ACS Pharmacology & Translational Science

pubs.acs.org/ptsci

Review

Emerging Landscape of Nanobodies and Their Neutralizing Applications against SARS-CoV-2 Virus

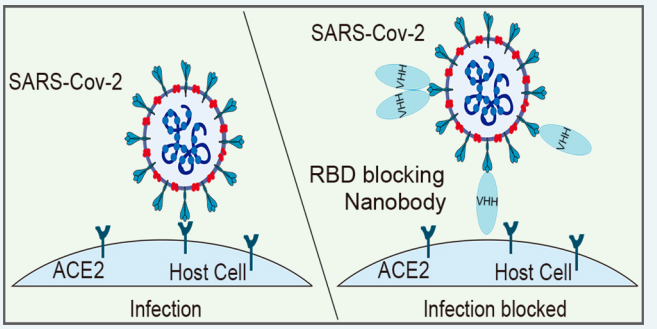
Xuemei Feng and Hu Wang*

Cite This: ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: The new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes the coronavirus disease 2019 (COVID-19) has significantly altered people's way of life. Despite widespread knowledge of vaccination, mask use, and avoidance of close contact, COVID-19 is still spreading around the world. Numerous research teams are examining the SARS-CoV-2 infection process to discover strategies to identify, prevent, and treat COVID-19 to limit the spread of this chronic coronavirus illness and restore lives to normalcy. Nanobodies have advantages over polyclonal and monoclonal antibodies (Ab) and Ab fragments, including reduced size, high stability, simplicity in manufacture, compatibility with genetic engineering methods, and lack of solubility and aggregation issues. Recent studies have shown that nanobodies that target the SARS-CoV-2 receptor-binding domain and disrupt ACE2 interactions are helpful in the prevention and treatment of SARS-CoV-2-infected animal models, despite the lack of evidence in human patients. The creation and evaluation of nanobodies, as well as their diagnostic and therapeutic applications against COVID-19, are discussed in this paper.



KEYWORDS: *COVID-19, SARS-CoV-2, nanobodies, neutralization, target, detection*

An extraordinary public health disaster has been brought on by the coronavirus disease 2019 (COVID-19), due to the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 infection manifests as flulike symptoms such as fever, coughing, dyspnea, runny nose, sore throat, headache, and exhaustion. Around 5% of COVID-19 patients develop severe cases of pneumonia, acute lung injury, or acute respiratory distress syndrome; these conditions are closely related to the high mortality rate of COVID-19. Around 80% of COVID-19 patients have mild or asymptomatic symptoms and do not need to be hospitalized.[1] More than 6.56 million people have died because of the COVID-19 epidemic, infecting 622 million individuals globally. However, it is anticipated that SARS-CoV-2 variations will remain in circulation for a number of years, increasing the potential of major illnesses and fatalities brought on by COVID-19 as viral evolution continues.[2,3]

In addition to the worldwide health issue brought on by COVID-19, the global financial system is also significantly impacted. Despite the fact that SARS-CoV-2 vaccines had previously been developed and given to people all over the world, recent research suggested that no vaccine can achieve 100% effectiveness to prevent COVID-19 in some conditions, such as no or weak immune response to a certain vaccine in some populations, as well as some fully vaccinated individuals still contracting COVID-19 through vaccine breakthrough infection.[4,5] Therefore, it is extremely vital to develop vaccinations, treatments, and diagnostics to stop the COVID-19 pandemic.

Nirmatrelvir (commercialized as Paxlovid, a combination with another HIV protease inhibitor ritonavir), a SARS-CoV-2 main protease (Mpro) inhibitor developed by Pfizer, was approved for treatment of COVID-19. Although it lowers the risk of hospitalization or death by 89% in individuals with mild to moderate COVID-19 and is effective against Omicron, more and more reports suggested that patients experienced a rebound of COVID-19 symptoms after treatment with Paxlovid and progressed to severe COVID-19.[6] There is currently limited information available on additional treatments for COVID-19 rebound following Paxlovid treatment.

Unprecedented amounts of governmental attention and financial support have been given over the past three years to the quick development of COVID-19 diagnostic, preventative, and treatment measures. The development of antibodies as

Received: March 2, 2023
Published: June 5, 2023

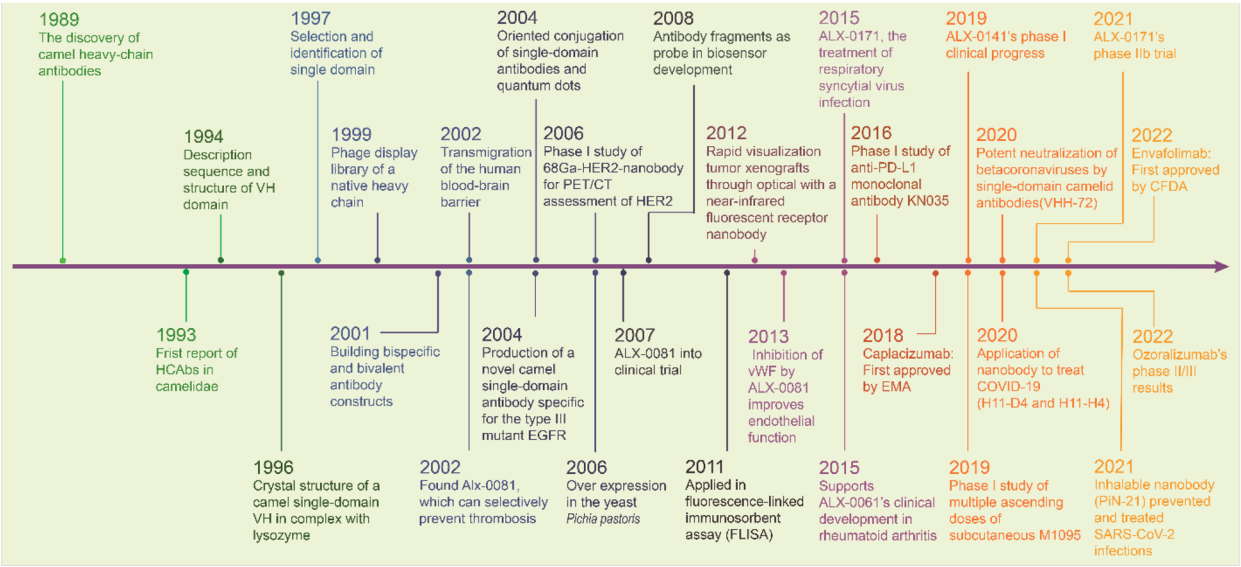


Figure 1. Timeline of historical nanobody development. This timeline demonstrates key time points in the development of nanobodies; major improvements in the same category are illustrated in the same color.

targeted molecules to fight off infections has a long history that dates back over a century (Figure 1). Due to their great specificity and adaptability, monoclonal antibodies (mAbs) are unquestionably in the vanguard of the fight against COVID-19, even though therapeutic mAbs have been widely used to treat a variety of illnesses, including viral infections, and in molecular imaging, drug discovery, and therapeutics for a variety of diseases, including autoimmune, cardiovascular, and infectious diseases, cancer, as well as inflammation (summarized in Figure 2).[7] However, compared to polyclonal antibodies and mAbs, nanobodies have distinct advantages, such as smaller size, high stability, ease of production, compatibility with genetic engineering techniques, strong antigen-binding affinity, water solubility, and natural origin, and lack of solubility and aggregation issues. They are particularly suited for the development of next generation biotherapeutics due to their outstanding features. The nanobody and its variants have been widely used in the biomedical research community, as well as in the biotech and pharmaceutical industries, over the past several decades (Figure 3). The United States Food and Drug Administration (FDA) approved the first nanobody from Sanofi named caplacizumab for acquired thrombotic thrombocytopenic purpura (a rare disease characterized by excessive blood clotting in small blood vessels) in 2019, and several other nanobodies targeting human diseases, including inflammation, cancer, respiratory, and infectious diseases, are currently being studied in clinical settings. This is exciting news that raises hopes for academia and industry that are exploring novel uses for COVID-19 control and prevention.

FEATURES OF SARS-COV-2

The betacoronavirus genus includes the single-stranded RNA virus known as SARS-CoV-2. When the viral S protein attaches to a host cell receptor like ACE2 or CD147,[8] that is the first crucial moment. Through the interaction of the spike glycoprotein with the angiotensin-converting enzyme 2 (ACE2) receptor, SARS-CoV-2 penetrates host cells where it multiplies and kills tissues and organs.[9] Since the SARS-CoV-2 receptor-binding domain (RBD) has a far greater affinity for ACE2 than it does for host cells, it is more likely to efficiently infect tissues and contribute to pathogenesis.[10] A crucial stage in the viral infection of humans is the attachment of the SARS-CoV-2 RBD to ACE2 in host cells. After the initial binding event, host proteases like furin, transmembrane serine protease 2 (TMPRSS2), and cathepsin L cleave the head of the S protein, transforming it into a springlike structure. This action enables the viral membrane to fuse with the host membrane and enables direct cell surface entry or via endosome by endocytosis.[11] When a virus enters a host cell, its RNA is translated, and the host then expresses type I/III interferon, chemokines, and cytokines like tumor necrosis factor (TNF), interleukin 1 beta (IL-1), interleukin 6 (IL-6), and granulocyte-macrophage colony-stimulating factor (GM-CSF) to immediately trigger the innate immune response (GM-CSF).[7,12,13] The cytokine levels may keep increasing with continuous virus replication, causing significant tissue damage and cytokine release syndrome (CRS) in certain individuals.[14]

CURRENT ANTIBODY TREATMENT FOR COVID-19

SARS-CoV-2 therapy approaches fall into three main categories: blocking viral entry, obstructing virus replication and survival in the host cell, and managing immune response. Current antiviral research on 2019-nCoV mostly focuses on neutralizing the virus, with antibodies primarily focusing on the RBD to inhibit specific virus recognition and ACE entry into host cells.[15]

Monoclonal antibodies (mAbs), which have great specificity and dependability, have become effective instruments for diagnosing and treating a wide range of disorders. As a result, several researchers worked rapidly to create Ab-based treatments and kits for the treatment and detection of SARS-CoV-2. Since the SARS-CoV-2 spike protein is essential for viral infection and has a known precise structure, the RBD has emerged as a prominent target for the creation of therapeutic antibodies. The use of Ab cocktails is anticipated to be a key component of an efficient COVID-19 treatment plan since SARS-CoV-2 is an RNA virus with a high mutation rate, particularly when subjected to the selection pressure of

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

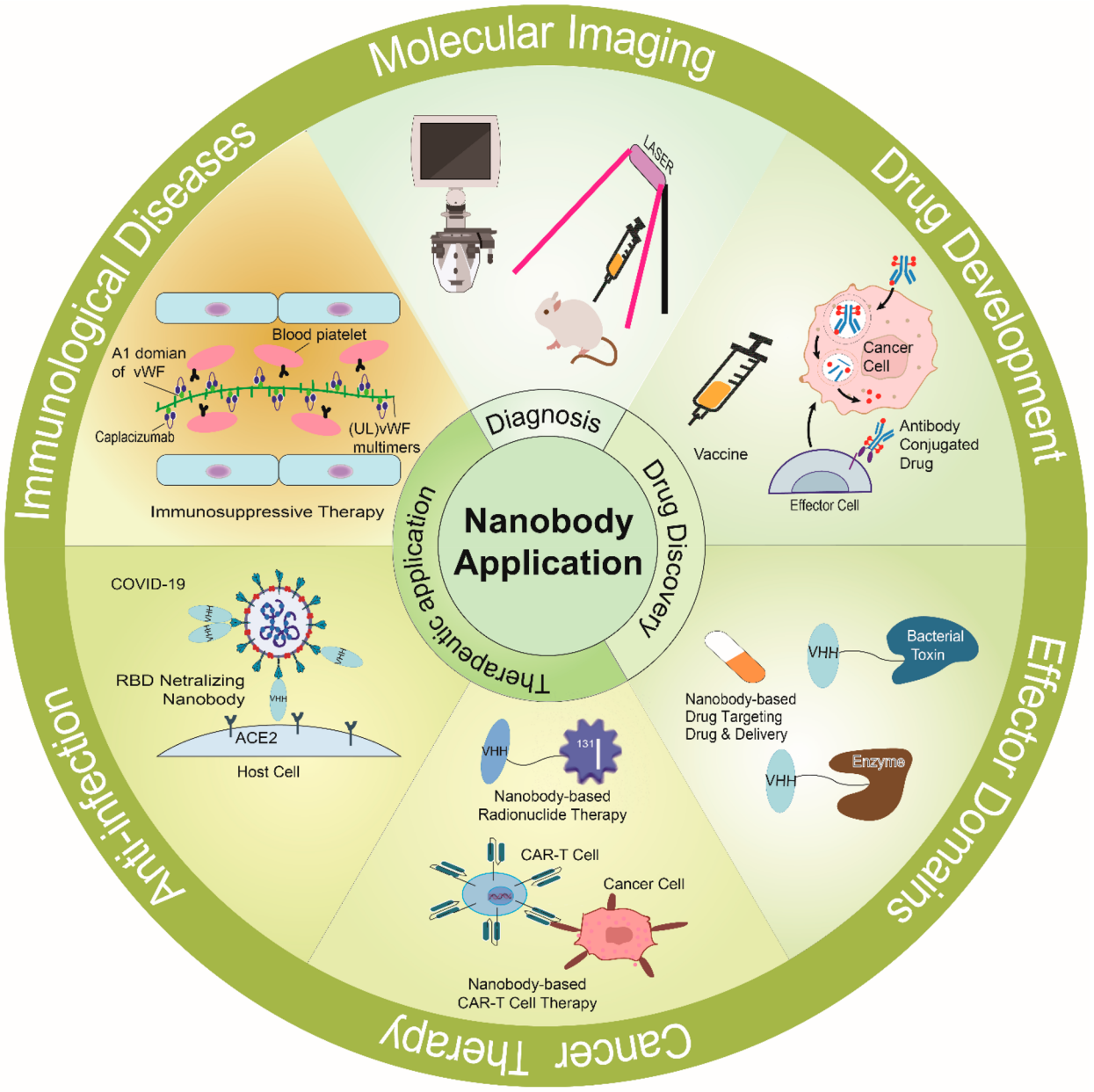


Figure 2. Summary of applications of antibodies and nanobodies in diagnostics and biotherapeutics. Current antibody-based bioproducts can be divided into different categories such as molecular diagnosis, drug development, and relative therapeutics based on immunoregulation in cancer, infection, and immunological diseases.

aggressively applied preventive vaccinations and neutralizing Abs.

