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Nanobodies as *in vivo*, non-invasive, imaging agents

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In vivo imaging has become in recent years an incredible tool to study biological events and has found critical applications in diagnostic medicine. Although a lot of efforts and applications have been achieved using monoclonal antibodies, other types of delivery agents are being developed. Among them, VHHs, antigen binding fragments derived from camelid heavy chain–only antibodies, also known as nanobodies, have particularly attracted attention. Indeed, their stability, fast clearance, good tissue penetration, high solubility, simple cloning and recombinant production make them attractive targeting agents for imaging modalities such as PET, SPECT or Infra-Red. In this review, we discuss the pioneering work that has been carried out using VHHs and summarize the recent developments that have been made using nanobodies for *in vivo*, non-invasive, imaging.

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1. Introduction

In vivo imaging allows the investigation of molecular and cellular events in intact living subjects. Studying the dynamics of biological processes non-invasively and in their native context may offer new insights and provide opportunities for the discovery of novel biology. *In vivo* imaging has therefore become an indispensable tool in both biomedical research and medical practice.[1–5]

A diverse set of *in vivo* imaging approaches has been developed. These include Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT), Computed Tomography (CT), Magnetic Resonance Imaging (MRI), Infra-Red and Near Infra-Red Imaging (IR/NIR), Ultrasound (US), and photoacoustic imaging.[1,3–8]

Regardless of the method used, *in vivo* imaging requires differential accumulation of the relevant signal at the target site to distinguish it from background.[9] The major challenge of

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Dr Ashraful Islam earned a Bachelor of Pharmacy (2008) from Northern University Bangladesh. He received an MSc in Biomedicine (2013) and a PhD in Cell Biology (2018) from UiT The Arctic University of Norway. In 2019, he moved to Boston Children's Hospital/Harvard Medical School, where he works as a postdoctoral fellow (grant from the Norwegian Cancer Society and an overseas grant from UiT) in the Ploegh lab. Currently, he is performing a PET imaging project, with an emphasis on scFv-based PET tracer development, to investigate the infiltration of immune cells into the tumor microenvironment.

non-invasive *in vivo* imaging of a target of interest is to achieve adequate selectivity and discrimination from the background signal in the targeted area. To achieve specificity, combining the imaging agent (radio)isotope or fluorophore with a carrier such as antibodies,[10] peptides,[11,12] small molecules,[13] and aptamers[14,15] has been the method of choice. In particular, immunoglobulins and immunoglobulin-derived fragments have long been considered attractive candidates, since they can be generated to target a wide range of biomolecules[16–18] and recognize them with exquisite specificity.

Nevertheless, monoclonal antibodies (mAbs) are large (150 kDa), which explains their comparatively poor tissue penetration when administered systemically, with a long circulatory half-life.[9,19] Efforts have been devoted to improving the pharmacokinetics of antibodies without compromising their unique affinity and specificity. Antibody fragments such as Fab, F(ab′)2, single chain Fv (scFv), or variants like diabodies and minibodies (molecular weight ranging from 25–100 kDa) were bioengineered specifically for the purpose of creating *in vivo* imaging agents.[20–22] More recently, other types of proteins have been investigated as alternatives to antibodies for *in vivo* imaging. These include nanobodies (Fig. 1), Affibodies, and anticalins.[20–22]

Of these, nanobodies have attracted growing interest for use in molecular imaging across various imaging platforms, including SPECT, PET, Infra-red (IR) and ultrasound.[9,23]

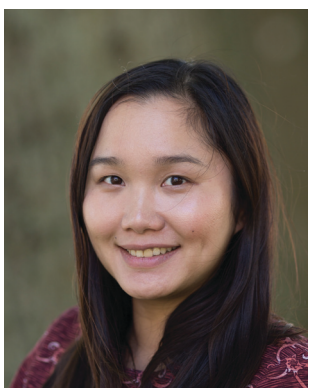
Nanobodies are antigen binding fragments derived from camelid heavy chain-only antibodies, which fold and function in the absence of light chains. These camelid immunoglobulin (Ig) heavy chains can be shrunk to just their variable domains to yield VHHs or nanobodies. The resulting VHHs retain antigen binding and can be produced in an active form in *E. coli*, often without the need of an intrachain disulfide bond. Typically, nanobodies have a molecular weight of ∼12–16 kDa and are considered the smallest immunoglobulin-derived antigen binding fragments.[24] Because of their much smaller size when compared to intact Ig (∼150 kDa), Fab (∼50 kDa) and scFv (∼25 kDa) fragments, nanobodies can penetrate tissues more efficiently and bind to antigens less accessible to conventional antibodies or their smaller derivatives.[19,25–27] Better tissue penetration is not the only advantage of nanobodies as compared to intact conventional Ig. Nanobodies are poorly immunogenic in humans,[19] and some nanobodies have been "humanized" to display even less immunogenicity.[25,28,29] Furthermore, their small size and the preponderance of beta sheet secondary structure make them highly stable, even at high temperatures and extremes of pH.[30] Finally, owing to their small size, nanobodies that fail to bind their target are rapidly cleared from the blood, mostly by renal elimination.[19,25,31–34] This, in tandem with their usually high affinity, can produce a high tumor-to-background ratio as early as 1 h after tracer injection.[32,35,36]

Although nanobodies possess the many advantages listed above, their relatively small size leads to rapid elimination from the circulation by renal clearance. Their rapid renal clearance from the body might prevent optimal binding at the desired site,[35] as the concentrations of the imaging agent in the circulation drop rapidly. This is often accompanied also by uptake of the imaging agent in the kidneys, making imaging experiments in neighboring tissues difficult because of the high background.[37] Finally, the binding properties of nanobodies may be altered when they are conjugated to produce imaging agents.[38]

In this review, we describe how nanobodies are equipped with the corresponding probes for each imaging modality and how rapid clearance and non-targeted organ uptake, problems inherent in the use of nanobodies, can be overcome.

2. Labeling of nanobodies for *in vivo* imaging

In order to be observable by any imaging modality (SPECT, PET, or IR), nanobodies must be equipped with a suitable radioisotope or fluorescent dye. Although some aspects of the



Novalia Pishesha

Dr Novalia Pishesha completed her BS in Bioengineering from the University of California at Berkeley and her PhD in Biological Engineering from Massachusetts Institute of Technology. Since 2018, she has been a Junior Fellow at the Harvard Society of Fellows. In the Ploegh lab, her project revolves around developing engineered proteins and cells to investigate and tackle autoimmune diseases and infectious diseases.

