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Analytical Biochemistry 640 (2022) 114546

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Outlook of therapeutic and diagnostic competency of nanobodies against SARS-CoV-2: A systematic review

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ARTICLE INFO

Keywords:
Nanobody
SARS-CoV-2
Therapeutic application
Diagnosis application
In-vivo
In-vitro

ABSTRACT

Purpose: The newly emerged coronavirus (SARS-CoV-2) continues to infect humans, and no completely efficient treatment has yet been found. Antibody therapy is one way to control infection caused by COVID-19, but the use of classical antibodies has many disadvantages. Heavy chain antibodies (HCAbs) are single-domain antibodies derived from the Camelidae family. The variable part of these antibodies (Nanobodies or VHH) has interesting properties such as small size, identify criptic epitopes, stability in harsh conditions, good tissue permeability and cost-effective production causing nanobodies have become a good candidate in the treatment and diagnosis of viral infections.
Methods: Totally 157 records (up to November 10, 2021), were recognized to be reviewed in this study. 62 studies were removed after first step screening due to their deviation from inclusion criteria. The remaining 95 studies were reviewed in details. After removing articles that were not in the study area, 45 remaining studies met the inclusion criteria and were qualified to be included in the systematic review.
Results: In this systematic review, the application of nanobodies in the treatment and detection of COVID-19 infection was reviewed. The results of this study showed that extensive and sufficient studies have been performed in the field of production of nanobodies against SARS-CoV-2 virus and the obtained nanobodies have a great potential for use in patients infected with SARS-CoV-2 virus.
Conclusion: According to the obtained results, it was found that nanobodies can be used effectively in the treatment and diagnosis of SARS-CoV-2 virus.

1. Introduction

The global pandemic of new coronavirus disease 2019 (COVID-19), due to infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a worldwide health issue and economic burden. As of October 2021, SARS-CoV-2 had led to 245,373,039 confirmed cases and 4,979,421 deaths globally (https://covid19.who.int/). The SARS-CoV-2 is made up of four structural proteins, including spike (S) protein, nucleocapsid (N), membrane (M) protein, and envelope (E) protein [1]. S protein is involved in virus entrance into host cells. S protein has a receptor-binding domain (RBD) that targets angiotensin-converting enzyme 2 (ACE2) as its specific receptor [2]. To overcome quick transmission of COVID-19, significant work is urgently required to develop more potent and cost-effective SARS-CoV-2 therapies and vaccines. Antibodies are safe to use as diagnostic and therapeutic agents against viruses because of their capacity to neutralize viruses. As a result, the discovery of neutralizing antibodies that inhibit the interaction between ACE2 and RBD is critical for preventing SARS-CoV-2 infection. Although therapeutic antibodies attach to their targets with great affinity and specificity, they are limited in their capacity to reach tissue due to their large size and low stability [3].

Nanobodies (Nbs) are single-domain antibodies (sdAbs) isolated

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https://doi.org/10.1016/j.ab.2022.114546
Received 4 December 2021; Received in revised form 29 December 2021; Accepted 2 January 2022
Available online 4 January 2022
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from camelids and sharks with high sequence similarity to the human VH gene. Nbs are valuable tools for numerous purposes in biomedical fields due to their small size, high permeability, thermostability, high binding specificity, cost-effective production, and minimal immunogenicity [4]. Because of these properties, nanobodies are considered for the diagnosis and treatment of various diseases, and many studies have reported promising results [5–10]. Nanobodies are also a viable option for controlling infections; many of them have been developed to suppress viruses such as the human immunodeficiency viruses (HIV), Hepatitis B virus, influenza virus, and respiratory syncytial virus [11]. Given that definitive treatment for COVID-19 is not yet available, so, using Nbs may be one of the most efficient treatments for treating and preventing COVID-19. They are able to recognize RBD or other SARS-CoV-2 receptors and prevent the virus from infecting the cell. The purpose of this study is to review researches that have applied nanobodies to diagnose or neutralize SARS-CoV-2(Figs. 1 and 2).

2. Methods

2.1. Study design

This study platform was designed to conduct a comprehensive review on specific nanobodies isolated against SARS-CoV-2 virus. Here the most related findings about title have been discussed. It is noteworthy that we aimed to categorize and shape released data to reach ease in review. To achieve this goal, two authors were assigned to search independently through the data sources.

2.2. Criteria eligibility

All studies discussing the feature of Anti-SARS-CoV-2 nanobody were qualified to be reviewed. Papers in languages other than English were put aside. In the present systematic review, we focally discussed about SARS-CoV-2 specific nanobodies and studies with other concepts were not of interest generally.

2.3. Sources of data

Data were mined by searching through electronic database of PubMed, LitCOVID, and Scopus. No time period limitation was considered for study selection.

2.4. Search

In order to find any paper related to the subject of interest, two researchers independently searched enjoying the following terms. SARS-CoV-2, COVID-19, Coronaviruses, Nanobody, Camelid antibody, Single chain antibody, Heavy chain anti body and VHH." to address all papers focusing on the topic Afterward, we used the following search constructs with Boolean operator of "OR" and "AND" to find the related data for different section of the present study: "(SARS-CoV-2 OR COVID-19 Or Coronaviruses) AND (Nanobody OR Single chain antibody or Camelid antibody OR Heavy chain anti body OR VHH)"

2.5. Study selection

Totally 157 records (up to November 10, 2021), were recognized to be reviewed in this study. 62 studies were removed after first step screening (titles and abstracts) due to their deviation from inclusion criteria. The remaining 95 studies were reviewed in details. 50 studies, addressing other aspects of title, were excluded. Remaining studies met the inclusion criteria and were qualified to be included in the systematic review. The search flow has been illustrated in Fig. 3.

3. Results

3.1. Nanobodies against SARS-CoV-2

Ahmad et al. [12]. was reported three synthetic nanobodies (sybodies) known as Sb16, Sb45, and Sb68 bind to the receptor-binding domain (RBD) of SARS-CoV-2 spike (S) protein. When these structures are superimposed on trimeric spike (S) protein models, it appears that

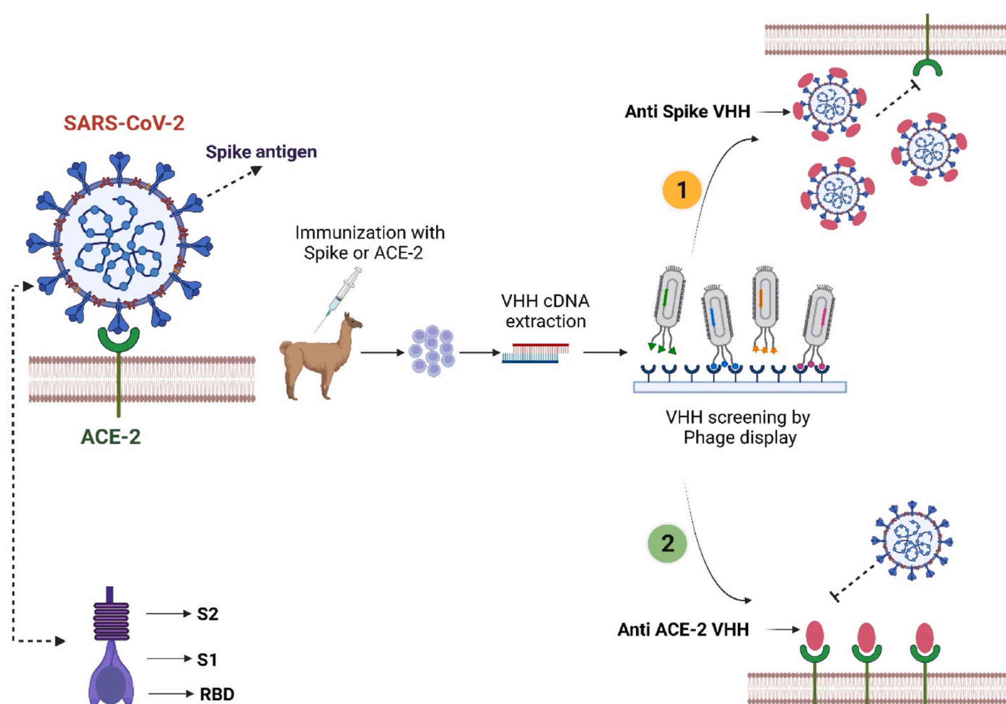

Fig. 1. Application of nanobodies in the treatment of SARS-CoV-2 virus. (1). Neutralization by binding to virus spikes. (2). Neutralization by binding to human cell receptors.

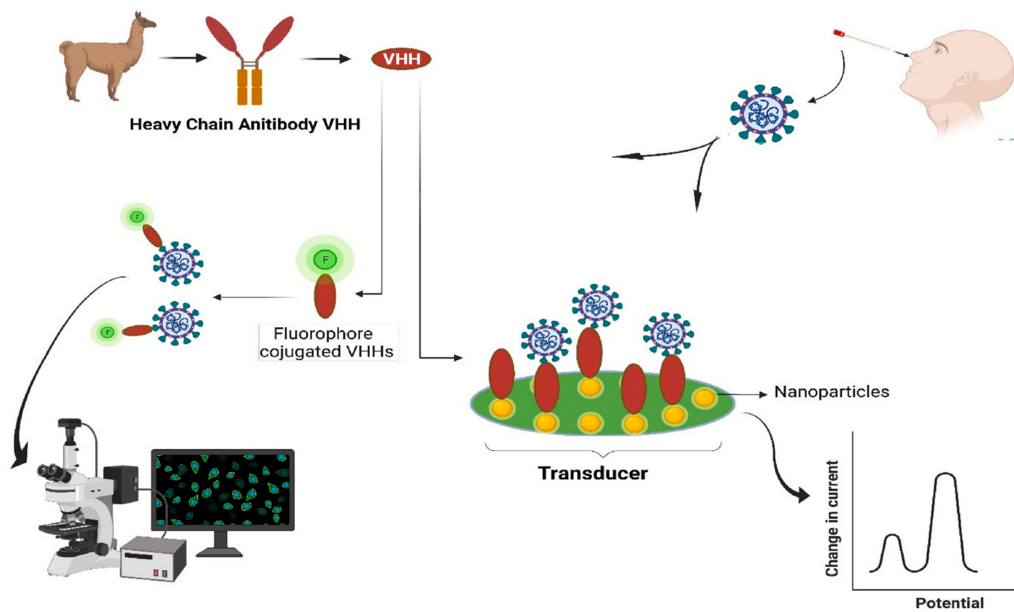

Fig. 2. Application of nanobodies in the diagnosis of infection caused by SARS-CoV-2 virus.

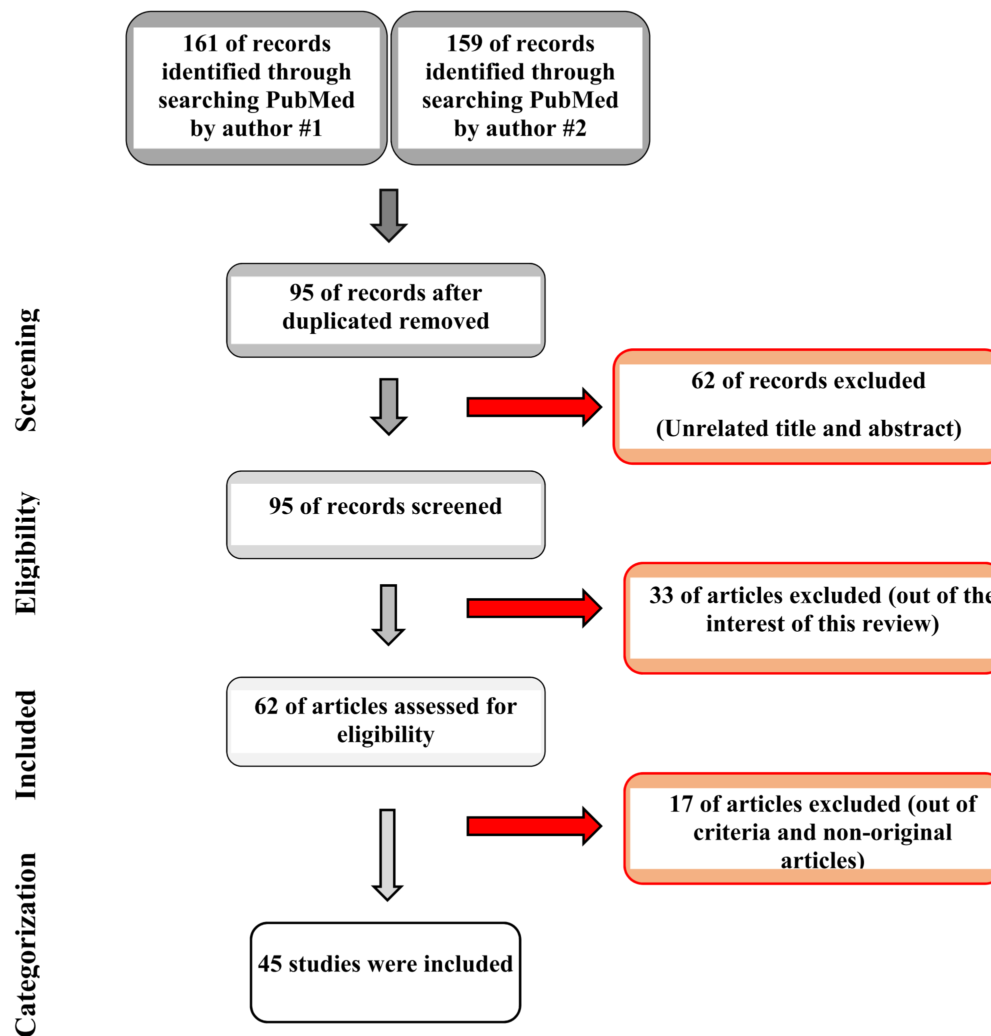

Fig. 3. Systematic review flow chart of the literature search according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines.

