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Nanobodies: emerging tools for clinical applications

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Abstract

Since the introduction of heavy-chain antibodies (HcAbs) two decades ago and the explanation of nanobodies, valuable biochemical properties of nanobodies have generated the interest of researchers on their use in diagnosis and therapy of tumor. Various specific nanobodies (VHHs or sdAbs) have been selected from library and detected high affinity with their antigens. The small size of nanobodies (~15 kDa, 4 nm long and 2.5 nm wide) make it easy for them to penetrate the tissue or get through the blood brain barrier as drugs. Furthermore, nanobodies have been offered in conjugates with other effector domains and in drug delivery systems as targeting for tumors. The nanobodies has the potential to make effective biomedical carriers in the fields of research, diagnostics and therapy. In this review we have explained the important advances in the field of nanobodies. Nevertheless, there are many potentials to further develop and improve nanobody-mediated tumor targeting. In the near future, new targets and their corresponding nanobodies must be identified. Currently, some researchers exploited available nanobodies against tumor-specific receptors for delivering drugs or toxins to tumors, therewith reducing nonspecific toxicity to normal cells and lessening side effects. In conclusion, nanobodies as targeting carrier appear to be a promising method of tumor targeting therapy and diagnosis.

***Keywords*:** Nanobodies, tumor targeting, clinical applications

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Introduction

The discovery of monoclonal antibodies has made a renaissance in biology and medical diagnostics research (1-3). However, the impact of this revolutionary technology on the development of therapeutic approaches has witnessed less-than-expected success. There are many reasons for the failure in using monoclonal antibodies as therapeutic agents (4).

Recombinant antibodies provide many advantages over monoclonal antibodies (5). These benefits might be attributed to two important features of recombinant antibodies: a) the ability to introduce the antibody-encoding DNA into microorganisms such as Escherichia coli and subsequent use of genetic engineering and b) using antibody fragments rather than whole immunoglobulin G (IgG) molecules. Antibody fragments are smaller in size and do not contain the Fc

region (Fig.1). Thus, the antibody molecule is inhibited from nonspecific binding to Fc receptors on the cell surface and as a result adverse reactions associated with the Fc region of a whole antibody do not occur (6-8).

Compared with whole antibodies, the exploitation of recombinant antibody fragments is growing rapidly in biomedicine, biotechnology, and pharmaceutical sciences (9). This ever increasing application of antibody fragments in diverse research areas is due to some intrinsic characteristics including efficient tissue penetration, high specificity and affinity for antigen recognition, small size and easy large scale production (10). The expression and purification of recombinant antibodies are performed inexpensively and conveniently through bacterial fermentation. Also, this procedure requires less time in comparison with production of monoclonal and polyclonal antibodies (11-14). Furthermore, recombinant antibodies have higher stability and solubility. Nowadays, recombinant nanobodies routinely serve for research purposes, diagnosis of infectious diseases, and treatment of pathological conditions such as cancer (15,16).

In current article, we present a viewpoint on the development of camelid single-domain antibodies (sdAbs or VHHs, also widely known as nanobodies) since their discovery and discuss the advantages and disadvantages of these unique molecules in various areas of research, industry, and medicine.

Recombinant antibodies

scFv fragments:

scFv fragments constitute a widely used format of recombinant antibody fragments in which VH and VL domains are linked together via a flexible peptide linker. This linker prevents two domains from being disrupted. The amino acid sequence of the linker is mainly composed of glycine and serine residues as well as charged residues such as glutamic acid and lysine scattered throughout the linker sequence (17). Glycine and serin are able to introduce flexibility into the three dimensional structure of the linker and charged amino acids increase its solubility (18).

scFv is only half the size of Fab. Accordingly, scFv has less residence time in off-target sites, is cleared faster from the blood, and penetrates more efficiently into tumors (19).

As scFv fragments are lacking the Fc region, they are less immunogenic and also tend to merge with other peptides and proteins. scFv antibody fragments are genetically stable and their production does not require large scale cell culture systems or lab animals (20). They act without dependence on glycosylation and as a consequence can be expressed actively in bacterial cells that do not have post-translational modification (PTM) system. These antibody fragments are able to bind to a variety of antigens including haptens, proteins, polysaccharides, and the whole pathogens (Fig.2). This makes them appropriate for being used in ELISA assays. Unlike natural immunoglobulins that are secreted by plasma cells and act extracellularly, scFv fragments can be expressed inside the eukaryotic cells (21).