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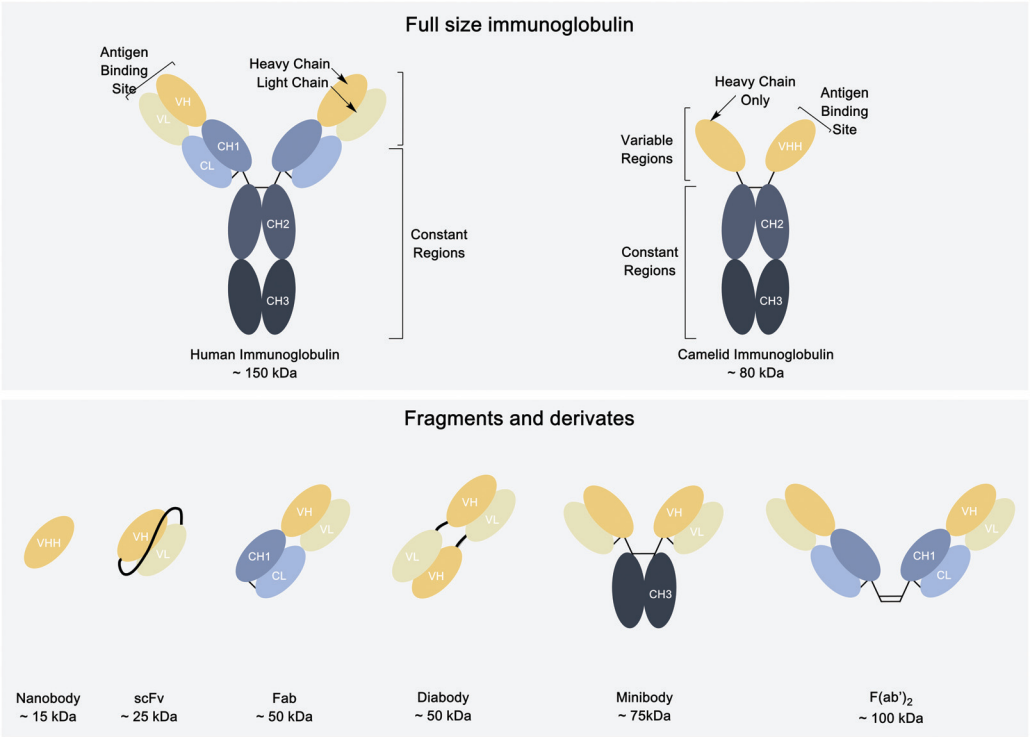

Fig. 1 Schematics of the different antibodies and antibody fragment structures.

requisite methods for modification are similar, the imaging modality chosen might require specific considerations with respect to the labeling method used.

2.1. Radio-labeling for SPECT and PET imaging

Radiohalogens, like $^{123/125/131}$I for SPECT or ^{124}I and ^{18}F for PET, must be covalently attached to the nanobody. Radiometals such as ^{67}Ga, ^{99m}Tc, and ^{111}In for SPECT and ^{64}Cu, ^{68}Ga, and ^{89}Zr for PET are complexed *via* a chelator and thus require a two-step process where the nanobody is first equipped with a chelator and then incubated with the radiometal in solution. The (His)6 tag often present on recombinantly expressed proteins to facilitate affinity purification readily coordinates site-specifically with ^{99m}Tc–tricarbonyl.[39,40]

In either case, a chelator or a prosthetic group must be attached to the nanobody of choice. Various labeling approaches can be divided into two major classes: uncontrolled or site-specific labeling. Uncontrolled labeling relies mainly on lysine and cysteine conjugation *via* carbodiimide/*N*-hydroxy-succinimide (DIC/NHS) chemistry or maleimide chemistry, respectively. Although this method is common, straightforward, and easily accessible, the heterogenous mixture of labeled products might lead to reduced binding of the nanobody, especially if the binding site itself is modified.[41] Site-specific strategies aim to obtain homogeneous and more consistent products, with the desired moiety attached to a specific site of one's choosing. Different site-specific methods to modify nanobodies have been described, relying on enzymatic methods, click chemistry, or meticulous and selective incorporation of an unpaired cysteine residue.[24,42]

There are a wide range of commercially available chelators with functionalized handles, such as maleimide, NHS ester, azido derivatives, *etc.* (Fig. 2A). However, this is not the case for the radiohalogens (obtained as salts) for both ^{18}F and $^{123/124/125/131}$I. The Iodogen method allows rapid but uncontrolled iodination of the desired protein *via* an oxidation reaction of tyrosine sidechains, but a more sophisticated synthetic chemistry is required to prepare radiohalogen reagents prior to their use for the labeling of nanobodies (Fig. 2B).[43,44]

2.2. Fluorescent dye labeling for IR and NIR

In vivo imaging using fluorescent dyes relies on fluorophores emitting in the near infrared (NIR) or infrared region (IR). An ideal dye should have a maximum emission in the 650 nm to 900 nm range (Fig. 2C). This particular range of wavelengths reduces scattering and nonspecific autofluorescence, produces a good signal to background ratio, and thus improves resolution.[23] The cyanine dye derivatives: Cy5, AF680, IRDye 680RD IRDye800CW, *etc.* are commonly used for this application.[45,46] However, their high hydrophobicity has pushed the development of more water-soluble dyes, such as the pyrrolopyrrole cyanine family (PPCy) (Fig. 2C).[47]

Conjugation of fluorophores can significantly affect pharmacokinetics, biodistribution, quality/stability and aggregation properties of the engineered construct. Uncontrolled and multiple-labeling of fluorophores to nanobodies yields products with abnormal distribution, can contribute adversely to the background signal, produces accumulation in non-targeted organs such as the liver and results in poor contrast for the target of interest.[36,48–51] Site-specific labeling of IRDye800CW

