The role of single-domain antibodies (or nanobodies) in SARS-CoV-2 neutralization

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Abstract
The severe acute respiratory syndrome (SARS-CoV-2), a newly emerging of coronavirus, continues to infect humans in the absence of a viable treatment. Neutralizing antibodies that disrupt the interaction of RBD and ACE2 has been under the spotlight as a way of developing the COVID-19 treatment. Some animals, such as llamas, manufacture heavy-chain antibodies that have a single variable domain (VHH) instead of two variable domains (VH/VL) as opposed to typical antibodies. Nanobodies are antigen-specific, single-domain, changeable segments of camelid heavy chain-only antibodies that are recombinantly produced. These types of antibodies exhibit a wide range of strong physical and chemical properties, like high solubility, and stability. The VHH's high-affinity attachment to the receptor-binding domain (RBD) allowed the neutralization of SARS-CoV-2. To tackle COVID-19, some nanobodies are being developed against SARS-CoV-2, some of which have been recently included in clinical trials. Nanobody therapy may be useful in managing the COVID-19 pandemic as a potent and low-cost treatment. This paper describes the application of nanobodies as a new class of recombinant antibodies in COVID-19 treatment.

Keywords
Single-domain antibodies · Nanobody · COVID-19 · SARS-CoV-2

Introduction
From December 2019, the new coronavirus disease 2019 (COVID-19) has spread quickly around the world. Being reported in over 200 countries, it has claimed a large number of lives [1, 2]. As there is no effective medication for the severe acute respiratory syndrome (SARS-CoV-2), treating COVID-19 patients, particularly those with severe pneumonia, is challenging [3]. Neutralizing antibodies are critical for SARS-CoV-2 immunity as well as COVID-19 prevention and treatment [4]. Although monoclonal antibody-based therapy can be useful for those with mild COVID-19 symptoms, it requires exceedingly large doses, typically a few grams intravenously [5, 6]. Moreover, they cannot be produced fast or economically, and are unable to target several epitopes at the same time [7]. Camelid antibodies with a single variable domain (also known as nanobodies/VHH) are a practical alternative, as they are tiny in size (13 to 15 kDa), and have excellent solubility, and stability. They are particularly well suited for pulmonary administration [8, 9]. VHH's high-affinity compete with the receptor-binding domain (RBD) of viral spike, enabling SARS-CoV-2 neutralization. Therefore, the S1 RBD has become a prominent target for vaccine research [10, 11]. The first stage of a viral life cycle is entering a host cell [12, 13]. The interaction of the RBD of the viral spike with the cellular receptor, known as angiotensin-converting enzyme 2 (ACE2), is required for SARS-CoV-2 cell entry [14, 15]. Besides, Nbs that target chemokines and cytokines can be customized to modify inflammation responses, which could be conducive to COVID-19 patients' recovery [16]. In this review, we elaborate on the use of nanobodies, a new type of recombinant antibody, as a treatment for COVID-19.
SARS-CoV-2 characteristics

An unknown respiratory tract infection began in Wuhan, China, in the autumn of 2019. Based on the patients’ symptoms, clinicians diagnosed virus-induced pneumonia [17]. According to genomic sequencing, this pneumonia was caused by a novel coronavirus [18]. The international committee on taxonomy of viruses (ICTV) named this unexpected coronavirus SARS-CoV-2 [19]. As the seventh coronavirus, SARS-CoV-2 is able to infect humans [20]. The SARS-CoV-2 is an enveloped, non-segmented, positive-sense single-stranded RNA virus with a genomic size of about 30 kb [22], which encodes both structural and non-structural proteins. The non-structural proteins such as Papain-like protease, 3C-like serine protease (3CL-protease), RNA-dependent RNA polymerase, Helicase, Endoribonuclease, and the structural proteins are spike glycoprotein (S), an envelope protein (E), membrane protein (M), and nucleocapsid protein (N). The coronavirus spiral nucleocapsid is covered by phospholipid bilayers with M/E proteins, and the trimmers of S protein are found on the virus particle’s surface [21].

