Rapid development of SARS-CoV-2 receptor binding domain-conjugated nanoparticle vaccine candidate

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Abstract

The ongoing of coronavirus disease 2019 (COVID-19) pandemic caused by novel SARS-CoV-2 coronavirus, resulting in economic losses and seriously threatening the human health in worldwide, highlighting the urgent need of a stabilized, easily produced and effective preventive vaccine. The SARS-COV-2 spike protein receptor

binding region (RBD) plays an important role in the process of viral binding receptor angiotensin-converting enzyme 2 (ACE2) and membrane fusion, making it an ideal target for vaccine development. In this study, we designed three different RBD-conjugated nanoparticles vaccine candidates, RBD-Ferritin (24-mer), RBD-mi3 (60-mer) and RBD-I53-50 (120-mer), with the application of covalent bond linking by SpyTag-SpyCatcher system. It was demonstrated that the neutralizing capability of sera from mice immunized with three RBD-conjugated nanoparticles adjuvanted with AddaVax or Sigma System Adjuvant (SAS) after each immunization was ~8- to 120-fold greater than monomeric RBD group in SARS-CoV-2 pseudovirus and authentic virus neutralization assay. Most importantly, sera from RBD-conjugated NPs groups more efficiently blocked the binding of RBD to ACE2 or neutralizing antibody in vitro, a further proof of promising immunization effect. Besides, high physical stability and flexibility in assembly consolidated the benefit for rapid scale-up production of vaccine. These results supported that our designed SARS-CoV-2 RBD-conjugated nanoparticle was competitive vaccine candidate and the carrier nanoparticles could be adopted as universal platform for future vaccine development.

1. Introduction

The unexpected outbreak of COVID-19 pandemic since 2019 has become a global public health crisis affecting 216 countries or regions, 26016839 confirmed cases and over 850000 confirmed death (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019>). SARS-CoV-2, the causative virus affirmed by laboratory evaluation and comprehensive sequencing, belongs to β -coronavirus in coronavirus family which comprises other highly-pathogenetic virus strains (SARS-CoV, MERS-CoV) for humans¹⁻³. The infection caused a diverse clinical characterization of respiratory syndrome and person-to-person transmission⁴, and even led to death⁵⁻⁶.

As a member of coronavirus, SARS-CoV-2 adopts a similar cell-entry mechanism relying on spike protein on viral membrane to fulfill the host cell recognition,

attachment and membrane fusion, while the spike protein of coronavirus also shared great similarity in structural appearance as a trimeric fusion protein⁷⁻⁹. Specifically, SARS-CoV-2 spike protein recognize the angiotensin converting enzyme 2 (ACE2) as the entry receptor like SARS-CoV and the key binding interface lies on the receptor binding domain (RBD) of the spike protein, which has been confirmed by both structural elucidation through high resolution Cryo-EM structure and interface mutation scanning in previous work¹⁰⁻¹¹.

With the basis of clear structural information and biological function of SARS-CoV-2 spike protein and the key region RBD, most neutralizing antibodies and potential therapeutic agent are found against the spike protein RBD, making this region an ideal target for vaccine development^{8, 12-13}. However, despite a comprehensive effort on RBD-based vaccine, application of RBD subunit is still hindered by the quite low immunogenicity due to a variety of reason¹⁴. To increase the immunogenicity, scientists endeavor to modify the RBD to achieve larger antigen-carrier complex size or multimerization, which would complicate the overall structure of RBD and prolong the production and validation process of the recombinant modified antigen¹⁵⁻¹⁶. To shorten the time cost of vaccine development during emergency, a more compact workflow of antigen-displayed nanoparticle production is in demand. Protein covalent bond linking strategy has enjoyed a rapid development in recent years, rendering greater easiness to protein modification and multimerization¹⁷⁻¹⁹. SpyTag-SpyCatcher system has been used as a strategy for antigen display on particles in hepatitis B²⁰ and HIV²¹ vaccine development, overcoming the obstacles in massive in vitro production of fusion protein of antigen and nanoparticle scaffold.

Here we reported our designed SARS-CoV-2 spike protein RBD-based nanoparticle vaccines in application of the covalent bond linking strategy. With SpyTag fused to the C-terminus of RBD, the antigen could covalently be linked to the SpyCatcher upon the nanoparticle scaffold. The RBD nanoparticles could elicit higher neutralizing antibody titers compared to monomer RBD in mice, confirmed by stronger sera RBD-competition with both ACE2 and neutralizing antibody. Besides, our work

validated 3 different nanoparticles platform with SpyCatcher in N-terminus viable for SpyTag-fused protein coupling, which could become general nanoparticle capture platform for other antigen in the future.

2. Results

2.1. Design and production of RBD-conjugated nanoparticles

Previous studies have demonstrated that immunization with receptor binding region (RBD) of SARS-CoV-2 Spike protein formulated with aluminum hydroxide adjuvant in mice elicited higher neutralization antibody titers in comparison with the extracellular domain protein (ECD), S1-subunit protein (S1) and S2-subunit protein (S2)²². In addition, RBD amino acid sequences from 24 representative SARS CoV-2 strains isolated in different countries were aligned and founded to be very conservative (Figure S1, Supporting Information). In the present study, we focused on the RBD of SARS-CoV-2 S glycoprotein to design the RBD-conjugated nanoparticle vaccine based on the SpyTag-SpyCatcher system. As mentioned in previous study¹⁷⁻¹⁸, the enhanced shortening form of SpyCatcher, Δ N1-SpyCatcher was used in our study. Therefore, we engineered and adapted three Δ N1-SpyCatcher-nanoparticles (Δ N1-SpyCatcher-NPs) conjugation platform, Δ N1-SpyCatcher-Ferritin, Δ N1-SpyCatcher-mi3 and Δ N1-SpyCatcher-I53-50, to display more antigen to the surface of NPs based on the formation of isopeptide bond between SpyTag peptide and Δ N1-SpyCatcher in vivo (Figure S2b, Supporting Information). 24-mers ferritin were self-assembled into a spherical particle and form an octahedral nanocage²³. Computational designed and optimized mi3 NP protein with mutation of C76A and C100A from KDPG aldolases to escape the potential disulfide bond-mediated heterogeneity was dodecameric cage engineered scaffold with 60 total subunits multiply display its on surface of NPs^{17, 24}. The I53-50 NPs was a computational designed icosahedral nanoparticle assembled with two components, 20 copies of trimeric I53-50A1.1PT1 and 12 copies of pentameric I53-50B.4PT1²⁵.

Previous studies have manifested that immunization with RBD of SARS-CoV S

119 protein expressed in the mammalian cells could elicited higher potent neutralizing
120 antibody responses in mice and provided completely protection following infection
121 with SARS-CoV compared with those expressed in insect cells and E.coli ²⁶.
122 RBD-SpyTag (residues 319–541) protein was firstly expressed by transient
123 transfection method into HEK293F cells and purified by Ni-NTA affinity
124 chromatography, and followed by SEC. As shown in Figure 1C and 1D, purified RBD
125 -SpyTag was identified uniform and highly pure demonstrated by a clear single blot in
126 SDS-PAGE and a single major peak in SEC chromatogram. As shown in Figure 1C
127 and 1D, Δ N1-SpyCatcher-NPs, Δ N1-SpyCatcher-Ferritin NP, Δ N1-SpyCatcher-mi3
128 NP and Δ N1-SpyCatcher-I53-50 NP were expressed in E.coli and purified by
129 Ni-NTA affinity chromatography followed by SEC, and the high yield and production
130 quality of protein could be observed also from the SDS-PAGE and SEC peak result.
131 Further, with the preparation of precursor proteins in high quality produced, assembly
132 of the RBD-conjugated nanoparticles, purified Δ N1-SpyCatcher-Ferritin, Δ
133 N1-SpyCatcher-mi3 and Δ N1-SpyCatcher-I53-50A1.1PT1 proteins was performed.
134 A 50 μ M subunit concentration of RBD-SpyTag was incubated with 8 times higher
135 excess molar of Δ N1-SpyCatcher-NPs overnight for in vitro bonding reaction, and
136 then applied to SEC assay to separate out RBD monomers and unlinked nanoparticles.
137 As shown in Figure. 1C RBD-conjugated Ferritin, mi3 and I53-50 NPs were verified
138 through the single band and an uniformly increase of molecular weight (around 35
139 kDa to 72 kDa) from reducing SDS-PAGE and further confirmed by peak forward
140 shifts from the SEC assay (Figure 1D) , which suggested that RBD-SpyTag could
141 completely conjugated with Δ N1-SpyCatcher-NPs at a high efficacy and full reaction
142 level. Collectively, we used the SpyTag-SpyCatcher system to guarantee a both
143 flexible and high-efficiency production of SARS-CoV-2 RBD-conjugated
144 nanoparticles.