various sybodies bind S protein conformations in distinct ways. The highest RBD affinity among sybodies was Sb45 with the K_D of 0.038 μM Sb16 and Sb45 block the ACE2 binding site competitively and directly, while Sb68 does so non-competitively. Also, Sb16 and Sb45 were superposed to the RBDs, while any RBD could not be superposed to Sb68. Sb45 and Sb68 interact at two distinct faces of the RBD. The binding of Sb16 and Sb45 to RBD through CDR2 and CDR3 is diametrical. Sb16 CDR2 and Sb45 CDR3 recognize the same epitopic sites. Both Sb16 and Sb45 have the ability to bind the RBD in the up and down position. Also, when binding the RBD, Sb16 exhibits a large movement of CDR2. Sb68's capacity to interfere with ACE2's interaction with RBD is most likely due to a steric clash of the N322- and N546-linked glycans. Virus neutralization test has not been performed, but steric clashes with glycans have been hypothesized to be a mechanism that neutralizes the virus [12].

Dong et al. [13]. described llama multi-specific nanobodies fused to human IgG1 Fc domains and exhibited efficacy against SARS-CoV-2. They employed biolayer interferometry to perform epitope binning. Biolayer interferometry is an optical analytical technology for analyzing the molecular interactions, wherein the interaction of an interest molecule with the bait can be observed in real-time, which correlates with changes in the thickness of the biolayer. Then they computed llama nanobodies models, docked to S1 RBD/ACE2, and predicted possible epitopes for each VHH model. Using five deletion mutants for each anticipated epitope, they confirmed the interaction of predicted epitopes in VHH/RBD binding. These structures show that S1 RBD has two central binding regions: N-terminal (del1: 340–358) and ACE2 binding surface (del3: 448–472, del4: 483–494, del5: 498–505). Tri-specific antibodies' VHH binders covered both of these regions. Tri-specific antibodies are promising treatment due to bind several epitopes inside the RBD simultaneously, boosting affinity and avidity of antigen binding, increased S1/ACE2 blocking and virus neutralization effect, reduced antibody-dependent enhancement (ADE), increased ability to target a range of viruses, and reduced loss of antibody binding owing to virus mutations. Because of these properties, tri-specific VHH could be used therapeutically at low doses and reducing human toxicity. The tri-specific VHH-Fcs 3F–1B-2A have shown binding affinities to RBD with K_D of 0.047 nM. The Fc domain provides Fc-dependent functions like antibody-dependent cell cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). These Fc-dependent activities would give the VHH-Fcs different mechanisms of action, increasing their effectiveness in neutralizing coronaviruses. Also, they revealed that at virus blocking, multi-specific antibodies are more effective than a mixture of monoclonal antibodies. These features of the multi-specific VHH revealed that they are more resistant to aggregation, can be purified in large quantities, facilitating their manufacture, and have good thermostability [13].

Margulies et al. [14], by using X-ray crystal structures and binding studies of sybodies that were designated previously [15] reported that these sybodies (S14, S16, S45, S68) interfaced ACE2-RBD interaction in specific ways and had a potent neutralization. They applied *E. coli* to express the sybodies and did the purification by metal-affinity chromatography. Their binding affinity (KD) was investigated by surface plasmon resonance (SRP). Each of these sybodies were shown to have a single disulfide-linked loop (75 or 76 amino acids) that stabilized the two β-sheets, which is characteristic of an IgV fold. To investigate how these sybodies impair RBD and ACE2 interaction in structural base, they enclosed sybody–RBD structures individually with the ACE2–RBD structure and then evaluated the steric clashes. They showed that each sybody employed its distinct CDR residues of each sybody interacted with RBD epitopic residues and arrested mainly a large epitopic area. For Sb16 and Sb45, CDR2 and CDR3 bestride RBD but Sb14 used more non-CDR residues to interact with RBD. Sb68 also used only one CDR1 but four CDR2 and nine CDR3 in binding surface. Moreover, the bivalent or multivalent binding of these sybodies exhibited significant potential in neutralization. Since for the major circulating variants, Alpha, Beta, Gamma and Delta, mutations in RBD enhance binding affinity to ACE2, they evaluated the efficacy of these sybodies on individual mutations K417 N, E484K, and N501Y in the RBD engineered by Site directed mutagenesis. They reported that the sybodies interacted with the wild type RBD showed different patterns of binding to the mentioned mutants. Although among these sybodies, few were able to bind to the RBD mutants, they failed to interact detectably and showed decreased recognition of K417 N and N501Y and E484K as compared to WT. As a result, these sybodies might be potent to neutralize the wild SARS-CoV-2 in contrary to its various variants such as Alpha, Beta, Gamma and Delta [14].

In another study, Xu and colleagues [16] isolate an anti-RBD nanobodies from llamas. In binding assays analysis, they identified two nanobodies with highly neutralizing potency. The VHHs could solve the RBD antigenic drift through recognizing a different section outside the ACE2-binding site [16].

3.2. Nanobodies inhibited SARS-CoV-2 in-vitro

Custodio et al. [17]. showed that a synthetic nanobody library (sybodies, Sb) was used to select specific Sb against SARS-CoV-2 with highly neutralizing activity. The Sb platform eliminates the need for immunization phases and for the whole selection phase only uses a portion of the purified antigen, so it can be done in less time. Sybodies have the potential to rapidly isolate and characterize nanobodies from a synthetic library to developing highly specific nanobody-based therapeutics with neutralization potential. From a single selection round, they find 85 different binders. Six of them had high affinity for RBD. RBD affinities vary among six sybodies: 24.2, 10.6, 5.0, 58.1, 43.9, and 38.7 nM, for Sb12, Sb23, Sb42, Sb76, Sb95, and Sb100 respectively (the most effective neutralizer was Sb23 with an IC50 of 0.6 μg/mL). Furthermore, Sb23 binds to the RBD with a higher affinity than the ACE2, and Sb23 has been shown to bind to the ACE2-binding site, preventing ACE2 from binding by steric hindrance. Sb23 bound two spike conformations, one "1-up" and the other "2-up." The "2-up" shape allows epitopes to be accessed. FAB-C105 has been used as a conventional antibody to evaluate and compare its Sb23. FAB-C105 and Sb23 bind to the RBD in two distinct but partially overlapping regions. It seems Sb23 could be used in combination with other SARS-CoV-2 protein binders to neutralize the virus. By combining Sb23 with a non-overlapping Sb12, they increase avidity against RBD [17].

Esparza et al. [18]. isolated various nanobodies that bind to spike protein RBD of the SARS-CoV-2 and prevent ACE2 interaction. B-cell cDNA sequences as nanobodies were extracted using standard llama immunizing procedures, and a phage display library with approximately 108 clones was generated. Thirteen distinct lead candidate nanobodies were known as NIH-CoVnb-101 to NIH-CoVnb-113 that bind to the RBD was identified from this phage library, several of which effectively inhibit the RBD-ACE2 interaction. To isolate suitable nanobodies that bound to the RBD and impede their interaction with ACE2, a novel screening technique was created and implemented. The highest affinity nanobody was NIH-CoVnb-112 with K_D of 4.9 nM. The NIH-CoVnb-112 has a significantly higher affinity for SARS-CoV-2 than previously reported nanobodies. In an infection pseudotyped model of SARS-CoV-2, they discover that NIHCoVnb-112 also effectively blocks viral transduction. They suggest inhalation therapy could be used to deliver these nanobodies specifically. They are particularly enthused about the prospect of developing inexpensive, safe, and stable nanobody-based treatments for inhalation use. The fact that NIH-CoVnb-112 has the ability to binds to and inhibits three recently discovered RBD mutations is promising [18].

Guttler et al. [19]. try to develop VHH antibodies that effectively neutralize SARS-CoV-2. They focused on the S1 segment of the Spike protein for this. Among the newly identified anti-RBD VHHs, 43 out of 60 nanobodies prevented infections. They use VHH72, a single-domain Camelid antibodies developed against the RBD by Wrapp et al. [20]. The virus was nearly entirely inhibited by pre-incubation with 500 nM

VHH-72 (7 mg/L), but lower concentrations had no impact. Re5D06 was another nanobody that all epitope-binders blocked the ACE2-RBD interaction (e.g., Re5D06, Re6H06, or Re9B09). Re9F06 prevents docking to ACE2. The structure reveals a remarkable affinity between this nanobody and the RBD. Four tyrosines (Y449, Y453, Y489, Y505) and three phenylalanines (F456, F486, F490) are particularly notable on the RBD side, implying that the nanobody covers a "sticky" portion of the RBD. The long CDR3 loop considerably enhances the shape complementarity with the RBD surface. CDR3 has three highly exposed tyrosines that are required for RBD binding. This indicates that the loop conformation has been strongly stabilized. Re6B06 and Re6H06 are both hyperthermostable, and Re6H06 is one of the most potent RBD binders (KD $\leq$ 1 pM) and neutralizers (at 50 pM). They designed an approach that increased avidity and discovered that homo-trimerization is a better way to gain avidity. The NC1 trimerization domains of human collagens XV and XVIII were identified as possibilities. They evaluated different designs and discovered that VHH-spacer-collagen XVIII NC1 fusions had the best neutralizing potency. The trimerization also reduced the nanobody Re9F06's minimal neutralizing concentration from 50 nM to 167 pM. The best VHHs for the trimerized format and thermostability were Re6B06-spacer-ColXVIII. The Alpha/UK B.1.1.7 variant with an N501Y mutation did not influence the binding of Re5D06 or any other nanobody that was examined. The Re5D06-RBD interaction was reduced by beta/South African or gamma/Brazilian mutations. They're looking for nanobodies with a high affinity for all SARS-CoV-2 variations and showed that Re9F06 isn't affected by the escape mutations. They fused two structures (Re9F06-R28) and demonstrated this tandem bound mutated RBD variant remarkably well. Re9F06-Re6H06 and the collagen XVIII trimer of the Re9F06 neutralization effect were more efficient (17 pM). It's showed that fusing nanobodies to an Fc-fragment will improve their antiviral activity and the trimerization domain of collagen XVIII looks to be a suitable alternative fusion partner. Re9F06, collagen XVIII-trimerized, tolerates RBD escape mutations better than the others and appears to be an ideal therapeutic candidate. Because of their exceptional potency, great mutation tolerance, and hyperthermostability, Re6H06 are excellent candidates [19].

Koenig et al. [21]. revealed four neutralizing nanobodies (VHHs E, U, V, and W) that target the RBD of the SARS-CoV-2 spike. The RBD was inoculated to camelids, nanobody libraries were identified by phage display, and it was shown that they are inhibitory. Based on epitope mapping information from X-ray crystallography and SPR as well as detailed data on the conformation of spike-nanobody interactions established by cryo-electron microscopy (cryo-EM) for the determination of biomolecular structures, they have rationally developed multivalent nanobody constructions. They created two types of nanobody fusions: multivalent nanobodies that target the ACE2 binding site on the RBD and biparatopic fusions of two nanobodies that target the non-overlapping binding interfaces that amplify neutralization by activating the spike protein. The nanobodies on the RBD bind to two different epitopes, interfaces "E" and "UVW," according to X-ray crystallography. The discovery of VHH E, U, and W with two distinct binding mechanisms that early spike activation (maintain the active conformation of the spike) could be a neutralizing mechanism and shows that this form of action is more prevalent than previously assumed. Bi- and trivalent nanobodies with better neutralizing capabilities were created. The replication-deficient vesicular stomatitis virus (VSV) method was used to measure the neutralizing activity. This method was showed that the trivalent VHH EEE prevented infection the most effectively with IC50 of 520 pM) and induced spike fusogenic activity the most significantly. They hypothesized that the nanobody VE attachment to the spike would alter the spike conformation in such a way as to induce an incomplete fusion, thereby deactivating the spike. This activity is called "fusogenic". Because of their improved neutralizing activity, the multivalent structure-based nanobodies reported here have significant therapeutic potential [21].