An excessive immune response may be triggered by SARS-CoV-2 infection, creating a cytokine storm that accelerates the onset of a severe illness. Ab therapies for cytokine storms have also been researched as COVID-19 therapy. Abs are now used in SARS-CoV-2 detection assays, such as antigen and immunoglobulin testing, in addition to their usage as medications. Such Ab-based detection assays are essential surveillance instruments that may be utilized to stop COVID-19 from spreading. Therefore, therapeutic Abs that block the biological activities of cytokines may lessen the negative consequences of an overactivated host immune response and provide COVID-19 therapies.

Rapid diagnostic testing for SARS-CoV-2 variations is also required for quick medical and public health choices, such as deciding who has to be hospitalized or placed in quarantine in order to stop unchecked propagation.[16] When employed in contact tracing, molecular assays based on viral antigens can be utilized to detect people with acute phase SARS-CoV-2 infection, restrict transmission, and enable repeat testing for disease screening. The most promising method to carry out this kind of extensive surveillance and manage COVID-19 outbreaks is testing employing Ab-antigen-formatted immuno-complexes.

The US FDA or European Medicines Agency (EMA) has authorized a total of 124 antibody-based therapies as of June 2023 (Table 1). Bebtelovimab, a crown-neutralizing antibody from Eli Lilly, is primarily prescribed for those with mild to moderate COVID-19 in adults and children 12 years of age and older as well as those who are at a high risk of developing severe COVID-19. Bebtelovimab has been demonstrated to

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

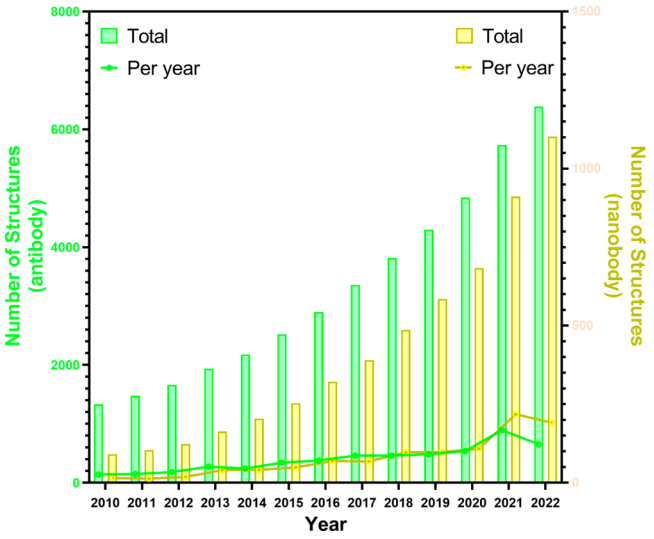


Figure 3. Number of antibody and nanobody-related therapies developed in the past decade (total and per year). Green bar indicates the total number of antibodies developed since 2010; green line indicates antibodies developed in single year; yellow bar indicates the number of nanobodies developed; and the dark yellow line indicates the nanobody number developed in single year.

have neutralizing action against the Omicron strain and its subtype variation strain BA in pertinent investigations.[17]

In comparison to poly- and monoclonal antibodies (mAb) and antibody fragments, nanobodies have distinct advantages, such as smaller size, high stability, ease of production, compatibility with genetic engineering techniques, lack of solubility and aggregation issues, and the absence of animal sacrifice.[11] Since the novel coronavirus infection is primarily confined to the lungs and nasopharynx and only 0.2% of conventional antibodies may enter the lungs by intravenous injection, the clinical effect is not substantial. Many scientists are now engaged in experimental work, and a variety of teams' investigations on the effectiveness and stability of nanobodies to neutralize SARS-CoV-2 have been published in a number of publications. Through the capture of mutant RBDs and SARS-CoV-2 spike proteins, antibodies suppress viral infection.[18] In both liquid and aerosol environments, the antibodies functioned effectively, showing that they are still potent after inhalation. Such efficient and affordable nanoantibodies have the potential to be a successful strategy for the prevention of neocoronavirus by choosing nanobodies with binding capacity to SARS-CoV-2 via phage and yeast display libraries.

NANOBODY CHARACTERISTICS

The serum of camelid and cartilaginous fish, including shark which, unlike human immunoglobulins, only has the heavy -chain part and the Fc section, nonetheless carries out typical biological tasks.[12] The first constant domain (CH1) of both light-chain and heavy-chain polypeptides is absent from the structural sequence of natural HCAb; a graphical representation of different antibody (conventional IgG, HCAbs, and nanobody) structures can be found in Figure 4A and Figure 4B, respectively. The heavy-chain variable domain (VHH) of a heavy-chain antibody is where nanobodies are produced from (Hcab).[13] It also goes by the name "Nanobody" because of its diminutive size (Nb). Nanobody VHH shares the same structural sequence as the VH domain of human immunoglobulins. Complementarity determining regions (CDR1, CDR2, CDR3) surround three hypervariable antigen-binding loops in four conserved framework regions.[14] The mouse VH structural domain CDR3 only includes 12 or 14 amino acids on average, but the CDR3 of nanobodies is an average of 18 amino acids;[19] this improves antigen recognition selectivity and relatively strong antigen-binding capacity. The Cys of CDR3 in VHH can establish disulfide connections with the Cys of CDR1 or FR2, in addition to the conserved disulfide bonds within the structural domain, increasing the structural stability of the nanobodies.[20] When exposed to chemical denaturants and proteases, at nonphysiological pH levels and at high temperatures of 60−80 °C, nanobodies are able to preserve the majority of their antigen-binding ability (pH range 3.0−9.0).[21] In the nanobodies, four highly conserved hydrophobic amino acids (V42, G49, L50, and W52 in human VH) were changed to hydrophilic amino acids, which improved the nanobodies' water solubility. The VHH's solvent-exposed location had a higher frequency of polar and charged amino acids, which improved the solubility of nanobodies in polar solvents.[22] For improved contact with the antigen surface, the expanded CDR3 loops in the nanobodies can form fingerlike structures or convex umbrella-like structures. The heavy-chain variable domain can serve as a standalone antigen-binding domain and exhibits a comparable affinity to human IgG antigens.[23] At present, 19 nanobodies-based diagnoses and therapies are under advanced clinical investigation for a wide spectrum of human diseases including infectious, cancer, and inflammatory diseases, as presented in Table 2 (data obtained from https://clinicaltrials.gov). Overall, the potential of nanobodies has generated a lot of interest in translational biomedical research as well pharmaceutical research and development, and ongoing research is likely to uncover new applications and opportunities.

Nanobodies may be made by *Escherichia coli* (abbreviated as *E. coli*), yeast, and ribosomes, which allows for far cheaper manufacturing costs and quicker production cycles than time-consuming and expensive monoclonal antibodies. In general, nanobodies are more suitable for harsh chemical modifications than other types of antibodies due to their special ability to enable recognition of hidden epitopes that are challenging for antibodies to recognize, as well as their small size for tissue penetration and high stability under high temperature and enhancer conditions. Furthermore, their low immunogenicity and humanization make them a safe option for long-term therapy,[24] although more study is needed to fully assess their potential as safe and effective long-term therapeutics in clinical trials for a range of diseases. The nanobody will easily pass through the kidney since its molecular weight (50−60 kDa) is substantially below the glomerular filtration threshold. Based on this trait, our researchers may decide how much medication to provide. This is a suitable option if quick clearing of nanobodies is necessary. In addition to altering repeated dosing in accordance with the half-life, one may employ multimeric nanobody structures or serum albumin-fused nanobodies to lengthen the half-life in the blood if there is a requirement to prolong the duration of action of nanobodies in the body.[25] The serum half-life of the nanobodies was extended to 2−3 days by fusing bivalent-EGFR nanobodies with human serum albumin-bound nanobodies.[26] Although the unique physicochemical and pharmacokinetic properties of nanobodies possess many desirable characteristics in bio-

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 1. List of Antibodies Approved for Therapy

name/identifier	brand name	target and type	application	year approval
muromonab-CO3	Orthoclone Okt3	CD3; murine IgG2a	reversal of kidney transplant rejection	1986
efalizumab	Raptiva	CD11a; humanized IgG1	psoriasis	2003
tositumomab-i131	Bexxar	CD20; murine IgG2a	non-Hodgkin lymphoma	2003
daclizumab	Zinbryta; Zenapax	IL-2R; humanized IgG1	multiple sclerosis; prevention of kidney transplant rejection	2016; 1997
olaratumab	Lartruvo	PDGFRα; human IgG1	soft tissue sarcoma	2016
abciximab	Reopro	GPIIb/IIIa; chimeric IgG1 Fab	prevention of blood clots in angioplasty	1994
rituximab	MabThera, Rituxan	CD20; chimeric IgG1	non-Hodgkin lymphoma	1997
basiliximab	Simulect	IL-2R; chimeric IgG1	prevention of kidney transplant rejection	1998
palivizumab	Synagis	RSV; humanized IgG1	prevention of respiratory syncytial virus infection	1998
infliximab	Remicade	TNF; chimeric IgG1	Crohn's disease	1998
trastuzumab	Herceptin	HER2; humanized IgG1	breast cancer	1998
adalimumab	Humira	TNF; human IgG1	rheumatoid arthritis	2002
ibritumomab tiuxetan	Zevalin	CD20; murine IgG1	non-Hodgkin lymphoma	2002
omalizumab	Xolair	IgE; humanized IgG1	asthma	2003
cetuximab	Erbitux	EGFR; chimeric IgG1	colorectal cancer	2004
bevacizumab	Avastin	VEGF; humanized IgG1	colorectal cancer	2004
natalizumab	Tysabri	a4 integrin; humanized IgG4	multiple sclerosis	2004
panitumumab	Vectibix	EGFR; human IgG2	colorectal cancer	2006
ranibizumab	Lucentis	VEGF; humanized IgG1 Fab	macular degeneration	2006
eculizumab	Soliris	C5; humanized IgG2/4	paroxysmal nocturnal hemoglobinuria	2007
certolizumab pegol	Cimzia	TNF; humanized Fab, pegylated	Crohn's disease	2008
ustekinumab	Stelara	IL-12/23; human IgG1	psoriasis	2009
canakinumab	Ilaris	IL-1β; human IgG1	Muckle-Wells syndrome	2009
golimumab	Simponi	TNF; human IgG1	rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009
ofatumumab	Arzerra	CD20; human IgG1	chronic lymphocytic leukemia	2009
tocilizumab	RoActemra, Actemra	IL-6R; humanized IgG1	rheumatoid arthritis	2010
denosumab	Prolia	RANK-L; human IgG2	bone loss	2010
belimumab	Benlysta	BLyS; human IgG1	systemic lupus erythematosus	2011
ipilimumab	Yervoy	CTLA-4; human IgG1	metastatic melanoma	2011
brentuximab vedotin	Adcetris	CD30; chimeric IgG1; ADC	Hodgkin lymphoma, systemic anaplastic large-cell lymphoma	2011
pertuzumab	Perjeta	HER2; humanized IgG1	breast cancer	2012
ado-trastuzumab emtansine	Kadcyla	HER2; humanized IgG1; ADC	breast cancer	2012
raxibacumab	(pending)	*Baccillus anthrasis* PA; human IgG1	anthrax infection	2012
obinutuzumab	Gazyva, Gazyvaro	CD20; humanized IgG1 glycoengineered	chronic lymphocytic leukemia	2013
siltuximab	Sylvant	IL-6; chimeric IgG1	Castleman disease	2014
ramucirumab	Cyramza	VEGFR2; human IgG1	gastric cancer	2014
vedolizumab	Entyvio	$\alpha4\beta7$ integrin; humanized IgG1	ulcerative colitis, Crohn's disease	2014
nivolumab	Opdivo	PD1; human IgG4	melanoma, non-small-cell lung cancer	2014
pembrolizumab	Keytruda	PD1; humanized IgG4	melanoma	2014
blinatumomab	Blincyto	CD19, CD3; murine bispecific tandem scFv	acute lymphoblastic leukemia	2014
alemtuzumab	Lemtrada; MabCampath, Campath-1H	CD52; humanized IgG1	multiple sclerosis; chronic myeloid leukemia	2014; 2001
evolocumab	Repatha	PCSK9; human IgG2	high cholesterol	2015
idarucizumab	Praxbind	dabigatran; humanized Fab	reversal of dabigatran-induced anticoagulation	2015
necitumumab	Portrazza	EGFR; human IgG1	non-small-cell lung cancer	2015
dinutuximab	Qarziba; Unituxin	GD2; chimeric IgG1	neuroblastoma	2015
secukinumab	Cosentyx	IL-17a; human IgG1	psoriasis	2015
mepolizumab	Nucala	IL-5; humanized IgG1	severe eosinophilic asthma	2015
alirocumab	Praluent	PCSK9; human IgG1	high cholesterol	2015
daratumumab	Darzalex	CD38; human IgG1	multiple myeloma	2015
elotuzumab	Empliciti	SLAMF7; humanized IgG1	multiple myeloma	2015
ixekizumab	Taltz	IL-17a; humanized IgG4	psoriasis	2016
reslizumab	Cinqaero, Cinqair	IL-5; humanized IgG4	asthma	2016
bezlotoxumab	Zinplava	*Clostridium difficile* enterotoxin B; human IgG1	prevention of *C. difficile* infection recurrence	2016
atezolizumab	Tecentriq	PD-L1; humanized IgG1	bladder cancer	2016
obiltoxaximab	Anthim	*B. anthrasis* PA; chimeric IgG1	prevention of inhalational anthrax	2016
brodalumab	Siliq, LUMICEF	IL-17R; human IgG2	plaque psoriasis	2017