The S protein of coronaviruses is a trimeric class I viral fusion protein. It consists of two subunits: S1 (in the amino-terminal) that contains RBD, and S2 (in the carboxy-terminal) that induces membrane fusion (Fig. 1) [22]. The RBD of the SARS-CoV-2 S protein mediates cell entry by attaching to human angiotensin-converting enzyme 2 (ACE2). The ACE2 is a transmembrane protein placed on the epithelial cells of the nasal mucosa, lungs, heart, kidneys, stomach, bladder, and intestine [23]. The dissociation of S1 from ACE2 is induced by receptor contact, causing the transition of S2 from a metastable pre-fusion mode to a stable post-fusion mode, which is essential for membrane fusion [24]. The S protein can be a target of antibody-mediated neutralization due to its crucial function, and the characterization of the pre-fusion S structure can provide useful information for vaccine formulation and development [24, 25].

Virus mutations and variants are major impediments to the control of the SARS-CoV-2 pandemic and the development of an effective vaccine. The coronavirus will probably mutate during the replication phase, which will alter the virus’s behavior as the genomes of RNA viruses are intrinsically unstable [26]. The mutation E484K, called an escape mutation, takes place in diverse variants identified in Brazilian (B.1.1.28), South African (B.1.351), and UK (B.1.1.7). The E484K mutation identified in the RBD is associated with the ability to evade neutralizing antibodies and the body’s immunological responses. This mutation can influence vaccine efficacy. The E484K mutation increases the number of serum antibodies needed to protect cells from infection [27, 28]. Another mutation in the spike is the N501Y, which in the case of SARS-CoV-2, appeared in Brazilian, South African, and United Kingdom variants [29]. The SARS-CoV-2 RBD, with the N501Y mutation, is linked to the improved receptor binding specificity and the virus growth speed. The SARS-CoV-2 with N501Y mutation was tested in the mouse model and increased infectivity and virulence were reported [29]. Several mutations in the Spike’s S subunits have been reported in India, including the D614G. In addition, G1124V in the Spike (S2 subunit) protein has been recognized as a nonsynonymous mutation [30]. SARS-CoV-2 features can be altered by any of these spike mutations.

What are nanobodies

Nanobodies represent a relatively new type of recombinant antibody. The Camelidae members, which include ancient species like dromedaries and camels as well as more recent species such as llamas and alpacas, can produce
non-conventional antibodies besides conventional antibodies [31, 32]. The conventional (classical) antibodies produced by all mammals have a heterotetrameric structure with two heavy and two light chains, but non-conventional antibodies are only composed of two heavy chains and have a single variable domain (VHH, k15kDa), which is the antigen-binding region (Fig. 2a and b) [33]. Heavy-chain antibodies (HcAbs) are the third generation of antibodies that provided a new approach to the creation of therapeutic antibodies [32, 34]. Even though the lack of a light chain variable domain (VL) may seem disadvantageous in terms of antigen binding, nanobodies have evolved to compensate, generating characteristics that improve stability [33]. These antibodies’ variable parts contain intriguing features compared to other antibodies including higher solubility, small size, greater resistance to denaturation, stability in high temperatures, and high/low pH, cost-effective production, high specificity, low immunogenicity, ease of manipulation, and identification of variable epitopes [35, 36]. Furthermore, tissue penetration and extravasation are better in nanobodies than in classical monoclonal antibodies, which is of great therapeutic value [37].

The overall-architecture framework regions (FR1/2/3/4) and complementarity determining regions (CDR1/2/3) of nanobodies are the same as VHs. The CDR3 is a key factor in antigen recognition and specificity and its length are significantly greater in nanobodies than in human VH domains. The CDR1 and CDR2 are proteins that contribute to the binding strength [38]. However, human immunogenic reactions could be triggered by nanobodies in humans. Thus, for therapeutic purposes, humanization protocols have been developed to identify the aminoacid sequences in the framework regions that correspond to their human heavy-chain variable domain counterparts [39, 40]. The first adult medication developed based on the single-domain antibody (sdAb) was approved in November 2018 by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) [38, 41]. The antiviral effects

![Fig. 2 Nanobodies neutralize the SARS-CoV-2.](image)

