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Review

Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer

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ABSTRACT

Since their discovery, nanobodies have been used extensively in the fields of research, diagnostics and therapy. These antigen binding fragments, originating from *Camelid* heavy-chain antibodies, possess unusual hallmarks in terms of (small) size, stability, solubility and specificity, hence allowing cost-effective production and sometimes outperforming monoclonal antibodies. In this review, we evaluate the current status of nanobodies to study, diagnose, visualize or inhibit cancer-specific proteins and processes. Nanobodies are highly adaptable tools for cancer research as they enable specific modulation of targets, enzymatic and non-enzymatic proteins alike. Molecular imaging studies benefit from the rapid, homogeneous tumor accumulation of nanobodies and their fast blood clearance, permitting previously unattainable fast tumor visualization. Moreover, they are endowed with considerable therapeutic potential as inhibitors of receptor-ligand pairs and deliverers of drugs or drug-loaded nanoparticles towards tumors. More *in vivo* and clinical studies are however eagerly awaited to unleash their full potential.

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1. Introduction

Over the last decades, monoclonal antibodies (mAbs) against cancer-related transmembrane receptors, or their ligands, have found their way to the clinic. mAbs can direct a cytotoxic payload towards tumor cells, or radioactive or fluorescent tracers for PET/SPECT or optical imaging, respectively. Their distribution and tumor penetration are however limited due to mAb dimensions (~150 kDa, 10–15 nm long and 7–9 nm wide). Moreover, their long half-life (ranging from days to up to 4 weeks) accounts for high background levels during molecular imaging. In addition, host immune responses still remain an issue.

The variable fragments of *Camelid* heavy-chain only antibodies (HcAbs), called nanobodies, may provide an answer to several of these concerns (Hamers-Casterman et al., 1993) (Fig. 1A). Nanobody hallmarks include small size (~15 kDa, 4 nm long and 2.5 nm wide), high solubility, stability, specificity and affinity, ease of cloning as well as thermal and chemical resistance. Moreover, recombinant production in microorganisms is very cost-efficient and nanobodies can easily be used as building blocks for multi-domain constructs (Muyldermans, 2013). These advantageous properties arise from their single domain nature and from crucial amino acid mutations in the framework 2 region, rendering the overall structure more hydrophilic compared to conventional antibody fragments (Fig. 1B). Their convex surface and

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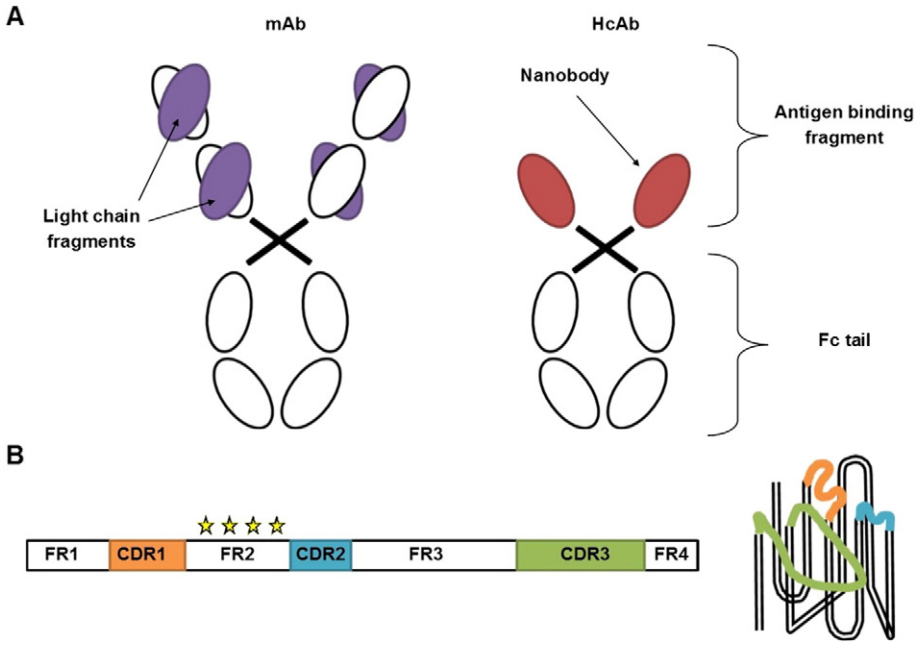

Fig. 1. Representation of a heavy-chain antibody (HcAb) and its antigen binding fragment, called nanobody. A. In contrast to a monoclonal antibody (mAb), which comprises two heavy and two light chains, an HcAb only contains heavy chains. As HcAbs also lack one constant domain, the antigen binding region only consists of a single fragment, called a nanobody. The tail region of the antibodies forms the Fc part and is able to trigger the immune system. B. Schematic representation (left) and conformation (right) of the nanobody entity, composed of framework regions (FR1–4) alternated with three complementary determining regions (CDR1–3). Mutations in FR2 (stars) render the structure more hydrophilic as compared to conventional antibody fragments. Moreover, the CDR3 loop is extended and enables recognition of hidden or buried epitopes.

extended CDR3 loop further enables recognition of cavities or hidden epitopes on the surface of the antigen (Fig. 1B). Combined with the fact that nanobodies are considered to be non-immunogenic due to their high similarity with human VH sequences, these unique properties triggered numerous applications in fundamental research, diagnostics and therapy (De Meyer et al., 2014; Chakravarty et al., 2014; Kijanka et al., 2015; Muyldermans, 2013; Oliveira et al., 2013).

