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REVIEW

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Discovery of nanobodies: a comprehensive review of their applications and potential over the past five years

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Abstract

Nanobodies (Nbs) are antibody fragments derived from heavy-chain-only IgG antibodies found in the Camelidae family as well as cartilaginous fish. Their unique structural and functional properties, such as their small size, the ability to be engineered for high antigen-binding affinity, stability under extreme conditions, and ease of production, have made them promising tools for diagnostics and therapeutics. This potential was realized in 2018 with the approval of caplacizumab, the world's first Nb-based drug. Currently, Nbs are being investigated in clinical trials for a broad range of treatments, including targeted therapies against PDL1 and Epidermal Growth Factor Receptor (EGFR), cardiovascular diseases, inflammatory conditions, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. They are also being studied for their potential for detecting and imaging autoimmune conditions and infectious diseases such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A variety of methods are now available to generate target-specific Nbs quickly and efficiently at low costs, increasing their accessibility. This article examines these diverse applications of Nbs and their promising roles. Only the most recent articles published in the last five years have been used to summarize the most advanced developments in the field.

Keywords Nanobody, Cancer, Cardiovascular, Nanobody-imaging, Display techniques, Production, Neurogenerative diseases, CRISPR, Diagnosis, Neurogenerative, Infectious-disease

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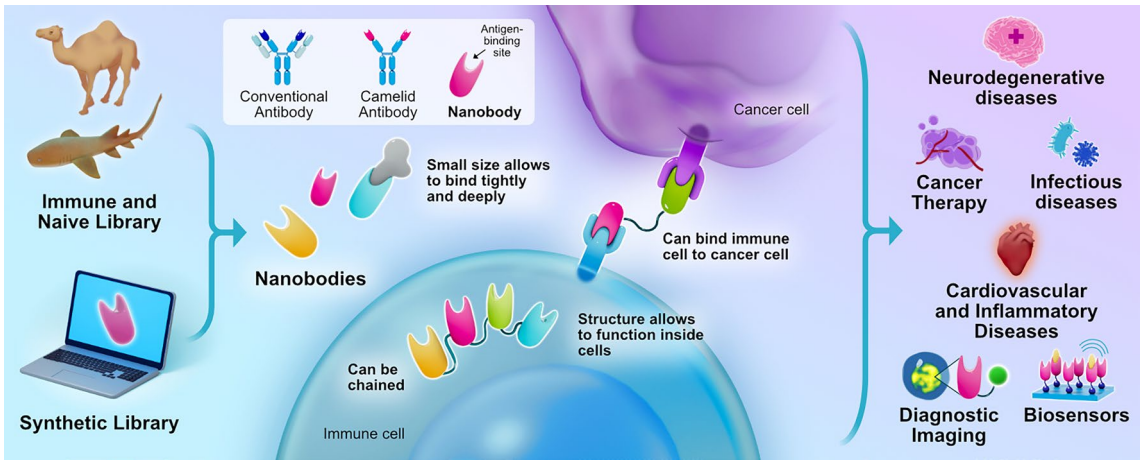
Graphical Abstract



Introduction

The discovery of nanobodies (Nbs) originated from a class of antibodies (Abs) found in dromedary camels, known as heavy-chain-only antibodies (HCAbs), which lack light chains and CH1 domains. Similar HCAbs have also been identified in cartilaginous fish [1]. This single-domain structure, lacking the light chain and Fc region of conventional antibodies, results in a much smaller size (approximately 15 kDa). While this smaller size can enhance tissue penetration, it does not inherently confer resistance to aggregation, as some nanobodies, like conventional antibodies, may still exhibit aggregation tendencies. However, as this review illustrates, engineering strategies could improve the aggregation resistance of Nbs, augmenting the properties that make them more attractive for therapeutic and diagnostic applications than conventional Abs [2–4].

This review explores the emerging field of Nbs, focusing on their advantages and challenges. Initially, we introduce a detailed description of the generation and production of Nbs, followed by a discussion of their characteristics, and highlighting the therapeutic applications of Nbs. The review further delves into the current and future potential of Nb technology, providing insights into the design methodologies and innovative ideas that could shape the development of subsequent generations of Nbs, setting the stage for its expanded use in medical science and therapeutics.

Structural characteristics and properties of nanobodies

Structural characteristics

Compared to conventional Abs, VHHs can have a longer CDR3 for a more diverse paratope, featuring hydrophilic amino acid substitutions that enhance solubility and prevent VL domain interaction while sharing a similar structural organization with differences in FR2 and CDRs to the VH in conventional Abs (Fig. 1) [1, 2]. These qualities enhance antigen-binding diversity and high solubility and resistance to aggregation due to their hydrophilic FR2 while remaining stable in the presence of proteases and pH changes, which suggests potential for alternative routes of administration, such as oral delivery, for therapeutic use [3]. In contrast, conventional Abs such as IgGs comprise two heavy chains and two light chains with a combined mass of 150 kDa, featuring three constant domains (CH1, CH2, and CH3) and a variable domain (VH) in each heavy chain, along with a constant domain (CL) and a variable domain (VL) in each light chain (Fig. 1). Therefore, Nbs stand out due to their structural simplicity, offering advantages such as efficient tissue penetration, high stability, resistance to extreme conditions [5], high solubility and particularly adept at recognizing and binding to some of epitopes, with a distinct preference for non-planar surfaces, such as cavities and grooves, enabling them to target these regions with high precision [2, 6].

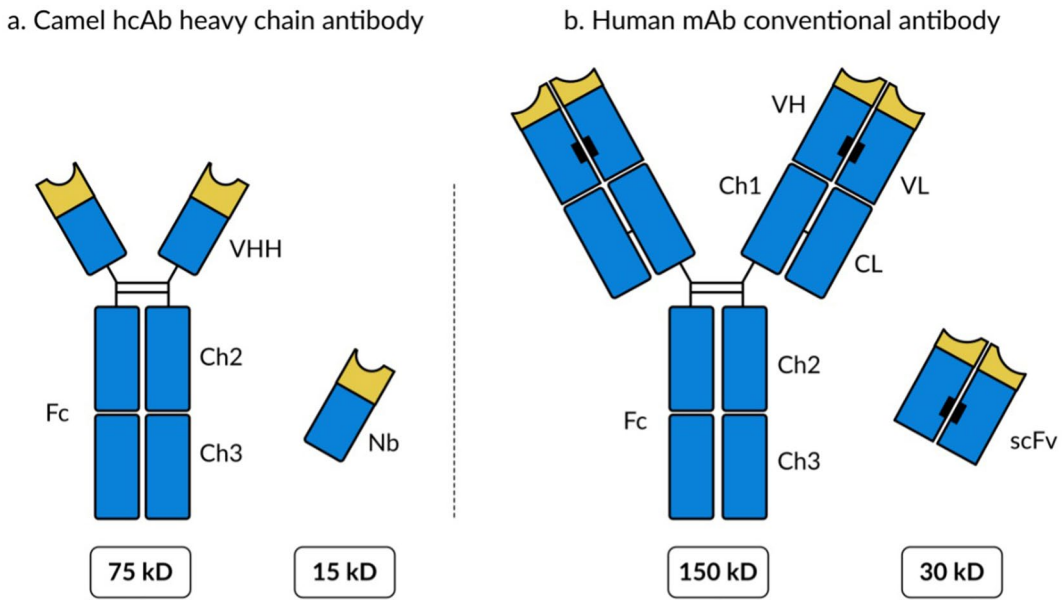


Fig. 1 Key Structural Features of Nanobodies Compared to Conventional Antibodies. Unlike conventional antibodies with both heavy and light chains, HCAbs lack light chains, enabling the isolation of VHH genetic fragments, also known as nanobodies (Nb). B. Conventional mAbs consist of two heavy and two light chains, together forming two identical antigen-binding sites. Each binding site comprises non-covalently linked variable domains, designated VH and VL. Adapted illustration [7]

Table 1 Properties of conventional Abs versus Nbs

Features	Nanobodies	Conventional antibodies
Immunogenicity	Generally less immunogenic but can exhibit immunogenicity due to specific sequences, such as the VTVSS motif in FW4 Humanization does not always eliminate the risk of ADA development [11]	Therapeutic IgGs are typically selected for low immunogenicity, but transgenic human antibodies can still be immunogenic [11]
Thermostability	Can withstand temperatures up to 90 °C [11]	Stability below 90 °C [11]
Nasal Administration	Possible via aerosol [11]	Not suitable for nasal administration [11]
Size	Approximately 15 kDa [1]	Approximately 150 kDa [1]
Tissue Penetration	Capable of penetrating the blood–brain barrier and reaching extracellular matrix, grooves, and clefts [9]	Limited tissue penetration [1]
Stability/Solubility	Generally exhibits high stability and solubility, but some VHHs can be prone to aggregation [9]	Soluble and stable, with a risk of aggregation for some IgGs. Highly stable IgGs can be concentrated to over 300 mg/mL [9]
Half-Life	Rapid clearance, but can be extended using techniques like albumin-binding VHH, Fc domain addition, or PEGylation. However, these modifications can affect tissue penetration [1]	Generally long half-life, but may be shortened by poor pharmacokinetics due to factors like hydrophobicity, target sink, or immunogenicity [1]

Nanobodies have highly desirable properties

Nbs, are the smallest functional antibody domains, can be bioconjugated for enhanced efficacy, improved pharmacokinetics, and broader applications, overcoming the limitations of conventional Abs [1]. Despite observations of Nb aggregation during heat denaturation, Nbs exhibit remarkable physical properties, including extended shelf life at + 4 °C and − 20 °C, resilience to high temperatures (60–80 °C) and proteolytic degradation, tolerance to nonphysiological pH (3.0–9.0), resistance to elevated pressure (500–750 MPa), and exposure to chemical denaturants (2–3 M guanidinium chloride, 6–8 M urea), with minimal impact on their antigen-binding capacity [8]. Certain nanobodies, particularly those that have been engineered or specifically selected for this purpose, have shown the ability to cross the blood–brain barrier, making them potential candidates for treating central nervous system disorders (Table 1). However, rapid renal clearance and surface charge limit CNS potential. Strategies to improve their

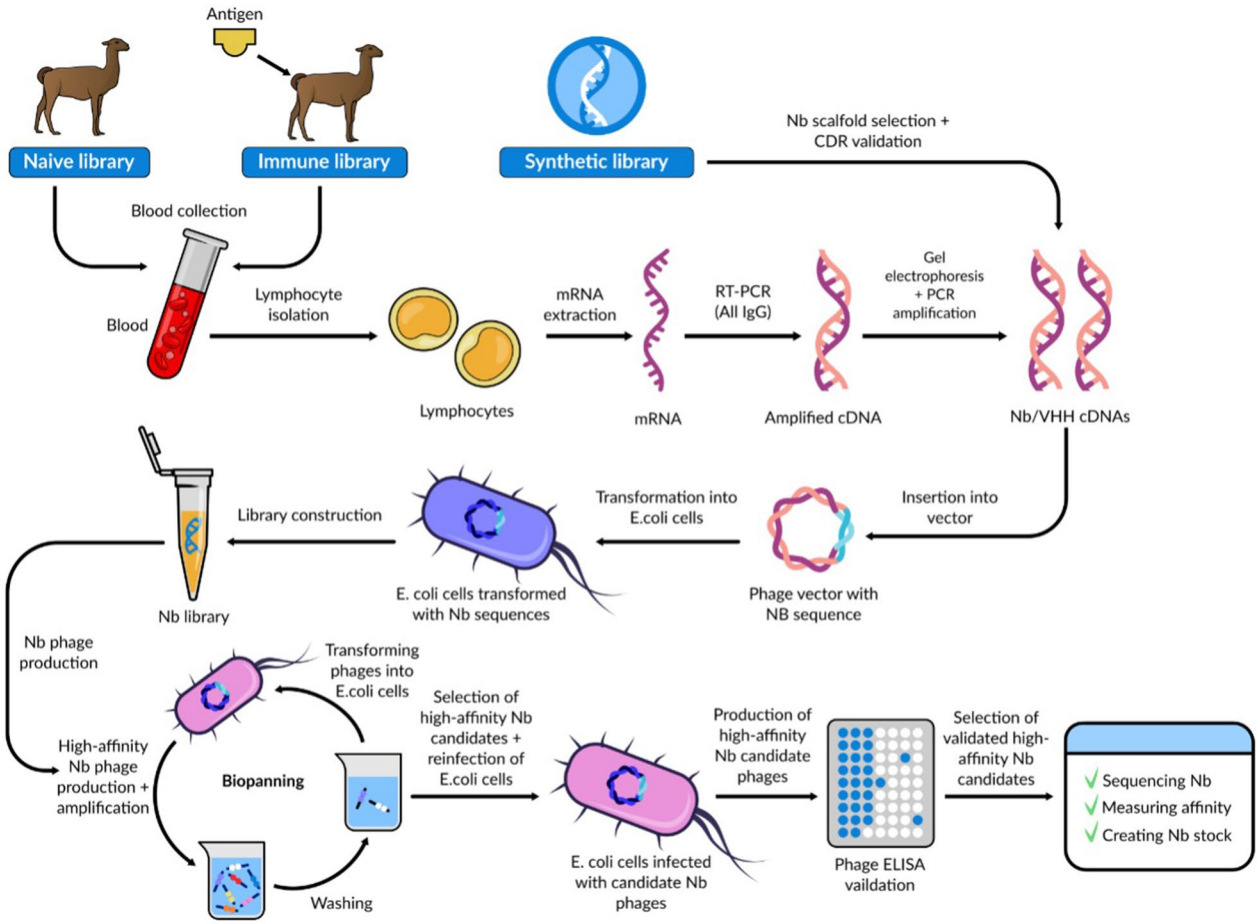


Fig. 2 Generating Antigen-Specific Nb: A Phage Display Library Approach. A schematic diagram of the different methods of generating Nbs. Camelids are immunized with an antigen to generate an immune library, while a naïve library does not require inoculation. Blood is collected to extract lymphocytes and mRNA, and Nb sequences are refined through PCR and gel electrophoresis. In synthetic libraries, a Nb scaffold is used, and CDRs are randomized. These libraries are transformed into phage vectors and *E. coli* to display Nbs and produce high-affinity phages via biopanning. Successful phages undergo phage-ELISA, sequencing, affinity testing, and are stored in glycerol for future production. Adapted illustration[3]

pharmacokinetics include attaching them to polyethylene glycol or fusing them with Fc domains [9]. For example, small nanobodies with a molecular weight around 15 kDa, which lack an Fc region, are rapidly cleared by the kidneys post-injection. This rapid renal clearance can lead to high kidney accumulation, resulting in an elevated kidney radiation dose, particularly in the context of radiolabeled nanobodies used for imaging or therapeutic purposes [10].