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 1. continued

name/identifier	brand name	target and type	application	year approval
dupilumab	Dupixent	IL-4R α; human IgG4	atopic dermatitis	2017
inotuzumab ozogamicin	BESPONSA	CD22; humanized IgG4; ADC	acute lymphoblastic leukemia	2017
guselkumab	TREMFYA	IL-23 p19; human IgG1	plaque psoriasis	2017
sarilumab	Kevzara	IL-6R; human IgG1	rheumatoid arthritis	2017
avelumab	Bavencio	PD-L1; human IgG1	Merkel cell carcinoma	2017
emicizumab	Hemlibra	factor Ixa, X; humanized IgG4, bispecific	hemophilia A	2017
ocrelizumab	OCREVUS	CD20; humanized IgG1	multiple sclerosis	2017
benralizumab	Fasenra	IL-5R α; humanized IgG1	asthma	2017
durvalumab	IMFINZI	PD-L1; human IgG1	bladder cancer	2017
gemtuzumab ozogamicin	Mylotarg	CD33; humanized IgG4; ADC	acute myeloid leukemia	2017; 2000
erenumab, erenumab-aooe	Aimovig	CGRP receptor; human IgG2	migraine prevention	2018
galcanezumab, galcanezumab-gnlm	Emgality	CGRP; humanized IgG4	migraine prevention	2018
burosumab, burosumab-twza	Crysvita	FGF23; human IgG1	X-linked hypophosphatemia	2018
lanadelumab, lanadelumab-flyo	Takhzyro	plasma kallikrelin; human IgG1	hereditary angioedema attacks	2018
mogamulizumab, mogamulizumab-kpkc	Poteligeo	CCR4; humanized IgG1	mycosis fungoides or Sézary syndrome	2018
tildrakizumab; tildrakizumab-asmn	Ilumya	IL-23 p19; humanized IgG1	plaque psoriasis	2018
fremanezumab, fremanezumab-vfrm	Ajovy	CGRP; humanized IgG2	migraine prevention	2018
ravulizumab, ravulizumab-cwvz	Ultomiris	C5; humanized IgG2/4	paroxysmal nocturnal hemoglobinuria	2018
cemiplimab, cemiplimab-rwlc	Libtayo	PD-1; human mAb IgG4	cutaneous squamous cell carcinoma	2018
ibalizumab, ibalizumab-uiyk	Trogarzo	CD4; humanized IgG4	HIV infection	2018
emapalumab, emapalumab-lzsg	Gamifant	IFNg; human IgG1	primary hemophagocytic lymphohistiocytosis	2018
moxetumomab pasudotox, moxetumomab pasudotox-tdfk	Lumoxiti	CD22; murine IgG1 dsFv immunotoxin	hairy cell leukemia	2018
caplacizumab, caplacizumab-yhdp	Cablivi	von Willebrand factor; humanized nanobody	acquired thrombotic thrombocytopenic purpura	2019
risankizumab, risankizumab-rzaa	Skyrizi	IL-23 p19; humanized IgG1	plaque psoriasis	2019
polatuzumab vedotin, polatuzumab vedotin-piiq	Polivy	CD79b; humanized IgG1 ADC	diffuse large-B-cell lymphoma	2019
romosozumab, romosozumab-aqqg	Evenity	sclerostin; humanized IgG2	osteoporosis in postmenopausal women at increased risk of fracture	2019
brolucizumab, brolucizumab-dbll	Beovu	VEGF-A; humanized scFv	neovascular age-related macular degeneration	2019
crizanlizumab; crizanlizumab-tmca	Adakveo	CD62 (aka P-selectin); humanized IgG2	sickle cell disease	2019
enfortumab vedotin, enfortumab vedotin-ejfv	Padcev	Nectin-4; human IgG1 ADC	urothelial cancer	2019
[fam-]trastuzumab deruxtecan, fam-trastuzumab deruxtecan-nxki	Enhertu	HER2; humanized IgG1 ADC	HER2+ metastatic breast cancer	2019
isatuximab, isatuximab-irfc	Sarclisa	CD38; chimeric IgG1	multiple myeloma	2020
belantamab mafodotin, belantamab mafodotin-blmf	BLENREP	B-cell maturation antigen; humanized IgG1 ADC	multiple myeloma	2020
sacituzumab govitecan; sacituzumab govitecan-hziy	TRODELVY	TROP-2; humanized IgG1 ADC	triple-neg. breast cancer	2020
tafasitamab, tafasitamab-cxix	Monjuvi, Minjuvi	CD19; humanized IgG1	diffuse large -B-cell lymphoma	2020
satralizumab, satralizumab-mwge	Enspryng	IL-6R; humanized IgG2	neuromyelitis optica spectrum disorder	2020
eptinezumab, eptinezumab-jjmr	VYEPTI	CGRP; humanized IgG1	migraine prevention	2020
inebilizumab, inebilizumab-cdon	Uplizna	CD19; humanized IgG1	neuromyelitis optica spectrum disorders	2020
teprotumumab, teprotumumab-trbw	Tepezza	IGF-1R; human IgG1	thyroid eye disease	2020
evinacumab	Evkeeza	angiopoietin-like 3; human IgG4	homozygous familial hypercholesterolemia	2021
dostarlimab, dostarlimab-gxly	Jemperli	PD-1; humanized IgG4	endometrial cancer	2021
amivantamab, amivantamab-vmjw	Rybrevant	EGFR, cMET; human bispecific IgG1	NSCLC w/EGFR exon 20 insertion mutations	2021
tralokinumab, tralokinumab-ldrm	Adtralza	IL-13; human IgG4	atopic dermatitis	2021
anifrolumab, anifrolumab-fnia	SAPHNELO	IFNAR1; human IgG1	systemic lupus erythematosus	2021
loncastuximab tesirine, loncastuximab tesirine-lpyl	Zynlonta	CD19; humanized IgG1 ADC	diffuse large-B-cell lymphoma	2021
atoltivimab, maftivimab, and odesivimab-ebgn	Inmazeb	Ebola virus; mixture of 3 human IgG1	Ebola virus infection	2020
naxitamab-gqgk	DANYELZA	GD2; humanized IgG1	high-risk neuroblastoma and refractory osteomedullary disease	2020
margetuximab-cmkb	MARGENZA	HER2; chimeric IgG1	HER2+ metastatic breast cancer	2020
ansuvimab-zykl	Ebanga	Ebola virus glycoprotein; human IgG1	Ebola virus infection	2020
aducanumab, aducanumab-avwa	Aduhelm	amyloid beta; human IgG1	Alzheimer's disease	2021

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 1. continued

name/identifier	brand name	target and type	application	year approval
sotrovimab	Xevudy	SARS-CoV-2; human IgG1	COVID-19	2021 [EUA] (revoked)
tisotumab vedotin, tisotumab vedotin-tftv	TIVDAK	tissue factor; human IgG1 ADC	cervical cancer	2021
casirivimab + imdevimab	REGEN-COV2, Ronapreve	SARS-CoV-2; human IgG1	COVID-19	NA
tezepelumab, tezepelumab-ekko	Tezspire	thymic stromal lymphopoietin; human IgG2	severe asthma	2021
faricimab, faricimab-svoa	Vabysmo	VEGF-A, Ang-2; human/humanized IgG1 kappa/lambda bispecific, with domain crossover	neovascular, age-related macular degeneration, diabetic macular edema	2022
sutimlimab, sutimlimab-jome	Enjaymo	C 1s; humanized IgG4	cold agglutinin disease	2022
tixagevimab, cilgavimab	Evusheld	SARS-CoV-2; human IgG1	COVID-19	2022 [EUA]
spesolimab	SPEVIGO	IL-36 receptor; humanized IgG1	generalized pustular psoriasis	2022
teplizumab, teplizumab-mzwv	TZIELD	CD3; humanized IgG1	delay onset of type 1 diabetes	2022
ublituximab	BRIUMVI	CD20; chimeric IgG1	multiple sclerosis	2022
tebentafusp, tebentafusp-tebn	KIMMTRAK	gp100, CD3; bispecific immunoconjugate	metastatic uveal melanoma	2022
relatlimab	Opdualag (relatlimab + nivolumab combo)	LAG-3; human IgG4	melanoma	2022
mosunetuzumab	Lunsumio	CD20, CD3; humanized IgG1 bispecific	follicular lymphoma	2022
teclistamab	TECVAYLI	B-cell maturation antigen, CD3; humanized/human bispecific IgG4	multiple myeloma	2022
tremelimumab	Imjudo	CTLA-4; human IgG2	NSCLC; liver cancer	2022
mirvetuximab soravtansine, mirvetuximab soravtansine-gynx	ELAHERE	folate receptor alpha; humanized IgG1 ADC	ovarian cancer	2022
lecanemab	Leqembi	amyloid beta protofibrils; humanized IgG1	Alzheimer's disease	2023

medical applications, they still have limitations and disadvantages that limit their use (Figure 4C).

TECHNOLOGY OF THE NANOBODY GENERATION

These surface display technologies have several advantages such as high expression efficiency, simple operation, and integration with other techniques to expand the diversity of nanobodies for different targets. The expression system can be easily optimized by selecting the appropriate bacterial host to improve the stability and solubility of displayed nanobodies. These technologies enable efficient screening of nanobodies for high affinity to the target, leading to the selection of high-quality nanobodies for various applications in research and development.

Phage Display. Phage display technology uses bacteriophages, a type of virus that infects bacteria, as a vehicle for displaying and selecting target proteins on their surface (Figure 5). The target protein, such as an antibody or a peptide, is genetically fused to the phage surface protein and displayed on the phage's surface. Through various selection and amplification steps, the phage display allows for the efficient identification of high-affinity target proteins with specific binding properties. This technology has been widely used in various fields, including antibody discovery and development, as well as protein engineering and drug discovery.[27] It is a powerful tool in molecular biology and biotechnology for the discovery and selection of target-specific antibodies. The phage display technology utilizes bacteriophages, which are viruses that infect bacteria, to display peptides or proteins on their surface. This allows for the rapid screening of large numbers of variants and the selection of those that specifically bind to a target molecule. The antibodies generated through phage display can then be used for various applications such as diagnostic tests, drug discovery, and basic research.[28] Phage-displayed antibody libraries are widely used for screening high-affinity antibodies against various targets including fibronectin, HPV16E7 virus, epidermal growth factor, and other proteins. The phage display technology allows for the rapid and efficient selection of highly specific and high-affinity antibodies from a large diverse library of antibody-encoding genes (Table 3).

Yeast Display. A similar eukaryotic protein expression method that is more suited for eukaryotic expression is yeast cell surface display technology (Table 4). In the library, correctly folded proteins are guaranteed. It does not result in clone loss or library bias. The yeast display technique uses FACS sorting technology to test for antibody affinity and display amount, eliminating expression-induced bias and differentiating clones with minute affinity differences. Antibody affinity may be assessed directly on the yeast cell surface using double-stained FACS, doing away with the necessity for time-consuming subcloning, expression, and purification.[29] Direct measurement of antibody affinity on yeast cell surfaces using double-stained FACS eliminates the need for time-consuming subcloning, expression, and purification.[30] The three main yeasts employed are lysoarolic bilobic yeast, *Saccharomyces cerevisiae* fermentation, and bilobic yeast. *S. cerevisiae* is also the yeast most frequently used for cell surface display. Many antibodies, notably those against the cholera toxin, have been produced using yeast display technology.[31] To find blocking active antibodies, yeast display technology has been used. Examples include the nanobody NB97, which inhibits the PD-L1 and PD-1 linker axis in cancer patients,[32] the anti-IL-5Ra antibody for the treatment of patients with severe eosinophilic asthma (SEA),[33] and the trivalent nanobody mNb6-tri,[34] which prevents the binding of a novel coronavirus spike to human ACE2 for the treatment of patients with new coronavirus.

Ribosome Display. Ribosome display is a technique that uses PCR *in vitro* cell-free display technology to pick and evolve proteins from large libraries.[35] The protein−mRNA combination is subsequently subjected to *in situ* reverse transcription of PCR (RT-PCR) to recover the DNA-encoding protein sequence. The promoter and transcription start sequences are the first step in this process, which then

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

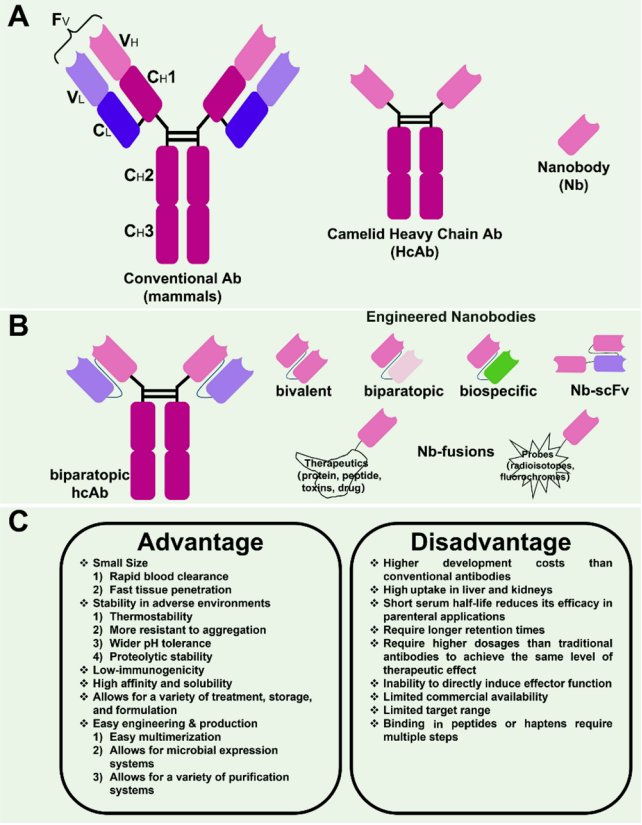


Figure 4. Graphical representation of the structure and comparison of the advantages and disadvantages of conventional Ab, HcAb, and nanobody. (A) Conventional antibodies (e.g., IgG) consist of two identical heavy and light (variable: V_H and V_L; constant: $C_H1/2/3$ and C_L) chains connected with disulfide bonds. Disulfide-stabilized variable fragment Fv composed of V_H and V_L is the smallest antigen-binding unit. Camelid heavy-chain antibodies (HcAb) consist of two identical heavy chains (variable: V_H and constant: $C_H2/3$). The nanobody consists of a single-variable domain V_H. (B) Overview of the different types of engineered HcAb and nanobodies with various modifications. (C) An outline of the advantages and disadvantages of nanobodies compared to conventional mAb formats.

develops into stable protein−ribosome−mRNA (PRM) complexes, connects various antibody fragments to their respective mRNAs, and chooses the appropriate protein−mRNA based on the affinity of the ligand. Finally, the target proteins were identified using ELISA. It is not bound by environmental cell transformation in terms of producing toxic or hydrolytically susceptible and unstable proteins.[36] During *in vitro* screening, the ribosome display reaction method, which makes use of PCR and expands the library's capacity as well as the possibility for affinity maturation of antibodies or peptides, could cause random mutations. Both prokaryotes and eukaryotes have ribosome display systems. Ribosomes produce high-affinity antibodies and have stronger and more stable antibody bonds.[37] It has become an essential tool for cost-effectively and productively manufacturing diagnostic antibodies. Using RNA isolated from peripheral monocytes of HIV-1 patient donors, Tang et al. employed ribosome display to hunt for recombinant gp120 (donor K350)-reactive monoclonal antibodies in two single-chain antibody fragments (scFvs) antibody libraries.[38]

Bacterial Surface Display. The bacterial surface display system is a powerful tool for discovering and selecting nanobodies, thanks to its high expression efficiency, ease of use, and the ability to improve stability and solubility of displayed nanobodies. This system also allows for efficient screening of nanobodies for high affinity to the target and can be combined with other techniques to increase the diversity of nanobodies for different targets.

