Nanobodies against SARS-CoV-2 RBD from a Two-step Phage Screening of Universal and Focused Synthetic Libraries

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Research Article

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Abstract

Coronavirus disease 2019 (COVID-19) is an evolving global pandemic, and nanobody (Nb) is recognized as a potential diagnostic and therapeutic tool for infectious disease. Here, we designed and synthesized a humanized and highly diverse phage Nb library hsNb-U (Humanized synthetic Nb Library - Universal). We expressed and purified the SARS-CoV-2 receptor-binding domain (RBD), and screened this universal library against the RBD protein target. Then, the CDR1 and CDR2 sequences of five leads obtained from the hsNb-U phage panning were combined with randomly mutated CDR3 to construct a targeted (focused) phage display library, hsNb-RBD, for subsequent phage panning and screening. From the obtained sequences, we expressed 45 unique anti-RBD candidate Nbs. Among the selected Nbs, eight were found to be highly expressed, and five of these show high-affinity to RBD (EC\textsubscript{50} less than 100nM). Finally, we found that Nb39 can compete with angiotensin converting enzyme 2 (ACE2) for binding to RBD. Overall, this two-step strategy of synthetic phage display libraries enables rapid selection of SARS-CoV-2 RBD nanobodies with potential therapeutic activity, and this two-step strategy can potentially be used for rapid discovery of Nbs against other targets.

Background

Since December 2019, a novel, highly transmissible severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, COVID-19) [1, 2] has erupted on a large scale worldwide and spread rapidly. As of May 2023, more than 750 million people have been infected and about 7 million lives have been claimed. These numbers are still rising. The global COVID-19 pandemic poses serious challenges to patients, health care systems, and economic and social activity.

Although the SARS-CoV-2 vaccine is widely used around the world, the vaccine’s protective effect is greatly reduced in people with weakened immunity system, such as elderly or people with immune-compromised conditions. Vaccine alone is not enough to end the pandemic. The development of neutralizing antibodies or related passive immunization molecules to prevent and treat SARS-CoV-2 will always be unmet needs [3].

Monoclonal antibodies (mAb) have had tremendous success in treating a variety of disease, and several mAb have been approved for the treatment of COVID-19 [4–9]. However, virus like SARS-CoV-2 continuously evolve through mutations during genome replication. Since the outbreak, multiple variants of SARS-CoV-2 have been identified [10], named in Greek letters by the World Health Organization (WHO). Omicron variant is more transmissible than other variants and is insensitive to some of the anti-SARS-CoV-2 mAbs that have been developed for treatment or prophylaxis [11, 12].

The high production costs, large doses needed, and low-temperature requirements for transportation and storage associated with traditional mAbs make it challenging to be cost-effective for large scale applications. Single-domain antibodies or variable domain of the heavy chain of HACbs (VHH) from camels, having a smaller molecular weight (15 kD) than traditional mAbs (150 kD), are commonly termed nanobodies (Nbs) [13, 14]. Nbs have shown great potential in biomedical applications, including cancer, infection, inflammation, and other diseases [15–17]. The first Nbs-based medicine was approved to treat acquired thrombotic thrombocytopenic purpura (aTTP) in 2018 [18].

Nbs have several important advantages over traditional mAbs, such as higher thermal stability, higher solubility, and easier penetration into tissues [19]. Due to their minimal size, they are particularly suited to reach hidden epitopes such as crevices of target proteins [20]. Nbs can be expressed in prokaryotic systems with lower production costs [21, 22]. Although for therapeutic applications, it will be necessary to produce under GMP, which may increase the production cost to a level comparable to mAbs production. Nbs can be easily bioengineered into novel bivalent / multivalent / multispecific and high-affinity molecules [23, 24]. Especially, Nbs are stable and small, and they can be aerosolized for direct delivery to the lungs. Nbs provide possible opportunities for rapid production of antiviral drugs.