SARS-CoV-2 neutralizing humanized nanobody constructions with sub-nanomolar affinities were developed and designed quickly and efficiently, according to fu et al. [22] using cryoEM structural study and molecular dynamics simulations. RBD-1-2G (NCATS-BL8125), the most effective nanobody, tolerates the N501Y RBD mutation while still blocking the Alpha (B.1.1.7) variant. RBD-1-2G-Fc reduced viral burden following WA1 and B.1.1.7 variants in an airway ex vivo model [22]. S protein and receptor-binding domain (RBD) induce neutralizing antibodies. These antibodies are predominant candidates for development of targeted therapeutics. Li et al. [23], used the typical phage display to create a phage display nanobody library from alpacas immunized with recombinant SARS-CoV RBD protein. They applied the fusion of recombinant SARS-CoV RBD protein with rabbit IgG-Fc tag to immunize the alpacas in which nanobodies naturally produced. Selected positive clone named S14 had significant binding affinity to SARS-CoV RBD (KD = 143 pmol/L) and the inhibition of pseudovirus entry with an IC50 of 4.93 ng/mL. The neutralization mechanism showed that in a dose-dependent manner, it blocked the binding between soluble ACE2 and SARS-CoV S1, which represented that S14 directly weaken the interaction between SARS-CoV RBD and ACE2. Since the major circulating variants contain mutations in RBD, which strengthen the binding affinity, they produced different mutations of RBD in some main residues (Y442A, L472A, N479A, D480A, and T487A) to investigate if these mutations were involved in S14-mediated neutralization. Nevertheless, these generated mutations were not related to Delta variant, the current dominant and more infectious mutants, the results demonstrated that S14 did the neutralization well with all mentioned mutants. Since these selected residues in RBD are important in host tropism and cross species infections and this nanobody showed the potent neutralizing impact, it might be a promising candidate to investigate its neutralizing effect on more contagious SARS-CoV variant named Delta [23].

Currently, most therapeutic antibodies impair the RBD-ACE2 interactions [24–27]. Interestingly, Lu et al. [28], identified five C-type lectins, DC-SIGN, L121SIGN, LSECtin, CLEC10A as well as TTYH2, plasma membrane proteins, that influence SARS-CoV-2 interactions beyond ACE2. In fact, they bound to non-RBD epitopes of SARS-CoV-2 S protein and were able to moderate the interactions and the attachment with myeloid cells though TTYH2 interacted weakly with RBD-Fc. Both outside and inside of the RBD in S protein is densely glycosylated. As glycosylation provides a shelter for viruses to diminish immunogenicity and expedite immune escape as well as accelerate viral entry [29–31]. Lu et al. [28], added mannan and indicated that it could competitively interface the S protein binding with DC-SIGN and L-SIGN but not to ACE2, LSECtin, ASGR1, CLEC10A, or TTYH2. They also showed that N-glycosidase F (PNGase F) predominately effected on S-Fc protein binding reduction to the myeloid cell receptors. To investigate which specified glycosylated residues played a main role in receptor binding, they employed a mutagenesis screen within the S1 subunit. They identified two types of functional mutation sites as the inhibitory groups. The first one was a single mutation causing major loss of S1-Fc binding to the host receptors in addition to enhancing group (N343 to 203 ACE2, N603 to almost all C-type lectins, and the majority of the mutants to both ASGR1 and CLEC10A). The second one leaded to enhance of S1- Fc interactions (N74, N149, N282, N603, N616, and N657 to ACE2, N165 to DC-SIGN/L-SIGN/LSECtin, 206 N234, N343 and N657 to DC-SIGN, and N122 to LSECtin). Their findings represented that C-type lectins and TTYH2 might play an ACE2 independent role in SARS-CoV-2 pathogenesis and pathological inflammation so applying antibodies that could block virus-myeloid receptor interactions could be promising for future treatment. In this research, two selected nanobodies, A8 and G11 fused with Fc, impaired the interaction between S1 and ACE2 as well as the six myeloid receptors. Not only did these two nanobodies recognize various S epitopes but also the both A8-Fc and G11-Fc showed large and significant blocking activity. Furthermore, the binary engineered nanobody, A8-G11-Fc, showed a potent neutralization (IC50 = 0.09 μg/mL) and high binding affinity (KD) of 180 pM in compare to A8-Fc or G11-Fc alone. Their results pinpointed the predominant role of

SARS-CoV-2-myeloid receptor interactions in the virus immune pathogenesis and implied that this nanobody can widely block the interaction between SARS-CoV-2 spike and ACE2 or myeloid cell receptor. Thereby, blockade of SARS-CoV-2 protein with this selected nanobody could lead to reduction of the myeloid cell hyper activation and could be a successful treatment for COVID-19 [28].

Lu et al. [32], used VHH (Single variable domain on a heavy chain) library of Bactrian camel to select specific nanobodies against SARS-CoV-2-S or RBD protein. They also investigated the effect of neutralization potency of the monovalent, bivalent and trivalent of the selected nanobodies. To achieve this goal, firstly, the Baculovirus Expression System employed to produce the recombinant spike protein and its RBD domain of SARS-CoV-2 to screen nanobodies. Secondly, they used camel's peripheral blood lymphocytes (PBLs) to construct the naïve VHH library. Then by doing nested-PCR, the VHH genes amplified and cloned into the vector. To select the nanobodies against spike and RBD, phage rescue performed for four rounds. In addition, the selected ones, cloned into the expression vector and finally, HEK293FT (Human embryonic kidney) mammalian system used to express and purification of the selected nanobody-hFc protein that bound to S and RBD protein. They identified two successful nanobodies Nb91-hFc and Nb3-hFc based on neutralization assays. Afterward, they highlighted that multivalent nanobodies improved the neutralization activity against SARS-CoV-2. Although the results showed that the homodimer (biNb91-hFc, biNb3-hFc), heterodimer (Nb91–Nb3-hFc) and homotrimer (triNb91-hFc, triNb3-hFc) bound with RBD protein with higher affinity in compare to monovalent, the heterodimer nanobody Nb91–Nb3-hFc demonstrated enhanced neutralizing ability IC50 at 1.54 nM. On the one hand, this Nb91-hFc could bind with both S protein and RBD, and Nb3–hFc recognized RBD protein, so this heterodimer could be a promising candidate for COVID-19, which could cover a wide region of spike protein. On the other hand, since the half-life of these nanobodies is short, these established nanobodies could be considered to prevent COVID-19 as aerosolized inhalation products [32].

Ma el al [33], developed and characterized nanobodies from immunized alpacas that had RBD-ACE2 blocking abilities. Primarily, they immunized the alpacas with recombinant SARS-CoV-2 RBD and then by generating a VHH library from peripheral blood mononuclear (PBM) and applying phage display, six nanobodies (aRBD-2, aRBD-3, aRBD-5, aRBD-7, aRBD-41, aRBD-54) against RBD were characterized. These nanobodies had a significant potent KD from 2.6 nM to 21.9 nM. Additionally, they applied expression vector in HEK 293F to express and purify the nanaobodies. So the C terminal of selected nanobodies were fused to a tobacco etch virus (TEV) protease cleavage site as well as a human IgG1 Fc in a mammalian vector. In this method, digestion of fusion proteins by TEV protease could accelerate the preparation of nanobody's monomers without Fc. In this research, all Nb-Fc fusions mostly showed high binding affinity, KD from 72.7 pM (aRBD-7-Fc) to 2.82 nM (aRBD-41-Fc), to not only RBD but also the whole S1 and S2 of SARS-CoV-2 spike protein. They also reported that the engineered aRBD-2-5 and aRBD-2-7 the hetero-bivalent Nbs greatly increased SARS-CoV-2 neutralizing and binding efficacy (ND50 of 0.043 nM, KD = 59.2 pM and ND50 = 0.111 nM, KD = 0.25) respectively. Lu et al., 2021, achieved the same results; they also reported that the hetero-bivalent nanobody had a better effect on blocking RBD and ACE-2 in compare to their homo nanobodies [32]. In contrast to their research, the current study, Ma et al. [33], reported that the Fc fusions of aRBD-2-5-Fc and aRBD-2-7-Fc did not enhance the neutralization potency (ND50 = 0.107 nM and 0.0606 nM respectively). Eventually, they evaluated the efficacy of these nanobodies against the N501Y point mutation, a prominent mutation in Alpha, Beta and Gamma variants of SARS-CoV-2, which binds to ACE2-Fc potentially in contrary to the original RBD. The data represented that the N501Y variant did not affect the selected Nbs binding activity. They also tested the effect of point mutations of RBD on binding site with the Nbs to check where Nbs bound on RBD. The specific mutations (Y449A, Q493A, Q498A, V503E, and Y505E) of RBD that interact with ACE2 were selected. They reported among the mentioned mutations only Y449A mutation significantly lessened the binding of one nanobody and the other four mutations did not affect the binding with the selected Nbs. Although any of these selected mutations were not include in current SARS-CoV-2 variants (Alpha, Beta, gamma, Delta), they might be a potent candidate for neutralization of original RBD in SARS-CoV-2 [33].

Mast et al. [34], developed their existing data [35], to generate many promising nanobody candidates. In the first step, they selected llamas, which normally had potent immune responses and then immunized the two selected llamas with SARS-CoV-2 S1 and S2 subunits expressed and purified in HEK293. They continued the immunization protocol to reach high HCAb. Furthermore, all the RNA was extracted from lymphocyte in bone marrow aspirates and subsequently the cDNA was synthesized; based on VHH sequences expressed in each llama the in silico library was produced. They lysed the VHH components and analyzed the peptides by LC-MS/MS. By using the improved Llama-Magic software package [35] they used the obtained data to investigate the VHH sequence library to characterize the selected nanobody sequences. Finally, by updating their Liama-Magic software, they could identify 347 unique sequences. They clustered CDR sequences to enhance sequence diversity and eventually 177 promising candidates with periplasmic secretion in bacteria were expressed. The screened nanobodies those with more than 20% binding affinity were selected. Among 113 filtered nanobodies, 71 nanobodies bound to RBD S1 binding. They also showed that six of these nanobodies (S1-1, S1-6, S1-RBD-9, S1-RBD-11, S1-RBD-15 and S1-RBD-35) were able to block Alpha (B.1.1.7/20I/501Y.V1) and Beta (B.1.351/20H/501Y.V2 variants) though the binding affinity of S1-RBD-11 nanobody for variant B.1.351 reduced (20 pM–161 pM). Besides that, they examined the stability of the nanobodies. Therefore, they applied DSF (differential scanning fluorimetry) to investigate their thermal stability, which was from 50 °C to 80 °C. In addition, they reported that lyophilization did not effect on neither their stability nor affinity. They pinpointed that the stability and binding affinity of these nanobodies, the same as those were reported [36,37] did not alter in different temperature or storage conditions. They also did neutralization assay and taken together, IC50 of 40% of the monomeric nanobody was less than 100 nM. They indicated that oligomerization could increase the neutralization activity as well as binding affinity of nanobodies. Take S1-RBD-35 and S1-23 as examples, S1-RBD-35, the monomeric, showed IC50 = 12 nM while the dimers and trimers forms enhanced the neutralization activity to 160pM and75pM respectively. S1-23 also showed 170pM and ~90pM for dimers and trimer. Since ACE2 is the same receptor for SARS-CoV-1, they also investigated the neutralization potency of the nanobodies for SARS-CoV-1. Among the selected ones, S1-1, S-39, S-1-RBD-5–S-1-RBD-6 showed similar IC50 for both SARS-CoV-2 and SARS-CoV-1. They used two nanobodies, S1-1 and S1-23 in a human ex vivo model system to investigate their potency to prevent SARS-CoV-2. Briefly they treated differentiated primary airway epithelial cells (AEC) with the mentioned nanobodies and then SARS-Cov-2 virus added to the cells and finally by doing q-PCR the virus replication measured. Both S1-1and S1-23 had a high neutralization but the trimer form of S1-23 demonstrated a robust efficacy for preventing the virus replication. In this research, they showed the synergistic activity with nanobody combinations. They selected the candidate based on structural data and biophysical performance and epitope mapping. The synergistic effect of S1-RBD- 15 with S1-23 enhanced by 300-fold. Eventually, based on Weisbun et al. method [38] they developed nanobody cocktails that were able to escape the probable variants. 35 mutants were resistant to the all-20 selected nanobodies. However, when they applied the cocktail of two nanobodies they could rescue of the variants. Taken together, some of the selected nanobodies showed the promising results against Alpha and Beta variants and the multimers form or the cocktails of nanobody showed the potential efficacy to SARS-CoV-2 [34].

