Supporting information

Synthesis and immunological evaluation of synthetic peptide based anti-SARS-CoV-2 vaccine candidates

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General experimental procedures and methods for synthesis

All chemicals are reagent grade unless otherwise stated. A centrifugal filter with a molecular weight cut-off of 30,000 was purchased from EMD Millipore. HRP-conjugated goat anti-mouse IgG, IgG1, IgG2b, IgG2c and IgG3 were purchased from Abcam. Two SARS-CoV-2 neutralization test kits (L00847 and SC2087A) were purchased from GenScript. The SARS-CoV-2 spike RBD-His recombinant protein (RBD-His) was obtained from Sino Biological. To characterize the CRM197-RBD conjugates, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed. Coomassie plus protein reagent (Bradford assay, Pierce) was used to measure protein concentration with bovine serum albumin (BSA) as the standard.

Synthesis of RBD peptides 1−3

The RBD peptides **1**−**3** were synthesized from Wang resin using Fmoc chemistry. The *N*-terminal protecting group Fmoc was deprotected by 20% piperidine in DMF. The amino acid coupling was carried out with Fmoc amino acids (5 eq.) using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU)/hydroxybenzotriazole (HOBt) (4.9 eq.) and DIPEA (10 eq). After the peptide was assembled, the *N*-terminal Fmoc group was removed. Afterward, the peptides were cleaved from resins by TFA/TIPS/H_2O = 95/2.5/2.5 for 2 h. The excess TFA was evaporated. The peptides were precipitated by diethyl ether and centrifuged to pellet the solid. The crude peptides were purified on a Shimadzu HPLC. The resulting peptides were characterized by HPLC and ESI HRMS (see Schemes S1,2 and Fig. S1)

RBD **1**, ESI ($C_{191}H_{271}N_{43}O_{52}S$), calculated $[M-4H]^{4-}$: 1007.23, found: 1007.50.

RBD **2**, ESI ($C_{115}H_{153}N_{25}O_{32}S$), calculated $[M-2H]^{2-}$: 1213.53, found: 1213.50.

RBD **3**, ESI ($C_{116}H_{178}N_{34}O_{34}S$), calculated $[M+3H]^{3+}$: 875.77, found: 875.75.

Synthesis of CRM197-RBD conjugates 5−7

For preparation of CRM197-RBD conjugates, CRM197-MAL **4** was first synthesized. In brief, 200 μl of maleimide-PEG2-succinimide ester (50 mM) in DMSO was added to the cooled CRM197 (5.6 mg mL^{-1}, 1 mL) in 0.1 M potassium phosphate (K-Phos) buffer (pH = 7.0). The reaction was carried out at room temperature (rt) for 3 h. Afterward, the product was purified by an Amicon Ultra 30 kDa MW cut-off against 0.1 M K-Phos to yield CRM197-MAL **4** bearing 20 maleimides per CRM197 characterized by MALDI-TOF (see Fig. S2). To synthesize CRM197-RBD conjugate **5**, 9.4 mg of peptide **1** was dissolved in 80 μL of DMSO, and then added to the cold solution of CRM197-MAL **4** (2.5 mg mL^{-1}) in K-Phos buffer (pH = 7.0, 0.1 M, 400 μL). The reaction was performed at rt overnight. For synthesis of CRM197-RBD conjugate **6** or **7**, 5 mg of peptide **2** or **3** was respectively dissolved in 20 μL of DMSO, and then added to the cold solution of CRM197-MAL **4** (5 mg mL^{-1}) in K-Phos buffer (pH = 7.0, 0.1 M, 200 μL). The reaction was carried out at rt overnight. Afterward, the product was purified by an Amicon Ultra 30 kDa MW cut-off against 0.1 M K-Phos. The total protein content was quantified by Bradford assay against BSA standards. The extent of modification was determined by gel electrophoresis. As shown in Fig. S3, conjugate **5** was synthesized with a smaller number of peptides per CRM197 due to the poor solubility of peptide **1**. Therefore, we can consider directly selecting E484-Q493 instead of peptide **1** as one of the B cell epitopes for CRM197 conjugation in future vaccine design.

Mouse Immunization

Pathogen-free C57BL/6 female mice aged 6−10 weeks were purchased from Shandong University Laboratory Animal Center. All animal experiments were conducted by the guidance of the Animal Care and Use Committee of Shandong University. In all studies, 0.1 mL of various

CRM197-RBD conjugates were injected subcutaneously into mice under the scruff as an emulsion in complete Freund's adjuvant on day 0. On days 14 and 28, boosters were given subcutaneously under the scruff mixed with incomplete Freund's adjuvant. Sera samples were collected on days 0 (before immunization), 21, 35, 49 and 63. All RBD vaccine conjugates administered have the same amounts of RBD peptide (21 nmol).

Evaluation of antibody titers by ELISA

To test the ELISA for antibody titers against RBD peptides, the 96-well microtiter plates (Nunc MaxiSorp) were coated with 10 μg mL^{-1} of the corresponding RBD peptides **1**−**3** (100 μL/well) in $NaHCO_3/Na_2CO_3$ buffer (0.05 M, pH 9.6) containing sodium azide (0.02 %) by incubation at 4 ℃ overnight. For testing ELISA of antibody titers against RBD protein, the plates were coated with 1 μg mL^{-1} of the SARS-CoV-2 spike RBD-His recombinant protein (RBD-His) (100 μL/well). After antigen coating, the plates were washed with PBS/0.5% Tween-20 (PBST) (200 μL × 4) and blocked with 1 % BSA in PBS (100 μL/well) at rt for 1 h. The plates were washed with PBST (200 μL × 4) and incubated with serial dilutions of sera in 0.1 % BSA/PBS (100 μL/well). The plates were incubated for 2 h at 37 °C and washed with PBST (200 μL × 4). To determine antibody titers against peptide **1** produced by the conjugate **5**, a 1:8000 dilution of HRP-conjugated goat anti-mouse IgG1, IgG2b, IgG2c and IgG3 in 0.1% BSA/PBS (100 μL) was added to the wells, respectively. For all other studies, a 1:2000 dilution of HRP-conjugated goat anti-mouse IgG, IgG1, IgG2b, IgG2c and IgG3 in 0.1% BSA/PBS (100 μL) was added. Subsequently, the plates were incubated for 1 h at 37 °C and washed with PBST (200 μL × 4). A solution of the enzymatic substrate 3,3',5,5'-tetramethylbenzidine (TMB, 200 μL) was added to the plates (for one plate: 5 mg TMB, 2 mL DMSO, 18 mL citric acid buffer and 20 μL H_2O_2). The color was developed for 15 min and quenched by the addition of 50 μL of 0.5 M H_2SO_4. The absorbance was

measured at 450 nm using a microplate reader. The titer was calculated by regression analysis with log10 dilution plotted with optical density and reported as the highest fold of dilution giving the absorbance value of 0.1 over those of the negative control sera before immunization (OD = 0.2). All samples were performed in triplicate.