At present, SARS-CoV-2 Nbs are mainly obtained through the "in vivo" method [25–31]. The recombinant spike (S) protein or receptor-binding domain (RBD) protein is used to immunize camellid animals such as camels or alpacas. However, "in vivo" screening methods require a long development period (usually > 3 months) from antigen to final specific Nbs. So, it is difficult to develop and generate antibodies against new virus mutant strains or new viruses quickly and at low cost. Therefore, rapid and efficient "in vitro" screening becomes an important approach by combining phage display technology with naive [32] or synthetic [33–35] Nbs libraries.

Here, we designed and synthesized a highly diverse phage library of humanized Nbs, and screened it to obtain Nbs binding to RBD. The CDR1 and CDR2 of the obtained lead sequences were then assembled with randomly mutated CDR3 to construct a focused (targeted) library, hsNb-RBD, specifically targeting RBD. By this two-stage method, Nbs can be selected within ten working days, which is considerably faster than "in vivo" method, which requires the repetitive immunization prior to binder selection by phage display. Finally, we screened and obtained several Nbs that bind to RBD with high affinity. Overall, we have established an efficient two-stage method for rapid development of humanized Nbs targeting SARS-CoV-2 RBD. This two-stage method has the potential to strengthen our ability to respond to the current COVID-19 pandemic, possible new variants and even other diseases.

Results

1. Design and construction of a synthetic Nb library hsNb-U.

We used a Nb framework (Fig. 1–2) based on a soluble human germline immunoglobulin heavy-chain variable region (IGHV3-66*01), which has been shown to be an ideal alternative to camel Nbs [36]. For highly variable complementary determine regions (CDRs), the sequence library (Fig. 1–2) was designed based on the work of McMahon et al [34].

A fixed size of 7 amino acids (AAs) was chosen for the CDR1 and 12 AAs was chosen for the CDR2. It is observed that Nbs CDR3 varies greatly in length, and contributes most to antigen-binding affinity and specificity. Therefore, three sizes (10 AAs, 14 AAs and 18 AAs) were chosen for CDR3. For variable positions not fully randomized (such as the first amino acid in CDR1 region), we designed degenerate codons using a web-based tool SwiftLib [37]. The designed DNA
sequence is further optimized for *E. coli* expression based on codon usage. The sub-library was constructed using NNC and NNK respectively for fully randomized amino acids, and then combined in the phage screening. The full-length Nb sequences were assembled by overlap-extension PCR from DNA fragments with degenerated codons. The full-length Nb sequences were cloned into the pComb3Xss vector, and then transformed into *E. coli* TG1 strain by electroporation in multiple batches. Finally, >1 10^9 clones were obtained. Quality control was carried out using Sanger sequencing of 50 randomly picked clones, and no redundant clone was found. In this way, we generated a large and highly diverse library of humanized Nbs library hsNb-U (Humanized synthetic Nbs Library-Universal) (as shown in Fig. 1–2).

### 2. Initial screening of anti-RBD Nbs

SARS-CoV-2 expresses a surface Spike (S) glycoprotein composed of two subunits, S1 and S2, and forms the homotrimeric S protein [38] which can interact with host cells. The interaction between the SARS-CoV-2 and the host cell is mediated by the RBD of the S1 subunit, which binds to the peptidase domain (PD) of Angiotensin-converting enzyme 2 (ACE2) [38]. After that, the S2 subunit undergoes a drastic conformational change and triggers membrane fusion [38]. Therefore, the S protein RBD has become one of the most important targets for developing SARS-CoV-2 antibodies.

We obtained the recombinant SARS-CoV-2 S protein RBD (as shown in Fig. 3A-B) through the Bac-to-Bac baculovirus expression system and purified by Ni–NTA affinity column followed by Superdex-75 gel filtration column. Two rounds of phage panning were performed against the recombinant RBD from the hsNb-U library. Enrichment of Nbs-displaying phages against the RBD were monitored by enzyme-linked immunosorbent assay (ELISA). Candidate phages enriched more than 5-fold over the bovine serum albumin (BSA) control protein were selected as initial leads. We identified 5 high-affinity clones: H1, F3, E5, A6, H6 (Fig. 3D).

