Haddad, F., Dokmak, G., Kanwal, S.,
Karaman, R. Al-Quds J Nat. Scie 2026: 2(4):6-26
https://doi.org/10.47874/AQUPP:6-26

Al-Quds Journal for
Academic Research

A Comprehensive Review of Therapeutic Potential of Nanobodies

REVIEW ARTICLE

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ABSTRACT

Recently, nanobodies have taken center stage in drug discovery and development research. Several nanobodies therapeutic possibilities are undergoing clinical trials and waiting for the Food and Drug Administration's approval. This study aimed to emphasize the potential of nanobodies as therapeutic agents by concentrating on the most recent published studies that examined their properties, manufacturing, and possible applications. This article demonstrates that the unique properties of nanobodies, compared to conventional antibodies, which are based on their small size and offer a number of benefits, make them seem to have a promising future. These advantages include accessing complex or hidden target sites that may be elusive to their larger antibody counterparts and increased resilience against extreme conditions like temperature changes and pH variations. As a result, nanobodies seem to have a bright future as adaptable tools for imaging in cancer and non-malignant diseases, as well as for in vitro and in vivo diagnostic and therapeutic potential for a variety of conditions, including oncology, infectious, metabolic, neurological, and other conditions like ophthalmologic, immune-mediated, and genetic disorders. More research is required to determine their effectiveness and safety in clinical applications. The current analysis offers a thorough overview of the therapeutic uses for nanobody products that are either on the market or undergoing clinical trials.

Keywords: imaging; nanobody; single-domain antibodies; therapeutic nanobody; VHHs

1. INTRODUCTION

Glycoproteins termed antibodies, commonly known as immunoglobulins (Ig), are created by B lymphocytes (Megha and Mohanan, 2021). They are vital components of the immune system that play a crucial role in the body's defense against disease by identifying and inhibiting invading antigens (Singh et al., 2018). Conventional antibodies are "Y"-shaped molecules made up of two heavy chains and two light chains (Igκ or Igλ), which are joined together by covalent disulfide bonds to form a tetrameric structure (Figure 1, (A)) (Lu et al., 2020). As seen in (Figure 1, (A)), both light chain classes have constant and variable domains (Wang et al., 2019a). Antibody light chains in humans come in two different varieties: kappa and lambda. They have a similar form and function while having different protein sequences (Rajpal et al., 2013). In contrast, human antibody heavy chains can be one of five isotypes: IgA, IgD, IgE, IgG, or IgM, each with a distinct function within the adaptive immune system (Chiu et al., 2019, Wang et al., 2019a). IgAs, IgDs, and IgGs contain three constant domains and one variable domain. IgEs and IgMs are composed of one variable domain and four constant domains. IgA and IgM can form dimers and pentamers, respectively, due to the presence of an additional joining chain (Chiu et al., 2019, Wang et al., 2019a). These moieties are symmetrical, with a variable region called the Fab (fragment antigen binding) and a constant region called the Fc (fragment crystallizable) (Figure 1, (A)) (Wang et al., 2019a, Lu et al., 2020).

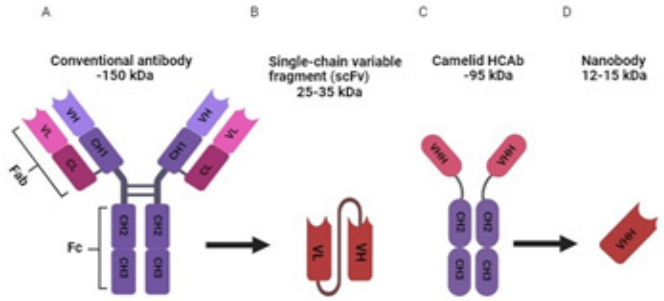

Figure 1: Structure of conventional antibody (A), single-chain variable fragment (scFv) (B), camelid heavy chain only antibody (HCAb) (C), and nanobody (D). CH1 (constant domain 1 of the heavy chain), CH_2 (constant domain 2 of the heavy chain), CH_3 (constant domain 3 of the heavy chain), CL (constant domain of the light chain), Fab (fragment antigen binding), Fc (fragment crystallizable), VH (variable domain of heavy chain), VL (variable domain of light chain), and VHHs (variable domain of HCAb).

The antigen recognition and binding specificity of the entire Ig molecule depend on the Fab region, especially on the two variable domains at the top. In contrast, the Fc domain initiates biological processes upon antigen binding (Asaadi et al., 2021). IgA, IgD, IgE, IgG, and IgM are the five main classes of antibodies. They are categorized into alpha, delta, epsilon, gamma, or mu, depending on the heavy chain they contain. These differ in terms of hinge structure, the valency of the antibody, and the number and sequence of constant domains, as aforementioned (Rajpal et al., 2013). The most abundant immunoglobulin isotype in human serum is IgG (Liu and Liu, 2021). Several stages can be identified in the evolution of antibodies. It is reported that Edward Jenner is responsible for developing the smallpox vaccination (Jenner, 1801). Louis Pasteur then unintentionally discovered the vaccine, opening up new avenues for antibody research (Rees, 2021). Early research mostly focused on observations of protective effects following direct injection of modest doses of virulent disease vectors; however, tests with crude vaccines intended to treat or prevent the disease were also carried out (Packer, 2021). However, it took until the 1880s for the germ hypothesis to replace the spontaneous generation and miasma theories before inoculation could successfully treat diseases other than smallpox (Packer, 2021). The concept of the "antibody" and the use of the antiserum to detect pathogens were inspired by research into natural immunity in the 1890s (Packer, 2021). Paul Ehrlich's contributions to the field of immunology, which included the development of the "side-chain theory" to explain the specificity of immune reactions, are largely responsible for the discovery of antibodies, which further clarified the specific interaction between antibodies and antigens in the blood (Davies and Chacko, 1993, Lemieux and Spohr, 1994). Emil Fischer added to Ehrlich's initial theory of immunological recognition by providing the framework for mechanisms akin to a lock-and-key system, which allowed for a better understanding of how antibodies bind to antigens (Lemieux and Spohr, 1994). The development of monoclonal antibodies (mAbs) technology by Köhler and Milstein in 1975, which made it possible to produce highly specific antibodies for a variety of uses, gave this idea even more support (Hosseini et al., 2018). Therefore, it is considered that Paul Ehrlich suggested antibodies might have been employed for therapeutic purposes more than a century ago (Grillo-López, 2013). In 1948, Astrid Fagraeus reported that plasma B cells have a specialized role in producing antibodies, and in 1957, Frank Burnet and David Talmage established the clonal selection theory (Hosseini et al., 2018). The molecular structure of immunoglobulin was separately described by Gerald Edelman and Rodney Porter in 1959, for which they eventually shared the Nobel Prize in 1972 (Hosseini et al., 2018). The first high-resolution structure of an antibody fragment was initially reported in 1973 (Köhler and Milstein, 1975, Hosseini et al., 2018). The discovery of mAbs by Georges Köhler and César Milstein in 1975 marked the beginning of the contemporary age of antibody research and discovery (Hosseini et al., 2018). The approval of the first monoclonal antibody (mAb) by the Food and Drug Administration (FDA) in 1986 marked the undeniable evolution of antibody engineering (Lu et al., 2020). In recent times, the therapeutic antibodies drug market has experienced an exceptional surge in growth, primarily attributed to the approval of novel drugs for treating a broad range of diseases, including cancer, neurological, infectious, autoimmune, metabolic, genetic, and others (Mahmuda et al., 2017). For instance, more than 100 mAbs have received official authorization for commercial purposes (Castelli et al., 2019, Erstad and Davis, 2023). In 2022, about 24% of novel medications authorized by the FDA were mAbs (Erstad and Davis, 2023). The therapeutic potential of mAbs is because they are developed via a single clone of B cells, a property that makes them monospecific and homogeneous compared with polyclonal antibodies that are produced in vivo (Castelli et al., 2019). The four types of therapeutic antibodies, depending on the source from which they are produced, are murine, chimeric, humanized, and fully human mAbs (Gupta and Chaudhary, 2022). Human anti-mouse antibodies have been created due to the frequent usage of murine antibodies. In order to lessen the immunogenicity of mouse antibodies, chimeric, humanized, and totally human antibodies have been developed (Gupta and Chaudhary, 2022). Depending on how they are utilized, there are various therapeutic mAbs types, such as unconjugated (naked), conjugated, and bi- and tri-specific (Behl et al., 2023).