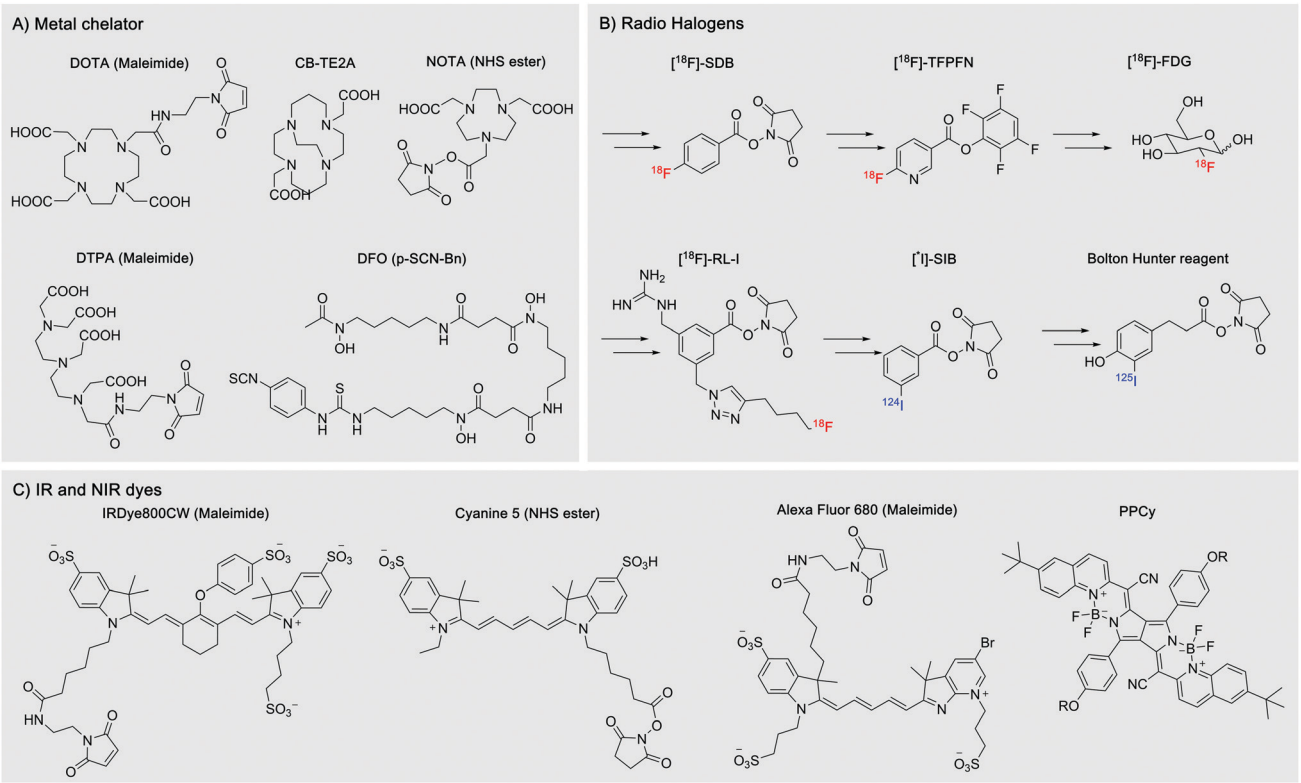

Fig. 2 Examples of potential probes for *in vivo* labeling. Most of them are commercially available as maleimide, NHS ester, azide, *etc.* derivatives. (A) Metal chelators used for SPECT and PET imaging. (B) Typical radio halogen-based reagents. (C) IR and near IR dyes for infra-red imaging.

and IRDye680RD dyes by conjugation to a selectively introduced C-terminal cysteine yields imaging agents with improved biodistribution: acceptable kidney uptake, rapid binding to the target of interest and minimal nonspecific uptake (Fig. 3).[49]

Nanobodies for NIR/IR *in vivo* imaging can also be prepared as fusions to a far-red fluorescent protein. This eliminates the need of further handling of the nanobody but comes with a drawback: the considerable size of the fluorescent protein (20–30 kDa) compared to the nanobody (~15 kDa) might disturb binding and the biodistribution of the resulting fusion protein.[23,51]

3. Overcoming nanobody challenges: renal accumulation and rapid clearance

Long-term renal retention of nanobodies, especially in a radiolabeled form, can cause nephrotoxicity. The bright kidney signals caused by such accumulation make the imaging of molecular targets in the adjoining areas difficult. Different strategies have been developed to reduce renal absorption. For example, co-injection with gelofusin or positively charged

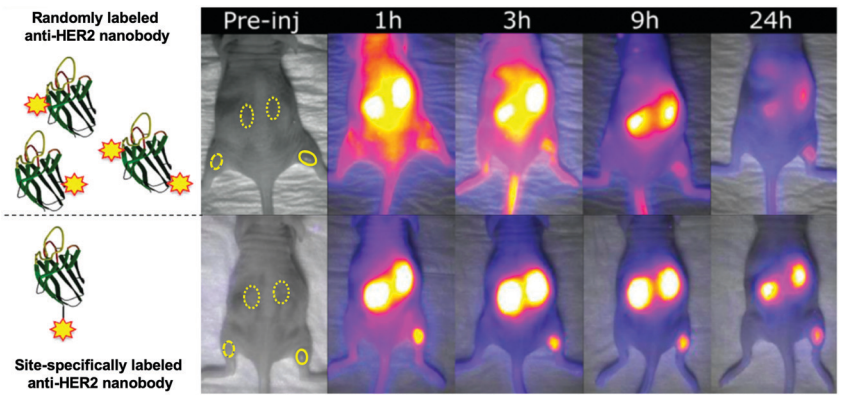

Fig. 3 Effects of the unspecific conjugation of NIR/IR dye on the biodistribution of a nanobody (solid ROI: tumor; dashed ROI: contra-lateral muscle; dotted ROI: kidney). In this tumor mouse model, the anti-HER2 nanobody (2Rs15d) was labeled with IRDye800CW in a uncontrolled and controlled manner (top and bottom panel respectively). Adapted with permission from.[49] Copyright (2017) American Chemical Society.

