The nanoparticle suspensions were subjected to UV-Vis spectroscopy where the spectra of all the ratio are presented in figure S2. Moreover, the size and zeta potential of each mixture was measured using the same instrument.

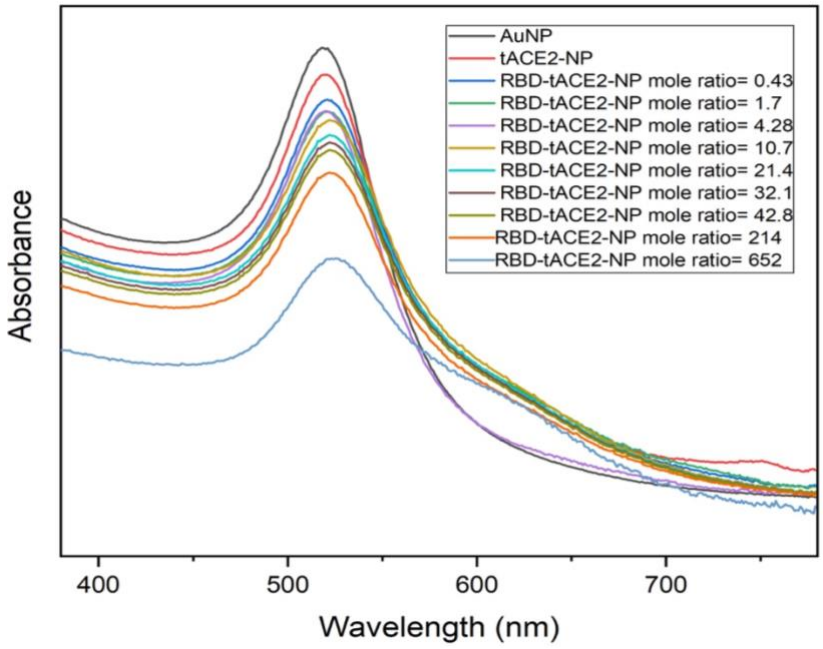

Figure S2. UV-Vis spectra of RBD-tACE2-AuNP in varying mole ratio

Time-dependent size measurements by dynamic light scattering

Two ratios representing low and high RBD levels were chosen to perform the particle size determination without the centrifugation and washing steps to monitor the progress of size increase as a function of time. The 21.4 and 214.0 RBD/tACE2-AuNP ratios were prepared and subjected to dynamic light scattering for particle size measurements. Hydrodynamic diameters were measured at the following time after the addition of RBD: 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 120, 240, 360, 480, 600, and 720 min using the ZetaPLUS Zeta Potential and Particle size Analyzer (Brookhaven Instruments, USA). The detection mode was set to 90º scattering with an active dust filter. Each reported value is an average of at least five measurements.

Binding experiments with interfering protein (BSA)

The time-dependent size measurements were conducted using the mixture of bovine serum albumin (BSA) protein and tACE2-AuNP at the BSA/tACE2-AuNP ratio of 214. In a separate setup, BSA and RBD were mixed with tACE2-NP such that each protein in the resulting mixture has ratio with respect to tACE2-AuNP, particularly RBD-BSA/tACE2-AuNP ratio of 214. Both mixtures were measured at the time intervals described in the previous section.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This approach applies slight modification to the previously reported SDS-PAGE protocol.[3] The resolving polyacrylamide gel was prepared using acrylamide solution (30%), 4X TRIS buffer (pH= 8.8), double deionized water, ammonium persulfate (10%), and tetramethylethylenediamine. The gel was casted between two plates with spacers and after drying the stacking gel made from the same components except for TRIS (pH= 6.8) was added to the plate. The resulting polyacrylamide gel cast was placed in a chamber with running buffer then filled with samples in different columns: marker, BSA, GST-tagged SARS-CoV-2 RBD, and supernatant solution of BSA-RBD-tACE2-AuNP mixture. The gel electrophoresis was conducted for 1 h with a current of 35 mA and an initial voltage of 150 V. The gel was subjected to staining with a Coomassie blue solution for 20 min thenm subjected to de-staining solution for 4 hrs. The resulting gel has four columns with visible bands that represent proteins.