Behl et al., 2023). Conjugated mAbs are linked with) chemotherapy or a radioactive compound. Bispecific mAbs pair two different mAbs and bind to two diverse antigens simultaneously. Meanwhile, tri-specific mAb can interact with three different antigens (Behl et al., 2023). Although conventional antibodies are useful for treating several illnesses (Suzuki et al., 2015), there are still a number of issues that need to be resolved. Particularly, drug resistance and poor stability, because mAbs are glycoproteins, remain to be the main drawbacks (Ioele et al., 2022).

Several structural alterations to their structure have been made using recombinant DNA technology and protein engineering to enhance conventional antibody features (Asaadi et al., 2021). As a result, smaller antibody fragments have been designed, including Fab, single-chain variable fragments (scFvs), mini-, dia-, and tria-bodies, and nanobodies (Nbs), which are also recognized as VHHs (variable domain of heavy-chain-only antibodies (HCAb)) or single-domain antibodies (Figure 1, (B, C, and D)) (Asaadi et al., 2021). Among them, scFvs and nanobodies are the most widespread (Figure 1, (B, and D)) (Asaadi et al., 2021). The scFvs, which are made up of the Ig heavy and light chain variable regions that bind by a peptide linker, were earlier thought to be the smallest antibody fragment with a molecular weight of around 25–35 kDa and the same antigen-binding selectivity as the entire Ig molecule (Figure 1, (B)) (Asaadi et al., 2021, Navabi et al., 2021). However, it was shown that a single variable-like domain can maintain the affinity of an entire antibody molecule via the discovery of the camelid VHH (Hamers-Casterman et al., 1993) and shark variable new antigen receptor (Streltsov et al., 2005). Although scFvs continue to rule the clinic, ten of them have received FDA approval (Asaadi et al., 2021); the superior features of Nbs have made them dominant in the field of recombinant antibody engineering in less than three decades since the discovery of HCAb (Asaadi et al., 2021).

Nbs were discovered in 1993 when Hamers et al. revealed for the first time that camelids such as llamas, alpacas, and camels naturally have HCAbs that lack light chains (Figure 1, (C, and D)) (Pillay and Muyldermans, 2021, Hamers-Casterman et al., 1993). Although single-domain antibodies were also detected in cartilaginous fish, camelids were used in most single-domain antibody biotechnological applications studies since they are easier to handle and immunize (Siontorou, 2013). In 2018, Caplacizumab received approval from the European Medicines Agency for treating patients with thrombotic thrombocytopenic purpura and became the first nanobody drug to be marketed (Duggan, 2018). Another nanobody called Ozoralizumab was approved in Japan in September 2022 to manage rheumatoid arthritis that is not adequately controlled by currently existing therapies (Keam, 2023). Several Nb-based therapeutic candidates, such as Sonelokimab and Gontivimab, are undergoing clinical studies and waiting for FDA approval (Asaadi et al., 2021).

Since research on therapeutic Nbs has dominated drug discovery and development lately (Lu et al., 2020), this study conducted a thorough review focusing on the latest studies that discussed the characteristics, production, and potential indications of Nbs to highlight the future directions of these novel agents.

2. METHOD

Literature electronic search was conducted using the most popular search engines: Google Scholar, PubMed, Scopus, and Science Direct, and the following keywords have been used: Nb, imaging, diagnosis, single-domain antibodies, VHHs, and indications of Nbs. The most recent research that met the following selection criteria was included in this review: (1) written in English and (2) discussed the features, production, or therapeutic potential of Nbs. Studies that have not explored the Nbs were excluded.