Sun, D et al. [39] conducted a study by using High-resolution

cryoelectron microscopy, the structure of eight nanobodies attached to the epitope was examined. These Nbs strongly neutralize the SARS-CoV-2 and are classified into three groups. group I Nbs (Nbs 20, 21) targets ACE2-binding sites, group II (Nbs 34, 95, 150) binds highly conserved epitopes includeds hydrophobic residues in the CDR3 region and group III recognizes unique epitopes includes residues 345–356 that are not traceable for classic antibodies. And also, has been evaluated the effect of point mutations in the RBD region on the ineffectiveness of Nbs. In summary, the results of this study indicate that the ability of group 1 Nbs to bind strongly to the RBS region is not altered by mutations in the alpha variant but the RBD binding of group I Nbs may be abolished by a single point mutation (E484K/Q) that is present in Beta, Gamma and Kappa variants. Since group II and III Nbs are attached to conserved and semi-conserved parts, the known point mutations so far do not affect their binding affinity. Structure-function analysis of Nbs indicates a variety of antiviral mechanisms. group I and II Nbs strongly neutralize SARS-CoV-2 by binding to the ACE2 binding site and group III nanobodies use the cell protease activity to destroy the function of the spike protein and do not act like the previous two groups by competing with ACE2. On the other hand, group I RBD Nbs for example, Nb21 can neutralize of the SARS-CoV-2 at 9.7 ng/mL, and group II, III Nbs can strongly neutralize SARS-CoV-2 below 150 ng/mL and 100 ng/mL, respectively [39].

Nieto, G et al. [40] described an alpaca monomer Nanobody, W25, which binds specifically to the receptor-binding-domain (RBD) of the SARS-CoV-2 Spike protein. Furthermore, is reported a rapid and a new easy method for Nanobodies separation from *E. coli* bacterial display libraries in a one-step and by using a simple density gradient centrifuge. Briefly, *E. coli* bacteria express intimin-Nanobody protein fusions that anchor in the outer membrane. Therefore, the bacteria expressing specific Nanobodies on their surface would bind NHS-beads coated with Spike protein of SARS-CoV-2 S and precipitate due to the density gradient, other bacteria remain in the supernatant. W25 binds to the SARS-CoV-2 S RBD with 0.3 nM affinities and efficiently competes with ACE-2 receptor binding and is potently neutralizes SARS-CoV-2 wild type and the D614G variant, a mutation to the kappa and delta strains, with IC50 values in the 9 and 5 nM, respectively. Also, fused W25 to an Fc fragment preserving most neutralization properties and potentially increasing the retention in the circulation for several days.

The method presented in this study is fast, simple, and inexpensive and seems to be useful for emergencies and epidemics conditions [40].

Moliner-Morro A et al. [41] reported an alpaca nanobody, Ty1, that binds to the receptor binding domain (RBD) region of the spike SARS-CoV-2 and strongly neutralizes the virus. In this study, a series of polyvalent structures of Ty1 has been created using a combination of Sortase A factorization and click chemistry. Sortase A has been used to bind the click chemistry of the functional groups to the c-terminal of the nanobodies before creating the nanobodies fusion and tetrameric nanobody structures of 4-arm polyethylene glycol (PEG). The results showed that divalent structures are significantly stronger than monovalent Ty1 and a 4-arm PEG tetramer equipped with four Ty1 molecules is very strong and can neutralize SARS-CoV-2 with IC50 in the low picomolar range (13 pM) Due to the low concentration (pM) and high neutralization efficiency (Up to 100%) of Nanobody reported in this study, this method can be used for producing more efficient Nanobodies against infectious viruses such as SARS-CoV-2 [41].

Wagner et al. [42]. have identified eleven nanobodies isolated from an immunized Alpaca with high-affinity against RBD. Multiple tests including a multiplexed ACE2 competition assay and virus neutralization test were performed to select high-affinity binding candidates blocking interactions of RBD: ACE2 in the various spike-derived antigens of SARS- CoV-2. Meanwhile, NM1226 and NM1230 Nbs with opposite binding sites on the RBD showed more neutralizing effect. NM1230 inhibits ACE2 interaction by binding to the spike protomer in the down and up conformation, while NM1226 binds to the up conformation on the RBD, and it was stronger against mutations into the RBD: ACE2 interface. Therefore, to have the advantages of both nanobodies, a biparatopic Nb NM1267 made of both of them showed IC50 values of binding affinity and neutralization potency at the picomolar range. NM1267 also showed high-affinity binding to both B.1.1.7 andB.1.351 RBD from UK and South Africa strains, respectively. Multiplex ACE2 competition assay of NM1267 illustrated the ACE2: RBD inhibition, ACE2:S1 inhibition, ACE2: spike inhibition and viral neutralization potency with 0.19 nm, 0.13 nm, 0.48 nm, and 0.89 nm IC50 values, respectively. Competitive multiplex binding assay ("NeutrobodyPlex") showed that bipNb NM1267 is a suitable candidate for evaluating efficiency of neutralizing antibodies against RBD in the serum of patients immunized through vaccination, recovered and infected. To investigate whether bipNb NM1267 can be a suitable nanobady for monitoring the presence and appearance of neutralizing antibodies in patients' serum, the NeutrobodyPlex method was applied. For this purpose, RBD, S1, S2, and Spike of SARSCoV-2 were incubated with NM1267 and also another well-specified nanobody as a control which targets the interface between RBD and ACE2 resulted in selection of NM1267 as a potent nanobody to detected neutralizing antibodies against RBD [42].

Yao H et al. [43] developed a study to design and develop single-chain nanobodies derived from llama to prevent ACE2-RBD binding by targeting receptor-binding motif (RBM). In the previous study, 99 sybodies from three extremely various synthetic libraries were produced against the RBD by phage display, which had neutralizing activity. 10 out of the 99 sybodies didn't show any neutralizing activity at 1 μM concentration, such as SR31, although able to bind to RBD. Yao et al. reported a synthetic nanobody (~14 kDa) named SR31 as a fusion partner to ameliorate the strength of antibodies interaction with RBM. The binding of SR31 to RBD in a protected location (an epitope) away from the RBM did not show any neutralizing activity. Since The Bio-layer Interferometry (BLI) technique did not show any competition between MR17 Nb and SR4 Nb in binding to RBD, they can bind simultaneously to RBD. Also, binding each of MR6 and MR17 Nbs to the SR31 led to the neutralization of SARS-CoV-2. Since crystallographic experiment showed that SR31 binding to RBM does not result in allosteric changes at RBM surface, so SR31can be compatible with other antibodies to bind to RBM. Though SARS-CoV-2 is not neutralized by SR31, its fusion with MR17 and MR6 leads to enhance in their affinity and neutralization function. The combination of SR31Nb as a fusion partner with other RBM-binders could improve their avidity and potency of attachment that is led to an increase in its neutralizing activity against SARS-CoV-2. The biparatopic fusion of MR17-SR31Nb revealed a significant improve in binding affinity compared with each of MR17or SR31 Nb alone. MR17-SR31 and MR6-SR31 are more capable of neutralizied SARS-CoV-2 pseudovirus than MR17 Nb and MR6 Nb by 13 and 27 folds respectively. In addition, the binding affinity and neutralizing activity of constructs such as MR6-MR6, MR17-MR17 and SR31-SR31 Nbs were also investigated and the binding affinity increased by 7, over 800 and 115 folds, respectively. Of course, SR31-SR31 Nb hadn't neutralizing activity. While this activity increased 324 and 29-fold for MR17-MR17 and MR6-MR6, respectively. Thus, although SR31 Nb alone does not have a neutralizing activity, but it can be biparatopic partner for other RBM binders because of having different epitope as of RBM [43].

Xiang et al. [44]. identified multiple neutralizing nanobodies with affinity of picomolar to femtomolar against the RBD using llama immunization with expressed recombinant RBD in human 293T cells. Serum obtained after immunization was potent with specific activities in binding and neutralizing of pseudotyped SARS-CoV-2 RBD at the neutralization titer of 310,000. Ensure these activities, the single chain VHH antibodies were separated from the IgG of the serum and the high affinity SCFVs binding to RBD with IC50 = 509pM was confirmed. 109 Nb sequence selected from the unique CDR3s repertoire to be expressed in Ecoli. The ELISA technique has been used to test the binding of 94 of them to RBD and 71 RBD-specific binders confirmed that among them, 49 Nabs have high solubility and affinity with IC50 less than 30 nM. The

antiviral activities of these Nbs determined by SARS-CoV-2-GFP pseudovirus neutralization assay. Neutralizing activity of Nbs 89, 20, and 21 were 0.129 nM, 0.102 nM, and 0.045 nM, respectively, based on the pseudovirus assay and 0.154 nM, 0.048 nM, and 0.021 nM, respectively, using SARS-CoV-2. surface plasmon resonance (SPR) has been applied to measure Binding kinetics of these Nbs. The analysis of Nbs interactions with RBD Showed a different binding state of Nb20 to the RBD compared with other reported the virus neutralizing nanobodies. Using X-ray crystallography and Cryo-Electron Microscopy, a dominant Epitope I identified and confirmed in Nbs20 and Nbs21 which has overlapping with the binding site of hACE2. Each of the two monomer nanobodies includes Nb21 or Nb20 separated with a 31 or 25 residues flexible linker in heterodimeric forms of engineered Nbs. These new multivalent Nb structures including $Nb21_3$ and $Nb20_3$ have up to 30 folds more neutralizing activity compared to the monomer forms with IC50 value of 1.3 pM and 3 pM, respectively. Also obtained up to a 4-fold enhancement of potency for the heterodimeric constructs such as Nb21–Nb34 [44].

In another study, Zupancic, et al. [45] report an interestingly directed evolution technique for producing nanobodies with great affinity against SARS-CoV-2. This Fc fusion nanobody was produced in HEK293 cell line (20–140 mg/L). The affinity maturation was done by error-prone PCR (1.2–1.5 mutations per nanobody on average), and binding affinity increased from 50 nM to 100 pM. One of the concerns about the affinity maturation of nanobodies is their reduced stability. In this study, it was found that the stability of nanobodies does not change during maturity. The antibody Tm before and after maturation was 69.8 $\pm$ 0.7 °C and 69.2 °C $\pm$ 0.8 respectively. Notably, they demonstrated the mature VHHs neutralized both live SARS-CoV-2 and pseudovirus effectively, and had biopharmaceutical-like biophysical properties. They expect that these procedures will advance VHH discovery and improve the generation of effective neutralizing nanobodies against various SARS-CoV-2 type [45].

3.3. Nanobodies inhibited SARS-CoV-2 in-vivo

Nambulli S et al. [46] used the previously introduced homotrimeric Pittsburgh inhalable Nanobody 21 (PiN-21) that efficiently neutralized SARS-CoV-2 In vitro. The therapeutic effect of PiN-21 Nanobody on SARS-CoV-2 has been investigated in the form of intranasal and aerosol delivery in Syrian hamsters. The results of this study showed that PiN-21 Nanobody targets pulmonary structures such as terminal alveoli lined with ACE2-receptor-rich alveolar cells, thus strongly neutralizing the SARS-CoV-2. Intranasal delivery of PiN-21 (at 0.6 mg/kg) reduces SARS-CoV-2 in the early stages of the disease. It also prevents severe hamster weight loss. On the other hand, the delivery of PiN-21 in the aerosol form (0.2 mg/kg) reduced the viral titer in all parts of the respiratory system and prevented severe lung damage and lung diseases such as pneumonia. In general, the properties of nanobodies, such as their small size, stability, and solubility, make it possible to deliver it to the deep parts of the lung by aerosolization. According to the results of this work, aerosolization of PiN-21 increases the concentration of Nanobodies in the deep parts of the respiratory system and makes it more effective and it will be useful for a large group of patients. In addition, due to the simplicity and cheapness of this method, it can be used in epidemic conditions [46].

