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Biochemical Pharmacology 208 (2023) 115401

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Review

Applications of nanobodies in the prevention, detection, and treatment of the evolving SARS-CoV-2

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ARTICLE INFO

Keywords:
Nanobody
SARS-CoV-2
COVID-19
Variants

ABSTRACT

Global health and economy are deeply influenced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its newly emerging variants. Nanobodies with nanometer-scale size are promising for the detection and treatment of SARS-CoV-2 and its variants because they are superior to conventional antibodies in terms of cryptic epitope accessibility, tissue penetration, cost, formatting adaptability, and especially protein stability, which enables their aerosolized specific delivery to lung tissues. This review summarizes the progress in the prevention, detection, and treatment of SARS-CoV-2 using nanobodies, as well as strategies to combat the evolving SARS-CoV-2 variants. Generally, highly efficient generation of potent broad-spectrum nanobodies targeting conserved epitopes or further construction of multivalent formats targeting non-overlapping epitopes can promote neutralizing activity against SARS-CoV-2 variants and suppress immune escape.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has posed unprecedented influence on global health and economy, mainly due to its high transmissibility [1], high mutation rate [2,3], asymptomatic infection [4], lack of effective prevention and treatment methods, as well as inappropriate management strategies. Moreover, patients who have recovered from COVID-19 are still affected. Recent studies have shown that about 1 out of 8 patients will experience "long-term COVID-19" symptoms after recovery [5]. In addition, the SARS-CoV-2 virus keeps evolving into various new variants, among which the variants with enhanced transmissibility or infectivity are classified as variants of concern (VOC), including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and the currently circulating Omicron (B.1.1.529) variant based on the latest information of World Health Organization (WHO) (https://www.who.int/activities/tracking-SARS-CoV-2-variants/) (Fig. 1), and this has brought great challenges to the prevention and control of the virus [6,7]. For the currently globally dominant Omicron variant, the coexistence of various strains has led to the emergence of Omicron XD, XE, XF, and other recombinants, which are more infectious and resistant to developed vaccines and antiviral drugs [2,8].

Nanobodies (Nbs), also known as VHHs or single-domain antibodies, are small antigen-binding fragments derived from unique heavy-chain-only antibodies (HCAbs) in camelids [9]. Compared with conventional

Abbreviations: ACE2, angiotensin-converting enzyme 2; ACET, alternating current electrothermal flow; AHEAD, autonomous hypermutation yeast surface display; AI, artificial intelligence; APCs, antigen-presenting cells; AuNPs, Au nanoparticles; bipNb, biparatopic nanobody; BSL-3, biosafety level 3; CDRs, complementary determining regions; Cmax, maximum concentration; COVID-19, the coronavirus disease 2019; DPV, differential pulse voltammetry; E, envelope proteins; ELISA, enzyme-linked immunosorbent assay; ERGIC, ER-Golgi intermediate compartment; FP, fusion peptide; HCAbs, heavy-chain-only antibodies; ISGs, interferon-stimulated genes; LAMP, loop-mediated isothermal amplification; LFIs, lateral flow immunoassays; LOD, limit of detection; M, membrane proteins; N, nucleocapsid proteins; NAbs, neutralizing antibodies; Nbs, nanobodies; NSPs, non-structural proteins; PD, peptidase domain; PEC, photoelectrochemical; PERx, proximity-enabled reactive therapeutic; POCT, point-of-care testing; PROTAC, proteolysis-targeting chimeric; PTMs, post-translational modifications; RBC, red blood cell; RBD, receptor-binding domain; RBM, receptor-binding motif; RdRp, RNA-dependent RNA polymerase; RER, rough endoplasmic reticulum; RTC, replication-transcription complex; RT-PCR, reverse transcription polymerase chain reaction; S, spike proteins; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Sybodies, synthetic nanobodies; TMPRSS2, transmembrane serine protease 2; VNT, viral neutralization test; VOC, variants of concern; WHO, World Health Organization.

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https://doi.org/10.1016/j.bcp.2022.115401
Received 25 October 2022; Received in revised form 23 December 2022; Accepted 27 December 2022
Available online 30 December 2022

antibodies, nanobodies are featured by small size (12–15 kDa, about one-tenth the size of conventional antibodies), single domain, high stability (resistance to high temperature and extreme pH conditions) and long shelf-life, high solubility, deep-tissue penetration, cryptic epitope accessibility, amenable to versatile modalities, lack of glycosylation as well as Fc-mediated immune activation, and high amount but cost-effective prokaryotic production, all of which make nanobodies excellent diagnostic and therapeutic tools [10] (Fig. 2). In particular, the robustness of nanobodies enables their inhaled delivery, which appears to be an ideal administration route for the treatment of pulmonary diseases including COVID-19 [11,12], this is because nebulized nanobodies increase their lung delivery efficiency and concentration in bronchoalveolar lavage fluid, and allow the nanobodies to reach their maximum concentration (Cmax) more quickly [13] (Fig. 2D). It has been reported that multimeric nanobodies retain their function after aerosolization, lyophilization, and heat treatment, enabling direct aerosolized delivery to the nasal and lung tissues, which are two key sites of SARS-CoV-2 replication [14].

2. Life cycle of SARS-CoV-2

SARS-CoV-2 is an enveloped virus with positive-sense single-stranded RNA (ssRNA) that encodes four structural proteins (the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins), which are required for viral assembly and cell entry, 16 non-structural proteins (NSPs) (nsp1-16) that are essential for viral replication, and 9 accessory proteins that may interfere with host immunity [15,16]. The coronavirus homotrimeric S glycoprotein consists of the receptor-binding domain (RBD)-containing S1 and S2 subunits that mediates viral attachment and subsequent fusion with host cell membranes [17,18]. Interestingly, the prefusion form of each RBD is in a dynamic equilibrium switching between two conformations: the receptor-inaccessible down (or closed) conformation and the receptor-accessible up (or open) conformation which is considered to be less stable [19,20], depending on the transient hide or exposure of the receptor binding determinants, and S protein is predominantly in the 3down or 1up2down conformation [19,21]. S protein with at least one RBD in the up conformation is accessible to ACE2 and their binding triggers the S protein trimer dissociation as well as fusion events, whereas S protein with all three RBDs in the down conformation is inactive [14,22–24]. Cryo-EM experiments showed that upon furin cleavage, RBD undergoes a transition from a compact inaccessible down conformation to a partially accessible form, followed by an ACE2-bound up conformation [25].