3. RESULTS AND DISCUSSION

3.1. Nbs characteristics and production

The primary characteristic of Nbs lies in their simplified structure, consisting of a sole variable domain encompassing the antigen-binding region. This key attribute results in their notably smaller size with a molecular weight of only 12–15 kDa compared to conventional antibodies that have a molecular weight of around 150 kDa, granting Nbs the unique capacity to access intricate or concealed target sites that may remain elusive to larger antibody counterparts (Figure 1, (A, and D)) (Muyldermans, 2013, Abbady et al., 2012, Wang et al., 2021). In addition, their compact size offers several benefits, including heightened resilience against extreme conditions like temperature fluctuations and pH variations (Muyldermans, 2013, Abbady et al., 2012). Significantly, Nbs exhibit remarkable binding affinity and specificity towards their target antigens, often surpassing the performance of traditional antibody molecules. Their solubility propensity reduces aggregation risk and facilitates simplified manufacturing processes. Moreover, Nbs' exceptional ability to penetrate tissues underscores their potential for diagnostic and therapeutic applications, and their amenability to genetic

manipulation allows for tailored enhancements) of their properties. Nbs also demonstrate reduced immunogenicity, minimizing the likelihood of undesirable immune responses (Pillay and Muyldermans, 2021, Tang et al., 2023). Despite the aforementioned Nbs advantages, they have some drawbacks, including short serum half-life since they are rapidly cleared by the kidneys due to their small size (Jovčevska and Muyldermans, 2020); however, multimeric Nbs, pegylated Nbs, or Nbs that bind to serum albumin can be manufactured to enhance their half-life in blood (Siontorou, 2013, Jovčevska and Muyldermans, 2020). In addition, it was believed that unmodified Nbs cannot permeate via the cell membrane (Beghein and Gettemans, 2017). Nevertheless, Singh et al. has recently described an unmodified cell penetrating Nb, SBT-100, that enters the cell membrane and gives a therapeutic effect against cancers. Further investigations are being conducted (Singh et al., 2022b). Nbs are typically acquired from immunological libraries created by animal immunization and involve the administration of the appropriate antigen through animal injection (Figure 2) (Muyldermans, 2021b). Naïve libraries, derived from the blood of non-immunized camelids, are also used in Nbs production. However, in recent years, synthetic libraries of various forms have gained traction as trustworthy Nb sources, delivering cost and speed advantages (Contreras et al., 2023). A synthetic Nb library is defined by two main features: framework selection and the construction of complementarity-determining regions (Contreras et al., 2023). A healthy young adult camelid is inoculated for an immune library with 50 to 200 μg of the selected antigen per injection, depending on antigen features. For two months, the animal must be injected with four to eight shots in total (Muyldermans, 2021b). After immunization, B-lymphocytes are isolated from the peripheral blood or lymph nodes of the immunized animal and are then used to extract mRNA (Muyldermans, 2021b). After using the mRNA for reverse transcription to create complementary DNA and polymerase chain reaction (PCR) with VHH-specific primers to amplify the VHH gene area, the nanobody sequences are chosen by phage display or any display method such as ribosome, yeast, and bacterial displays, or NestLink-based selection (Muyldermans, 2021b). Phage display is the oldest and still the most durable of these selection technologies (Muyldermans, 2021b). The chosen nanobodies are recombinantly expressed at the industrial level in either eukaryotic or prokaryotic systems, including mammalian cells, bacteria such as Escherichia coli, or yeasts such as Pichia pastoris (de Marco, 2020, Jin et al., 2023). Finally, it is extracted and purified (Figure 2) (de Marco, 2020).

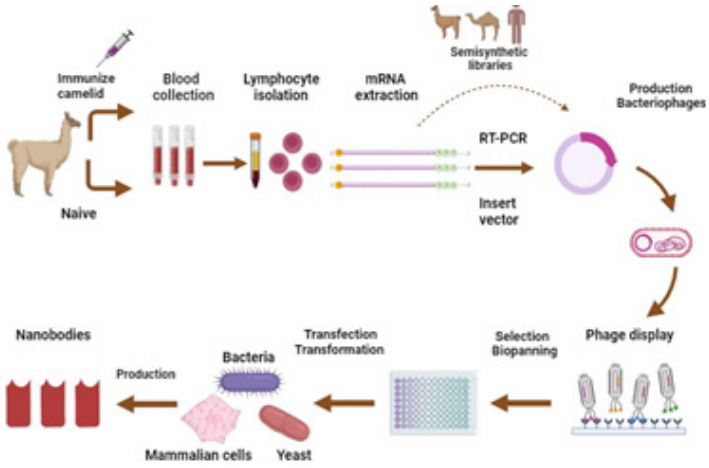

Figure 2: Representation of nanobody production procedure

3.2. Nbs characteristics and production

Nbs emerge as a promising and versatile molecular tool applicable across diverse domains such as imaging, diagnostics, therapeutics, and research, with their unique attributes distinguishing them from conventional antibody formats and other antibody fragments (Pillay and Muyldermans, 2021, Tang et al., 2023).

3.2.1. Nbs for Imaging

Nbs have lately been considered incredible tools for confocal and high-resolution imaging due to their small size, reducing the distance between the probe and the antigen (Harmand et al., 2021). Nbs enable acquiring early high-quality images that allow personalized precision therapy by providing a total estimation of diseases such as cancer and many other diseases (Bao et al., 2021). A molecular tracer compositing of targeting integrity and a detection label are required to achieve the direction and specific accumulation of the probe and allow the visualization of the target tissue, respectively (Küppers et al., 2021). Therefore, Nbs must be supplied with appropriate radioisotope or fluorescent dye (Küppers et al., 2021). Numerous labeling methods of Nbs have been applied to enable in vivo imaging including radio-labeling for single photon emission computed tomography (SPECT) and PET imaging (Harmand et al., 2021). For nuclear imaging uses, positron-emitting isotopes are applied for PET, whereas a gamma-emitting isotope is employed for SPECT (Küppers et al., 2021). Several approaches have been used for radiolabeling of Nbs, including direct and indirect radiohalogenation and radiometals using synthetic or proteinogenic chelators (Küppers et al., 2021). In addition to radiolabeling of Nbs, fluorescent dye labeling of the Nbs has also been used for near-infrared or infrared region imaging (Harmand et al., 2021).

3.2.1.1. Nbs for imaging in cancer

Nbs have been used for imaging cancer cell markers (Xavier et al., 2016). For instance, a modern PET probe, anti-HER2 Nb labeled with 18F for imaging HER2 overexpression in cancer cells, has been shown to have great potential for clinical translation (Xavier et al., 2016). The bi-paratopic Nb construct (MSB0010853) is another example of using Nbs for imaging cancer cell markers, but this complex was formulated to target HER3 (Warnders et al., 2017). It consists of three domains, two of them are directed against the HER3 receptor, and the third is developed to bind to albumin, resulting in extending its serum half-life (Warnders et al., 2017). This Nb was radiolabeled with 89Zr (Warnders et al., 2017). It has shown potential to be employed for both therapeutic and imaging applications in cancer (Warnders et al., 2017). NJB2 is another Nb that was designated specifically to target the Fibronectin-EIIIB splice variant expressed in the extracellular matrix of cancer and fibrosis (Jailkhani et al., 2019). They proposed that NJB2 can be used as a powerful tool for the non-invasive detection and targeting of tumors and other metastatic lesions (Jailkhani et al., 2019).