145

146 2.2. Structural characterization of SARS-CoV-2 RBD-conjugated

147 **nanoparticles**

148 We next observe the structural characterization of RBD-conjugated nanoparticles
 149 by using negative stain electron microscopic (EM). As shown in Figure 2A, RBD was
 150 conjugated with Ferritin, mi3 and I53-50 nanoparticles and presented on the surface
 151 of monodispersed particles. As shown by the EM graphs, a burred exterior surface of
 152 nanoparticles could be observed among RBD-conjugated NPs, especially for
 153 RBD-Ferritin NP. The hydrodynamic diameters of RBD monomer, Δ
 154 N1-SpyCatcher-NPs and RBD-conjugated NPs were further measured by Dynamic
 155 Light Scattering (DLS). As displayed in Figure 2B, particle characteristic of both
 156 unconjugated NPs and RBD-conjugated NPs were validated and uniform distribution
 157 of particle sizes were rendered. Moreover, consistent with the results of negative stain
 158 EM, the hydrodynamic diameter of RBD-conjugated nanoparticles was larger than the
 159 unconjugated NPs from DLS.

160 In order to explore the physical stability of the nanoparticles to verify the
 161 compatibility of antigen and the nanoparticle platform,
 162 nano differential scanning fluorimetry (nanoDSF) was performed to RBD monomer,
 163 Δ N1-SpyCatcher-NPs and RBD-conjugated NPs, and detailed thermostability
 164 parameters were given (Figure 2B). A close Tm1 of RBD and RBD-conjugated NPs
 165 primarily indicated that the overall structure of RBD upon the nanoparticle was not
 166 affected by the conjugation. The risen Tm1 for mi3-NP after conjugation with RBD
 167 may be ascribed to RBD-buried unsatisfied exterior surface, which even strengthened
 168 the structural viability of covalent bond linking strategy for RBD with the
 169 nanoparticles. Except for melting temperatures, no aggregation was observed for RBD
 170 monomer, RBD-Ferritin NP and RBD-mi3 NP during the process of thermal
 171 denaturation (Figure 2B). Comparatively, RBD-I53-50 NP underwent aggregation
 172 under approximately 70°C, close to the aggregation temperature of empty I53-50 NP
 173 and significantly higher than the Tm1 of the RBD monomer. The above results
 174 demonstrated that under general medicine or vaccine storage environment at 4°C, the
 175 designed conjugated vaccine could maintain a similar stability behavior as the RBD

monomer which highly benefited commercial production and distribution.

2.3. In vitro antigenicity validation of SARS-CoV-2 RBD-conjugated nanoparticles

We next expressed and purified recombinant human ACE2 ectodomain and RBD-specific neutralizing antibody (CB6), and then characterized the antigenicity of RBD-conjugated NPs by detecting the binding affinity with the receptor and antibody. CB6 neutralization antibody was isolated from a COVID-19 convalescent patient and recognized an epitope that overlap with the hACE2-binding site of RBD, a critical character enabling a potential effect to neutralize the authentic SARS-CoV-2 virus²⁷. ELISA profiles showed that RBD-SpyTag monomer and three RBD-conjugated NPs bound to hACE2 and CB6 antibody in a dose-dependent manner. Analogous to soluble RBD-SpyTag monomer, three RBD-conjugated NPs bound to purified hACE2, suggesting that the conformation of RBD monomer was retained on the conjugated nanoparticles (Figure 3A). However, the binding between three RBD-conjugated NPs and CB6 antibody was significantly higher than RBD-SpyTag monomer (Figure 3B). Bio-layer interferometry assay was then applied to further examine the binding kinetics of RBD-conjugated NPs. As illustrated in Figure 3B and 3D, the measured binding affinity constant (kD) of RBD monomer and two RBD-conjugated NPs, RBD-Ferritin NP and RBD-I53-50 NP, with hACE2 receptor were 4.34E-09, 1.74E-08 and 1.00E-09 M. However, the dissociation was very slowly between RBD-mi3 NP and hACE2, and kD reached up to 1.0E-12 M, indicating RBD-mi3 NP showed an even higher antigenicity in comparison to RBD-Ferritin NP and RBD-I53-50 NP. The binding kinetics of RBD-conjugated NPs to CB6 antibody were also measured. The binding capability between three RBD-conjugated NPs and CB6 antibody were significantly stronger than RBD monomer (Figure 3C and 3D), suggesting that three RBD-conjugated NPs may showed a higher affinity to specific BCR targeting the RBD of SARS-CoV-2.

2.4. Immunogenicity of RBD-conjugated nanoparticles in BALB/c mice

206 To compared the immunogenicity of three RBD-conjugated NPs and soluble
 207 monomeric RBD, mice were immunized with 5 µg monomeric RBD or corresponding
 208 weights of RBD-mi3 NP, RBD-Ferritin NP and RBD-I53-50 NP with equimolar RBD
 209 formulated with 50 % (v/v) AddaVax or SAS adjuvant at weeks 0, 2 and 4 (Figure
 210 4A). PBS was used as the negative group. As expected, after prime immunization,
 211 almost no binding antibody response was detected in groups immunized with
 212 monomeric RBD adjuvanted with both AddaVax and SAS. However, immunized with
 213 three RBD-conjugated NPs, RBD-Ferritin, RBD-mi3 and RBD-I53-50, formulated
 214 with AddaVax adjuvant elicited 71.8 to 168.4-fold higher binding antibody (ED_{50} :
 215 $10^{3.8 \pm 0.4}$, $10^{3.9 \pm 0.2}$, $10^{4.2 \pm 0.2}$, respectively) than RBD (ED_{50} : $10^{2.0}$). Analogous to the the
 216 Addvax adjuvant, immunized with RBD-Ferritin, RBD-mi3 and RBD-I53-50
 217 adjuvanted with SAS adjuvant induced virtually the same antigen-specific binding
 218 antibodies in mice (ED_{50} : $10^{4.1 \pm 0.3}$, $10^{4.0 \pm 0.2}$, $10^{4.3 \pm 0.2}$, respectively) after the prime
 219 immunization. Next, the RBD-specific antibody titers were substantially increased
 220 among groups immunized with monomeric RBD and three RBD-conjugated NPs in
 221 the following 1st boost and 2nd boost immunization. ELISA profiles showed that
 222 throughout the whole immunization three RBD-conjugated NPs induced significantly
 223 higher RBD-specific binding antibody compared to the monomeric RBD, while the
 224 titer levels among groups of RBD-conjugated NPs adjuvanted or groups between the
 225 two adjuvants were generally similar (Figure 4B). In order to explore the detailed
 226 immune response during immunization, we further evaluated the IgG subtype of the
 227 antibody elicited and the results showed that a similar trend of antibody titer among
 228 different immunization groups could be observed regardless of subtypes of IgG.
 229 Moreover, the titer ratio of IgG1:IgG2a among groups were greater than 1 throughout
 230 the whole immunization process, indicating a Th2-favored antibody response ²⁸
 231 (Figure 4B).

232 Besides the intensity of antibody production, the neutralizing capability of
 233 generated antibody would be another critical factor impacting the quality of
 234 immunization. Thus, for further evaluation of immunogenicity of RBD-conjugated
 235 NPs, serum competition assay through BLI was performed. Sera of mice immunized

with antigen adjuvanted with after 2nd boost was retrieved and mixed within each group for a general evaluation. After serial dilution, sequentially-diluted sera were applied to the RBD captured on the biosensors for blocking. It was observed that sera from RBD-conjugated NPs groups can priorly hinder the binding of ACE2 and CB6 antibody to RBD under each dilution level in comparison with the RBD monomer (Figure 4C). As the binding signals were recorded, the non-competing binding curve height R_o and competing binding curve of each dilution level R_c could be used for quantitative analysis. The heatmap of relative competition level of mice sera further displayed that competitive levels of RBD-conjugated NP groups were 4- to 16-fold stronger to the monomeric RBD group (Figure 4D). And as the copies of RBD presented on the surface increased, a stronger competition could be observed when comparing the RBD-Ferritin NP with RBD-I53-50/mi3 NPs. A stronger relative competition level may indicate a more lasting occupation of RBD of the spike protein on the virus intruding and hinder its binding with ACE2 to prevent cell infection, which would further confirmed by neutralizing assays.