Generation, production and characteristics of nanobody libraries

Generation and production of nanobody libraries

Antigen-specific Nbs can originate from immune, naïve, or synthetic libraries (Fig. 2). Immune libraries are typically generated by immunizing animals from the Camelidae family with the target antigen or using transgenic mice when the antigen is scarce. Blood was then extracted from the animal, and lymphocytes were then purified for mRNA extraction and cDNA. A polymerase chain reaction (PCR) approach is employed to amplify the heavy-chain variable domain (VHH) sequence, followed by ligation into a vector and transformation into various expression systems, with an ideal immune library containing at least 10^7 unique transformants. The standard production of Nbs utilizes *E. coli* as the expression system, although alternative systems may be employed based on specific requirements (Fig. 3) [3]. A large Nb library enhances the likelihood of finding high-affinity binders with strong specificity and high affinity for antigens, maximizing the chances of discovering suitable Nb candidates [3,

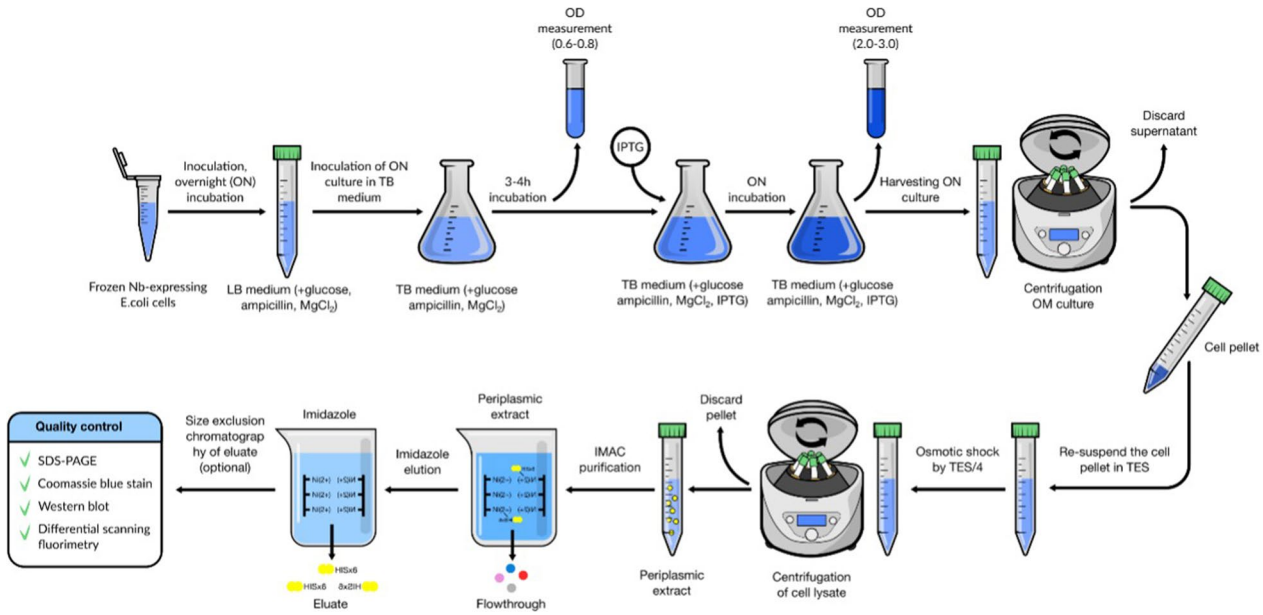


Fig. 3 From Glycerol Stock to Lab-Scale Production: A Conventional Nb Production Protocol. Various systems are available for nanobody production, including bacterial, yeast, mammalian, and plant expression systems. Among these, the bacterial expression system, particularly using E. coli, is the most common due to its simplicity, cost-effectiveness, and high yield. In this system, LB medium supplemented with glucose, ampicillin, and MgCl2 is inoculated with glycerol stock and incubated overnight. The culture then seeds TB medium for a few hours before nanobody (Nb) expression is induced with IPTG overnight. Post-expression, cells are centrifuged and lysed to extract Nbs. The extract undergoes IMAC purification, followed by imidazole elution, with an optional size exclusion chromatography. Quality is confirmed through SDS-PAGE, Coomassie staining, and western blotting. Adapted illustration [3]

12]. Two-step PCR, while useful in certain contexts, can introduce biases that may lead to decreased diversity and the overrepresentation of specific clones due to PCR bias. Therefore, careful consideration is needed when selecting between one-step and two-step PCR methods, depending on the primer system employed and the specific application at hand [13–15]. In addition to phage display, yeast display offers distinct advantages, particularly in maintaining the structural integrity of nanobodies during the selection process. This method is especially beneficial in applications that require more complex post-translational modifications, making it a valuable alternative in certain cases [16].

Instead of camelids, Japanese topesharks or sharks are a viable source for developing Variable New Antigen Receptor (VNAR) Abs [17, 18]. In immunization studies, Venus fluorescent protein has been used as a model antigen, and it has been observed to significantly enhance the production of antigen-specific IgM and IgNAR antibodies in these sharks. This increase is attributed to the strong immune response elicited by the Venus fluorescent protein, which serves as a potent antigen in these species. Phage-display and yeast-display libraries can then be constructed from RNA obtained from the immunized shark splenocytes, allowing for the identification of multiple high-affinity VNAR antibodies with exceptional stability [18].

The isolation of HCAbs from camelid serum uses affinity chromatography and differential adsorption techniques with protein A and protein G, resulting in three fractions: "IgG-1", which contains conventional Abs, and "IgG-2" and "IgG-3", which consist exclusively of HCAbs. HCAbs have adapted to bind antigens independently of the light chain (VL) using a specific domain called VHH, which is functionally equivalent to the Fab fragment in conventional Abs and consists of four conserved regions (frameworks, FRs) connected by three hypervariable Complementarity-Determining Regions (CDRs), with CDR3 being the main contributor to binding specificity [2, 3].

Characteristics of different nanobody libraries

Synthetic and naïve libraries do not require immunized animals, which can involve the collection of several liters of blood from at least 10 animals to match the size of a typical synthetic library. The NaLi-H1 phage-displayed library is the most well-known synthetic library [19]. In this setting, framework selection and CDR design are key features. The scaffold should be highly stable, especially under reducing conditions such as the cytoplasm, and the framework should be compatible with diverse CDRs,

Table 2 Advantages and disadvantages of naïve, synthetic and immune libraries

	Naïve libraries	Synthetic libraries	Immune libraries
Advantages	No prior immunization needed [3] [25]	Versatile, can target diverse antigens [3]	Enriched for antigen-specific Nbs due to affinity maturation [3] [12]
	Simpler and quicker library construction [3] [25]	No need for immunization or animal components [12] [25]	High affinity Nbs can be obtained [3, 12, 25]
	Avoids animal experimentation [3]	Shorter time to obtain Nb binders [3]	Can yield high-quality Nbs [12]
	Less complex procedure, no need for animals [25]	Can be used in multiple projects [3]	Proven track record of success [12]
Disadvantages	Limited antigen-specificity [3, 12, 25]	Potential lower affinity compared to immune libraries [3, 12, 25]	Costs associated with animal immunization [3, 12, 25]
	May require additional affinity maturation [12, 25]	Larger libraries needed for diversity [3]	Longer and more tedious immunization process [12]
	Lower probability of high-affinity Nbs [25]	Requires thorough framework and CDR design [12, 25]	Limited antigen variety in a single immune library [12]
	Lower diversity compared to synthetic libraries [3]	May have weaker physicochemical properties [3]	Limited to animals' immune responses [12]
	Less effective for nonimmunogenic or toxic targets [3]	Optimizing framework is essential for desired properties [25]	May involve ethical concerns related to animal use [12]

Table 3 Nanobody selection techniques

Selection method	Advantages
Phage display	Robust, fast, versatile, offers various selection strategies [34–40]
Yeast display	Highly selective, allows for FACS gating for specific functions [27, 36, 41–45]
Bacterial display	Sporadically used, rapid screening, cost-effective [26, 46, 47]
Two hybrid	Can be used with bacterial or yeast systems, sporadically used [12]
Ribosome display	In vitro technique, high diversity, rapid selection cycles, sporadically used for Nbs [48–50]
CIS display	In vitro technique, high diversity, no dependence on ribosomes, sporadically used [12]
Phenotypic selection	Highly promising for selecting function-modulating Nbs [12, 31]
NestLink-based selection	Amenable to sophisticated screenings, e.g., panning in organisms [12, 33]

making established camelid VHH frameworks the safest choice due to current limitations in protein stability prediction algorithms [20, 21]. These Nb libraries offer vast diversity, yielding numerous unique sequences (up to 10^{10}–10^{12} variants). In contrast, immune libraries naturally undergo in vivo affinity maturation, potentially resulting in higher affinity and specificity than synthetic libraries because they possess unique binding characteristics and epitope recognition profiles shaped by the natural immune system, defeating synthetic libraries (Table 2) [19, 22, 23]. Synthetic libraries are ideal for nonimmunogenic or toxic molecules that may not elicit an immune response in heavy-chain Ab classes or are too hazardous for animals (Table 2) [19]. Naïve Nb libraries use cDNA from nonimmunized humans or animals. Tsukahara et al. used a CDR shuffling protocol with peripheral lymphocytes from nonimmunized and human serum albumin-immunized alpacas. They showed that this approach effectively generates high-affinity VHHs against nonimmunized antigens such as cardiac troponin T (cTnT), cardiac troponin I (cTnI), EGP1, and human IgG [24].

Advancements in screening technologies have led to the discovery of novel Nbs for diverse applications. Phage display is widely used to select high-affinity binders from Nb libraries, while yeast display offers improved efficiency and structural preservation. Innovative platforms such as ribosome display and mammalian cell display are also expanding Nb selection capabilities [26–28].

Recent innovations in nanobody discovery have been significantly advanced by the work of Andrew Kruse and Chang Liu. Kruse's lab has developed high-throughput screening methods that leverage protein engineering and next-generation sequencing, enabling rapid identification of nanobodies with high affinity for challenging targets, including membrane proteins [29, 30]. Chang Liu has made significant contributions to the field of nanobody discovery and optimization through the application of directed evolution and synthetic biology techniques. His

work has been pivotal in creating ultra-diverse nanobody libraries, which have greatly enhanced the ability to discover and fine-tune nanobodies with superior stability and specificity [31].

Display techniques

After obtaining the Nb library, specific Nbs for the target antigen are typically selected and retrieved using the widely employed method of phage display. It is a robust technique for selecting Nbs in immune libraries. However, despite its widespread use, phage display has several limitations. These challenges involve the complex manipulation required in phage production, low levels of Nb display, and significant background binding by phage particles, complicating the selection process against complex, non-purified antigens such as intact cells and tissues [26].

An improvement in the phage display system could be an enhanced yeast display system for Nb selection. One system involves fusing the Nb to the N-terminal end of Aga2p, a component of yeast mating adhesion, to avoid steric hindrance and ensure better antigen binding. A novel aspect of this system is the use of an orthogonal acyl carrier protein (ACP) tag. This allows for the covalent attachment of a fluorophore to the displayed Nb, enabling easy monitoring and analysis of the Nb on the yeast cell surface. The use of ACP tagging for covalent fluorophore attachment simplifies and speeds up the process of analyzing displayed Nbs, which can be beneficial in high-throughput screening scenarios [27].

Other alternative selection systems include ribosome display, bacteria display, and NestLink (Table 3). In vitro selection (panning) should be performed under conditions that maintain the desired conformation of the protein [28, 32]. NestLink is an innovation involving the ligation of the Nb library with genetically encoded barcoding peptides, followed by deep sequencing and expression of representative fractions as soluble proteins mixed with the antigen (Ag), purification of the Nb-Ag complex, and identification by mass spectrometry, enabling the ranking of binders based on k-off rates and offering the potential for in vivo panning in whole organisms [12, 33].

NestLink could characterize numerous individual binder candidates directly within an ensemble, offering a significant increase in throughput compared to existing phage and yeast display methods. This technique efficiently ranks hundreds of binder candidates based on their off-rates, which is crucial for evaluating Nb binding kinetics. Unlike yeast display, NestLink associates binder sequences with their respective off-rates. It outperformed classical phage display and ELISA screening in identifying camelid Nb families that recognize a membrane transporter. Overall, NestLink overcomes the biases and limitations of traditional methods, enabling the identification of a more diverse set of Nbs with improved detection sensitivity [33].

Nanobody technology and CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based drugs can potentially target any gene, and utilizing effective delivery systems such as Nb nanoparticles could ensure that they reach their target cells without inducing an immune response [51].

CRISPR interference (CRISPRi) enhances sdAb production in *E. coli* by decoupling growth and production phases. Furthermore, using CRISPRi-based growth switches targeting nucleotide biosynthesis genes, significant growth decoupling effects were observed, leading to increased sdAb production. Proteomic analysis revealed downregulation of RNA polymerase sigma and upregulation of ribosome-associated proteins in the growth-decoupling CRISPRi strains. This approach stalls growth without inducing a stationary phase response, maintaining ribosome content and enhancing sdAb accumulation [52].

This technology can also be used in the manufacturing process to generate CAR-T cells that target CD105, which is a molecule in neoangiogenic endothelial cells and many cancer cells. For example, a CD105-specific Nb (C184) was generated from a camel immunization, and a CAR was designed by fusing C184 to key human domains. CRISPR/Cas9 (CRISPR-associated proteins) was used to insert this CAR construct into a safe harbor locus in T cells, ensuring stable CAR expression without affecting essential genes [53].

Additionally, CRISPR, an innovative split-Cas9 system, can play a crucial role, as demonstrated by Deng et al., in enabling targeted and controlled editing of the Survivin gene in cancer cells. Precisely regulating critical proteins in cancer cells is crucial but challenging. This study combined photoactivatable split-Cas9 gene editing (paCas9) for nuclear control and light-induced protein degradation (paProtacL) for cytoplasmic control of the Survivin gene. This precise gene editing method combined with protein degradation strategies represents a novel approach for cancer treatment, potentially leading to more effective therapies with fewer side effects [54] (Fig. 4).

Applications of nanobodies

Applications in cancer therapeutics

The potential for advancing tumor therapies is ever expanding; Nbs are targeting the tumor microenvironment through a variety of agents (Fig. 5).

Immune Libraries: Camels receive an inoculation of the desired antigen, while naïve libraries skip this step. Blood is then drawn, and lymphocytes are isolated. From these, mRNA is extracted. This mRNA serves as the blueprint for creating a library of Nbs. Typically, PCR is performed to amplify the Nb sequences, and gel electrophoresis is used to isolate the Nb sequences, which are smaller in size compared to conventional antibody sequences due to the absence of light chains and the reduced size of the heavy-chain variable domain.

Synthetic Libraries: A suitable Nb scaffold is required, and the CDRs of the scaffold are diversified using a design approach that incorporates specific biases and exclusion criteria to avoid developability issues, such as glycan motifs, free cysteines, deamidation, or isomerization sites. The resulting library is then inserted into phage vectors, which are carriers of genetic material, and introduced into *E. coli*. Within these cells, the phages are produced, with each carrying the Nb nucleotide sequence on its surface.