Besides using Nbs for imaging cancer cell markers, they have also been used for imaging immune checkpoint markers. For example, an Nb-based probe is a potential agent for PET imaging for future clinical measurement of expression of human programmed death-ligand 1, an immune checkpoint, by PET imaging (Bridoux et al., 2020). Envafolimab is a novel anti-programmed death ligand 1 Nb (Li et al., 2018a, Li et al., 2019, Berland et al., 2021). It has lately been used for immuno-PET imaging with 89Zr-Df-KN035 and is considered a powerful tool for non-invasive in vivo evaluation of programmed death-ligand 1 expression in non-small-cell lung cancer and other tumors (Li et al., 2018a, Li et al., 2019). A cytotoxic T lymphocyte antigen 4 specific Nb fluorescent carbon quantum dots complex (QDs Nb36) has currently been designed to detect the cytotoxic T lymphocyte antigen 4 positives T cell, cytotoxic T lymphocyte antigen is a checkpoint inhibitory molecule via flow cytometry or immunofluorescent staining (Wang et al., 2019b). Lymphocyte-activation gene 3 is another frequent target in immune checkpoint inhibition (Lecocq et al., 2019). Most recently, Nbs have been developed and radiolabeled to non-invasively measure lymphocyte-activation gene 3 expression by SPECT/CT imaging in tumor-bearing mice (Lecocq et al., 2019). They have demonstrated that 99mTc-labeled Nb is an excellent SPECT probe to assess lymphocyte-activation gene 3 on immune cells and tumor cells (Lecocq et al., 2019). They later assessed the ability of Nb to image lymphocyte-activation gene 3 on tumor-infiltrating lymphocytes by SPECT/CT in various tumor-bearing mice (Lecocq et al., 2021). Their findings suggest that Nbs could be used to detect the lymphocyte-activation gene 3 upregulation of tumor-infiltrating lymphocytes non-invasively (Lecocq et al., 2021). A follow-up trial in tumor-carrying mice treated with anti-PD-1 antibodies concluded that Nb-lymphocyte-activation gene 3 imaging could have prognostic value and could be used to predict therapy outcomes both before and during therapy (Lecocq et al., 2021).

Nbs have also been used to target immune markers to image immune responses by imaging particular subgroups of immune cells, such as T cells and macrophages (Berland et al., 2021). This can help in predicting the response to treatment. In addition, tracking the infiltration of these immune cells would be beneficial in detecting tumors (Berland et al., 2021). An anti-CD8 Nb was designed and radiolabeled with 89Zr to image the animals non-invasively, with PET showing clearly the lymphoid organs (Rashidian et al., 2017). A pegylated moiety was attached to this radiolabeled Nb, which enhanced signal-to-noise and reduced kidney absorption (Rashidian et al., 2017). The above-mentioned 89Zr labeled Nb has been later used to investigate the dynamics of the CD8+ T cells and CD11b+ myeloid cells in response to anti-PD-1 therapy in the MC38 colorectal mouse adenocarcinoma model (Rashidian et al., 2019). They demonstrated that non-invasive immuno-PET imaging can track intratumoral CD8+ T cells even when their specificity is unknown and track the development of the anticancer response to anti-PD-1 medication (Rashidian et al., 2019). These findings may help specialists to make better choices regarding the patients' treatment options (Rashidian et al., 2019). Newly, high radiochemical purity and affinity Nb has been designed to target human CD8 antigen (Zhao et al., 2021). This novel Nb (68Ga-NOTA-SNA006a) has shown a long retention period, low background, and impressive tumor-to-background ratios compared with other Nbs, allowing precisely gauging the human CD8 positive T cells in mice models, demonstrating the excellent ability for immunotherapy observations and effectiveness measurement (Zhao et al., 2021).

has undergone a phase I clinical study and shown high safety and tolerability and allowed high contrast to noise imaging at 1:30 hours after injection (Gondry et al., 2023). This Nbs will be moved to a phase II clinical study (Gondry et al., 2023).

3.2.1.2. Nbs for imaging of non-malignant diseases

Nbs have also been designed to image the biomarkers that are overexpressed in other diseases, such as atherosclerosis, fibrosis, arthritis, and some neurodegenerative disorders (Berland et al., 2021). For example, the expression level of vascular cell adhesion molecule-1 has been detected both in mouse models of atherosclerosis and in ex vivo human endarterectomy specimens via anti-vascular cell adhesion molecule-1 Nbs that is linked to microbubbles (Punjabi et al., 2019). This complex is translatable into the clinic and could enhance risk stratification for atherosclerosis (Punjabi et al., 2019). The aggregation of α-synuclein protein underlies the development of several neurodegenerative disorders (Iljina et al., 2017). Therefore, two anti-α-synuclein Nbs were successfully designed to link to the C-terminal domain of α-synuclein (Iljina et al., 2017, Gerdes et al., 2020). These anti-α-synuclein Nbs also hold promise in the diagnosis and management plans of neurodegenerative diseases (Iljina et al., 2017). Another study has demonstrated that 64Cu-anti-EIIIB Nb has the promise to image and detect pulmonary fibrosis in a clear, non-invasive, and specific way (Jailkhani et al., 2019). Moreover, a 99mTc-labeled anti-DPP6 Nb successfully detects high amounts of EndoC-βH1 cells or human islets grafted in severe combined immunodeficient mice. Moving this Nb to the clinic may enable non-invasive, in vivo monitoring of diabetic management progress (Demine et al., 2020).

Overall, Nbs have demonstrated great success as imaging tools in cancers and non-malignant diseases.

3.2.2. Nbs for diagnosis

The adaptability of Nbs in diagnostics has drawn interest (Jin et al., 2023). They are promising for a variety of diagnostic systems in both in vivo and in vitro experiments due to their compact structure, stability, and strong binding to target antigens (Jin et al., 2023, Muyldermans, 2021a). Additionally, Nbs are made specifically for screening chemicals and pesticides in food, to ensure safety (Muyldermans, 2021a).