VHH fragments:

In 1993, Harmer et al. discovered a novel class of antibodies in the Camelidae family of organisms. These antibodies were demonstrated to constitute fifty percent of functional antibodies in camel and harbor no light chains (22). Further studies revealed that only three domains exist in the structure of this antibody: two constant domains at the C-terminus that are homologous with terminal constant domains of the human antibody. These constant regions contain large sequences and account for similar functions in both species. The N-terminal variable domain is only composed of heavy chain variable region and is called VHH to be distinguished from known VH domains. The affinity of this antibody is at nanomolar levels reflecting its highly specific binding to the target antigen (Fig.1).

Antibodies without light chains are known as heavy-chain antibody in camel and new antigen receptor antibody in shark. These antibodies with a single-chain variable domain, called VHH in camel and V-NAR in shark, display high affinity for a wide variety of antigens. These short fragments, approximately 13 kDa, can be expressed easily in bacterial and yeast cells and are referred to as domain antibodies, single-domain antibodies or nanobodies (23-25). These fragments show binding specificity and affinity similar to whole antibodies. Also, they are highly stable and easy to produce owing to their small size. Small size makes them capable of penetrating efficiently into tissues and recognizing cryptic antigens (22). Nanobodies are naturally soluble in aqueous environments, do no aggregate and show resistance to high temperature. In addition, they are stable against proteolytic enzymes and pH extremes (27). The stability of nanobodies in the gastrointestinal tract allows them to be used via oral administration. Nanobodies display a high degree of similarity to human-derived antibodies (almost 90 %). Amino acid alteration may increase this similarity to 95-99% (3).

Production of recombinant nanobodies

Currently, recombinant nanobodies are produced by using genetic engineering techniques that is performed mainly through rapid cloning and selection of antigen-specific nanobodies or VHH fragments (28). Within the past decade, cloning of the repertoire of antigen-binding fragments from an immunized animal into a phage vector and subsequent selection of antigen-specific clones have been applied routinely to identify antigen-specific molecules (3,29). At present, the use of Nanoclone technology has allowed researchers to directly clone nanobodies from specific B lymphocytes. Soluble nanobodies may be efficiently produced in bacterial or eukaryotic host cells (30-34).

Therapeutic applications

With recent advancements in the design of antibody structures and methods used for the selection and construction of antibody gene repertoires and libraries, production of recombinant antibody fragments with high specificity and binding affinity for any desired antigen has become a routine and rapid procedure (35-37). After one decade of focused engineering followed by preclinical and clinical trials, recombinant antibody fragments are poised to be added as potent diagnostic and therapeutic agents to the toolbox of clinically valuable antibodies (38). These fragments are receiving increasing attention in tumor targeting, diagnosis and treatment of inflammatory conditions, autoimmune diseases, viral infections, discovery of novel tumor biomarkers, development of sensitive microarrays, and powerful nanosensors. For some clinical applications, small antibody fragments provide several advantages over whole antibodies (Fig.3). Smaller size facilitates their penetration into solid tumors and tissues. In this regard, VHH fragments have been the subject of some investigations (39). In addition, small size accelerates their clearance from the blood and yields difference in selectivity. Bifunctional molecules serve to deliver therapeutic proteins into target cells with on-demand activity. In this context, the therapeutic dose is limited, detrimental side effects on normal tissues are significantly reduced and the body's spontaneous immune response to the protein drug is minimized. Also, physical interaction between target and effector molecule leads to enhanced capacity of the effector molecule. Fused proteins are considered as ideal tools for diagnostic and therapeutic purposes. Bifunctional tumor-specific antibodies are a prime example of such molecules (38-40). These antibodies deliver potent cytotoxic molecules specifically into tumor cells resulting in the death of diseased cells without damage to healthy ones. Like other water-soluble compounds, antibodies cannot cross the blood brain barrier (BBB).

This poses some problems to the antibody-based treatment of neurological disorders (41). By contrast, VHH fragments are able to cross BBB. This valuable feature has been applied to develop efficient and ideal antibody-based delivery vehicles that are capable of transporting macromolecules to nerve cells, thereby treating neurological diseases (42). The hypervariable regions of VHH are longer than VH. Therefore, VHH is able to penetrate deeply into the cavities of VH active site of enzymes and bind to novel epitopes that cannot be recognized by routinely used antibodies. Accordingly, VHH fragments represent potential to be used as enzyme inhibitors (43).