Bacterial surface display is a method used for the expression and presentation of target proteins or peptides on the surface of bacteria. This system can be used with different types of bacteria such as *E. coli*, Gram-negative bacteria, Gram-positive bacteria, and lactic acid bacteria, depending on the specific requirements of the experiment. It provides a convenient and efficient method for studying the interactions between target proteins and potential ligands and can be used in a variety of applications, such as vaccine screening, polypeptide library screening, and expression and purification of foreign proteins.[39]

The cell surface display system is a way to present target proteins or peptides on the surface of bacteria, such as *E. coli*, Gram-negative bacteria, Gram-positive bacteria, and lactic acid bacteria. The target protein or fragment is expressed as a fusion protein with a carrier protein and expressed on the surface of the bacteria. The location of the fusion protein can be N-terminal, C-terminal, or three-piece fusion, and the expression can be confirmed by functional analysis and FACS analysis. This technology can be used for various purposes, including protein engineering, antibody discovery, and drug development.

The study from Nienberg et al. found that the B2 variant peptide had the highest activity against CK2 holoenzyme among the screened peptides, with a significant increase in activity compared to the wild-type peptide. The results showed that the bacterial surface display technology is a valuable tool for the discovery of highly potent and selective inhibitors of enzymatic targets.[40]

The bacterial surface display system is a useful tool for isolating unknown receptors/ligands of human noroviruses (HuNoVs) by capturing and easily isolating them from various samples. It provides a simpler alternative method compared to traditional methods.[36]

Bacterial surface display has a wide range of applications in various fields including biotechnology, medicine, and bioengineering. In addition to vaccine screening and polypeptide library screening, the bacterial surface display system is also used for the expression and purification of different foreign proteins. The system is also used for cell adsorption of target proteins and ligand interactions, biocatalysis, and the production of biological adsorbents. The bacterial surface display system offers a highly efficient and cost-effective method for protein expression, purification, and study of protein−protein interactions.

THERAPEUTIC NANOBODY AGAINST SARS-COV-2 INFECTIONS

Nanobodies have several advantages over monoclonal antibodies[41] in the treatment of SARS-CoV-2. They are easier to produce, have high thermal stability, and are soluble, making them easier to scale up. They are also small and have a low molecular weight, which makes them easy to manipulate and express. Additionally, they maintain the specificity and affinity of conventional antibodies but are easier to clone and produce. All these factors make nanobodies a promising option for the

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 2. Active Nanobodies in Clinical Trials

nanobody name	conditions	target	clinical trial	status
M6495	healthy volunteers	anti-ADAMTS-5	NCT03583346	phase 1 (completed)
	knee osteoarthritis		NCT03224702	phase 1 (completed)
MSB0010841	psoriasis	anti-interleukin-17A/F1	NCT02156466	phase 1 (completed)
18F-FDG	solid tumor	anti-CLDN18.2	NCT05436093	phase not applicable (recruiting)
68Ga-THP-APN09	lung cancer; melanoma; PD-L1; PET/CT	anti-PD-L1	NCT05156515	phase not applicable (recruiting)
18F-FDG	lung cancer; colorectal cancer	anti-PD-L2	NCT05803746	phase not applicable (recruiting)
CD19/CD20 bispecific CAR T cells	B-cell lymphoma stage I; refractory; relapsed	anti-CD19/20	NCT03881761	phase 1 (unknown)
caplacizumab	acquired thrombotic thrombocytopenic purpura	anti-vWF	NCT01151423	phase 2 (completed)
αPD1-MSLN-CAR T cells	non-small-cell lung cancer; mesothelioma	anti-PD1	NCT04489862	early phase 1 (unknown)
ALX-0651	healthy volunteers	anti-CXCR4	NCT01374503	terminated (phase 1)
ALX-0061	rheumatoid arthritis	anti-IL6R	NCT01284569	ALX-0061: phase 1 (completed)
				placebo: phase 2 (completed)
BCMA nanobody CAR T cells	relapsed/refractory myeloma	anti-BCMA	NCT03664661	phase 1 (unknown)
αPD1-MSLN-CAR T cells	solid tumor	anti-PD1	NCT05373147	early phase 1
αPD1-MSLN-CAR T cells	colorectal cancer	anti-PD1	NCT04503980	early phase 1
	ovarian cancer			
SAR442970	hidradenitis suppurativa	anti-TNF-OX40L	NCT05849922	phase 2 (not yet recruiting)
JS014	neoplasm malignant neoplasm; experimental solid tumor; adult lymphoma	antihuman serum albumin; anti-PD-1	NCT05296772	phase 1 (recruiting)
VHH batch 203027	male children; 6−24 months old with rotaviral diarrhea; noncholera patients	antirotavirus VHH Fragment	NCT01259765	phase 2 (completed)
sonelokimab (M1095) and adalimumab	hidradenitis suppurativa	anti-IL-17 A/F; anti-TNF	NCT05322473	phase 2 (active, not recruiting)
ALXN1720	generalized myasthenia gravis	anti- AChR	NCT05556096	phase 3 (recruiting)

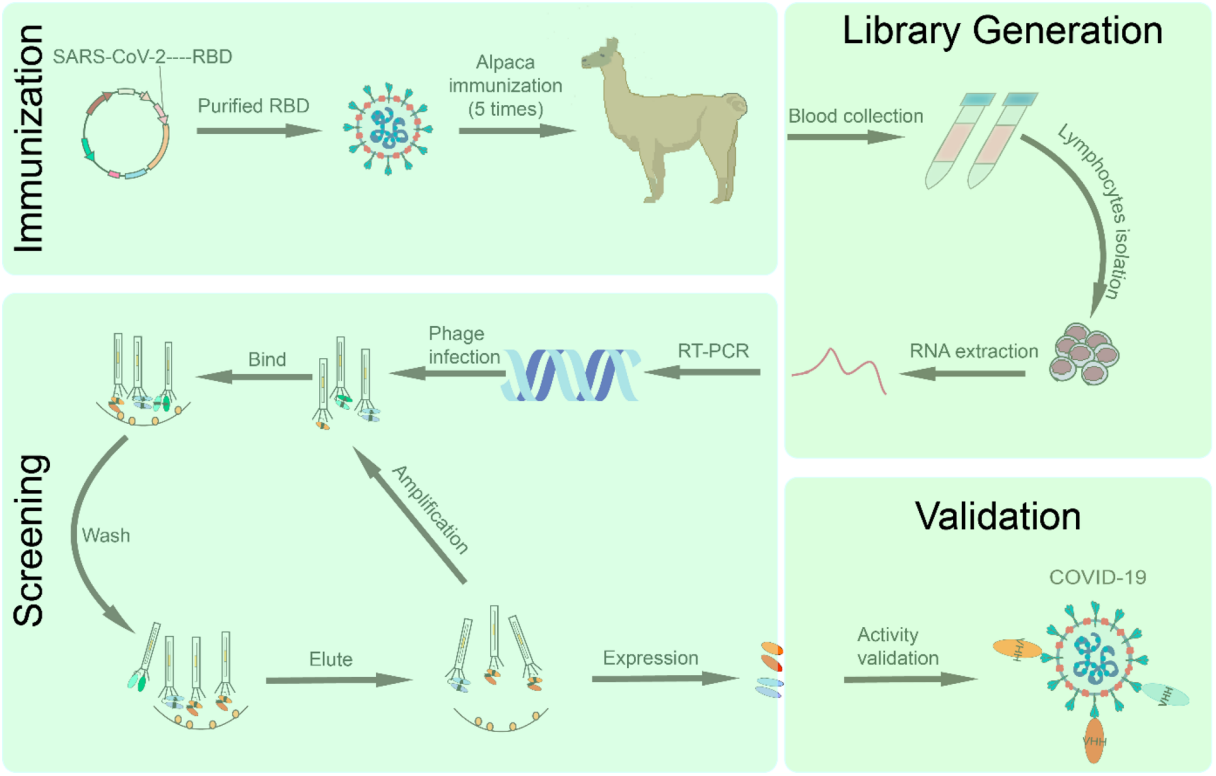


Figure 5. Schematic illustration of nanobody generation. Nanobody screening and generation through phage display can be divided into immunization, library preparation, screening, and final validation before application in COVID-19 diagnosis and treatment.

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 3. Nanobodies Generated by Phage Display Technology

nanobody name	antigens of interest	endogenous location of epitope	dissociation constant	testing methods (*in vitro* and *in vivo*)	application	ref
anti-SEB-specific nanobodies	SEB	extracellular region	ELISA test affinity constant of 3.6 nm	SDS-PAGE, Western blotting, and ELISA	immunoassay for staphylococcal enterotoxin B (SEB) in suspect foods	65
microcystin-resistant LR nanobodies	microcystins	extracellular region	IC-ELISA (IC50) of 0.87, 1.17, and 1.47 μg/l, respectively	spiking tests, the three nanobodies ANAb12, ANAb9, and ANAb7	detection of MC-LR in tap water samples	66
NB28, NB31	fungal toxin deoxynivalenol	extracellular region	N-28: 78.89 ± 1.77; N-31: 68.18 ± 1.15 ng/mL	commercial ELISA kit of DON	immunoassay for various chemical contaminants in food	67
nanobody V2-5	aflatoxin	extracellular region	the IC50 for aflatoxin B1 was 0.16 ng/mL; for B2, G1, and G2 it was 90.4%, 54.4%, and 37.7% respectively	ELISA	the VHH-based immunoassay was successfully applied	68
anti-EGFR nanobodies	EGFR	cytoplasm	no data	ELISA	anti-EGFR Nbs can serve as targeted drug delivery applications	69
anti-TNF-αNb nanobodies	TNF-α	cell membrane	IC50 values NT-3 (0.804 μM), NT-5 (1.372 μM)	ELISA *in vitro* assay *in vitro* cytotoxicity assay	provides a new alternative to Nbs for targeted therapy of TNF-α-mediated disease	70
VHH17	HER2	nucleus	VHH17 inhibits HER2-positive cells and induces cell death	TK activity inhibition test	promising alternative HER2 kinase inhibitor to reduce breast cancer cell viability	71
nanobody SHMR4	botulinum neurotoxin E	extracellular region	affinity of 1.43×10^{-7} mol/L	ELISA SHMR4 nanosomes for recombinant BoNT\/E Hc	biosensor for the detection of *Clostridium botulinum* type E	72
nanobody 3C3	Cry1Ie toxin	extracellular region	IC50:0.73 ng/mL	fluorescence Iimmunoassay (IC-TRFIA)	detection of Cry1Ie toxin in agricultural and environmental samples	73
antibrucella nanobodies	brucella	cell membrane	the mean ELISA signal colonies are approximately 3 times higher	detection of Brucella Ag specificity of soluble Nbs in all PCR screened colonies	vaccination, diagnosis, and treatment of brucellosis	74
nanobody VHH 15	BVDV virus	cell membrane	100 μg/mL VHH has a blocking effect on BVDV	ELISA test and qRT-PCR	special competence in the prevention and treatment of BVDV	75
nanobodies of γ-H2AX	γ-H2AX	nucleus	at 2 ng/mL can visualize discrete amounts of γ-H2AX in cells.	bivalent nanobody detection of γ-H2AX	for the study of drug-induced replication stress in fixed and live cancer cells	76
SARS-CoV-2 and N501Y D614G variant	SARS-CoV-2 RBD	cell membrane	strong neutralizing activity against SARS-CoV-2 (down to 0.1 nM)	fluorescence resonance Energy transfer (FRET)	effective neutralization of wild-type SARS-CoV-2 and N501Y D614G variants	77
antithyroglobulin single-domain antibody	thyroglobulin	extracellular membrane	dissociation constant KD ~ 18 pmol	ion body resonance (SPR) systems	immunoreactivity of anti-Tg - sdab (KT75) in thyroglobulin immunoassays	78
CAR-T cells in nanosomes	CAR-T cells	cell membrane	Nb CAR-T cells can identify and kill all primary tumor cells *in vitro*	assessment with Cell Trace Far Red	Nb CAR-T cells may be a promising strategy for treating tumor cells	79
anti-VEGF-VHH display immuno real-time PCR assay	VEGF	cytoplasm	IPCR has good accuracy and high reproducibility for the quantitative detection of VEGF	ELISA-based detection of phage display-mediated immuno-PCR	provides a specific and sensitive immunoassay protocol for the detection of low levels of VEGF	80
nanobody Nb2	HPV16E7	cell membrane	Nb2 inhibited the proliferation of HPV16-positive CasKi cells by MTT assay	ELISA	great application in the treatment of HPV16-related disease	81
nanosota-1C-Fc	SARS-CoV-2 RBD	cell membrane	KD of 15.7 μM (~3000 times tighter than ACE2 with RBD) and ND50 of 0.16 μg/mL (~6000 times tighter than ACE2)	ELISA	prevention and treatment of SARS-CoV-2 infected hosts	82

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 4. Nanobodies Generated by Yeast Surface Display Technology

name	antigens of interest	endogenous location of epitope	dissociation constant	testing methods	application	ref
AT1Bi4	AT1R	cell membrane	AT1Bi4 significantly attenuates the increase in blood pressure caused by angiotensin II	radioligand binding test	nanosome antagonists may offer an alternative treatment for hypertensive disorders	83
nanobody NB7-14	H7 hemagglutinin	cell membrane	the IC50 of bivalent NB7-14 is 130 pM	ELISA and viral microneutralization assays	monitoring, treatment, and prevention of H7N9	84
novel PD-1 specific nanobody Nb97	somatic blockade of PD-1-PD-L1 axis	cell membrane	MY2935 nanobody expressed in *Pichia pastoris* IC50 1.217 mg/mL	flow cytometry	potential therapies for immunotherapy	32
trivalent nanobody mNb6 tri	SARS-CoV-2 spike	cell membrane	Nb6-tri was shown with average IC50 of 160 pM	flow cytometry of yeast	disrupting the interaction between spike and ACE2	34
cetuximab and matuximab against unique vNAR	EGFR	cytoplasm	good target recognition in the case of human and mouse sera	ESDS-PAGE	antiunique vNAR variants can be used as effective capture tools	85
antiricin antibody	ricin A chain	extracellular region	yeast surface expression and FACS can isolate high-affinity natural pairs	surface plasmon resonance (SPR) analysis	antiricin A-chain antibodies provide diverse reagents for diagnosis and treatment	86
nanobody AT110i103	angiotensin II receptor type 1 (AT1R)	cell membrane	anti-RBD nanobodies are approximately 925-fold higher than their parental sequences	FACS	providing templates for large-scale simplified antibody generation	82
glioblastoma stem cell-like cells antibody VH-9.7	glioblastoma stem cell-like cells (GSC)	cell membrane	purified VH-9.7 recognizes human GSC in *in situ* NOD-SCID mouse xenografts	plasmid recovery, sequencing, and analysis	facilitates GSC isolation, diagnostic imaging, and therapeutic targeting	87
CfaE-resistant nanobody	enterotoxigenic *E. coli*	extracellular region	2R215 showed broad protection against all tested strains	ELISA assay	induces potent immunity against conserved ETEC epitopes	88
against influenza B hemagglutinin nanobody	influenza B hemagglutinin	cell membrane	IC50 values of 0.2 nM and 15.5 nM on B-Vic and B-Yam spectrum viruses	trace neutralization analysis method	applications in IBV diagnosis, vaccine efficacy testing, and immunotherapy	89

treatment of SARS-CoV-2 and could lead to the development of more effective and affordable therapies (Table 5).