Biopanning: This process involves several rounds of selecting phages with the strongest binding affinity to the target antigen. Selected phages are then used to infect *E. coli* once again, generating a new batch of phages enriched with high-affinity Nbs. Biopanning selects high-affinity Nbs through multiple rounds with stringent conditions, such as high antigen concentrations or the presence of competitor proteins. It is important to note that maintaining diversity and functionality is critical during the initial library construction, particularly in synthetic libraries, to ensure a wide range of potential high-affinity binders.

Phage-ELISA: The authenticity of these enriched phages is confirmed using ELISA, which detects the presence of specific proteins or peptides.

Nb Candidate Selection: Next, the Nbs from the enriched phage population are sequenced and evaluated for their affinity to the target antigen.

Glycerol Stock Preparation: Finally, promising Nb candidates are preserved as glycerol stocks, ensuring their availability for future production and applications.

Immune libraries yield high affinity Nbs via in vivo affinity maturation, but access to camelids may be limited, and some antigens may not elicit a strong immune response

Naïve libraries have lower diversity and require larger sizes (10^6-10^{10} clones) due to lacking affinity maturation.

Synthetic libraries can be designed to target a wide range of antigens, including non-immunogenic or toxic ones, and offer higher diversity. However, their construction requires a deeper understanding of antibody engineering and more advanced techniques. Both synthetic and naïve libraries can be effectively used for challenging targets, depending on the specific requirements of the study.

Fig. 4 Sidebar 1 Comparative Analysis of Antibody Library Construction Methods [22, 55]

Examples include CD38-specific Nbs for Nb-based chimeric antigen receptors (Nb-CARs) eliminating multiple myeloma (MM) cells [56]; CD38-specific Nb-based killer cell engagers (nano-BiKEs) promoting NK cell-mediated MM cell killing [57]; high-affinity PD-1-targeted Nbs enhancing the T-cell response [58]; EGFR-targeted Nbs improving tumor spheroid penetration [59]; HER2-targeted Nbs facilitating tumor regression in resistant patients [60]; engineered anti-MUC1 (Mucin 1) Nbs sensitizing breast cancer cell

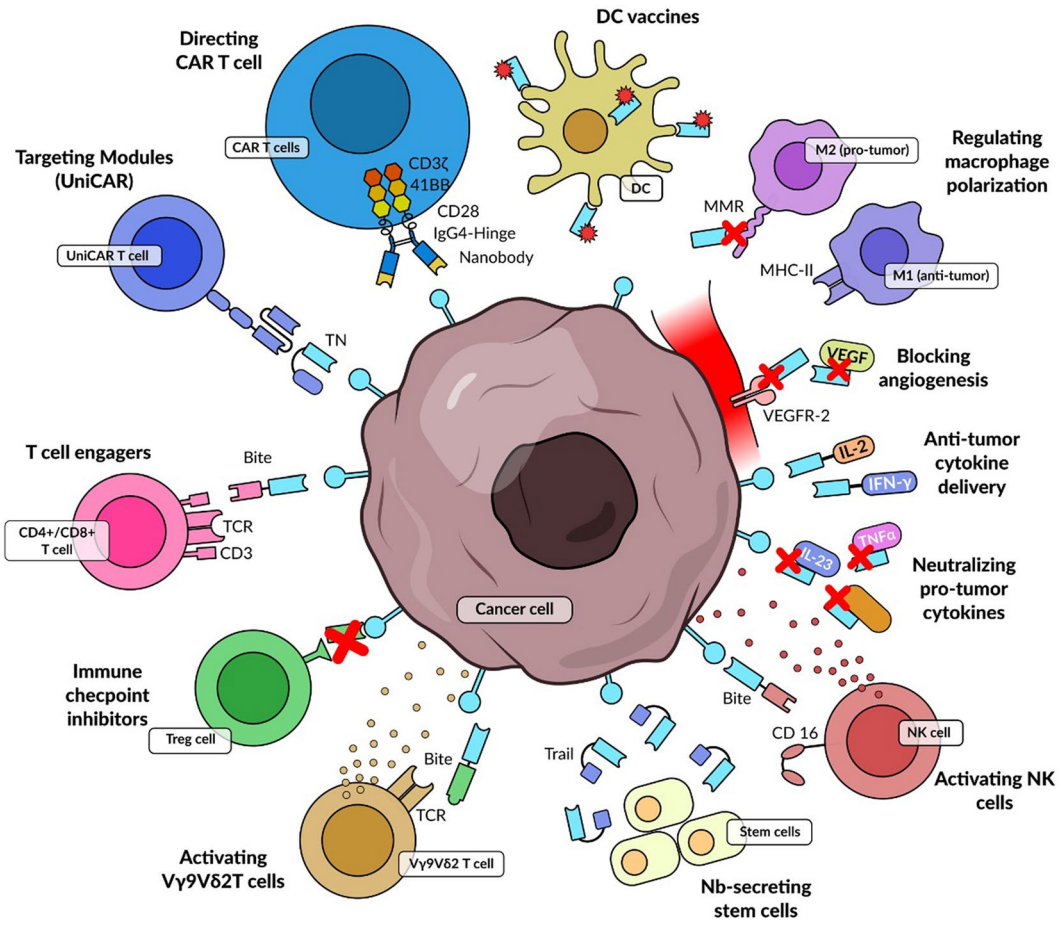


Fig. 5 Nb targets in the tumor microenvironment

lines to drugs [61]; Nb-based HER2-targeted Abs for synergistic use with trastuzumab [62]; trispecific killer engagers (CLEC12A TriKE) for Acute Myeloid Leukemia (AML) cell elimination while preserving healthy cells [63]; silk fibroin nanoparticles modified with Nb 11C12 targeting the proximal membrane end of carcinoembryonic antigen on colorectal cancer [64]; humanized BsNb, BI 836880, inhibiting vascular endothelial growth factor and angiopoietin-2 [65]; and EpCAM-targeted Nbs inhibiting tumor growth [66]. Additionally, VHHs in CD19-targeted CAR-T cells exhibit efficacy similar to that of scFvs, suggesting that VHHs are a promising alternative to CAR therapies [67].

In cancer therapy, humanization strategies for camelid-derived Nbs could minimize the risk of immune responses [68]. One approach involves grafting the Nb with antigen specificity of interest onto a universal humanized Nb scaffold (h-NbBcII10FGLA) or replacing nonhuman Nb CDRs with human Ab CDRs [68]. These methods preserve antigen-binding properties while increasing human content. Humanized anti-EGFR Nbs fused with the tumor-penetrating peptide "iRGD" demonstrated improved antitumor activity, indicating maintained functionality. Similarly, humanized anti-c-MET Nbs show promise for treating multiple myeloma [68, 69].

Similarly, fully human single-domain antibodies (UdAbs) can target carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) for anticancer therapy. The Ab candidate B9 demonstrated strong binding to CEACAM5, with excellent biophysical properties. When conjugated with the cytotoxic agent monomethyl auristatin E (MMAE), B9 exhibited potent antitumor efficacy against CEACAM5-expressing cancer cell lines, including gastric, pancreatic, and colorectal cancer cell lines. In mouse xenograft models, B9-MMAE administration significantly inhibited tumor growth [70].

Nanobodies as targeted cancer therapies

Numerous Nbs have been created to specifically target key tumor markers involved in tumor-promoting

signaling pathways. The first CAR-T-cell therapy with an Nb used anti-MUC1 VHH fused with a human IgG3 hinge, an IgG3-Fc spacer, CD28, and CD3ζ. Jurkat cells cocultured with MUC1 + MCF7 tumor cells exhibited increased proliferation and increased cell lysis and IL2 secretion [71]. Additionally, Nbs targeting various antigens, including MUC-1, CD7, CD38, VEGFR2, PSMA, GPC2, and TCR-like nanobody-CARs [56], have shown effectiveness in different B-cell tumor models [71, 72]. CD7, a transmembrane glycoprotein and significant marker for T-lineage tumors commonly found on natural killer and peripheral T cells, represents a promising target for immunotherapy for T-ALL/LBL patients. Preclinical and phase I studies have shown that autologous CD7 CAR-T cells derived from Nbs are effective and have long-lasting antitumor effects on patients with relapsed/refractory T-ALL/LBL, with manageable toxicity [73].

However, CAR-T cells targeting CD7 may destroy each other, known as "fratricide", but these Nb-engineered CAR-T cells are fratricide resistant, enhancing their persistence and efficacy [73]. Furthermore, bispecific CAR-T cells can prevent antigen escape, with superior affinity and specificity for Nb-derived bispecific CAR-T cells targeting CD5 and CD30, showing potent antitumor efficacy [74]. Sequentially, tumor-selected Ab and antigen retrieval (STAR) system-engineered CAR-T cells bispecific for CD13 and TIM3 effectively eliminated patient-derived AML in preclinical models with reduced toxicity [75].

In mouse models, CAR-T-Nbs targeting B7-H3 in solid tumors show potent activity against large tumors [76]; PD-L1-specific CAR-T cells exhibit cytotoxicity and combat aggressive tumor models through direct cytotoxicity, cytokine production, and checkpoint molecule binding, reducing immune suppression and exhaustion [77] (Table 4).

PD-L1 as a target: immune checkpoint inhibition

Envafolimab, a fusion of a humanized single-domain PD-L1 Ab and an IgG1 Fc fragment connected by disulfide bonds, is approved for subcutaneous (SC) administration for treating various solid tumors, soft tissue sarcomas and biliary tract cancer. Its efficacy is facilitated by single-domain Abs, allowing for SC administration due to improved solubility and tissue penetration. Phase I trials involving 28 patients with advanced solid tumors demonstrated favorable safety and pharmacokinetics, while phase II trials involving 103 patients with MSI-H/dMMR tumors showed satisfactory therapeutic effects of envafolimab monotherapy [78].

However, clinical data reveal that less than 30% of patients benefit from anti-PD-1/PD-L1 Ab monotherapy, primarily due to insufficient intratumoral T cells, especially tumor-specific CD8 + T cells capable of direct tumor killing. To enhance immune checkpoint therapy, engineered bivalent anti-PD-L1/VEGF Nbs can inhibit angiogenesis in vitro [79], and bispecific T-cell engagers (BiTEs) are emerging as a promising approach to activate and redirect cytotoxic T cells, increasing intratumoral T-cell levels in both blood and solid tumors [58].

A potential novel BiTE candidate for enhancing the treatment of PD-L1-overexpressing melanoma could be developed using bispecific PD-L1 xCD3 Nb structural modeling and molecular docking-informed binding site predictions. In vitro tests using A375PD-L1 target cells and Peripheral blood monocytes (PBMC) effector cells highlighted its promise as a novel BiTE candidate for melanoma therapy [80].

EGFR as a target

The epidermal growth factor receptor (EGFR) and its family members (ERBB) play a critical role in malignant tumor development through the activation of intracellular tyrosine kinase pathways, particularly when mutations or overexpression occur. However, EGFR is a challenging target due to the significant risk of on-target off-tumor toxicity, as seen with therapies like Cetuximab, which can cause severe skin toxicity. This limitation is also relevant for VHH-based therapies targeting EGFR and HER2/3, as VHHs alone cannot inherently overcome these challenges.

To address resistance in solid tumors, a tetravalent biparatopic anti-EGFR Nb-drug conjugate (S7 ADC) was developed, demonstrating significant antitumor activity in A431 xenograft models. The multivalent binding approach employed by this conjugate enhances efficacy by driving down receptor expression more effectively than monovalent binding, leading to synergistic antitumor effects [81].

Additionally, synergism can be achieved through bispecific VHHs that target CD16 on NK cells and EGFR on epithelial tumor cells. This strategy facilitates cell lysis irrespective of KRAS mutational status and has shown enhanced cytotoxicity against colorectal cancer cells when used in combination with autologous or allogeneic CD16-expressing NK cells [82].

Colorectal cancers are also targeted by immunotoxins (ITXs), which couple antibody targeting with toxin-mediated cytotoxicity. One promising approach involves an EGFR-targeting nanobody (VHH 7D12) linked to the fungal ribotoxin α-sarcin, forming a nanoITX. The small size and specific binding properties of the nanobody enhance the treatment's effectiveness, with trimeric and bispecific nanoITXs demonstrating superior antitumor activity [83].

Table 4 Selected nanobody clinical trials in cancer therapy

Nb	Sponsor	Disease	Target	MoA	Trial No	Phase	Status
ALX-0651	Ablynx/Sanofi Sanofi	NHL, MM	CXCR4	Inhibition	NCT01374503	I	Discontinued
TAS266	Ablynx/Sanofi Sanofi	Advanced solid tumor	DR5	Agonism	NCT01529307	I	Terminated
DR30303	Doer Biologics	Advanced solid tumor	Claudin 18.2	ADCC	NCT05639153	I	Recruiting
BI 836,880	Boehringer Ingelheim	Advanced solid tumor	VEGF + Ang-2	Inhibition	NCT02674152 NCT02689505 NCT03972150	I	Completed
Envafolimab/KN035	Alphamab	Advanced solid tumor	PD-L1	Inhibition	NCT02827968	I	Completed
		Advanced carcinoma			NCT03101488	I	Completed
		Advanced solid tumor			NCT03248843	I	Completed
		dMMR/MSI-H advanced solid tumor			NCT03667170	II	Recruiting
KN044	Alphamab	Advanced solid tumor	CTLA-4	Inhibition	NCT04126590	I	Recruiting
KN046	Alphamab	Multiple disorders	PD-L1 + CTLA-4	Inhibition	> 20 clinical trials	IIIII/II II II/II	Recruiting
131I-GMIB-Anti-HER2-VHH1	Precirix	HER2-positive breast cancer patients	HER2	Targeted radionuclide therapy	NCT02683083	I	Completed
		Advanced HER2-positive Breast, Gastric, GEJ Cancer			NCT04467515	I/II	Recruiting
LDOS-47	Helix BioPharma	NSCLC	CEA	Immunoconjugate	NCT02340208	I/II	Completed
		NSCLC	CAM6		NCT02309892	I	Completed
		Lung adenocarcinoma			NCT03891173	II	Terminated
		Advanced pancreatic cancer			NCT04203641	I/II	Recruiting
LAVA-051	LAVA Therapeutics	Relapsed/refractory CLL, MM and AML	CD1d	BiTE	NCT04887259	I/II	Recruiting
LAVA-1207	LAVA Therapeutics	Refractory metastatic prostate cancer	PSMA	BiTE	NCT05369000	I/II	Recruiting
SAR444200	Ablynx/Sanofi Sanofi	Advanced solid tumor	GPC3	BiTE	NCT05450562	I/II	Recruiting
BCMA CAR-T Cilta-cel	Nanjing Legend Biotech	Relapsed/refractory MM	BCMA	CAR T-cell	NCT03090659	I/II	Completed
	Janssen	MM			NCT03548207 NCT04133636 NCT04181827 NCT04923893 NCT05257083 NCT05201781	Ib/II to IV	Active/ recruiting/ completed
CD19/20 Bispecific CAR-T Cells	Henan Cancer Hospital	B-cell lymphoma	CD19 CD20	CAR T-cell	NCT03881761	I	Recruiting
BCMA CAR-T	Henan Cancer Hospital	Relapsed/refractory MM	BCMA	CAR T-cell	NCT03664661	I	Recruiting
TC-110	TCR2 Therapeutics	Relapsed/refractory NHL ALL	CD19	CAR T-cell	NCT04323657	I/II	Completed
CD7 CAR-T Cells	PersonGen Bio-Therapeutics	TLL, NK/T-Cell Lymphoma, ALL	CD7	CAR T-cell	NCT04004637	I	Recruiting