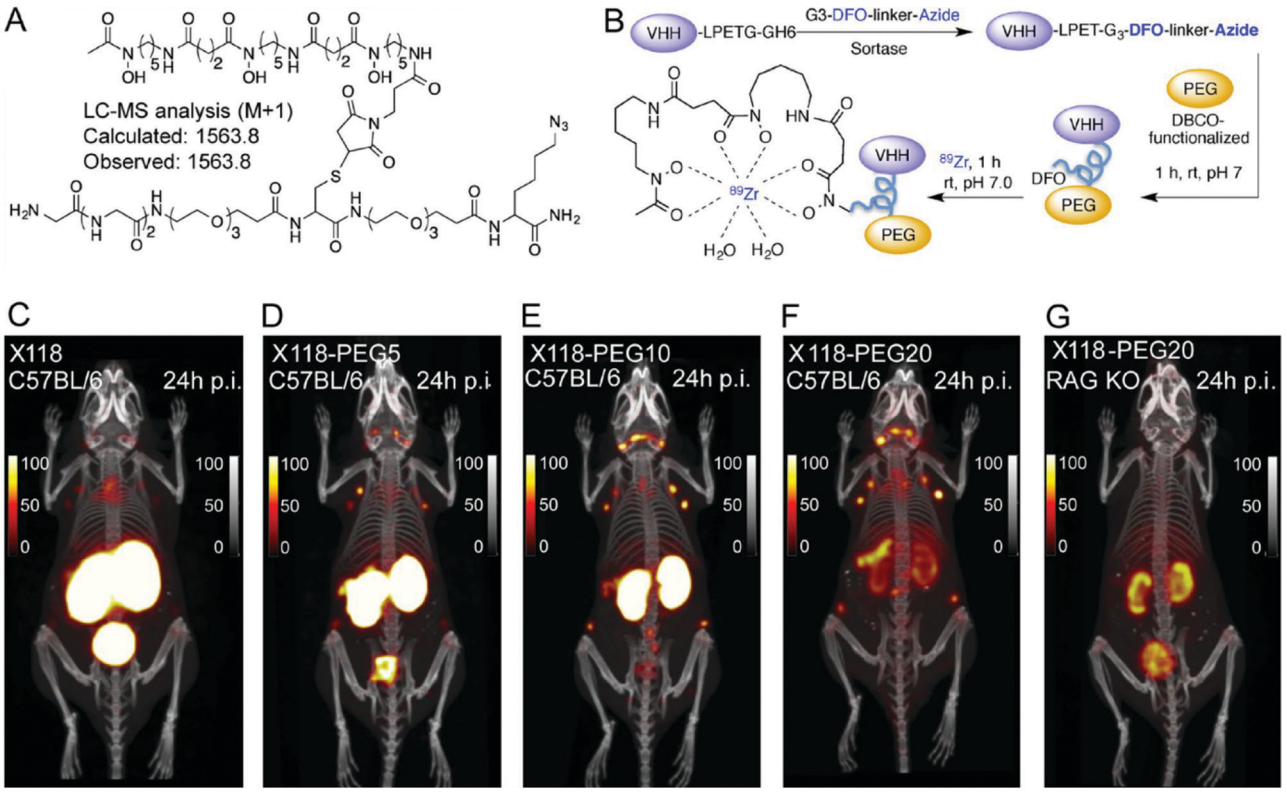

Fig. 4 Selective labeling, using sortase A, of a nanobody with a metal chelator for ^{89}Zirconium-based PET imaging. (A) Structure of the bioorthogonal sortase substrate. (B) Schematic representation of preparing PEGylated ^{89}Zr-labeled VHHs for PET imaging. (C–G) PET-CT images of anti-CD8 ^{89}Zr-labeled (VHH_{X118}) with and without different-size PEG in wild-type C57BL/6 and RAG−/− mice. Images were acquired 24 h post-injection of the radiolabeled VHHs. Adapted from ref. 56.

amino acids like lysine can reduce renal uptake for radiolabeled biomolecules.[52,53] This strategy has also been applied to nanobodies.[31,54] Another approach consists of removing the affinity tags often used for purification purposes: these tags, typically myc or (His)6 tags, consist of negatively- and positively-charged amino acids respectively, and can directly impact the degree of renal accumulation of the nanobody.[54,55] Attachment of PEG moieties can decrease renal uptake. The availability of differently sized PEGs allows simple screening and tuning of the desired construct to optimize its imaging characteristics. Rashidian *et al.* used nanobody constructs bearing differently sized PEG moieties and showed a direct correlation between the size of the PEG moiety and the amount of tracer accumulating in the kidney (Fig. 4).[56]

Unmodified VHH X118, a nanobody that targets CD8+T cells, fails to cleanly show the spleen (located near the kidneys), although the spleen is a main reservoir of CD8+T cells. In contrast, the 20 kDa PEG-derivative of VHHX118 readily allows visualization of secondary lymphoid organs.

PEGylation tackles yet another variable associated with the use of nanobodies, which is their rapid clearance from the circulation. While quick elimination from the body allows nanobodies to be used for same-day imaging, it also can lead to poor accumulation in the area of interest. Attachment of PEG of different sizes, *e.g.* 5, 10 or 20 kDa, increases their molecular weight/Stokes' radius and thus their circulatory dwell time, with an improvement in the signal to noise ratio.[56] Increasing the half-life of nanobodies by increasing their size has been accomplished by methods other than PEGylation. Wardners *et al.* used a tri-paratopic nanobody construct, consisting of two nanobodies that bind to two different HER3 epitopes and a third that binds to albumin in order to extend the circulatory half-life of the construct. This construct showed less renal accumulation than a 'regular' HER3-targeting nanobody and circulated longer, as measured by radioactivity remaining in the blood.[57] Size and targeting properties must be properly balanced, parameters that cannot be predicted and therefore require systematic experimentation.

Interestingly, the degree of kidney retention for radiohalogenated (fluorinated and iodinated) nanobodies is significantly lower than their radiometal-labeled counterparts. Catabolites of radiohalogenated compounds formed in the kidneys are thought to be non-residualizing and hydrophobic. They are rapidly excreted *via* the urine.[23]

With the exception of this kidney accumulation – which can be overcome at least in part by the methods described here– accumulation in other non-targeted organs is usually very low. The unique characteristics of nanobodies (efficient tissue penetration, fast tissue diffusion and fast clearance) thus allow same-day imaging with high target-to-background ratios.[9,23]

This is impossible to achieve with conventional antibodies, where several days of equilibration between circulation and target tissues are required after administration before it is possible to acquire high quality images.

4. Application and examples of nanobodies for *in vivo* imaging

4.1. Radio imaging: SPECT and PET

Radionuclide-based techniques such as PET and SPECT are commonly used because they are sensitive, quantitative, and clinically relevant.[58,59] SPECT and PET have the sensitivity needed to visualize most interactions between physiological targets and ligands, enabling non-invasive detection of tracers down to the picomolar level.

4.1.1. SPECT imaging using nanobodies. Single-photon imaging exploits the γ rays emitted by a radioactive atom. A *γ*-emitting element with an energy of 100–250 keV is ideal.[1] The spatial resolution of single-photon imaging is on the order of 8–10 mm, although somewhat better resolution can be obtained with recently developed special-purpose imaging systems.[60–63]

Although ^{99m}Tc, $^{123/131}$I, ^{111}In and ^{155}Tb are the four radionuclides suitable and commonly used for SPECT imaging,[64] only two of them have been used with nanobodies: ^{99m}Tc and ^{111}In. A few examples describe nanobody-based SPECT imaging using ^{111}In as a radionuclide: Chatalic *et al.* developed an anti-PSMA nanobody conjugated to the metal chelator diethylenetriaminepentaacetic acid (DTPA), which showed good tumor targeting and minimal uptake by the non-targeted organs.[54] Bala *et al.* used ^{111}In in combination with a VCAM-1 targeting nanobody in a study to compare the pharmacokinetics of the imaging agent with the use of the different tracers: ^{68}Ga, ^{18}F, ^{111}In and ^{99m}Tc.[65]

The reason for the success of ^{99m}Tc as a radionuclide for SPECT imaging derives not only from its favorable nuclear decay characteristics, but also from its simple and straightforward radiolabeling chemistry. This has allowed the labeling of a wide range of biomolecules. For example, ^{99m}Tc(CO)$_3$ can be used to directly label nanobodies equipped with a (His)6 tag without further modifications.[32,66] Additionally, the long half-life of ^{99}Mo and the low cost associated with the ^{99}Mo/^{99m}Tc generator make the production of ^{99m}Tc widely available.[67]

Nanobodies against many different markers have been used in combination with ^{99m}Tc for SPECT imaging of cancers. Examples include epidermal growth factor receptor 1 (EGFR)[31,32,66,68,69] and 2 (HER-2),[70] prostate-specific membrane antigen (PSMA),[71] the Macrophage Mannose Receptor (MMR),[37] carcinoembryonic antigen (CEA),[72,73] mesothelin[74] and the M-protein.[75] Nanobodies that target non-cancer markers have also found application for SPECT imaging. De Vos *et al.* immunized a dromedary with the Lectin-like oxidized low density lipoprotein receptor (LOX-1).[76] This protein is a biomarker for detection and phenotyping of atherosclerotic plaques. They obtained a nanobody, LOX-sdAb, that binds LOX-1 with picomolar affinity. Recombinant expression in *E. coli* of this nanobody equipped with a C-terminal (His)6-tag, followed by direct radiolabeling using ^{99m}Tc(CO) allowed SPECT imaging in apoliprotein E-deficient mice (ApoE−/−). It showed a clear signal of atherosclerotic plaques in the aortic arch. This nanobody is an example of a potentially powerful tool for cardiovascular prognostic and diagnostic use.