The five high-affinity RBD-binding Nb clones were sequenced. Their CDR1 sequences were: 1. NTFLLRS, 2. NTFFPART, 3. TISAGPR, 4. NTSTRHP, 5. STFRVR; and their CDR2 sequences were: 1. FLSAINDGSTTY, 2. FGSTIADGASTN, 3. FVSGIGPGSITY, 4. FVSTIGRGEITY, 5. LVASIGPGRSTI (Fig. 3C). These CDR1 and CDR2 sequences are consistent with the sequence characteristics of Nbs (Fig. 3C). Then, we combined these CDR1s and CDR2s with fully randomized CDR3 library (as in Fig. 1–2) to construct an RBD-specific Nbs library. We synthesized the five CDR1 fragments and the five CDR2 fragments, randomly combined them (5 x 5 = 25 combinations) with the randomly mutated CDR3 library to construct a new library, hsNb-RBD (Fig. 3C).

### 3. Anti-RBD Nbs screening from the focused library

From the hsNb-RBD library, two rounds of panning were performed against the recombinant RBD. Dozens of candidate Nbs-displaying phage clones with more than 30-fold enhanced ELISA signals over BSA were selected (Fig. 4). On average, the Nbs phage clones screened from the targeted hsNb-RBD library had stronger affinity to the RBD than those from the universal hsNb-U library. These results showed that hsNb-RBD library, with CDR1 and CDR2 optimized according to the lead sequences, had improved neutralizing capacity against RBD compared with the initial library hsNb-U.

### 4. Binding affinity of the top Nb candidates

For *E. coli* expression of phage-screened Nbs, we selected 45 highest-affinity clones with diverse CDR sequences to encompass a variety of biophysical, structural and potentially different antiviral properties. We found that 13 of them had no expression (28.8%), 23 had low expression (51%), and only 8 candidates had high expression (17.7%) (Fig. 5A). We purified these 8 Nbs and tested their RBD binding by ELISA, from which we identified five high affinity RBD-specific Nbs (Fig. S8-E). Amino acid sequences of the five highest affinity RBD Nb candidates were shown as Fig. 5E. Among them, Nb39 and Nb42 had the highest affinity to RBD, however, the Nb42 protein was less stable and precipitated after freezing and thawing. Nb39 has good stability, high affinity to RBD, and the half-inhibitory concentration reaches about 10 nM (Fig. 5B-E).

### 5. Candidate Nbs compete with ACE2 for RBD binding

To test whether the Nb candidates compete with angiotensin converting enzyme 2 (ACE2) for RBD binding. We obtained the extracellular domain of ACE2 protein through the Bac-to-Bac baculovirus expression system, and purified it by Ni–NTA affinity column followed by Superdex-75 gel filtration column (Fig. 6A).

To investigate whether the binding of candidate Nbs to RBD is competitive with ACE2, we developed a competition assay to determine the binding of Nb39 to RBD with and without ACE2 by microscale thermophoresis (MST). The binding affinity of Nb39 to RBD is similar as the one (Kd = 14.0 ± 11.6 nM) determined by the ELISA method without ACE2 (Fig. 6B). However, the observed affinity of Nb39 to RBD drops 10-fold (Kd = 140.3 ± 91.8 nM) in the presence of 5nM ACE2 (Fig. 6B). The Kd values were analyzed using the MO Affinity Analysis software (NanoTemper Technologies). This indicates that Nb39 can bind the same site on RBD as ACE2 does. Also, the results indicate Nb39 and ACE2 have a similar binding affinity to RBD, and Nb39 binds RBD of the SARS-CoV-2 spike protein and inhibits the interaction between RBD and ACE2.

To understand the structural basis of the binding of Nb39 to RBD, a Nb39-RBD complex model was predicted by the SnugDock [39] method in Rosetta 3 (Fig. 6C-E). According to the docked model, Nb39 binds to RBD via key interactions involving mainly CDR1 and CDR3. The CDR1 forms a hydrogen bond with RBD (H32-E166 in Fig. 6D). The long CDR3 “folds” back to the framework region by forming a hydrophobic core involving V37, L45, L47, V96, L99, and W103 within the Nb39 itself. For RBD binding, the CDR3 contributes a hydrogen bond (R100F-T152) and several hydrophobic interactions (F95-F172, Y102-F172, Y102-I154, and F98-I150), as shown in Fig. 6E.