Bradford protein assay

This a colorimetric approach for measuring total protein concentration based on the Bradford dye-binding method. It is easy to adapt the assay from the standard-concentration range to a low-concentration (<25 µg/ml; 1–20 µg total) microassay.[4] This study used the microassay protocol because the RBD concentrations involved are lower than the scope of the standard protocol. BSA was used as standard solution for calibration procedure in which 500 µL of BSA in a series of concentrations (0.1, 0.2, 0.3, 0.4 µM) were prepared from the stock solution, it is followed the addition of 200 µL of the Bio-Rad protein dye solution. The solution was mixed and stand for at least 5 min prior to spectrophotometric analysis at 595 nm. Similarly, the same volume of samples comprised of the supernatant solutions of RBD-tACE2-AuNP were treated with 200 µL of the Bio-Rad reagent and subjected to spectrophotometric analysis. The concentrations of RBD in the supernatant solution were derived using the linear equation generated by calibration standards as shown in figure S3. These concentrations represent the unbound RBD and hence the difference between this and the initial RBD concentration from each ratio gives the amount bound in µM, eq. 1. Note that the initial concentration of RBD varies from each ratio and since the total volume (RBD + tACE2-AuNP) must be considered the concentration will be less than that of the added RBD, refer to table S1 for the list of initial RBD concentrations. In addition, the ratio between the amount bound and the initial amount presents the reduction efficiency of the tACE2-AuNP against RBD in solution, eq. 2. The detailed results from concentration of samples until reduction efficiency are presented in table S2. The method detection limit is based on the 8 blank measurements from which the average and standard deviations were derived and then subsequently plug in to eq. 3. The calculated MDL (2×10^{-3} µM**)** is less than the initial concentration of each RBD-tACE2-AuNP mixture. Hence, a sample that gives a non-significant absorbance value means that the RBD concentration is lower than the detection limit, labeled as below MDL. For that reason, the bound RBD is assumed to be equal the initial RBD concentration and thus have an approximately 100% reduction efficiency.

Equation 1.

$$\textit{Bound RBD} = \textit{Initial RBD} - \textit{RBD in the supernatant solution}$$

Equation 2.

$$\%\ \textit{Sequestration} = \frac{\textit{Bound RBD}}{\textit{Initial RBD}} * 100$$

Equation 3.

$$\textit{Method detection limit} = \textit{mean}_{\textit{blank}} + 2.998 * \textit{standard deviation}$$

For N=8 (blank measurements)

RBD-to-tACE2-AuNP ratio	Concentration of RBD, μM	Volume of RBD, μL	Volume of tACE2-NP, μL	Initial concentration of RBD in the mixture, μM ($V_{RBD} * C_{RBD} / V_{total}$)
10.7	0.25	50	700	0.017
21.4	0.5	50	700	0.033
32.1	0.75	50	700	0.050
42.8	1	50	700	0.067
214	5	50	700	0.33
652	5	50	233 + 467 H_2O	0.33

Table S1. Initial concentration of RBD for each ratio of RBD and tACE2-AuNP based on the mixture composition