Other examples of diseases/conditions imaged using nanobodies for SPECT include diabetes,[77,78] liver inflammation,[79,80] rheumatoid arthritis,[81,82] Gelsolin amyloidosis,[83] imaging of immune cells,[84] or more generally immune checkpoints in cancer.[85] Nanobodies used for SPECT imaging, labeled with ^{99m}Tc, are summarized in Table 1 and show the breadth of nanobodies deployed for such studies.

While these preclinical examples show the utility of nanobodies for SPECT imaging in mouse models, their application for *in vivo* imaging in humans is not as straightforward. Nanobodies, because of their non-human origin, might induce an immune response when applied in clinical settings. ''Humanization'' of nanobodies is usually considered to advance their application to the clinic. Vaneycken *et al.* described an interesting approach in which they genetically grafted the CDRs of an anti-CEA nanobody, NbCEA5, onto a humanized nanobody scaffold (Fig. 5).[73]

The ''transplant'' was successful, and the grafted nanobody displayed imaging properties similar to the original. This promising work opens the door to wider application of nanobodies in a human setting.

4.1.2. PET imaging using nanobodies. PET is considered the more attractive non-invasive imaging modality over its single-photon cousin, owing to its higher spatial resolution (usually 2–3 mm or lower for PET), higher sensitivity (typically 10^{-8} to 10^{-10} M tracer concentrations for PET, compared to $\sim10^{-6}$ M for SPECT), and the wide availability of positron-emitting radioisotopes.[90] In the context of immuno-oncology, nanobodies and PET have been partnered to investigate the expression and tracking of immunologically relevant molecular targets, such as effector molecules, immune cell populations, and checkpoint molecules within the tumor microenvironment (TME). The application of nanobodies to cancer-relevant models, both for imaging and therapeutic purposes has been reviewed recently,[9,23,91,92] but some highlights are presented in the following.

Nanobody-based PET tracers that target cancer biomarkers, such as human epidermal growth factor receptor 2 (HER2),[33,93,94] HER3,[57] CD20,[95] epidermal growth factor receptor (EGFR)[34] and hepatocyte growth factor (HGF),[96] have been used as imaging agents to evaluate the presence or absence of these biomarkers in preclinical models. The results from these studies showed high tumor-specific uptake of the nanobody-based probes into the TME. A major step towards clinical translation of nanobody-based PET tracers was the phase I clinical trial of ^{68}Ga-NOTA-labeled anti-HER2 nanobody in breast carcinoma patients.[97] PET/CT images taken at 90 min post-injection were chosen as the optimal imaging time point, showing less background than at earlier time points. Irrespective of non-specific accumulation of tracer in the liver, kidneys

Table 1 Overview of nanobody-based tracers for non-invasive SPECT imaging using ^{99m}TC

Condition	Target	Nanobody	Disease model	Ref.
Cancer	EGFR	D10	Human epidermoid carcinoma (A431)	66
		7C12		31, 68 and 69
		8B6	Human epidermoid carcinoma (A431), human prostate carcinoma (DU145)	32
	PSMA	PSMA30	Prostate cancer (LNCaP)	71
	HER-2	2Rs15d	Breast Cancer (SKBR3), ovarian cancer (SKBR3)	70
	MMR	a-MMR	Mammary adenocarcinoma (TS/A), Lewis lung carcinoma (3LL-R)	37
	CEA	CEA1	Human colon adenocarcinoma (LS174)	72
		NbCEA5 & humanized variant		73
	M-Protein	R3B23	Multiple Myeloma (5T33MM)	75
	Mesothelin	A1	Triple negative breast cancer (HCC70)	74
Immune Checkpoint	PDL-1	C3, E2	PDL1 immune checkpoint	85
Atherosclerosis	LOX-1	LOX-sdAb	Atherosclerosis (ApoE-deficient mice)	76
	VCAM1	cAbVCAM1-5		65, 86–88
	MMR	a-MMR		89
Rheumatoid arthritis	MMR	a-MMR	Rheumatoid arthritis	81
	CRIg	NbV4m119	Collagen-induced arthritis (CIA)	82
Immune cells	Unknown	Nb-DC2.1	Myeloid cells	84
		Nb-DC1.8	Immature bone marrow-derived dendritic cells	
Diabetes	DPP6	4hD29		77 and 78
Liver disease	Vsig4	NbV4m119	Concanavalin A induced hepatitis	79
	Clec4F	C4m22	Concanavalin A induced hepatitis and methionine choline deficiency-induced non-alcoholic steatohepatitis	80
Gelsolin amyloidosis	8 kDa of MT1-MMP	FAF Nb1-3	AGel mice	83

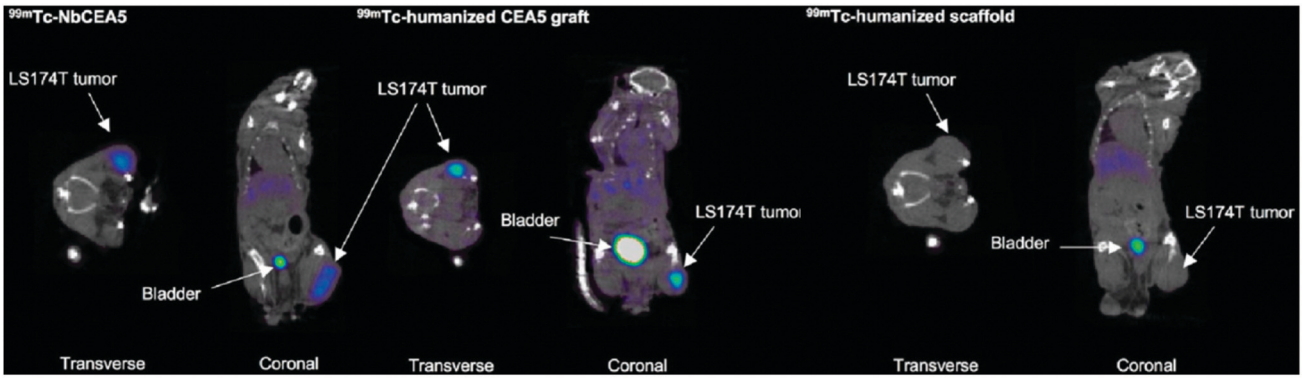

Fig. 5 SPECT imaging 1 h after injection of intravenously injected ^{99m}Tc-labeled nanobodies. Both ^{99m}Tc-NbCEA5 and ^{99m}Tc-humanized CEA5 graft show high uptake in CEA-positive LS174T tumors in contrast to the ^{99m}Tc-humanized scaffold alone. Adapted from ref. 73.

and intestines, tumor-specific uptake was above background (Fig. 6). In this clinical trial, Keyaerts *et al.* achieved safe, non-invasive visualization of HER2 status of both primary lesions and/or metastases. A phase II study of this tracer is underway (NCT03331601).