Epitope profiling of induced antibodies by CRM197-RBD conjugates

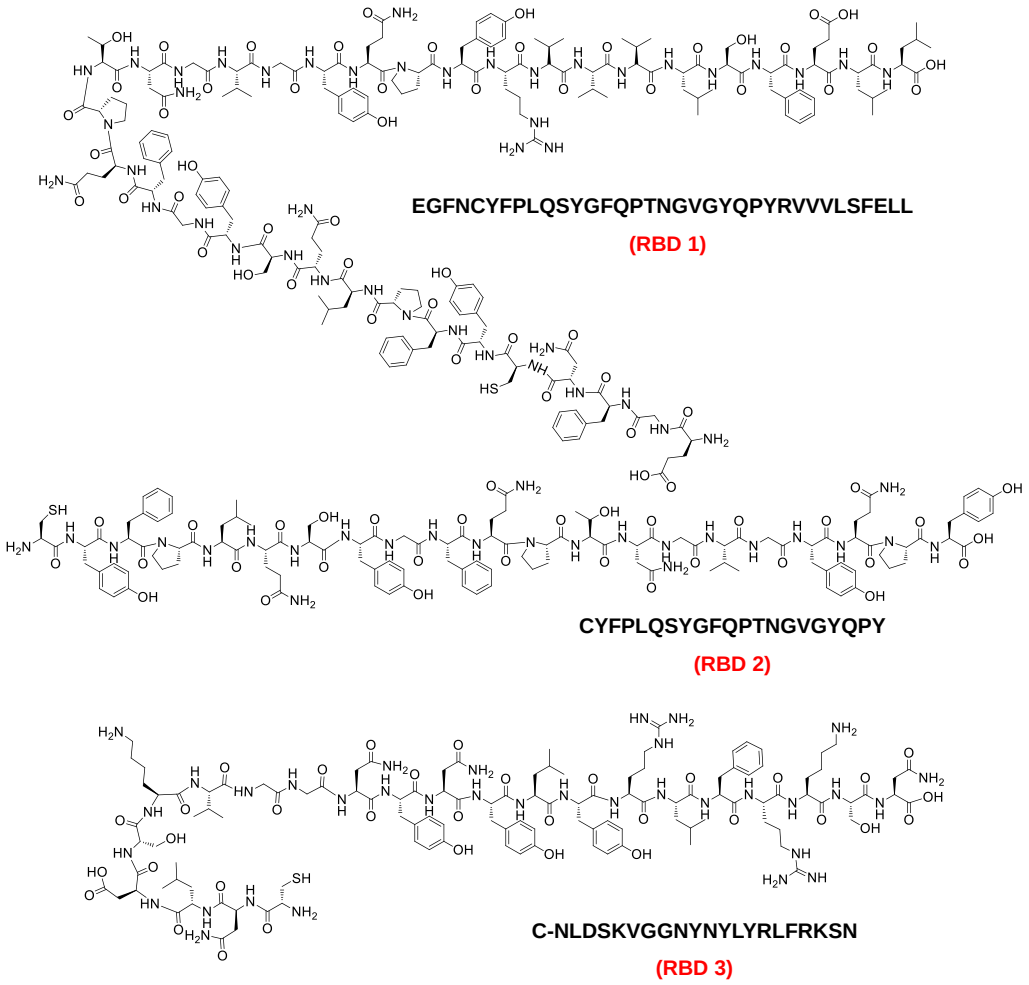
The ELISA plate was coated with RBD peptides **8–12**, **2** or **1** in $NaHCO_3$ (pH 9.6) buffer. This was followed by incubation with 1:12800 dilution of serum generated by the conjugate **5** and **6**, respectively, and HRP-conjugated goat anti-mouse IgG (1:4000, Abcam), washed and then incubated with TMB solution, Finally, 0.5 M H_2SO_4 was added to quench the reaction. The absorbance was measured at 450 nm.

SARS-CoV-2 surrogate virus neutralization test by ELISA

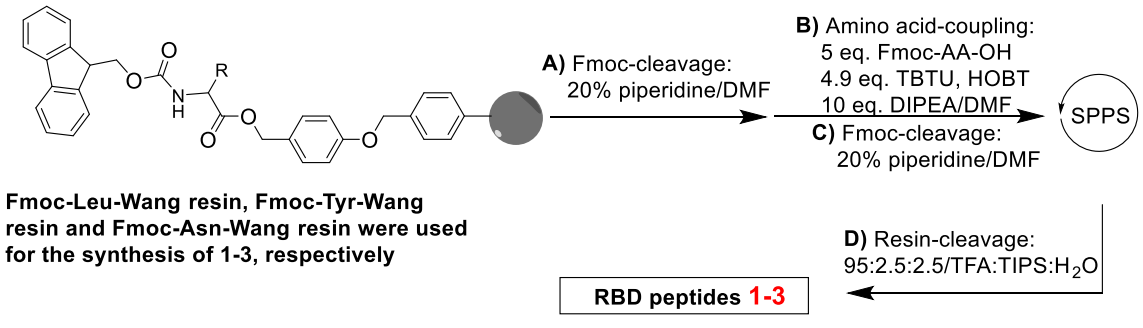
The SARS-CoV-2 surrogate virus neutralization test kit from GenScript was used for this study. In brief, the sera, positive and negative controls were diluted with sample dilution buffer at 1:10 dilution. Then the diluted positive control, negative control and sera were mixed with the same volume (50 μL) of HRP-RBD solution, respectively (the final dilution is 1:20). The mixture was incubated for 30 min at 37 ℃. Afterward, the mixed solution was added to 96-well plates precoated with hACE2. After 15 min of incubation at 37 ℃, plates were washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) solution (100 μL) for 15 min in the darkness. The reaction was stopped by the addition of 50 μL of H_2SO_4 stop solution. The absorbance at 450 nm was immediately collected using a microplate reader. Besides, other dilutions of sera (1:40 and 3:40, respectively) were also evaluated. The formula is as follows: $\text{Inhibition}\,\% = \left(1 - \frac{\text{OD value of sample}}{\text{OD value of negative control}}\right) \times 100.$

SARS-CoV-2 pseudovirus neutralization test

The SARS-CoV-2 pseudovirus neutralization detection kit (SC2087A, GenScript) from GenScript was used in this study. In brief, the positive control was diluted with Opti-MEM reduced serum medium (Opti-MEM) to a concentration of 20 μg mL^{-1}. 65 μL of post-immune sera (mixed with five mice sera) from **5**−**7** and pre-immune sera were respectively mixed with 65 μL of Opti-MEM to a volume of 135 μL. Then the sera were respectively diluted with Opti-MEM in a 3-fold gradient. Add 25 μL of positive control, Opti-MEM only, blank control (Opti-MEM) and serial dilutions of sera to the wells of 96 cell culture microplate (Corning, 3788). 25 μL of pseudovirus solution (2.0E+7 IFU mL^{-1}) was added to each well containing the positive control/Opti-MEM only or serial dilutions of sera. 25 μL of Opti-MEM was added to the blank control group. The pseudovirus neutralization process was carried out at rt for 1 h. Then, add 50 μL of Opti-HEK293/ACE2 cells (6×10^5 cells / mL) to each well. 24 h later, 50 μL of DMEM with 10% FBS was added to each well. After 48 h of infection, the supernatant in the 96-well plate was carefully aspirated and discarded. Then 50 μL of freshly prepared luciferase color reagent was added to each well. After 3 min of color development, the signal was collected on a chemiluminescence instrument. Besides, we found that a low concentration of negative (pre-immune) serum can slightly improve the pseudovirus infection, which may be due to certain factors in the serum. To get the most value is positive, negative serum (1:36 dilution) sample signal was used as a negative control signal. The formula is as follows: $\text{Inhibition } \% = \left(1 - \frac{\text{sera sample signal} - \text{blank control signal}}{\text{negative control signal} - \text{blank control signal}}\right) \times 100.$