Neutralization antibody titers were determined in vitro using SARS-CoV-2 pseudovirus and authentic SARS-CoV-2 virus neutralizing assays. The neutralization titer of sera collected with the RBD-conjugated nanoparticles formulated with AddaVax adjuvant after the 2nd boost immunized mice was ~10- to 120-fold greater than that of sera from the monomeric RBD control group during SARS-CoV-2 pseudovirus-based assay. Analogical result was also observed when immunized with antigen formulated with SAS adjuvant (Figure. 5A). Therefore, we further performed authentic SARS-CoV-2 virus to detected the neutralization activity of sera by method of cytopathic effect (CPE) evaluation and focus reduction test. As shown in Figure. 5B, 90 % focus reduction neutralization antibody titers ($FRNT_{90}$) of post-2nd boost sera from mice immunized with RBD-Ferritin NP, RBD-mi3 NP and RBD-I53-50 NP formulated with AddaVax adjuvant ($FRNT_{90}$: $10^{4.1 \pm 0.5}$, $10^{4.0 \pm 0.6}$, $10^{4.1 \pm 0.3}$, respectively) was ~10- to 40-fold higher than monomeric RBD ($FRNT_{90}$: $10^{2.1 \pm 0.8}$). As was similar to AddaVax, three RBD-conjugated nanoparticles adjuvanted with SAS also showed significantly higher $FRNT_{90}$ than RBD monomer (Figure 4B). Otherwise we

266 compared the difference of neutralization activity of sera from all groups after each
 267 immunization procedure according the CPE-based microneutralization assay. Results
 268 manifested that after the first prime, little neutralizing effect could be observed among
 269 all groups. As the immunization procedures progressed, neutralizing effect of sera
 270 samples of groups from RBD-conjugated NPs was significantly overwhelming the
 271 monomeric RBD group in regardless of adjuvants used. Specially, comparative serum
 272 neutralizing activity could be observed after 1st boost for the RBD-conjugated groups
 273 while an equal strength of neutralization was postponed to post-2nd boost for
 274 monomeric RBD group, during which the neutralizing activities of nanoparticle
 275 groups were nearly 10-fold higher. It was interesting to point out that the
 276 RBD-Ferritin NP showed a relatively inferior effect compared to the other two
 277 nanoparticles when we made a parallel contrast between groups, which was in
 278 accordance to the competition assay.

279

280 **2.5. Cellular reponse during the immunization**

281 To explore whether a difference in immune cell level of immune response to
 282 antigen manipulate the above effect and determine the T cell immune response
 283 elicited by RBD-conjugated NPs, analysis of germinal centers (GCs) B cells, T
 284 follicular helper (Tfh) cells and immune cells containing intracellular cytokine was
 285 performed. Compared to monomeric RBD, analysis of responding cells in draining
 286 lymph nodes following a 2nd boost immunization shown that no significant difference
 287 was observed in Tfh and GC cell responses when immunized with three
 288 RBD-conjugated NPs (Figure S3A and 3B, Supporting Information). In addition,
 289 consistent with the RBD-dimer results as previously reported ¹⁶, compared with
 290 monomeric RBD immunized mice, no substantially increase of T cell responses of
 291 collected draining lymph nodes and spleen was detected by flow cytometry in
 292 RBD-conjugated NPs immunized mice (Figure S3C and S3D, Figure S4, Supporting
 293 Information).

294

295 **3. Discussion**

296 With the worldwide collaboration in SARS-CoV-2 research, various vaccine
 297 candidates were raised and validated in preclinical or clinical trials ²⁹⁻³². And to
 298 enhance the immunogenicity of antigen used in vaccine, strategies including
 299 live-virus platform ³³⁻³⁴, viral vector vaccine using vesicular stomatitis virus ³⁵ or
 300 adenovirus ³⁶, lipid nanoparticle-encapsulated mRNA ³⁷, or whole inactivated virus
 301 ³⁸⁻³⁹ were adopted, among which the multivalent presentation of antigen on
 302 nanoparticle protein was regarded as one of the most rapidly-developing method for
 303 vaccine design ⁴⁰⁻⁴¹. However, the increased structural redundancy during the de novo
 304 design of antigen constructed on the multimeric component pulled apart the gap
 305 between well-designed blueprint structure and the actual production ⁴². Hence, we
 306 reported the design of RBD-based nanoparticles using covalent bond linking strategy
 307 as the example for a potent method for rapid antigen-nanoparticle design.

308 As the global awareness of the urgent need of fast-responding vaccine
 309 development grew under the pandemic, a delicate selection of antigen able to elicit
 310 competent intensity of neutralizing antibody was demanded. Thus, spike protein as
 311 the major viral membrane protein became the focused antigen candidate for vaccine
 312 design, leading to selection of different protein subsection used (full ectodomain S
 313 protein, S1 segment and RBD) based on the co-structure and functional mate, ACE2 ⁸,
 314 ^{13, 43}. Despite a full display of available antigenic site, full length S protein bear
 315 uncertainty of prospective immune response due to increasing evidence of versatile
 316 mutations ⁴⁴, unpredictable presenting efficacy of neutralizing epitope and
 317 antibody-dependent enhancement (ADE) effect ⁴⁵. The shortening of ectodomain of
 318 spike protein to S1 maintains a balanced characteristic between full length protein and
 319 RBD domain but still carried the inherited shortage from the full-length spike protein
 320 ⁴⁶. As the co-structure of RBD and ACE2 has been elucidated, growing attention was
 321 put on the RBD as primary antigen for vaccine design and variable strategies were
 322 adopted to enhance the immunogenicity including dimerization, nanoparticlization or
 323 simple combined use of adjuvant ¹²⁻¹³. Among all the strategies, multivalency of
 324 antigen and enlargement of antigen size gained most effort due to that increased
 325 antigen size and antigen saturation of BCR prolongs the antigen-presentation retention

and foster the recognition of antigenic epitope from the immunogen⁴⁷. To achieve multimeric display of RBD existed as monomer, necessary component was introduced to the original RBD sequence to form commutative bond between RBDs or to add additional scaffold to initiate multimerization, which required skilled structure-guided modification on corresponding antigen and iterations of ideal-to-real test production^{31, 48}. The two time-costing prerequisites for fine design of antigen-nanoparticle would expose disadvantages during emergent spread of infectious diseases, especially under pandemic. Therefore, we validated the utilization of covalent bond linking strategy during rapid development of SARS-CoV-2 vaccine and confirmed the viability by comprehensive evaluation.

Ascribing to separation of the expression of antigen RBD and nanoparticles used for antigen capture, the construction and production of proteins could be achieved in different optimal expression systems (Figure 1B). Later the covalent bond linking would be performed easily by incubation of RBD-SpyTag and Δ N1-SpyCatcher-NPs, yielding fully multivalent RBD-conjugated NPs with high structural uniformity and stability, and little sacrifice in assembling efficiency (Figure 1C and 1D, Figure 2). Both monomer RBD and RBD-conjugated NPs underwent further antigenicity inspection and results showed that multivalency RBD-conjugated NPs exhibited stronger affinity to receptor ACE2 and neutralizing antibody CB6 (Figure 3). We immunized Balb/C mice with monomer RBD and RBD-conjugated NPs adjuvanted with AddaVax or SAS. Serum anti-RBD antibody titers of RBD-conjugated NPs were significantly higher than monomer RBD (Figure 4A and 4B) regardless of adjuvant used, indicating the achieved target of nanoparticle design. Neutralizing assay of pseudovirus or authentic SARS-CoV-2 virus proved that elicited neutralizing antibody titers of RBD-conjugated NPs were also far away higher than monomer (Figure 5), which could be explained by the competition assay of immunized mice sera to ACE2 or CB6 antibody (Figure 4C and 4D). Stronger competition behavior from higher dilution level of sera guaranteed a more perfect protection of recognition by RBD from intruding virus. Moreover, it seemed to be creditable that with the increase of

valency of RBD upon the nanoparticle surface, a more favorable immunization effect could be induced, as we compared 24-mer RBD-Ferritin NP with the other 2 nanoparticles (Figure 4C and 4D, Figure 5C).

Here, we reported three RBD-based nanoparticle design using a universal strategy in vaccine development and validated three different nanoparticles platform for future need in rapid and general vaccine design. It's completely viable for replacement of RBD to other antigens from potential risky pathogen as the immunogen core. The independence of antigen screening and expression, nanoparticle scaffold selection, particle assembly and immunogenicity validation would bring helps to researchers devoted to contribute to vaccine development without setting an excess threshold of required structural information and experimental skills, and to manufacturer in commercial production due to a high yield of protein components and shortening process in upper-stream research and development, not only for the current pandemic but for the future battle with unknown pathogens.

4. Experimental Section

Cells and viruses

Vero-E6 (clone E6) and Vero cells were kidney epithelial cells from African green monkey and purchased from ATCC. The HEK293T cell is a human embryonic kidney epithelial cell line and obtained from ATCC. The HEK293F cells were purchased from Life Technologies and maintained in Union 293 medium (Union-Biotech) at 37 °C with 5% CO₂ and shaking at 120 rpm. The HEK293T cell expressing human angiotensin-converting nzyme 2, HEK293T-hACE2, is deposited in our lab. All adherent cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C with 5% CO₂. All cell lines were confirmed free of mycoplasma contamination. In this study, both SARS-CoV-2 strains we used were isolated from COVID-19 patients in Guangzhou (Genbank: MT123290 and GISAID: EPI_ISL_413859).

385 **Mice**

386 Specific pathogen-free (SPF) six to eight weeks old female BALB/c mice were
387 obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All
388 experimental animal studies were approved by the ethics committee of Sun Yat-sen
389 University Cancer Center (approve number: L102042020000A).