In addition to imaging the tumor's antigen profile, monitoring the immunological landscape in the TME could provide insight into the prognosis of a therapeutic response. Composition of the myeloid compartment, other immune cell types, cellular distribution and activation status could all be monitored A nanobody against macrophage mannose receptor (MMR, CD206), a marker for M2-polarized macrophages the presence of which correlates with an immunosuppressive TME and poor prognosis, showed the presence of tumor-associated macrophages (TAMs) in preclinical models.[98,99] A phase I clinical trial with ^{68}Ga-NOTA-labeled anti-MMR nanobody is ongoing (NCT04168528). Nanobody-based PET tracers that target CD11b and major histocompatibility complex (MHC) class II positive cells, can track immune infiltrates in both xenogeneic and syngeneic tumor models.[100] In line with these findings, an anti-human MHC class II nanobody detects inflammation in a humanized graft-versus-host disease mouse model.[101]

The distribution of cytotoxic CD8+T cells could play a pivotal role in predicting the outcome of immune checkpoint blockade (ICB) therapy. Rashidian *et al.* demonstrated promising results in tracking CD8+T cell responses to ICB in preclinical models, using a ^{89}Zr-labeled anti-CD8 nanobody.[56,102] Indeed, PET imaging showed a positive correlation between homogeneous

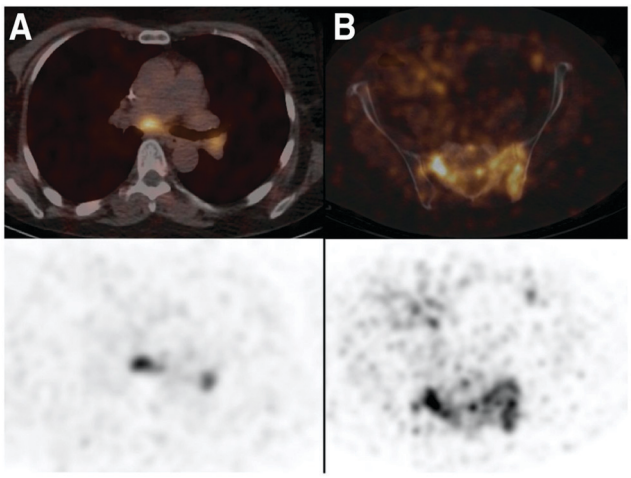

Fig. 6 PET/CT images from the phase I clinical trial of ^{68}Ga-anti-HER2-Nanobody in breast cancer patients. (A) Patient 18, with invaded lymph nodes in the mediastinum and left hilar region. (B) Patient 20, with bone metastasis in the pelvis. PET/CT images (top) and PET images (bottom). Adapted from ref. 97.

distribution of CD8+T cells and responders to ICB therapy (Fig. 7). Similar to tracking of immune cells, non-invasive imaging of the targets of ICB may lead to better understanding of the intratumoral immunological landscape. Nanobody-based PET tracers against PD-L1 and CTLA-4, developed by Ingram *et al.*, showed distribution of checkpoint molecules in melanoma.[103,104] In yet another example, the extracellular matrix (ECM) of tumors is a critical determinant for understanding the response to therapy. An anti-EIIIB nanobody that recognizes an alternatively spliced variant of fibronectin in the ECM was developed and used for PET imaging of primary, metastatic and fibrotic lesions in preclinical cancer models.[105] An overview of some of the nanobody-based PET tracers is provided in Table 2.

4.2. Infra-red imaging *in vivo*

Infra-red imaging is less sensitive than SPECT and PET. The amount of probe that must accumulate in the targeted area in order for it to be visible is much larger than that for SPECT and PET. Yet, recent development of fluorescent dyes in the infra-red and near infra-red region has led to the emergence of optical imaging as a viable technology for *in vivo* imaging.[106–108] IR/NIR imaging is flexible, sensitive, fast and relatively inexpensive compared to the radiolabeling approach for SPECT and PET, not to mention the advantage of avoiding radioactivity altogether.[9] Nanobodies conjugated to such dyes are now being used for *in vivo* imaging.[23,48,109–112] For example, Bannas *et al.* used a nanobody (s+16a) against the toxin-related ADP-ribosyltransferase ART2, an enzyme present on the surface of mouse T cells, to image lymph nodes in ART2-TG and ART2−/− mice using AlexaFluor 680.[50] For comparison, they also compared images of the cervical and axillary lymph nodes obtained with a nanobody-Fc fusion (s+16mFc) and the monoclonal antibody Nika102 (Fig. 8). Although all three constructs show *in vivo* fluorescence of the lymph nodes, s+16a shows no

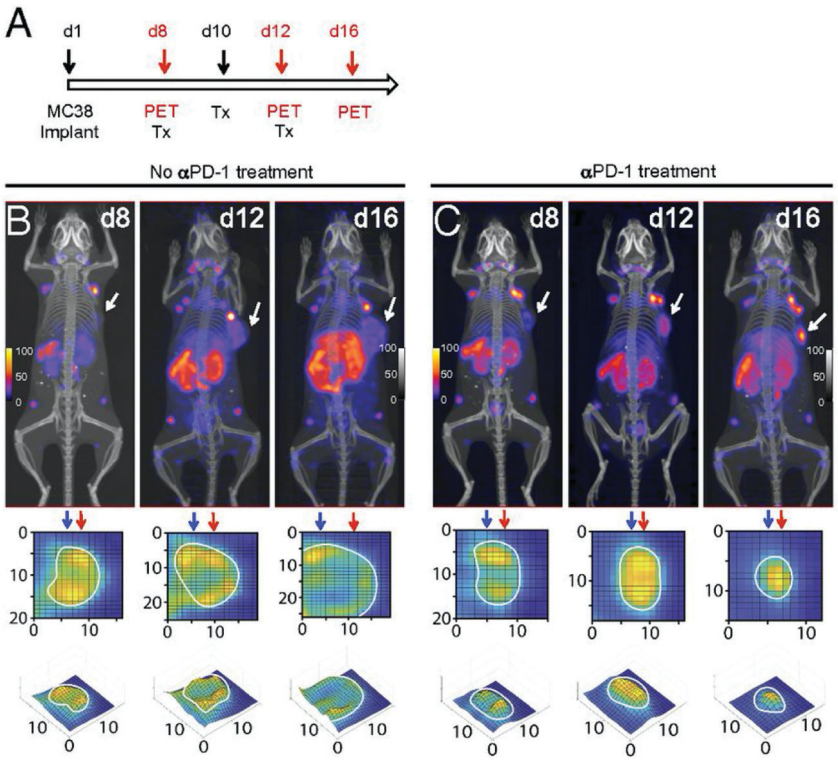

Fig. 7 Longitudinal monitoring of CD8$^+$ T cells in response to anti-PD-1 (αPD-1) treatment. (A) Schematic for the treatment and PET imaging. (B and C) PET/CT images of tumor-bearing mice in the no treatment and treatment group. Adapted from ref. 102.