- **Fig. 2** Nanobodies neutralize the SARS-CoV-2. **FC** Fragment crystallizable, **Fab** fragment antigen-binding, **VH** Heavy chain variable domain, **VL** Light chain variable domain, **CH** heavy chain constant region, **CL** Light chain constant region, **ACE2** angiotensin-converting enzyme 2. SARS-CoV-2 neutralization was made possible by inhibiting the target cell entrance. VHHs bind to the spike RBD and prevent the ACE2 engagement with RBD.
of nanobodies against respiratory viruses such as coronavirus have been demonstrated in a host of investigations [42]. The SARS-CoV-2 spike protein has been used to immunize some animals and discover nanobodies that interact with the virus’s receptor-binding region [43].

Nanobodies against SARS-CoV-2

There are some effective diagnostic procedures for the diagnosis of SARS-CoV-2 infection, but no therapy that can interfere with SARS-CoV-2 replication has been identified so far [44]. Antibody treatment is one of the different methods for controlling COVID-19 infection [35]. The therapeutic potential of nanobodies against coronaviruses has been established in recent studies [45–47]. A primary target for anti-SARS-CoV-2 neutralizing antibodies involves blocking the interaction of the SARS-CoV-2 S1 protein with ACE2 [46–48]. The S1 RBD has been the main target of vaccine development, as SARS-CoV-2 can be neutralized by VHH’s high-affinity competition with the spike RBD (Fig. 2c) [10, 11]. Several techniques are currently used to identify nanobodies that neutralize SARS-CoV-2, including llama immunization, the phage display of a naive llama nanobody library or humanized synthetic nanobody library, as well as the yeast surface display of synthetic nanobodies [23, 39, 40, 43, 49–54]. The developed nanobodies effectively neutralized both SARS-CoV-2 pseudovirus and live virus [55]. Recently, researchers in the United States have isolated nanobodies that bind to the receptor-binding domain of the SARS-CoV-2 spike protein and prevent the interaction of spike protein with ACE2 [50]. Furthermore, the results of an in vitro analysis suggest two nanobodies (H11-D4 and H11-H4) with high affinity to spike RBD protein that can prevent the spike’s attachment to ACE2 [40]. In another study, Hanke et al. discovered Ty1, a SARS-CoV-2 RBD-specific nanobody that effectively neutralizes the virus. The Ty1 nanobody has several practical advantages, including high-yield production in bacteria, low cost, and scalability. As suggested in the literature, future research needs to explore a range of techniques to improve Ty1’s potency and efficacy [54]. Three VHHs, H11-D4, H11-H4, and Ty1, have been identified to target the SARS-CoV-2 spike RBD and disrupt its interaction with ACE2 [56, 57].

Nanobodies have special biophysical characteristics, such as thermostability and tiny size, which enable their aerosolized administration. They can be readily nebulized and inhaled straight into the lungs by an inhaler [8, 58]. One of the advantages of inhalable nanobodies is antibodies like PiN-21 and Nb11-59, which can successfully target deep and local lung tissues such as terminal alveoli that share a border with alveolar cells rich in ACE2 receptors [34, 59]. Thus, inhalation therapy can be used to administer nanobody therapeutics [8], aside from other routes of delivery such as intravenous, intramuscular, or subcutaneous treatment [50].

Synthetic nanobodies (or sybodies)

Traditional nanobodies are extracted from immunized camelids, but creating libraries of synthetic nanobodies (sybodies) is a new technique for fast drug development that produces highly selective binders with neutralizing potentials in a short time [60]. In this context, another option is the chimeric nanobodies-Fc (in which the variable area of nanobody is bonded to Fc of human immunoglobulin). The synthetic nanobodies are tiny, aerosolizable and heat stable, which makes them a feasible option for COVID-19 prevention and therapy [60].