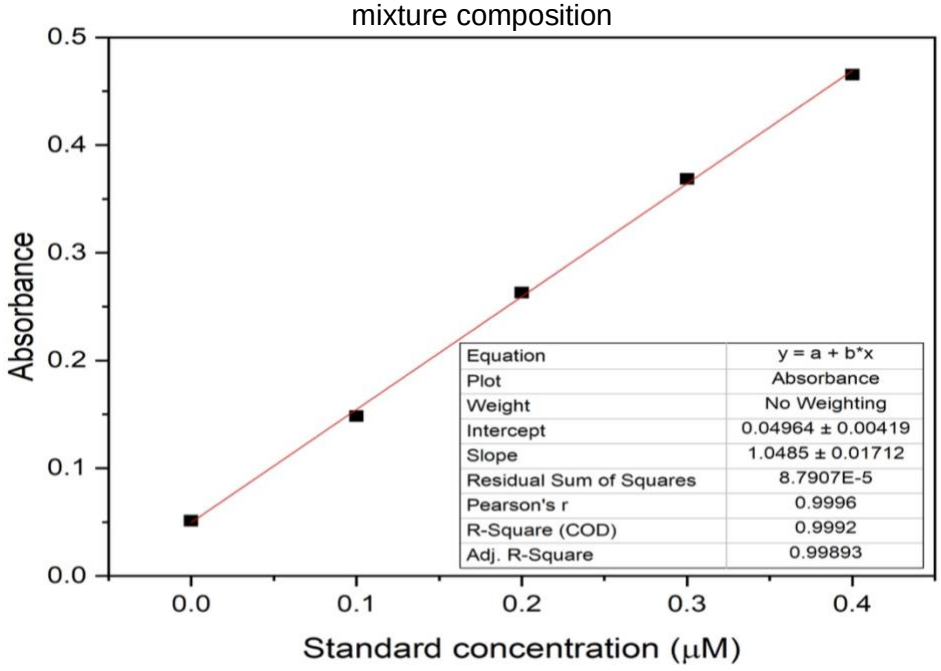

Figure S4. Bradford protein assay calibration curve

RBD-to-tACE2-AuNP ratio	Absorbance	Concentration of RBD in the supernatant, μM [x= (y-b/m)- blank]	Bound RBD, μM	Sequestration, %
Mean of the blank reading		0.001964711	**----**	**----**
10.7- A	*0.0017*	-0.04572246	Below MDL	~99
10.7- B	*0.0038*	-0.0437196	Below MDL	~99
10.7- C	*0.0047*	-0.04286123	Below MDL	~99
21.4- A	*0.0482*	-0.00137339	Below MDL	~99
21.4- B	*0.05*	0.000343348	0.03266	99
21.4- C	*0.0488*	-0.00080114	Below MDL	~99
32.1- A	*0.023*	-0.02540773	Below MDL	~99
32.1- B	*0.0261*	-0.02245112	Below MDL	~99
32.1- C	*0.0223*	-0.02607535	Below MDL	~99
42.8- A	*0.0529*	0.003109204	0.06389	95
42.8- B	*0.0531*	0.003299952	0.06370	95
42.8- C	*0.0525*	0.002727706	0.06427	96
214- A	*0.1201*	0.067200763	0.26609	80
214- B	*0.1118*	0.059284692	0.27402	82
214- C	*0.1048*	0.052608488	0.28069	84
652- A	*0.1521*	0.097720553	0.23558	70
652- B	*0.1493*	0.095050072	0.23825	71
652- C	*0.1487*	0.094477825	0.23882	71

Table S2. Bradford protein assay detailed calculation of the RBD in the supernatant, bound RBD, and reduction efficiency.

The approximation symbol (~) is placed on the efficiency of the replicate or mixtures with below MDL detection, the value is based on the sample 21.4-B.

Model equations and Fitting methods

Langmuir Isotherm

The plot of size vs. tACE2 concentration was fitted with a Langmuir model to verify the monolayer assumption, derive the association constant of the peptide to the gold nanoparticle, and the maximal hydrodynamic diameter. Langmuir model for DLS data was previously reported to monitor the conjugation coverage and it is presented in eq. 4 where C is the concentration of the peptide, q represents the size, K is the association constant between the nanoparticle and the peptide, and Q represents maximum size.[5] Subsequently, y-axis was set as the tACE2 peptide concentration-to-size ratio whereas the x-axis was the tACE2 peptide concentration, this plot was subjected to Linear fitting using the OriginPro software. Fitting details and results are shown in figure S4.

Equation 4.