Table 2 Some nanobody-based tracers for non-invasive PET imaging

Application	Target	Nanobody	Disease model	Ref
Tumor biomarkers	EGFR	7D12	Skin cancer	34
	HER2	2Rs15d 5F7	Breast cancer	33, 93 and 94
	HER3	MSB0010853	Non-small cell lung cancer, head and neck cancer	57
	CD20	9079	Non-Hodgkin lymphoma	95
	HGF	1E6-Alb8, 6E10-Alb8	Glioma	96
Immune cells	CD8	VHH-X118	Tumor immunology and inflammatory diseases	56 and 102
	CD11b	DC13		100
	MHC II	VHH7, VHH4		100 and 101
	MMR	MMR 3.49		98 and 99
Checkpoint molecules	PD-L1	B3	PD-L1 immune checkpoint	103
	CTLA-4	H11	CTLA-4 immune checkpoint	104
ECM	Fibronectin EIIIB	NJB2	Breast cancer, PDAC, and melanoma	105

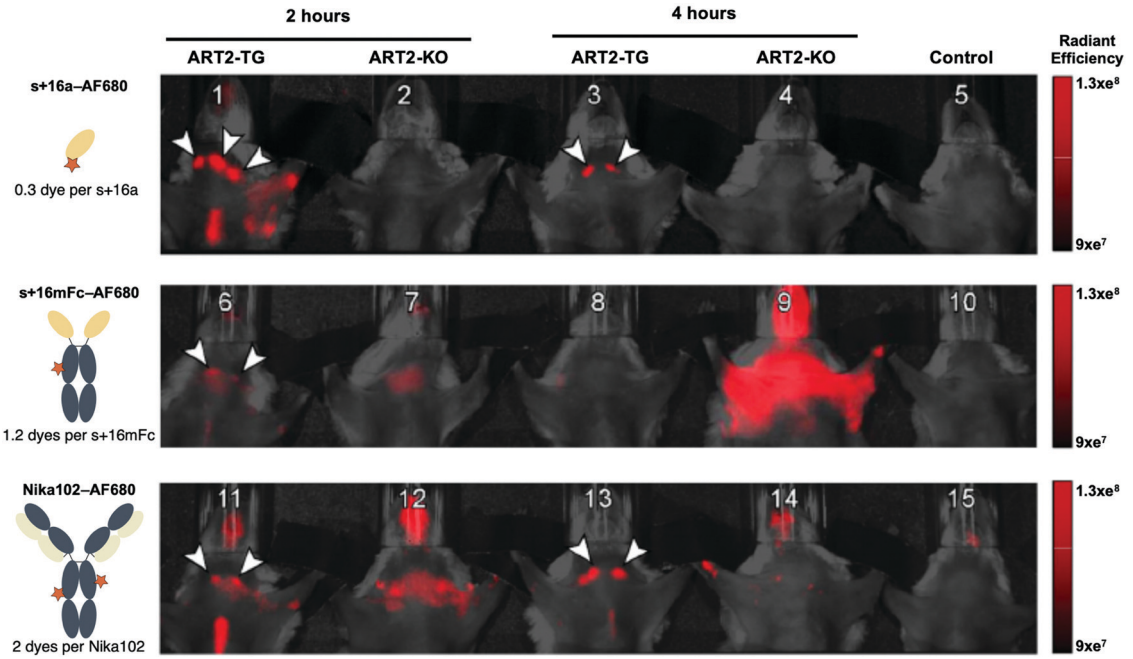

Fig. 8 Fast clearance of nanobodies allows rapid *in vivo* imaging after injection. ART2-TG mice and ART2−/− mice were injected with: buffer (control), 50 mg s+16a680, 5mg s+16mFc680 or 10 mg Nika102680. Control and antibody-injected mice (2 and 24 h after injection) were subjected to simultaneous imaging to obtain visually comparable images. Arrowheads indicate cervical and axillary lymph nodes. Adapted from ref. 50.

background signal, while strong background fluorescence was observed for Nika 102 and s+16mFc, particularly visible in the ART2−/− mice (Fig. 8).

Light scattering and light absorption by tissues limits the depth of penetration for NIR/IR imaging, ranging from microns to a few centimeters only.[113,114] Bleaching and blinking of fluorescent dyes also diminish the imaging signal strength over time. For these reasons, NIR/IR *in vivo* imaging is not suitable for whole-body imaging. Other systems that overcome these limitations are being developed and are being used for *in vivo* imaging. One such example is single-walled carbon nanotubes (SWCNTs). These nanomaterials present ideal optoelectronic characteristics for imaging in the IR region by emitting in the 850 to 1700 nm region.[115] This spectral window is highly beneficial for biological and especially deep-tissue imaging, due to its reduced absorbance, phototoxicity, reduced background fluorescence and scattering as compared to traditional fluorescent dyes. SWCNTs do not bleach or blink, allowing longer exposure and imaging times. Mann *et al.* successfully used this type of imaging agent by conjugating it to a GFP-specific nanobody. To link SWCNTs to the nanobody a two-step process was deployed, using a maleimide-bearing oligonucleotide that wrapped itself around the SWCNT, followed by conjugation to the nanobody bearing a free cysteine residue. This construct was then used to monitor and track GFP-modified Kinesin-5 motor proteins in a Drosophila embryo (Fig. 9).[116]

One promising area where NIR/IR imaging may find application is in the context of intraoperative imaging to discriminate