### Discussion

Nbs have several important advantages over traditional antibodies, including low cost, high thermal stability, small molecular weight, and nebulization for direct delivery to the lungs. Nbs can be nebulized, inhaled, and administered directly to the site of infection, with rapid onset of action, high local drug
concentration/high bioavailability, and high patient compliance (needle-free) [40, 41], making them very attractive agents against respiratory viruses. In recent years, research on their application against respiratory pathogens has also accelerated. For example, Nbs against MERS-CoV [42], H1N1 [43], H5N1 [44], influenza [45] and so on has been documented. ALX-0171, a trivalent Nb that neutralizes Respiratory syncytial virus (RSV), directly prevents or treats RSV infection in the lungs of cotton rats [41].

High-quality Nbs are promising candidates for the treatment of COVID-19 pneumonia [46–48]. The development of highly effective anti-SARS-CoV-2 Nbs may provide an important means for multifunctional, cost-effective prevention, treatment and immediate diagnosis. Xiang et al. immunized camels with recombinant RBD and identified several high-efficiency SARS-CoV-2 neutralizing Nbs using proteomics methods [28]. These heat-stable Nbs can be mass-produced rapidly by microorganisms, and are resistant to freezing, drying and aerosolization [28]. They further developed the most efficient tri-valent Tri-Nb21 into PiN-21 aerosol, which can effectively prevent and treat Syrian hamsters infected by SARS-CoV-2 at an ultra-low dose, greatly reducing viral load and preventing lung damage and viral pneumonia [27, 28].

The synthetic Nb library uses gene synthesis technology to introduce random DNA sequences at specific sites, which is highly controllable and allows fast and efficient screening against target proteins. However, due to combinatorial explosion, the possible Nbs sequences space is extremely vast and much larger than the capacity of phage library. In this study, we first screened universal hsNb-U library against the target protein, and obtained five high-affinity lead sequences. We further assembled the CDR1 and CDR2 sequences from these five initial leads with randomly mutated CDR3 to construct a second library hsNb-RBD, which is more focused on the target protein (RBD). Multiple Nb candidates were obtained from the hsNb-RBD library. Among them, Nb39 has good stability, high binding affinity to RBD with the EC50 reaching 3nM, and Nb39 can compete with ACE2 for binding to RBD.

The probability of screening high-affinity RBD-binding Nbs from the focused hsNb-RBD library is higher than from the universal hsNb library, and the identified Nbs have higher affinities than the lead sequences. These results validate the feasibility and effectiveness of this two-stage screening strategy, where a universal and diverse library is initially screened to obtain lead sequences for construction of an antigen-specific library for a second-stage screening (Fig. 7). This strategy allows screening of high affinity SARS-CoV-2 Nbs within ten working days, which is relatively quickly compared to immunized animals. Moreover, compared to the traditional one-step synthetic library screening (universal library in the first step), the second step focused library is more efficient in screening. This strategy can be extended to the screening of other targets.

In addition, the current rapid development of computational technology and artificial intelligence (AI) has facilitated the development of protein structure prediction and computer-aided drug design. At present, large-scale co-evolution analysis is the commonly used algorithms for predicting the 3D structure of proteins based on gene sequences and performs quite well [49]. This algorithm is used by Google's AlphaFold, which can accurately predict protein structure from protein sequence within minutes [50, 51]. With the development of theoretical chemistry and computational biophysics, our understanding of the physical nature of protein folding and interactions has improved, and various software has been developed to model and simulate proteins and other biomolecules. David Baker et al. used their Rosetta software to design a variety of proteins with unnatural structures or proteins with high affinity to specific targets [52]. The Rosetta software has also been extended to perform high-precision modeling of antibodies [53]. Computer-aided nanobody development may become a very important tool in the future.