The life cycle of SARS-CoV-2 starts with host cell entry, a critical step and a key target for COVID-19 preventive and therapeutic drugs. Upon reaching the cell surface, RBD in the up conformation binds to the peptidase domain (PD) of angiotensin-converting enzyme 2 (ACE2), resulting in a conformational change in the S1 subunit and the subsequent exposure of the S2 subunit located S2′ proteolytic site [25], which is cleaved by highly expressed host transmembrane serine protease 2 (TMPRSS2) in lung epithelial cells, and this leads to the rapid cell entry of wild-type or previous variants of SARS-CoV-2 [26]. Otherwise, for Omicron, the virus mainly infects the upper airway epithelial cells with low TMPRSS2 expression, followed by being slowly endocytosed into endolysosomes, where the S2′ site is cleaved by cathepsin L [26,27]. Cleavage at the S2′ site further results in exposure of the fusion peptide (FP) that induces fusion between the viral and host cellular membranes [28]. Viral RNA is released through fusion pore into the cytoplasm of host cell and then hijacks the host ribosomal machinery to produce viral polypeptides (pp1a and pp1ab), which are cleaved by Mpro and PLpro proteins [15,29]. The resulting replication-transcription complex (RTC) which contains RNA-dependent RNA polymerase (RdRp) replicates the viral ssRNA [30]. The N protein is biosynthesized in the cytoplasm, while other structural proteins including S, M, and E proteins are translated on the rough endoplasmic reticulum (RER) to confer post-translational modifications (PTMs), and the S protein is cleaved at the S1-S2 boundary by furin or furin-like proprotein convertase in the Golgi apparatus, resulting in the non-covalently associated S1 and S2 subunit [31,32]. Viral structural proteins and genomic RNA are then transported for virion assembly to the ER-Golgi intermediate compartment (ERGIC), where the N protein is packaged internally along with ssRNA, while the S, E, and M proteins are incorporated into the viral membrane. The virions are then released from host cells through exocytosis to restart the life cycle [33] (Fig. 2D).

3. Diagnosis of COVID-19 with nanobodies

Since SARS-CoV-2 is an evolving virus which has and may further

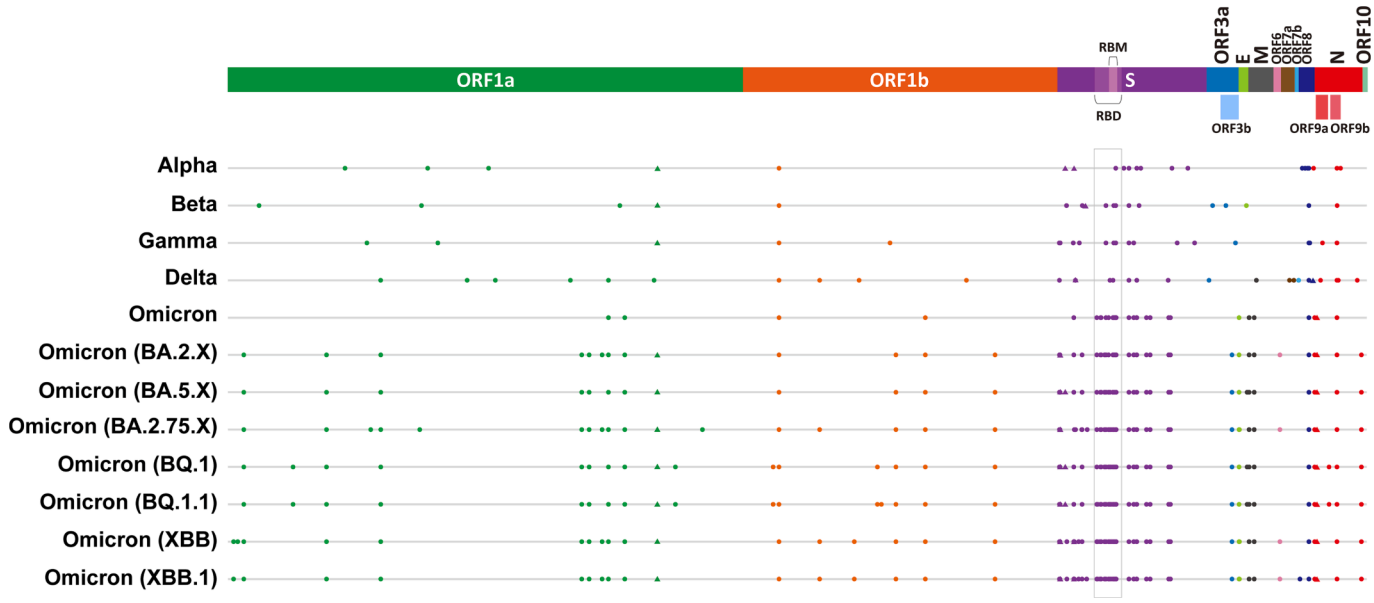

Fig. 1. Mutations of SARS-CoV-2 variants. Mutations of SARS-CoV-2 variants are depicted (amino acid substitutions are indicated as dots (●), while deletions as triangles (▲) with different colors representing different proteins of the virus. The rectangular box indicates RBD, which is the convergent region of the SARS-CoV-2 variants mutations. Generated based on sequences from GISAID (https://gisaid.org/wiv04/, and https://outbreak.info/situation-reports/).

