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Engineering antibodies and proteins for molecular *in vivo* imaging

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The rapid and ongoing discovery of new disease related biomarkers leads to a dramatic paradigm change in human healthcare and constitutes the basis for a truly personalized medicine. Molecular imaging enables early detection and classification of human diseases and provides valuable data for optimized, target-oriented therapies. By now, the biochemical and physiological properties of antibody derivatives or alternative protein scaffolds can be engineered for the detection of a wide range of target structures. The successful application of these reagents in animals, xenograft models and cells in preclinical research clearly demonstrate their utility for molecular imaging. Despite these promising perspectives, only a few antibodies and recombinant proteins are used yet for molecular imaging in human medicine. Especially the high safety demands and the need to eliminate off target effects in humans require extensive research and development efforts.

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Introduction

The rapid progress in genome and proteome technologies has enabled screens for molecular changes in human diseases and yields an ever-growing list of candidates that are currently being evaluated for their suitability as biomarkers. In parallel, imaging technologies are being developed and refined for increased sensitivity as well as improved temporal and spatial resolution. The challenge is now to develop reliable and safe reagents for molecular imaging for early detection and accurate classification of human diseases that provide valuable additional diagnostic and prognostic information.

Antibody derivatives coupled to detectable tracers like radionuclides or dyes represent the most promising tools for target-specific molecular imaging, since antibodies are naturally evolved, highly selective binding molecules. However, although the potential of antibodies for molecular imaging is obvious, it has barely been utilized for *in vivo* imaging applications as there are severe obstacles to overcome like insufficient tissue penetration and immunogenicity. The recent progress in recombinant antibody engineering and the development of alternative protein scaffolds with custom tailored biochemical properties enables a broad application of recombinant proteins for molecular *in vivo* imaging and will likely have a profound impact on human healthcare in the following decades.

Antibodies for *in vivo* imaging

Antibodies are arguably the most powerful tools in biomedical research, as they can detect and identify practically any cellular structure. Generated by the highly sophisticated immune response system, antibodies are naturally secreted or presented at the cell surface as part of a defense strategy against foreign compounds and pathogens. Antibodies per se are large and complex molecules (~150 kDa) comprising two heavy and two light chains linked by disulfide bridges (Figure 1). Each heavy chain consists of a variable domain (VH) and three constant regions (CH) each light chain consists of a variable domain (VL) and one constant region. Antigen binding is mediated by the three complementarity determining regions (CDR) located in the VH and VL, respectively [1].

Currently, intense researches focus on the development of new antibodies for diagnostic imaging. Antibodies can be engineered to fulfil several criteria for *in vivo* imaging. First, they need to bind their target antigen with high specificity and affinity. Second, their blood clearance should be optimized to enable high contrast imaging in short time. Third, they need to have a low immunogenicity.

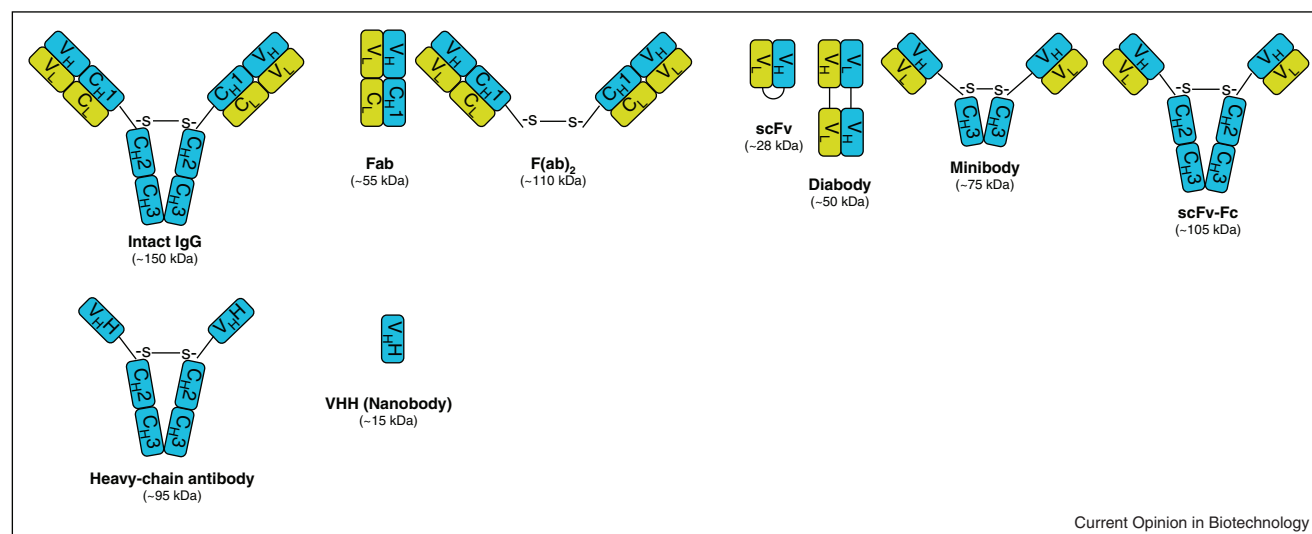
Since 1990 only four antibodies are on the diagnostic imaging market targeting Tag-72 (OncoScint[®]), EGP-1 (Verluma[®]), CEA (CEA-Scan[®]) and PSMA (ProstaScint[®]). However, recent progress in antibody engineering renewed the interest in using antibodies as diagnostic imaging agents (see Table 1).