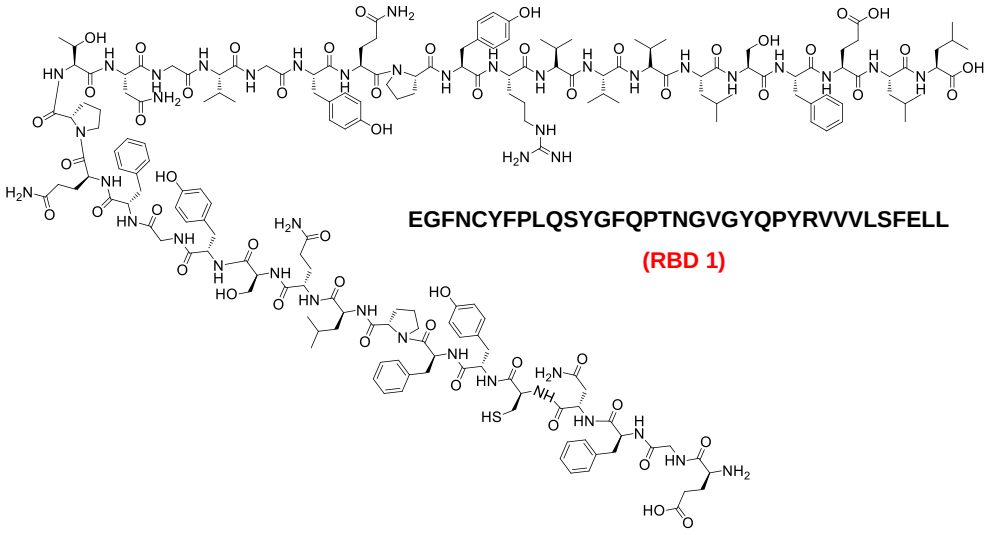


Scheme S1 Structures of RBD peptide epitopes **1**–**3**.



Scheme S2 Synthesis of RBD peptide epitopes **1**–**3**.

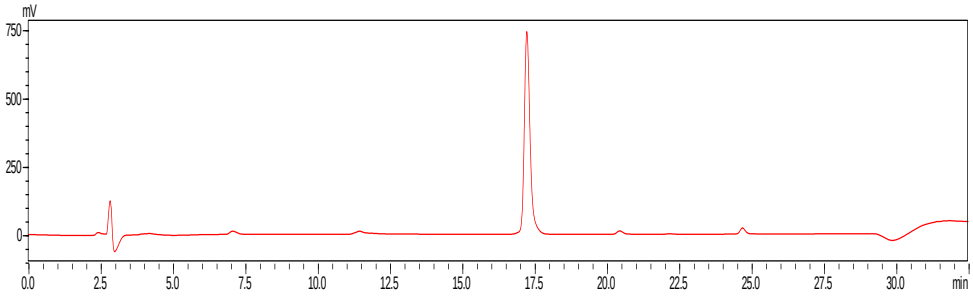
Fig. S1 Characterization of synthetic RBD peptides.



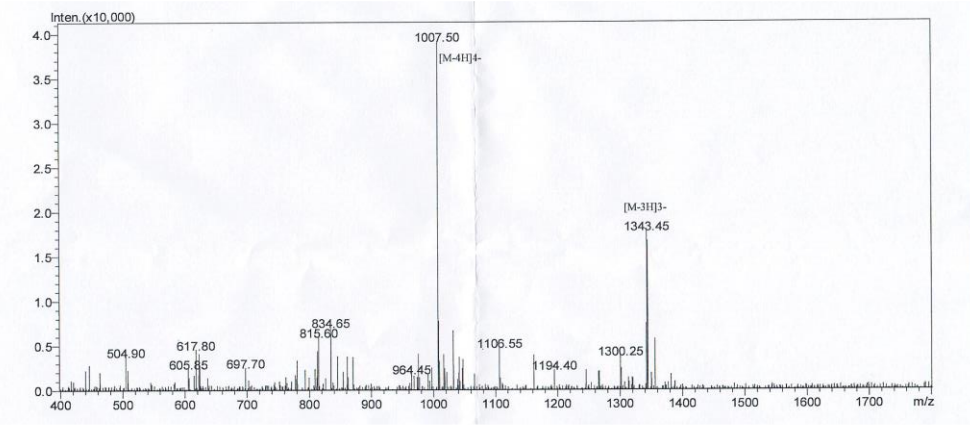
EGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELL

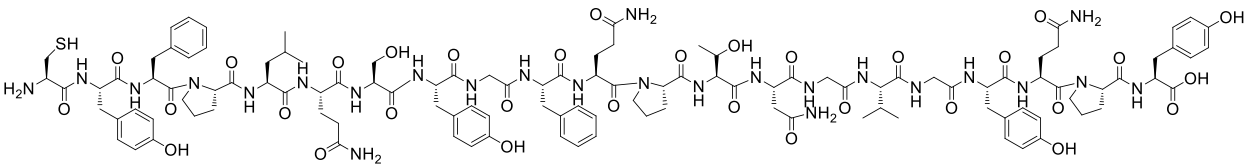
(RBD 1)