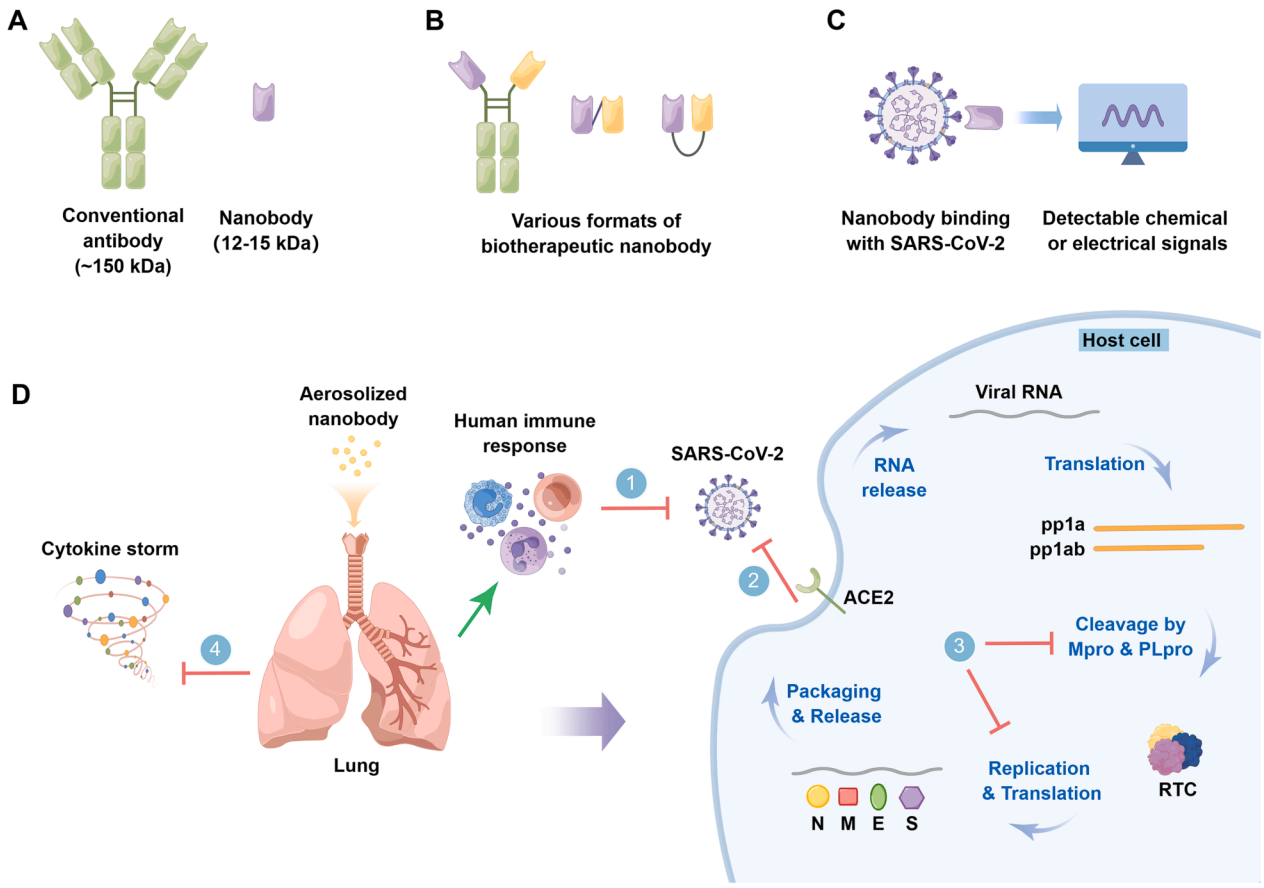

Fig. 2. Prevention, Detection, and treatment of the SARS-CoV-2 and its variants with nanobodies. A. Schematic representation of conventional antibody and nanobody which is the variable domain of heavy chain-only antibody (HCAb) and only one tenth the size of the former. B. Nanobodies are adaptable to different multivalent formats, enabling enhanced avidity, simultaneous engagement of multiple spatially discrete non-overlapping epitopes, and mitigation of viral escape, especially when using conserved epitopes as targets. Here, multivalent formats of nanobodies are exemplified by dimerization achieved by genetic immunoglobulin (Ig) Fc fusion or C-to-*N*-terminal assembly, or otherwise C-to-C-terminal fusion of nanobodies through chemical approach. C. Application of robust nanobodies in SARS-CoV-2 detection, especially in point-of-care testing (POCT), during which binding events are translated into detectable chemical or electrical signals. D. Local pulmonary delivery of aerosolized nanobodies is promising in prevention and treatment of the SARS-CoV-2 and its variants by inducing human immune response (1), blocking their life cycle at different stages, such as viral entry (including viral binding and the subsequent membrane fusion) (2) and viral replication (3), or by modulating host inflammation (cytokine storm) (4). Green line with arrow indicates "Induction", and red "T" shaped lines indicate "Inhibition". By Figdraw.

strengthen its airborne transmission [2,34], high transmissibility, infectious asymptomatic infections [35], and other characteristics, readily and highly sensitive detection of the virus plays a pivotal role in curbing the ongoing pandemic. The current molecular detection of SARS-CoV-2 mainly utilizes real-time reverse transcription polymerase chain reaction (RT-PCR) or loop-mediated isothermal amplification (LAMP) with high sensitivity [36,37]. Nevertheless, this gold-standard method not only relies heavily on laboratory equipment, but also is costly and time-consuming, which is not conducive to mass screening and timely interruption of viral transmission [38]. Alternatively, viral antigen proteins can be directly detected through a cost-effective and time-saving antibody-based immunoassay without sample processing [39]. The specificity, sensitivity, and stability of antibodies are critical for the viral detection performance in immunoassays [40], and nanobodies are excellent reagents mainly due to their improved shelf-life, and capacity of high-density coupling to raise detection sensitivity resulted from small size [41], especially in point-of-care testing (POCT) which provide on-the-spot results, such as lateral flow immunoassays (LFIs).

Currently, many nanobody-based immunoassays target different proteins of SARS-CoV-2 to convert binding events into detectable chemical or electrical signals (Fig. 2C) (Table 1). Based on the immunoreaction between an antigen and its antibody, laboratory-based sandwich enzyme-linked immunosorbent assay (ELISA) with labelled immunosensor is a commonly used quantitative method. However, its application in high-throughput mass screening and portable use is limited by time-consuming, high cost, as well as complex operation, while all of these are overcome in LFIs, in which binding events are amplified and converted into electrical signals readout through electrochemical transistors [42,43]. Au nanoparticles (AuNPs) anchored with S protein-specific nanobodies have been used as photoelectrochemical (PEC) immunosensors with a reported limit of detection (LOD) of ~ 5 fg/mL for S protein, in which the capture of S proteins by nanobodies increases the steric effect, resulting in impediment of photoinduced charges diffusion and reduced photocurrent signal [44]. In another research, AuNPs were coated with nanobodies targeting non-overlapping epitopes of RBD and served as multivalent antigen-binding sensors called Nano2RED, in which antigen binding signals are converted into detectable physical AuNP aggregation with a LOD for RBD as low as ~ 1.3 pM in diluted human serum [45]. Both AuNP-based methods described above can obtain readout from a sample within 30 min. Nanobody-functionalized electrodes bioconjugated with RBD binding nanobody VHH72 fused SpyCatcher with high density and controlled orientation, were formatted into sensors by combining with solution-processable conjugated polymer in the transistor channel and give single-molecule-sensitive readouts within 15 min using unprocessed saliva or nasopharyngeal swab samples [46]. In another research, Pagneux et al. introduced a cysteine mutant in a loop between frameworks 1 and 2 of VHH72 to make it perpendicular orientated on the

Table 1
Summary of nanobody-based immunoassay methods for SARS-CoV-2 detection.

Nanobody name	Nanobody target	Affinity to target (K_D)	Method	Sample source	Specificity	Sensitivity	Sample to-result time	Features	References
NTD-E4, NTD-B6	N protein	23.8 ± 2.1 nM (NTD-E4), 46.5 ± 2.6 nM (NTD-B6)	ELISA	Nasal swabs	–	–	Several hours	–	[51]
E2, B6, C2	N protein	0.8 nM (E2), 1.6 nM (B6), 1.1 nM (C2)	ELISA	–	–	LOD of 50 pg/mL for N and 1.28×10^3 pfu/mL for the killed virus	Several hours	–	[50]
C5, F2	S protein	99 pM (C5), 40 pM (F2)	ELISA	–	–	103 ffu/ml for inactivated virus	Several hours	–	[116]
–	RBD of S protein	–	ELISA	Saliva, nasopharyngeal swabs	100 %	Saliva - 93.3 % (Ct ≤ 30), 87.5 % (Ct ≤ 35), 86 % (Ct ≤ 40); Nasopharyngeal swabs - 90 % (Ct ≤ 30), 80 % (Ct ≤ 35), 88 % (Ct ≤ 40)	Several hours	–	[117]
–	S protein		Photoelectrochemical (PEC) immunosensor	–	–	LOD = 2.8×10^{-17} M (5 fg/mL)	30 min	Label-free, convenient, rapid, low cost	[44]
RBD8/ RBD10	RBD of S protein	–	Nanobody-functionalized nanoparticles for rapid, electronic detection (Nano2RED)	Serum, blood	–	LOD=~1.3×10^{-12} M, for RBD in diluted human serum	5–20 min	Label-free, rapid detection, low cost	[45]
VHH72-C13	RBD of S protein	12.1 nM	Nanobody-functionalized electrochemical platform	Saliva, nasopharyngeal swab	Saliva-85 %, nasopharyngeal swab-90 %	Saliva-80 %, nasopharyngeal swab-90 %, LOD = 1.2×10^4 viral RNA copies mL^{-1}, 2×10^{-17} M	10 min	Portable, quick, accurate, user-friendly	[47,118]
VHH72	RBD of S protein	23 nM	Organic electrochemical transistors (OECTs)	Saliva, nasopharyngeal swab, serum	–	Single-molecule, sensitivity comparable to RT-PCR, saliva (LOD = 1.2×10^{-21} M), nasopharyngeal swab (LOD = 1.9×10^{-14} M)	<15 min	Label-free, rapid, sensitive, reusable, simple, robust	[46,118]
Ty1	RBD of S protein	24.9 ± 10.3 nM	Alternating current electrothermal flow (ACET) integrated OECT-based immunosensor	Saliva	–	LOD = 1×10^{-16} M	~2 min	Label-free, partially reusable, speedy detection	[48]