3.2.2.1. Nbs for in vitro diagnosis

Nbs have gained prominence in in vitro diagnostictests, particularly in lateral flow immunoassays (LFIA) and enzyme-linked immunosorbent assays (ELISA) (Jin et al., 2023). LFIA employs Nbs to swiftly detect specific antigens, forming a visual “sandwich” at the test line (Koczula and Gallotta, 2016). Nbs’ stability, broad binding capabilities, absence of a cross-reactive Fc region, multivalency, and affinity for gold nanoparticles enhance LFIA’s sensitivity (De Genst et al., 2006, Mohseni et al., 2018, Pinto Torres et al., 2018, Vincke et al., 2012, Goossens et al., 2017). The Cellular and Molecular Immunology research unit has significantly advanced LFIA development. Notably, they have successfully detected Trypanosoma congolense pyruvate kinase, a glycolytic enzyme, potentially diagnosing active trypanosomosis infections (Pinto Torres et al., 2018). This involves generating Nbs against Trypanosoma congolense secretome, creating a sandwich ELISA, and adapting it to LFIA, demonstrating high sensitivity (80%) and specificity (92%) (Pinto Torres et al., 2018). Similar strategies extend to Nbs targeting glycolytic enzymes enolase and aldolase, enhancing Trypanosoma evansi and Trypanosoma congolense diagnosis (Li et al., 2020, Odongo et al., 2016, Pinto et al., 2017). Nb-based sandwich ELISAs also exhibit promise across various antigens, such as cancer biomarkers, toxins, and bacteria, expanding diagnostic possibilities (Jin et al., 2023) and a reported competitive Nbs-based ELISA for diagnosing foot and mouth disease antibodies in cattle (Gelkop et al., 2018).

Nbs perform well as bioreceptors in biosensors, anchoring onto platforms like nanoparticles or semiconductors. This biosensing approach involves Nbs binding to target molecules, triggering electric potential changes converted into measurable signals (Katz, 2021). This rapid response enables real-time detection, surpassing traditional methods like ELISAs and LFIAs (Jin et al., 2023). For instance, Nbs were applied in a fibrinogen-detecting biosensor, a cardinal cardiovascular disease biomarker (Campuzano et al., 2014). Nbs also show promise as biosensors for identifying spike proteins of SARS-CoV-2 and Middle East respiratory syndrome coronavirus (Guo et al., 2021).

3.2.2.2. Nbs for in vivo diagnostic imaging

In vitro diagnostics are crucial for disease detection, yet their utility is limited,

especially for localized abnormalities in cases like cancer within bodily fluids (Jin et al., 2023). In contrast, fluorescent or radioactively labeled antibodies are used in in vivo diagnostics, such as optical and nuclear imaging, to detect abnormalities like tumors, as aforementioned. Due to their size, ease of modification, and quick clearing, Nbs are an ideal option for making target-specific tracers (Jin et al., 2023). Although optical imaging may not be as accurate as nuclear imaging, it is safer and provides data immediately (Noltes et al., 2021). A fluorescent Nb named 7D12 demonstrated a quicker and deeper response than the commercial mAb Cetuximab, indicating potential for optical imaging (Oliveira et al., 2012). Nbs against HER2 and carbonic anhydrase IX revealed increased target-to-background ratios for improved tumor diagnosis in a dual tracer method (Kijanka et al., 2016). Nbs benefit cardiovascular molecular imaging due to their lack of an Fc region and quick clearance, as demonstrated by a 99mTc-labeled Nb against vascular cell adhesion molecule-1, a biomarker linked to susceptible atherosclerotic plaques (Broisat et al., 2012, Broisat et al., 2014). In the field of nuclear imaging (PET/SPECT), Nbs as radioactive tracers against HER2 exhibit high potential (Noltes et al., 2021). For instance, 68Ga conjugate Nbs against HER2 showed high-contrast PET imaging results (Xavier et al., 2013). Thus, this promising Nb is undergoing phase 2 trials, which are expected to be completed by 2023 (Keyaerts et al., 2019). Moreover, radioactively labeled anti-HER2 Nbs like 18F and 177Lu show promise in detecting biomarkers not only in cancer but also in other conditions (Xavier et al., 2016, Jin et al., 2023, D'Huyvetter et al., 2014). Nbs' size, clearance, and ease of functionalization position them as excellent candidates for both in vitro and in vivo imaging, advancing disease diagnosis (Jin et al., 2023).

3.2.3. Nbs for oncology

Several strategies have been employed to improve the efficacy and targeting of anticancer agents in recent years (Najjar et al., 2017). Nbs' outstanding properties, such as their small size and ability to penetrate tumors in vivo, enhanced their tumor-targeting ability (Bannas et al., 2017). Compared with traditional antibodies, Nbs possess the same antigen binding features but with a single Ig variable domain for antigen recognition (Verhaar et al., 2021). Therefore, they can reach epitopes that conventional antibodies cannot access, such as cleft on a protein's surface (Verhaar et al., 2021). Anticancer Nbs can be divided into 3 types: naked monomeric or multimeric Nbs, Nbs as targeting moieties to effector domains, and Nbs binding the surface of liposomes or other nanoparticles that encapsulate compounds (Bannas et al., 2017).

Regarding oncology therapy, Nbs have been used to target tumor antigens and they have first been examined as antagonists. For instance, Nbs against epidermal growth factor receptor (EGFR) and c-mesenchymal-epithelial transition factor have been developed (Roovers et al., 2011, Kijanka et al., 2015). They have shown in vitro antagonistic effects for either EGF or hepatocyte growth factor (Kijanka et al., 2015). Tumor growth in vivo was suppressed with trivalent biparatopic anti-EGFR Nb 7D12-9G8-Alb (Roovers et al., 2011, Kijanka et al., 2015). In addition to the antagonistic effect of Nbs, they can be produced as allosteric inhibitors to modify the enzymatic action of their interest protein (Kijanka et al., 2015). On the other hand, when only one ligand is responsible for stimulating the target receptor, Nbs can bind to the ligands directly (Kijanka et al., 2015). Nbs have also been demonstrated to have potential effects in enhancing immune checkpoint inhibitors (Broos et al., 2019, Yang and Shah, 2020). Several trials have developed Nb immune checkpoint inhibitors for programmed death-ligand 1 that can exhibit significant antitumor effects (Broos et al., 2019, Yang and Shah, 2020). It has also shown promising activity in inhibiting angiogenesis, such as recombinant anti-vascular endothelial growth factor bivalent Nb developed with extended plasma half-life properties that holds promise to inhibit angiogenesis within solid cancers (Yang and Shah, 2020, Sadeghi et al., 2020). As demonstrated by these findings, Nbs-based treatment in oncology holds tremendous potential.

3.2.4. Nbs for infectious diseases

Nbs have become a promising therapeutic approach with the potential to revolutionize the treatment of infectious diseases against viruses, bacteria, and parasites by focusing on different agents (Sanaei et al., 2020).