Stefan et al. designed a huge synthetic VHH library (3.18 × 10^10), finding over 50 VHH candidates that can bind to SARS-2 [61]. Schoof et al. (2020) identified a panel of Nbs that can bind to various epitopes on Spike from a synthetic Nbs library. These Nbs are divided into two groups. Class I binds directly to the RBD and competes with the ACE2 receptor on human cell surfaces. Class II, identified as a different binding site, contributes to the alteration of the RBD’s structural conformation, preventing it from the recognition of the ACE2 receptor [52]. Researchers have identified a new synthetic Nb called SR31. Stronger binding affinities and neutralizing activities of this synthetic nanobody have been reported in fusion with additional sybodies. Additionally, to reinforce affinity and efficacy, it can be coupled with monoclonal antibodies or other antibody fragments [62]. In the context of synthetic nanobodies, a recent study has used a mix of ribosome display and phage display. Accordingly, it managed to develop 99 distinct sybodies against the SARS-CoV-2 S-RBD from three libraries, which neutralized SARS-CoV-2 pseudo-viruses efficiently and selectively [63]. Synthetic or naive nanobodies including IE2, 2F2, 3F11, 4D8, 5F8, H11–D4, and H11–H4 can block RBD–ACE2 binding, and neutralize the pseudotyped and live SARS-CoV-2 infections under in vitro conditions through SARS-CoV-2 RBD-targeting [39, 40]. Custodio et al. reported that sybody 23 (Sb23) neutralized SARS-CoV-2 spike pseudovirus by competing with ACE2 binding. This type of synthetic nanobody, displaying a high affinity for the recombinant RBD and the prefusion spike glycoprotein, has strong neutralizing activities [64]. However, synthetic nanobodies have several limitations. As monomers, they often lack the high binding affinity required for therapeutic use. In this way, multivalent or multi-paratopic nanobodies might offer a faster way to exploit avidity in order to improve affinity and effectiveness [65, 66].
Multivalent nanobodies

In certain cases, SARS-CoV-2 undergoes rapid mutations, which seem to have avoided the antibody response [67–69]. The developing variations, B.1.1.7, B.1.351, and P.1 (first reported in the UK, South Africa, and Brazil, respectively) have undermined the effectiveness of serum in COVID-19 patients and immunotherapies approved for emergency use [70–72]. Multivalent nanobodies or variable domains of heavy-chain Abs have been developed by investigating the precise architectures of SARS-CoV-2 epitopes and binding modalities to the S protein of the virus, as well as fusing virus to the cell membrane through the S protein [43]. An in-silico technique that leads to the fusion of VHVs to Fc domains is currently utilized to create multi-specific antibodies with elevated avidity and affinity as well as enhanced S/ACE2 blocking [49]. The mutations of SARS-CoV-2 variant are countered by multivalent nanobodies via two mechanisms: amplified avidity for the binding domain of ACE2 and detection of preserved epitopes, which are chiefly not accessible to human antibodies [69]. Moreover, the serum half-life could be improved by the oligomerization of nanobodies [73]. In the S1 RBD and S/ACE2 inhibition of SARS-CoV-2, the bi-specific VH-FFc antibodies are significantly more effective than monoclonal VH-FFcs [49]. Ma et al. reported the isolation of seven anti-RBD Nbs from alpacas immunized with SARS-CoV-2 RBD. In their analysis, combining two Nbs with different epitopes led to the creation of two hetero-bivalent Nbs with high affinity, producing antibodies with significant SARS-CoV-2 neutralizing efficacy [11]. In line with these findings, the heterodimer nanobody Nb91-Nb3-hFc with an IC50 of 1.54 nM displayed the highest RBD-binding affinity and neutralizing activity against SARS-CoV-2 pseudo-viruses. By limiting the interaction of spike protein with ACE2 through RBD targeting, the neutralizing nanobodies were able to drastically reduce SARS-CoV-2 pseudo-virus infection in the host HEK293T-ACE2 cells [47]. Pymm et al. used four bivalent nanobodies to neutralize SARS-CoV-2 (WNbFc 2, WNbFc7, WNbFc 15, and WNbFc 36). They discovered that nanobody cocktails containing two noncompeting nanobodies could prevent ACE2 engagement with RBD variations commonly found in human populations and neutralize both wild-types of SARS-CoV-2 and the N501Y D614G RBD variant at low quantities [4]. Another study suggested that tri-specific VH-FFc antibodies as promising therapeutics for COVID-19 treatment and prevention. These tri-specific VH-FFcs were found to be particularly effective in binding to SARS-CoV-2 S1 RBD and blocking S/ACE2, while inhibiting the infection of human target cells by a SARS-CoV-2 pseudo-virus [44].