390

391 **Gene synthesis and plasmid construction**

392 The SARS-CoV-2 RBD (residue 319-541, GenBank: MN908947) construct for
393 preparation of RBD-based nanoparticle vaccine were human codon-optimized and
394 synthesized by Genscript, and further cloned into mammalian expression vector
395 VRC8400 with a N-terminal Kozak consensus sequence, signal peptide for protein
396 secretion and a C-terminal octa-histidine tag followed by a 13-residues SpyTag¹⁸
397 using the BamHI restriction sites. The SARS-CoV-2 RBD construct for ELISA assay
398 was basically same as the above but without the 13-residue SpyTag. The human
399 ACE2 (residue 19-615, GenBank: NM_021804.2) was synthesized by Genscript and
400 cloned into VRC8400 with a N-terminal Kozak consensus sequence and signal
401 peptide and with a C-terminal octa-histidine tag.

402 The IgG heavy and light chain variable genes of CB6 mAb (GenBank: MT470196
403 and MT470197) were human codon-optimized and synthesized by Genscript and
404 cloned into antibody expression vectors.

405 The Δ N1-SpyCatcher-mi3 construct (GenBank: MH425515) with mutations C76A
406 and C100A based on pentameric I3-01(60-mer)^{17, 24} was E. coli codon-optimized and
407 synthesized by Genscript and cloned into a modified pET28a+ vector with a
408 N-terminal hexahistidine tag. The Δ N1-SpyCatcher was fused to the N-terminal
409 trimeric Ferritin (24-mer)²³ or trimeric I53-50A1.1PT1 (60-mer)²⁵ using the (GGG)₄
410 spacer, and E. coli codon-optimized and synthesized and then cloned into a modified
411 pET28a+ vector with a N-terminal hexahistidine tag or a C-terminal hexahistidine tag,
412 respectively, by Genscript.

413 The I53-50B.4PT1 was codon-optimized and synthesized and then cloned into a

414 modified pET28a+ vector with a C-terminal hexahistidine tag by Genscript.

415

416 **Protein expression and purification in HEK293F cells**

417 The expression plasmid was transformed into DH5a competent cell for plasmid
418 DNA extraction using the NucleoBond Xtra Maxi kit according to the manufacture
419 protocol. The SARS-CoV-2 RBD monomer and hACE2 proteins were produced in
420 HEK293F cells. The HEK293F cells were cultured in Union 293 medium at 37 °C,
421 80-90% humidity, 5% CO₂ with rotation at 120 rpm for expansion. Then cells were
422 transiently transfected with 2mg expression plasmid per 1 liter using the
423 Polyethylenimine (PEI) MAX (Polysciences) at a density of 1.0 x 10⁶ cells/ml in
424 fresh Union 293 medium. After 5 days culture, the cell culture was collected and
425 centrifuged at 4°C, 8000 g for 1h. Collected supernatant was further filtered using
426 Steritop (0.22 µm pore size; Guangzhou Jet Bio-Filtration Co., Ltd) and concentrated
427 to 1/10 volume using tangential flow filtration system (10 kD retention molecular
428 weight, Millipore). Then concentrated supernatant was purified by immobilized
429 metal-affinity chromatography with Ni-NTA resin (Roche) stocked in WET FRED
430 gravity flow columns (IBA) and beads were eluted with buffer composed of 50 mM
431 HEPES, pH 7.3, 300mM imidazole and 300 mM NaCl. The eluate was concentrated
432 and further purified with size exclusion chromatography using Superose 6 Increase
433 10/300 GL gel filtration column (GE Healthcare) in a buffer composed of 50 mM
434 HEPES, pH 7.3 and 300 mM NaCl. Fractions of the target peak were and pooled and
435 concentrated using centrifuge tubes (10KDa MWCO, Millipore), and followed by
436 store in 4 °C for further use.

437 CB6 antibody was expressed and purified as previously reported ²⁷. Briefly, the
438 plasmids encoding the IgG heavy and light chain gene were transiently tansfected into
439 HEK293F cells at a ratio of 5:6. The supernatant of culture was collected 5 days after
440 transfection, centrifuged and purified by affinity chromatography with protein A resin
441 (Genscript), and subjected to desalt in a composed buffer 50 mM NaPO₄, 150 mM
442 NaCl, pH 7.3 using HiTrap Desalting column (GE Healthcare) with AKTA pure
443 chromatography system (GE Healthcare).

444

445 **Protein expression and purification in E. coli**

446 The modified pET28a+ expression plasmid of Δ N1-SpyCatcher-mi3, Δ
 447 N1-SpyCatcher-Ferritin, Δ N1-SpyCatcher-I53-50A1.1PT1 and I53-50B.4PT1 was
 448 transformed into Rosetta™ (DE3) competent cells (TIANGEN). After incubation for
 449 overnight at 37 °C on TB-agars culture plate supplemented with 50 mg/mL
 450 kanamycin and 33 mg/mL chloramphenicol, a single positive colony was selected and
 451 inoculated into 10 mL TB medium in the presence of 50 mg/mL kanamycin and 33
 452 mg/mL chloramphenicol and was grown overnight at 37 °C with shaking at 220 rpm.
 453 The culture was added to the 3L baffled triangle shake flasks containing 1 L TB
 454 medium and 50 mg/mL kanamycin, and grown at 37 °C with shaking at 150 rpm.
 455 When OD600 value of the culture reached up to 0.6~0.8, isopropylthiogalactoside
 456 (IPTG) was added to a final concentration of 1 mM for induction at 20 °C for 16-20 h
 457 with shaking at 150 rpm. The bacterial cultures were harvested and centrifuged at
 458 20 °C, 2450 g for 15 min

459 For Δ N1-SpyCatcher-mi3 purification, cell pellets were resuspended in lysis
 460 buffer (250 mM Tris, pH 8.5, 300 mM NaCl, 30 mM imidazole, 1 μ M DNases, 0.75%
 461 CHAPS, 5 mM MgCl₂ and EDTA-free protease inhibitor cocktail [Roche]), and
 462 lysed with high pressure cell homogenizer (Union-Biotech) at a pressure of 800 MPa.
 463 The suspensions were centrifuged for supernatant collection, filtered with Steritop
 464 (0.22 μ m pore size), and purified with the gravity flow columns containing Ni-NTA
 465 resin. Beads were eluted with 50 mM HEPES, pH 8.0, 300 mM NaCl, 300 mM
 466 imidazole and 0.75% CHAPS, and the elution was concentrated to 1mL and loaded
 467 onto the size exclusion chromatography using Superose 6 Increase 10/300 GL gel
 468 filtration column (GE Healthcare) pre-equilibrated with 50 mM HEPES, pH 8.0 and
 469 300 mM NaCl. Peak fractions were identified with
 470 SDS-polyacrylamide gel electrophoresis (PAGE) analysis to determine whether target
 471 protein was collected. After a concrete confirmation of purity and yield of protein, the
 472 fractions were pooled, concentrated, and stored at 4 °C.

For Δ N1-SpyCatcher-Ferritin and Δ N1-SpyCatcher-I53-50A1.1PT1 proteins purification, cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.3, 300 mM NaCl, 30 mM imidazole, 1mM DTT, 0.75% CHAPS, 1 μ M DNases, 5 mM $MgCl_2$ and EDTA-free protease inhibitor cocktail [Roche]). Purification was similar as the above instead of the elution buffer used (50 mM HEPES, pH 7.3, 300 mM NaCl, 300 mM imidazole, 1mM DTT and 0.75% CHAPS), and equilibration buffer for SEC (50 mM HEPES, pH 7.3 and 300 mM NaCl).

Endotoxin of all purified proteins was removed with ToxinEraserTM Endotoxin Removal Kit (Genscript) in accordance to the manufacturer's instruction. The remnant endotoxin was identified with ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript) and no more than 0.1 EU/mL of endotoxin was detected.

484

485 **Preparation of RBD-conjugated nanoparticles**

To assemble the RBD-conjugated nanoparticles, RBD-SpyTag should be incubated with Δ N1-SpyCatcher-NPs to form a covalent peptide bond in between due to an automatic reaction of SpyTag-SpyCatcher system (Banerjee and Howarth, 2018; Bruun et al., 2018). Purified Δ N1-SpyCatcher-Ferritin or Δ N1-SpyCatcher-I53-50A1.1PT1 protein presented at a subunit concentration of 50 μ M were incubated in a 1:8 molar excess ratio with RBD-SpyTag for overnight at room temperature buffered with 50 mM HEPES, pH 7.3, 300 mM NaCl. For Δ N1-SpyCatcher-mi3, 50 μ M subunit of concentration of protein was mixed in a 1:8 molar excess ratio with RBD-SpyTag for overnight at room temperature buffered with 50 mM HEPES, pH 7.5, 300 mM NaCl, 5% glycerol. In order to separate the conjugated nanoparticles with the empty-NPs and monomer RBD, all incubated substrates were applied to size exclusion chromatography using Superose 6 Increase 10/300 GL gel filtration column pre-equilibrated with 50 mM HEPES, pH 7.3, 300 mM NaCl. Fraction were collected for SDS-PAGE analysis. The protein of interest was selected, concentrated and stored at 4 °C.