Li et al. [47] developed a synthetic nanobody called sybody (MR3) which had a high neutralizing activity. The in-vivo study (in hamster and mice) also showed significant protection from SARS-CoV-2 infection. They applied Concave, Loop, and Convex libraries once for ribosome display and RBD for phage display (three rounds) to select the SARS-CoV-2 RBD binders. Among all 99 sybodies, MR3 bound RBD at RBM (receptor binding motif) surface with relatively high affinity of KD 1.0 nM. To investigate the potency of this sybody, they did in vitro studies as well as in vivo study applied in hamster and mice. Firstly, in vitro study, they showed that MR3 blocked the ACE2–RBD interaction competitively. Indeed, it targeted RBD in RBM surface and did the neutralization of SARS-CoV-2. They engineered MR3 to enhance its affinity and neutralizing activity. Therefore, to achieve that, they did three different divalent engineering; 1- Selected two sybodies bound to the RBD and then fused to N-terminal of MR3. Since this dimer could recognize two distinct epitopes, it might be potential to escape mutants hence it could be a prominent candidate for neutralization of various SARS-CoV-2 variants. 2- Fused MR3 with the dimeric human IgG Fc. It showed a binding affinity of KD of 0.22 nM, which represented better affinity contrary to MR3 alone. 3- They used the divalent engineering MR3-MR3 that indicated higher neutralization activity. In the next step, for in vivo study, they worked on two different animal models mice and hamsters to show the protection of potent divalent MR3 for SARS-CoV-2. Since serum half-life of nanobodies is around several minutes, ABD (Albumin-binding domain) fused to MR3-MR3 divalent. The result showed that this fusion extended its stability in mice comparing to MR3 alone or MR3-MR3. Besides mice model, finally, they developed hamster model, a suitable model for COVID-19, to further investigation of MR3 efficacy. Although based on analyzing the histopathology results MR3-MR3-ABD protected the hamsters from lung damage moderately, attachment of Fc to MR3 caused fully protection of hamsters from SARS-Cov-2. Interestingly, the result of in-vitro study demonstrated that MR3-MR3-ABD had the same potency and efficacy as Fc-MR3 but in-vivo study, hamster model, showed that Fc-MR3 was more potent in hamsters. This might be because of different half-life for Fc and ABD. This study represented that divalent sybody whether with addition of ABD or Fc could be a robust nanobody to neutralize SARS-CoV-2 in compare to its monovalent [47].

Pymm P et al. [48] by Using the phage display method and Nanobodies obtained from two alcapas treated with coronavirus spike and RBDs, identified and isolated a collection of Nanobodies that strongly neutralize the SARS-CoV-2 virus. The Nanobody binds to the RBDs with IC50 values $<$ 1.0 nM (nM) and prevents RBDs from binding to the ACE2, thus strongly neutralizing the SARS-CoV-2 virus. In this study, by using epitope binning experiments, cryogenic electron microscopy (cryo-EM), and X-ray crystallography were identified two different antigenic sites on the spike trimer protein. Nanobodies from two different epitope classes bound to the two antigenic site on the spike protein in a non-competitive manner and strongly neutralized the SARS-CoV-2 virus. To increase the half-life of Nanobody, Nanobody was integrated with the Fc fragment of IgG. The results of this study showed that the Nanobody-FC compound with a high affinity of about 0.1 nM (KD $<$ 0.55 nM) neutralizes both the SARS-CoV-2 and D614G variant (a mutation to the kappa and delta strains). Also, Nanobody-FC was able to protect mice against both wild type and D614G variants and play a prophylactic role against the SARS-CoV-2 virus.

According to the results of Nanobody-Fc fusions can be used as a strong and prophylactic compound against the SARS-CoV-2 virus. However, since the Nanobody attaches to the RBD region and the highest rate of mutation occurs in this region of the spike protein, its effect on the delta and other variants needs to be further investigated [48].

Stefan et al. [49] described several SARS-CoV-2 humanized nanobody (VHH)-based antibodies (VHH-huFc) for further therapeutic evaluation. By using the VHH-based phage library, several SARS-CoV-2 virus neutralizing nanobodies were identified that bind to RBDs with high affinity and prevent the formation of the RBDs-ACE2 complex. The best VHH candidates were produced as human sdAbs with the crystallizable fragment (Fc) domain of a human IgG1 (VHH-huFc) and were named SP1B4, SP1D9, SP3H4. Of all the antibodies investigated, SP1B4, SP1D9, and SP3H4 were finally introduced that strongly neutralizes the SARS-CoV-2 virus with EC50 0.1–0.4 nM. The results of the study showed that SP1D9 and SP3H4 antibodies had therapeutic and prophylactic properties, but SP1B4 could not protect mice against wild type SARS-CoV-2 infection. While all three of these antibodies In-vitro strongly neutralized the wild type SARS-CoV-2 virus. It was also

reported that the alpha variant was detected by three selected antibodies with lower affinity than wild-type. But the beta variant was not detected by any of these antibodies. SP3H4 detected the L452R point mutation present in the Epsilon, Kappa, and Delta variants. It appears that the SP3H4 Nanobody is connected to the RBD from two different sites [49].

K Haga and colleagues [50] introduced a new Nanobody called K-874A. K-874A is derived from a VHH Nanobody without any modification and inhibits the SARS-CoV-2 well by Intranasal delivery to the lung. This Nanobody neutralized the SARS-CoV-2 well in human lung-derived alveolar organoid cells, VeroE6/TMPRSS2 cell line, and Syrian hamsters. Intranasal delivery of K-874A to the lungs of virus-infected Syrian hamsters also prevented weight loss and increased cytokines [50].

J Hong et al. [51] developed two Nanobodies, 7A3 and 8A2, by using VHH Nanobody phage libraries derived from dromedary camels. 7A3 and 8A2 bind to a highly conserved region in the coronavirus spike protein and RBD, respectively, to neutralize the SARS-CoV-2. The results of this study showed that 7A3 Nanobody at a dose of 5 mg/kg could protect K18-hACE2 mice against Delta and Beta SARS-CoV-2 variants [51].

In another study, four homotrimers Nanobodies named H3, F2, C5, C1 were reported. These Nanobodies neutralize the SARS-CoV-2 by binding to the RBD region (with pM affinity). The results of this study showed that C1 homotrimers neutralized the alpha, beta and Victoria SARS-CoV-2 variants. H3 and C5 nanobodies were also effective on Victoria, and Alpha variants. C5 Nanobody in the form of nasal delivery and intraperitoneal injection in SARS-CoV-2-infected Syrian hamsters has shown good therapeutic and preventive properties [52].

Ye et al. [53], developed a nanobody (Nanosota-1A) as anti-SARS-CoV-2 from a camelid nanobody phage display library (diversity 7.5×10^{10}). In order to increase affinity, random mutations were generated through error-prone PCR in the Nanosota-1A (Kd = 2.28×10^{-7} M) gene, and this nanobody was subjected to two rounds of affinity maturation. As a result, Nanosota-1C (Kd = 1.42×10^{-8} M) was produced, with one mutation in CDR2 and another mutation in FR2. Structural data indicated that the nanobody was able to attach to the often hidden RBD of SARS-CoV-2 spike protein, thus inhibiting the binding of the virus to the ACE2 receptor. In the following, they developed an Fc-tagged type of Nanosota-1C (Nanosota-1C-Fc) to generate a bivalent molecule with increased affinity (Kd = 1.57×10^{-11} M). The result showed that Nanosota-1C-Fc bound to RBD approximately 3000 times more strongly than ACE2, and inhibited the pseudovirus about 160 times more efficiently than ACE2. Examination of the in vitro stability of Nanosota-1C-Fc was produced in bacteria (40 mg/L) and purified on protein A column and gel filtration (nearly 100%). Nanosota-1C-Fc at different temperatures (−80 °C, 4 °C, 25 °C, or 37 °C) for 1 week showed that this nanobody is able to maintain nearly all of its RBD bonding capacity at the studied temperatures. Moreover, the in vivo stability of Nanosota-1C-Fc was much higher than Nanosota-1C. After injecting Nanosota-1C-Fc into mice via tail vein and examination of serum at different time points, it was shown that Nanosota-1C-Fc retains RBD-binding ability after 10 days. In contrast, the in vivo stability of Nanosota-1C was only a few hours. Finally, the biological distribution of Nanosota-1C-Fc in mice showed that this nanobody remained at high levels in blood, lung, heart, kidney, liver and spleen after three days. These tissues are all targets of SARS-CoV-2 [53].

In a study by Wu et al. [54] obtain several RBD-specific nanobodies, which derived from an alpaca immunized against SARS-CoV-2 spike antigen. To increase the efficacy of the nanobodies, various configurations of nanobodies are designed in this study. Nb(15)-Nb(H)–Nb(15) is created for the simultaneous identification of RBD and human serum albumin. This bispecific nanobody demonstrate acceptable neutralization potency against the SARS-CoV-2 (Including Delta variants) with a long half-life for the treatment and prevention of SARS-CoV-2, via intranasal administration in transgenic hACE2 mice. Nb15–NbH–Nb15 showed the most potent neutralization of the pseudotyped virus with an IC50 of 0.4 ng/mL (9.0 pM). Moreover, the Kd value of this hybrid nanobody to RBD is 0.54 nM [54].

3.4. Nanobodies that specifically detected SARS-CoV-2

Anderson et al. [55] used a llama library to select single-domain antibodies (sdAbs) specific for three separate epitopes on the SARS-CoV-2 nucleocapsid (N) protein. A llama was inoculated with a recombinant N protein, and a variable domains phage-display library was created to achieve this. The sdAbs that were chosen from this collection were divided into five sequence families. As revealed by surface plasmon resonance, three of these families (NRL-N-C2, NRL-N-E2, and NRL-N-B6) bind to distinct epitopes with significant affinity. MagPlex fluid array assays, carboxylated polystyrene microparticles, were used to test the efficacy of the sdAbs in detecting nucleocapsid protein. Specificity tests revealed that these three sdAbs bind to exclusively N protein of SARS-CoV-2 and SARS-CoV, showing their high affinity. E2 sdAbs appears to have higher selectivity than the other sdAbs with the K_D of 0.8 nM. So, they fused E2 with C2 and B6, demonstrating that the E2-B6 and E2-C2 heterobivalent structures outperformed other sdAbs. The limit of detection (LOD) suggests that this method applied in this study may be efficient in identifying SARS-CoV-2. Only the N of SARS-CoV and SARS-CoV-2 showed substantial binding to sdAbs in specificity tests, showing extremely selective binding ability. As COVID-19 moves from a pandemic phase to an endemic phase, the ability to detect cases and local outbreaks will become increasingly critical, particularly those in developing countries. These three sdAbs bind to N protein with high affinity, implying that they could be employed in antigen assays to detect SARS-CoV-2 infections. Novel antigen assays based on these sdAbs can be generated in large quantities, recognize the N protein with ideal specificity and affinity, and help control the current pandemic as a diagnostic test [55].

Guo et al. [56] describe nanobody-based organic electrochemical transistors (OECTs) for quickly detecting SARS-CoV-2 spike protein RBD in complex physiological fluids. The sensor was fast and used a conjugated polymer in the transistor as well as nanobody-SpyCatcher fusion proteins with high density to detect GFP, MERS-CoV, and SARS-CoV-2 spike proteins. Sensors were initially validated on nasopharyngeal swab samples, and so, it is suitable for COVID-19 screening with serum or unprocessed saliva samples. After 10 minutes of incubation of the nanobody–OECT biosensor with the electrode, it recognizes its targets. The nanobody used in this transistor was VHH72, developed against the SARS-CoV-2 RBD by Wrapp et al. with high selectivity and K_D of 7 nM [20]. They also developed MERS-CoV VHH83, VHH04, and VHH55 detection structures. They believe the nanobody–OECT method can be quickly adapted to detect any antigen for which nanobodies are accessible. They overcome protein detection difficulties using a bioelectronic technique that develops OECT technology on multiple levels, from chemical materials to biological identification. The utilization of an individual organic semiconductor and custom-engineered nanobodies were critical components in this technique. The OECT confirmed that all nasal samples that had previously been identified as positive were positive. In all samples, on the presence or absence of SARS-CoV-2, mean OECT signals were confirmed with RT-qPCR. Preliminary testing with samples from healthy volunteers and COVID-19 patients shows that the sensor is as accurate and sensitive as RT-PCR. The use of nanobodies instead of conventional antibodies (or antibody fragments) boosted the biorecognition layer's density and robustness even further. Their nanobody functionalized OECT's speed, performance, and versatility, as well as its compatibility with a wide range of sample sources, suggest that a wide range of clinical or non-clinical diagnostic procedures can be supplemented or replaced with this biosensor technology [56].