$$\frac{C}{q} = \frac{1}{K\,Q} + \frac{C}{Q}$$

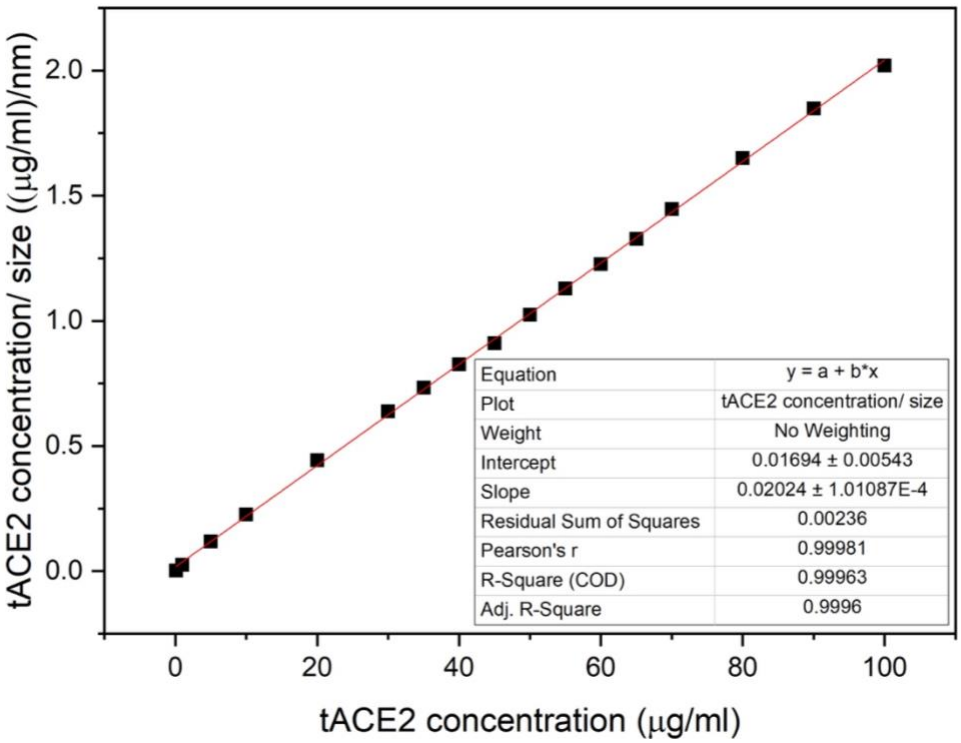

Figure S4. Fitting details for the Langmuir isotherm

Single exponential function

Binding kinetics from the time-dependent response measurements like surface plasmon resonance or fluorescence are usually derived using a single exponential function presented in eq. 5.[6] We can describe a response variable r, given by the size increase at a particular time t, while R is the maximum response in terms of size increase, and k_{obs} is the observed rate constant for binding molecules. The time-resolved plots from t=0 to t= 60 min generated from the time-dependent size measurements were fitted with the same equation using the OriginPro software with a Levenberg Marquardt iteration algorithm. Successful iterations are indicated by the fit convergence between the plots of the data and the model equation. Fitting details and results are shown in figure S5.

Equation 5.