Researchers developed a VH-phage library and targeted the binding interface of the SARS-CoV-2 Spike receptor-binding domain’s angiotensin-converting enzyme 2 (ACE2) (Spike-RBD). They discovered VH binders for two non-overlapping epitopes, which were combined into multivalent and biparatopic forms. Compared to stand-alone VH domains, they demonstrated an increased affinity for spike (up to 600-fold) and neutralization efficacy (up to 1400-fold) on pseudo-typed SARS-CoV-2 virus [66]. Koenig et al. engineered multivalent nanobodies using more than 100 neutralizing activities of monovalent nanobodies, which targeted the receptor-binding domain of the SARS-CoV-2 spike protein. The biparatopic nanobody fusions inhibited the scope of viral mutants. By targeting two distinct epitopes, these multivalent nanobodies prevented SARS-CoV-2 infection and inhibited mutational escape [43].

A recent study by Zupancic et al. has suggested that a hexavalent VH-72 nanobody with excellent stability, solubility, and non-specific binding was successfully attached to spike proteins in SARS-CoV-2 variants with high transmission (B.1.1.7 and B.1.351) and neutralizes them effectively [74]. Another study discovered three new bispecific nanobodies (Nb15-Fc, Nb22-Fc, and Nb31-Fc) that could significantly inhibit the wild-type and variations of SARS-CoV-2, such as circulating forms, as was the case in mutant viruses found in the UK and South Africa with the N501Y mutation [75]. Sun et al. reported that SARS-CoV-2 neutralizing Nbs can be grouped into three epitope classes. Class I includes some of the most effective SARS-CoV-2 neutralizing Nbs discovered so far. However, a single point mutation (E484K/Q) found in Gamma (P.1), Beta (B.1.351), or Kappa (B.1.617.1) variations can prevent class I Nbs from binding to RBD. Class II Nbs are designed to target conserved epitopes resistant to existing variants of concern (VOCs) and are expected to resist mutation. Class III Nbs can bind to formerly unidentified high-affinity epitopes with strong affinities, such as the Nb17 epitope, which is likely inaccessible to conventional antibodies [76]. Thus, nanobodies can be a potential tool to neutralize SARS-CoV-2 variants, even if new mutations continue to develop. Table 1 outlines a summary of similarities and differences in published nanobodies.

Nanobodies modulate inflammation

COVID-19 hyper-inflammation, one of the uncontrolled systemic inflammations and a key process in acute respiratory distress syndrome (ARDS), is also known as a cytokine storm. It takes place when copious numbers of pro-inflammatory cytokines (e.g. TNF, IL-1, and IL-6) and chemokines (e.g. CXCL-10) are released [77–79]. SARS-CoV-2 S-interacting proteins expressed in myeloid cells can operate as signaling receptors to activate particular hyperinflammatory responses. Also, they play a key role in COVID-19 immunopathogenesis and immunological dysregulation. Researchers manufactured a bispecific anti-spike nanobody,
A8-G11-Fc, which inhibited both ACE2-mediated infection and myeloid receptor-mediated proinflammatory responses [80].

The use of monoclonal antibodies to target and neutralize proinflammatory cytokines provides a potential therapeutic approach for inflammatory disorders. Nanobodies offer novel tools to modulate inflammatory responses in COVID-19 patients [81]. By targeting chemokines and cytokines, they can be adjusted to control inflammation responses, which contributes to COVID-19 patients' recovery [16]. For example, the isolation of anti-CXCL10 polyclonal HcAbs has been conducted to create specific Nbs, which can selectively target CXCL10 for in vivo therapies [82]. TNF-alpha is a critical cytokine and TNF-blocking sdAbs have been reported in a llama immunized with human and mouse antigens. It has been shown to be more effective than TNF-blocking antibodies like Infliximab and Adalimumab in TNF neutralization [83]. Furthermore, antigen presenting through antigen presentation cells (APCs) enhances antibody formation, CD4+ T cell activation, and CD8+ T cell responses, all of which help boost immunological responses. APCs contain high levels of MHC-II products, integrins (CD11b), and scavenger receptors (CD36). VHHs targeting these molecules improved immune responses in dendritic cells (DCs), induced humoral immunity, and detected inflammation in order to cure or prevent SARS-CoV-2 infection [16, 84].