In summary, here we report a synthetic Nb platform for rapid screening of anti-RBD Nbs (Fig. 7), and this pipeline can be extended to screening of other targets. These Nbs may be promising candidates for COVID-19 prevention, treatment, or as reagents to facilitate SARS-CoV-2 vaccine development. This two-stage strategy can be used to rapidly developed new Nbs against mutant virus strains, and address the need for continues virus mutation in a pandemic. We believed that ongoing research will surely pave the way to a safer world.

Methods

1. Construction of humanized Nb library

The full-length Nb DNA sequences were obtained by a series of overlap-extension PCR (OE-PCR) using oligonucleotides (with degenerated codons) purchased from external primer synthesis services. PCR was performed using high fidelity DNA polymerase (Phusion Green DNA polymerase, Thermoﬁsher) for 20–32 cycles (with annealing temperature chosen according to Thermoﬁsher's online Tm calculator). Finally, the full-length Nb DNA fragments were digested with SfiI (New England Biolabs) and cloned into phagemid pComb3Xss (NBbiolab, China). The recombinant vector was electro-transformed (Bio-Rad MicroPulser electroporator) into TG1 bacteria at 2.5kV (0.2cm cuvette) and 49~52ms time constant, Pre-warmed SOC medium was added and incubated at 37°C with shaking at 250 rpm for 1 h. 10 μl of the culture was 10-fold serially diluted and plated on 2×TY agar plates containing 1.5% glucose (final concentration, the same below) and 75mg/mL carbenicillin. The plates were incubated overnight at 37°C, and the diversity of the library was calculated next day by counting the colonies.

To prepare phage library, the cultures were inoculated (1:20) to 2×TY medium with 75mg/mL carbenicillin and 1.5% (w/v) glucose, and incubated at 37°C with constant shaking. When the culture reach OD600 = 0.5, TG1 cells were infected with M13KO7 helper phages (NBbiolab, China), and incubated without shaking for 45min at 37°C. The TG1 were harvested and resuspended in 2×TY medium with carbenicillin (75mg/mL) and kanamycin (15mg/mL), and cultured overnight (~10hr) at 30°C with constant shaking. Next day, the cultures were centrifuged and phages were precipitated from the supernatant by adding PEG-NaCl (final concentration: 4% PEG8000, 0.5M NaCl). Precipitated phages were collected by gentle centrifugation, and resuspended in sterile PBS buffer.

2. Nb screening from phage library
96-well plates (Corning, high binding surface) were coated with 100 μl of 100 μg/ml purified protein (RBD or BSA) for 2 hours at room temperature (RT), and blocked with PBS buffer containing 2% milk powder (w/v) for 1 hour at RT. Phages library were incubated with immobilized antigen for 1 hour and then washed with PBST (PBS buffer supplemented with 0.5% Tween 20). Bound phages were eluted with 100μl of 20μg/ml trypsin, and were used to infect TG1 bacteria culture (OD = 0.2 ~ 0.8) at 37°C for 45 min. The eluted phage library was amplified according to the protocol described in above section. The antigen-specific-binding of phages library after each round of panning was assessed by polyclonal phage ELISA. Single-clone phage ELISA were also carried out using colonies on phage titration plates.

3. Enzyme-linked Immunosorbent Assay (ELISA)

The entire ELISA procedure was carried out at room temperature. 96-well plates (Corning #3690) were coated with 100 μl of 100 μg/ml purified protein (RBD or BSA) for 2 hours, and blocked with PBS buffer containing 2% milk powder (w/v) for 1 hour. For polyclonal phage ELISA, phages from each round of panning were incubated with immobilized antigen and bound phages were detected with anti-M13-horseradish peroxidase (HRP) polyclonal antibody (Thermofisher, MA5-29950). For the purified antibody binding assay, serially diluted Nbs (with HA-tag) solutions were added and incubated for 1.5 h, and bound Nbs s were detected with monoclonal anti-HA-HRP antibody. The enzyme activity was measured with the subsequent addition of substrate EL-TMB and signal reading was carried out at 450 nm using a Microplate Spectrophotometer.