Table 4 (continued)

Nb	Sponsor	Disease	Target	MoA	Trial No	Phase	Status
CD7 CAR-NK cells	PersonGen Bio-Therapeutics	Relapsed/refractory Leukemia and Lymphoma	CD7	CAR NK-cell	NCT02742727	I/II	Recruiting
TC-110	TCR2 Therapeutics	Relapsed/refractory NHL ALL	CD19	CAR T-cell	NCT04323657	I/II	Completed
Gavo-cel	TCR2 Therapeutics	Advanced MSLN + solid tumors	MSLN	CAR T-cell	NCT03907852	I/II	Recruiting
TC-510	TCR2 Therapeutics	Advanced MSLN + solid tumors	MSLN	CAR T-cell	NCT05451849	I/II	Recruiting
αPD1-MSLN-CAR T Cells	Shanghai Cell Therapy	Advanced MSLN + solid tumors	MSLN	CAR T-cell	NCT05373147 NCT04503980 NCT04489862 NCT05089266	I	Recruiting

Source: www.clinicaltrials.gov

Table 5 Nanobodies and their mechanisms of action against SARS-CoV-2

Nb	Source	Mechanism of action
Ty1	Alpaca	Targeting RBD of spike protein with a greater affinity
H-11 D4	Llama	Blocking attachment of ACE2 receptor to the spike protein
H-11 H4	Llama	Blocking attachment of ACE2 receptor to the spike protein
Sb23	Library of synbodies	Blocking attachment of ACE2 receptor to the spike protein
K-874A	Camels (no modifications are included)	Inhibiting fusion of the viral membrane, preventing viral replication
Nb11–59	Pichia pastoris	Blocking interaction of ACE2 receptor and RBD
VNAR	Sharks	Targeting S1-RBD and breaking the salt bridge between ACE2 and RBD
n3113.1-Fc	Unknown	Binding to RBD of the spike protein without involving ACE2 receptor

Source: Adapted from Bhattacharya et al. [91]

Nanobodies in infectious disease therapies

COVID-19 sparks interest in Nbs as potential therapies with ongoing clinical trials and further developments [84]. A review of 45 studies revealed that Nbs combat SARS-CoV-2 through mechanisms such as blocking the angiotensin-converting enzyme 2 (ACE2) binding site, binding to multiple receptor-binding domain (RBD) epitopes, disrupting the ACE2-RBD interaction, preventing viral entry, and exhibiting high-specificity neutralization via specific interactions (Table 5) [85].

Candidates that can neutralize SARS-CoV-2 include Nb-H6 and Nb4, with the latter demonstrating specificity against Omicron strains [86, 87]. The crystal structure of Nb4 in complex with the virus spike protein interacts with an RBD epitope that competes with ACE2 binding, which is crucial for virus entry into human cells. Similarly, Nb-H6 competes with ACE2 for S1 and RBD binding [86, 87]. The interaction between the RBD and ACE2 initiates SARS-CoV-2 infection [88]. These Nbs have potential for prophylactic and therapeutic intranasal administration [87, 89]. Additionally, Nanosota-2, -3, and -4 Nbs also demonstrate efficacy against SARS-CoV-2 viruses, with Nanosota-2 being the most potent SARS-CoV-2 entry inhibitor known [90].

Interestingly, Gauhar et al. presented shark-derived VNAR Abs as novel neutralizing sdAbs against SARS-CoV-2 [92]. Normally, an Nb phage library is established by immunizing a camel with the RBD protein, yielding specific Nbs against the SARS-CoV-2 spike protein RBD [93]; however, in this study, by screening shark VNAR libraries and reforming them into bivalent human IgG Fc fusions, researchers obtained three VNAR-hFc Abs (3ID10_16, 6ID10_75, 3ID10_99) targeting the SARS-CoV-2 RBD. These Abs blocked the ACE2-RBD interaction, neutralized authentic SARS-CoV-2, and blocked the RBD mutants E484K and N501Y in virus variants, expanding potential COVID-19 therapeutic options [94].

A significant challenge in treating SARS-CoV-2 infections is the ability of the virus to mutate and evade neutralization, especially with the emergence of variants. However, Mast et al. showed that synergy in countering

Table 6 Nanobody therapies for infectious diseases

Nanobody	Disease	Target	Structure features	Stage of development	References
MR3	SARS-CoV-2	RBD	Bivalent Nb; bispecific Nb; VHH-Fc	Preclinical	[99]
D7,D3	Ehrlichia infection	TASS effector	CPP-Nb conjugation	Preclinical	[99]
CeVICA	SARS-CoV-2	RBD	Nb-His tag	Preclinical	[99]
Fu2	SARS-CoV-2	RBD	Bispecific; bivalent; trivalent; VHH-Fc	Preclinical	[99]
125 s	Chronic hepatitis B infection	Hepatitis B surface antigen	VHH-Fc	Clinical Trials	[99]
VUN100	Latent human cytomegalovirus (HCMV) infection	Signaling of the viral receptor US28	Nb–photosensitizer conjugates; bivalent Nb; Nb-His tag	Preclinical	[99]
NB7-14	Influenza H7N9 virus	HA	Bivalent Nb	Preclinical	[100]
anti-TMPRSS2	Human coronaviruses causing common colds	HKU-1	VHH-Fc	Preclinical	[101]
Nb-M4	Diarrhea due to norovirus	GII.4 HuNoV	Complex with GII.4 capsid protein	Preclinical	[100}
NbCXCR4	HIV	HIV coreceptor CXCR4	FITC-conjugated siRNA	Preclinical	[99]
Pfs230-specific Nb	*P. falciparum* malaria	Pfs230	Binds to distinct sites of the 6-cysteine domains of Pfs230	Preclinical	[102]
Nb6,Nb8,Nb10,Nb11	Septicemia	*Pseudomonas aeruginosa* Exotoxin A	Neutralization Nbs for PEA	Preclinical	[103]

SARS-CoV-2 involves combining individual Nbs that bind to the spike protein. Moreover, multiple Nbs can bind to the same RBD, allowing up to nine Nbs to target a single spike. By strategically selecting Nb combinations with synergistic, distinct neutralization mechanisms, it is possible to create powerful and broadly effective agents that are resistant to viral escape [95].

Beyond SARS-CoV-2 infection, engineered Nbs targeting CXCR4 efficiently deliver siRNA to inhibit HIV infection in T lymphocytes through receptor-mediated endocytosis (Table 6) [96]. In addition, Nbs inhibit respiratory infections and infectious diseases through multiple mechanisms of action. In infants, respiratory syncytial virus (RSV) is a major cause of severe respiratory infections. ALX-0171 is an anti-RSV F-specific Nb that binds specifically to RSV F site II and is emerging as an efficacious treatment. The therapeutic efficacy of ALX-0171 compared to that of palivizumab, an existing treatment for RSV, or a placebo showed that ALX-0171 treatment significantly reduced RSV titers, especially when ALX-0171 was administered at higher concentrations. ALX-0171 was found to be approximately three times more potent than palivizumab [97, 98].

Exploring the use of nanobodies in neurogenerative diseases

Currently, while there has been ample innovation in the use of Nbs for treating neurogenerative diseases, most of these compounds are in preclinical trials. One preclinical model identified an Nb targeting the mouse transferrin receptor (mTfR) through mutagenesis. Among the variants, Nb62 bound to the chimeric receptor. Researchers fused Nb62 to neurotensin (NT), which induces hypothermia in the brain. The intravenous administration of Nb62-NT to mice resulted in dose-dependent hypothermia, as measured externally, indicating brain penetration [104]. This model offers a clear assessment of brain target engagement through the direct effect of NTs on body temperature and represents a noninvasive preclinical study that could lead to therapeutic applications. For example, Nb delivery across the BBB is facilitated by an adeno-associated virus (AAV) vector, where AAV vectors transport antibody-coding sequences into CNS cells for in situ Nb production, enhancing therapeutic efficacy [105].

Treating Alzheimer's disease (AD)

AAV vectors can deliver VHH-B9 into the CNS of AD model mice, resulting in brain-wide, long-lasting VHH-B9 production with positive effects on the pathology of AD, namely, amyloid load, neuroinflammation, synaptic function, and cognition [106, 107]. Emerging cutting-edge treatment strategies for AD involve multivalent Nb PNBILs, which mitigate amyloid-β (Aβ) aggregation and oxidative stress. After being modified with Aβ and interleukin-1β (IL-1β) fragments, the PNBIL

recognizes and inhibits Aβ aggregation and aids in microglia-mediated clearance, reducing AD symptoms in mouse models [108]. Furthermore, Aβ aggregates are a therapeutic target in AD. An in vivo study revealed that FC5-mFc2a-ABP, a brain-penetrating anti-amyloid Nb fusion protein, reduced brain Aβ, increased the Aβ42/40 ratio in cerebrospinal fluid, and improved neurological measures [109]. Likewise, tau protein aggregation is characteristic of this disease [110]. VHH Z70, which is expressed in the brain of a tauopathy mouse model, mitigates pathological Tau accumulation [111]. Another approach involves inhibitory Nbs targeting tau aggregation with sequences (VDW, W3, and WIW) grafted onto a Nb scaffold. These Nbs bind the amyloid-driving sequences of tau, VQIINK and VQIVYK [110]. Furthermore, reduced sortilin-related receptor with A-type repeats (SORLA) levels in AD necessitate reliable detection; Nbs from an alpaca immunized with TCR's extracellular domain demonstrate high affinity and epitope mapping potential for disease research and diagnostics [112].

Treating Parkinson's disease (PD)

Mutations in leucine-rich repeat kinase 2 (LRRK2) are associated with PD, leading to decreased (guanosine triphosphate) GTPase activity and increased kinase activity. Nbs—NbRoco1 and NbRoco2—bind to a bacterial homolog of LRRK2 (CtRoco) in a conformation-specific manner. NbRoco1 increases CtRoco's GTP turnover rate, potentially reversing decreased GTPase activity caused by a PD-analogous mutation. Targeting the dimer-monomer cycle of LRRK2 may correct dysfunctional GTPase activity caused by these mutations. Additionally, Nbs selectively inhibit specific LRRK2 activities, making them promising for drug discovery without the side effects of current kinase inhibitors [113, 114].

In addition, alpha-synuclein (α-syn) aggregation is associated with various neurodegenerative disorders, including PD, dementia with Lewy bodies [115], and multiple system atrophy, which are collectively termed α-synucleinopathies [107]. Nbα-syn01, a Nb, targets the N-terminal region of α-syn and is prone to mutations in early PD. Nbα-syn01 and BivNbα-syn01 recognize α-syn fibrils, inhibit aggregation in vitro, and reduce toxicity, indicating their therapeutic potential [116].

Treating amyotrophic lateral sclerosis (ALS)

Mutations in ALS can cause superoxide dismutase 1 (SOD1) protein to adopt abnormal conformations, potentially contributing to the disease. Engineering specific anti-SOD1 Nbs for mutant and misfolded versions of human SOD1 in the laboratory setting results in two Nbs stabilizing mutant SOD1, enhancing its expression and restoring its subcellular localization in immortalized cells. Furthermore, in ALS motor neurons with the SOD1 A4V mutation, anti-SOD1 Nb promoted neurite outgrowth, suggesting protection. One of the Nbs selectively targeted human mutant SOD1 over murine SOD1, supporting preclinical testing in human and mouse models of ALS [117].

TDP-43 protein misbehavior and aggregation in neurons are also implicated in ALS pathology. TDP-43 is essential for nucleic acid metabolism. Sequestration of TDP-43 aggregates is a possible therapeutic strategy that could alleviate or block pathology in ALS, and this could be achieved by an engineered Ab (intrabody) against TDP-43 from a Llama Nb library. This intrabody recognizes the RNA binding regions of TDP-43, offering a potential strategy for mitigating TDP-43 proteinopathy in ALS patients and serving as a diagnostic tool [118].

Nanobodies in treating autoimmune diseases

Nbs in autoimmune therapies mark a successful bridge between research and approved medications. Caplacizumab is a bivalent, humanized, single-variable-domain immunoglobulin or Nb that targets the A1 domain of von Willebrand factor (vWF), preventing interactions between vWF and platelets and the formation of microvascular thrombi [119]. It is known to cause immune-mediated thrombotic thrombocytopenic purpura (iTTP), which is caused by autoantibodies against ADAMTS-13, leading to microangiopathic hemolytic anemia due to impaired processing of ultralarge vWF multimers in the absence of ADAMTS13 activity [119]. By inhibiting the interaction of ultralarge vWF multimers with platelets, caplacizumab prevents the formation of microthrombi, which are responsible for the severe complications observed in acquired thrombotic thrombocytopenic purpura aTTP [120]. Randomized controlled trials, including the pivotal HERCULES trial and real-world evidence [121, 122], have shown that caplacizumab reduces the time to platelet count normalization, refractoriness, and exacerbation of the disease, with an acceptable safety profile [123, 124]. The long-term safety profile of caplacizumab for recurrence treatment is consistent with that of the HERCULES trial, in which most bleeding events were nonserious and no major cases of organ dysfunction were observed [125, 126].

Recent studies have successfully uncovered the mechanism of activation of Caplacizumab, with the crystal structures being independently resolved by two groups. These findings have provided detailed insights into how Caplacizumab interacts with its target, shedding light on its inhibitory mechanism and offering valuable information for further therapeutic development [127, 128].

Ozoralizumab is an anti-TNFα Ab designed as a 38-kDa trivalent Nb compound. It consists of two humanized anti-human TNFα VHH Abs and one humanized anti-human serum albumin VHH Ab. In the context of rheumatoid arthritis (RA), TNFα plays a significant role in the pathogenesis of this disease, contributing to the chronic inflammation and joint damage observed in affected patients [129]. By neutralizing TNFα, ozoralizumab can reduce the inflammatory response in patients with RA, leading to a reduction in symptoms and improvement in joint function. Ozoralizumab has significant therapeutic effects with or without MTX in patients with active RA and is well tolerated [129–131].

M1095 (sonelokimab), a novel trivalent bispecific anti–IL-17A/F Nb, comprehensively blocks psoriasis inflammation by targeting both IL-17A and IL-17F [132]. It contains three Nbs, one for IL-17F, one for both IL-17A/F, and one for human serum albumin, to extend its half-life [133]. Furthermore, as a promising future therapy, anti-FGF-2 Nbs effectively inhibited the proliferation and migration of psoriatic keratinocytes in an in vitro model and reduced psoriatic lesion severity in an imiquimod-induced mouse model. It also improves the inflammatory environment by suppressing cytokines (IL-1β, IL-6, IL-23, and TNF-α), chemokines (CXCL1 and CCL20), and neutrophil infiltration. This approach holds promise for treating psoriasis by targeting FGF-2-mediated mitogenic signaling [134].