sensor surface, resulting in a nanobody-functionalized electrochemical platform using differential pulse voltammetry (DPV) as a readout, which detects saliva and nasopharyngeal swab samples rapidly within 10 min [47]. Nevertheless, the capture of analyte molecules by biosensors commonly relies on diffusion-dominated transport or manual mixing, which is still time-consuming and labor-intensive. To address this issue, Koklu et al. introduced alternating current electrothermal flow (ACET) with micro-stirring effects to accelerate the device operation, thereby reducing the sample-to-result time to approximately only 2 min [48]. Apart from improving intrinsic sensitivities, efforts have also been made to capture and concentrate SARS-CoV-2 virus using nanobody-functionalized cellulose to improve detection capability, which can also be used for virus sampling and virus removal [49]. It is worth noting that although the S protein is currently the most commonly used target for antigen detection, the N protein is also used [50,51] due to its abundant expression and presence in nasopharyngeal swabs [52,53]. More importantly, the N protein is a potential universal target because it is more conserved than the S protein with convergent mutations [54] (Fig. 1).

In addition to direct detection of the virus, diagnostic testing of human neutralizing antibodies (NAbs) against SARS-CoV-2 is also of great significance to monitor the spread of viral infection or the effectiveness of vaccination at individual and population scales. While currently used detection methods are usually immunoassays (ELISA, LFI, etc.) that do not distinguish NAbs from non-NAbs, or viral neutralization test (VNT) that require the use of live SARS-CoV-2 virions and biosafety level 3 (BSL-3) facility, which are often time-consuming, laborious, and relatively expensive [55]. Wagner et al. established a competitive multiplex binding assay (NeutrobodyPlex) using a biparatopic nanobody (bipNb) consisting of two RBD-specific inhibitory nanobodies and succeed in high-throughput screening and detailed analysis of NAbs against RBD [56]. Nanobodies have also been applied in POCT, enabling speedy, highly portable, semi-quantitative, and cost-effective NAb measurements. Redecke et al. constructed a bi-specific protein comprising a red blood cell (RBC) binding nanobody and RBD to mediate instantaneous hemagglutination in the presence of RBD-targeted NAbs, and they used artificial intelligence (AI) to analyze the digital images captured by cell phones, promoting its application in

POCT [57]. Notably, these methods are adaptable to other pathogens.

4. Prevention of SARS-CoV-2 infection

Effective vaccines are significant in blocking the spread of COVID-19, while most of the vaccines currently in use are sophisticated to manufacture and entail specialized storage and transportation conditions, which limits their distribution, especially in remote areas. It is promising to develop vaccines based on nanobodies due to their superior stability. Pishesha et al. fused RBD with a nanobody targeting class II MHC antigens to develop a candidate vaccine, which delivers RBD directly and specifically to antigen-presenting cells (APCs), resulting in robust humoral and cellular immunity against SARS-CoV-2 as well as its variants (Alpha, Beta, and Gamma) in both young and aged mice. Moreover, this vaccine remains stable with full efficacy for room temperature storage or lyophilization [58]. Besides, innate anti-viral immune response was also used. IFN-β inhibits SARS-CoV-2 replication by upregulating the expression of interferon-stimulated genes (ISGs) and has been used to treat patients with COVID-19. Lyu et al. generated exosomes conjugated with IFN-β fused by S protein-targeting nanobody to selectively deliver IFN-β to virus-infected cells, which may also avoid the side effects caused by systemic administration of IFN-β [59].

5. Treatment of SARS-CoV-2

All strategies that can effectively disrupt the life cycle of SARS-CoV-2 in humans have potential therapeutic value for the treatment of COVID-19. In addition, nanobodies also have the potential to modulate host immune response in hospitalizations associated with severe COVID-19 (Fig. 2D) (Table 2).

5.1. Blocking of viral invasion

Currently, S protein is the most prominent target of antibody-based targeted therapy for COVID-19 [60], mainly due to its vital role in viral recognition and fusion with host membranes, as previously described. Neutralizing nanobodies block viral invasion in several ways.

First, interfering with ACE binding. Most of the identified nanobodies showed efficacy by directly engaging epitopes of RBD. Another class of nanobodies, such as NB1A7 [61], do not bind to receptor-binding motif (RBM) of RBD, but they sterically hinder ACE2 binding. NB1B11 was reported to bind to RBM in both its up and down conformation and buries the ACE2-binding site, thereby blocking RBD/ACE2 attachment [61]. Apart from NB1B11, several nanobodies such as Ty1 [62], Sb23 [63], P17 and P86 [64]), have similar performance of binding both conformations, demonstrating their excellent antiviral efficacy. Second, stabilizing the epitope-inaccessible/inactive form of S protein. These nanobodies recognize RBD epitopes overlapping with the ACE2 binding site, and they bind the S protein in its fully inactive conformation (3downs), locking its receptor binding domains into an inaccessible state [14,65,66]. Third, inhibiting SARS-CoV-2 S protein-mediated membrane fusion with target cells or premature inducing of the post-fusion conformation to preclude bona fide fusion events. It has been reported that RBD bound to nanobody K-874A can still interact with ACE2, resulting in blocking viral membrane fusion but not viral attachment [67]. Growing evidence suggests that neutralizing nanobody binding can stabilize S protein in its up conformation, leading to transitions from premature to an irreversible post-fusion state and inactivation [61,68] or aberrant activation of the fusion machinery that renders virions noninfectious [69,70], probably due to exposure of proteolysis site and the subsequent shedding of S1.

5.2. Inhibition of viral replication

After viral entry, an effective strategy is to block viral replication mediated by RTC, which is composed of a number of NSPs [71]. For example, it is recently reported that a novel mechanism for nsp9-mediated SARS-CoV-2 RNA capping, which plays essential roles in viral translation and proliferation [72], suggesting that nsp9 is a promising target. Nanobodies targeting and inactivating key proteins in viral translation are potential anti-coronaviral targeted drugs. Recently, several studies have developed nanobodies against nsp3 [73], nsp7 [74], nsp8 [74], nsp9 [75], RdRp [74], and Mpro [76]. Unfortunately, these studies did not provide direct evidence of inhibition of viral replication and further research is needed. In fact, as opposed to preventing viral entry outside the cell, interfering with viral replication likely requires delivery of nanobodies into host cells, which may be critical to their efficacy.

5.3. Modulation of host immune response

Direct antiviral strategies including interventions of blocking SARS-CoV-2 entry or replication show efficacy only in the early stage, but relatively ineffective in hospitalizations associated with severe COVID-19, which may be largely driven by hyperactive immunopathology resulted from uncontrolled systemic inflammation including cytokine storm [77]. Studies have begun using nanobodies to inhibit proinflammatory responses by blocking the interaction of proinflammatory mediators with their receptors. A report focused on myeloid cells that produce proinflammatory cytokines and chemokines (IL-1, IL-6, IL-8, CXCL10, etc.), and constructed a bispecific nanobody A8-G11-Fc using two S protein-specific nanobodies (A8 and G11) with complementary capacity to block interactions between the S1 protein and host ACE2 as well as myeloid cell receptors, and then demonstrating its capacity of blocking ACE2-mediated infection as well as the myeloid receptor-mediated proinflammatory responses [78]. In another research, Ettich et al. developed a bispecific inhibitor, c19s130Fc, involving an RBD-targeting nanobody VHH72 to block viral infection, and a nanobody VHH6 targeting IL6:sIL-6R complexes to block IL-6 *trans*-signaling, which has been demonstrated to be a primary driver of ongoing inflammatory reactions [79].