Sherwood et al. [57] described a single-pot phage display repertoire "Nomad", a semisynthetic assembly of nanobodies (sdAb or VHH) reporter fusion specific for the Nucleocapsid (N) protein with potential diagnostic applicability The Nucleocapsid (N) protein is with several

functions that is completely conserved over time in the SARS-CoV family. The Nucleocapsid (N) is also the most abundant viral component in cells infected with SARS-CoV. Because the nucleocapsid (N) is much less mutated and conserved over time and in all places, it can be used as a suitable marker for SARS-CoV classification. N protein is present in large amounts in serum early during SARS-CoV infection and has been used as a biomarker of infection in SARS-CoV-2. In this study, *E. coli* were used to produce sdAb fusion with fluorescent proteins. fluorescent proteins are produced at large quantities of the *E. coli* cytoplasm. sdAb is produced in large amounts in the periplasm. To solve this problem and generate innately fluorescent immunoreagents, novel periplasmic sdAb fusions made with mScarlet-I and mNeonGreen were produced at milligram amounts and were reported in this article for the first time. The results of this article indicate that sdAB-FP easily detects the target antigen and the infected cell, and enable single-step high resolution fluorescence microscopy. According to the results reported in this study sdAB-FP can be produced in *E. coli* for relatively low expense production. With one-step immunoprobing, there is no need to buy secondary antibodies at an additional cost. This method also speeds up the imaging process. In addition to these features, targeting the N protein with a conserved sequence could make sdAb a suitable option for use in epidemic conditions [57].

Two major structural proteins in the SARS-CoV-2 are Spike (S), and Nucleocapsid (N) antigens. Due to the presence of N antigen on the surface of SARS-CoV-2 (such as antigen S) and infected cells, this antigen can be a common choice for antigen tests. The N protein has a modular structure with a C-terminal dimerization domain (CTD) and a RNA binding domain (RBD). Ye and colleagues [58], report high-resolution crystal structures of three single domain nanobody from an immunized llama. Two of them are bound to the N-terminal of the RBD and one of them bound to the N-terminal of the CTD (Kd = 0.8–1.6 nM). Molecular analysis of these nanobodies showed there is no obvious similarity in CDR region of these nanobodies. As a result, each of the three nanobodies detects distinct epitopes on the N protein. Given the mutations that may occur in SARS-CoV-2, the use of antibodies that can detect different epitopes of an antigen can be very useful in detecting all circulating SARS-CoV-2 variants [58].

Gransagne et al. [59] inoculated alpaca with recombinant SARS-CoV-2 N protein and produced the VHH library. Sandwich ELISA was used to identify 7 VHHs that recognize the N protein after phage display selection. So that, the highest affinity nanobody have Kd of 0.206 nM. In addition, the VHHs were able to identify SARS-CoV2 variants alpha (B.1.17), beta (B.1.351), and gamma (P1) [59].

Huo et al. [60] reported nanobodies produced from a parent llama VHH discovered that bind to the Spike protein via affinity maturation. For example, H3, H4, C1, F2, and C5 was shown efficacy in neutralizing SARS-CoV-2 RBD and blocking binding to ACE2 in the Syrian hamster COVID-19 model [60]. Girt et al. [61]. tested C1, F2, C5, and H4-Fc nanobodies in a sandwich ELISA to detect the SARS-CoV-2 Spike protein and found the ideal combination of nanobodies (C5-Fc-SS-biotin and F2-Fc-HRP) with better antigen capture [61].

3.5. Nanobodies developed using special techniques and sources

3.5.1. Engineered mice

In a study by Xu J et al. [62] nanobodies against RBD was isolated from llamas and also from engineered mice with ability of generating VHHs antibody isolated from alpacas, dromedaries and Bactrian camels. To this end, 30 VHHs antibody elected from Bactrian camel, alpaca and dromedary genes were replaced the mouse VH locus using CRISPR–Cas9 and obtained nanomice for generating very specific nanobodies against RBD of SARS-CoV-2. Therefore, two groups of potent and functional neutralizing nanobodies were identified. The first group of nanobodies extracted from nanomice such as Nb30andNb12act by recognizing a highly conserved RBD region. The second group, llama nanobodies, such as Nb56 and Nb15inhibit the RBD–ACE2 interface and just in the form of homotrimers can be neutralized variants of SARS-CoV-2 containing N501Y or E484K substitutions. Measuring binding affinity of RBD by the Bio-layer interferometry (BLI) methods showed that the designed nanobodies of llama origin such as Nb15, Nb17, Nb19 and Nb56 and also Nb12 and Nb30 nanobodies from nanomouse showed KD value less than 30 nm. Neutralizing assay was measured by pseudotyped of lentviral particles with the spike of the virus and the highest value was obtained for Nb12 (11.7 nM) and the lowest value was obtained for Nb19 (0.335 nM). By creating bivalent antibodies, they found that as the number of linked monomers increased, the neutralizing activity of Nb15 and Nb12 increased up to 3-fold and 180-fold, respectively. According to this report, although, Nb15, Nb17, Nb19, and Nb56 in the monomer forms could neutralize wild-type viruses, they did not withdraw any mutations in RBD of SARS-CoV-2 variants. Therefore, multimerization of nanobodies as bivalent or trivalent forms mentioned as a potent technique to develop antibodies with higher potency and neutralizing effects. The neutralization potencies of nanomouse Nb12 and Nb30 in comparison to llama nanobodies were not altered by RBD mutations which indicates their different functional mechanisms. Investigating the neutralizing of trivalent Nb15, Nb56 and Nb12 as well as bivalent Nb30 nanobodies showed the neutralizing potency of all of them against wild and B1.1.7, B1.351 and P.1 variants. Nb15 and Nb30 are able to neutralize B1.1.7 variant with IC50 values 4 pM and 538 pM, respectively. Nb56 and Nb30 are able to neutralize B.1.351 variant with IC50 values 18 pM and 2,755 pM, respectively. Study have shown these antibodies have moderate neutralization potency against P.1 variant [62].

3.5.2. AHEAD methods

Wellner et al. [63] used a synthetic recombinant antibody generation technology named Autonomous Hypermutationy East surfAce Display (AHEAD) methods to produce nanobodies against the S glycoprotein of SARS-CoV2 using engineered yeast. In this technology an error-prone orthogonal DNA replication system with ability of continues mutation on the surface displayed antibodies has been used to generate some repertoires of antibody fragments in Saccharomyces cerevisiae as a host. Simple cycles of culturing and screening for antigen binding resulted in the evolution of antibody clones with high-affinity in a short time. To this start, eight clones that bound to the RBD of virus were selected from the source naïve nanobody YSD library and each of them was encoded on a cytosolic plasmid. After 3–8 cycles of AHEAD, multi-mutation nanobodies were produced with higher affinity for RBD including RBD1i13, RBD6id, RBD10i10, RBD3i17, RBD11i12 and RBD10i14 which showed up to ~580-fold improvement in RBD-binding affinity. Due to the importance of Anti-RBD monoclonal antibodies in blocking ACE2 interaction with this virus, they were evaluated as therapeutic candidates. The nanobodies were fused to the human IgG1 antibody isotype's Fc-region. Most anti-RBD nanobodies showed up to ~925-fold neutralizing potency using neutralization assays against pseudotypes of the SARS-CoV-2. Of all the evolved variants, RBD1i13 and RBD11i12 were nanobodies with the highest neutralizing potencies. Competitive evaluation of potent neutralizers, such as RBD1i13, RBD6id, and RBD11i12, showed moderate to strong competition in ACE2 binding, but RBD10i10 did not compete. This phenomenon indicates different RBD binding sites and neutralization mechanisms in the mentioned nanobodies. So, it can be used in nanobody fusions between different nanobodies. This property is important for the production of fusions or cocktails nanobodies and resulted in the neutralization of viral mutations [63].

3.5.3. Engineering Antibodies

Yang et al. [64] study provided using biparatopic nanobody (Nbs) sequences specific for the spike receptor-binding domain of SARS-CoV-2 could inhibit its interaction with ACE2 as a combination of neutralizing antibodies of virus receptor binding domain (RBD) of three corona virus; MERS-Co V, SARS-CoV and SARS-CoV-2 viruses on to stable nanobody (Nb) scaffold. The grafting CDR regions of developed specific

neutralizing antibodies (nAbs) against SARS-CoV, MERS-CoVs including monoclonal antibodies and mAbs of SARS-CoV-2 onto a known nanobody scaffold with stable structure resulted in a VHH, 16Nbs sequences (CS01– CS16). After modeling Nbs using modeler, protein–protein docking by Rosetta docking and molecular dynamic simulation were performed to evaluate the binding energy and select the most suitable nanobody with specificity and high affinity.CS10, CS16, CS01, CS02 and CS03 Nbs out of 16 designed nanobodies were selected with the best free energy. They occupied the ACE2 binding site, that may block the RBD: ACE2 interaction. While CS10 and CS16 Nbs inhibit this interaction through an allosteric mechanism. Molecular dynamic simulation studies showed that CS02 do not bind to the RBD of this virus stably. To increase their binding affinity and stability, redesign was performed on the residues of the five Nbs located on the interface region in complex with RBD and generated 7 redesigned Nbs including (CS16_RD01, CS16_RD02, CS03_RD01, CS03_RD02, CS03_RD03, CS03_RD04, and CS02_RD01) which have high affinities to RBD of SARS-CoV2 due to the increase of hot spots number on the interfaces of nanobody-RBD complex. For example, the CS02binding free energy in the RBD complex enhanced −40 kcal/mol with a change of (CS02-RBD) to (CS02_RD01-RBD). Their study showed that the RBD loop region plays an important role in the connection to Nbs. Nbs that are connected to RBD through two regions CDR3 and CDR2, due to have the greater number of key residues involved in this connection, form more stable complexes. In addition, the recognized hot spots of the aimed Nbs are placed in these two regions. The hot spot residues number on the interface of each complex indicated that CS03_Nbs-RBD complex and four brand-new Nbs derived from it, as compared with other complexes, can have high binding affinities and efficient agent to prevent the SARS-CoV-2 infection. Collectively, all redesigned Nbs except to CS02 showed stabilities and high affinities for binding to RBD of the virus [64].

Wu et al. [65] tried to developed human single-domain antibodies (SCFVs) against the RBD as a target that led to 18 unique SCFVs with high potency against RBD. They reported that the single-domain antibodies of human are more suitable compared to camelid nanobodies in therapeutic benefits. In this study, scaffold nanobody called germline 3–66*01 was used to produce libraries from the antibodies in the healthy adult donor's blood. This library was panned against the S1 subunit and RBD of SARS-CoV-2which were classified into 4 competition groups and thus, single-domain antibodies of human were recognized that could be targeting 5 different epitopes on RBD of SARS-CoV-2 in the low nanomolar/subnanomolar ranges affinities. n3072 was the only type of antibody that had high binding affinity to S1 but did not bind to RBD. Measuring the neutralizing activity of these antibodies by the pseudovirus neutralization evaluate showed that n3113and n3086 antibodies have modest neutralization activities, and they could block infection of SARS-CoV-2 pseudovirus with 26.6 and 18.9 mg/mL IC50 values, respectively. Antibodies n3088 of the Group D with IC50 values of 3.3 and IC80 values of 10.5 or n3130 of the Group D with 3.7 μg/mL IC50 values and 11.5 μg/mL IC80 values. The combination of n3113 with either n3088 or n3130 showed improved SARS-CoV-2neutralization activity with 0.70 and 0.51 μg/mL IC50 values and 3.6 and 3.2 μg/mL IC80 values, respectively. The n3088 and n3130 antibodies of group D neutralized the virus with an IC50 of 2.6and 4.0 μg/mL, respectively. The n3088 and n3130 antibodies of group D neutralize the virus by targeting an epitope placed in the interface of spike trimetric. It is possible that n3088 and or n3130 are two ideal antibodies for concomitant use with the ACE2-competing neutralizing antibodies. Antibody n3113 of the group E did not bind to RBD of SZTH-004SARS-CoV-2 strain (N354D/D364Y), but it bound with high potency with the RBD of IDF0372 strain (V367F). This reveals that the n3113 epitopes are in a distinct region from the ACE2 binding site. Comparison of binding kinetics represent that among the SCFVs determined from antibody library against RBD of the virus, n3010 has strong binding affinity to RBD. Concomitant use of single domain and other neutralizing antibodies create perfect structures named multi-specific antibodies to avoid the viral mutant's appearance. For example, one trivalent nanobody such as ALX-0171, have 6,000 fold and more than 10,000-fold neutralization potency against RSV-B and RSV-A versus its monovalent antibodies [65].

3.5.4. A cell-free platform

Chen et al. [66] design CeVICA, a rapid cell-free nanobody approach that employs ribosome display to isolate nanobodies from a large library in vitro, and utilize this to produce high-affinity nanobodies binder to RBD (Kd = 2.18 nM). They identified 30 RBD binder families and 11 families that block the infection of the pseudovirus. For example, SR38 binds to RBD and neutralizes N501Y variant pseudovirus strongly, SR6c3 with high refolding capacity after thermal challenge, and SR6v15 and SR6v15.d with high affinity and neutralization capacity [66].