The available anti-viral agents face several drawbacks, particularly poor pharmacokinetics and the development of resistance (Sinokrot et al., 2017).

Therefore, the scientific community consistently investigates strategies to address these problems and create more effective and appealing agents (Sinokrot et al., 2017, Breijyeh and Karaman, 2021, Karaman, 2014). Nbs showed considerable potential in treating viral infections by neutralizing a variety of viruses, such as influenza, respiratory syncytial virus, HIV, and SARS-CoV-2. They disrupted the infection cycle by successfully preventing viral entrance, fusion, and replication (Mei et al., 2022, Sanaei et al., 2020). With their improved affinity and virus-blocking properties, polyvalent Nbs have the potential to treat a variety of infectious diseases and improve global health outcomes (Mei et al., 2022, Sanaei et al., 2020). Nbs that target the virus's receptor-binding domain are quite successful in the context of SARS-CoV-2. The viral entry is facilitated by the interaction of SARS-CoV-2 with the human angiotensin-converting enzyme 2 receptor via the receptor binding domain. Multivalent Nbs targeting the SARS-CoV-2 receptor binding domain bind multiple epitopes simultaneously, countering viral mutation evasion (Xiang et al., 2020). These Nbs can block the interaction, preventing viral invasion and positioning them as prime candidates for immunotherapy (Mei et al., 2022). Notable examples include Nb variants like "VHH-72 Fc fusion" and "VHH-114," which demonstrate strong neutralization potency against multiple SARS-CoV-2 variants (Zupancic et al., 2021, Saied et al., 2022). Beyond SARS-CoV-2, multivalent Nbs show efficacy against infections like HIV and respiratory syncytial virus. Notably, Nb ALX-0171, under clinical trials, targets respiratory syncytial virus and significantly reduces viral load (Detalle et al., 2016). Nbs also hold potential for influenza, Shigella spp., and other pathogens. Multivalent Nbs offer the ability to target pathogens and receptors in vivo simultaneously. For example, Nbs developed against specific proteins on the H5N1 influenza virus have shown remarkable potential in mice (Ibanez et al., 2011). Moreover, Nbs' potential extends to hepatitis C therapy customization and fighting Rift Valley Fever virus and Schmallenberg virus infections (Wichgers Schreur et al., 2020, Kennedy et al., 2018, Li and Lo, 2015, Liver, 2017). Overall, these de novo studies highlighted the significant potential of Nbs in combating viral infectious diseases. Their precise targeting, specificity, and potency lead the way for novel therapeutic and diagnostic strategies, potentially revolutionizing a new approach to viral infections.

In bacterial infections, Nbs have displayed effectiveness by targeting virulence factors essential for the invasion of pathogens like Clostridium difficile, Staphylococcus aureus, and Escherichia coli into host cells. Through their binding, Nbs prevent bacterial attachment, toxin activity, and invasion (Wilken and McPherson, 2018). An important example is Nbs that target Shigella spp.'s Type III secretion system (T3SS), decreasing their hemolytic activity and possibly enabling novel therapeutic approaches against bacillary dysentery (Barta et al., 2017). Furthermore, an Nb has been created to target a bacterial surface antigen as a substitute for antibiotics in the context of treating Streptococcus mutants, the cause of tooth decay (Wilken and McPherson, 2018). However, the combined Nb and glucose oxidase hybrid (S36-VHH-GOx) exhibited a reduction in bacterial levels in infected rats (Wilken and McPherson, 2018). Similarly, efforts to create Nb-based treatments for Neisseria and Escherichia coli-related infections aimed to provide alternatives for antibiotic-resistant individuals (Wilken and McPherson, 2018). The Neisseria-targeting VHH-5G Nb effectively blocked lipopolysaccharide receptor interaction, whereas the treatment targeting Escherichia coli F4 fimbriae did not show success in preventing disease or improving mortality rates in a mouse model (Wilken and McPherson, 2018). On the other hand, Nbs created specifically for Clostridium difficile infections have shown promise in focusing on bacterial toxins and inhibiting receptor connections (Wilken and McPherson, 2018). The investigation of Nbs as a potential therapeutic tool opens up new possibilities for treating bacterial infections in immunocompromized and antibiotic-resistant individuals.

Nbs have been used to treat parasitic infections, including Trypanosoma brucei and Plasmodium species. Researchers designed Rift Valley fever virus and Schmallenberg virus-specific Nbs, which effectively neutralized the respective viruses, reducing morbidity and mortality in mice (Wichgers Schreur et al., 2020). In particular, Nbs targeting the Duffy antigen receptor for chemokines inhibited the binding of Plasmodium vivax and Plasmodium knowlesi to human erythrocytes (Smolarek et al., 2010). For Trypanosomes causing sleeping sickness and Chagas disease,

Nbs targeting variable surface glycoproteins have been developed, including NbAn33 for parasite detection and cross-reactive Nb392 for diagnosing trypanosomiasis by targeting the paraflagellar rod protein of Trypanosoma brucei (Stijlemans et al., (2011, Obishakin et al., 2014).

In conclusion, multivalent Nbs offer a powerful tool for combating infectious diseases, with diverse strategies to enhance their affinity, neutralization, and therapeutic potential. While challenges remain in designing optimal Nb combinations and forms, ongoing research holds promise for expanding their applications and impact.

3.2.5. Nbs for metabolic disorders

Nbs are increasingly offering novel therapeutic and diagnostic possibilities for complex disorders in the field of cardiovascular research, such as atherosclerosis, coronary artery disease, and thrombosis (Bocancia-Mateescu et al., 2023). As part of the treatment of heart conditions, Nbs are being explored for targeting amyloidosis-related cardiotoxicity, inhibiting ryanodine receptor 2 phosphorylation to mitigate cardiac dysfunction, and inhibiting G-protein coupled receptors to regulate hypertension. Furthermore, plasminogen activator inhibitor-1 (PAI-1), controls blood clot formation through its crucial role in plasminogen activation (Broggini et al., 2022, Wingler and Feld, 2022, McMahon et al., 2020, Li et al., 2018b, Sillen and Declerck, 2020). A high plasminogen activator inhibitor-1 level increases the risk of developing thrombosis and cardiovascular diseases such as heart attacks and strokes. Plasminogen activator inhibitor-1 activity is inhibited by Nb called VHH-2w-93. Ultimately, this will reduce the excessive clotting and the associated cardiac complications (Broggini et al., 2022, Wingler and Feld, 2022, McMahon et al., 2020, Li et al., 2018b, Sillen and Declerck, 2020). In addition, Nbs may enhance heart function by optimizing calcium ion balance in heart muscle cells by influencing sarco-endoplasmic reticulum Ca2+-ATPase and phospholamban levels (De Genst et al., 2022). Anti-von Willebrand factor bivalent Nbs, such as Caplacizumab, show potential for disrupting von Willebrand factor activity and platelet aggregation in thrombotic thrombocytopenic purpura (Callewaert et al., 2012). It has received approval from the European Medicines Agency and the US FDA for the management of patients with thrombotic thrombocytopenic purpura (Jovčevska and Muyldermans, 2020). Furthermore, Nbs targeting matrix metalloproteinase-2 and glycoprotein VI exhibit potential in inhibiting platelet aggregation and thrombus formation, indicating their potential in anti-thrombotic therapy (Marturano et al., 2020, Jooss et al., 2022). In general, Nbs hold immense promise for revolutionizing diagnostic approaches and unveiling new therapeutic avenues for addressing a range of cardiac challenges (Bocancia-Mateescu et al., 2023).