These multivalent nanobodies or variable domains of heavy-chain antibodies have the potential to overcome the challenges posed by the constantly evolving virus, as well as to improve the therapeutic efficacy of the antibody. They can bind multiple sites on the virus and increase the binding affinity and specificity, resulting in a longer half-life in the serum and improved efficacy against the virus.[42] In addition, the small size and high stability of these nanobodies make them suitable for different delivery methods, including inhalation, injection, and oral administration, allowing for a more flexible and convenient approach to treating COVID-19. These multivalent nanobodies have the potential to be a promising new tool in the fight against SARS-CoV-2 and other viruses in the future.

This is promising for the potential use of Nb15 as a prophylactic or therapeutic agent for the treatment of COVID-19, as its multivalent and dual-targeting nature provides broad-spectrum protection against different strains of the virus and its good thermal stability makes it more suitable for production and storage. The results suggest that Nb15 may be a valuable tool for the prevention and treatment of COVID-19 and other coronavirus outbreaks in the future. The nanobody Nb15-NbH-Nb15 has a high stability, which means it can remain effective even after exposure to high temperatures. This can be an important factor in the storage and transportation of the nanobody as well as in its application in the treatment of COVID-19.[43] The bivalent Nb15-NbH-Nb15 has been shown to be effective in preventing and treating SARS-CoV-2 infection in mouse experiments. The improved binding ability and increased stability *in vivo* result in a 100% protection against the virus. The inhaled form of this nanobody has important clinical significance as a low-cost, portable treatment option for postvaccination breakthrough infections caused by delta viruses.

The inhaled trimeric nanobody PiN-21 has shown to be effective in reducing the replication of the novel coronavirus in host cells. In a study, when hamsters infected with the virus were given PiN-21 6 h after infection, it was shown to have a significant therapeutic effect in reducing virus replication in the lungs and reducing severe necrotizing bronchiolitis. Early administration of PiN-21 had a good prophylactic effect and reduced the chance of the virus entering the lungs. The minimum effective dose of PiN-21 was found to be 0.2 mg/kg, and it was shown to be effective when inhaled, reaching the lungs effectively.[44]

NIH-CoVnb-112 is a highly potent nanobody against the SARS-CoV-2 virus that has been shown to have ultrahigh binding capacity compared to other monomeric nanobodies. It has a binding affinity of 0.01 μg/mL and blocks 50% of ACE2 binding and has a half-maximum effective concentration (EC50) of 0.02 μg/mL. The nanobody can block the interaction between the RBD of human ACE2 and high affinity variants of SARS-CoV-2. The EC50 of NIH-CoVnb-112 is 0.116 μg/mL after nebulization with an affinity of 2.1 nM and expression amount of 40 mg/mL.[45] The nanobody Nb11-59 has been shown to be a promising molecule for the prevention and treatment of COVID-19 due to its high activity against the virus and its stability for inhalation administration. Its IC50 is 0.55 μg/mL, the functional block rate of inhibiting the interaction of RBD and ACE2 is 98.9%, and the 50% neutralizing dose (ND50) is 0.55 μg/mL. The ability to reach deep lungs through inhalation administration may reduce

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 5. List of SARS-CoV2 Targeting Nanobodies

name	resources	targeting epitope	neutralizing ability	characteristics	ref
trivalent nanobodies (Nb15-NbH-Nb15)	immunized alpacas	RBD	suppress new crown prevalent strains such as Alpha, Epsilon, and Delta	drug nebulization does not affect stability and increase therapeutic effect.	43
trimeric nanobodies (PiN21)	immunized alpacas	RBD	the minimum effective dose of 0.2 mg/kg can inhibit the replication of the virus in host cells; the IC50 is 0.022 nM	nebulized administration of 0.2 mg/kg can effectively suppress the virus	44
monovalent nanobody (NIH-CoVnb-112)	immunized alpacas	RBD	three variants of RBD with binding intensity of 0.01 μg/mL and EC50 of 0.02 μg/mL with an affinity of 2.1 nM	stable structure, high yield, effective for a variety of new crown variants	45
58G6 13G9 510A5	SARS-COV-2 monoclonal antibody library	RBD	strong neutralizing ability for both SARS-COV-2 and variant B.1.351, with IC50 as low as 2 ng/mL for 58G6	significant reduction in viral load of SARS-CoV-2or B.1.351 in lung tissue	50
bivalent structure Nanosota-1C-Fc	immune alpaca (phage display)	RBD	KD was 15.7 μM (~3000 times tighter than ACE2 with RBD), and ND50 was 0.16 μg/mL (~6000 times tighter than ACE2).	it has a faster binding and slow dissociation with RBD, high yield production, high stability	90
Nb11−59	immunized alpaca (produced by Bichmann's yeast)	RBD	inhibits the binding of RBD to ACE2 with a blocking rate of 98.9%, Kb = 21.6 nM, ND 0.55 μg/mL	high purity, high yield, good stability	46
unit price VHH E multivalent VHH VE	immunized alpacas	RBD	IC for VHH E is 48−185 nM; EC50 is 60 nM	polyvalent can stop immune escape	47
Ty1	immunized alpacas	RBD	Ty1 neutralizes pseudovirus and blocks ACE2 binding; IC50 = 12 ng/mL	low cost and good scalability	49
H11−H4 H11-D4	natural database	RBD	they bind RBD (KD 12 and 39 nM, respectively) and block their interaction with ACE2	both nanobodies bind to the three rbd in the spike-in trimer	91
H11−H4−Fc			h11-H4-Fc has a high potency ND50 at 4−6 nM		
fusion of IgG1 Fc with sdAbs	immunized alpacas	RBD	the fusion of human IgG1 Fc with sdAbs increases their neutralizing activity by 10-fold	meaningful for research	92
VHH72	immunized alpacas	RBD	novel coronavirus KD = 40 nM, ND50 = 0.2 μg/mL	VHH72-Fc and bivalent VHH72-VHH72 bind better to SARS-CoV-2	52
Sb23 Sb23-Fc	three Sb libraries	RBD	high RBD affinity: Sb23, KD = 10 nM; Sb23-Fc, KD = 225 pM	binds RBD in the up and down conformation, inhibits ACE2 binding RBD	93
Nb6 mNb6 tri	yeast surface-displayed library	S protein (mutant form)	Nb6 binds to spike S S2P KD = 210 nM, IC50 = 370 nM trimers enhance inhibition of pseudovirus Nb6-tri (2000-fold, IC50 = 1.2 nM)	mNb6-tri was stable and functional after heat exposure, lyophilization, and aerosolization	34
WNb 2, WNb 7, WNb 15, WNb 36, WNbFc	alpaca immunization	S protein and RBD	Nb (WNb 2, WNb 7, WNb 15, and WNb 36) have high affinities (KD = 0.14−19.49 nM) WNbFc bind to most RBD variants (EC50 = 0.7−14 nM)	WNbFc inhibits wild-type RBD-ACE2 engagement and most RBD variants	77
C5, F2, H3, C1, trimeric C5	llama immunization	S protein and RBD	high binding affinity of H3, F2, C5, and C1 (KD = 20−615 pM). C5-Fc: high RBD binding affinity (KD = 37 pM)	intranasal administration seems to promote faster recovery than intraperitoneal	94

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

Table 5. continued

name	resources	targeting epitope	neutralizing ability	characteristics	ref
BipNb	alpaca immunization	RBD	NM1267, strong neutralization potency for B.1 (IC50 = 0.33 nM), B.1.351 (IC50 = 0.78 nM), and B.1.617.2 (IC50 = 52.55 nM)	targets different RBD epitopes and has high stability, purity, and production yields	95
NM1267 and NM1268			NM1268, strong neutralization potency for B.1 (IC50 = 2.37 nM), B.1.351 (IC50 = 6.06 nM), and B.1.617.2 (IC50 = 0.67 nM)		
ShAb01 and ShAb02	nurse shark immunization	RBD	ShAb01: IC50 = 188−873 ng/mL (SARS-CoV-2 WA-1, Alpha, Beta, and Delta variants)	ShAbs potently cross-neutralize SARS-CoV-2 WA-1, Alpha, Beta, Delta, Omicron BA.1 and BA.5, and SARS-CoV-1 pseudoviruses and confer protection against SARS-CoV-2 challenge in the K18-hACE2 transgenic mouse model	96
			ShAb02: IC50 = 15−52 ng/mL (WA-1, Alpha, Beta, and Delta variants)		
			ShAb02: IC50 = 178 ng/mL (BA.1), 1 μg/mL (BA.4/5)		
Vnarbody 20G6 and 17F6	bamboo shark immunization	RBD	20G6-Fc dimer: IC50 = 11.79 × 10^{-9} M (Wuhan strain), 9.36 × 10^{-9} M (Beta variant), and 10.26 × 10^{-9} M (Delta variant).	Vnarbodies 20G6 and 17F6 exhibit potent and broadly neutralizing activities against SARS-CoV-2 variants	97
			17F6-Fc dimer: IC50 = 34.36 × 10^{-9} M (Wuhan strain), 19.87 × 10^{-9} M (Beta variant), and 33.85 × 10^{-9} M (Delta variant)	20G6 exhibit therapeutic protection in mice challenged with SARS-CoV-2	
nurse shark VNAR antibody	nurse shark single-domain antibody sequence libraries	S1, S1-RBD	6ID10_5: IC50 = 1.0 × 10^{-07} M(S1), 8.3 × 10^{-08} M (S1-RBD); 6ID10_6: IC50 = 6.2 × 10^{-08} M (S1), 3.4 × 10^{-08} M (S1-RBD); 3ID10_16: IC50 = 7.3 × 10^{-08} M (S1), 8.9 × 10^{-08} M (S1-RBD); 6ID10_75: IC50 = 3.6 × 10^{-07} M (S1), 4.5 × 10^{-08} M (S1-RBD); 3ID10_99: IC50 = 6.4 × 10^{-08} M (S1), 1.5 × 10^{-08} M (S1-RBD); 6ID10_113: IC50 = 7.0 × 10^{-08} M (S1), 5.0 × 10^{-08} M (S1-RBD)	VNAR-hFc exhibit neutralizing activities against Wuhan variant virus in *in vitro* cell infectivity assays	98

systemic reactions and improve the safety and efficacy of the product.[46]

VHH E is a nanobody that can trigger spike-fusion activity without the presence of the receptor ACE2. It also neutralizes SARS-CoV-2, reducing spike-ACE2-mediated fusion by 50% and blocking 50% of ACE2 binding with an EC50 of 60 nM. The multivalent form of VHH E (VHH-VE) can target two independent epitopes, reducing the risk of drug resistance.[47] Nanobody Nb12 has overlapping antigenic epitopes with previously identified epitopes of SARS-CoV-1 and SARS-CoV-2. This increases its potential to prevent the spread of new mutant strains of SARS-CoV-2. Nb12 and Nb30 multimers have been shown to effectively neutralize pseudotyped viruses carrying mutations in the PANGO variants of SARS-CoV-2.[48] Ty1 is a single-domain antibody isolated from animals that were specifically immunized with the SARS-CoV-2 protein. It directly prevents the binding of the SARS-CoV-2 RBD to the host cell receptor ACE2, neutralizing the pathogen and reducing the rate of infection. Ty1 binds an epitope on the RBD that is accessible in both conformations, blocking RBD−ACE2 binding in multiple ways. Ty1 can be easily produced in high yields in bacteria, making it a candidate for a low-cost, scalable antiviral drug against SARS-CoV-2. Fusing Ty1 to human IgG1 Fc increased its IC50 to approximately 12 ng/mL.[49]

The nanobodies 58G6, 13G9, and 510A5 have a high neutralizing effect against the B.1.351 virus. They also showed strong preventive effects in hACE2 transgenic mice infected with SARS-CoV-2 and B.1.351, with IC50 values ranging from 1.285 to 9.174 ng/mL. These nanobodies were not affected by the RBD escape mutation of B.1.351 and significantly reduced the viral load in lung tissue. Additionally, they prevented body weight loss induced by B.1.351 on day 3.[50] H11-D4 and H11-H4 are nanobodies that bind to the 2019-nCoV spike RBD, blocking its interaction with ACE2. Single-particle cryo-EM showed that both nanobodies bound to the three RBDs in the spike trimer. Fused with immunoglobulin-G (IgG) Fc, both nanobodies showed neutralizing activity against the 2019-nCoV virus, with H11-H4-Fc showing particularly high potency (ND50 4−6 nM). H11-H4 also showed additive neutralization with CR302216. The nanobodies were shown to bind RBD via surface plasmon resonance (SPR) with KDs of 5 and 10 nM for H11-H4 and H11-D4, respectively.[51] Nanobody VHH72 binds to the novel coronavirus with a KD of 40 nM. It blocks ACE2 binding and has neutralizing activity against the novel coronavirus pseudovirus 25, with an ND50 of 0.2 μg/mL.[52]

NANOBODY-BASED SARS-COV-2 DETECTION

Developing effective and rapid diagnostic tests for SARS-CoV-2 variants is essential for timely medical and public health decisions in controlling COVID-19 transmission and reducing its health burden. Along with vaccines and therapeutic antibodies, these diagnostic tests play a critical role in slowing the spread of the virus and reducing the impact of the pandemic.

Identifying infected individuals through the detection of SARS-CoV-2 antigens is a crucial method for interrupting the source of transmission and preventing the spread of the virus. This is currently the primary method for detecting the presence of the virus and controlling its transmission. SARS-CoV-2 is a positive-stranded RNA virus with a genome size of 30 kb and encodes four key structural proteins, including the

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

nucleocapsid (N) protein, envelope (E) protein, membrane (M) protein, and spike (S) protein. The spike protein on the viral surface is the key to the virus infecting host cells and is the target for many of the vaccines and treatments developed for COVID-19. The spike protein binds to the ACE2 receptor on host cells through its receptor-binding domain (RBD).[53] Nucleic acid testing is one of the most used methods for SARS-CoV-2 detection, which relies on the reverse transcription polymerase chain reaction (RT-PCR) to amplify and detect viral RNA in a sample. The RT-PCR method is a highly sensitive and specific diagnostic test for SARS-CoV-2.