Less amount of the purified antigen is required to produce recombinant antibody fragments. Also, all antigen types cannot be used for the production of monoclonal antibodies (44). However, the generation of highly toxic antibodies or antibodies specific for non-immunogenic antigens – that is not possible by current animal immunization technologies – can be achieved through antibody engineering techniques (45). The target-binding affinity of recombinant antibodies may be raised to levels out of reach of the animal's natural immune system (46). The capacity for large scale production has raised interest in the application of recombinant antibody fragments in proteomics. As these antibody fragments are derived from human or synthetic genes, they do not elicit sever immune responses in patients. This property makes them highly well-matched for translation into the clinic (47-50).

VHH fragments have presented efficacy in crossing BBB and targeting brain epitopes. Therefore, they offer potential to be used for detecting the activity of cellular proteins in vivo (8,51). Compared with routinely used antibodies, VHH fragments possess a higher capacity for penetration into fixed cells. This facilitates the production of anti-idiotypic antibodies for vaccination purposes (52).

Targeting pathogenic organisms

Pathogenic bacteria may be detected and removed from biological specimens by using antibodies against their specific surface antigens (53). The major benefit of antibody fragments for bacterial identification is the lack of the Fc region. Fc is a highly conserved component of IgG and might be recognized by proteins that different bacterial species express. As a result, routine antibodies are not appropriate for specific identification of a distinctive bacterial sub-population within a complex microbial community. However, recombinant antibody fragments can serve as valuable tools for this purpose (54). VHH fragments have been produced with specificity against cell wall protein of the fungus Malassezia furfur, one of the most common causes of dandruff. This VHH fragment can be used as an anti-dandruff agent in shampoos if it can withstand harsh chemical condition of the shampoo formulation. Recombinant antibody fragments can also be exploited for the diagnostic identification of parasitic glycoprotein epitopes in vitro and in vivo. For example, one VHH fragment has been produced against Trypanosoma species that can be applied to develop a fast flow cytometry-based diagnostic system for quantitative determination of pathogen concentration in blood samples (55). Recombinant antibody fragments can also be used, as a low-cost alternative to IgG, for viral infections. These antibody fragments have been shown to be capable of binding to viral proteins involved in interaction with the host cell receptor leading to the inhibition of infection in vivo. Another application of antibody fragments in the treatment of viral infections is the exploitation of intracellular antibodies that are synthesized by the cell and abolish the activity of specific intracellular proteins (56).

Toxin identification and detoxification

Within the recent years, recombinant antibody fragments have received increasing attention for toxin

identification and neutralization (12,57). Routinely used antibodies cannot tolerate harsh environmental conditions such as high temperature, potent detergents, and high concentrations of reducing agents. Recombinant antibody fragments are considered optimal for harsh conditions because their stability can be increased through approaches such as N-glycosylation and introduction of disulfide bonds (58). In this context, antibody fragments have been applied to neutralize the Salmonella-produced toxin SpvB. This toxin is directly secreted from the bacterium to the cytoplasm of the host cell and therefore is not accessible by extracellular antibodies, whereas VHH fragment produced against this toxin can act as a highly specific intrabody. This fragment neutralizes the toxin and inhibits its pathogenic effects on target cells (18,24).

Recombinant antibodies as tools for biosensor development

Biosensor technology that combines the principles of biology and electronics has emerged as a new scientific field. This novel technology may provide an alternative to many routine laboratory techniques in a variety of sciences (59). The sensitivity of a biosensor is dictated by attachment of its biological element(s) to a specific substrate. High specificity and affinity have turned antibodies into ideal biological agents for diagnostic purposes. When using as biosensors, several key characteristics of antibodies deserve further consideration including sensitivity, selectivity, stability, directional immobilization to the surface, the capacity to be labeled, and size (18-21).

Recombinant antibodies are considered as excellent means for optimization of these parameters (60). The structure of antibodies can be modified by using genetic engineering tools leading to improved selectivity, stability, size, and immobilization capacity of recombinant antibodies (14,51). Furthermore, the sensitivity of antibodies may be increased through high throughput screening of ribosome and phage display libraries (43). These libraries, made by recombinant technology methods, provide the possibility to screen very large libraries in search of ligands with high sensitivity. Single-domain antibodies have been used successfully to develop immunosensors for the diagnosis of HIV-1 virions.