3.2.6. Nbs for neurological disorders

Nbs hold promise as therapeutic agents for neurological disorders due to their ability to cross the blood-brain barrier (BBB) and target central nervous system (CNS) antigens (Jovčevska and Muyldermans, 2020). Nbs' small size and specificity address the challenge of BBB delivery, offering the potential for conditions like Alzheimer's and Parkinson's diseases (De Genst et al., 2022). Methods like attaching BBB-crossing peptides and receptor-mediated transcytosis show potential for targeted drug delivery (De Genst et al., 2022). Additionally, the "Trojan horse" strategy, utilizing Nbs to target BBB receptors, extends their potential (De Genst et al., 2022).

3.2.6.1. Nbs for Alzheimer's disease

Nbs hold significant promise for diagnosing and treating Alzheimer's disease. They hold the potential for detecting Alzheimer's disease biomarkers in body fluids, such as cerebrospinal fluid, through simple ELISA tests (Knopman et al., 2021), and identifying early Alzheimer's disease changes in the retina (Habiba et al., 2021). Nbs also present a targeted strategy for addressing Alzheimer's disease pathology, inhibiting amyloid-β and tau aggregation, neutralizing toxic amyloid-β oligomers, and even directly degrading amyloid-β. These Nbs can also be fused with therapeutic molecules for precise brain delivery (Danis et al., 2022, Marino et al., 2022, Marino and Holt, 2022). Additionally, Nbs hold therapeutic potential for tau pathology, with Nbs like VHH Z70 effectively inhibiting tau aggregation. Nbs' delivery into the brain via viral vectors like adenoviruses offers a gene therapy potential for Alzheimer's disease (Danis et al., 2022, Marino et al., 2022, Marino and Holt, 2022). Furthermore, a new investigation introduces a novel therapeutic strategy in the form of a multivalent Nb conjugate equipped with a robust scaffold capable of scavenging reactive oxygen species (Zhao et al., 2023).

By integrating an amyloid-β segment into an Nb, it recognizes and inhibits amyloid-β aggregation. Further enhancements involve modifying the Nb with a human interleukin-1β fragment to aid amyloid-β clearance (Zhao et al., 2023). The resulting conjugate shows significantly improved binding to amyloid-β aggregates and long-term capabilities in reducing oxidative stress and inflammation. Notably, this conjugate mitigates symptoms in Alzheimer's mouse models, validating its potential as a multi-target therapeutic approach (Zhao et al., 2023). Although some Nbs showed promise in preclinical studies, further research is crucial to establish their effectiveness and safety in clinical applications. Nonetheless, Nbs' distinct characteristics make them promise for innovative Alzheimer's diagnosis and treatment methods.

3.2.6.2. Nbs for Parkinson's disease

Nbs have demonstrated significant potential for both diagnosing and treating Parkinson's disease (Bloem et al., 2021). Nbs have the potential to target Parkinson's disease-related proteins such as Nbsyn2, Nbsyn87, and Nbalpha-syn01, which have shown promise in inhibiting α-synuclein aggregation and toxicity (Vuchelen et al., 2009, De Genst et al., 2010, Guilliams et al., 2013, El-Turk et al., 2016, Hmila et al., 2022). Additionally, Nbs targeting other Parkinson's disease-related proteins like Leucine-rich repeat kinase 2 have been developed as a potential therapeutic option (Singh et al., 2022a). In summary, Nbs hold great potential for both diagnostic and therapeutic applications in Parkinson's disease, particularly in targeting α-synuclein and other related proteins. Further research and clinical studies are needed to fully realize their potential in improving Parkinson's disease diagnosis and treatment (Zheng et al., 2022).

3.2.6.3. Nbs for Multiple sclerosis

Multiple sclerosis is a demyelinating disease that involves inflammatory responses, potentially linked to genetic, environmental, and infectious factors (Zheng et al., 2022). Nbs have been recently evaluated as a potential therapeutic option for multiple sclerosis. Studies reported that Nbs such as anti-tumor necrosis factor receptor 1 Nb and 3Nb12 Nb that are targeting proteins like tumor necrosis factor receptor 1 and C-X-C motif chemokine ligand 10, respectively, have demonstrated anti-inflammatory effects in experimental models. These Nbs showed promise in reducing neuroinflammation and preserving myelin and neurons (Steeland et al., 2015, Steeland et al., 2017, Sadeghian-Rizi et al., 2019).

3.2.6.4. Nbs for Brain tumor

The therapeutic effects of Nbs in brain tumor therapy is a promising and rapidly developing field due to their small size, which allows them to cross the BBB and selectively target tumor cells (Zheng et al., 2022). Conjugating Nbs with therapeutic agents enables precise drug delivery, minimizing damage to healthy brain tissue. Nbs can also be engineered to inhibit tumor growth by targeting specific signaling pathways or antigens. While early preclinical studies show promise, further research and clinical trials are needed to fully explore Nbs' potential in brain tumor treatment (Zheng et al., 2022).