Engineered antibody fragments

Despite high affinity and target specificity, slow blood clearance of intact antibodies presents a problem for molecular imaging. Often, high contrast images are only obtained several days after tracer injection. To address

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Figure 1



Schematic representation of antibody formats.

this issue, antibody fragments of smaller size with comparable binding properties were engineered and tested in imaging applications during the last decade.

Fab (50 kDa) and F(ab)₂ (100 kDa) fragments are generated by proteolytic digestion of full length antibodies. Diagnostic imaging using Fab fragments started nearly twenty years ago with a Fab fragment derived from the murine monoclonal antibody IMMU-4 directed against carcinoembryonic antigen (CEA-Scan[®]). Coupled to Technetium (^{99m}Tc) it is used for the diagnostic imaging of colorectal cancers [2]. Recently, Fab and F(ab)₂ frag-

ments of the monoclonal antibody 3/A12 against prostate-specific membrane antigen (PSMA) [3] were tested for use in *in vivo* imaging. Both fragments showed faster blood clearance compared to the parental intact antibody. However, although the intact anti-PSMA antibodies labelled with ⁶⁴Cu showed specific tumour uptake 24 h after tracer injection in PET imaging of prostate carcinoma xenografts, both Fab fragments showed only minimal tumour uptake and were not suitable for PET imaging [4].

Single chain variable fragments (scFvs) are even smaller (~30 kDa) than Fab fragments and comprise a variable

Table 1

Binding proteins used for *in vivo* imaging applications described 2009 and 2010.

Protein	Format	Antigen	Preclinical/clinical	References
Antibody	Intact IgG	PSMA	Preclinical	[4]
Antibody	Intact IgG	PSMA	Preclinical	[39]
Antibody	Humanized intact IgG	PSMA	Preclinical	[40]
Antibody	Minibody	Prostate stem cell antigen	Preclinical	[8]
Antibody	Humanized intact IgG	Epidermal growth factor receptor	Preclinical	[41]
Antibody	Intact IgG	Vascular endothelial growth factor receptor-3	Preclinical	[41]
Antibody	Intact IgG	Vascular endothelial growth factor receptor-2	Preclinical	[42]
Antibody	Diabody	Epithelial cell adhesion molecule	Preclinical	[43]
Antibody	Intact IgG	vascular cell adhesion molecule-1	Preclinical	[44]
Antibody	Intact IgG	CD133/Prominin	Preclinical	[45]
Antibody	Minibody/scFv-Fc	CD20	Preclinical	[9]
Antibody	Intact IgG	Lymphatic vessel endothelial hyaluronan receptor-1	Preclinical	[46**]
Nanobody		Insulin-like growth factor-binding protein 7	Preclinical	[47]
Nanobody	Multimer	Epidermal growth factor receptor	Preclinical	[48]
Nanobody	Humanized nanobody	Carcino embryonic antigen	Preclinical	[14]
Nanobody		dendritic cells	Preclinical	[15]
Affibody		HER2	Clinical	[18**]
Affibody		Epidermal growth factor receptor	Preclinical	[49]
DARPin		HER2	Preclinical	[19**]

region of light and heavy chains genetically linked by a flexible polypeptide. scFvs can be expressed in large quantities in heterologous systems and provide fast tissue penetration. However, the monovalency of scFvs and an accelerated blood clearance can be substantially disadvantageous. Therefore, scFvs are used as building blocks to generate bimeric, trimeric or multimeric antibody derivatives like diabodies (~60 kDa) and minibodies (~80 kDa) comprising a scFv and a single constant domain (C_H3) or scFv-Fc (~105 kDa) containing an intact Fc region, as reviewed in [5**] (Figure 1).

In a recent study, imaging behaviour of a minibody (scFv-CH3) against Prostate stem cell antigen (PSCA) was compared to the parental intact antibody. Using the minibody, best imaging results were obtained 21 h after tracer injection compared to one week using the intact antibody. However, the minibody showed a nine-fold decrease in affinity when used *in vitro* [6,7]. In a follow up study, the binding affinities of minibodies were engineered by error prone PCR and selection. Although three of the minibody candidates exhibited higher affinities, only one of them exhibited improved qualities for *in vivo* imaging and is currently being tested in a clinical imaging study [8].

In a second study, ¹²⁴I labelled minibodies (scFv-CH3) and scFv-Fc fragments based on the anti-CD-20 monoclonal antibody (rituximab) were generated. Reliable imaging of CD20-positive B-cell lymphoma xenografts by PET was possible 21 h after tracer injection with both fragments [9].