Diverse applications for nanobodies

The versatility of Nbs paves the way for their application in many therapies via diverse modalities.

BsNb inhibitors, which are based on sgp130Fc and could receive approval as inflammatory bowel disease (IBD) treatments, such as cs130-TNFVHHFc and cs130-IL-12/23VHHFc, show therapeutic potential for IBD. These inhibitors exhibit strong binding to IL-6/sIL-6R complexes, human TNFα, and IL-12/IL-23. They effectively block IL-6/sIL-6R trans-signaling, offering promise for IBD patients with fewer adverse events [135]. Similarly, a drug platform called probiotic type 3 secretion *E. coli* therapeutic, which uses engineered *E. coli* to secrete therapeutic proteins directly at disease sites, successfully prevents inflammation in a colitis model [136].

No current treatments for liver fibrosis exist, necessitating the urgent development of antifibrotic therapies. One promising target is connective tissue growth factor (CTGF), which plays a pivotal role in the development of liver fibrosis. The use of anti-CTGF Nbs, which are specifically designed to act against human CTGF, is a potential solution. These Nbs can effectively inhibit TGFβ1-induced LX-2 cell proliferation, activation, and migration while promoting apoptosis. Additionally, they significantly reduced TGFβ1, Smad2, and Smad3 expression in LX-2 stellate cells stimulated with TGFβ1, demonstrating their antifibrotic potential in vitro [137]. Moreover, Pannexin1 (Panx1) proteins play a pivotal role in acetaminophen-induced liver injury and inflammation by releasing ATP when their channels open. These channels are significant in inflammatory conditions but targeting them therapeutically has been challenging due to the lack of specific inhibitors. Fortunately, three Panx1 Nbs (Nb1, Nb3, and Nb9) effectively blocked ATP release via Panx1 channels and exhibited anti-inflammatory effects by reducing interleukin 1 beta (IL-1β) levels in vitro [138].

In the context of eliminating excess cytokines in blood purification therapy for hypercytokinemia-related diseases, Nbs, along with static mixers (Nb-SMC) in a modified conduit, effectively remove surplus cytokines from the bloodstream. Nb-SMC is compatible with human blood composition and holds promise for the clinical treatment of hypercytokinaemia in high-risk patients [139].

Among other innovative approaches, a trivalent BsNb comprising three VHHs that bind to two different epitopes of IL-5 and one epitope of albumin from immunized phage display libraries has potential as a therapeutic agent that targets IL-5 in severe eosinophilic asthma. This trivalent IL-5-HSA Nb exhibits similar IL-5/IL-5R blocking activity as the approved IL-5 monoclonal Ab mepolizumab (Nucala). Additionally, IL-5-HSA Nb is producible at scale in a *P. pastoris* X-33 yeast system, boasting high purity and thermal stability [140].

Snake envenomation is a significant global health issue, and conventional antivenoms have limitations in treating local tissue damage. Nbs—M28, M35, M43, M67, M85, and M88—from a llama immunized with Bothrops atrox snake venom components can effectively neutralize the hemorrhagic and myotoxic effects of the venom, showing their potential as innovative therapeutic agents against envenomation by viperid snake species [141].

Studies have demonstrated that purinergic receptors can serve as therapeutic targets to modulate the inflammatory response in various brain disease models. P2X7-specific Nbs have been developed to effectively block the P2X7 channel, which represents a promising target for modulating brain inflammation in ischemic stroke models. These Nbs show promise in reducing stroke size when administered intracerebroventricularly by blocking P2X7 receptors on microglia, suggesting a potential therapeutic approach for ischemic stroke [142]. Additionally, erroneous activation of the human complement system contributes to serious inflammatory disease. Two high-affinity anti-C5 Nbs, UNbC5-1 and UNbC5-2, which target the C5 protein of the human

complement system, can efficiently inhibit C5 cleavage and its harmful effects in the human serum environment. These Nbs hold potential for diagnostic, research, and therapeutic applications in modulating complement system activation [143].

In dermatology, Nbs can also enhance current therapies. SAR442970 is a drug candidate designed to target immune system molecules for potential anti-inflammatory action and was tested in a study of Hidradenitis Suppurativa. Similarly, another study evaluated the clinical efficacy and safety of the Nb sonelokimab for treating moderate to severe hidradenitis suppurativa (NCT05322473; NCT05849922).

Emerging applications

Overall, Nbs are not only substitutes for monoclonal Abs; their unique characteristics broaden the scope of biotherapeutic applications, including molecular imaging [144], targeting and visualization via microscopy [145], diagnostic tests for pharmaceuticals, including recombinant human interferon α2b [146], and tunable drug targeting systems [147, 148].

Nbs have sparked research in neuromodulation and regenerative medicine. Many nervous system diseases, such as multiple sclerosis (MS), involve inflammation and are treated by reducing inflammation and promoting myelin regeneration. Nbs targeting TNFR1 and CXCL10 have been evaluated for MS treatment. TROS, an anti-TNFR1 Nb, inhibits inflammation and preserves myelin and neurons in MS models. The activity of Nb 3Nb12 against CXCL10 blocks CXCL10-CXCR3 binding, inhibiting cell chemotaxis and showing potential for use in regenerative medicine [149]. Neuromodulation affects groups of neurons by adjusting synaptic strength. Inhibitory neurotransmitters activate anion channels and G Protein-Coupled receptors (GPCR), while excitatory neurotransmitters activate cation channels and relevant GPCR. In this neuromodulator approach, researchers used a conformation-specific Nb to investigate the activation of β2-adrenoceptors. This Nb80 specifically targets the activated form of β2AR. As a result, when β2AR is activated, the Nb80-EGFP (Enhanced Green Fluorescent Protein) moves from the cytoplasm to the plasma membrane. Although difficult to achieve in vivo, this represents another emerging application but still speculative for neuromodulating medicine studies [149, 150].

Applications in agriculture and biotechnology

Nbs have diverse applications in agriculture, biotechnology, food and plant pathology [151, 152]. They are fused with fluorescent proteins for immunocytochemistry [153] or facilitate real-time in vivo protein tracking for endogenous plant proteins, enabling minimal interference with cellular activities compared to traditional green fluorescent protein (GFP) fusion methods [154], as demonstrated by Rocchetti et al. in visualizing tobacco leaf cell actin dynamics with less disruption to Golgi body movement [151]. Nbs can be produced against tomato leaf curl Sudan virus, which is the most widespread tomato-infecting begomovirus in Saudi Arabia, offering potential for begomovirus diagnostics and the development of nanomaterials, as well as Nb-mediated begomovirus resistance in key crops such as tomato, potato, and cucumber [152]. Another example involves the use of Nbs in sandwich enzyme-linked immunosorbent assays (ELISAs) designed for pathogen detection, such as for *Salmonella enteritidis.* The Nb SE-Nb9 captures *S. enteritidis*, allowing sensitive and specific pathogen monitoring [155]. Similarly, using an organism-bispecific Nb (BsNbs) scaffold, genetically engineered BsNbs can detect *Salmonella* spp. and *Vibrio parahaemolyticus*, with one Nb immobilizing inactivated bacteria and the other acting as the capture Ab, enabling highly sensitive detection of both pathogens [156]. BsNbs can also be engineered for determining carbaryl and its metabolite 1-naphthol, allowing for risk assessment of pesticide exposure in agricultural and environmental samples [157]. Nbs are also suitable for novel passive immunization, such as in-feed oral delivery of VHH-IgAs, which has demonstrated effective protection against gastrointestinal infections in piglet models [158].

Modification of nanobodies for specific applications

For therapeutic purposes, humanizing the lead Nb is standard practice to avoid potential immunogenicity issues when it is administered to humans. During humanization, preserving specific VHH hallmark amino acids in framework region 2 is crucial. To simplify this process, ordering a synthetic entirely humanized Nb coding sequence that considers the preferred codon usage of the expression host is often preferred [12].

Furthermore, chimeric antigen receptor (CAR)-modified T cells in adoptive immunotherapy have achieved remarkable responses. These strains can be constructed from an Nb library generated from llama immunization. One engineering involves the use of Nb sequences that are PCR-amplified and cloned and inserted into retroviral vectors with second-generation CAR constructs. Transduced T cells display strong nanoCAR expression. These nanoCAR-T cells exhibit robust activation, cytokine production, and tumor cell lysis in response to antigen-positive cells both in vitro and in vivo. These results demonstrate the potential of VHH-based CAR-T cells for targeted immunotherapy, which can target a wide range of tumor-associated

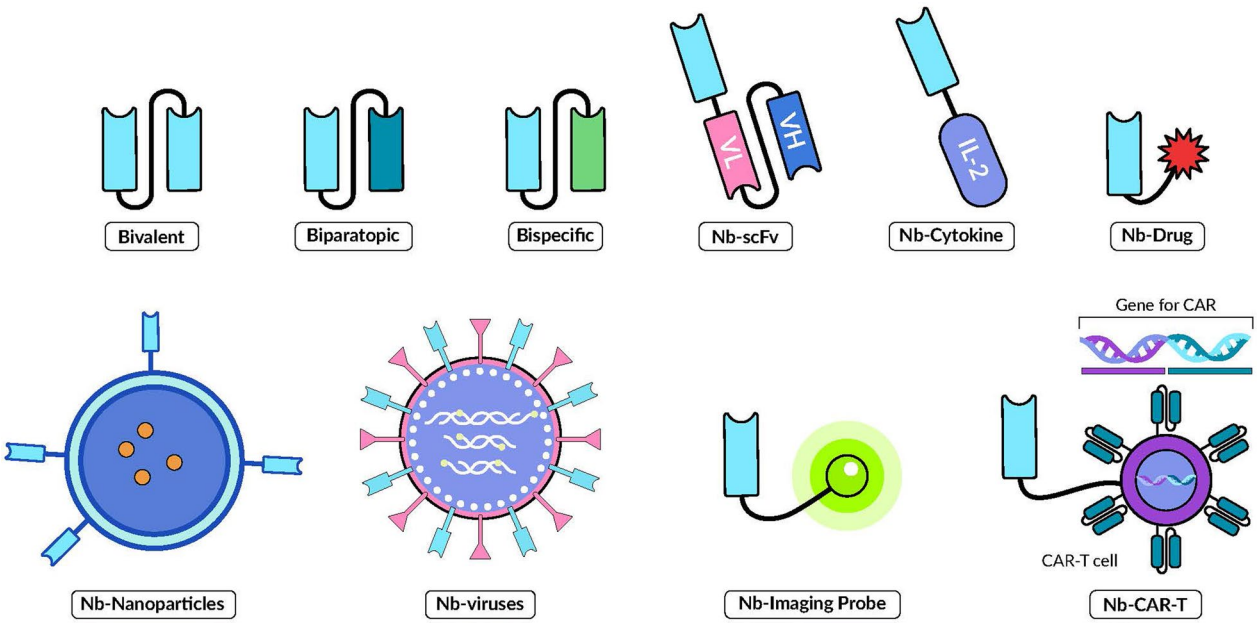


Fig. 6 Exploring the Versatility of Engineered Nanobodies: Types and Modifications. Schematic representation of the different types of engineered nanobodies showcasing their adaptability for a wide range of applications. Adapted from [161]

antigens in adoptive T-cell immunotherapy for solid tumors (Fig. 6) [159, 160].

Diagnostic applications

Ongoing research has explored the unique advantages of Nbs in diagnostics, highlighting their use as labeled probes that bind to certain surface receptors or other target molecules (Fig. 7) [3, 162]. For example, Harmsen et al. showed that Llama VHHs could be produced against Botulinum neurotoxin (BoNT) serotypes C and D, recognizing 10 antigenic sites and exhibiting cross-reactivity with mosaic variants. The researchers created 52 VHH multimers, 15 of which had high affinity for BoNT/C, D, DC, and CD, making them suitable for sensitive Endopep-MS assays and potential use in diagnostics [163].

Compared to full-length antibodies (mAbs), nanobodies offer several distinct advantages and some limitations in the context of disease diagnosis. Nanobodies are significantly smaller than full-length antibodies, which allows for better tissue penetration and access to hidden or recessed epitopes, a crucial feature in precise diagnostic targeting. They are also highly stable under extreme conditions such as low pH or high temperatures, making them ideal for diagnostic assays in variable environments. However, due to their smaller size, nanobodies have a shorter half-life in the bloodstream, which may require modifications or frequent administration in certain diagnostic contexts. In some cases, nanobodies may also exhibit lower binding affinity compared to full-length antibodies, particularly where multivalent binding is essential (Fig. 8).

Nbs are ideal replacements for Lateral Flow Immunoassay (LFIA) autoabs due to their stability, diverse binding capabilities, absence of cross-reactive Fc regions, easy multivalency, and compatibility with gold nanoparticles, expanding LFIA detection possibilities [3]. Using ELISA and LFIA, Maeda et al. demonstrated that a panel of Nbs can detect the spike proteins of five SARS-CoV-2 variants, including Omicron. Antigen test kits based on a lateral flow membrane assay in which two Nb clones, specifically P158 and P86, conjugated to labeled beads were able to detect 300 ng of the spike delta variant [166].

ELISA setups offer laboratory-based diagnostic methods, providing advantages such as a higher limit of detection for the target antigen. Nb-based sandwich ELISAs have been developed to quantify the protein toxins TcdA and TcdB of *Clostridioides difficile* in vitro as well as in the cecal and fecal contents of infected mice [167]. Additionally, selected serum ferritin-specific Nbs have been successfully employed in a high-sensitivity and reproducible heterologous Nb-pair-based sandwich ELISA for diagnostic purposes, achieving a limit of detection of 1.01 ng/mL [168]. Additionally, competitive ELISA tests using *E. coli*-produced Nbs that target Abs against the hepatitis E virus (HEV) ORF2 protein, its main antigen, demonstrated both high sensitivity and

Fig. 7 Nanobodies in Disease Diagnosis: Applications and Impact

From Bench to Bedside...

The overexpression of HER2 in breast cancer, linked to aggressive tumors and poorer outcomes, necessitates developing HER2-targeted radiotracers using Nb, utilizing their favorable kinetics and intratumoral distribution, to non-invasively assess HER2 for selecting suitable therapies and monitoring therapeutic response and resistance.

2Rs15dHis6 Nb was identified as the lead compound, and was conjugated with the 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) chelator to enable labeling with the positron-emitting radioisotope gallium-68 (68Ga).

The Nb was conjugated with the NOTA chelator to enable labeling with the positron-emitting radioisotope gallium-68 (68Ga).

Then, NOTA-conjugated Nb was labeled with 68Ga at room temperature and pH 5.0; radioactivity of 68Ga reached over 97% within 5 minutes, with a specific activity of 55-200 MBq/nmol. After labeling, the 68Ga-NOTA-Nb was purified and characterized using analytical techniques, including ITLC-SG, SEC, and RP-HPLC.