Nanobodies are stable in the reducing cytoplasmic environment and when expressed as an intrabody they can modulate, trace and visualize antigens (Muyldermans, 2013; De Meyer et al., 2014). Moreover, they can serve as biomarker probes and when fused to radionuclides or near-infrared fluorophores they represent ideal non invasive *in vivo* imaging agents (Chakravarty et al., 2014; Oliveira et al., 2013). Therapeutically, they can be utilized as neutralizing agents, as receptor-ligand antagonists and as vehicles for effector delivery or targeted vehicle-based drug therapy (Kijanka et al., 2015; Oliveira et al., 2013). Their development as antagonists of extracellular disease-related targets is currently undergoing phase I, II and III clinical trials by Ablynx, the company of which nanobodies are the trademark (http://www.ablynx.com). Although nanobodies also aid in identifying new interesting intracellular targets, their penetration through the cell membrane remains a problematic issue for therapeutic targeting of cytosolic proteins. In this review, we provide insight into the current status, ongoing developments and future challenges towards successful implementation of nanobodies in the diagnosis and treatment of cancer.

2. Therapeutic Nanobodies Directed Against Extracellular Targets

In addition to 'classical' receptor targets such as EGFR (Roovers et al., 2007,2011; Schmitz et al., 2013; Omidfar et al., 2013), HER2 (Jamnani et al., 2012; Even-Desrumeaux et al., 2012), c-MET (Slordahl et al., 2013) and VEGFR (Behdani et al., 2012), nanobodies against new targets such as the DR5 death receptor (Huet et al., 2014; Papadopoulos et al., 2015) and the chemokine receptors CXCR4 (Jahnichen et al., 2010) and CXCR7 (Maussang et al., 2013; Blanchetot et al., 2013) come into play. Alternatively, nanobodies can be generated against the cognate receptor ligands, such as HGF (for c-MET) (Vosjan et al., 2012), VEGF (for VEGFR) (Kazemi-Lomedasht et al., 2015; Ebrahimizadeh et al., 2015; Farajpour et al., 2014), uPA (for uPAR) (Kaczmarek and Skottrup, 2015) or CXCL11/12 (for CXCR7) (Blanchetot et al., 2013) (Table 1).

Generally, one starts from a pool of nanobodies against the desired target. Further selection is based on nanobody affinity (nM) and the capacity to inhibit receptor-ligand binding or receptor activity *in vitro*. Higher affinity or avidity may be obtained by using a mixture of nanobodies recognizing different epitopes at the surface of the same antigen (oligoclonal) (Jamnani et al., 2012) or by using multivalent nanobodies (Even-Desrumeaux et al., 2012; Huet et al., 2014), which are usually linked in tandem via flexible glycine-serine linkers (Maussang et al., 2013; Huet et al., 2014). In addition, the nanobodies are often evaluated against characterized mAbs by competition assays. Remarkably, an anti-EGFR nanobody did not compete with Cetuximab but structural studies demonstrated that it targets an epitope that would not be accessible for the flatter mAb paratope, pointing to the advantage of nanobodies to reveal new intervention points (Schmitz et al., 2013).

To predict nanobody therapeutic efficacy, preclinical cancer cell line models are utilized in diverse experimental settings such as cell adhesion, proliferation, migration, angiogenesis-like properties or perturbation of specific signaling pathways. The small size of nanobodies is conducive to deep(er) and homogenous tumor penetration but disadvantageous in terms of *in vivo* half-life (few hours). Therefore, nanobodies are often linked to an anti-albumin nanobody, enabling binding to serum albumin (~66 kDa) (Tijink et al., 2008; Vosjan et al., 2012; Slordahl et al., 2013; Roovers et al., 2011; Maussang et al., 2013). Several successful nanobody-based *in vivo* xenograft studies with bispecific or multivalent nanobodies were reported, resulting in delay of tumor growth (Vosjan et al., 2012; Roovers et al., 2011) or inhibition of angiogenesis (Maussang et al., 2013). Such constructs sometimes outperform the corresponding mAb (Huet et al., 2014) but in other cases they don't. For instance, a CONAN-1 nanobody could not outperform Cetuximab, probably due to the lack of an Fc region and associated immune effector functions (Roovers et al., 2011). Adding an Fc tail, as done before for other nanobodies (De Buck et al., 2013), could provide a solution, but is not yet generally established. Although