Although RT-PCR has high sensitivity, specificity, and reliability, its detection process is complex, necessitating a lot of staff and equipment. This, combined with the viral sample load and target region mutation, which have a significant impact on detection, leads to a high detection difficulty and a high likelihood of false negative detection results. An effective substitute for RT-PCR is thought to be loop-mediated isothermal amplification (LAMP). It has six to eight distinct primer sequences that can recognize eight different regions. Without isolating the RNA, it may detect SARS-CoV-2 in saliva or swabs. The method of detection is quick and very precise, and the sensitivity varies with the amount of virus.[54]

The colloidal gold method, a rapid detection test strip, determines if a person is infected with a novel coronavirus by detecting the presence of IgM against the novel coronavirus in the blood. This means that its detection range is smaller than the nucleic acid test, but the test results are available in about 15 min and are portable. The second form, which is appropriate for small-scale laboratory testing, is the ancestor of the laboratory sandwich enzyme-linked immunosorbent assay (ELISA). A highly sensitive approach for detecting antigens in solution is the sandwich ELISA, or enzyme-linked immunosorbent assay. The target analyte is captured using one ligand and detected using a different ligand. The typical method of detection is a colorimetric readout produced by reacting the substrate with a secondary ligand that has been enzyme-labeled. The spike protein of SARS-CoV-2 was detected using sandwich ELISA, which combines manufactured nanobodies. Due to the spike's tiny size and inadequate plate adhesion, binding nanobodies isolated from the original alpaca VHH library were able to bind to it. We linked human IgG1 Fc (diacylated) to VHH as predicted, increasing the total size (still smaller than normal antibodies) but introducing a region that alters the VHH distal structural domain in a specific way to make it compatible with the plate. The antigen−antibody binding affinity determines how sensitive an ELISA test is.[53]

To create a heavy-chain variable domain (VHH) antibody library, Gransagne et al. inoculated alpacas with recombinant SARS-CoV-2 N protein. Following phage display selection, ELISA was used to determine the N-terminal domain (NTD) specific VHH (NTD E4-3 and NTD B6-1). The best detection signal at a concentration as low as 4 ng/mL is generated by the mixture of Nb in C-terminal domain (CTD) Nb G9-1 and N-terminal domain (NTD) Nb E4-3. Sandwich ELISA NTD E4-3/G9-1 can identify the N protein in cells with the B.1.1.7 and B.1.351 variants and can identify the SARS-CoV-2 virus and its variant.[55] Using immunological alpaca, phage display technology, and *in vitro* binding tests, Wagner et al. screened the double-labeled Nb (bipNb) (NM1267) that can successfully disrupt the interaction between RBD, S1, homotrimeric spike protein (spike protein), and ACE2. Nc (NM1226 and NM1230) that simultaneously target two separate RBD epitopes make up NM1267. NM1267 has a high affinity for various RBDs, and both RBDs in their wild-type and mutant forms—RBDs with KDs of 0.5 and 1.15 nM, respectively—inhibit the binding of ACE2 to antigen and efficiently neutralize viruses (IC50 = 0.9 nM). To detect neutralizing antibodies and examine the immune response of infected and immunized individuals both subjectively and quantitatively, Wagner et al. developed NeurobodyPlex utilizing NM1267.[56]

PERSPECTIVES AND CONCLUSION

Several intracellular targets involved in different stages of the viral life cycle have been identified as potential targets for nanobodies to inhibit SARS-CoV-2 replication and packaging, such as Mpro, RdRp, and viral N protein.[57,58] Although nanobodies' small size enables us to effectively target intracellular proteins and other targets involved in various cellular pathways, delivering nanobodies into the cytoplasm of target cells is a significant challenge due to the presence of cellular membranes that prevent their entry. There are several approaches to deliver nanobodies into the cytoplasm, including chemical conjugation,[59] cell-penetrating peptides,[60−62] and nanoparticle-based delivery systems.[63,64] These methods have been used successfully for the delivery of other therapeutic molecules. Thus, the development of nanobodies targeting intracellular targets involved in SARS-CoV-2 replication still holds promise for the development of new therapeutics to combat COVID-19.

In conclusion, the use of nanobodies in the treatment and diagnosis of SARS-CoV-2 has great potential due to their ability to neutralize the virus by blocking the binding of the spike protein RBD to human ACE2, as well as their advantages in production, preservation, and scalability. The results of experiments with nanobodies against the virus have shown promising results, and their application as drugs and imaging probes holds great promise in the future. Further studies are needed to establish universal principles for nanobody-based treatments and diagnoses of diseases, including SARS-CoV-2.

ASSOCIATED CONTENT

Data Availability Statement

Data sharing is not applicable to this article as no data sets were generated or analyzed during the current study.

AUTHOR INFORMATION

Corresponding Author

Hu Wang − *Department of Microbiology and Immunology, College of Medicine and Health Science, China Three Gorges University, Yichang 443002, China; Institute of Cell Engineering, School of Medicine, Johns Hopkins University, Baltimore 21215, United States;* orcid.org/0000-0001-6132-8788; Email: biomed_wang@yahoo.com

Author

Xuemei Feng − *Department of Microbiology and Immunology, College of Medicine and Health Science, China Three Gorges University, Yichang 443002, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsptsci.3c00042

Author Contributions

H.W. created the concepts, X.F. and H.W. performed the literature search, manuscript drafting, table preparation, and

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

figure illustration. H.W. and X.F. elaborated on and consolidated the manuscript. All authors have read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Fan, E.; Beitler, J. R.; Brochard, L.; Calfee, C. S.; Ferguson, N. D.; Slutsky, A. S.; Brodie, D. COVID-19-associated acute respiratory distress syndrome: is a different approach to management warranted? *Lancet Respir Med.* **2020**, *8* (8), 816−821.

(2) Bubar, K. M.; Reinholt, K.; Kissler, S. M.; Lipsitch, M.; Cobey, S.; Grad, Y. H.; Larremore, D. B. Model-informed COVID-19 vaccine prioritization strategies by age and serostatus. *Science* **2021**, *371* (6532), 916−921.

(3) Focosi, D.; Maggi, F. Neutralising antibody escape of SARS-CoV-2 spike protein: Risk assessment for antibody-based Covid-19 therapeutics and vaccines. *Rev. Med. Virol* **2021**, *31* (6), e2231.

(4) Lange, B.; Gerigk, M.; Tenenbaum, T. Breakthrough Infections in BNT162b2-Vaccinated Health Care Workers. *N Engl J. Med.* **2021**, *385* (12), 1145−1146.

(5) Hacisuleyman, E.; Hale, C.; Saito, Y.; Blachere, N. E.; Bergh, M.; Conlon, E. G.; Schaefer-Babajew, D. J.; DaSilva, J.; Muecksch, F.; Gaebler, C.; Lifton, R.; Nussenzweig, M. C.; Hatziioannou, T.; Bieniasz, P. D.; Darnell, R. B. Vaccine Breakthrough Infections with SARS-CoV-2 Variants. *N Engl J. Med.* **2021**, *384* (23), 2212−2218.

(6) Yang, D. W.; Ju, M. J.; Wang, H.; Jia, Y. C.; Wang, X. D.; Fang, H.; Fan, J. Proxalutamide for the treatment of COVID-19 rebound following Paxlovid treatment: Report of four cases and review of the literature. *J. Clin Lab Anal* **2023**, e24880.

(7) Hwang, Y. C.; Lu, R. M.; Su, S. C.; Chiang, P. Y.; Ko, S. H.; Ke, F. Y.; Liang, K. H.; Hsieh, T. Y.; Wu, H. C. Monoclonal antibodies for COVID-19 therapy and SARS-CoV-2 detection. *J. Biomed Sci.* **2022**, *29* (1), 1.

(8) Fenizia, C.; Galbiati, S.; Vanetti, C.; Vago, R.; Clerici, M.; Tacchetti, C.; Daniele, T. SARS-CoV-2 Entry: At the Crossroads of CD147 and ACE2. *Cells* **2021**, *10* (6), 1434.

(9) Walls, A. C.; Park, Y. J.; Tortorici, M. A.; Wall, A.; McGuire, A. T.; Veesler, D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* **2020**, *181* (2), 281−292.e6.

(10) Shang, J.; Wan, Y.; Luo, C.; Ye, G.; Geng, Q.; Auerbach, A.; Li, F. Cell entry mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (21), 11727−11734.

(11) Jovčevska, I.; Muyldermans, S. The Therapeutic Potential of Nanobodies. *BioDrugs* **2020**, *34* (1), 11−26.

(12) Hamers-Casterman, C.; Atarhouch, T.; Muyldermans, S.; Robinson, G.; Hammers, C.; Songa, E. B.; Bendahman, N.; Hammers, R. Naturally occurring antibodies devoid of light chains. *Nature* **1993**, *363* (6428), 446−448.

(13) Bannas, P.; Hambach, J.; Koch-Nolte, F. Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics. *Front Immunol* **2017**, *8*, 1603.

(14) Ingram, J. R.; Schmidt, F. I.; Ploegh, H. L. Exploiting Nanobodies' Singular Traits. *Annu. Rev. Immunol.* **2018**, *36*, 695−715.

(15) Najmeddin, A.; Bahrololoumi Shapourabadi, M.; Behdani, M.; Dorkoosh, F. Nanobodies as powerful pulmonary targeted biotherapeutics against SARS-CoV-2, pharmaceutical point of view. *Biochim Biophys Acta Gen Subj* **2021**, *1865* (11), 129974.

(16) Li, X.; Wang, J.; Geng, J.; Xiao, L.; Wang, H. Emerging Landscape of SARS-CoV-2 Variants and Detection Technologies. *Mol. Diagn Ther* **2023**, *27* (2), 159−177.

(17) Bebtelovimab. In *Drugs and Lactation Database (LactMed)*; National Library of Medicine (US): Bethesda, MA, 2006.

(18) Xu, J.; Xu, K.; Jung, S.; Conte, A.; Lieberman, J.; Muecksch, F.; Lorenzi, J. C. C.; Park, S.; Schmidt, F.; Wang, Z.; Huang, Y.; Luo, Y.; Nair, M. S.; Wang, P.; Schulz, J. E.; Tessarollo, L.; Bylund, T.; Chuang, G. Y.; Olia, A. S.; Stephens, T.; Teng, I. T.; Tsybovsky, Y.; Zhou, T.; Munster, V.; Ho, D. D.; Hatziioannou, T.; Bieniasz, P. D.; Nussenzweig, M. C.; Kwong, P. D.; Casellas, R. Nanobodies from camelid mice and llamas neutralize SARS-CoV-2 variants. *Nature* **2021**, *595* (7866), 278−282.

(19) Kolkman, J. A.; Law, D. A. Nanobodies - from llamas to therapeutic proteins. *Drug Discov Today Technol.* **2010**, *7* (2), e139−e146.

(20) Govaert, J.; Pellis, M.; Deschacht, N.; Vincke, C.; Conrath, K.; Muyldermans, S.; Saerens, D. Dual beneficial effect of interloop disulfide bond for single domain antibody fragments. *J. Biol. Chem.* **2012**, *287* (3), 1970−1979.

(21) De Vos, J.; Devoogdt, N.; Lahoutte, T.; Muyldermans, S. Camelid single-domain antibody-fragment engineering for (pre)-clinical in vivo molecular imaging applications: adjusting the bullet to its target. *Expert Opin Biol. Ther* **2013**, *13* (8), 1149−1160.

(22) Muyldermans, S. Nanobodies: natural single-domain antibodies. *Annu. Rev. Biochem.* **2013**, *82*, 775−797.

(23) Dumoulin, M.; Conrath, K.; Van Meirhaeghe, A.; Meersman, F.; Heremans, K.; Frenken, L. G.; Muyldermans, S.; Wyns, L.; Matagne, A. Single-domain antibody fragments with high conformational stability. *Protein Sci.* **2002**, *11* (3), 500−515.

(24) Sun, S.; Ding, Z.; Yang, X.; Zhao, X.; Zhao, M.; Gao, L.; Chen, Q.; Xie, S.; Liu, A.; Yin, S.; Xu, Z.; Lu, X. Nanobody: A Small Antibody with Big Implications for Tumor Therapeutic Strategy. *Int. J. Nanomedicine* **2021**, *16*, 2337−2356.

(25) Van Audenhove, I.; Gettemans, J. Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer. *EBioMedicine* **2016**, *8*, 40−48.

(26) Tijink, B. M.; Laeremans, T.; Budde, M.; Stigter-van Walsum, M.; Dreier, T.; de Haard, H. J.; Leemans, C. R.; van Dongen, G. A. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. *Mol. Cancer Ther* **2008**, *7* (8), 2288−2297.

(27) Qi, H.; Ma, M.; Lai, D.; Tao, S. C. Phage display: an ideal platform for coupling protein to nucleic acid. *Acta Biochim Biophys Sin (Shanghai)* **2021**, *53* (4), 389−399.

(28) Ledsgaard, L.; Kilstrup, M.; Karatt-Vellatt, A.; McCafferty, J.; Laustsen, A. H. Basics of Antibody Phage Display Technology. *Toxins (Basel)* **2018**, *10* (6), 236.

(29) Chao, G.; Lau, W. L.; Hackel, B. J.; Sazinsky, S. L.; Lippow, S. M.; Wittrup, K. D. Isolating and engineering human antibodies using yeast surface display. *Nat. Protoc* **2006**, *1* (2), 755−768.

(30) Cherf, G. M.; Cochran, J. R. Applications of Yeast Surface Display for Protein Engineering. *Methods Mol. Biol.* **2015**, *1319*, 155−175.

(31) Tanaka, T.; Yamada, R.; Ogino, C.; Kondo, A. Recent developments in yeast cell surface display toward extended applications in biotechnology. *Appl. Microbiol. Biotechnol.* **2012**, *95* (3), 577−591.

(32) Xian, Z.; Ma, L.; Zhu, M.; Li, G.; Gai, J.; Chang, Q.; Huang, Y.; Ju, D.; Wan, Y. Blocking the PD-1-PD-L1 axis by a novel PD-1 specific nanobody expressed in yeast as a potential therapeutic for immunotherapy. *Biochem. Biophys. Res. Commun.* **2019**, *519* (2), 267−273.

(33) Drick, N.; Seeliger, B.; Welte, T.; Fuge, J.; Suhling, H. Anti-IL-5 therapy in patients with severe eosinophilic asthma - clinical efficacy and possible criteria for treatment response. *BMC Pulm Med.* **2018**, *18* (1), 119.