HPLC of RBD **1**



HRMS of RBD **1**

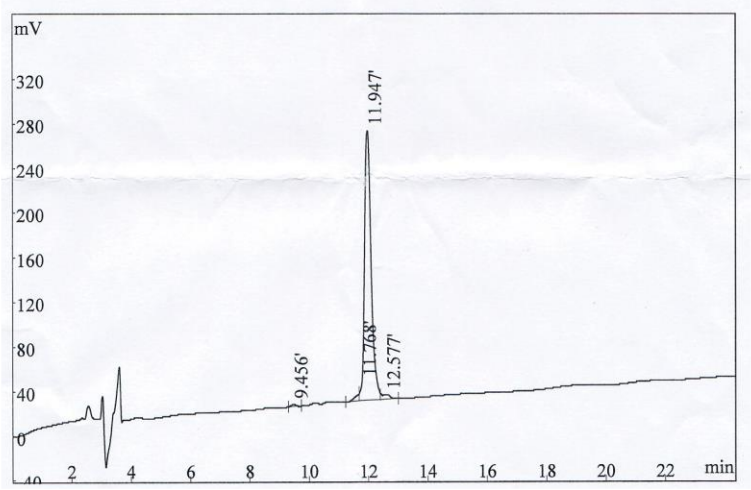




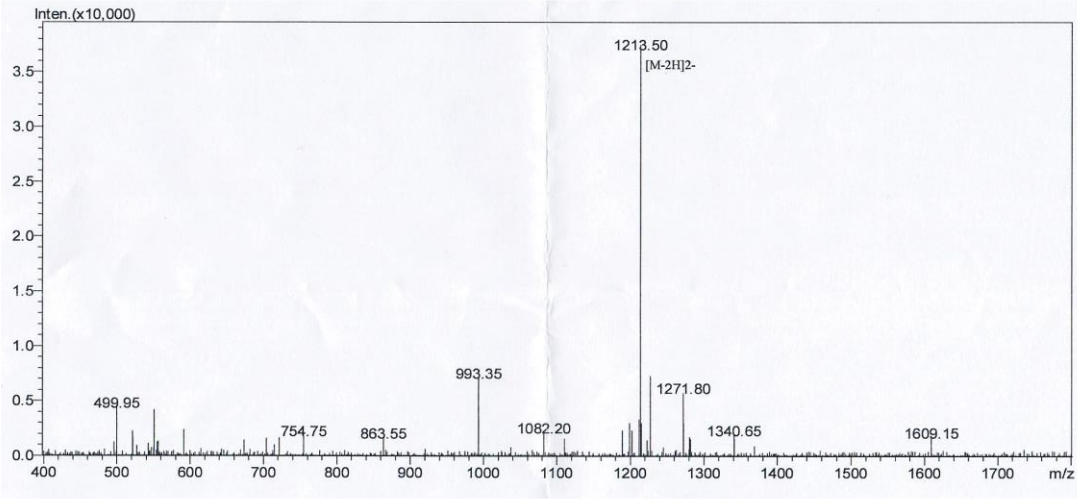
CYFPLQSYGFQPTNGVGYQPY

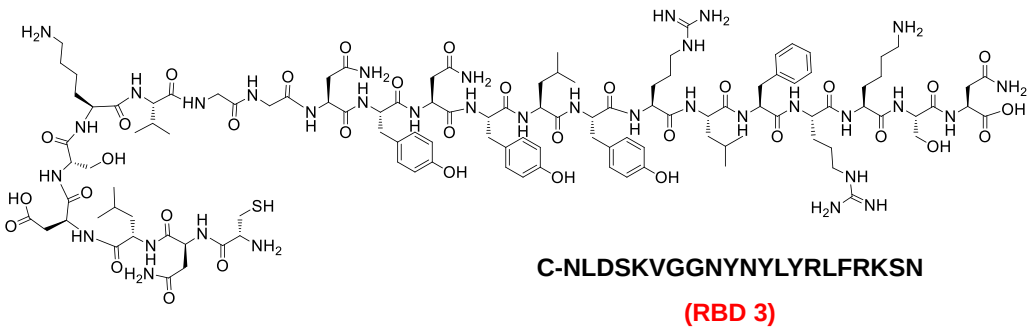
(RBD 2)

HPLC of RBD **2**



HRMS of RBD **2**

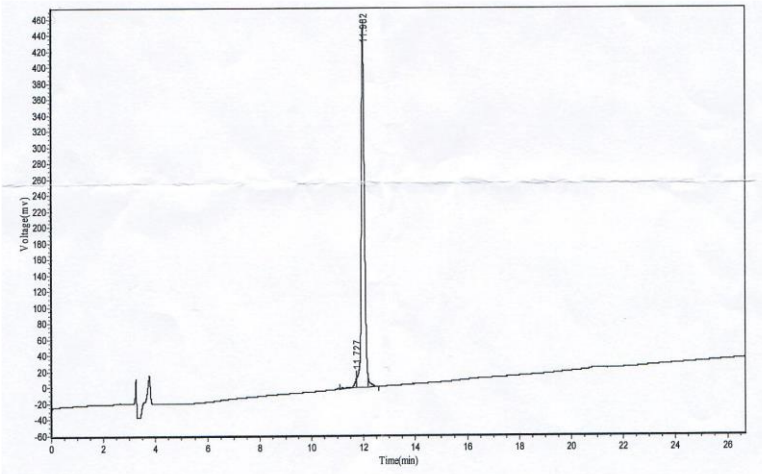




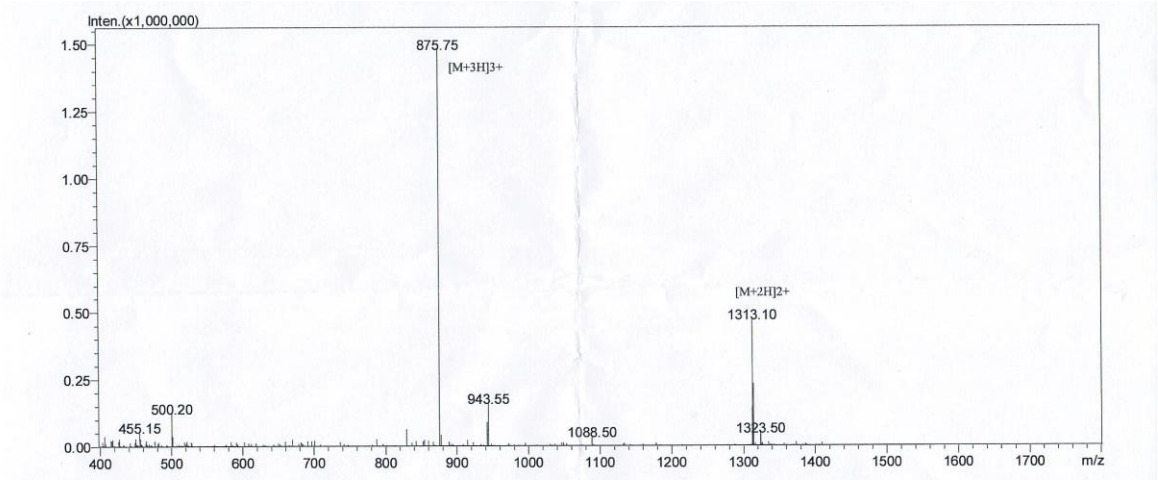
C-NLDSKVGGNYNYLYRLFRKSN

(RBD 3)