Concluding remarks

Recent advancements in genetic engineering and recombinant technology have facilitated DNA manipulation, cloning, and expression of antibody genes in different hosts. Today, antibodies are widely used as therapeutics for the treatment of a variety of diseases. Clinical trials have revealed that mouse-derived monoclonal antibodies suffer from some major limitations for therapeutic purposes. These drawbacks mainly include immunogenicity, distribution in normal tissues, and low penetration into solid tumors. Genetic engineering developments have provided a framework to engineer smaller binding units that both retain the specificity and binding affinity of conventional antibodies and possess less immunogenicity. Nanobodies offer several characteristics turning them into ideal tools for diagnostic and therapeutic purposes both in basic research and in clinical studies. These features include low immunogenicity, high expression capacity in microbial systems, small size, and appropriate biochemical properties such as good solubility, stability, specificity, and affinity for target antigen. Substantial advancements made in antibody engineering and optimization of expression systems have led to significant improvements in the large scale production of antibody fragments for a wide variety of practical areas. Here, we provided an overview of these applications. Without doubt, current situation heralds more progress in the future for extending the scope of nanobody applications to other not-yet-addressed areas.

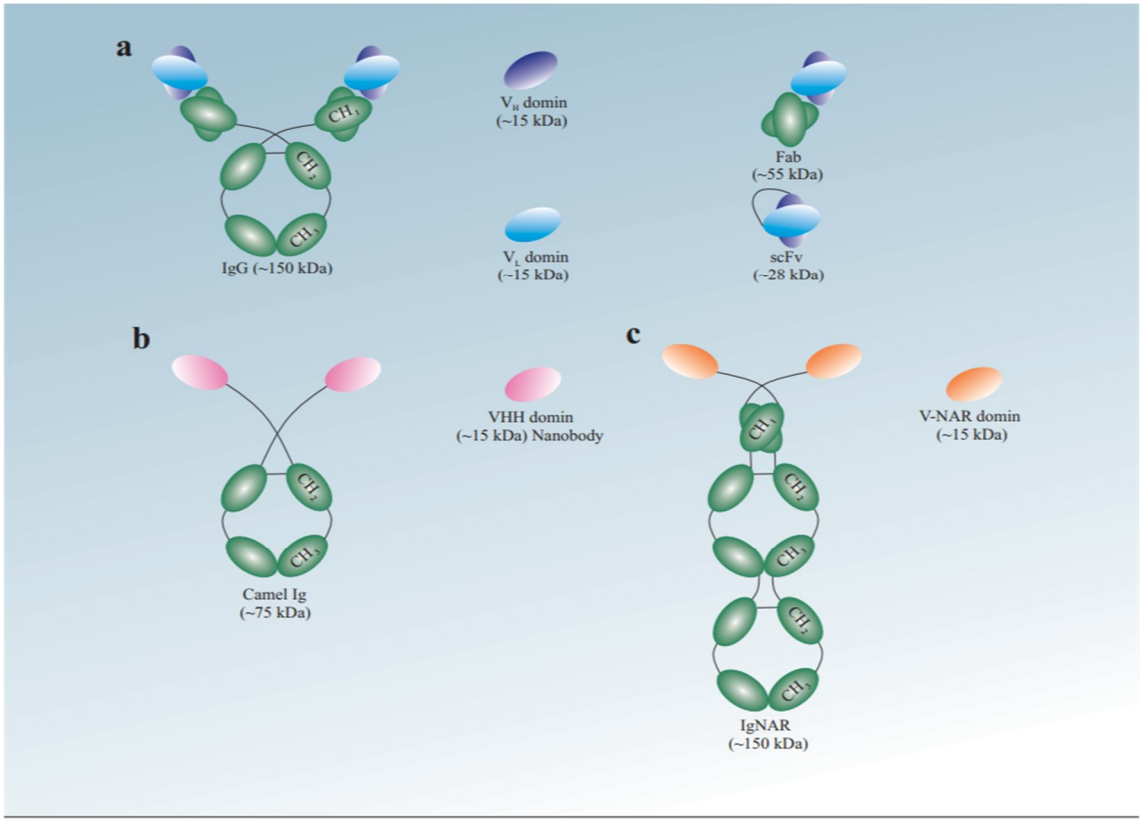

Fig 1. Different types of Antibody fragments. Depiction of a full size antibody and various antibody fragment types. CH, constant heavy chain; CL, constant light chain; IgG, immunoglobulin; Fab, antigen binding fragment; scFv, single chain variable fragment, VH, variable heavy chain; VL, variable light chain.