Stability testing by incubation in human plasma showed high metabolic stability and resistance to transchelation, with over 98% of the activity corresponding to the intact radio compound after 1 hour at 37°C.

Animal Model (Pre-clinical)

To assess biodistribution and tumor uptake, female athymic nude mice (5 weeks old) and female athymic nude rats (7 weeks old) were subcutaneously injected with SK-OV-3 or MDA-MB-435D cells in phosphate-buffered saline. Tumor growth was monitored for 1-2 weeks until reaching specific sizes.

Phase I Clinical Trial

Phase I Study of 68Ga-HER2-Nanobody for PET/CT Assessment of HER2 Expression In Breast Carcinoma. Marleen Keyaerts, Catarina Xavier, Johannes Heemskerk, et. Al. Journal of Nuclear Medicine Jan 2016, 57 (1) 27-33; DOI: 10.2967/jnumed.115.162024

This is the first-in-human Phase I clinical trial was conducted to assess the safety, biodistribution, and tumor-targeting potential of the 68Ga-HER2-Nb in 20 breast cancer patients. The Nb showed favorable biodistribution, with the highest uptake in the kidneys, liver, and intestines, but very low background levels in other organs, and tracer accumulation in HER2-positive metastases was high compared to normal surrounding tissues, indicating the potential for tumor imaging.

Phase II Clinical Development

Based on Phase I results, a Phase II trial is in progress to further quantify the 68Ga-HER2-Nb uptake in breast cancer patients, including those with brain metastases; similarly, a Phase I trial evaluating a 99mTc-labeled HER2 Nb for SPECT imaging was also initiated.

Fig. 8 Sidebar 2 The use of radiolabeled Nb exemplifies the potential of a variety of Nbs in diagnostic applications [164, 165]

specificity for HEV genotypes 3 and 4 across diverse samples, competing with serum Abs for binding to the HEV ORF2 protein [169].

Conjugated Nbs can also be used in the detection of cytotoxic T-lymphocyte antigen-4-positive T cells (CTLA-4+). Wang et al. demonstrated the highly sensitive, specific and effective use of fluorescent carbon quantum dot-conjugated Nb (QDs-Nb36) for CTLA-4+ [170]. Similarly, Nbs were engineered for enhanced tetrabromobisphenol-A (TBBPA) binding by linking multiple specific Nbs in tandem and biotinylating the C-terminus of the Nb chain, improving their binding affinity and enabling conjugation to streptavidin (SA)-derivatized materials to increase their binding capacity. The binding affinity of the resulting BMP-SA-biotin-Nbs for TBBPA was evaluated using a competitive ELISA [171].

Nbs have excelled in biosensor development as bioreceptors, offering swift detection via electric potential changes, outpacing ELISA and LFIA in speed, and their durability under variable conditions, making them ideal for storage in less-than-ideal environments. Examples include the following: detection of *Toxocara canis* antigens causing human toxocariasis using Nbs in an electrochemical magnetosensor with amperometric read-out [172]; the creation of diagnostic tools for early cancer and sepsis detection by introducing a capacitive electrochemical impedance spectroscopy platform using gold surfaces modified with anti-IL6 Nbs or IL-6 aptamers for IL-6 detection in human serum [173]; the integration of a hand-held breath aerosol collector with a Nb specific to the SARS-CoV-2 spike protein bound to an ultrasensitive microimmunoelectrode biosensor [174]; and the development of Nb-functionalized organic electrochemical transistors (OECTs) for rapid quantification of SARS-CoV-2 [175].

Consequently, Nbs show promise as accurate and cost-effective diagnostic devices, particularly for early disease detection in conditions such as cardiovascular and infectious diseases, and could revolutionize clinical medicine by integrating advances in biological understanding with imaging technology progress [3, 164]. Nbs meet the ASSURED criteria; ineffective diagnosis of infectious diseases often results from tests failing to meet affordability, accessibility, or efficacy and specificity standards. For example, the detection of the *Trypanosoma congolense* glycolytic enzyme pyruvate kinase in plasma for the diagnosis of active trypanosomosis infections showed 80% sensitivity and 92% specificity for LFIAs, indicating their potential for commercial production. Similar methods were used to identify Nbs against glycolytic enzymes for diagnosing *T. evansi and T. congolense,* with LFIA detecting human norovirus exhibiting 80% sensitivity and 86% specificity against four strains [3]. However, Nb development, primarily in mammalian cells, is costly and time-consuming, prompting exploration in microorganisms such as yeasts or *E. coli*, especially for pharmaceuticals. In addition, challenges include validating in vivo models, addressing ethical and regulatory concerns, and resolving issues such as low binding efficiency and selectivity in advanced imaging techniques [162].

Nanobodies as imaging agents

Disease diagnostics and in vivo diagnostic techniques, such as optical and nuclear imaging (e.g., PET and SPECT), use fluorescent or radioactive labeled Abs to detect and monitor diseases. However, Nbs are well suited for in vivo imaging due to their small size, rapid tissue penetration, ease of modification, and quick renal clearance. While optical imaging is safer and faster, nuclear imaging offers deeper penetration and sensitivity. Labeled NBs have shown promise in PET and SPECT imaging, especially for cancer biomarkers such as HER2, demonstrating their potential in in vivo diagnostics across various diseases. Table 7 lists examples of applications for Nbs in the experimental phase or in clinical use [3, 176].

Nanobodies as research tools

Due to their remarkable tumor penetration properties, capacity to specifically target distinct antigens, notably their exceptional selectivity toward a particular target, ability to stabilize dynamic proteins [201], and ability to facilitate single-particle cryo-electron microscopy and focused drug discovery efforts [202], Nbs are highly promising research tools [203]. Nbs also offer unique advantages for studying fast protein dynamics using nuclear magnetic resonance (NMR) spectroscopy, particularly for smaller proteins, overcoming challenges associated with larger proteins in solution NMR, and X-ray crystallography or electron microscopy, due to slower tumbling rates and resulting in improved spectral information [204]. Furthermore, synthetic Nb libraries offer a versatile solution to the widespread need for antigen-specific binders in research applications [25]. For example, single-domain Abs (DesAbs) are research tools for investigating the biophysical and cytotoxic properties of Aβ peptide oligomers. DesAb18-24 and DesAb34-40 demonstrate the structure–toxicity relationship of Aβ oligomers, focusing on Zn2+-stabilized Aβ40 oligomers as models. These Abs induce structural changes in oligomers, increasing their size and hydrophobicity, with both changes offsetting their impact on cytotoxicity, providing insights into Aβ oligomer properties [205].

Harfmansa et al. overcame the lack of suitable research tools by developing a synthetic receptor

Table 7 Examples of Nanobodies in imaging

Nanobody name	Target	Imaging modality	Application	References
Nanobodies (A1 and E8)	Cadherin 17 (CDH17)	IVIS imaging system	Gastric cancer	[177]
MIRC208 and MIRC213	HER2-positive breast tumors	SPECT/CT imaging	Breast cancer patient selection before trastuzumab	[178]
[68 Ga]-Ga-HNI01	LS174T tumors	Targeted PET imaging	Colorectal carcinoma	[179]
99mTc-NM-02	HER2-targeting	CT scan & SPECT imaging	HER2 status in metastatic (breast) cancer	[180]
sdAb 3187	huLAG-3	PET-tracer	Human lymphocyte activation gene-3, (huLAG-3 expression)	[181]
99mTc-labeled anti-mTIGIT-Nb 16,988	TIGIT	SPECT/CT imaging	T-cell Ig and ITIM domain receptor (TIGIT) expression	[182]
89Zr-ssHN3	Glypican-3 (GPC3)	PET/CT	Hepatocellular carcinoma	[183]
68 Ga-NODAGA-SNA006	CD8 + T cells	Positron emission tomography (PET)	CD8 expression for individualized cancer immunotherapy	[184]
Cy7-Nb119	V-set and Ig domain-containing 4 (Vsig4)	Near-infrared NIR fluorescence (NIRF) imaging	Rheumatoid arthritis	[185]
[99mTc]-NM-01	PD-L1	SPECT/CT	Immunotherapy management in NSCLC	[186]
68 Ga-NOTA-Nb109	PD-L1	PET	Detection and quantification of PD-L1 expression in cancers	[187, 188]
[68 Ga]-Ga-NOTA-anti-MMR-sdAb	Macrophage Mannose Receptor (MMR)	PET/CT	Protumorigenic macrophages in the tumor microenvironment	[189]
68 Ga-NOTA-anti-MMR Nb	MMR	PET/CT	Noninvasive imaging of atherosclerotic plaques	[190]
[68 Ga]-Ga-Anti-CD206-sdAb	CD206	PET/CT	Quantification of CD206-expressing macrophages in the tumor microenvironment	[191]
99mTc-NM-01	PD-L1	SPECT/CT	PD-L1 expression in primary tumors and metastatic malignancies	[192]
2Rs15d & 5F7	HER2	PET	HER2 levels in breast carcinoma	[193]
Anti-PD-L1 99mTc-nanobodies	PD-L1	SPECT/CT	Efficacy of CD8 + T-cell and iNKT cell activating mRNA vaccine	[194]
sdAbs-7C12 & cAbBCII10	EGFR	OI & PET	Diagnostic Cancer Imaging	[195]
99mTc-A1	Mesothelin	SPECT-CT	Pancreatic ductal adenocarcinoma	[196]
anti-MSLN-Nb- S1	Mesothelin	PET/CT	Imaging of Mesothelin positive tumors	[197]
sdAb-K2	PD-L1	SPECT/CT	PD-L1 expression	[198]
68Ga-anti-HER2-VHH1	HER2	PET/CT	Imaging of breast cancer brain metastases	[176]
64Cu-VCAM 64Cu-MMR 64Cu-LOX	VCAM-1 MMR LOX-1	Integrated PET/magnetic resonance imaging (MRI)	Atherosclerosis	[199]
NJB2	Disease-associated Extracellular matrix proteins	PET/CT	Noninvasive detection of tumors, metastatic lesions, and fibroses	[200]

called morphotrap, which consists of vhhGFP4 (green fluorescent protein) fused with the mouse CD8 transmembrane protein, to study the secretion and dispersal of GFP-tagged Decapentaplegic morphogen in Drosophila wing disc tissue and introduced a toolbox, including the morphotrap and five synthetic GFP traps for different cellular compartments, enabling interference with target proteins in both extracellular and intracellular spaces [206]. Similarly, LlamaTags, a protein tag, enables the visualization of transcription factor concentration dynamics in live embryos of the fruit fly *Drosophila melanogaster* [207]. Loreau et al. introduced an Nb toolbox for precise protein labeling in dense muscle tissues, facilitating the study of sarcomere morphogenesis in Drosophila and overcoming the difficulties associated with measuring protein positions and dynamics within

intact tissues or whole animals, particularly the limited accessibility of large Abs to dense tissues [208]. Camelid antibody-derived GFP-binding proteins could also create a library of hybrid transcription factors, allowing control of gene expression exclusively in the presence of GFP, enabling selective manipulation of GFP-labeled cells in various organisms [209]. A split GFP Nb strategically positioned at complementarity-determining regions, combining split-protein technology with light-responsive proteins, could also be used to engineer an optogenetic system to control intrabodies. The result is an optogenetically activated intracellular Ab (optobody) consisting of split Ab fragments and blue-light inducible heterodimerization domains, allowing precise regulation of endogenous target proteins in living cells [210].

Furthermore, the cell-specific modulation of cellular activity can be achieved via magnetic activation of ferritin-tagged ion channels. This could be achieved by fusing an anti-ferritin Nb with transient receptor potential cation channel subfamily V member 1. This fusion allows direct binding of the channel to endogenous ferritin in both mouse and human cells. It can be efficiently delivered using a single AAV, inducing magnetic cell activation in vitro [211].

Electronic biosensors rely on selective biomolecule binding to alter their properties, facilitating the detection of specific biomolecules in solution. OECTs functionalized with antigen-specific Nbs can detect SARS-CoV-2 and Middle East Respiratory Syndrome Coronavirus spike proteins within 10 min, even at the single-molecule level, in diverse sample types, such as saliva, serum, and nasopharyngeal samples [212]. To expedite biosensor detection, alternating current electrothermal flow (ACET) could be integrated into an OECT-based sensor to speed up its operation. ACET is specifically applied to a gate electrode functionalized with Nb fusion proteins to detect the SARS-CoV-2 spike protein in human saliva in just 2 min [213]. Different Nbs could also functionalize a ClyA nanopore using a 5–6 nm DNA linker. Ty1, 2Rs15d, 2Rb17c, and nb22 Nbs can specifically recognize the large protein SARS-CoV-2 Spike, a medium-sized HER2 receptor, and the small protein murine urokinase-type plasminogen activator, respectively. The modified nanopores could effectively sense these proteins, making them promising tools for clinical protein biomarker detection [214]. Furthermore, Nbs could be optimized for efficient immobilization on sensing substrates to detect antigens by strategically mutating lysine residues—Nbs immobilized in an orientation accessible to the antigen tend to have high binding activity—by adding a Lys-tag to the C-terminus and maintaining favorable physical properties [215].

During SARS-CoV-2 infection, the IgG Fc glycoforms related to the infection can be identified and manipulated via Nbs, which can distinguish different protein glycoforms without reacting with unrelated glycoproteins or glycans. These Nbs are applied to differentiate glycoforms IgG, including IgG Fc glycoforms related to SARS-CoV-2 infection. This method has potential clinical applications in characterizing infections, disrupting IgG-Fcγ receptor binding, and studying B-cell receptor glycan structures [216].

Leveraging the power of phage display technology, a remarkable array of innovations are possible, including the generation of VNARs targeting O-GlcNAc transferase (OGT), which now stand as invaluable tools for advancing OGT and O-GlcNAcylation research [217]. Additionally, Nbs targeting extracellular matrix proteins in metastases, using preparations from triple-negative breast cancer and colorectal cancer as immunogens, have been developed using phage-display libraries, showing potential for cancer-specific imaging and therapies [218]. Furthermore, the use of an alpha-type anti-idiotypic Ab (Ab2α) via phage display significantly improves the sensitivity of an ELISA for detecting the cyanobacterial toxin microcystin-LR, indicating that this method is promising for enhancing immunoassays for environmental pollutants [219].

Combining chromatin immunoprecipitation (ChIP) with DNA microarray analysis (ChIP-chip) or high-throughput sequencing (ChIP-Seq) currently provides a valuable method for studying DNA-binding events of transcription factors. In this context, Nbs, which are small and easily produced Ab fragments, are being utilized in ChIP-chips to identify DNA–protein interactions on a genome-wide scale, showing their potential to streamline ChIP experiments, especially in organisms with limited genetic manipulation capabilities [220].

Delivery of nanobodies

Direct injection of nanobodies

In addition to maximizing the advantageous small size of Nbs, intertumoral or direct injections could also maximize efficacy and therapeutic outcomes [221, 222].