$$r(t) = R^*[1 - \exp(-k_{obs}^*t)]$$

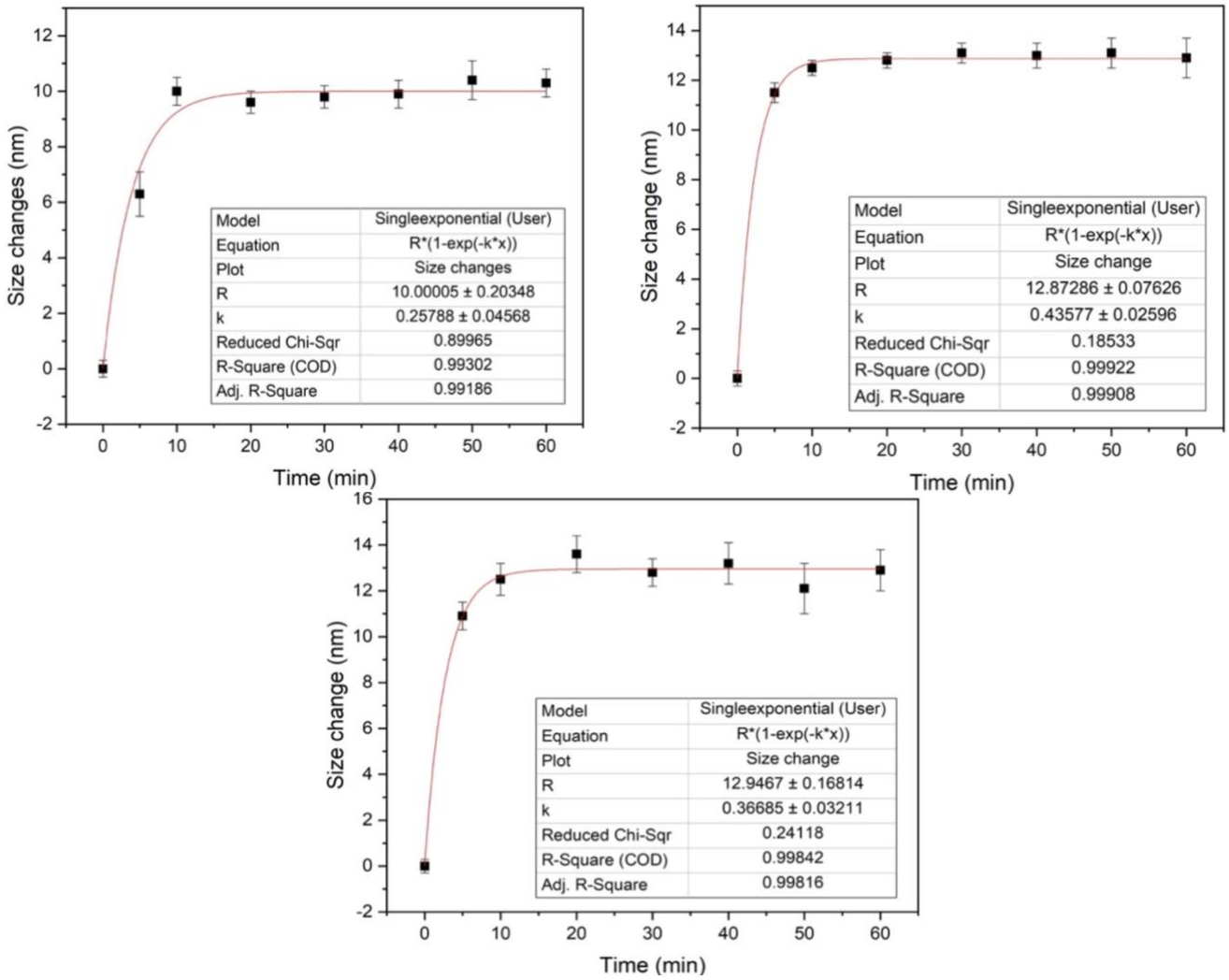

Figure S5. Single exponential fitting details for the time-resolved plots of RBD/tACE2-AuNP ratio of 21.4 (top left), 214 (top right), and RBD-BSA-tACE2-AuNP (bottom).

Modified Hill equation

DLS-monitored protein binding wherein a fixed amount of the conjugated nanoparticles was incubated with varying concentrations of the protein target and the resulting size was measured are previously reported.[7] A modified Hill equation shown in eq. 6 was used to derive the dissociation constant K_D which measures the binding affinity, B as the maximum size increase, and the Hill constant h that indicates the mode of binding. Inputs such as Y and X represent the size increase and RBD concentration, respectively. The plot of size increase vs. RBD concentration (0.01 µM to 3.0 µM) was fitted with the same equation using the OriginPro software with a Levenberg Marquardt iteration algorithm. Successful iterations are indicated by the fit convergence between the plots of the data and the model equation. Figure S6 presents the Hill equation fitting details of the size increase vs. RBD concentration plot.

Equation 6.

$$Y = \frac{B\,X^h}{X^h + K_D^h}$$

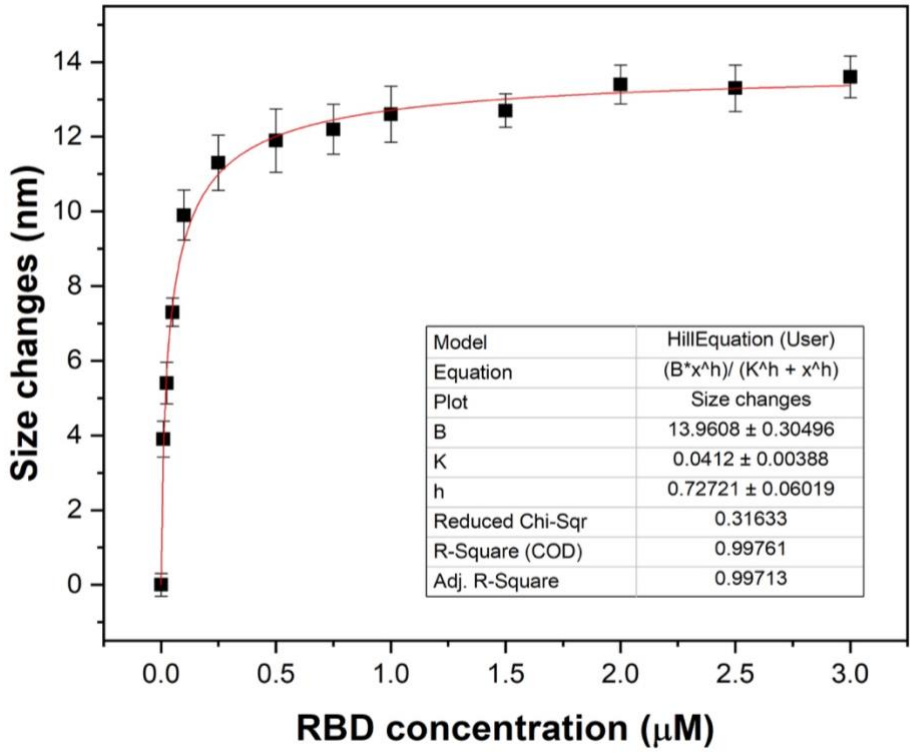

Figure S6. Hill equation fitting details for the size increase vs. RBD concentration plot.

The experiment was repeated to check the performance of this assay. Fitting results in figure S7 shows a binding affinity value of 40.7 nM which is in agreement with the reported value in figure S6 but with slightly higher value of reduced chi-squared.