6. Strategies for coping with the emerging SARS-CoV-2 variants

SARS-CoV-2 is an RNA virus, which is featured by large population size and short viral generation time [80], and it has a high mutation rate despite the proofreading activity conferred by nsp14 [81]. Moreover, recombination occurs between different circulating variants when they infect the same host [2], and RNA editing is also induced by human immune system [82]. Besides, human NAbs, as well as other drugs including vaccines, may act as an additive selective pressure, supported by evidence that mutations converge in RBD, especially the RBM region, which is a major target of NAbs [61,83,84] (Fig. 1). All of the above result in mutations accumulation, viral evolution, and finally the emergence of new variants [2,85]. These variants, especially the Omicron variant including its subvariants, confer S protein mutations, among which R346T, K444T, N460K, and F486S represent key neutralization escape mutations [86–88], while the N460K and F486 mutations enhance fusogenicity [88], and some of the convergent hotspots render initially developed therapeutic antibodies ineffective [6,7,84,89,90].

6.1. Enhancing avidity of nanobodies

Since mutations in variants reduce their binding to neutralizing nanobodies, increasing affinity of nanobodies reversely may be a strategy for dealing with variants. Fc region fusion has been used to enhance their binding activity as well as neutralizing activity [91,92], and interestingly, it is reported that nanobodies fused to the Fc region of human mucosal IgA may be more potent against Omicron variants compared to the IgG equivalent, possibly due to the longer hinge region of IgA-Fc [93]. In an excellent recent study, Xu et al. isolated a set of

Table 2
Summary of nanobodies in treatment of SARS-CoV-2 variants.

Nanobody name	Nanobody target	Nanobody format	Source	Screening method	Expression system	Affinity to target (K_D)	Neutralizing pseudovirus (IC50)	Neutralizing SARS-CoV-2 (IC50)	Mechanism	Virus variants	Features	References
NB1A2, NB2B4	Mpro	Monovalent	Mpro-immunized camel	Phage display	Top10F' cells	2.43 nM, 0.46 nM	–	–	Targeting Mpro dimer and dissociate it into monomers, engaging the catalytic and substrate binding sites of Mpro	–	–	[76]
Nb15-NbH-Nb15	S protein	Heterotrimeric	Alpaca immunized with the extracellular domain of S protein (S1 + S2 ECD, S)	Phage display	293F cells	0.541 nM	0.0004 ± 0 μg/ml	–	–	Wild type, Alpha, Delta, Epsilon, B.1.429	Nb15-NbH-Nb15 formatting; intranasal administration	[119]
See original literature	S protein	Monomeric, dimeric, trimeric	Llamas immunized with S1 and S2 Fc fusion proteins	Combining *in vivo* antibody affinity maturation and proteomics	Arctic Express (DE3) cells	*See original literature*	*See original literature*	*See original literature*	Multiple modes (*see original literature*)	Alpha, Beta, Gamma	Combining *in vivo* antibody affinity maturation and proteomics, synergistic viral-neutralizing effects analysis	[96]
See original literature	S protein (RBD, S2, or NTD)	Monovalent, bivalent (Fc fusion)	Llamas immunized with S protein (and boosted with RBD)	Phage display	*E. coli* (Monovalent) HEK293-6E cells (Fc fusion)	*See original literature*	*See original literature*	*See original literature*	*See original literature*	Wild type, Alpha, Beta, Omicron	Targets besides RBD	[92]
saRBD-1	RBD of S protein	Monovalent, bivalent	Spike RBD immunized alpaca	Phage display	Bacteria, 293F cells	750 pM (monovalent), 302 pM (bivalent)	4.26 nM (monovalent), 100 pM (bivalent)	–	Competitive binding to RBD of the S protein	Wild type, Alpha, Beta, Gamma, Delta, Epsilon	–	[120]
Sb#15, Sb#68	RBD of S protein	Monovalent, bivalent	Synthetic sybody libraries	Ribosome display + phage display	*E. coli* MC1061 cells	12 nM (Sb#15), 9 nM (Sb#68), 0.3 nM (GS4),	2.3 μg/ml (Sb#15), 2.3 μg/ml (Sb#68), 0.02 μg/ml (G4S), 0.01 μg/ml (Tripod-GS4r)	–	Interfering with ACE2 interaction	Wild type, Alpha, Beta, Delta,	–	[69]
VHH72	RBD of S protein	Bivalent, VHH72-Fc	Llama immunized with SARS-CoV-2 S glycoprotein	Phage display	HEK293 cells, *Pichia pastoris*	23 nM	0.2 μg/mL	–	Disrupting RBD dynamics and receptor binding	Wild type, Delta, Beta, Kappa	Extracellular vesicles-targeting platform	[118,121]
1B-3F-Fc, 3F-1B-2A-Fc, 1B-3F-2A-Fc	RBD of S protein	Bispecific, trispecific	Naïve and synthetic humanized llama VHH libraries	Phage display	Expi293 cells	0.25 nM (1B-3F-Fc), 46.8 pM (3F-1B-2A-Fc), 94.7 pM (1B-3F-2A-Fc)	3.00 nM (3F-1B-2A-Fc), 6.44 nM (1B-3F-2A-Fc)	–	Interfering with ACE2 interaction	Wild type, Alpha, Beta, Delta, Lambda, Omicron	–	[100,112,122]
NB1A7, NB1B11	RBD of S protein	Monovalent, multivalent	Recombinant RBD-immunized camel	Phage display	Periplasm of *E. coli* BL21 (DE3) (monovalent), HEK293F cells (multivalent)	0.74 ± 0.003 nM (NB1A7), 6.76 ± 0.027 nM (NB1B11)	131.3 ± 1.39 nM (NB1A7), 303.9 ± 1.46 nM (NB1B11)	1.0 ± 1.5 nM (Format I: NB1A7-Fc + NB1B11-Fc), 0.6 ± 1.19 nM (Format II: NB1A7-NB1B11-Fc)	Blocking the interaction between ACE2 and RBD	–	–	[61]
n3113v, n3130v, bn03,	RBD of S protein	Monovalent, bivalent	Grafted fully human single-domain antibody	Phage-display, in vitro affinity maturation	*E. coli* HB2151	0.81–2.54 nM (n3113v); 1.16–6.04 nM	0.28 μg/mL (bn03)	–	Inducing S trimer to unstable wide-up state, inhibiting	Wild type, Alpha, Beta,	Human origin single-domain antibody library	[68,99,123]

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Table 2 (*continued*)