501

502 **SDS-PAGE analysis**

503 SDS-PAGE was performed as previously described ⁴⁹. Briefly, five micrograms
504 of purified protein by added the 5x loading buffer were heated at 95 °C for 5 min, and
505 loaded on 12 % Tris-glycine gels for 30 min at 300 V. Gels were stained with
506 coomassie brilliant blue (Beyotime, China) and destained with 30% methanol ,10%
507 glacial acetic acid in double distilled water and subjected to film by ChemiDoc
508 system (BioRad).

509

510 **Dynamic light scattering**

511 DLS was carried out to characterize the hydrodynamic diameter and
512 polydispersity index of RBD-conjugated nanoparticles and individual nanoparticles
513 using Zetasizer Ultra instrument (Malvern Panalytical). Briefly, purified proteins were
514 centrifuged at 16,250 g, 4 °C for 10 min to remove any aggregates, and diluted to at a
515 concentration of 0.5 mg/mL in PBS and loaded onto the disposable solvent resistant
516 micro cuvette.

517 The particle distribution of the purified protein was determined by measuring the
518 intensity of the light scattered by the sample using the avalanche photodiode detector
519 placed at a measurement angle of 173° at 25 °C. Each sample was measured in
520 triplicate and the average values of the hydrodynamic diameter and polydispersity
521 index of the sample were recorded and analyzed using the manufacturer's software
522 (Malvern Panalytical).

523

524 **Negative stain electron microscopy**

525 Approximately 5 µL aliquot of purified nanoparticle at a concentration of 0.05-2
526 mg/mL was applied to freshly glow-discharged 300-mesh copper grids and incubated
527 for 1 min. Excess liquid were blotted with filter paper. The grids were washed twice
528 by double distilled water and blotted, and then negatively stained freshly 2 % (w/v)
529 uranyl acetate for 45s, and followed by air dried. Grids were imaged with FEI Tecnai
530 T12 transmission electron microscope (FEI, USA) operating at 120 kV. The digital

micrographs were obtained at 150,000x magnification.

Nano differential scanning fluorimetry

NanoDSF Systems were conducted to measure the thermostability and aggregation of purified protein using Prometheus NT.48 instrument (NanoTemper Technologies). A 10 μ L aliquot of sample diluted to a concentration of 0.5 mg/mL were applied to quartz capillary cassette and placed into card slot. The scan temperature was increased linearly starting from 20.0 $^{\circ}$ C to 95 $^{\circ}$ C at a scan rate of 1 $^{\circ}$ C. The thermal transition midpoint (T_m) and aggregation results from start to finish (Tagg) were reported and analyzed in PR.ThermControl software (NanoTemper Technologies). Three replicates are measured for each sample.

Enzyme-linked immunosorbent assay

ELISA assay was performed to examine the binding ability of purified protein to the receptor ACE2 and SARS-CoV-2 RBD-specific CB6 Antibody. Purified RBD monomer and RBD-conjugated NPs was diluted to a concentration of 1 μ g/mL and precoated on 96-well microplates (Corning) (100 μ L/well) in triplicate overnight at 4 $^{\circ}$ C. The plates were washed three times with PBS with 0.05 % Tween 20 (PBS-T), and blocked with ELISA blocking buffer (2 % gelatin, 5 % casein and 0.1 % proclin 30 in PBS) for 1 h at 37 $^{\circ}$ C. The plates were then incubated with sequentially 1:5 diluted ACE2 starting from 5×10^{-4} ng/mL to 10^{-2} ng/mL in ELISA blocking buffer. After 1 h incubation, the plates were washed with PBS-T for five times, and 100 μ L hACE2-specific rabbit antibody (Sino Biological Inc) diluted at a ratio of 1:5000 was added to wells at 37 $^{\circ}$ C for 1 h. The plates were washed with PBS-T 5 times and a 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (Promega) was added for 45 min at 37 $^{\circ}$ C. After the final round of wash to remove all disassociated antibody, substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Sangon Biotech) was added to generate chromogenic reaction for 15 min at room temperature, and the reaction would be suspended by a followed addition of 2M H_2SO_4 . The well-absorbance at 450 nm and 630 nm was immediately recorded by SpectraMax Plus plate reader.

(Molecular Devices, USA).

As for the assay of CB6 antibody binding, the coating, incubating and chromogenic reaction generating were similar to the ACE2 assay instead of that primary antibody binding to antigen was not required and that HRP-conjugated goat anti-human IgG antibody (Promega) would be used as the secondary antibody to detect binding of CB6 antibody to the antigens.

Bio-layer Interferometry

BLI analysis were performed on an Octet Red 96 (Fortebio) instrument at 30 °C with shaking at 1000 rpm. Signals were collected at a standard frequency as default (5.0 Hz).

Kinetic assay: Firstly, the Streptavidin (SA) biosensors (Fortebio) were pre-incubated in PBS (ThermoFisher) containing 0.05 % Tween 20 (Sigma-Aldrich), the assay buffer used throughout the whole procedures, for 15 minutes. To couple the RBD protein on the biosensors, EZ-link-Sulfo-NHS-biotin biotinylation kit (ThermoFisher) was used to biotinylate the RBD/ACE2/CB6 antibody in the following of the instruction as below.

Step1: Calculation of the amount of biotinylation agent used

1. Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mM protein}}{\text{mg protein}} \times \frac{20 \text{ mM Biotin}}{\text{mM protein}} = \text{mM Biotin}$$

20 = Molar fold excess of biotin

2. Calculate microliters of 10 mM biotin reagent solution to add to the reaction:

$$\text{mM Biotin} \times \frac{1,000,000 \text{ } \mu\text{L}}{\text{L}} \times \frac{\text{L}}{10 \text{ mM}} = \text{ } \mu\text{L Biotin}$$

Step2: Biotinylation

Add the calculated amount of 10 mM biotin reagent into the RBD/ACE2/CB6 protein in PBS (5 mg/mL, 200 μ L) and incubated the reaction system at room temperature for 30 minutes.

588 Step3: Desalting

589 Equilibrate the desalting column PD-10 (GE Pharmacia) with 10 mL of PBS. After
590 the equilibration, the reaction solution would be added in the column and then washed
591 and eluted with 400 μ L PBS separately.

592 To perform kinetic assay, after 60 s baseline, ACE/CB6-biotin protein diluted with the
593 buffer was captured on the SA sensor at 2 μ g/mL for 120 s. Then 2 fold-diluting
594 RBD-copy molar concentrations of RBD monomer or different RBD-conjugated NPs
595 were associated to the biosensor for 180 s, followed by a 300 s disassociation and 3
596 rounds of regeneration with 10 mM Glycine pH 1.5. The curve data was analyzed by
597 ForteBio data analysis software. Raw curves aligned at association were adjusted with
598 baseline signals before a 1:1 binding model fitting was performed. Then a global fit to
599 all binding curves was conducted to render overall kinetic parameters (kD, kon, kdis
600 etc.)

601 **Serum competition assay:** Mice serum from AddaVax-adjuvanted immunization
602 group harvested after 2nd boost were collected and equal volume (5 μ L) of sera from
603 each mouse within the same immunogen group was mixed together in representative
604 of overall characteristic of the group. To perform the competition assay, RBD-biotin
605 protein was captured on the biosensor as the above at 5 μ g/mL. Then to saturate the
606 RBD, 2 fold-diluting mice sera mixtures from each group and control PBST were
607 loaded to the biosensor for 300 s. After the end of sera loading, 400 mM ACE2 or
608 CB6 antibody were associated to the biosensors for 300 s to detect the competitive
609 binding signal under the saturation of each dilution level of mice sera. Sensors were
610 also regenerated with 10 mM Glycine pH 1.5. Real-time signal data was collected and
611 the competition behavior was displayed by the ACE2/CB6 binding signal of different
612 curves. Binding signal data were retrieved from curve, Ro represented the saturated
613 non-competing binding curve height and Rc represented saturated competing binding
614 curves of each dilution level. Relative competition levels of each serum dilution level
615 could be calculated as $(Ro-Rc)/Rc$.