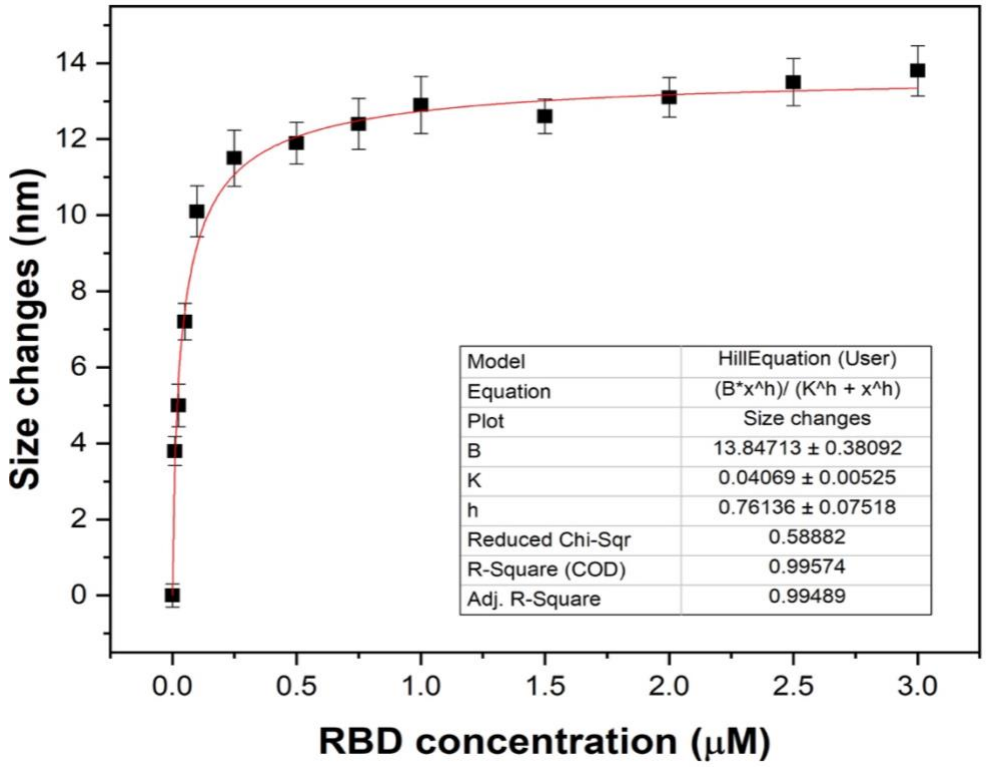

Figure S7. Hill equation fitting details for the size increase vs. RBD concentration plot from the parallel experiment under the same condition.

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy has been widely used to characterize the secondary structures of proteins and peptides.[8] Here, we used this technique to verify the alpha helical structures of the tACE2 in PBS, tACE2-AuNP, and RBD-tACE2-AuNP. The peptide concentration utilized here was significantly higher than that of the original conjugation and binding protocol to generate a quality signal. Specifically, we used 60 µM of tACE2 solution and 20 µM RBD solution for this process. The CD spectra were obtained using the Chirascan Spectrometer (Applied Photophysics, UK) with solvent background correction.