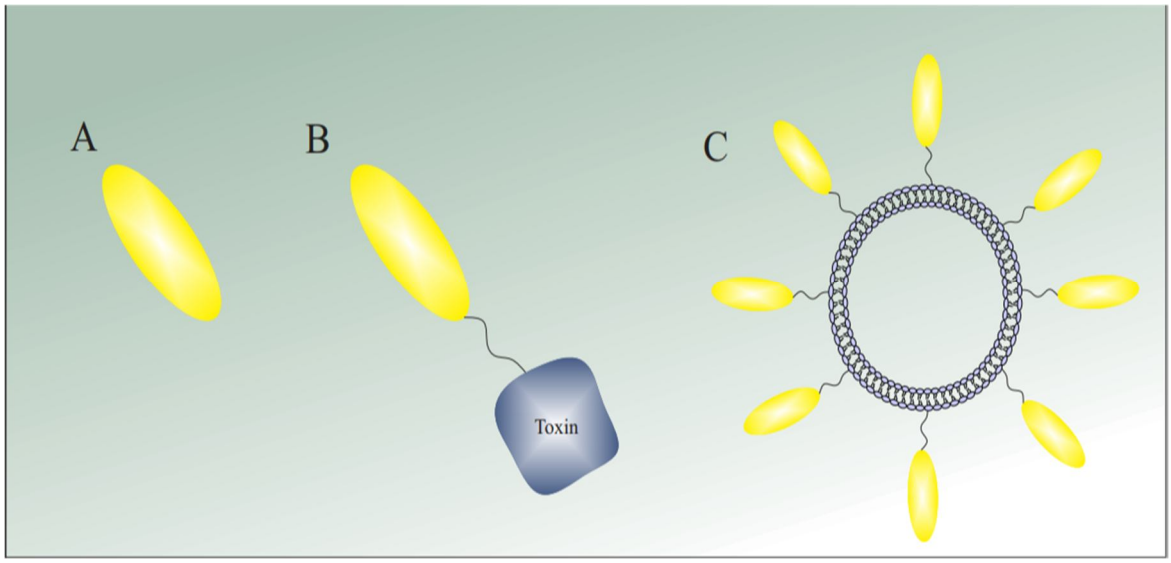

Fig 2. Application of single domain antibody with synthetic vehicles unite toxins, cytokines, etc. It is easy to couple with other molecules, such as Immunotoxin.

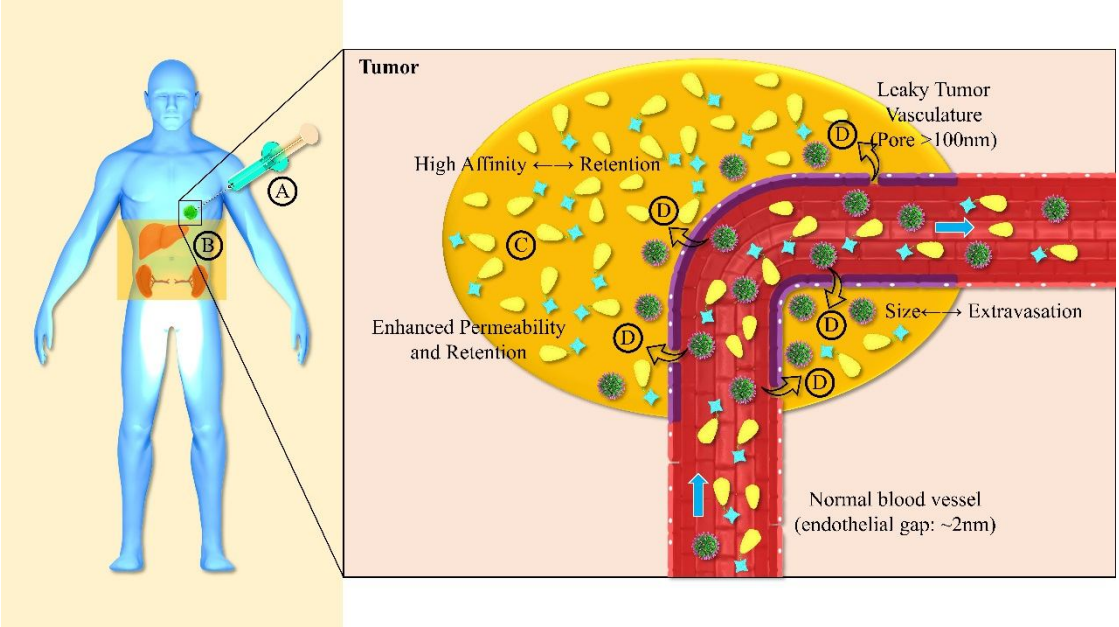

Fig 3. Anti-tumor mechanism of VHH. The small molecular weight of VHH makes it easy to penetrate barriers to achieve the tumor site. The VHH can action as a pure blocker. Moreover, coupled with toxins, it can form nanoparticles in the tumorous region and infiltrate into the organization.

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Conflict of interest disclosure

None of the authors has any conflict of interest to declare.

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