(34) Schoof, M.; Faust, B.; Saunders, R. A.; Sangwan, S.; Rezelj, V.; Hoppe, N.; Boone, M.; Billesbølle, C. B.; Puchades, C.; Azumaya, C. M.; Kratochvil, H. T.; Zimanyi, M.; Deshpande, I.; Liang, J.; Dickinson, S.; Nguyen, H. C.; Chio, C. M.; Merz, G. E.; Thompson, M. C.; Diwanji, D.; Schaefer, K.; Anand, A. A.; Dobzinski, N.; Zha, B. S.; Simoneau, C. R.; Leon, K.; White, K. M.; Chio, U. S.; Gupta, M.; Jin, M.; Li, F.; Liu, Y.; Zhang, K.; Bulkley, D.; Sun, M.; Smith, A. M.; Rizo, A. N.; Moss, F.; Brilot, A. F.; Pourmal, S.; Trenker, R.; Pospiech, T.; Gupta, S.; Barsi-Rhyne, B.; Belyy, V.; Barile-Hill, A. W.; Nock, S.; Liu, Y.; Krogan, N. J.; Ralston, C. Y.; Swaney, D. L.; García-Sastre, A.; Ott, M.; Vignuzzi, M.; Walter, P.; Manglik, A. An ultrapotent synthetic nanobody neutralizes SARS-

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942

CoV-2 by stabilizing inactive Spike. *Science* **2020**, *370* (6523), 1473−1479.
(35) Groves, M. A.; Nickson, A. A. Affinity maturation of phage display antibody populations using ribosome display. *Methods Mol. Biol.* **2012**, *805*, 163−190.
(36) He, M.; Taussig, M. J. Ribosome display: cell-free protein display technology. *Brief Funct Genomic Proteomic* **2002**, *1* (2), 204−212.
(37) Kunamneni, A.; Ogaugwu, C.; Bradfute, S.; Durvasula, R. Ribosome Display Technology: Applications in Disease Diagnosis and Control. *Antibodies (Basel)* **2020**, *9* (3), 28.
(38) Tang, J.; Wang, L.; Markiv, A.; Jeffs, S. A.; Dreja, H.; McKnight, Á.; He, M.; Kang, A. S. Accessing of recombinant human monoclonal antibodies from patient libraries by eukaryotic ribosome display. *Hum Antibodies* **2012**, *21* (1−2), 1−11.
(39) Liu, D.; Geng, H.; Zhang, Z.; Xing, Y.; Yang, D.; Liu, Z.; Wang, D. An Effective Platform for Exploring Rotavirus Receptors by Bacterial Surface Display System. *Virol Sin* **2020**, *35* (1), 103−109.
(40) Nienberg, C.; Garmann, C.; Gratz, A.; Bollacke, A.; Götz, C.; Jose, J. Identification of a Potent Allosteric Inhibitor of Human Protein Kinase CK2 by Bacterial Surface Display Library Screening. *Pharmaceuticals (Basel)* **2017**, *10* (1), 6.
(41) Kreuzberger, N.; Hirsch, C.; Chai, K. L.; Tomlinson, E.; Khosravi, Z.; Popp, M.; Neidhardt, M.; Piechotta, V.; Salomon, S.; Valk, S. J.; Monsef, I.; Schmaderer, C.; Wood, E. M.; So-Osman, C.; Roberts, D. J.; McQuilten, Z.; Estcourt, L. J.; Skoetz, N. SARS-CoV-2-neutralising monoclonal antibodies for treatment of COVID-19. *Cochrane Database Syst. Rev.* **2021**, *9* (9), Cd013825.
(42) Kontermann, R. E. Strategies for extended serum half-life of protein therapeutics. *Curr. Opin Biotechnol* **2011**, *22* (6), 868−876.
(43) Wu, X.; Cheng, L.; Fu, M.; Huang, B.; Zhu, L.; Xu, S.; Shi, H.; Zhang, D.; Yuan, H.; Nawaz, W.; Yang, P.; Hu, Q.; Liu, Y.; Wu, Z. A potent bispecific nanobody protects hACE2 mice against SARS-CoV-2 infection via intranasal administration. *Cell Rep* **2021**, *37* (3), 109869.
(44) Nambulli, S.; Xiang, Y.; Tilston-Lunel, N. L.; Rennick, L. J.; Sang, Z.; Klimstra, W. B.; Reed, D. S.; Crossland, N. A.; Shi, Y.; Duprex, W. P. Inhalable Nanobody (PiN-21) prevents and treats SARS-CoV-2 infections in Syrian hamsters at ultra-low doses. *Sci. Adv.* **2021**, *7* (22), No. eabh0319.
(45) Esparza, T. J.; Martin, N. P.; Anderson, G. P.; Goldman, E. R.; Brody, D. L. High affinity nanobodies block SARS-CoV-2 spike receptor binding domain interaction with human angiotensin converting enzyme. *Sci. Rep* **2020**, *10* (1), 22370.
(46) Gai, J.; Ma, L.; Li, G.; Zhu, M.; Qiao, P.; Li, X.; Zhang, H.; Zhang, Y.; Chen, Y.; Ji, W.; Zhang, H.; Cao, H.; Li, X.; Gong, R.; Wan, Y. A potent neutralizing nanobody against SARS-CoV-2 with inhaled delivery potential. *MedComm (2020)* **2021**, *2* (1), 101−113.
(47) Koenig, P. A.; Das, H.; Liu, H.; Kümmerer, B. M.; Gohr, F. N.; Jenster, L. M.; Schiffelers, L. D. J.; Tesfamariam, Y. M.; Uchima, M.; Wuerth, J. D.; Gatterdam, K.; Ruetalo, N.; Christensen, M. H.; Fandrey, C. I.; Normann, S.; Tödtmann, J. M. P.; Pritzl, S.; Hanke, L.; Boos, J.; Yuan, M.; Zhu, X.; Schmid-Burgk, J. L.; Kato, H.; Schindler, M.; Wilson, I. A.; Geyer, M.; Ludwig, K. U.; Hällberg, B. M.; Wu, N. C.; Schmidt, F. I. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. *Science* **2021**, *371* (6530),.
(48) Xu, J.; Xu, K.; Jung, S.; Conte, A.; Lieberman, J.; Muecksch, F.; Cetrulo Lorenzi, J. C.; Park, S.; Wang, Z.; Tessarollo, L.; Bylund, T.; Chuang, G. Y.; Olia, A. S.; Stephens, T.; Teng, I. T.; Tsybovsky, Y.; Zhou, T.; Hatziioannou, T.; Bieniasz, P. D.; Nussenzweig, M. C.; Kwong, P. D.; Casellas, R. Multimeric nanobodies from camelid engineered mice and llamas potently neutralize SARS-CoV-2 variants. *bioRxiv* **2021**, DOI: 10.1101/2021.03.04.433768.
(49) Hanke, L.; Vidakovics Perez, L.; Sheward, D. J.; Das, H.; Schulte, T.; Moliner-Morro, A.; Corcoran, M.; Achour, A.; Karlsson Hedestam, G. B.; Hällberg, B. M.; Murrell, B.; McInerney, G. M. An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat. Commun.* **2020**, *11* (1), 4420.
(50) Li, T.; Han, X.; Gu, C.; Guo, H.; Zhang, H.; Wang, Y.; Hu, C.; Wang, K.; Liu, F.; Luo, F.; Zhang, Y.; Hu, J.; Wang, W.; Li, S.; Hao, Y.; Shen, M.; Huang, J.; Long, Y.; Song, S.; Wu, R.; Mu, S.; Chen, Q.; Gao, F.; Wang, J.; Long, S.; Li, L.; Wu, Y.; Gao, Y.; Xu, W.; Cai, X.; Qu, D.; Zhang, Z.; Zhang, H.; Li, N.; Gao, Q.; Zhang, G.; He, C.; Wang, W.; Ji, X.; Tang, N.; Yuan, Z.; Xie, Y.; Yang, H.; Zhang, B.; Huang, A.; Jin, A. Potent SARS-CoV-2 neutralizing antibodies with protective efficacy against newly emerged mutational variants. *Nat. Commun.* **2021**, *12* (1), 6304.
(51) Zupancic, J. M.; Schardt, J. S.; Desai, A. A.; Makowski, E. K.; Smith, M. D.; Pornnoppadol, G.; Garcia de Mattos Barbosa, M.; Cascalho, M.; Lanigan, T. M.; Tessier, P. M. Engineered Multivalent Nanobodies Potently and Broadly Neutralize SARS-CoV-2 Variants. *Adv. Ther (Weinh)* **2021**, *4* (8), 2100099.
(52) Wrapp, D.; De Vlieger, D.; Corbett, K. S.; Torres, G. M.; Wang, N.; Van Breedam, W.; Roose, K.; van Schie, L.; Team, V.-C. C.-R.; Hoffmann, M.; Pohlmann, S.; Graham, B. S.; Callewaert, N.; Schepens, B.; Saelens, X.; McLellan, J. S. Structural Basis for Potent Neutralization of Betacoronaviruses by Single-Domain Camelid Antibodies. *Cell* **2020**, *181* (6), 1436−1441.
(53) Girt, G. C.; Lakshminarayanan, A.; Huo, J.; Dormon, J.; Norman, C.; Afrough, B.; Harding, A.; James, W.; Owens, R. J.; Naismith, J. H. The use of nanobodies in a sensitive ELISA test for SARS-CoV-2 Spike 1 protein. *R Soc. Open Sci.* **2021**, *8* (9), 211016.
(54) Fernandes, Q.; Inchakalody, V. P.; Merhi, M.; Mestiri, S.; Taib, N.; Moustafa Abo El-Ella, D.; Bedhiafi, T.; Raza, A.; Al-Zaidan, L.; Mohsen, M. O.; Yousuf Al-Nesf, M. A.; Hssain, A. A.; Yassine, H. M.; Bachmann, M. F.; Uddin, S.; Dermime, S. Emerging COVID-19 variants and their impact on SARS-CoV-2 diagnosis, therapeutics and vaccines. *Ann. Med.* **2022**, *54* (1), 524−540.
(55) Gransagne, M.; Aymé, G.; Brier, S.; Chauveau-Le Friec, G.; Meriaux, V.; Nowakowski, M.; Dejardin, F.; Levallois, S.; Dias de Melo, G.; Donati, F.; Prot, M.; Brûlé, S.; Raynal, B.; Bellalou, J.; Goncalves, P.; Montagutelli, X.; Di Santo, J. P.; Lazarini, F.; England, P.; Petres, S.; Escriou, N.; Lafaye, P. Development of a highly specific and sensitive VHH-based sandwich immunoassay for the detection of the SARS-CoV-2 nucleoprotein. *J. Biol. Chem.* **2022**, *298* (1), 101290.
(56) Wagner, T. R.; Ostertag, E.; Kaiser, P. D.; Gramlich, M.; Ruetalo, N.; Junker, D.; Haering, J.; Traenkle, B.; Becker, M.; Dulovic, A.; Schweizer, H.; Nueske, S.; Scholz, A.; Zeck, A.; Schenke-Layland, K.; Nelde, A.; Strengert, M.; Walz, J. S.; Zocher, G.; Stehle, T.; Schindler, M.; Schneiderhan-Marra, N.; Rothbauer, U. Neutrobody-Plex-monitoring SARS-CoV-2 neutralizing immune responses using nanobodies. *EMBO Rep* **2021**, *22* (5), e52325.
(57) Antonopoulou, I.; Sapountzaki, E.; Rova, U.; Christakopoulos, P. Inhibition of the main protease of SARS-CoV-2 (M(pro)) by repurposing/designing drug-like substances and utilizing nature's toolbox of bioactive compounds. *Comput. Struct Biotechnol J.* **2022**, *20*, 1306−1344.
(58) Mercorelli, B.; Desantis, J.; Celegato, M.; Bazzacco, A.; Siragusa, L.; Benedetti, P.; Eleuteri, M.; Croci, F.; Cruciani, G.; Goracci, L.; Loregian, A. Discovery of novel SARS-CoV-2 inhibitors targeting the main protease M(pro) by virtual screenings and hit optimization. *Antiviral Res.* **2022**, *204*, 105350.
(59) Schumacher, D.; Helma, J.; Schneider, A. F. L.; Leonhardt, H.; Hackenberger, C. P. R. Nanobodies: Chemical Functionalization Strategies and Intracellular Applications. *Angew. Chem., Int. Ed. Engl.* **2018**, *57* (9), 2314−2333.
(60) Geng, J.; Wang, J.; Wang, H. Emerging Landscape of Cell-Penetrating Peptide-Mediated Organelle Restoration and Replacement. *ACS Pharmacol Transl Sci.* **2023**, *6* (2), 229−244.
(61) Geng, J.; Xia, X.; Teng, L.; Wang, L.; Chen, L.; Guo, X.; Belingon, B.; Li, J.; Feng, X.; Li, X.; Shang, W.; Wan, Y.; Wang, H. Emerging landscape of cell-penetrating peptide-mediated nucleic acid delivery and their utility in imaging, gene-editing, and RNA-sequencing. *J. Controlled Release* **2022**, *341*, 166−183.
(62) Liu, H.; Zeng, F.; Zhang, M.; Huang, F.; Wang, J.; Guo, J.; Liu, C.; Wang, H. Emerging landscape of cell penetrating peptide in