3.5.5. Shark nanobodies

Gauhar et al. [67] used phage display and found 56 single domain shark variable new antigen receptor (VNAR) antibodies that neutralize the SARS-CoV-2 spike protein with high specificity. Ten of them were able to prevent both the Wuhan and the N501Y variant RBD from binding to ACE2 receptor. The VNAR-hFc antibodies also could neutralize virus N501Y or E484K variants [67].

4. Discussion

Nanobodies (Nbs) are single-domain antibodies (sdAbs) isolated from camelids and sharks that valuable tools for numerous purposes in biomedical fields due to their small size, high permeability, thermostability, high binding specificity, cost-effective production, and minimal immunogenicity [4]. Because of these properties, nanobodies are considered for the diagnosis and treatment of various diseases. As demonstrated in this systematic review study, Since the binding domain of the spike protein receptor is involved in binding to host cells and thus infecting them, therefore considered as an immunological hotspot for diagnostic and therapeutic of viral infection and therapeutic purposes. Nanobodies are also a viable option for controlling infections; many of them have been developed to suppress viruses such as the human immunodeficiency viruses (HIV), Hepatitis B virus, influenza virus, and respiratory syncytial virus [11]. Using Nbs may be one of the most effective treatments for treating and preventing COVID-19. They are able to recognize RBD or other SARS-CoV-2 receptors and prevent the virus from infecting the cell. However, the small size of the nanobodies causes these valuable molecules may lead to rapid renal clearance, and to solve this problem in therapeutic use, the nanobodies are attached to other molecules, such as FC domain of antibodies or attach the nanobody to the polyethylene glycol (PEG). On the other hand, the small size of nanobodies can be a diagnostic advantage and make it easier to build a Covid-19 detection kit. A potential way to against emerging variants viral include B.1.1.7 (N501Ymutation), B.1.351 (N501Y, K417 N, and E484K mutations of RBD) and P.1 (N501Y, K417T and E484K substitutions) is the use of VHHs. Nanobodies can identify inaccessible epitopes. The results of this study show that very powerful nanoparticles with picomolar affinity and high specificity against SARS-CoV-2 virus have been obtained (Table 1) that can be used in the treatment and diagnosis of Covid-19 disease.

5. Conclusion

Various treatments for SARS-CoV-2 virus have been approved today, but none of these treatments have been completely efficient against the virus. It seems that the use of nanobodies along with other approved treatments can be more efficient against Covid-19. The nanobodies presented here have the benefits of extreme stability for cost-effective manufacture, prolonged plasma half-life, and excellent for transportation and storage due to their high stability and large-scale production technique, made it suitable for therapeutic and detection usage.

Table 1
List of nanobodies studied in this research and their properties.

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)	Target	Authors &Year
Sb16	0.10 μM ± 0.02	NR	RBD	Ahmad. et al. (2021)
Sb45	0.038 μM ± 0.0005			
Sb68	0.041 μM ± 0.006			
Sb16	0.41 nM ± 0.12		spike	
Sb45	0.82 nM ± 0.013			
Sb68	2.60 nM ± 1.4			
Sb23	10.6 nM ± 2.0	0.6	RBD	Custódio. et al. (2020)
Sb23-Fc	0.22 nM ± 0.0001	0.007		
tri-specific VHH-Fc 3F–1B-2A	0.047 nM	0.71 nM (3.00 nM for cells)	RBD	Dong. et al. (2020)
tri-specific VHH-Fc 1B–3F-2A	0.095 nM	0.74 nM (6.44 nM for cells)		
NIH-CoVnb-112	4.94 nM	**EC50** = 0.02 (1.11 nM)/0.323 μg/mL (23.1 nM) and 0.116 μg/mL (8.3 nM) for pre and post-nebulization	RBD	Esparza. et al. (2020)
VHH-72	27 nM	NR	RBD	Guttler. et al. (2021)
Re6B06	12 nM			
Re9F06	4 nM			
Re5D06	0.002 nM			
Re9B09	≤0.001 nM			
Re6H06	≤0.001 nM			
Re5F10	0.029 nM			
Re9H01	0.01 nM			
Re9H03	0.024 nM			
VHH E	1.86 nM	48 nM	RBD	Koenig. et al. (2021)
VHH U	21.4 nM	185 nM		
VHH V	8.92 nM	142 nM		
VHH W	22.2 nM	81 nM		
SR6v15	2.18 nM		RBD	Chen. et al. (2021)
SR6v15.m	NR	3.591 nM ± 0.043		
SR6v15.d	NR	0.329 nM ± 0.019		
SR6v15.t	NR	1.392 nM ± 0.186		
Nb21.m	NR	0.577 nM ± 0.031		
Nb21.d	NR	0.382 nM ± 0.012		
Nb21.t	NR	0.244 nM ± 0.007		
SR6c3	NR	71.410 nM ± 0.553		
SR6v7	NR	27.573 nM ± 2.078		
6ID10_5	5 nM	NR	RBD	Gauhar., (2021)
6ID10_6	6.4 nM			
3ID10_16	7.5 nM			
3ID10_40	22 nM			
6ID10_70	7.7 nM			
6ID10_71	12 nM			
6ID10_75	15 nM			
3ID10_96	8.8 nM			
3ID10_99	92 nM			
6ID10_113	6.1 nM			
RBD-1-1E	0.9 nM		RBD	Fu., (2021)
RBD-2-1F	4.9 nM	470 nM		
RBD-1-2G	9.4 nM	490 nM		
RBD-1-3H	14.3 nM			
RBD-1-1G	35.5 nM			
RBD-2-1E	73.4 nM			

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Table 1 (*continued*)

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)		Target	Authors &Year
RBD-2-1B	81 nM				
RBD-2-5A	135.5 nM				
RBD-2-3A	440.8 nM				
RBD-2-1C	5683 nM				
Nanobody	Binding Affinity (Kd)	Neutralization IC50		Target	Authors &Year
Nb20	0.48 nM	29.37 ng/mL		ACE2-binding domain (RBD)	Sun, D. et al. (2021).
Nb21	0.42 nM	9.7 ng/mL		RBD	
Nb34	0.63 nM	150 ng/mL		spike protein (S)	
Nb105	0.97 nM	150 ng/mL			
Nb95	6.05 nM	150 ng/mL			
Nb17	0.69 nM	25 ng/mL			
Nb36	1.70 nM	100 ng/mL			
W25	0.295 nM	D614	G614	RBD	Valenzuela Nieto, G. et al. (2021)
		9.82 nM	5.09 nM		
W25Fc	NR	7.39 nM	3.69 nM		
W25FcM		27.40 nM	12.36 nM		
VHH-72- FcM		1287.7 nM	1233.9 nM		
Ty1-Ty1	NR	0.125 nM		RBD	Moliner-Morro, A. et al. (2020).
Ty1-PEG-Ty1		0.125 npM			
4arm-PEG Ty1		0.013 nM			
WNb 67	0.61 (±0.16) nM	31.8 nM		RBD	Pymm, P. et al. (2021).
WNb 41	0.55 (±0.27) nM	49.4 nM			
WNb 36	0.43 (±0.21) nM	3.3 nM			
WNb 2	0.36 (±0.05) nM	23.5 nM			
WNb 70	0.81 (±0.13) nM	155.5 nM			
WNb 4	4.44 (±1.54) nM	213.1 nM			
WNb 3	2.08 (±0.68) nM	95.0 nM			
WNb 59	1.21 (±0.34) nM	355 nM			
WNb 61	12.74 (±1.07) nM	1432.2 nM			
WNb 69	19.4 9 (±1.56) nM	541.6 nM			
WNb 46	6.20 (±0.1 4) nM	153.1 nM			
WNb 49	4.31 (±0.05) nM	159.1 nM			
WNb 30	1.07 (±0.03) nM	167 nM			
WNb 20	2.59 (±0.18) nM	166.9 nM			
WNb 32	3.92 (±0.26) nM	136.4 nM			
WNb 31	1.25 (±0.09) nM	94.9 nM			
WNb 21	NR	36108 nM			
WNb 5	8.52 (±2.80) nM	215 nM			
WNb 53	0.64 (±0.15) nM	57.4 nM			
WNb 33	0.73 (±0.15) nM	161.2 nM			
WNb 11	1.96 (±0.39) nM	97.5 nM			
WNb 68	1.75 (±0.56) nM	215.5 nM			
WNb 54	4.57 (±0.34) nM	217.0 nM			
WNb 14	8.89 (±3.37) nM	389 nM			
WNb 22	1.88 (±0.11) nM	86.8 nM			
WNb 9	2.35 (±0.99) nM	98 nM			
WNb 7	0.26 (±0.14) nM	54.5 nM			
WNb 6	1.21 (±0.19) nM	109.1 nM			
WNb 8	6.62 (±2.60) nM	218.4 nM			
WNb 52	3.57 (±0.26) nM	175.5 nM			
WNb 35	12.64 (±0.98) nM	1322.6 nM			
WNb 50	0.63 (±0.15) nM	60.1 nM			
WNb 13	2.12 (±0.67) nM	97.1 nM			
WNb 58	1.91 (±0.63) nM	559.6 nM			

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Table 1 (*continued*)

Name	Binding Affinity (Kd)				Neuralization IC50 (μg/ml)	Target	Authors &Year
WNb 45	0.68 (±0.09) nM				158.3 nM		
WNb 60	0.51 (±0.45) nM				149.3 nM		
WNb 15	0.14 (±0.06) nM				43.6 nM		
WNb 63	2.83 (±1.05) nM				322.6 nM		
WNb 10	0.68 (±0.27) nM				68.1 nM		
WNb 1	0.79 (±0.10) nM				96.4 nM		
WNb 23	9.03 (±0.21) nM				1812.5 nM		
WNb 62	6.41 (±0.93) nM				1208.5 nM		
WNb 19	0.46 (±0.14) nM				71.2 nM		
WNb 17	3.77 (±0.21) nM				280.2 nM		
WNb 34	11.05 (±0.73) nM				1709.8 nM		
WNb 24	7.33 (±0.22) nM				749.9 nM		
WNb 27	12.33 (±0.61) nM				370.5 nM		
WNb 26	7.38 (±0.22) nM				869.4 nM		
WNb 25	6.02 (±0.28) nM				370.9 nM		
WNb 29	2.33 (±0.08) nM				170.7 nM		
WNb2-Fc	0.59 (±0.07) nM				NR		
WNb7-Fc	0.70 (±0.12) nM						
WNb15-Fc	0.44 (±0.15) nM						
WNb36-Fc	0.61 (±0.13) nM						
WNb2-Fc	<0.01 nM						
WNb7-Fc	<0.01 nM						
WNb15-Fc	<0.01 nM						
WNb36-Fc	<0.01 nM						
WNb2-Fc	NR				0.33 nM		
WNb7-Fc					3.18 nM		
WNb15-Fc					2.55 nM		
WNb36-Fc					0.10 nM		
WNb2-Fc					0.30 nM		
WNb7-Fc					5.04 nM		
WNb15-Fc					4.91 nM		
WNb36-Fc					0.11 nM		
SP1D9	8.9 nM				0.33 EC50 (nM)	RBD	Stefan, M. A. et al. (2021)
SP1B4	39.5 nM				0.45 EC50 (nM)		
SP3H4	Biphasic nM				0.14 EC50 (nM)		
C5	0.099 nM				NA	RBD	J. Huo. et al. (2021)
C1	0.615 nM						
H3	0.025 nM						
F2	0.04 nM						
	Wuhan-hu-1	B.1.1.7	B.1.351	P1			
1B5	0.1 nM	0.03 nM	0.26 nM	0.5 nM	3.2 nM	RBD	J. Hong et al. (2021)
7A3	0.2 nM	0.2 nM	0.1 nM	0.8 nM		highly conserved region in the coronavirus spike protein	
8A4	0.16 nM	5.6 nM	NA	NA	8 nM	RBD	
2F7	0.85 nM	3.2 nM	NA	NA			
8A2	0.26 nM	>0.001 nM	>0.001 nM	0.5 nM			
1H6	37 nM	17 nM	NA	NA			
K-874A	1.4 nM				5.74 μg/mL	RBD	K. Haga et al. (2021)
Nanobody	Binding Affinity (Kd)				**Neutralization IC50**	**Target**	**Authors &Year**
S14	0.143 nM				0.00493 μg/mL	RBD (SARS-CoV)	Li, J. F. et al. (2021)
MR3	1.00 nM				0.42 μg/mL	RBD	Li, T. et al. (2021)
SR4	14.5 nM				4.85 μg/mL		
MR17	83.7 nM				13.18 μg/mL		
MR4	23.3 nM				0.77 μg/mL		