616

617 **SARS-CoV-2 pseudovirus production**

618 The SARS-CoV-2 pseudovirus were produced as previously described⁵⁰, with
 619 some modification. Briefly, gene encoding SARS-CoV-2 S (GenBank: QHU36824.1)
 620 with a 19-amino-acids deletion in C-terminal was human codon-optimized and cloned
 621 into the expression vector pCMV14-3×Flag, a generously gift from Zhaohui Qian,
 622 Chinese Academy of Medical Sciences. HEK293T cells were co-transfected with the
 623 plasmids of PsPAX2, pCMV14-SARS-CoV-2 S Δ CT-3×Flag and pLenti-GFP at a
 624 ratio 1:2:1 using PEI-MAX (mentioned above). After 5 hours, the supernatant was
 625 replaced with fresh DMEM supplemented with 10% FBS. Sixty hours
 626 post-transfection, the supernatant containing SARS-CoV-2 pseudovirus were
 627 harvested and centrifuged at 4 °C, 3000 g for 10 min to remove cell debris, followed
 628 by a filter process with Steritop (0.45 μ m pore size,
 629 Guangzhou Jet Bio-Filtration Co., Ltd). Sterile PEG-8000 solution was added to the
 630 clarified supernatants and the solution was incubated for 1 hour at 4 °C. The mixture
 631 was then centrifuged, concentrated, and resuspended with DMEM for final collection
 632 of viruses. The storage of virus was at -80 °C.

633

BALB/c mice immunization

635 Forty-five 6-8 weeks-old female BALB/c mice were purchased from the Beijing
 636 Vital River Laboratory Animal Technology Co., Ltd, and arbitrarily divided into 9
 637 groups. Before immunization, purified immunogen was diluted with PBS and gently
 638 formulated with an equal volume of AddaVaxTM adjuvant (InvivoGen) or Sigma
 639 Adjuvant System (SAS, Sigma), and incubated overnight to achieve full absorption of
 640 antigen upon the surface of adjuvant particles at 4 °C with shaking at 40 g. Each
 641 group of mice received three immunizations at weeks 0, 2 and 4 via a subcutaneous
 642 route. The immunization dose was 5 μ g of RBD monomer, or corresponding weights
 643 of RBD-conjugated nanoparticle immunogens containing equal molar of RBD as the
 644 monomers which were RBD-mi3 (9.51 μ g), RBD-Ferritin (9.34 μ g) and RBD-I53-50
 645 (11.91 μ g). PBS were served as a negative control. Blood samples were harvested 10
 646 days following each immunization, and were placed at 37 °C for 30 min to reach
 647 ample coagulation. Then blood samples were centrifuged at 16,250 g, 4 °C for 10 min

and the upper layer serum was gently extracted, heat-inactivated at 56 °C for 30 min to deactivate the complement factors and pathogens, and then stored at -20 °C for future analysis.

Serum ELISA

1 µg/mL RBD monomer without SpyTag in PBS was precoated on 96-well maxiSorp ELISA microplates (Corning) overnight at 4 °C. Plates were blocked with ELISA blocking buffer for 1 h at 37 °C. Mouse serum samples were 5-fold sequentially diluted with blocking buffer starting at 1:50, and were added to the coated plates, and incubated at 37 °C for 1 h. After incubation, the plates were washed with PBS-T five times and added with a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG antibody (Promega) in blocking buffer at 37 °C for 45 min. For analysis of RBD-specific IgG isotype titers, a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG1 and IgG2a antibody was incubated at this step. Plates were washed, colored by TMB, and quenched with H₂SO₄. The absorbance at 450 nm and 630 nm of each well was immediately determined by SpectraMax Plus plate reader (Molecular Devices, USA). Results were plotted and fitted by GraphPad Prism 8 and EC₅₀ value were calculated using 4-parameters nonlinear regression fitting from fitted curve.

Pseudovirus-based neutralization assay

Pseudovirus-based neutralization assay was performed to evaluate the neutralization ability of the sera by immunized mice. In brief, approximately 1.75x10⁴ HEK293T-hACE2 cells were seeded onto 96-well culture plates overnight at 37 °C in 5 % CO₂. Sera were 4-fold serially diluted with complete DMEM medium starting at 1:20, and pre-incubated with an equal volume of pseudovirus of SARS-CoV-2 at 37 °C for 2 h. The mixtures were then added to the HEK293T-hACE2 cells for infection. After 2 hours incubation, the mixtures were replaced with fresh DMEM medium containing 2 % FBS and 1 % penicillin and streptomycin, and incubated for 48 hours at 37 °C, 5 % CO₂. Wells treated with only medium or virus without

incubation with serum were set as negative control and positive control in each plate, respectively. Afterwards, the cells were lysed with lysis buffer and luciferase activity was immediately measured by dual-glo luciferase assay system (Promega). The inhibition rate was calculated as (sera signals – blank control signals)/ (virus signals – blank control signals) * 100 %. The 90 % neutralization antibody titers (NT₉₀) were determined using 4-parameter s nonlinear regression fitting from fitted curve using GraphPad Prism 8.

Authentic SARS-CoV-2 virus-based neutralization assay

All of microneutralization assay for authentic SARS-CoV-2 virus we used in this study were performed in a BSL-3 facility. Two methods, the authentic SARS-CoV-2 virus-induced cytopathic effect (CPE) and focus reduction neutralization test (FRNT) were used to evaluate the neutralizing antibody titers of sera from immunized mice. Briefly, sera were 4-fold serially diluted starting at 1:4 with DMEM supplemented with 2 % FBS and 1 % penicillin and streptomycin, and mixed with the equal volumes of 100 half tissue culture infective doses (100 TCID₅₀) SARS-CoV-2-virus of 2020XN4276 strain at 37 °C for 2 h. Afterwards, the sera-virus mixture was added to pre-plated Vero-E6 cells in 96-well culture plate, and incubated for an additional 96 h at 37 °C in 5 % CO₂ to observe the CPE at 40X magnification. Wells with pure virus treated, pure diluted sera treated or cell only was set as controls for each plate. Virus back titration was performed in each plate. All diluted serum samples were tested in duplicate. The neutralization antibody titers of all of sera were defined as the reciprocal of serum dilution that could neutralize 50 % of virus infection at 4 days post-infection.

As for the FRNT method, serum samples were 5-fold serially diluted starting at 1:10, and mixed with the equal volumes of 100 focus forming unit (FFU) SARS-CoV-2 virus of human CHN/IQTC01/2020 strain in 96-well culture plate, and incubated for 1 h at 37 °C. The mixtures were then added to the 96-well plates that pre-seeded with Vero-E6 cells. After incubation for 1 h at 37 °C, 5 % CO₂, mixtures were removed and replaced with 100 µL MEM containing 1.2 %

carboxymethylcellulose pre-warmed to 37 °C for an additional 24 hours culture. Thereafter, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS, and then incubated with rabbit anti-SARS-CoV-2 nucleocapsid protein antibody (Sino Biological, Inc) for 1 hour at ambient temperature, followed by adding of a 1:4000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Plates were colorated using TrueBlue™ Peroxidase Substrate (KPL). Foci were counted on an ELISPOT reader (Cellular Technology Ltd. Cleveland, OH). The 90 % neutralization antibody titers (NT₉₀) were defined as the reciprocal of serum dilution that could inhibit 90 % FFU of virus-infection, and was calculated with a 4-parameter nonlinear regression fitting from fitted curve using GraphPad Prism 8.

Germinal center and Tfh analysis of mice drained lymph nodes

Immunized mice were sacrificed by CO₂ inhalation 12 days after the 2nd boost immunization (40 days). To simultaneously identify germinal center B cells and T follicular helper cells of mice drained lymph nodes, cell suspensions of draining lymph nodes from mice sacrificed were stained with fixable viability stain 780 (BD Biosciences) and blocked with anti-CD16/32 antibody (BD Biosciences), followed by labeling with anti-B220-BV421 (BD Biosciences), anti-IgD-PE (BD Biosciences), anti-GL7-Alexa Fluor 647 (BD Biosciences), anti-CD95-FITC (BD Biosciences), anti-CD4-BV510 (BD Biosciences), anti-CD44-BV786 (BD Biosciences), anti-ICOS-PE-Cyanine7 (BD Biosciences), anti-CXCR5-PE-CF594 (BD Biosciences) and anti-PD-1-APC-R700 (BD Biosciences) in PBS in the presence of 2 % BSA. The fluorescence signal of labeled samples was acquired on a CytoFLEX S flow cytometry (BECKMAN COULTER).

Intracellular cytokine staining

Drained lymph nodes and spleen were harvested and washed with RPMI 1640 medium. Then tissues were abraded into cell suspensions by the piston handle of 2 ml syringe in the culture medium (RPMI 1640 containing 10 % FBS and 1 % antibiotics).