According to the evidence, nanobodies can act as an ion-channel blocking agent. P2X7 is a ligand-gated ion channel expressed by lymphocytes and monocytes. This channel triggers a pro-inflammatory signaling cascade when detecting adenosine 5′-triphosphate produced by injured cells. It involves the production of pro-inflammatory cytokines like interleukin-1beta (IL-1β), IL-18, and IL-33 [85–87]. This ion channel is a potential therapeutic target in inflammatory diseases [88]. A variety of nanobodies capable of regulating the function of human and mouse P2X7 have been identified [87]. For example, the nanobody Dano1 has exhibited complete effectiveness in blocking human P2X7 [89]. This nanobody was found to be 1000 times more effective than small molecule inhibitors of P2X7 at blocking ATP-induced IL-1β release from monocytes [87]. In this way, nanobodies pave the way for novel experimental and therapeutic immunomodulation approaches.

| Nanobody       | Method                        | Neutralizing pseudovirus (IC50) | Affinity to RBD   | Function                                                                 | References |
|----------------|-------------------------------|---------------------------------|-------------------|--------------------------------------------------------------------------|------------|
| VHH-E          | Immunized phage display library | 60 nM                           | 1.86 nM           | Blocking RBD-ACE2 interaction/prevent the emergence of viral escape mutants | [90]       |
| Nb11-59        | Immunized phage display library | 36.7 nM                         | 21 nM             | Blocking RBD-ACE2 interaction/high binding activity to the RBD            | [34]       |
| Sybody (n3021) | Ribosome and phage display    | –                               | 0.63 nM           | Blocking RBD-ACE2 interaction/binding to the full-length SARS-CoV-2 spike protein | [91]       |
| Sybody (MR3)   | Ribosome and phage display    | 40 nM                           | 24.22 nM          | Blocking RBD-ACE2 interaction                                            | [92]       |
| Nb6            | yeast surface-displayed library | 2 uM                            | 210 nM            | Blocking RBD-ACE2 interaction/Binding Spike in a fully inactive conformation with its receptor binding domains | [93]       |
| H11-D4         | naive llama single-domain antibody library | –                             | 39 nM             | Blocking RBD-ACE2 interaction/Binding to all three RBDS in the spike trimer | [94]       |
| H11-H4         | naive llama single-domain antibody library | –                             | 12 nM             | Blocking RBD-ACE2 interaction/Binding to all three RBDS in the spike trimer | [94]       |
| Ty1            | Immunized phage display library | 54 nM                           | 5–10 nM           | Blocking RBD-ACE2 interaction/Binding to the RBD with high affinity       | [95]       |
| Nbs 89         | Immune library and MS proteomic | 0.133 nM                        | 108 pM            | Blocking RBD-ACE2 interaction/inhibit viral infection                    | [96]       |
| WNe            | Immunized phage display library | –                               | ≤ 80 nM           | Blocking RBD-ACE2 interaction/neutralize both wildtype SARS-CoV-2 and the N501Y D614G variant | [4]        |
Conclusion
Tiny, stable, and easy to make, the Nbs show great potentials as COVID-19 therapeutic proteins. To develop suitable nanobodies against SARS-CoV-2, the bulk of studies have employed the spike protein, especially RBD domains. Given that nanobodies can be utilized as an inhaler, it seems that the produced nanobodies can be applied to inhibit the infection of the lungs by the virus. Developing methods with effective nanobodies, high-expression yield and reasonable costs is crucial to control the COVID-19 pandemic.

Data availability
The data used to support the findings of this study are included in the article.

Declarations
Conflict of interest
The authors declare that there is no conflict of interests.

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

Consent for publication
All of the authors consent for publication.

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