Nanobody name	Nanobody target	Nanobody format	Source	Screening method	Expression system	Affinity to target (K_D)	Neutralizing pseudovirus (IC50)	Neutralizing SARS-CoV-2 (IC50)	Mechanism	Virus variants	Features	References
n3113.1-Fc (Y58L)				through error-prone PCR		(n3130v); ≤1 nM (bn03)			S protein mediated membrane fusion	Gamma, Delta, Omicron		
RBD-1-2G	RBD of S protein	Monovalent, bivalent (RBD-1-2G-Fc), trivalent	Synthetic humanized nanobody libraries	Phage-display	Vibrio natriegens (Monovalent);	9.4 nM (monovalent toward RBD); 1.9 nM (bivalent RBD-1-2G-Fc); 0.1 nM (trivalent)	490 nM (RBD-1-2G); 88 nM (RBD-1-2G-Fc); 4.1 nM (RBD-1-2G-Tri)	1211 nM (RBD-1-2G); 255 nM (RBD-1-2G-Fc); 182 nM (RBD-1-2G-Tri)	Interfering with ACE2 interaction	Wild type, Alpha	Synthetic humanized nanobody libraries	[124]
P2C5, P2G1, P5F8	RBD of S protein	Monovalent, bivalent	Bactrian camel immunized with SARS-CoV-2 RBD	Phage-display	*E. coli* BL21 cells	3.97 nM (P2C5), 5.36 nM (P2G1) and 1.94 nM (P5F8)	–	–	Competing with ACE2 for RBD binding	SARS-CoV-2B.1.1.1, Alpha, Beta, Gamma, Delta and Omicron	–	[125]
NIH-CoVnb-112	RBD of S protein	Monovalent	Llama immunized with SARS-CoV-2 S1 protein	Phage-display	BL21 (DE3) competent *E. coli* strain; X-33 Pichia pastoris	1–5 nM	–	–	Competing with ACE2 for RBD binding	Wild type, Alpha, Beta, Gamma, and Delta.	Nebulized delivery of NIH-CoVnb-112	[126,127]
Nb1, Nb2	RBD of S protein	Bivalent (Nb1–Nb2), tetravalent (Nb1–Nb2-Fc)	Synthetic library	Phage-display	*Escherichia coli* (Nb1–Nb2), Expi293F cells (Nb1–Nb2-Fc)	≤0.001 nM	Nb1-Nb2 (All variants ≤ 0.0865 nM)	Nb1-Nb2 (WT-1.207 nM, Alpha-0.8149 nM, Beta-1.776 nM, Gamma-13.01 nM, Delta-0.7317 nM); Nb1-Nb2-Fc (WT-0.0168 nM, Alpha-0.0117 nM, Beta-0.0097 nM, Gamma-0.0987 nM, Delta-0.0232 nM, Omicron-1.46 nM)	Competing with ACE2 for RBD binding	Alpha, Beta, Gamma, Delta, Lambda (C.37), Kappa (B.1.617.1), Mu (B.1.621), and Omicron	–	[128]
1.10, 1.26, 1.29, 2.15	RBD of S protein	Monospecific and bispecific	Dromedary camels immunized with RBDmFc	Surface Display in Bacteria	HEK-293F cells	Nb - 3.96 nM (1.10), 0.26 nM (1.26), 12.9 nM (1.29), 0.39 nM (2.15); HCAb - 0.77 nM (1.10), 0.08 nM (1.26), 0.63 nM (1.29), 0.08 nM (2.15)	–	–	Competing with ACE2 for RBD binding	WA1, Alpha, Beta, Gamma, Delta, Kappa, Zeta	–	[129]
VHH72	RBD of S protein	Multivalent (bivalent, tetravalent, hexavalent)	Llama immunized with SARS-CoV-2 S glycoprotein	Phage display	HEK293-6E cells	23 nM	3.3 ± 1.8 nM (bivalent), 0.34 ± 0.072 nM (tetravalent), 0.035 ± 0.003 nM (hexavalent)	–	Competing with ACE2 for RBD binding	Wild-type, Alpha, Beta	High valencies formatting of nanobody (hexavalent)	[103,118]
Nb12, Nb15, Nb17, Nb19,	RBD of S protein	Monomer, bivalent, trimer	Llama or nanomice immunized with	Phage display	WK6 cells (ATCC, 47078), Expi293 cells (Fc	Monomer - 8.15 nM (Nb15), 5.59 nM (Nb17),	Monomer - 11.7 nM (Nb12), 0.4 nM (Nb15), 0.6 nM	–	Interfering with ACE2–RBD associations	WA1, Alpha, Beta, Gamma	Creation of nanobody-	[91]

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Table 2 (*continued*)

Nanobody name	Nanobody target	Nanobody format	Source	Screening method	Expression system	Affinity to target (K_D)	Neutralizing pseudovirus (IC50)	Neutralizing SARS-CoV-2 (IC50)	Mechanism	Virus variants	Features	References
Nb30, and Nb56			RBD and/or S protein		conjugated nanobodies)	4.72 nM (Nb19), 3.26 nM (Nb56), 30.0 nM (Nb12), 6.55 nM (Nb30); Trimer - 16.4 pM (Nb15), <1 pM (Nb17), 35.1 pM (Nb19), 38.2 pM (Nb56), <1 pM (Nb12), 820 pM (Nb30)	(Nb17), 0.3 nM (Nb19), 6.9 nM (Nb30), 0.9 nM (Nb56); Bivalent - 0.7 nM (Nb12-llama), 0.5 nM (Nb12-human), 0.3 nM (Nb15-human), 79 pM (Nb17-human), 66 pM (Nb19-human), 1.9 nM (Nb30-llama), 1.8 nM (Nb30-human), 0.1 nM (Nb56); Trimer - 0.1 nM (Nb12-human), 65 pM (Nb12-llama), 0.1 nM (Nb15-human), 18 pM (Nb17-human), 9 pM (Nb19-human), 55 pM (Nb56-human)				producing mice - nanomice	
RBD1i13, RBD3i17, RBD6id, RBD10i10, RBD10i14, RBD11i12	RBD of S protein	Monovalent, bivalent (Fc fusion)	A synthetic yeast-displayed library of nanobodies	Autonomous hypermutation yeast surface display	Expi293 cells	32.2 nM (RBD1i13), 230 nM (RBD3i17), 263 nM (RBD6id), 2.14 nM (RBD10i10), 0.72 nM (RBD10i14), 316 nM (RBD11i12)	0.05 μg/ml (RBD1i13), 0.116 μg/ml (RBD3i17), 0.056 μg/ml (RBD6id), 0.19 μg/ml (RBD10i10), 0.42 μg/ml (RBD10i14), 0.04 μg/ml (RBD11i12)	–	–	–	Autonomous hypermutation yeast surface display (AHEAD)	[109]
W25	RBD of S protein	Monovalent (nanobody, monomeric Fc fusion), bivalent (Fc fusion)	Alpaca immunized with full S protein	bacterial display	Priplasmic expression the *E. coli* wk6 strain (W25) ExpiCHO cells (W25Fc, W25FcM)	~ 0.3 nM (RBD)	–	W25 - 9.82 ± 1.92 nM (D614), 5.09 ± 1.09 nM (G614 variant); W25FcM - 27.40 ± 8.38 nM (D614), 12.36 ± 2.84 nM (G614); W25Fc - 7.39 ± 2.39 nM (D614), 3.69 ± 0.96 nM (G614)	Competing with ACE2 for RBD binding	D614 variant, G614 variant	–	[109,130]
Nb20, Nb21, Nb34, Nb105, Nb95, Nb17, Nb36	RBD of S protein	Monovalent, homodimeric, homotrimeric, heterodimeric	Llama immunized with RBD-Fc fusion protein	Proteomic identification of high-affinity RBD-Nbs	BL21 (DE3) cells	10.4 pM (Nb20), <1 pM (Nb21)	0.102 nM (Nb20), 0.045 nM (Nb21), 1.991 nM (Nb34), 5.105 nM (Nb105), 10.05 nM (Nb95), 1.106 nM (Nb17), 1.563 nM (Nb36)	0.048 nM (Nb20), 0.022 nM (Nb21), 1.125 nM (Nb34), 5.070 nM (Nb105), 5.105 nM (Nb95), 1.500 nM (Nb17)	Sterically interfering with ACE2 binding (directly for class I nanobodies, or indirectly for class II nanobodies), lock the spike in an all-RBD-up conformation or destabilize it (for class III nanobodies)	Alpha, Delta	Proteomic identification of high-affinity RBD-Nbs, resist resistance of nanobody to lyophilization and aerosolization	[101,105]