4. Protein expression and purification

The gene sequences of the Nbs were amplified with PCR and subcloned into a pET-21(a+) expression vector, which contains a C-terminal 6xHis+HA tag. The expression construct was transformed into a BL21(DE3) chemically competent E. coli for protein expression.

The overnight culture with the selected colony was inoculated in one Liter LB media with correct antibiotics. The temperature was decreased to 18°C when OD600 of culture reached 0.6, the recombinant Nbs protein expressing was induced overnight with 0.5mM IPTG. Bacterial was harvested and resuspended in lysis buffer (50mM PBS, 2mM PMSF, pH 7.4). Protein was purified with Ni column (HiTrap Excel, GE Healthcare) and gel filtration (Superdex S75 column, GE Healthcare).

RBD (R319-F541) and human ACE2 ectodomain(S19-D615) protein were expressed with Bac-to-Bac Baculovirus Expression System (Invitrogen). The corresponding gene of two proteins were subcloned into a modified pFastBac1 vector (Invitrogen), which contains a N-terval GP67 secreting signal peptide sequence and a C-terminal 6xHis purification tag. The expressing construct was transformed into bacterial DH10bac competent cell, the recombinant bacmid was extracted and transfected into sf9 insect cell with Cellfectin II reagent (Invitrogen). After two-rounds amplification, the recombinant baculovirus with high-titer were harvested and mixed with Hi5 insect cell (2x10^6 cells per mL). After 60 hours infection, the cell culture containing the secreted proteins was harvested. Protein purified with Ni-column (HiTrap Excel, GE Healthcare) and gel filtration column (Superdex 200, GE Healthcare); PBS buffer was used for all purification steps.

5. Micro Scale Thermophoresis (MST)

The binding affinity between Nbs proteins and RBD was measured with the MST NT.115 device (NanoTemper Technologies). RBD was labeled with MonolilithTM RED-NHS labeling kit with the manufacturer's protocol. The labeled RBD protein was diluted with binding buffer (PBS-T: 20mM PBS, 0.05% Tween-20, pH=7.4) before it was used in the experiment. A 20nM final concentration of the labeled protein was mixed (1:1) with the sequentially diluted nanobodies. For the competition assay, the same procedure was followed except the PBST-buffer was containing 5nM ACE2 ectodomain. All measurements were triplicated with 50% LED medium MST power. The Mo. Affinity Analysis software (NanoTemper Technologies) was used for the data analysis.

6. Nb39-RBD complex structure prediction with Snugdock [39]

We performed nanobody-antigen docking with the SnugDock module in Rosetta 3. Before running SnugDock, we prepared models for Nb39 and RBD, respectively, as below.

For Nb39, an initial model was first obtained from Alphafold2 [54] prediction (monomer mode with Nb39 sequence as input and default settings, see below) by selecting the model with the highest average pLDDT score. The PDB residues were renumbered according to the Chothia antibody-numbering scheme. Then the initial model's CDR3 conformation was further optimized by the Antibody_H3 module in Rosetta 3 with default settings. 1000 conformations were generated in total and the 5 top-scored conformations were selected for docking.

For RBD, to account for possible conformational flexibility, we prepared multiple models from three different sources: (i) the RBD structure extracted from the complex of RBD with the Fab fragments of two neutralizing antibodies (PDB 6xdg); (ii) a model predicted by Alphafold2 (monomer mode with RBD sequence as input and default settings, see below); (iii) a model extracted from the Nb39-RBD complex model predicted by Alphafold-multimer [55] (with both RBD and Nb39 sequences as input and default settings) by selecting the model with the highest average pLDDT score.

For nanobody-antigen docking, the Snugdock method [39] in Rosetta 3 was employed with ensemble docking to mimic conformer selection and induced fit by performing simultaneous optimization of the antibody (nanobody)-antigen rigid-body positions and the CDR loops. The best complex conformation was selected according to the ranking score.