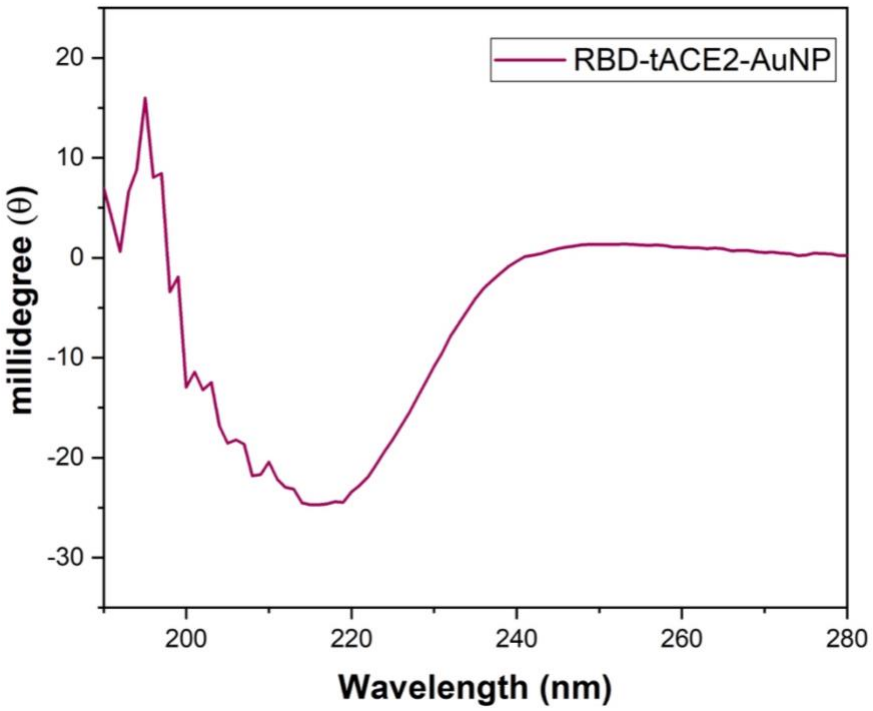

Figure S8. CD spectrum of RBD-tACE2-AuNP in buffer solution (PBS).

Raman spectroscopy of tACE2

Raman spectroscopy is another biophysical tool to monitor the structural changes in peptide or protein solution, particularly its amide band signals determine the protein secondary structures.[9] We examined the Raman spectra of solid tACE2 and 4mg /mL solution to evaluate any structural changes upon solvation. Spectral scan using the Invia (Renishaw) Micro-Raman spectrometer with the following laser parameters: 514nm, 12.42 mW, 40 s for the solid sample and 514nm, 24.84 mW, 150 s for the solution sample. Figure S8 shows the Raman spectra of the solid tACE2 and its solution form.

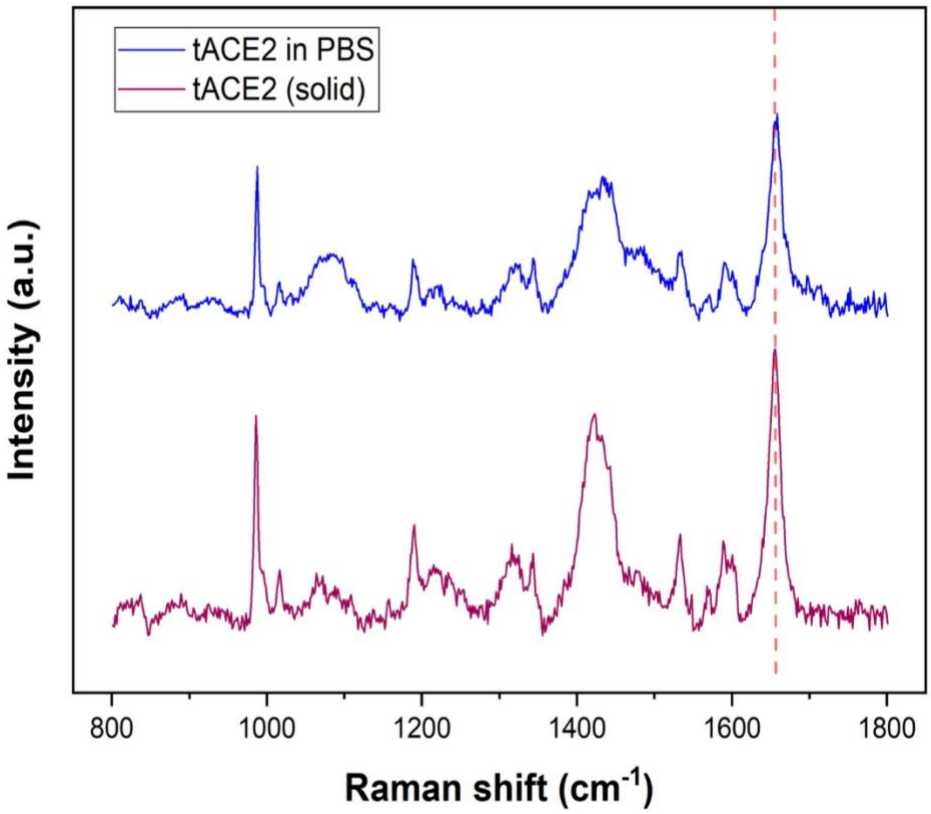

Figure S9. Raman spectra of tACE2 in its solid form (bottom) and solution form (top)

Fluorescence Anisotropy assays

Fluorescence anisotropy is one of standards in measuring the binding constant of a particular protein-protein or protein-ligand interaction. The freely moving ligand labelled by a fluorophore will give a less polarized signal, but a highly polarized signal is obtained when the ligand is bound to a larger molecule which indicates binding.[10] In this case, the tACE2 peptides were chemically labelled by the fluorescein isothiocyanate isomer 1 (FITC) according to the established protocol, this is followed by a conjugation to AuNP then added to different RBD concentrations. The anisotropy signals (Y) are plotted with varying concentrations of RBD (X) then fitted with an equation below,[11] where A represents the anisotropy of unbound tACE2-AuNP and B is the anisotropy of the fully bound state.