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Table 2 (*continued*)

Nanobody name	Nanobody target	Nanobody format	Source	Screening method	Expression system	Affinity to target (K_D)	Neutralizing pseudovirus (IC50)	Neutralizing SARS-CoV-2 (IC50)	Mechanism	Virus variants	Features	References
WNb 2, WNb 7, WNb 15, WNb 36	RBD of S protein	Monovalent, bivalent (Fc fusion)	Alpacas immunized with coronavirus spike and receptor-binding domains (RBD)	Phage display	–	0.14 - 19.49 nM		Wild type - 0.33 nM (WNbFc 2), 3.18 nM (WNbFc 7), 2.55 nM (WNbFc 15), 0.10 nM (WNbFc 36); N501Y D614G variant - 0.30 nM (WNbFc 2), 5.04 nM (WNbFc 7), 4.91 nM (WNbFc 15), 0.11 nM (WNbFc 36)	Interfering with ACE2–RBD associations	Wild type and the N501Y D614G variant	–	[131]
C5, H3, C1, F2	RBD of S protein	Monomeric, dimeric (Fc fusion), trimeric	Llama immunized with RBD, RBDFc, and/or S protein	Phage display	WK6 *E. coli* strain (monomeric), Expi293® cells (dimeric, trimeric)	RBD - 615 pM (C1), 99 pM (C5), 25 pM (H3), 40 pM (F2), 18 pM (C5 trimer), 0.3 pM (H3 trimer), 53 pM (C1 trimer)	–	18 pM (C5 with Victoria), 25 pM (C5 with Alpha)	Interfering with ACE2–RBD associations	Victoria, Alpha, Beta	–	[65]
DL28	RBD of S protein	Monomeric, dimeric (Fc fusion)	Alpaca immunized with RBD	Phage display	*E. coli* (monomeric), Expi293 suspension cells	1.56 nM	–	5.39 nM (Wild type), 4.61 nM (Alpha), 13.95 nM (Beta), 17.16 nM (Gamma), 21.88 nM (Delta), 8.68 nM (Omicron)	Keeping an RBD loop in a conformation incompatible with ACE2-binding	Wild type, Alpha, Beta, Gamma, Delta, Omicron	–	[65,66]
H7, G12	RBD of S protein	Dimeric (Fc fusion)	Synthetic humanized VHH library	Phage display, affinity maturation by CDR1 and CDR2 shuffling	FreeStyle™ 293-F cells	4.485 nM (H7-Fc), <1 pM (G12 × 3-Fc)	–	D614G variant - 133.8 ng/ml (H7-Fc), 13.1 ng/ml (G12 × 3-Fc); Delta - 12.3 ng/ml (H7-Fc), 0.9 ng/ml (G12 × 3-Fc); Omicron BA.1 - 106 ng/ml (H7-Fc), 9.6 ng/ml (G12 × 3-Fc)	–	Wild type, D614G variant, Delta, Omicron BA.1	–	[66,132]
C5G2	RBD of S protein	Monovalent	Synthetic nanobody library	Phage display-screening and affinity maturation	*E. coli* BL21 (DE3)	1.62 nM	1.59 nM (Wild type), 0.95 nM (Alpha), 0.56 nM (Beta), 1.06 nM (Gamma), 0.3 nM (Omicron)	–	Binding to RBD at a conserved region and the neighboring NTD domain through its CDR3, and inhibit ACE2 binding by FR2 due to steric hindrance	Wild type, Alpha, Beta, Gamma, Omicron	One nanobody wit triple functions	[132,133]

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Table 2 (*continued*)

Nanobody name	Nanobody target	Nanobody format	Source	Screening method	Expression system	Affinity to target (K_D)	Neutralizing pseudovirus (IC50)	Neutralizing SARS-CoV-2 (IC50)	Mechanism	Virus variants	Features	References
Nb-007	RBD of S protein	Monomeric, dimeric (Fc fusion)	Alpaca immunized subcutaneously with RBD	Phage Display	*Brevibacillus choshinensis* SP3 cells/ *E. coli* BL21 (DE3) (monomeric), 293 T cells (Fc fusion)	67.4 pM	Nb-007 - 37.6 nM (Wild type), 8.13 mM (Beta), 1.07 mM (Delta); Nb-007-Fc - 1.64 nM (Wild type), 42.6 nM (Delta)	–	Competing with ACE2 for RBD binding	Wild type, Beta, – Delta		[133,134]

RBD-ACE2 interface-targeting nanobodies that are ineffective against E484 or N501 substitution-carrying variants, but their homotrimeric versions showed fully neutralization activity against these variants, probably due to approximately-two orders of magnitude improvement in affinity [91]. However, high affinity nanobodies will still undergo dissociation from the targets because of their reversible interaction. To address this issue, Yu et al. designed latent bioreactive amino acid (FFY)-incorporated covalent nanobodies, which bind irreversibly to viral S protein due to the FFY-mediated proximity-enabled reactive therapeutic (PERx) mechanism, and their viral neutralization potency was enhanced drastically by the FFY incorporation [94].

6.2. Simultaneous engagement of multiple spatially discrete non-overlapping epitopes

As mentioned above, nanobodies targeting different epitopes block the SARS-CoV-2 invasion through different mechanisms. The simultaneous application of nanobodies with different epitopes as multivalent formatting or cocktails can exert a synergistic effect, resulting in greatly improved avidity and neutralization potency, as well as broad-spectrum protection against SARS-CoV-2 variants, and ultimately mitigates viral escape [14,69,70,95]. It is known that single-domain nanobodies, which are only one tenth the size of conventional antibodies, are superior in simultaneously binding discrete non-overlapping epitopes because of the lower chance of steric clash [96]. In fact, it is rare to see conventional antibodies binding to the same RBD simultaneously, as they will clash with each other and/or with other RBDs [96]. Notably, multivalent nanobodies are typically generated by genetic fusions that allow only C-to-*N*-terminal assembly, which may interfere with the function of their complementary determining regions (CDRs). Site-specific C-to-C-terminal fusion of nanobodies can be achieved by chemical approaches mediated by π-Clamp [97], or 4-arm PEG [98], etc. (Fig. 2B). It is worth noting that although both multivalent nanobodies and cocktails can be effective in suppressing mutational escape, the neutralization efficacy of multivalent nanobodies is generally more potent than the latter, and in one report they differ by as much as fourfold [99,100]. Moreover, the order of the nanobodies in the multivalent construct is also critical for efficacy, as this may affect how it binds to the trimeric S protein [70].