Collagen is an abundant matrix protein in tumors and serves as an effective target for locally administered immunotherapies. Nbs, in this approach, are tailored for intratumoral therapies targeting collagen within tumors, utilizing the collagen-binding ectodomain of murine leukocyte-associated immunoglobulin-like receptor-1 (LAIR) to engineer specific Nb variants [221]. These engineered Nbs are then fused with IL-2, a cytokine known for activating immune cells, with the aim of minimizing systemic toxicity and localizing the therapeutic effect to the tumor site [221].

Likewise, combining small-format IL-2 immunocytokines with high-affinity Nbs targeting the tumor-specific EIIIB domain of fibronectin, which is found in the tumor extracellular matrix, could lead to a delay in tumor growth. Intravenously, these immunocytokines exhibit effects on tumor growth similar to those of untargeted IL-2. However, intratumoral delivery, dependent on the affinity for EIIIB, results in improved survival outcomes [223].

There is also the possibility of engineering sdAbs for injection into human cells by *E. coli* bacteria equipped with a type III protein secretion system [222], potentially bypassing the need for the Abs to penetrate the plasma membrane, which is a significant barrier in targeting intracellular proteins for therapeutic purposes [222]. These sdAbs are engineered using two specific sdAbs, Vamy and Vgfp, which target amylase and GFP, respectively [222].

Conjugation of nanobodies with nanoparticles or liposomes

An alternative strategy involves the conjugation of Nbs for effective targeting [224]. One example involves conjugating Nbs targeting the hepatocyte growth factor receptor (MET-Nbs) to PEGylated liposomes at varying densities, ranging from 20 to 800 Nbs per liposome. A MET-Nb density above 300 Nbs per liposome results in a twofold increase in interactions with phagocytic cells in ex vivo human blood compared to nontargeted liposomes, demonstrating that adjusting the MET-Nb density increases the specificity of the nanoparticles toward their intended cellular target and reduces nanoparticle interactions with phagocytic cells [225].

Another example is tumor-associated macrophages (TAMs), which are promising targets for inhibiting tumor growth, overcoming drug resistance, and combating metastasis. TAMs also play a pivotal role in regulating the tumor microenvironment. A dual-targeting liposomal system modified with an anti-PD-L1 Nb and TfR-binding peptide T12 has been developed for the codelivery of simvastatin/gefitinib to treat brain metastasis (BMs) of non-small cell lung cancer (NSCLC). These dual-targeting liposomes efficiently penetrate the BBB and enter BMs, leading to TAM repolarization and the reversal of $EGFR^{T790M}$-associated drug resistance. Treatment mechanisms involve elevated ROS levels and suppression of the EGFR/Akt/Erk signaling pathway [226].

Versatile and efficient drug targeting is also possible with natural extracellular vesicles (EVs). They are ideal drug carriers due to their high biocompatibility. A novel method involving protein ligases allows for the efficient covalent conjugation of EVs with targeting moieties, such as an EGFR-targeting peptide or anti-EGFR Nb. This enables the accumulation of EVs in EGFR-positive cancer cells, enhancing drug delivery. This application in the delivery of paclitaxel by EGFR-targeting EVs at a low dose significantly increased drug efficacy in a xenograft mouse model of EGFR-positive lung cancer [227]. Similarly, single-walled carbon nanotubes, amantadine, and a specific Nb (NNV-Nb) can target nervous necrosis virus (NNV), demonstrating the potential of nanocarriers as promising approaches for CNS viral disease therapy [228]. In addition to exhibiting greater drug entrapment efficiency, nanotechnology-based nanocarriers exhibit lower cytotoxicity, greater stability, and better pharmacokinetics and pharmacodynamics than traditional therapies [229, 230].

Nanobody in genetic fusion or gene therapy

In addition to current immune-oncology therapies, immune checkpoint blockade could also employ different formats of a human PD-L1 sdAb, such as K2. Different formats of K2, which target the PD-1/PD-L1 immune checkpoint axis, have potential as immune checkpoint inhibitors via gene-based delivery. Compared with those of monovalent K2, bivalent and trivalent K2 formats significantly enhanced T-cell receptor signaling. Additionally, a fusion protein called K2-Fc, similar to an IgG1 Ab, demonstrated superior tumor cell killing efficacy in a 3D melanoma model versus avelumab, highlighting the potential of K2-based immune checkpoint therapies, particularly when they are fused with an IgG1 Fc domain for enhanced efficacy [231].

Further cancer therapy enhancement is possible via optimization through the utilization of Nbs and a self-amplifying RNA (saRNA) vector based on Semliki Forest virus (SFV). These Nbs, which target PD-1 and PD-L1, effectively inhibit interactions in both human and mouse models, and incorporating a dimerization domain significantly improves their inhibitory potential. The SFV particles delivering these dimeric Nbs demonstrate strong antitumor effects, surpassing the effectiveness of conventional Ab vectors, and stimulate proinflammatory reactions within tumors, bolstering the infiltration of immune cells. Local plasmid electroporation further enhances this potent antitumor effect, suggesting potential clinical applications [232].

Genetic engineering allows VHH against norovirus to be expressed on the surface of *Lactobacillus*, a natural gut microbiome component. The Nb VHH 1E4 effectively neutralizes GII.17 norovirus when administered orally to germ-free mice. These engineered lactobacilli continue to exhibit neutralizing activity in the intestines for at least 10 days, highlighting their potential as oral Nb delivery vectors for passive immunization against norovirus infections [233]. A similar technique can deliver

Nbs against necrotic enteritis caused by *Clostridium perfringens* in poultry. Recombinant *Limosilactobacillus reuteri* can efficiently produce and secrete Nbs that neutralize *C. perfringens* NetB toxins [234]. In addition, a Nb fused with porcine IgG Fc (Fcγ), known as Nb6-pFc, has been developed to inhibit the replication of the highly contagious porcine reproductive and respiratory syndrome virus (PRRSV) in susceptible cells through Fcγ-receptor-mediated endocytosis, offering a promising strategy for controlling and preventing PRRSV infections [235].

As previously discussed, AAVs are nonpathogenic in humans and have been developed into gene therapy vectors. Genetically modified AAV2 capsid proteins, VP1 and VP2, with Nbs that have a high affinity for the human CD4 receptor improve the targeting of human CD4 + cells, including primary human peripheral blood mononuclear cells and purified human CD4 + T lymphocytes [236].

Furthermore, allowing for efficient and specific in vivo gene delivery involves pairing antibody-based targeting with adenoviral-mediated gene transfer. This method employs capsid modifications to achieve cell-specific adenoviral tropism, particularly for cancer gene therapy. The engineering of sdAb into the chimeric fiber of the adenoviral vector, which consists of adenovirus serotype 5 fiber, fibritin, and the anti-CD276 sdAb, plays a pivotal role. The modified adenoviruses demonstrate effective gene transfer in vitro, independent of CAR, and exhibit high gene delivery levels to human tumor cells that overexpress CD276 [237].

Clinical translation of nanobody-based therapeutics and safety considerations

Clinical translation of nanobody-based therapeutics

Caplacizumab's approval has sparked greater interest in domain Abs, demonstrating that camelid-based VHH domains have potential for regulatory approval [238, 239]. The FDA also approved Ciltacabtagene autoleucel (Carvykti), a second-line treatment, as a CAR-T-cell therapy for advanced multiple myeloma patients who are refractory or have relapsed after at least four lines of therapy [240]. In Japan, Ozoralizumab, a trivalent anti-TNFα, received approval for treating inadequately managed RA [241]. However, among the 675 active Ab programs in clinical development, only 11 are domain Abs, highlighting their limited presence despite the rapid growth in Ab research [238]. Identifying niche areas where Nbs can excel among other biologics is important for an increasing number of clinical trials (Table 8) [238].

Safety considerations

While Nbs offer many advantages, safety concerns remain a crucial aspect of their application. For example, rapid renal clearance of Nbs can lead to high kidney accumulation post-injection, resulting in an elevated kidney radiation dose. Less than 10% of the injected dose circulates in the blood one hour after injection, which necessitates modifications to prolong circulation and reduce potential side effects [261]. One potential solution is the co-injection of positively charged amino acids and gelofusin, which competes for renal reabsorption and reduces kidney activity. Furthermore, removing the histidine tag has been shown to decrease kidney retention in biodistribution studies with 68 Ga-NOTA-anti-HER2 Nbs, possibly due to changes in the charge of the acidic environment in the kidney [164].

Nbs have shown low immunogenicity risk in clinical trials, especially when humanized [262], although non-human sequences alone may not predict immunogenicity [263, 264]. Two notable examples are tetrameric DR5-specific VHH TAS266 (targeting the DR5 receptor) and (tissue necrosing factor) TNFR1-specific VH GSK1995057 (targeting the TNFR1 receptor), which can trigger Ab responses [264]. Despite not being humanized, in a phase I study, a Nb targeting the HER2 antigen in breast carcinoma was labeled with 68 Ga and injected into patients, and no adverse effects were observed. The radiolabeled Nb effectively targeted HER2-positive primary breast carcinoma lesions and metastatic lesions while showing minimal background accumulation in other organs commonly associated with breast carcinomas or tumor metastasis [261]. Additionally, while challenging, single-domain Abs can be conjugated with a radiolabel or a fluorescent dye to create hybrid tracers suitable for surgical guidance [265].

Conclusion

Future challenges and opportunities for nanobodies

Nb technology presents diverse opportunities, emphasizing the future of therapeutic advancements involving bioinformatics, screening protocols, and delivery techniques. Intrabodies, an innovation derived from Nbs, have versatile applications across neurological disorders and show potential synergy with gene therapy in complex disease treatment. Breakthroughs in machine learning, artificial intelligence (AI), bioinformatics, screening protocols, and gene therapy techniques will enhance Nb-derived intrabody therapy across a wide spectrum of disorders [266].

Engineering and bioengineering, along with high-throughput sequencing, allow for animal immunization with the antigen of interest. PBMCs can be collected

Table 8 Nanobodies in clinical trials

Clinical trial ID/ Phase	Title	Study design	Eligibility criteria	Intervention	Primary outcome measures	Secondary outcome measures	Results	References
NCT05639153/ Phase I	A Trial to Evaluate the Safety, Tolerability, Pharmacokinetics and Preliminary Efficacy of DR30303 in Patients With Advanced Solid Tumors	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with advanced solid tumors	DR30303-a recombinant humanized monoclonal antibody that targets Claudin18.2	Safety and tolerability of DR30303, including incidence and severity of adverse events	Objective response rate (ORR), Progression-free survival PFS, OS (RECIST 1.1)	Completion date-30/04/2024	[242]
NCT03972150/ Phase I	A Study to Find the Best Dose of BI 836880 Alone and in Combination With BI 754091 in Japanese Patients With Different Types of Advanced Cancer	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with advanced solid tumors	BI 836880 BI 754091	Maximum tolerated dose (MTD) and/ or recommended Phase 2 dose (RP2D) of BI 836880 alone and in combination with BI 754091	Objective response rate (ORR) and disease control rate (DCR)	Maximum tolerated dose was not reached. BI 836880 alone and in combination with ezabenlimab had a manageable safety profile with preliminary clinical activity in Japanese patients with advanced solid tumors	[243]
NCT03248843/ Phase I	A Study of PD-L1 Antibody KN035 in Japanese Subjects With Locally Advanced or Metastatic Solid Tumors	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with advanced solid tumors	Single-domain anti-PD-L1 monoclonal antibody KN035	Safety and tolerability of KN035, including incidence and severity of adverse events	Objective response rate (ORR) and disease control rate (DCR)	Well tolerated with efficacy. Pharmacokinetics data and preliminary antitumor response support dose regimens	[244]
NCT03667170/ Phase I	KN035 in Subjects With Advanced Solid Tumors	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with advanced solid tumors	Single-domain anti-PD-L1 monoclonal antibody KN035	Objective response rate (ORR) and disease control rate (DCR)	Duration of response (DOR), Progression-free survival (PFS), Overall survival (OS)	Completion date-15/12/2025	[245]
NCT02827968/ Phase I	Phase 1 Study of Anti-PD-L1 Monoclonal Antibody KN035 to Treat Locally Advanced or Metastatic Solid Tumors	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with advanced solid tumors	Single-domain anti-PD-L1 monoclonal antibody KN035	Incidence of dose limiting toxicities (DLTs) Percentage of participants with adverse events (AEs), serious adverse events and AEs of special interest	Duration of response (DOR), Progression-free survival (PFS), Overall survival (OS)	Favorable safety and pharmacokinetic profile, with promising preliminary antitumor activity in patients with advanced solid tumors	[246]

Table 8 (continued)

Clinical trial ID/ Phase	Title	Study design	Eligibility criteria	Intervention	Primary outcome measures	Secondary outcome measures	Results	References
NCT02683083/ Phase I	[131I]-SGMIB Anti-HER2 VHH1 in Patients With HER2 + Breast Cancer	Interventional, Single Group Assignment, Open Label, Safety Study	Female patients with HER2 + breast cancer	[131I]-SGMIB Anti-HER2 VHH1	Number of participants with treatment-related adverse events as assessed by CTCAE v4.0	Tumor targeting potential will be visually scored on the planar total body scan	No drug-related adverse events with 131I-GMIB-anti-HER2-VHH1, primarily eliminated through the kidneys, stability in circulation, exhibited specific uptake in metastatic lesions in advanced breast cancer patients	[247]
NCT02340208/ Phase I/II	A Phase I/II Open-Label, Non-Randomized Dose Escalation Study of Immunoconjugate L-DOS47	Interventional, Open Label, Safety Study	Adults with histologically or cytologically confirmed nonsquamous NSCLC	Urease conjugated to a camelid monoclonal antibody- L DOS47	The incidence and severity of drug-related adverse events as a measure of safety and tolerability of L-DOS47	L-DOS47 related toxicity during the first 2 h after infusion, incidence and severity of all reported adverse events and serious adverse events	One dose-limiting toxicity (spinal pain) observed, no complete or partial responses were seen, 32 patients achieved stable disease after two treatment cycles, one patient in cohort 9 remained on treatment for 10 cycles without disease progression	[248]
NCT02309892/ Phase I	A Phase I, Open Label, Dose Escalation Study of Immunoconjugate L-DOS47 in Combination With Pemetrexed/Carboplatin in Patients With Stage IV (TNM M1a and M1b) Recurrent or Metastatic NSCL Lung Cancer	Interventional, Single Group Assignment, Open Label, Safety Study	Adults with histologically or cytologically confirmed nonsquamous NSCLC	L-DOS47	Number of patients with adverse events as a measure safety and tolerability of L-DOS47 in combination treatment with pemetrexed/ carboplatin	ORR	L-DOS47 combined with standard pemetrexed and carboplatin chemotherapy is well tolerated in patients with recurrent or metastatic nonsquamous NSCLC	[249]
NCT04887259/ Phase I/IIa	Trial of LAVA-051 in Patients With Relapsed/Refractory CLL, MM, or AML	Interventional, Sequential Assignment, Open Label, Treatment	Adults with relapsed or refractory CLL, MM, or AML	Bispecific gamma-Delta T-Cell engager-LAVA-051	Frequency and severity of AEs, Frequency and type of DLT	Number of participants with an anti-tumor response, Pharmacokinetics of LAVA-051, area under the plasma concentration versus time curve (AUC)	Completion – 30/12/2024	[250]