SEQUENCES USED FOR ALPHAFOLD2 AND ALPHAFOLD - MULTIMER PREDICTIONS

RBD:
RVQPTESIRFPNITLCPGEVFNVATRFASVYWNRKRSICNVDADSVLNYNASFTKFCYGVSPTKLDLCFTNYADSFVIRGDEVRQIAPGQTKIADYNKLPDDFTGCVIAYNSNNLSKVGGNYNYLYRFRKSNLKFERDISTIEYQAGSTPCNGVEGFCNCYFPLQSYGFGQPTNGVGYQPYRVVVLSEFLHAPATCVPKXSTNLVKNKCVNFFn39:
EVQLVESGGGLVQPGGSLRLSCAASGNTSTRHPMSWRQAPGKLELVASIGRGEITTYYADSVKGRFTISRDNSKNTLYLMNSLRAEDTAVYVCAAFVDLSRHNSRRPYPPRIMERS

Primers for hsNb-U
FR1: CACTGGCTGGTTTCGCTACCGTGGCCCAGGCGGCCGAAGTGCAGCTGGTGGAAAGCGGCGGCGGCCTGGTGCAGCCGGGTGGTAGCCTGCGTCTGAG
H1: AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H2: CGGCGAAAGGTCTGCGRSCATACVTRVCCNKKGGGRSCACACCWACTATGGCGGATAGCGTGAAAGGCCGCTTTACCATTGCTGACGC
FR3: CCGCGCAATAAATACCGCGCTATCTTCCGGCGCCGAGCTGTTCTACTTGCAAGATACGGGTTTTTGCTGTTATCGCGGCTAATGGTAAAGCGGCCT
H3A: CGGTGTATTCTGCGGGYGNKNNKNNNNKKNKNNKKNNKYWTNKTATTGGGCCAGGTACCCCTGTTGACGC
H3B: CGGTGTATTCTGCGGGYKNKNKNNKNNKNNKNNKNNKNKNNKNNKNKNNKNNKNNKNNKNNKNKNNKYWTNKTATTGGGCCAGGTACCCCTGTTGACGC
H3C: CGGTGTATTCTGCGGGYKNKNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKYWTNKTATTGGGCCAGGTACCCCTGTTGACGC
FR4: CATGGTGATGGTGATGGGTGCTGGCCGGCCTGGCCGCTGCTCACGGTCACCAGGGTACCC

Primers for hsNb-RBD
H1A (NTFFLRS):
AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H1B (NTFPART):
AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H1C (TISAGPR):
AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H1D (NTSTRHP):
AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H1E (STRFRVRI):
AATTCCAGACCTTTGGCCCGGCGCTAGCACCACAGTCATGGAGCGGAGAAAGGTGTATTACGGCTTGCTGCACAGCTCAGACGCAGGCTACCA
H2A (FLSAINDGSTTY):
CGGCGAAAGGTCTGGAAATTCTTTGTGGCCATTAAGCGACGCGACCAACTACTATGGGAGATTGGAAGGGCCGCTTTACCATTAGC
H2B (FGSTIADGSTY):
CGGCGAAAGGTCTGGAAATTCTTGTCGACCATTGGCGGATGGGCGACCAATATTGAGGGATTGGAAGGGCCGCTTTACCATTAGC
H2C (FVSGISPAGS):
CGGCGAAAGGTCTGGAAATTCTTGTCGACCATTGGCGGATGGGCGACCAATATTGAGGGATTGGAAGGGCCGCTTTACCATTAGC
H2D (FSTIGPGRSEY):
CGGCGAAAGGTCTGGAAATTCTTGTCGACCATTGGCGGATGGGCGACCAATATTGAGGGATTGGAAGGGCCGCTTTACCATTAGC
H2E (LVASIGPGRST):
CGGCGAAAGGTCTGGAAATTCTTGTCGACCATTGGCGGATGGGCGACCAATATTGAGGGATTGGAAGGGCCGCTTTACCATTAGC
Abbreviations
COVID-19, Coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Nbs, nanobodies; hsNb-U, Humanized synthetic Nbs Library - Universal; RBD, receptor-binding domain; CDRs, complementary determine regions; mAb, Monoclonal antibodies; WHO, World Health Organization; VOC, Variants of Concern; VHHs, variable domains of heavy-chain-only antibodies; sdAb, single domains antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; ACE2, angiotensin converting enzyme 2.