Equation 7.

$$\mathbf{Y= A + \frac{B*[X]}{K + [X]}}$$

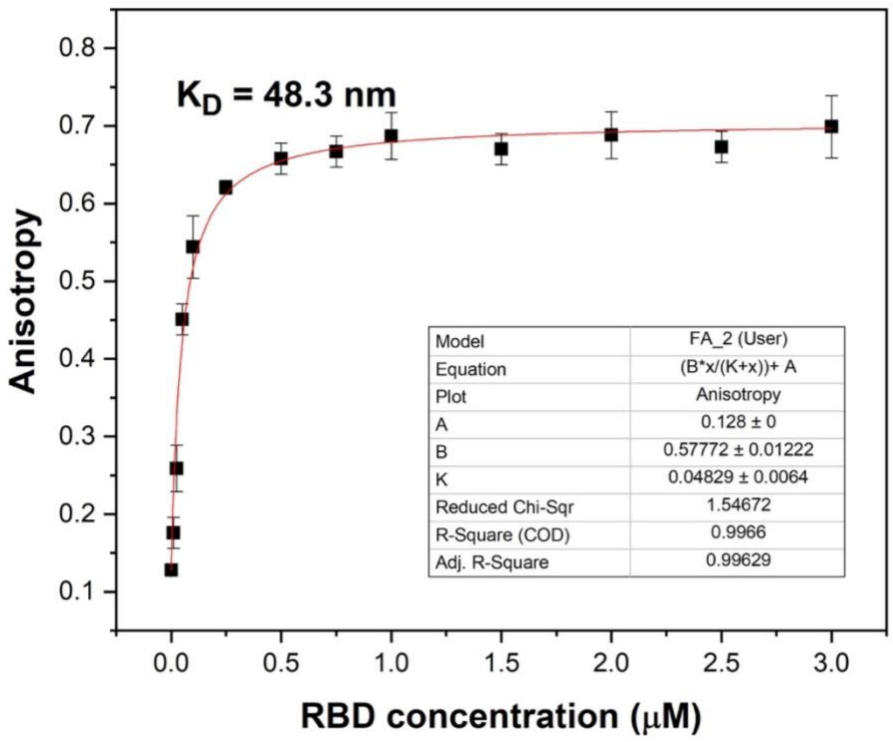

Figure S10. The fitting result for the Fluorescence anisotropy analysis.

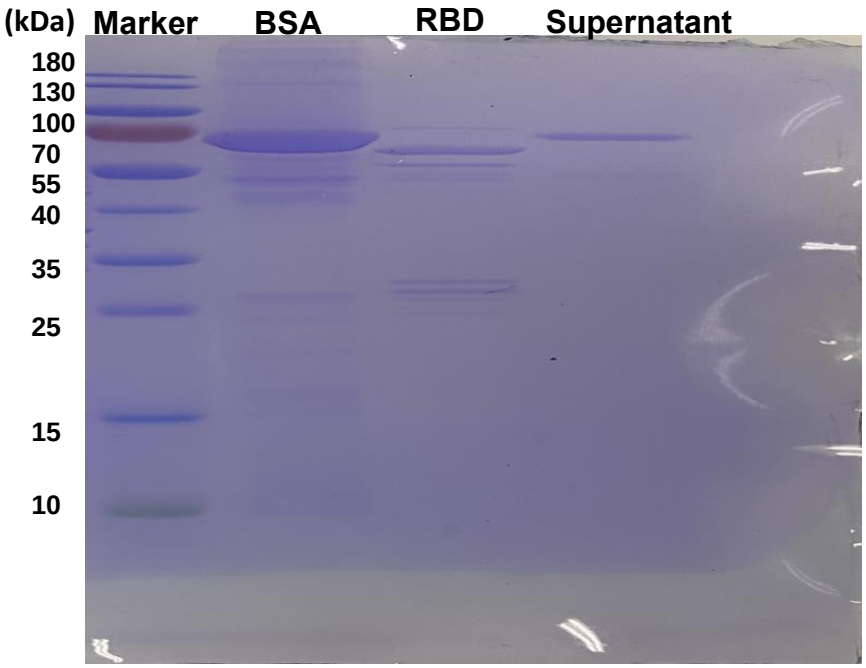

Figure S11. Raw image of the SDS-PAGE

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