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Table 1 (*continued*)

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)	Target	Authors &Year
LR1	47.5 nM	2.28 μg/mL		
LR5	22.8 nM	2.10 μg/mL		
MR3-MR3(34 GS)	0.22 nM	0.010 μg/mL		
FC-MR3	0.22 nM	0.042 μg/mL		
FC-MR17	<0.001 nM	0.464 μg/mL		
LR5-MR3(13 GS)	NR	0.112 μg/mL		
LR5-MR3(19 GS)		0.111 μg/mL		
LR5-MR3(34 GS)		0.118 μg/mL		
MR3-MR3(13 GS)		0.133 μg/mL		
MR3-MR3 (19 GS)		0.121 μg/mL		
MR3-MR3 (24 GS)		0.114 μg/mL		
MR17m-MR17 m(13 GS)		0.029 μg/mL		
MR17m-MR17 m(16 GS)		0.121 μg/mL		
MR17m-MR17 m(19 GS)		0.072 μg/mL		
MR17m-MR17 m(24 GS)		0.193 μg/mL		
A8-G11-Fc	0.180 nM	0.09 μg/mL	SARS-CoV-2 S1/ACE2 SARS-CoV-2 S1/myeloid cell receptor	Lu, Q. et al. (2021)
aRBD-2	2.6 nM	17.56 ± 2.99 nM	RBD	Ma, H. et al. (2021)
aRBD-3	3.33 nM	16.83 ± 3.62 nM		
aRBD-5	16.3 nM	182.5 ± 69.30 nM		
aRBD-7	3.31 nM	110.5 ± 35.89 nM		
aRBD-41	21.9 nM	300–900 nM		
aRBD-54	5.49 nM	10.80 ± 1.07 nM		
aRBD-42	113 nM	NR		
aRBD-2-Fc	1.57 nM	2.68 ± 0.37 nM		
aRBD-3-Fc	1.12 nM	2.59 ± 0.68 nM		
aRBD-5-Fc	1.25 nM	1.89 ± 0.27 nM		
aRBD-7-Fc	0.0722 nM	1.42 ± 0.29 nM		
aRBD-41-Fc	2.82 nM	5.76 ± 1.02 nM		
aRBD-54-Fc	0.573 nM	2.07 ± 0.25 nM		
aRBD-42-Fc	4.49 nM	NR		
aRBD-2-5	0.0592 nM	~0.043 nM		
aRBD-2-7	0.25 nM	~0.111 nM		
aRBD-2-5-Fc	0.0123 nM	0.0118 ± 0.0060 μg/mL		
aRBD-2-7-Fc	0.217 nM	0.00676 ± 0.0012 μg/mL		
NB91-hFc	NR	54.07 nM		Lu, Q. et al. (2021)
biNb91-hFc		11.27 nM		
triNb91-hFc		4.89 nM		
monoNb3-hFc		32.36 nM		
biNb3-hFc		6.87 nM		
triNb3-hFc		4.70 nM		
Nb91–Nb3-hFc		1.54 nM		
S1-RBD-35	0.188 nM	12 nM		Mast, F. D. et al. (2021)
S1-RBD-9	0.430 nM	NR		
S1-RBD-11	0.0197 nM	<20 nM		
S1-RBD-15	0.0658 nM	<20 nM		
S1-6	0.565 nM	NR	S1 SARS-CoV-2	
S1-49	1.86 nM	NR		
S1-23	0.0174 nM	7 nM		
S1-1	0.072 nM	<20 nM		
S1-3	0.850 nM	NR		
S1-30	2.38 nM			
S2-7	0.0562 nM		S2-SARS-CoV-2	
S2-10	3.37 nM			

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Table 1 (*continued*)

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)	Target	Authors &Year
S2-62	0.0354 nM			
S1-RBD-35		0.180 nM	S1 501Y.V1	
S1-23	0.229 nM			
S1-1	0.044 nM			
S1-6	0.177 nM			
S1-RBD-9	0.801 nM			
S1-RBD-11	0.074 nM			
S1-RBD-15	0.042 nM			
S1-RBD-35	0.446 nM		S1501Y.V2	
S1-1	0.045 nM			
S1-6	0.320 nM			
S1-RBD-9	0.713 nM			
S1-RBD-11	0.161 nM			
S1-RBD-15	0.055 nM			
S1-62	NR	<20 nM	RBD	
S1-48				
S1-23				
S1-37				
S1-35				
S1-27				
S1-6				
S1-6				
S1-RBD-23	NR	<20 nM	RBD	
S1-RBD-29				
S1-RBD-20				
S1-RBD-21				
S1-RBD-41				
S1-RBD-4				
Nanobody	Binding Affinity (Kd)	**Neutralizing activity**	**Target**	**Authors &Year**
NM1230	8.23 nM	~ 37 nM (strong neutralization potency with IC 50 values)	RBD	Wagner et al. (2021)
NM1226	3.66 nM	~ 15 nM (strong neutralization potency with IC 50 values)		
bipNb NM1267	0.48 nM	0.89 nM (strong neutralization potency with IC 50 values)		
NM1220	37.09 nM	NR		
NM1221	22.7 nM			
NM1222	17.96 nM			
NM1224	8.34 nM	~ 256 nM (strong neutralization potency with IC50 values)		
NM1225	53.68 μM	NR		
NM1229	53.19 nM			
NM1228	1.37 nM	~ 7 nM (strong neutralization potency with IC50 values)		
NM1227	3.72 nM	NR		
NM1223	3.82 nM	Not applicable		
RBD1i1	48.1 nM	0.18 (μg ml^{-1})	RBD	Wellner et al. (2020)
RBD1i13	32.2 nM	0.05 (μg ml^{-1})		
RBD3i2	128 nM	5.52 (μg ml^{-1})		
RBD3i17	230 nM	0.116 (μg ml^{-1})		
RBD6id	263 nM	0.056 (μg ml^{-1})		
RBD6i10	NR	1.14 (μg ml^{-1})		
RBD6i13		≥28 (μg ml^{-1})		
RBD10i10	2.14 nM	0.19 (μg ml^{-1})		
RBD10i14	0.72 nM	0.42 (μg ml^{-1})		
RBD11i12	316 nM	0.04 (μg ml^{-1})		
RBD7i12	NR	7.37 (μg ml^{-1})		
RBD7i13		≥63 (μg ml^{-1})		
SR31	5.6 nM	not applicable	RBD	Yao et al. (2021)

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Table 1 (*continued*)

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)	Target	Authors &Year
SR31-SR31	0.7 nM	not applicable		
MR6	23.2 nM	77.5 nM		
MR6-SR31	0.5 nM	2.7 nM		
MR6+SR31	NR	77.6 nM		
MR6-MR6	0.2 nM	2.6 nM		
MR17	83.7 nM	747 nM		
MR17-SR31	0.3 nM	52.8 nM		
MR17+SR31	NR	>3 μM		
MR17-MR17	0.1 nM	2.3 nM		
MR3	1 nM	24.4 nM		
MR3-SR31	NR	76.4 nM		
n3021	0.63 ± 0.01 (Mean ± SD) nM	NR	RBD	Wu et al. (2020)
n3063	29.30 ± 0.23			
n3010	71.6 ± 0.72			
n3072	No binding to RBD	NR	S1	
n3086	88.97 ± 1.88	(IC50) values of 26.6 μg/mL		
n3113	57.01 ± 1.52	(IC50) values of 18.9 μg/mL		
n3088	3.7 ± 0.09	neutralized live SARSCoV-2 with: IC50 value 3.3 μg/mL & IC80 values of 10.5 μg/mL		
n3130	55.39 ± 0.98	neutralized live SARSCoV-2 with IC50 of 3.7 μg/mL & IC80 values of 11.5 μg/mL		
mixture of n3130 with n3113	NR	neutralization of SARS-CoV-2 in a synergistic fashion IC50 values of 0.7 μg/mL & IC80 value 3.6 μg/mL	NR	
mixture of n3088 with n3113		neutralization of SARS-CoV-2 in a synergistic fashion IC50 values: IC50 values of 0.51 μg/mL & IC80 value of 3.2 μg/mL		
Nb20	Binding kinetics by SPR 1.04 × 10–11 (M)	neutralizing activity value (neutralization potency) (nM) 0.066	RBD	Xiang et al. (2020)
Nb21	<1 pM	0.022 nM		
Nb89	1.08 × 10 -10 (M)	0.154 nM		
Nb 9	NR	1.722 nM		
Nb 11		0.800 nM		
Nb 16		1.233 nM		
Nb 17		1.500 nM		
Nb 28		NR		
Nb 34		1.125 nM		
Nb 36		NR		
Nb 64		4.875 nM		
Nb 78		0.766 nM		
Nb 82		NR		
Nb 93		NR		
Nb 95		5.105 nM		
Nb 99		0.452 nM		
Nb 105		5.070 nM		
Nb107	NR	0.181 nM		
ANTE-COV2-Nab20T $_{GS}$		5.43 nM		
ANTE-COV2-Nab21T $_{GS}$		6.04 nM		
ANTE-COV2-Nab20T $_{EK}$		5.75 nM		
ANTE-COV2-Nab21T $_{EK}$		9.75 nM		
D20		NR		
D21				
D21-34				
D21-36		25.45 nM		
D21-93		NR		
D21-95				

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Table 1 (*continued*)

Name	Binding Affinity (Kd)	Neuralization IC50 (μg/ml)	Target	Authors &Year
D21-105				
Nb15	Monomer (8.15e-9) Trimer (1.64e-11)	16 pM	Wt	Xu et al. (2021)
Nb15	NR	4 pM	U.K. (B.1.1.7)	
Nb15		106 pM	S.A. (B1.351)	
Nb15		28 pM	BR (P.1)	
Nb12	Monomer (3.00e-8) Trimer (<1.0e-12)	249 pM	Wt	
Nb12	NR	64 pM	U.K. (B.1.1.7)	
Nb12		286	S.A. (B1.351)	
Nb12		65	BR (P.1)	
Nb56	Monomer (3.82e-11) Trimer (3.26e-9)	3	Wt	
Nb56	NR	8	U.K. (B.1.1.7)	
Nb56		18	S.A. (B1.351)	
Nb56		3	BR (P.1)	
Nb30	Monomer (6.55e-9) Trimer (8.20e-10)	9374	Wt	
Nb30	NR	538	U.K. (B.1.1.7)	
Nb30		2755	S.A. (B1.351)	
Nb30		1874	BR (P.1)	
Nb 17	Monomer (5.59e-9) Trimer (<1.0e-12)	NR	NR	
Nb19	Monomer (4.72e-9) Trimer (3.51e-11)			
trivalent Nb56	<1e-12 (M)		RBD	
trivalent Nb12	2.2e-12 (M)			
bivalent Nb30	<1e-12 (M)			
Nanobody	Binding Affinity (Kd)	LD50 or IC50	Target	Authors &Year
Nanosota-1A	2.28 × 10-7 M	NR	RBD	Ye et al. (2020)
Nanosota-1C	1.42 × 10-8 M	3230 ng/mL	RBD	
Nanosota-1C-Fc	1.57 × 10–11 M	160 ng/mL	RBD	
KC3.ep3-Fc	5.1 × 10-9 M	39 ng/mL	RBD	Zupancic et al. (2021)
Ty1-Fc	18.6 × 10-9 M	211 ng/mL	RBD	
sdAb-N3	5 × 10-10 M	44 nM	Sars-CoV-2 N protein	Sherwood et al. (2021)
sdAb-B6	0.8–1.6 × 10-9 M	NR	Sars-CoV-2 N protein	Anderson et al. (2021)
sdAb-C2			Sars-CoV-2 N protein	
sdAb-E2			Sars-CoV-2 N protein	
Nb(15)-Nb(H)–Nb(15)	5.4 × 10-10 M	0.4 ng/mL	RBD	Wu et al. (2021)

It seems that comprehensive and appropriate research has been done on the separation of nanobodies and their effect against Covid-19. The results of this study show that scientists have succeeded in producing high-performance nanobodies against the SARS-Co-2 virus but in this time, research in this area needs to be taken one step further, and researchers have used nanobodies with outstanding characteristics in animal and human tests to provide a suitable treatment using nanobodies against SARS-Co-2 as soon as possible.

Author contributions

HB and HZ gave the idea, drafted some part of the manuscript and drafted figures. HB drafted some parts of the manuscript and provided, comprehensive revising and editing to the manuscript. MH, HA, FM, FMN and HSG did, the literature searches and screening, writing of the manuscript. All authors read and approved the final version.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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