Declarations
ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

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Author Contributions
F.J. and Z.L. perceived the project. W.K. and F.J. designed the nanobody libraries, and performed theoretical studies. F.J., F.C. and Z.L. conducted most of the experiments and data analysis, and wrote the manuscript. All authors reviewed the results, assisted in writing the manuscript, and approved the final version of the manuscript.

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Notes
The authors claim no conflict of interest.

Consent for publication
We confirm that authors involved have revised the manuscript and agreed this submission.

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(Word Style "Section_Content"). Generally the last paragraph of the paper is the place to acknowledge people (dedications), places, and financing (you may state grant numbers and sponsors here). Follow the journal’s guidelines on what to include in the Acknowledgement section.

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43. Hufton SE, Risley P, Ball CR, Major D, Engelhardt OG, Poole S: The breadth of cross sub-type neutralisation activity of a single domain antibody to influenza hemagglutinin can be increased by antibody valency. PloS one 2014, 9(8):e103294.


Figures
Figure 1

HsNb-U library construction pipeline.
A. Primers:

![Diagram of framework selection and diversity design of the hsNb-U library.](image)

B. FR1: EVQLVESGGLVQPGGLRLSCAASG
FR2: MSWVRQAPGKGE
FR3: YADSVKGRFTISRDSNKLTLQLRPMQSLRAEDTAVYYC
FR4: YWGQGTLVTVSS

![Sequence details](image)

Figure 2

**Overview of framework selection and diversity design of the hsNb-U library.** (A) The full-length Nb sequences were obtained by overlapping extension PCR. (B) Sequence of the full-length Nbs.
Figure 3

Initial screening of anti-RBD Nbs. (A-B) Expression and purification of the recombinant RBD through baculovirus expression system and purified by Ni–NTA followed by gel filtration. C. The sequences of the CDR1 and CDR2 of the five high-affinity RBD-binding Nbs. D. The results of phage ELISA of candidate phages. The error bars represent S.D. from three independent experiments. The statistical difference was measured by paired two-sided Student’s t test.
Figure 4

The results of phage ELISA of candidate phages. The error bars represent S.D. from three independent experiments. The statistical difference was measured by paired two-sided Student's t test.

Figure 5

A. Expression

Low solubility

No expression

Expression

High solubility

High expression

Med-affinity

High expression

B. O.D.450

D. C.

E. E.

<table>
<thead>
<tr>
<th>No.</th>
<th>CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
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<td>NTFPRT</td>
<td>LVASIGGERSTI</td>
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RBD binding affinity of the Nb candidates. (A) Pie chart of RBD Nbs’ affinity and solubility. High expression indicated more than 5mg Nbs protein could be purified from 1L of *E. coli* broth. (B) RBD binding affinity analysis of the Nb candidates by ELISA. (C) EC$_{50}$ of the Nb candidates binding to RBD. The EC$_{50}$ is the concentration of Nbs that gives half-maximal RBD-binding respond. (D) Five highest affinity RBD Nb candidates. (E) Amino acid sequence of the five highest affinity RBD Nb candidates.

![Figure 6](image)

**Figure 6**

**Candidate Nb39 compete with ACE2 for RBD binding.** (A) Purified ACE2 recombinant protein with >90% protein purity. Protein purity was quantified by Gel-Pro analyzer. (B) The competition of candidate Nbs with ACE2 for RBD binding by MST. (C) Predicted model of Nb39-RBD complex by SnugDock. The CDR1, CDR2, and CDR3 of Nb39 are shown in blue, green, and magenta, respectively, whereas the rest of Nb39 are in gray, and RBD in light orange. (D) The hydrogen bond between His32 (H32) of CDR1 and Glu166 (E166) of RBD. (E) The binding interface between CDR3 and RBD where key residues were shown in sticks.
Figure 7
Strategy and time-line of the two-step Nb phage screening.

Supplementary Files
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