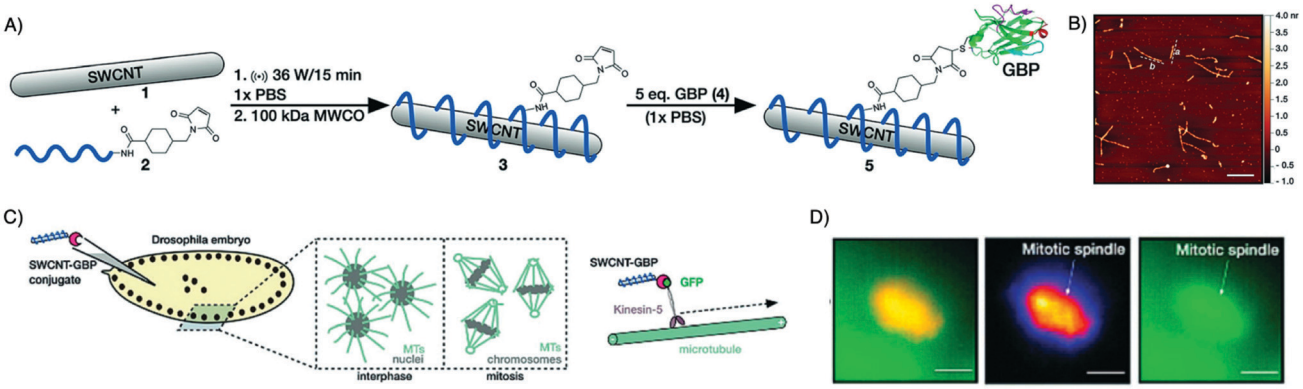

Fig. 9 Alternative to small molecule dyes for NIR/IR *in vivo* imaging. (A) Conjugation method for attaching a GFP-binding nanobody to DNA-wrapped SWCNTs. (B) Atomic force microscopy images of GBP-conjugated SWCNTs. Scale bar = 500 nm. (C) General scheme describing the injection of 5 into live Drosophila embryos expressing GFP proteins on Kin-5 motors. (D) GFP and SWCNT channel images showing colocalization of Kin-5 and conjugate 5 at a mitotic spindle (scale bars = 10 mm). Adapted from ref. 116.

malignant from healthy tissue and facilitate proper excision of a tumor.[23] This has dramatically improved surgery for removal of liver metastases, ovarian, cervical and breast cancer and melanoma.[111] Incomplete tissue penetration, which limits nanobody applications for whole body imaging, is less of a problem in this set-up. Indeed, nanobodies have been successfully used for such applications.[23,48,110–112]

Van Brussel *et al.* used a CAIX-specific nanobody, B9, modified *via* maleimide chemistry with a IRDye800CW dye. CAIX, carbonic anhydrase IX, is a tumor-specific membrane-bound protein expressed in hypoxic tumors.[117,118] Using a xenograft breast cancer mouse model of a ductal carcinoma *in situ* and mimicking a surgery set-up, strong accumulation of B9 was seen in the tumor, well differentiated from surrounding healthy tissue (Fig. 10A).[110]

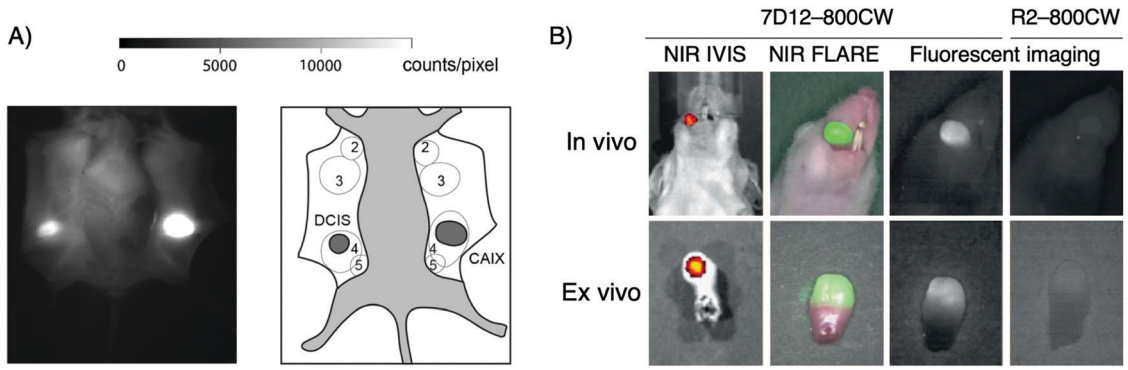

Fig. 10 Intraoperative imaging for cancer surgery. (A) Intra-operative imaging of DCIS and DCIS + CAIX tumors, 3 h post injection of fluorescently labeled VHH B9 (left). Schematic overview of mammary glands (2–5) and tumors as seen intra-operatively. DCIS + CAIX tumor indicated as "CAIX" in dark gray (right). (B) Real-time fluorescence imaging of orthotopic tongue tumor 24 hours after injection of the EGFR-specific nanobody 7D12 conjugated with IRDye800CW. Adapted from ref. 110.

Table 3 Overview of nanobody-based tracers for non-invasive IR/NIR imaging and intraoperative imaging

Target	Nanobody	Fluorescent Dye	Disease model	Ref
EGFR	7D12	IRDye800CW	Human epidermoid carcinoma (A431)	36
HER-2	11A4	IRDye800CW	Breast cancer (SKBR3),	38
			Breast cancer (MCF10DCIS)	30
		IRDye680RD		109
	2Rs15d	IRDye800CW	Breast cancer (SKOV3)	49 and 112
		IRDye680RD		49
		Cy5	Breast cancer (BT474M1)	119
CAIX	B9	IRDye800CW	Breast cancer (MCF10DCIS)	109
			Breast cancer (DCIS)	110
ART2	s+16a	AlexaFluor 680	ART2-TG	50
CEA	Anti-CEA	IRDye800CW	Human pancreatic cancer (BxPC-3 and MiaPACA-2)	120
Kin-5-GFP	GBP	SWCNT	GFP fusion proteins of Kin-5 motors	116

Another example reported by van Driel *et al.* used a nanobody that targets EGFR, 7D12, conjugated to IRDye800CW for the imaging of a tongue tumor (Fig. 10B). Not only were they able to clearly identify the primary tumor, the imaging agent also detected cervical lymph node metastases.[48]

Although NIR/IR *in vivo* imaging might not be as sensitive as the radiolabeling methods, it represents an appealing alternative, especially in the case of intraoperative imaging for cancer surgery. A summary of the different nanobodies used for NIR/IR *in vivo* imaging and for intraoperative imaging is shown in Table 3.

5. Conclusions

Here we reviewed recent advances in the field of molecular imaging using nanobody-based probes. The unique properties of nanobodies make them ideal targeting agents, and not just for *in vivo* imaging. Their rapid clearance from the circulation, their tissue penetration properties, and their low background retention enable highly specific imaging at early time points after administration, even on the same day, with a reduced risk of nonspecific toxicity. Unfortunately, this advantage over the regular antibodies leads generally to an important kidney uptake. However, methods, such as PEGylation, are emerging to overcome this challenge. Although most of the applications reported to date using nanobodies have employed SPECT or PET as the imaging modality, other techniques such as infrared imaging, especially in the case of intraoperative surgery, ultrasound,[121–123] photoacoustic imaging,[124,125] and Magnetic Resonance Imaging (MRI)[126–128] deserve attention.

The results obtained to date with nanobodies for molecular imaging show that these tracers will find many applications in the laboratory as well as in the clinic. The preclinical success of nanobodies justifies further exploration and development to expand molecular imaging, ultimately with a view to clinical success.

Conflicts of interest

There are no conflicts to declare.

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