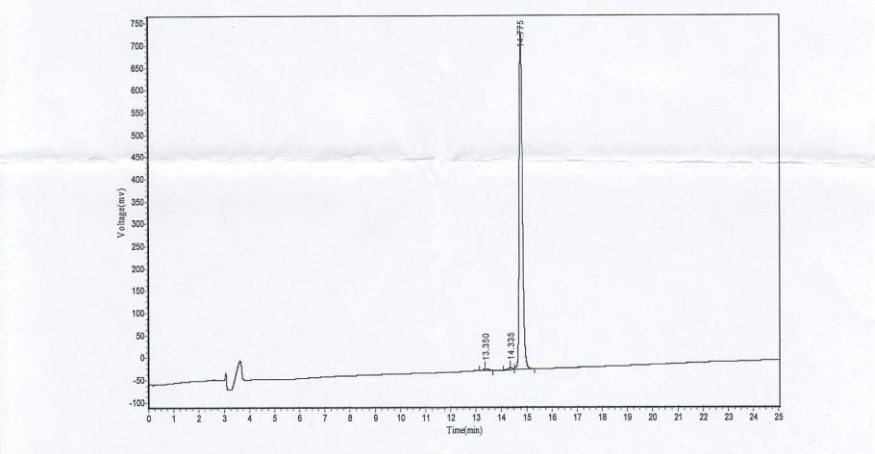
HPLC of RBD **3**



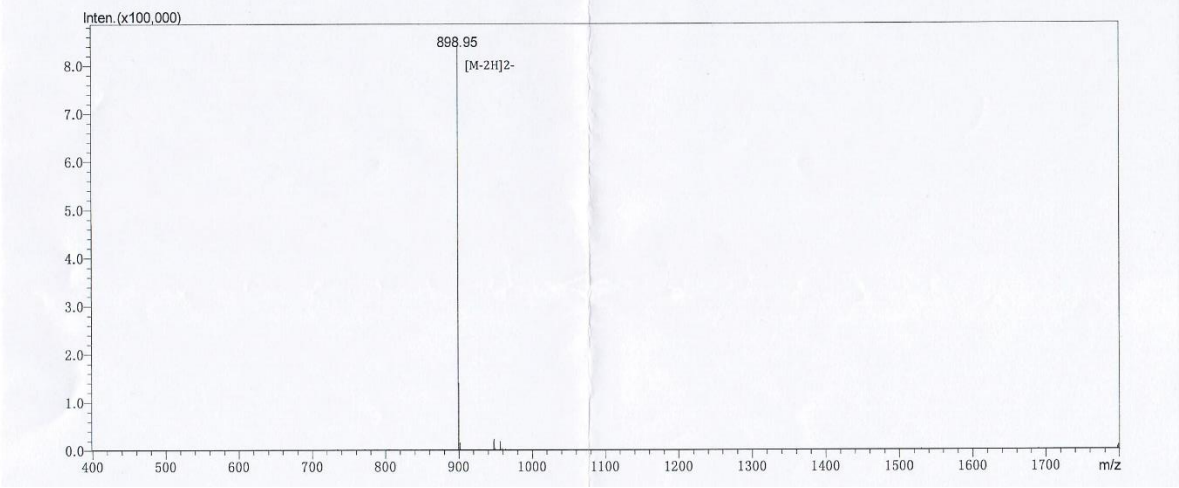
HRMS of RBD **3**



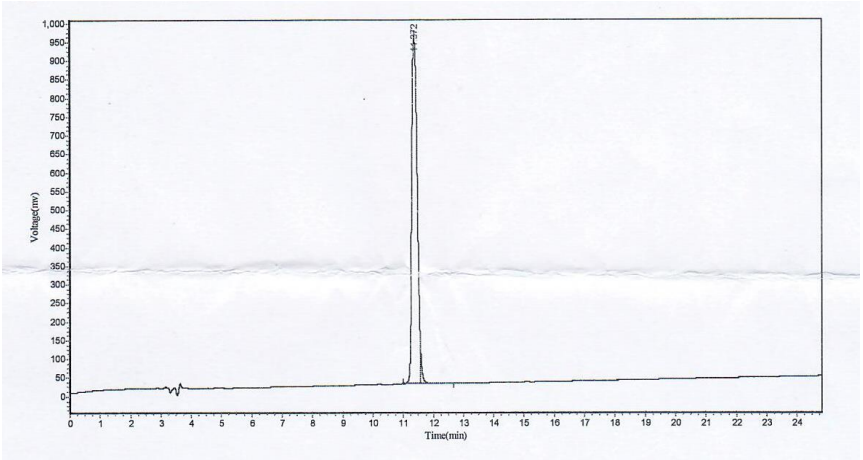
HPLC of **8** with the sequence of EGFNCYFPLQSYGFQ



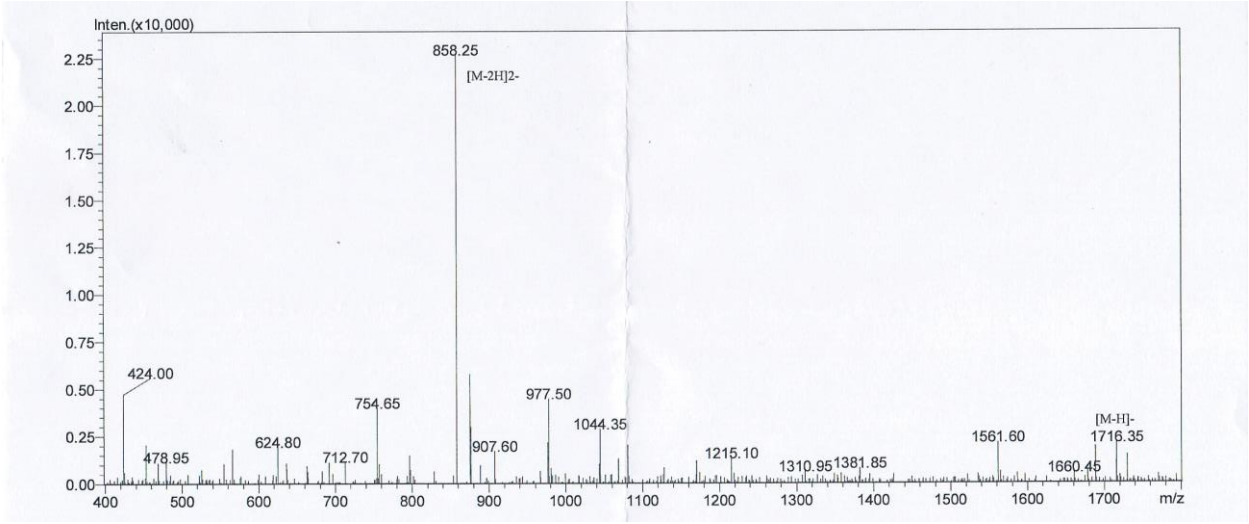
HRMS of **8** with the sequence of EGFNCYFPLQSYGFQ



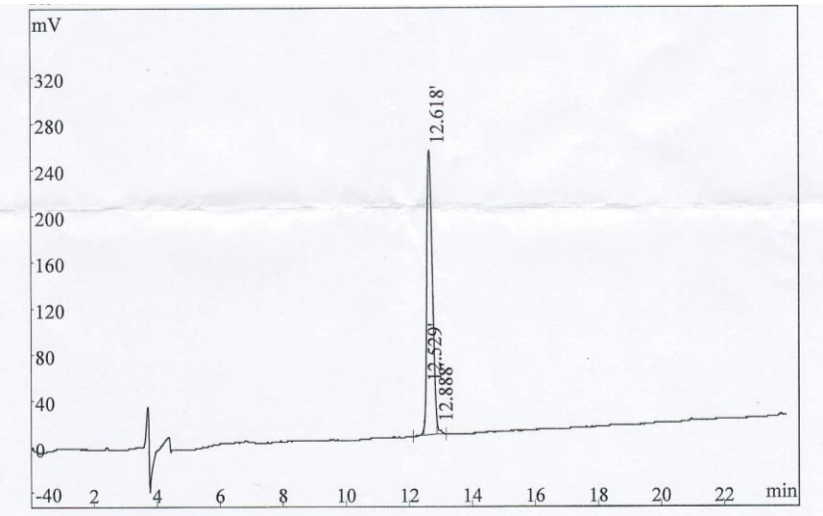
HPLC of **9** with the sequence of YFPLQSYGFQPTNGV