6.3. Targeting evolutionarily conserved epitopes of SARS-CoV-2 variants

As previously mentioned, under the selective pressure of NAbs, the ACE2-binding region RBM is the primary region for convergent mutations in current circulating variants and likely to be the case for future new variants [61,83,101]. Therefore, nanobodies targeting this region may easily lose their neutralizing activity, suggesting the importance of isolating nanobodies targeting conserved epitopes. Epitopes of SARS-CoV-2 NAbs can be classified into three types: ACE2 RBM, cryptic epitopes in the trimeric interface, and lateral surface epitopes [99,102]. Binding of non-RBM-targeting NAbs was reported to be highly correlated with Omicron neutralizing potency, suggesting conservatism of non-RBM epitopes. Indeed, the potency of the lateral surface epitope targeting nanobody n3113v and the trimeric interface cryptic epitopes targeting nanobody n3130v were less affected by the Omicron mutations [99]. In addition, several other studies have also confirmed that inhibition of mutational escape and the broadening of neutralizing potency against variants can be achieved by nanobodies targeting conserved epitopes [61,91,99,101,103,104], some of which are inaccessible to conventional antibodies [91,101].

6.4. Convenient and effective methods for generating potent neutralizing nanobodies

Typically, nanobody generation entails immunizing camelids and the subsequent isolation of peripheral blood lymphocytes, from which the variable regions of nanobodies are cloned to generate a phage display

immune library, which involves large animal rearing. Moreover, all the above steps will take approximately 9–15 weeks, which is time consuming [10]. Therefore, it is challenging to cope with the rapidly evolving SARS-CoV-2 variants using traditional ways of nanobodies generation. Because of the intense arms race between virus evolution and the development of effective vaccines and targeted drugs, it is crucial to develop reliable new methods for rapid screening and mass production of effective nanobodies against the evolving SARS-CoV-2 variants.

In addition to typical display-based library screening methods, proteomics has also been used to identify high-affinity nanobodies. Nanobody-containing HCAbs were purified from RBD-immunized llama plasma using protein G and protein A Sepharose beads and analyzed by mass spectrometry after proteolysis. Finally, thousands of RBD specific candidate nanobodies were identified, of which 109 nanobodies were characterized with picomolar to femtomolar affinities [105]. Similarly, Mast et al. generated a large repertoire of nanobodies against SARS-CoV-2 as well as the emerging VOCs using proteomics combined with *in vivo* antibody affinity maturation, that is, the LC-MS/MS results were used to search against the sequence library generated by amplifying nanobody domain sequence using lymphocyte RNA derived from bone marrow aspirates [96].

To avoid the use of large animals that are expensive and potentially involved in animal welfare, Xu et al. created a nanobody-producing mouse (referred to as nanomice) by replacing the VH domain with 30 VHH genes from alpacas, dromedary, and Bactrian camel in mouse embryonic stem cells, which were transferred into the uteri of pseudo-pregnant C56BL/6 recipients, followed by mating and isolation of VHH-homozygous nanomice, from which they isolated high-affinity RBD-targeting nanobodies [91]. Alternatively, synthetic nanobodies (sybodies) libraries allow for faster and cheaper animal-free isolation of nanobodies without using animals with a higher degree of control [106]. Several sybodies with high neutralization activity [63,107], some of which are humanized, have been selected against RBD [108].

Commonly, synthetic nanobody library screening requires large library size and well-established screening methods, mainly due to the lack of *in vivo* affinity maturation mediated by somatic hypermutation. To overcome these limitations while maintaining the animal-free advantage, researchers reported autonomous hypermutation yeast surface display (AHEAD), a highly accessible synthetic recombinant nanobody generation technology that emulates hypermutation using yeast. The core component - an orthogonal error-prone DNA polymerase, replicated nanobody fragments containing cytosolic plasmid (p1) to produce durable continuous hypermutation of p1-encoded nanobody, and the resulting nanobodies were surface displayed on yeast for antigen binding screening. High affinity nanobodies against S protein were rapidly generated within two weeks using AHEAD [109]. Notably, this technology not only facilitates the rapid evolution of previously discovered nanobodies, but also enables isolation of brand-new nanobodies against a given antigen.

Most cell-based screening methods are often restricted by the efficiency of DNA library transformation, which is not present in cell-free approaches such as ribosome display. Chen et al. reported CeVICA, a ribosome display based cell-free nanobody engineering platform, which enables the rapid isolating diverse nanobodies with favorable biophysical properties, especially high refolding capability, probably due to the lack of factors like chaperones in the cell-free environment, which favors the selection of antigen-binding nanobodies with strong inherent folding stability [110].

To accelerate the generation of potent neutralizing nanobodies, affinity maturation can be applied based on isolated nanobodies with low or moderate affinity, primarily by error-prone PCR-mediated random mutagenesis [110], or site saturation mutagenesis generated by incorporation of degenerate base combinations [14], or a newly reported CDR-swapping mutagenesis [111] through which high-affinity nanobodies can be generated simply using common-framework, non-immune nanobody libraries. In addition, computational methods have been used to predict, refine, or design nanobodies with high efficacy based on structural information, however, there is still a long way to go [100,104,112,113].

7. Concluding remarks

Nanobodies are promising for detection and treatment of SARS-CoV-2 due to their small size, high stability, cryptic epitope accessibility, and low production costs. At present, a large number of nanobodies with excellent performance have been isolated against SARS-CoV-2, and studies have shown that strategies such as targeting cryptic and conserved epitopes, simultaneous targeting different epitopes, and nanobody multimerization can enhance their efficacy and reduce mutational escape. However, to date, the vast majority of nanobodies are directed against the same target - RBD, which makes it imperative to explore the details of the SARS-CoV-2 life cycle, especially for the emerging variants, including their invasion, replication, packaging, etc., and to discover new target proteins. An excellent recent study probed into SARS-CoV-2–human protein–protein interactome network using high-throughput yeast two-hybrid and mass spectrometry, and obtained high-confidence interactions which provides potential therapeutic targets for nanobodies [114].

This paper summarizes the strategies currently used, and it is worth noting that efficacy can often be maximized by combining different strategies, including new methods and technologies that are constantly emerging. For example, proteolysis-targeting chimeric (PROTAC) technology has been reported to be used to generate a live attenuated influenza A vaccine with high efficacy [115], and PROTAC-mediated attenuation of SARS-CoV-2 and its variants may also be achieved by conserved epitopes-targeting nanobodies. All in all, although not an easy way to go, the dawn has come, and we believe the conquest of COVID-19 is near in the future.

CRediT authorship contribution statement

Wenyi Wang: Supervision, Funding acquisition, Conceptualization, Investigation, Visualization, Methodology, Writing - original draft, Writing - review & editing. **Yue Hu:** Investigation, Visualization, Writing - original draft. **Bohan Li:** Investigation, Visualization, Writing - original draft. **Huanan Wang:** Methodology, Writing - review & editing. **Jinhua Shen:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Fundamental Research Funds for the Central Universities, South-Central Minzu University (No. CZQ21001), and the Scientific Research Fund from South-Central Minzu University (No. YZZ20010). In particular, Dr. Wenyi Wang is grateful to Ms. Jie Dong for her full support of this work.

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