Table 8 (continued)

Clinical trial ID/ Phase	Title	Study design	Eligibility criteria	Intervention	Primary outcome measures	Secondary outcome measures	Results	References
NCT05369000/ Phase I/IIa	Trial of LAVA-1207 in Patients With Therapy Refractory Metastatic Castration Resistant Prostate Cancer	Interventional, Sequential Assignment, Open Label, Treatment	Male patients 18 years and older with metastatic castration resistant prostate cancer	Humanized bispecific antibody of two single domain antibody (VHH)-LAVA-1207	Frequency and severity of Adverse Events (AEs), Frequency and type of Dose-Limiting Toxicity (DLT)	Objective response rate (ORR), Duration of response (DOR), Progression-free survival (PFS)	Completion—30/03/2024	[251]
NCT03548207/ Phase Ib/2	A Phase 1b-2, Open-Label Study of JNJ-68284528, A Chimeric Antigen Receptor T-Cell (CAR-T) Therapy Directed Against BCMA in Subjects With Relapsed or Refractory Multiple Myeloma	Single Group Assignment, Open Label	Adults with multiple myeloma	A Chimeric Antigen Receptor T-Cell—JNJ-68284528	Phase 1b: Number of Participants with Adverse Events; Number of Participants with Adverse Events by Severity; Phase 2: Overall Response Rate (ORR)	Levels of B-Cell Maturation Antigen (BCMA) Expressing Cells and Soluble BCMA, Level of JNJ-68284528 T-Cell Expansion (proliferation), and Persistence, Levels of CAR-T Cells	With a median follow-up of 18 months, results show significant, long-lasting responses in heavily treated multiple myeloma patients, the treatment maintained a manageable safety profile without any new safety concerns	[252, 253]
NCT03090659/ Phase 1/2	A Clinical Study of Legend Biotech BCMA-chimeric Antigen Receptor Technology in Treating Relapsed/Refractory (R/R) Multiple Myeloma Patients	Single Group Assignment, Open Label	Patients with refractory multiple myeloma. Clear BCMA expression must be detected on malignant plasma cells from either bone marrow or a plasmacytoma by flow cytometry or immunohistochemistry	LCAR-B38M CAR-T-cell injection	Occurrence of treatment related adverse events as assessed by CTCAE v4.0	Anti-myeloma responses to LCAR-B38M cell treatment	Completion-31/12/2023	[254]
NCT04133636/ Phase 2	A Phase 2, Multicohort Open-Label Study of JNJ-68284528, a Chimeric Antigen Receptor T-Cell (CAR-T) Therapy Directed Against BCMA in Subjects With Multiple Myeloma	Single Group Assignment, Open Label	Adults with multiple myeloma	A Chimeric Antigen Receptor T-Cell—JNJ-68284528	Percentage of Participants with Negative Minimal Residual Disease (MRD), Percentage of Participants with Sustained MRD Negative Complete Response (CR)	Overall Response Rate (ORR), Duration of Response (DOR)	Completion-13/11/2028 Interim results—responses with manageable safety, responses in pts with ineffective or insufficient response to autologous stem cell transplantation	[255–258]

Table 8 (continued)

Clinical trial ID/ Phase	Title	Study design	Eligibility criteria	Intervention	Primary outcome measures	Secondary outcome measures	Results	References
NCT03924466/ Phase II	Quantification of 68-GaNOTA-Anti-HER2 VHH1 Uptake in Metastasis of Breast Carcinoma Patients and Assessment of Repeatability (VUBAR)—Pilot Study	Single Group Assignment, Open Label	Patients with locally advanced or metastatic breast cancer	68GaNOTA-Anti-HER2 VHH1	Repeatability of lesional PET/CT characteristics, Tracer update of 68GaNOTA-Anti-HER2 VHH1 in different cancer types, Feasibility and added value of 68GaNOTA-Anti-HER2 in neoadjuvant setting of breast carcinoma	Within-patient tumor heterogeneity for HER2 expression using PET/CT imaging, Immunogenicity, Histopathological results of biopsied lesions and correlation with PET/CT results	Completion-31/12/2024	[259]
NCT05556096/ Phase III	A Phase 3, Randomized, Double-blind, Placebo-controlled, Parallel, Multicenter Study to Evaluate the Safety and Efficacy of ALXN1720 in Adults With Generalized Myasthenia Gravis	Double-blind, randomized	Diagnosis of MG with generalized muscle weakness	ALXN1720	Change From Baseline in Myasthenia Gravis-Activities of Daily Living (MG-ADL) Total Score at Week 26	Change From Baseline in Quantitative Myasthenia Gravis (QMG) Total Score at Week 26, Percentage of Responders Based on Reduction of the MG-ADL Total Score at Week 26	Completion-07/07/2027	[260]

Source: www.clinicaltrials.gov

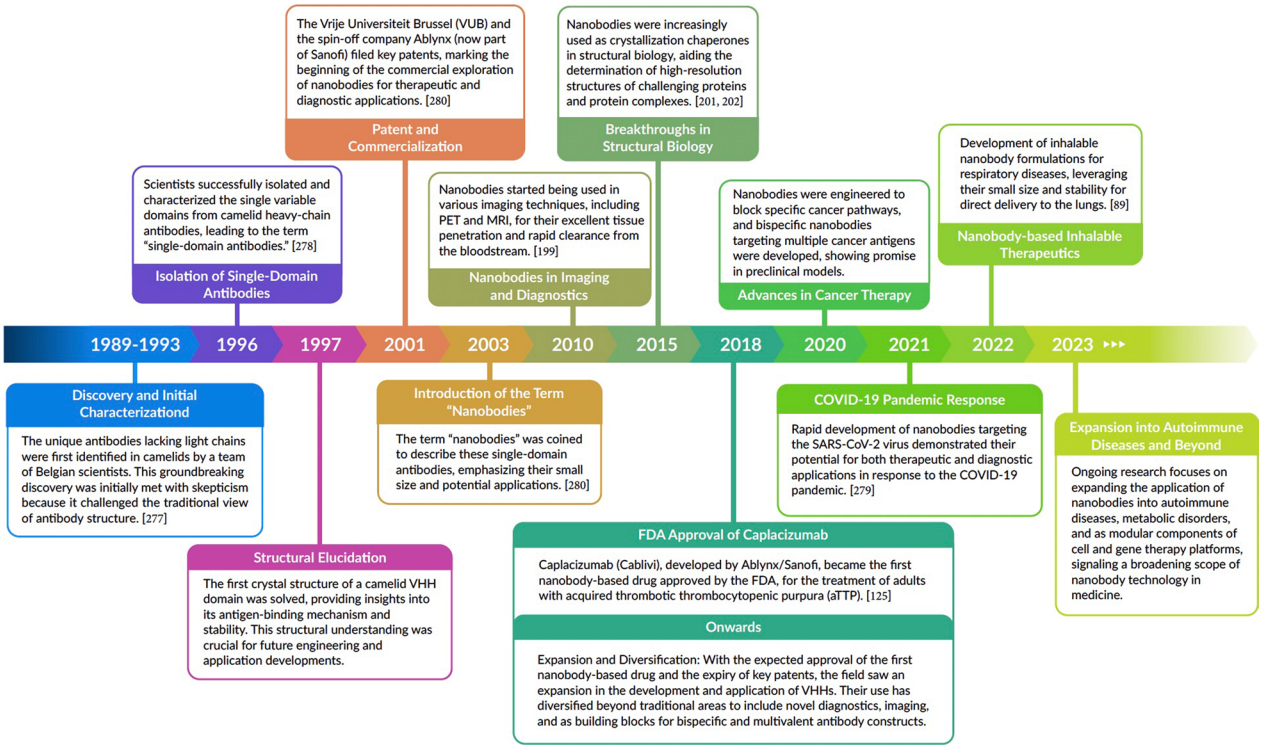


Fig. 9 From Discovery to Development: The Evolution of Nanobodies (original illustration) [89, 125, 199, 201, 202, 277–280]

during peak immune response and can be directly deep sequenced without the need for a phage or yeast library. Subsequently, bioinformatics can be used to identify prevalent clones that are then tested against antigens. Multiple studies have confirmed the superiority of AI programs over conventional methods in modeling protein structures, especially for Nbs. They can be the preferred choice for Nb modeling due to their speed and superior results; these programs offer adaptable, efficient modeling suited for low- to mid-range hardware, demonstrating a multidisciplinary approach in Nb research [266, 267].

Among the challenges for this emerging technology is the development of T-cell receptor-like (TCR-like) Nbs for high-affinity binding to major histocompatibility complex peptide (MHC-peptide) complexes. Moreover, bringing these Nbs into clinical trials is a multifaceted and demanding process, demanding rigorous validation at every step. Nevertheless, the promise of TCR-like Nbs in cancer therapy, particularly their efficacy in targeting intracellular tumor-associated antigens, cannot be overlooked [268]. While VHHs have shown promise in targeting MHC-peptide complexes, it remains a challenging task to achieve the high specificity that TCRs naturally possess. This difficulty highlights the need for ongoing research and development to improve the specificity of antibody-based pHLA targeting in immunotherapy.

In cancer treatment modalities, including surgery, chemotherapy, radiation therapy, and immunotherapy, Nbs show significant potential in enhancing treatment by serving as tracers for precise surgery and targeted toxin or radionuclide delivery. Additionally, ongoing clinical trials and research highlight Nbs as promising biological drugs with the potential for patient-targeted therapy stratification through noninvasive imaging, suggesting a future where Nb-based cancer therapy surmounts current challenges [269]. For example, innovative approaches can surmount challenges in delivery. Nb188, an anti-human TfR VHH, has potential for transporting therapeutic agents across the BBB. When Nb188 is fused with neurotensin, it successfully crosses the CNS, a feat unattainable by the neuropeptide alone. Furthermore, the creation of heterodimeric Abs by fusing the anti-β-secretase 1 (BACE1) 1A11 Fab with Nb62 or Nb188 has been shown to be effective in reducing Aβ1–40 levels in the brain, highlighting its promise as a carrier of therapeutic agents to the CNS [270].

Future directions for nanobody research and development

An abundance of applications for Nbs can be developed from diverse Ab repertoires obtained through animal immunization [209, 271]. Next-generation sequencing (NGS) offers a cost-effective approach, enabling the identification of potent sequences with improved developability profiles by integrating sequence-activity-relationship, frequency, and enrichment analyses [272]. Artificial intelligence/machine learning techniques applied to Ab NGS data have demonstrated versatility in designing sequences with enhanced potency and developability. These techniques, such as long short-term memory, have been used to diversify specific Ab regions in combinatorial mutagenesis libraries, generating models from NGS data to optimize Abs, as seen in studies on a kynurenine binding Ab [273, 274]. This technology could accelerate the development of intracellular Abs (iDAbs) that can target a wide range of cellular proteins, including those in the nucleus, cytoplasm, and plasma membrane, and can be modified with warheads to induce functional changes. iDAbs have the unique ability to target challenging proteins such as undruggable and hard-to-drug proteins [275].

Variable domains of shark Abs, such as AdAlta's i-bodies, are emerging therapies due to their unique properties. AdAlta's lead candidate, AD-214, is set to enter clinical trials for idiopathic pulmonary fibrosis, and the company is also exploring inhalable preparations for improved bioavailability. Other companies developing VNARs include Ossianix and Elasmogen, with AdAlta's Ad-214 being the only treatment in clinical practice [276].

In conclusion, the discovery of camelid Nbs has paved the way for groundbreaking advancements in diagnostics and therapeutics, as they possess the ability to traverse biological barriers and access solid organs. In the three decades since their discovery in 1989, nanobodies (Nbs) have been widely adopted in research disciplines and have been applied in both therapeutic and diagnostic applications due to their unique attributes. Figure 9 provides a detailed time line from the discovery of Nbs and their subsequent development to present day. Their adaptable manufacturing and conjugation capabilities have enabled the creation of highly specific and effective compounds.

Abbreviations

AML	Acute myeloid leukemia
ACP	Acyl carrier protein
AAV	Adeno associated virus
A-syn	Alpha-synclein
ACET	Alternating current electrothermal flow
AD	Alzheimer's disease
Aβ	Amyloid Beta
ALS	Amyotrophic lateral sclerosis
ACE2	Angiotensin converting enzyme 2
Ab	Antibody
AI	Artificial intelligence
BsNb	Bispecific nanobody
BiTES	Bispecific T-cell engagers
BBB	Blood brain barrier
BoNT	Botulinum neurotoxin
BM	Brain metastasis
cTnT	Cardiac troponin T
CNS	Central nervous system
CAR	Chimeric antigen receptor
CRISP	Clustered Regularly Interspaced Short Palindromic Repeats
CDRs	Complementarity-Determining Regions
CTGF	Connecting tissue growth factor
CTGF	Corrective tissue growth factor
Cas	CRISPR-associated proteins
CTLA-4 +	Cytotoxic-T-lymphocyte antigen-4-positive T cells
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EV	Extracellular vesicles
FR	Frameworks
GPCR	G protein-coupled receptor
GFP	Green fluorescent protein
VHH	Heavy chain-only antibodies
HEV	Hepatitis E virus
iTTP	Immune-mediated thrombocytopenic purpura
IgG	Immunoglobulin G
IBD	Inflammatory bowel disease
IL	Interleukin
iDAbs	Intracellular Abs
LFIA	Lateral flow immunoassay
LRRK2	Leucine-rich repeat kinase
MHC	Major histocompatibility complex
MMAE	Monomethyl auristatin E
MUC-1	Mucin 1
MM	Multiple myeloma
MS	Multiple sclerosis
Nb	Nanobody
Nb-SMC	Nanobody static mixers
NNV	Nervous necrosis virus
NGS	Next generation sequencing
NSCLC	Non-small cell lung cancer
NMR	Nuclear magnetic resonance
OECTs	Organic electrochemical transistors
PD	Parkinson's disease
PBMC	Peripheral blood monocytes
PCR	Polymerase chain reaction
PRRSV	Porcine reproductive and respiratory syndrome virus
PET	Positron Emission Tomography
RBD	Receptor binding domain
RA	Rheumatoid arthritis
SFV	Semliki forest virus
SARs-Cov-2	Severe acute respiratory syndrome coronavirus 2
SPECT	Single photon emission computed tomography
SORLA	Sortilin-Related Receptor with LDLR Class A Repeats
SOD-1	Superoxide dismutase 1
TCR	T-cell receptor like
TBBPA	Tetrabromobisphenol-A
TNF	Tissue necrosing factor
TfR	Transferrin receptor
TAMs	Tumor associated macrophages
VNAR	Variable new antigen receptor
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

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