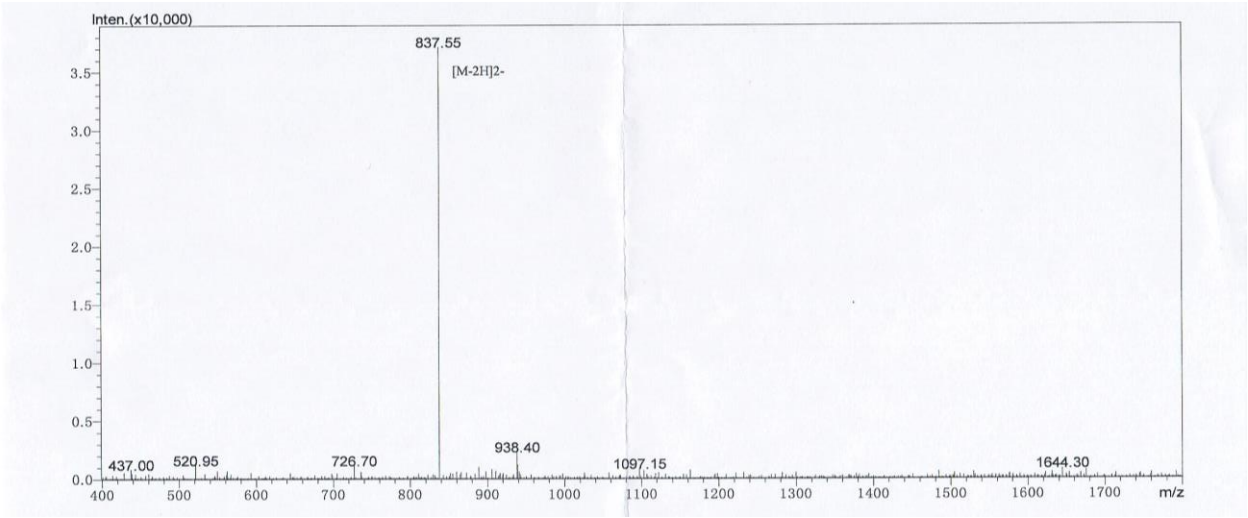
HRMS of **9** with the sequence of YFPLQSYGFQPTNGV



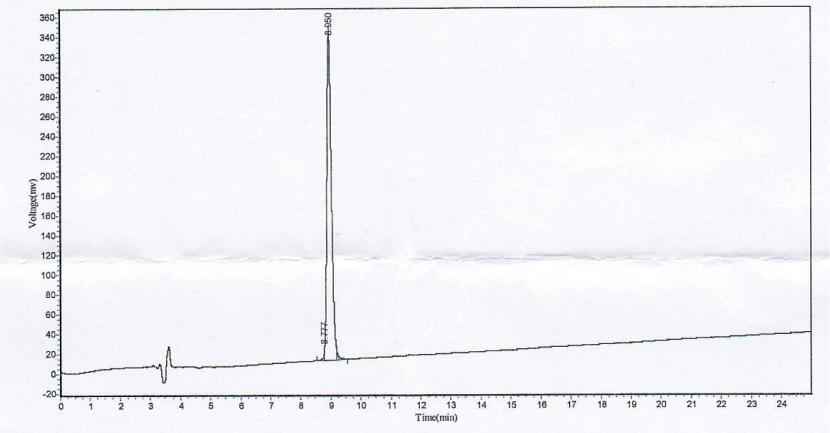
HPLC of **10** with the sequence of SYGFQPTNGVGYQPY



HRMS of **10** with the sequence of SYGFQPTNGVGYQPY



HPLC of **11** with the sequence of PTNGVGYQPYRVVVL



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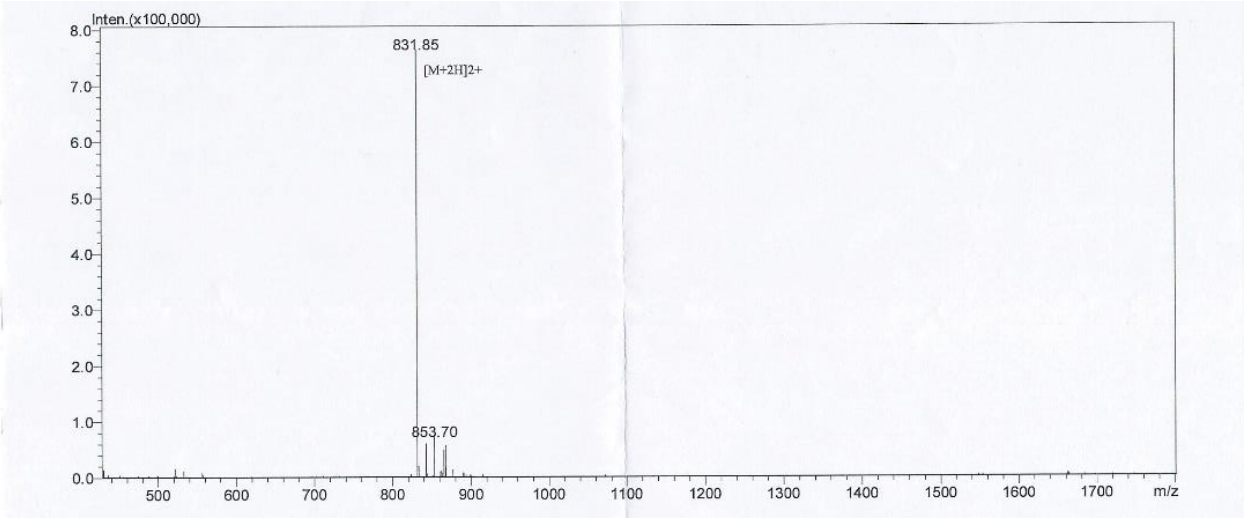
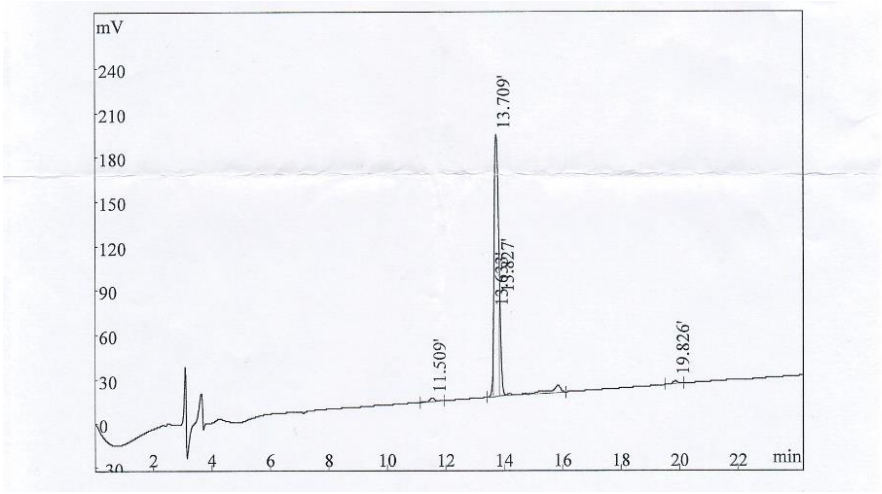
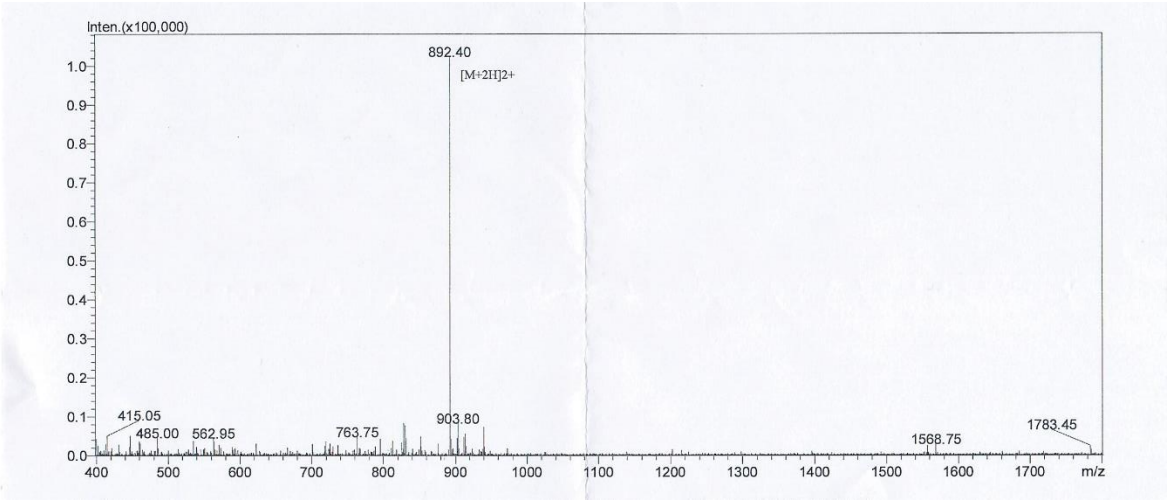