reprogramming and gene editing. *J. Controlled Release* **2016**, *226*, 124−137.
(63) Wang, Y.; Fan, Z.; Shao, L.; Kong, X.; Hou, X.; Tian, D.; Sun, Y.; Xiao, Y.; Yu, L. Nanobody-derived nanobiotechnology tool kits for diverse biomedical and biotechnology applications. *Int. J. Nanomedicine* **2016**, *11*, 3287−3303.
(64) Ruiz-López, E.; Schuhmacher, A. J. Transportation of Single-Domain Antibodies through the Blood-Brain Barrier. *Biomolecules* **2021**, *11* (8), 1131.
(65) Zanganeh, S.; Rouhani Nejad, H.; Mehrabadi, J. F.; Hosseini, R.; Shahi, B.; Tavassoli, Z.; Aramvash, A. Rapid and Sensitive Detection of Staphylococcal Enterotoxin B by Recombinant Nanobody Using Phage Display Technology. *Appl. Biochem. Biotechnol.* **2019**, *187* (2), 493−505.
(66) Xu, C.; Yang, Y.; Liu, L.; Li, J.; Liu, X.; Zhang, X.; Liu, Y.; Zhang, C.; Liu, X. Microcystin-LR nanobody screening from an alpaca phage display nanobody library and its expression and application. *Ecotoxicol Environ. Saf* **2018**, *151*, 220−227.
(67) Qiu, Y. L.; He, Q. H.; Xu, Y.; Bhunia, A. K.; Tu, Z.; Chen, B.; Liu, Y. Y. Deoxynivalenol-mimic nanobody isolated from a naïve phage display nanobody library and its application in immunoassay. *Anal. Chim. Acta* **2015**, *887*, 201−208.
(68) Wang, Y.; Li, P.; Majkova, Z.; Bever, C. R.; Kim, H. J.; Zhang, Q.; Dechant, J. E.; Gee, S. J.; Hammock, B. D. Isolation of alpaca anti-idiotypic heavy-chain single-domain antibody for the aflatoxin immunoassay. *Anal. Chem.* **2013**, *85* (17), 8298−8303.
(69) Xi, X.; Sun, W.; Su, H.; Zhang, X.; Sun, F. Identification of a novel anti-EGFR nanobody by phage display and its distinct paratope and epitope via homology modeling and molecular docking. *Mol. Immunol* **2020**, *128*, 165−174.
(70) Nie, J.; Ma, X.; Hu, F.; Miao, H.; Feng, X.; Zhang, P.; Han, M. H.; You, F.; Yang, Y.; Zhang, W.; Zheng, W. Designing and constructing a phage display synthesized single domain antibodies library based on camel VHHs frame for screening and identifying humanized TNF-alpha-specific nanobody. *Biomed Pharmacother* **2021**, *137*, 111328.
(71) Lamtha, T.; Tabtimmai, L.; Bangphoomi, K.; Kiriwan, D.; Malik, A. A.; Chaicumpa, W.; van Bergen En Henegouwen, P. M. P.; Choowongkomon, K. Generation of a nanobody against HER2 tyrosine kinase using phage display library screening for HER2-positive breast cancer therapy development. *Protein Eng. Des Sel* **2021**, *34*, No. gzab030.
(72) Shahi, B.; Mousavi Gargari, S. L.; Rasooli, I.; Rajabi Bazl, M.; Hoseinpoor, R. Random mutagenesis of BoNT/E Hc nanobody to construct a secondary phage-display library. *J. Appl. Microbiol.* **2014**, *117* (2), 528−536.
(73) Xu, C.; Liu, X.; Zhang, C.; Zhang, X.; Zhong, J.; Liu, Y.; Hu, X.; Lin, M.; Liu, X. Establishment of a sensitive time-resolved fluoroimmunoassay for detection of Bacillus thuringiensis Cry1Ie toxin based nanobody from a phage display library. *Anal. Biochem.* **2017**, *518*, 53−59.
(74) Abbady, A. Q.; Al-Mariri, A.; Zarkawi, M.; Al-Assad, A.; Muyldermans, S. Evaluation of a nanobody phage display library constructed from a Brucella-immunised camel. *Vet. Immunol. Immunopathol.* **2011**, *142* (1−2), 49−56.
(75) Li, T.; Huang, M.; Xiao, H.; Zhang, G.; Ding, J.; Wu, P.; Zhang, H.; Sheng, J.; Chen, C. Selection and characterization of specific nanobody against bovine virus diarrhea virus (BVDV) E2 protein. *PLoS One* **2017**, *12* (6), e0178469.
(76) Moeglin, E.; Desplancq, D.; Stoessel, A.; Massute, C.; Ranniger, J.; McEwen, A. G.; Zeder-Lutz, G.; Oulad-Abdelghani, M.; Chiper, M.; Lafaye, P.; Di Ventura, B.; Didier, P.; Poterszman, A.; Weiss, E. A Novel Nanobody Precisely Visualizes Phosphorylated Histone H2AX in Living Cancer Cells under Drug-Induced Replication Stress. *Cancers (Basel)* **2021**, *13* (13), 3317.
(77) Pymm, P.; Adair, A.; Chan, L. J.; Cooney, J. P.; Mordant, F. L.; Allison, C. C.; Lopez, E.; Haycroft, E. R.; O'Neill, M. T.; Tan, L. L.; Dietrich, M. H.; Drew, D.; Doerflinger, M.; Dengler, M. A.; Scott, N. E.; Wheatley, A. K.; Gherardin, N. A.; Venugopal, H.; Cromer, D.; Davenport, M. P.; Pickering, R.; Godfrey, D. I.; Purcell, D. F. J.; Kent, S. J.; Chung, A. W.; Subbarao, K.; Pellegrini, M.; Glukhova, A.; Tham, W. H. Nanobody cocktails potently neutralize SARS-CoV-2 D614G N501Y variant and protect mice. *Proc. Natl. Acad. Sci. U. S. A.* **2021**, *118* (19), No. e2101918118, DOI: 10.1073/pnas.2101918118.
(78) Kumarasamy, J.; Ghorui, S. K.; Gholve, C.; Jain, B.; Dhekale, Y.; Gupta, G. D.; Damle, A.; Banerjee, S.; Rajan, M. G. R.; Kulkarni, S. Production, characterization and in-vitro applications of single-domain antibody against thyroglobulin selected from novel T7 phage display library. *J. Immunol Methods* **2021**, *492*, 112990.
(79) Wang, H.; Wang, L.; Li, Y.; Li, G.; Zhang, X.; Jiang, D.; Zhang, Y.; Liu, L.; Chu, Y.; Xu, G. Nanobody-armed T cells endow CAR-T cells with cytotoxicity against lymphoma cells. *Cancer Cell Int.* **2021**, *21* (1), 450.
(80) Rezaei, Z. S.; Shahangian, S. S.; Hasannia, S.; Sajedi, R. H. Development of a phage display-mediated immunoassay for the detection of vascular endothelial growth factor. *Anal Bioanal Chem.* **2020**, *412* (27), 7639−7648.
(81) Li, S.; Zhang, W.; Jiang, K.; Shan, H.; Shi, M.; Chen, B.; Hua, Z. Nanobody against the E7 oncoprotein of human papillomavirus 16. *Mol. Immunol* **2019**, *109*, 12−19.
(82) Wellner, A.; McMahon, C.; Gilman, M. S. A.; Clements, J. R.; Clark, S.; Nguyen, K. M.; Ho, M. H.; Hu, V. J.; Shin, J. E.; Feldman, J.; Hauser, B. M.; Caradonna, T. M.; Wingler, L. M.; Schmidt, A. G.; Marks, D. S.; Abraham, J.; Kruse, A. C.; Liu, C. C. Rapid generation of potent antibodies by autonomous hypermutation in yeast. *Nat. Chem. Biol.* **2021**, *17* (10), 1057−1064.
(83) McMahon, C.; Staus, D. P.; Wingler, L. M.; Wang, J.; Skiba, M. A.; Elgeti, M.; Hubbell, W. L.; Rockman, H. A.; Kruse, A. C.; Lefkowitz, R. J. Synthetic nanobodies as angiotensin receptor blockers. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (33), 20284−20291.
(84) Gaiotto, T.; Ramage, W.; Ball, C.; Risley, P.; Carnell, G. W.; Temperton, N.; Engelhardt, O. G.; Hufton, S. E. Nanobodies mapped to cross-reactive and divergent epitopes on A(H7N9) influenza hemagglutinin using yeast display. *Sci. Rep* **2021**, *11* (1), 3126.
(85) Könning, D.; Rhiel, L.; Empting, M.; Grzeschik, J.; Sellmann, C.; Schröter, C.; Zielonka, S.; Dickgießer, S.; Pirzer, T.; Yanakieva, D.; Becker, S.; Kolmar, H. Semi-synthetic vNAR libraries screened against therapeutic antibodies primarily deliver anti-idiotypic binders. *Sci. Rep* **2017**, *7* (1), 9676.
(86) Wang, B.; Lee, C. H.; Johnson, E. L.; Kluwe, C. A.; Cunningham, J. C.; Tanno, H.; Crooks, R. M.; Georgiou, G.; Ellington, A. D. Discovery of high affinity anti-ricin antibodies by B cell receptor sequencing and by yeast display of combinatorial VH:VL libraries from immunized animals. *MAbs* **2016**, *8* (6), 1035−1044.
(87) Zorniak, M.; Clark, P. A.; Umlauf, B. J.; Cho, Y.; Shusta, E. V.; Kuo, J. S. Yeast display biopanning identifies human antibodies targeting glioblastoma stem-like cells. *Sci. Rep* **2017**, *7* (1), 15840.
(88) Amcheslavsky, A.; Wallace, A. L.; Ejemel, M.; Li, Q.; McMahon, C. T.; Stoppato, M.; Giuntini, S.; Schiller, Z. A.; Pondish, J. R.; Toomey, J. R.; Schneider, R. M.; Meisinger, J.; Heukers, R.; Kruse, A. C.; Barry, E. M.; Pierce, B. G.; Klempner, M. S.; Cavacini, L. A.; Wang, Y. Anti-CfaE nanobodies provide broad cross-protection against major pathogenic enterotoxigenic Escherichia coli strains, with implications for vaccine design. *Sci. Rep* **2021**, *11* (1), 2751.
(89) Ramage, W.; Gaiotto, T.; Ball, C.; Risley, P.; Carnell, G. W.; Temperton, N.; Cheung, C. Y.; Engelhardt, O. G.; Hufton, S. E. Cross-Reactive and Lineage-Specific Single Domain Antibodies against Influenza B Hemagglutinin. *Antibodies (Basel)* **2019**, *8* (1), 14.
(90) Ye, G.; Gallant, J. P.; Massey, C.; Shi, K.; Tai, W.; Zheng, J.; Odle, A. E.; Vickers, M. A.; Shang, J.; Wan, Y.; Drelich, A.; Kempaiah, K. R.; Tat, V.; Perlman, S.; Du, L.; Tseng, C. T.; Aihara, H.; LeBeau, A. M.; Li, F. The Development of a Novel Nanobody Therapeutic for SARS-CoV-2. *bioRxiv* **2020**, DOI: 10.1101/2020.11.17.386532.
(91) Huo, J.; Le Bas, A.; Ruza, R. R.; Duyvesteyn, H. M. E.; Mikolajek, H.; Malinauskas, T.; Tan, T. K.; Rijal, P.; Dumoux, M.; Ward, P. N.; Ren, J.; Zhou, D.; Harrison, P. J.; Weckener, M.; Clare, D. K.; Vogirala, V. K.; Radecke, J.; Moynié, L.; Zhao, Y.; Gilbert-

Jaramillo, J.; Knight, M. L.; Tree, J. A.; Buttigieg, K. R.; Coombes, N.; Elmore, M. J.; Carroll, M. W.; Carrique, L.; Shah, P. N. M.; James, W.; Townsend, A. R.; Stuart, D. I.; Owens, R. J.; Naismith, J. H. Neutralizing nanobodies bind SARS-CoV-2 spike RBD and block interaction with ACE2. *Nat. Struct Mol. Biol.* **2020**, *27* (9), 846−854.
(92) Chi, X.; Liu, X.; Wang, C.; Zhang, X.; Li, X.; Hou, J.; Ren, L.; Jin, Q.; Wang, J.; Yang, W. Humanized single domain antibodies neutralize SARS-CoV-2 by targeting the spike receptor binding domain. *Nat. Commun.* **2020**, *11* (1), 4528.
(93) Custódio, T. F.; Das, H.; Sheward, D. J.; Hanke, L.; Pazicky, S.; Pieprzyk, J.; Sorgenfrei, M.; Schroer, M. A.; Gruzinov, A. Y.; Jeffries, C. M.; Graewert, M. A.; Svergun, D. I.; Dobrev, N.; Remans, K.; Seeger, M. A.; McInerney, G. M.; Murrell, B.; Hällberg, B. M.; Löw, C. Selection, biophysical and structural analysis of synthetic nanobodies that effectively neutralize SARS-CoV-2. *Nat. Commun.* **2020**, *11* (1), 5588.
(94) Huo, J.; Mikolajek, H.; Le Bas, A.; Clark, J. J.; Sharma, P.; Kipar, A.; Dormon, J.; Norman, C.; Weckener, M.; Clare, D. K.; Harrison, P. J.; Tree, J. A.; Buttigieg, K. R.; Salguero, F. J.; Watson, R.; Knott, D.; Carnell, O.; Ngabo, D.; Elmore, M. J.; Fotheringham, S.; Harding, A.; Moynié, L.; Ward, P. N.; Dumoux, M.; Prince, T.; Hall, Y.; Hiscox, J. A.; Owen, A.; James, W.; Carroll, M. W.; Stewart, J. P.; Naismith, J. H.; Owens, R. J. A potent SARS-CoV-2 neutralising nanobody shows therapeutic efficacy in the Syrian golden hamster model of COVID-19. *Nat. Commun.* **2021**, *12* (1), 5469.
(95) Wagner, T. R.; Schnepf, D.; Beer, J.; Ruetalo, N.; Klingel, K.; Kaiser, P. D.; Junker, D.; Sauter, M.; Traenkle, B.; Frecot, D. I.; Becker, M.; Schneiderhan-Marra, N.; Ohnemus, A.; Schwemmle, M.; Schindler, M.; Rothbauer, U. Biparatopic nanobodies protect mice from lethal challenge with SARS-CoV-2 variants of concern. *EMBO Rep* **2022**, *23* (2), e53865.
(96) Chen, W. H.; Hajduczki, A.; Martinez, E. J.; Bai, H.; Matz, H.; Hill, T. M.; Lewitus, E.; Chang, W. C.; Dawit, L.; Peterson, C. E.; Rees, P. A.; Ajayi, A. B.; Golub, E. S.; Swafford, I.; Dussupt, V.; David, S.; Mayer, S. V.; Soman, S.; Kuklis, C.; Corbitt, C.; King, J.; Choe, M.; Sankhala, R. S.; Thomas, P. V.; Zemil, M.; Wieczorek, L.; Hart, T.; Duso, D.; Kummer, L.; Yan, L.; Sterling, S. L.; Laing, E. D.; Broder, C. C.; Williams, J. K.; Davidson, E.; Doranz, B. J.; Krebs, S. J.; Polonis, V. R.; Paquin-Proulx, D.; Rolland, M.; Reiley, W. W.; Gromowski, G. D.; Modjarrad, K.; Dooley, H.; Joyce, M. G. Shark nanobodies with potent SARS-CoV-2 neutralizing activity and broad sarbecovirus reactivity. *Nat. Commun.* **2023**, *14* (1), 580.
(97) Feng, B.; Chen, Z.; Sun, J.; Xu, T.; Wang, Q.; Yi, H.; Niu, X.; Zhu, J.; Fan, M.; Hou, R.; Shao, Y.; Huang, S.; Li, C.; Hu, P.; Zheng, P.; He, P.; Luo, J.; Yan, Q.; Xiong, X.; Liu, J.; Zhao, J.; Chen, L. A Class of Shark-Derived Single-Domain Antibodies can Broadly Neutralize SARS-Related Coronaviruses and the Structural Basis of Neutralization and Omicron Escape. *Small Methods* **2022**, *6* (7), e2200387.
(98) Gauhar, A.; Privezentzev, C. V.; Demydchuk, M.; Gerlza, T.; Rieger, J.; Kungl, A. J.; Walsh, F. S.; Rutkowski, J. L.; Stocki, P. Single domain shark VNAR antibodies neutralize SARS-CoV-2 infection in vitro. *FASEB J.* **2021**, *35* (11), e21970.

https://doi.org/10.1021/acsptsci.3c00042
ACS Pharmacol. Transl. Sci. 2023, 6, 925−942