738 The suspension was filtered with 40 µm nylon mesh cell strainer (Sangon Biotech).
 739 Cells were washed with culture medium and sterile erythrocyte lysis buffer was added
 740 (1.5 M NH₄Cl, 100 mM NaHCO₃, 10 mM EDTA in deionized water, pH 7.4) to
 741 remove red blood cells, followed by staining with fixable viability stain 780 (BD
 742 Biosciences) for 30 min at ambient temperature. Approximately 1.0 x10⁶ cells were
 743 added to the 6-wells plates and treated with anti-CD16/32 antibody (BD Biosciences)
 744 to block the Fc receptor, and then stimulated with 15 µg/mL purified RBD monomer
 745 for 3 h at 37 °C. After incubation, GolgiStop and GolgiPlug (BD Biosciences) was
 746 added to each well for an additional 15 hours at 37 °C. Next, the cells were harvested
 747 and washed twice with culture medium, and labeled with anti-CD3e-PerCP-Cy5.5
 748 (BD Biosciences), anti-CD4-BV510 (BD Biosciences) and anti-CD8a FITC (BD
 749 Biosciences) in PBS in the presence of 2 % BSA, after which cells were further fixed
 750 with 4 % paraformaldehyde and permeabilized with permeabilization buffer (2 %
 751 BSA, 0.1 % saponin, 0.05 % Na₃N in PBS). Finally, cells were washed with PBS in
 752 the presence of 2 % BSA and incubated with anti-IFN-γ-PE-CY7 (BD Biosciences),
 753 anti-IL-2-APC (BD Biosciences), anti-TNF-α-PE (BD Biosciences) and control
 754 anti-IgG1 antibody for 30 min at 4 °C. The fluorescence signal of labeled samples
 755 was acquired on a CytoFLEX S flow cytometry (BECKMAN COULTER).

756

757 **BALB/c mice challenge**

758 For SARS-CoV-2 challenge experiment, the female 6-8 weeks old mice were
 759 arbitrarily divided into 5 groups in each group. All purified antigens were prepared by
 760 mixed 100 µL protein solution diluted in PBS with an equivoluminal AddaVax
 761 adjuvant. Groups of twenty mice were immunized subcutaneously with a total protein
 762 dose corresponding to 10 µg of the RBD antigen on week 0 and 3. Purified
 763 gp350D123 protein of Epstein-Barr virus, including the RBD, formulated with
 764 AddaVax adjuvant was used as negative control. Blood were collected at week 2 and
 765 5 for analysis. Sixty days after the second immunization, the mice were lightly
 766 anesthetized with isoflurane and intranasally transduced with 2.5x 10⁸PFU of
 767 Ad5-hACE2 virus. Five days following transduction, the transduced mice were

768 challenged with 1×10^5 PFU of SARS-CoV-2 via the intranasal route. Weight changes
769 of the challenged mice were observed for ten consecutive days. At 1 and 3 days after
770 challenge, 4 mice in each group were sacrificed and their lung tissues were collected
771 for titration of the virus titers. On day 4 after challenge, 2 mice in each group were
772 sacrificed and necropsied, and lung tissues were collected for histopathological
773 analysis.

774

775 **Sequence alignment and analysis**

776 Except the sequence of Wuhan-Hu-1 which was first identified from a COVID-19
777 patient in Wuhan city ⁵, obtained from National Center for Biotechnology Information
778 (NCBI) database, other sequences are obtained from the
779 Global Initiative on Sharing All Influenza Data (GISAID). The accession numbers of
780 the RBD sequences of representative SARS CoV-2 strains isolated in different
781 countries are as follows: Wuhan-Hu-1 (Genbank: MN908947),
782 South_Korea/KCDC2489/2020 (EPI_ISL_514892), Thailand/NIH-2492/2020
783 (EPI_ISL_430841), Japan/Hu_DP_Kng_19-027/2020 (EPI_ISL_412969),
784 India/OR-RMRC25/2020 (EPI_ISL_455308), USA/WA-UW-1762/2020
785 (EPI_ISL_424245), Mexico/CMX-IMSS_01/2020 (EPI_ISL_424731),
786 Canada/ON_PHL2294/2020 (EPI_ISL_418384), Australia/VIC546/2020
787 (EPI_ISL_426809), Greece/218_35009/2020 (EPI_ISL_437886),
788 Greece/218_35009/2020 (EPI_ISL_437886), Greece/218_35009/2020
789 (EPI_ISL_437886), Greece/218_35009/2020 (EPI_ISL_437886),
790 Russia/SCPM-O-08/2020 (EPI_ISL_451970), Spain/Madrid_LP24_5999/2020
791 (EPI_ISL_428680), Sweden/20-50261/2020 (EPI_ISL_469078),
792 Switzerland/ZH-1000477102/2020 (EPI_ISL_413019), Portugal/PT0533/2020
793 (EPI_ISL_454257), Scotland/CVR138/2020 (EPI_ISL_425681),
794 Denmark/SSI-101/2020 (EPI_ISL_415646), England/20134020004/2020
795 (EPI_ISL_423108) and Iceland/348/2020 (EPI_ISL_424372),
796 Nigeria/OS085-CV14/2020 (EPI_ISL_455424) and Venezuela/VEN-95072/2020
797 (EPI_ISL_476704).

798

799 **Quantification and statistical analysis**

800 Kinetic parameters of Biolayer interferometry was rendered by Octet Data
801 Analysis software (Fortebio), and detailed curve fitting method could be found in
802 methods. Statistical analyses of all experimental results were performed with
803 GraphPad Prism 8.01 software. Except the results of flow cytometry are expressed as
804 a percentage of positive cells, all of results are presented as mean $\pm$ SEM. Method
805 used for statistical difference between groups could be found in figure legends or
806 corresponding methods for details.

807

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818

819 **Author contributions**

820 Y.K., C.S., Z.Z., R.Y., C.K., J.Z., and M.Z. conceived and designed the project; M.Z.
821 supervised the project; Y.K., C.S. and M.Z. wrote and edited the manuscript; Y.K.
822 purified the RBD-conjugated NPs protein and performed the DLS and DSC
823 experiment; Y.K. and Q.Z. performed the negative-stain EM; Y.K. and C.S.
824 performed the mice experiment and analyzed the results; X.C. and C.S. performed the
825 BLI assay; Y.K., R.Y., P.Z. and Z.Z. performed the neutralization assay.

826

827 **Competing interests**

828 Mu-Sheng Zeng has filed patent applications for the development of
829 SARS-CoV-2 RBD-conjugated nanoparticle vaccine candidate. The authors declare
830 no competing financial interests.

831

832 **Keywords:** SARS-CoV-2, RBD, Spytag-SpyCatcher, nanoparticle

833

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1025

1026

1027 **Figure legend**

1028

1029 **Figure 1. Construction and structural characteristics of RBD-conjugated**

1030 **nanoparticles.**

1031 (A) Sketch of RBD nanoparticle design. The left flow diagram shows a brief
1032 introduction to the modification to RBD and nanoparticle scaffolds with fusion of
1033 SpyTag-SpyCatcher system. The right schema display ideal nanoparticles with full
1034 valency of RBD. Colors of each nanoparticle is accordant to the displayed palette
1035 of the following charts.

1036 (B) Construction of target protein expression plasmid in different expression system,
1037 E. coli and HEK293F.

1038 (C) Reduced SDS-PAGE of the RBD monomer, RBD-conjugated NPs and unbonded
1039 nanoparticles. A high covalent bond linking efficiency is achieved as the blot of
1040 RBD monomer and unlinked nanoparticle scaffold disappear in the lane of
1041 RBD-conjugated NPs.

1042 (D) Size exclusion chromatography (SEC) of RBD monomer, RBD-conjugated NPs
1043 and unbonded nanoparticles on Superose 6 increase 10/300GL. Peak forward
1044 shifts of retention are observed after bond linking of RBD-SpyTag and Δ
1045 N1-SpyCatcher-NPs.

1046 (E) Dynamic light scattering (DLS) of RBD monomer, RBD-conjugated NPs and
1047 unbonded nanoparticles. Increased hydrodynamics diameters of nanoparticles
1048 after bond linking are shown.

1049

1050 **Figure 2. Assembly validation and physical evaluation of nanoparticles.**

1051 (A) Negative stain electron micrographs of unlinked nanoparticles and
1052 RBD-conjugated NPs.

1053 (B) Detailed information of DLS and nano DSF results.

1054 a. R_d : Hydrodynamics diameter

1055 b. PDI: Polydispersity index, PDI lower than 0.2 indicates a uniform particle size.

1056 c. T_{m1} : the first melting temperature

1057 d. T_{m2} : the second melting temperature

1058 e. T_{aggr} : the aggregation temperature

1059 f. Melting temperature and aggregation temperature are given by the analysis
1060 software of nanoDSF

1061

1062 **Figure 3. Antigenicity characterization of RBD monomer and RBD-conjugated**
1063 **nanoparticles.**

1064 (A) ELISA assay of ACE2 and CB6 antibody binding capability. Statistical analysis of
1065 binding titers between RBD monomer and the three RBD-NPs was performed
1066 using 2-way ANOVA corrected with Dunnett method.

1067 (B) (C) Biolayer interferometry (BLI) kinetic assays of RBD monomer and RBD-NPs.

1068 (D) Detailed information of BLI assay.

1069 a. k_D : binding affinity constant calculated by k_{on}/k_{dis} , smaller values generally
1070 indicate stronger binding capability

1071 b. k_{on} : association rates

1072 c. k_{dis} : dissociation rates

1073

1074 **Figure 4. Immunogenicity characterization of RBD monomer and**
1075 **RBD-conjugated nanoparticles.**

1076 (A) Schematic flow diagram of animal immunization procedures.