HPLC of **12** with the sequence of GYQPYRVVVLSFELL



HRMS of **12** with the sequence of GYQPYRVVVLSFELL



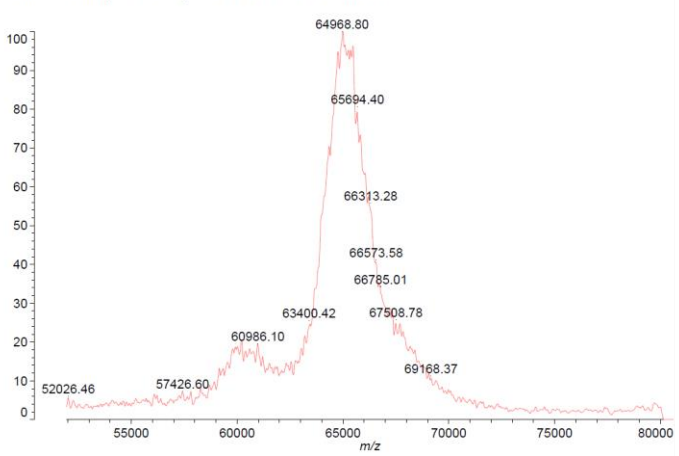
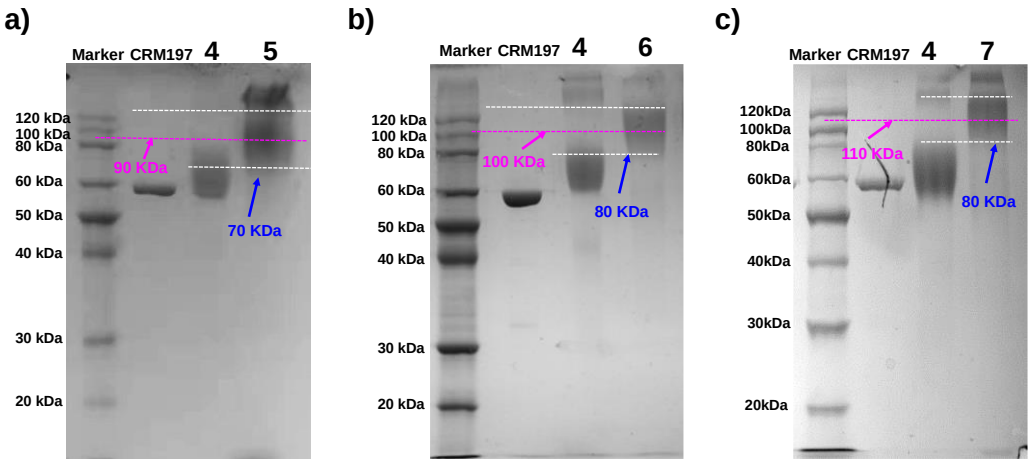


Fig. S2 MALDI-TOF spectra of CRM197-MAL **4** showed that the average numbers of maleimide per CRM197 are 20 (the molecular weight of CRM197 is 58.4 KDa).



d)

Conjugates	The mean molecular weights of conjugates	The mean numbers of peptides per CRM197
4	65000	0
5	90000	(90000-65000) / 4033 ≈ 6
6	110000	(100000-65000) / 2429 ≈ 14
7	120000	(110000-65000) / 2624 ≈ 17

Fig. S3 SDS-PAGE results obtained for CRM197-RBD conjugates **5**−**7**. Electrophoresis analysis showed that the mean numbers of peptides per CRM197 were 6, 14 and 17, respectively, for the conjugates **5**−**7** based on the increase of molecular weights following peptide conjugation.