Glioblastomas often exhibit amplification of the EGFR gene, playing a crucial role in their development (van de Water et al., 2012). Nbs targeting alpha EGFR have been developed to inhibit EGF-induced signaling and cell proliferation in vitro (Figarella-Branger et al., 2022). Combination strategies involving Nbs specific to EGFR and a pro-apoptotic molecule TNF-related apoptosis-inducing ligand delivered by neural stem cells effectively reduced glioblastoma growth and invasiveness in laboratory and animal studies (van de Water et al., 2012, Jovčevska and Muyldermans, 2020). Brain metastases, originating from primary tumors in other body parts, present significant clinical challenges (Ellingson et al., 2015). Nbs offer new approaches, such as using them for imaging and therapy in brain metastases originating from HER2-positive breast cancer. Nbs like 2Rs15d, an Nb targeting HER2 labeled with radioactive isotopes, enable improved imaging and treatment outcomes. Nbs-based innovations in liposomal systems have the potential to improve the identification of brain metastases and treatment outcomes by overcoming obstacles, including drug resistance and BBB penetration (Zhou et al., 2017, Vaneycken et al., 2011).

3.2.6.5. Nbs for infectious disease affecting CNS

Nbs are rarely employed in CNS infectious diseases, but the latest studies have shown promising outcomes in this field (Kulkarni et al., 2020). Notably, Nbs VHHG9 and VHHF3 were designed to target Neisseria adhesin A,

which can disrupt the interaction with its cell receptors. These Nbs reduced Neisseria meningitidis adhesion to human microvascular endothelial cells and hindered its BBB penetration in vitro, offering a potential treatment approach (Kulkarni et al., 2020). Additionally, for diseases like rabies, an infectious disease that primarily affects the CNS, Nbs have gained attention. They offer a cost-effective solution with high affinity. Novel bispecific Nbs extend albumin's bloodstream longevity, potentially enhancing post-exposure vaccine prophylaxis (Terryn et al., 2014, Terryn et al., 2016).

3.2.6.6. Nbs for other neurological disorders

Nbs hold potential benefits for other neurodegenerative disorders, including Huntington's disease. This disease is caused by genetic mutations leading to abnormal Huntingtin protein accumulation in nerve cells. In 2015, the first Nb targeting the N-terminal domain of the Huntingtin protein was developed, showing strong affinity for both mutant and wild-type forms of the protein (Schut et al., 2015, Colby et al., 2004). Considering the intracellular aggregation of the Huntingtin protein, Nbs used as intrabodies could be a promising avenue for development, with early evidence of feasibility from other sources of intrabodies (Schut et al., 2015, Colby et al., 2004).

Overall, Nbs offer a versatile and promising approach to diagnosing and treating neurological disorders. However, further studies are needed to fully validate their safety and effectiveness in clinical applications.

3.2.6.7. Nbs for other disorders

Nbs are expanding their therapeutic applications in fields like ophthalmic, genetic, immune-mediated, and other diseases (Wang et al., 2022, Beltran et al., 2023, Liu et al., 2023). In ophthalmologic conditions, their unique features reported promise in targeting ocular components like vascular endothelial growth factor and immune-related elements for treating retinal diseases and neovascular disorders (Beltran et al., 2023). In fungal keratitis, a severe corneal infection, a specialized Nb targeting β-glucan, effectively disrupted fungal growth when combined with natamycin, surpassing traditional treatments in safety and efficacy (Liu et al., 2023). Nbs also demonstrated potential in treating autosomal dominant retinitis pigmentosa, stabilizing P23H mutant rhodopsin proteins, and presenting a novel therapeutic approach for this genetic eye condition (Wu et al., 2022).

In immune-mediated disorders such as psoriasis, inflammatory bowel disease, spondyloarthritis, and knee osteoarthritis Nbs show potential in targeting inflammation and key molecules (Wang et al., 2022, Navarro-Compán et al., 2022). For instance, in spondyloarthritis, studies indicate their potential to target interleukin-17 (e.g. Sonelokimab) and TNF, offers effective therapeutic options (Navarro-Compán et al., 2022). This trivalent novel Nb, Sonelokimab, exhibited protective effects against cartilage degradation and aggrecan turnover in knee osteoarthritis, as shown in preliminary results from a phase I study (Li et al., 2023). Moreover, Sonelokimab has shown promising safety and effectiveness in other immune-mediated disorders, such as psoriasis (Wang et al., 2022). On the other hand, Ozoralizumab is a trivalent bispecific albumin-conjugated Nb that targets TNF-α (Wang et al., 2022). It has currently undergone a Phase III trial as a potential therapy for rheumatoid arthritis (Wang et al., 2022) and was authorized in Japan in September 2022 for the treatment of rheumatoid arthritis that is not adequately controlled by currently existing therapies (Keam, 2023).

In the field of genetic diseases, some researchers focused on voltage-gated sodium channels, which play a role in initiating rapid action potentials in skeletal and cardiac muscles (Bocancia-Mateescu et al., 2023, Srinivasan et al., 2022). Developing Nbs that target voltage-gated sodium channels presents a challenge due to potential cross-reactivity. However, specific Nbs (Nb17 and Nb82) binding with nanomolar affinity to voltage-gated sodium channel 1.4 and voltage-gated sodium channel 1.5 isoforms, crucial for skeletal and cardiac muscles, have been developed, highlighting potential therapeutic methods (Bocancia-Mateescu et al., 2023, Srinivasan et al., 2022).

From naturally occurring HCAb found in camelids in 1993, a particular class of small antibody fragments known as Nbs, VHHs, or single-domain antibodies was created. Their streamlined structure with just one variable domain encompassing the antigen-binding region serves as their primary point of differentiation. Their diminutive size offers a variety of benefits, including the ability to reach intricate or difficult-to-reach target sites that may be inaccessible to larger antibody competitors and enhanced tolerance to harsh environmental conditions, including temperature and pH fluctuations.

This in-depth examination emphasizes how important Nbs' therapeutic potential is in discovering and creating novel medicines.

It emphasizes that Nbs have therapeutic potential in a variety of conditions, including oncology, infectious (viral, bacterial, and parasitic), metabolic, neurological (Alzheimer's, Parkinson's, multiple sclerosis, brain tumor, and other disorders affecting the CNS), ophthalmologic, immune-mediated, and genetic disorders. To fully prove their effectiveness and safety in clinical settings, more study is needed. By providing an up-to-date and comprehensive assessment of the therapeutic indications of Nbs, this study serves as a valuable resource for academics, medical experts, and pharmaceutical industry professionals.

4. CONCLUSION

To conclude, Nbs have recently dominated drug discovery and research. Despite the fact that Nbs and other antibody fragments share the same ability to bind antigens, their unique characteristics—such as their small size—allow them to be more soluble, long-lasting, and capable of penetrating deeper into tissues. Nbs have a promising future ahead of them as versatile molecular tools that may be applied in a range of domains, including imaging, therapies, and diagnosis.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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