1077 (B) Serum antibody titers of mice immunized by immunogen adjuvanted with
1078 AddaVax or SAS determined by ELISA. Statistical difference between RBD
1079 monomer and RBD-NPs are calculated with Two-way ANOVA corrected by
1080 Dunnett method with setting the monomer as control group. * $p < 0.05$; ** $p <$
1081 0.01 ; *** $p < 0.001$; **** $p < 0.0001$.

1082 (C) BLI serum competition assay of sera from immunized with RBD monomer and
1083 RBD-NPs adjuvanted with AddaVax against ACE2 or CB6 antibody. R_c represent
1084 the binding signal of ACE2 or CB6 under each dilution level. R_o represent the
1085 binding signal of serum-free binding signal of ACE2 or CB6.

1086 (D) Heatmap overview of competition assay. The competition level presented by ratio
1087 $(R_o - R_c)/R_o$. Brighter color indicates stronger competition against receptor ACE2
1088 or neutralizing antibody CB6 under each dilution level.

1089

1090 **Figure 5. Neutralizing capability of mice sera of RBD monomer and**
 1091 **RBD-conjugated nanoparticles.**

1092 (A) SARS-CoV-2 pseudovirus neutralizing assay shows the NT₉₀.

1093 (B) SARS-CoV-2 live virus neutralizing assay show the focus reduction NT₉₀
 1094 (FRNT₉₀).

1095 Statistical difference of neutralizing titers of mice immunized by immunogen
 1096 adjuvanted with AddaVax or SAS are calculated with unpaired two-tailed
 1097 non-parametric Mann-Whitney U test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
 1098 **** $p < 0.0001$.

1099 (C) Table of SARS-CoV-2 live virus neutralizing titers determined by induced
 1100 cytopathic effect (CPE). Deeper red color represents a higher dilution ratio.

1101

1102 **Supporting Information**

1103

1104 **Figure S1. Sequence alignment of RBD from 24 representative SARS-CoV-2**
 1105 **strains isolated from six continents.** The sequence of 24 RBD was downloaded from
 1106 Genbank and GISAID. The strain isolated from Asia, North America, Oceania,
 1107 Europe, Africa and South America was colored by brown, orange, olivedrab, black,
 1108 green and cyan, respectively. Conserved residues are highlighted in red. Multiple
 1109 sequences were aligned by MAFFT (<https://mafft.cbrc.jp>). The sequence alignment
 1110 was converted with Clustal X2 (<http://www.clustal.org>) and visualized with ESPript
 1111 3.0⁵¹.

1112

1113 **Figure S2a. Co-structure of SARS-CoV-2 spike protein RBD with human ACE2.**
 1114 The SARS-CoV-2 spike protein trimer (Marine blue for chain with up-conformation
 1115 RBD and grey with down-conformation RBD) (PDB code: 6VSB) is aligned to the
 1116 complex of RBD (red) and human ACE2 (Light green) (PDB code: 6M0J) at the
 1117 up-conformation RBD to display the binding interface.

1118

1119 **Figure S2b. Schematic presentation of covalent bond linking strategy used in**
 1120 **RBD-conjugated nanoparticle construction.**

1121 The RBD fused with SpyTag would automatically links to the Δ N1-SpyCatcher-fused
 1122 nanoparticle scaffolds ferritin (PDB code: 3BVE), mi3^{17, 24} and I53-50 (PDB code:
 1123 6P6F) to form a complex of SpyTag-SpyCatcher (PDB code: 4MLI) in between as the
 1124 bridge. The linked nanoparticles would present RBD (red cartoon with grey surface)
 1125 on the surface as shown by alignment of C-terminus of RBD with the N-terminus of
 1126 scaffolds.

1127

1128 **Figure S3. Flowcytometry assay of immune spectrum of drained lymph nodes of**
 1129 **immunized mice.**

1130 (A) Germinal center B cells are marked out from the drained lymph node using B220+
 1131 (CD45R), IgD-low, GL7+ and CD95+ as cell marker. Ratio of the positive cells
 1132 are presented.

1133 (B) T follicular helper (Tfh) cells are marked out using CD4+, CD44+, PD-1+ and
 1134 CXCR5+ as cell marker. Ratio of the positive cells are presented.

1135 (C)(D) Cytokine-secreting CD4+ and CD8+ T cells are marked out using CD4+ and
 1136 IFN- γ + /IL-2+ /TNF- α + as cell markers.

1137

1138 **Figure S4. Flowcytometry assay of immune spectrum of spleen of immunized**
 1139 **mice.**

1140 (A)(B) Cytokine-secreting CD4+ and CD8+ T cells are marked out using CD4+ and
 1141 IFN- γ + /IL-2+ /TNF- α + as cell markers.

1142

Figure. 1

Figure. 2

A

B

	Particle size (DLS)		Thermostability (nano DSF) ^f		
	<i>R_d</i> (nm) ^a	<i>PDI</i> (%) ^b	<i>T_{m1}</i> (°C) ^c	<i>T_{m2}</i> (°C) ^d	<i>T_{aggr}</i> (°C) ^e
RBD	8.98±0.03	0.190±0.07	46.83±0.08	NA	NA
Ferritin NP	28.75±0.18	0.184±0.01	53.65±1.00	NA	34.74±1.14
RBD-Ferritin NP	32.99±0.04	0.175±0.01	49.54±0.18	NA	NA
mi3 NP	41.87±0.39	0.094±0.02	33.31	58.99	29.38
RBD-mi3 NP	55.19±0.49	0.163±0.01	42.55±0.18	NA	NA
I53-50 NP	46.54±0.40	0.151±0.01	78.16±0.25	89.90±0.03	77.15±0.29
RBD-I53-50 NP	50.67±0.11	0.135±0.01	41.99±0.12	77.42±0.29	69.73±0.67

Figure. 3

A

D

Loading arrangement		Kinetic values			
Coupled	Analyte	Sensors (n)	k_D (M) ^a	k_{on} (1/Ms) ^b	k_{dis} (1/s) ^c
ACE2	RBD	5	4.34E-09	1.18E+05	5.11E-04
	RBD-Ferritin NP	4	1.74E-08	3.28E+04	5.68E-04
	RBD-mi3 NP	5	<1.0E-12	1.57E+04	<1.0E-07
	RBD-I53-50 NP	4	1.00E-09	8.75E+05	8.77E-04
CB6 antibody	RBD	5	4.86E-09	5.97E+05	2.90E-03
	RBD-Ferritin NP	4	9.77E-10	6.82E+06	6.66E-03
	RBD-mi3 NP	4	5.25E-11	3.57E+05	1.88E-05
	RBD-I53-50 NP	4	4.31E-10	4.98E+05	2.15E-04

B

C

Figure. 4

Figure. 5

C

Immunogen (Addavax adjuvanted)	Mouse ID	NT50 of different immunization procedures			Immunogen (SAS adjuvanted)	Mouse ID	NT50 of different immunization procedures		
		Prime	1st boost	2nd boost			Prime	1st boost	2nd boost
RBD	1	< 4	< 4	32	RBD	21	< 4	8	1024
	2	< 4	64	1024		22	< 4	32	> 1024
	3	< 4	4	128		23	< 4	4	1024
	4	< 4	4	256		24	< 4	16	1024
	5	< 4	32	512		25	< 4	< 4	128
RBD-mi3 NP	6	< 4	512	1024	RBD-mi3 NP	26	< 4	512	4096
	7	< 4	1024	> 8192		27	< 4	> 1024	> 8192
	8	< 4	1024	6144		28	< 4	1024	> 8192
	9	< 4	512	6144		29	< 4	> 1024	8192
	10	< 4	1024	8192	RBD-Ferritin NP	30	< 4	512	3072
RBD-Ferritin NP	11	< 4	> 1024	> 8192		31	< 4	256	6144
	12	8	1024	6144		32	< 4	> 1024	3072
	13	8	> 1024	8192		33	< 4	1024	4096
	14	< 4	512	1536		34	< 4	> 1024	3072
RBD-I53-50 NP	15	< 4	> 1024	6144	RBD-I53-50 NP	35	< 4	256	2048
	16	< 4	1024	> 8192		36	32	1024	> 8192
	17	8	> 1024	4096		37	8	1024	> 8192
	18	8	128	4096		38	8	> 1024	> 8192
	19	8	> 1024	> 8192		39	4	> 1024	8192
	20	< 4	> 1024	8192	PBS	40	8	1024	3072
						41	< 4	< 4	< 4
						42	< 4	< 4	< 4
						43	< 4	< 4	< 4
						44	< 4	< 4	< 4
						45	< 4	< 4	< 4

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[illegible]

- Asia
- North America
- Oceania
- Europe
- Africa
- South America

Figure S2A

Figure S2B

Figure S3

Figure S4