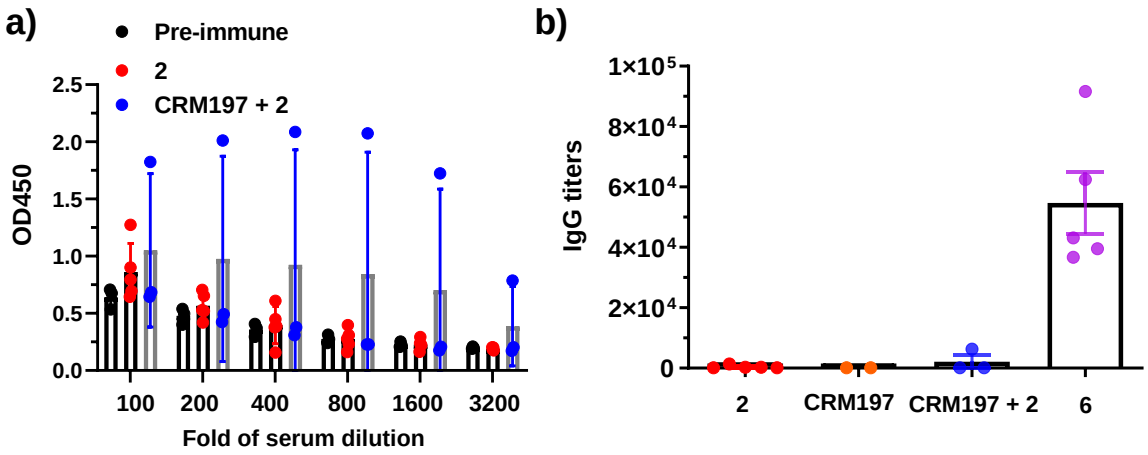


Fig. S4 (a) ELISA curves of pre-immune mouse sera (black), sera from mice immunized with peptide **2** (red), and sera from mice immunized with an admixture of peptide **2** and CRM197 (blue). (b) Titers of anti-RBD peptide IgG antibodies against peptide **2**. All ELISA measurements were performed in triplicate. Each symbol represents one mouse serum collected on day 21. It is critical that the peptide is covalently conjugated with CRM197, as peptide **2** only or an admixture of peptide **2** and CRM197 was less effective in generating anti-RBD peptide IgG antibodies compared to the covalent conjugate of CRM197-RBD **6**.

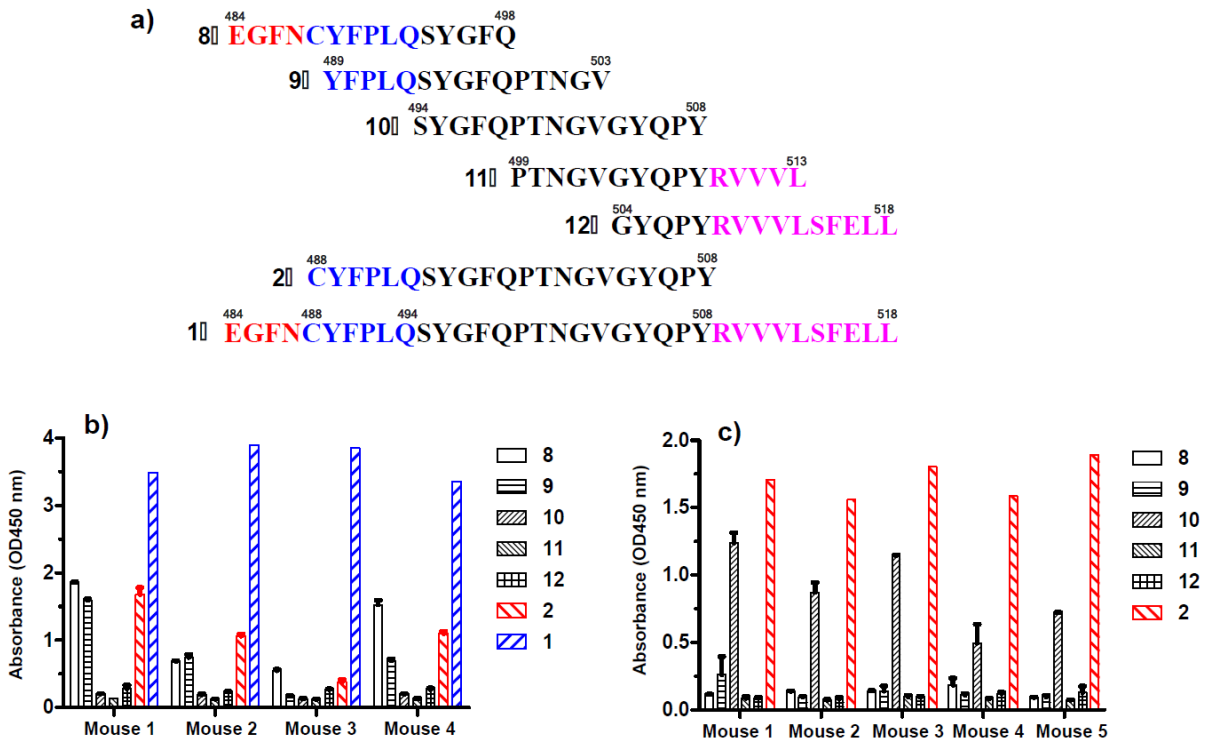


Fig. S5 Comparison of epitope profiles of sera from CRM197-RBD (b) **5** and (c) **6** immunized mice. Epitope profiles of IgG antibodies were determined through binding with RBD peptides **8**−**12** in an ELISA assay. (a) **8**: EGFNCYFPLQSYGFQ (E484-Q498) **9**: YFPLQSYGFQPTNGV (Y489-V503), **10**: SYGFQPTNGVGYQPY (S494-Y508), **11**: PTNGVGYQPYRVVVL (P499-L513), **12**: GYQPYRVVVLSFELL (G504-L518). The error bars show the standard error of the mean (s.e.m.) of three replicates. This revealed that the recognition epitopes of the antibodies generated by the conjugates **5** and **6** were EGFNCYFPLQ (E484-Q493) and SYGFQPTNGVGYQPY (S494-Y508), respectively.

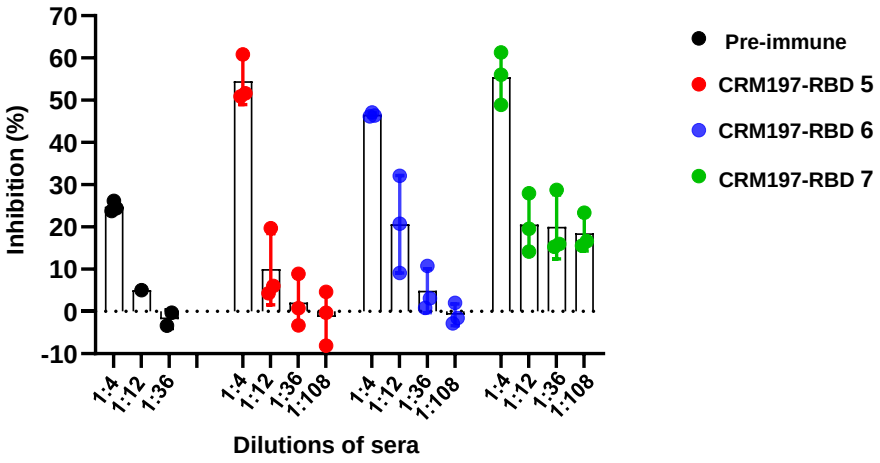


Fig. S6 Neutralization of pseudovirus by antibodies elicited by CRM197-RBD **5**−**7** showed the induced antibodies effectively neutralize SARS-CoV-2. The mixed post-immune sera and pre-immune sera of 5 mice were serially diluted and respectively incubated with the same volume of pseudovirus. Then, Opti-HEK293/ACE2 cells (3×10^5 cells per well) were added. After 48 h of infection, the supernatant in the 96-well plate was carefully aspirated, followed by the addtion of freshly prepared luciferase color reagent to generate a chemiluminescence signal. This experiment has been performed in triplicate.