Intermediate size fragments like minibodies and scFv-CH3 exhibit faster blood clearance than intact antibodies, but are still above the renal clearance limit (~60 kDa). Same day imaging requires even smaller antibody fragments that are below the renal clearance limit, in order to achieve faster blood clearance and higher tumour to blood ratios in shorter time. High quality imaging results could be obtained after 8 h using iodinated diabodies against CD20. Diabodies were either generated by introducing a short linker between VH and VL of a scFv (scFv-8) resulting in dimer formation of two scFvs, or by introducing a C-terminal cysteine at the scFv that allowed for disulfide bridge formation [10] (Figure 1).

Single domain antibodies

Up to now, single domain antibodies (sdAbs) are the smallest engineered antibody fragments still capable of binding antigens. sdAbs consist of only the variable domain of light chain or heavy chain. Naturally occurring antibodies consisting of heavy chains only were detected in camelids [11] and cartilaginous fish [12]. Since their discovery, camelid sdAbs (nanobodies) have been evaluated for binding of their targets *in vitro* as well as *in vivo* (reviewed by Vaneycken *et al.*, this issue). Because of their small size and high tumour penetration rate several nanobodies were already developed for diagnostic

purposes (see Table 1). Since low immunogenicity is highly important for clinical imaging in patients, the effect of humanization of nanobodies on imaging quality was investigated. In a proof of principle study, three CDRs of a nanobody against CEA were grafted onto a humanized antibody scaffold [13]. Although humanized CEA nanobody showed a 30-fold decrease in affinity compared to the parental nanobody, no quality difference of imaging data between parental anti-CEA and humanized anti-CEA nanobody was observed [14].

In vivo imaging is not only suitable for imaging of cancer, but it is also of great interest to image immune and inflammatory diseases. In a proof of principle study a nanobody against dendritic cells labelled with ^{99m}Tc was injected into healthy mice and animals were imaged 2 h after injection. A clear enrichment at locations of dendritic cells was observed, demonstrating that nanobodies can be used for imaging the distribution of specific cell types [15].

Engineered protein scaffolds for *in vivo* imaging

As an alternative to engineered antibody formats, recombinant DNA technology enables the development of new classes of binding proteins (reviewed in [16**]). Two prominent examples for protein scaffolds are affibodies and designed ankyrin repeat proteins (DARPs) (reviewed by Löfblom *et al.*, and Boersma *et al.*, this issue).

Probably the best-studied affibody is one that targets the human epidermal growth factor receptor 2 (HER2), a tumour marker overexpressed in breast cancer. In 2006, the HER2 affibody was described for imaging of breast cancer xenografts [17], and in 2010 it was shown that HER2 affibodies labelled with ¹¹¹In and ⁶⁸Ga are suitable for *in vivo* imaging of breast cancer patients with SPECT and PET [18**].

Besides affibodies, also DARPs have become highly interesting for molecular imaging. In a proof of principle study, a ^{99m}Tc labelled DARPin against HER2 was used for *in vivo* imaging studies. The monovalent DARPin (14.5 kDa) showed high accumulation of radioactivity in the tumour. To test the influence of the molecular weight on imaging properties a bivalent DARPin (30 kDa) and a PEGylated DARPin (over 300 kDa) were also generated. Bivalency of the DARPin resulted in lower accumulation of radioactivity in the tumour, whereas the PEGylated DARPin showed higher tumour accumulation. However, because of the slower elimination of the PEGylated DARPin from blood, the tumour to tissue ratio remained lower compared to the monovalent DARPin [19**].

Intracellular antibodies

Understanding of cellular processes and their pathophysiology requires not only information on biomarkers

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exposed on the cellular surface but also information on abundance, localization, post-translational modifications and dynamic interactions of intracellular components. Antibodies, commonly used for detection of cellular components, for example, by immunofluorescence, cannot be used to target their antigen in living cells as their application is limited by impaired disulfide bond formation in the reducing environment of the cytoplasm, difficult subcellular targeting, and inefficient assembly of epitope recognizing parts of the variable light and heavy chain [20,21].

However, methods like 3-SPLINT (Single Pot Library of Intracellular Antibodies) or IACT (Intracellular Antibody Capture Technology) were developed to select functional intrabodies [22–24]. Improvement of IACT (IAC³) allows direct selection of intracellular functional single domain fragments. IAC³ is based on a modified yeast two hybrid assay expressing an antigen-bait fused to the DNA-binding domain and a library of single domain antibody fragments (VH or VL) fused to the transcriptional activation domain. Interaction of the antigen with corresponding binding domains in the cytoplasm of yeast cells activates reporter genes which enables the isolation of the yeast cells carrying the cDNA encoding the antibody fragment [25]. This technique allows intracellular screening of a series of VH and VL libraries with randomized CDRs in a high throughput fashion without preparation of native antigen *in vitro*. In a recent study the IAC³ screen was applied to isolate a VH binding to Lmo2. After intracellular expression in cultivated murine erythroleukemia (MEL) cells this VH interferes with Lmo2 function in erythropoiesis by blocking the Lmo2-Lbd1 interaction [26]. Recently, several other studies have demonstrated an inhibitory or modulating effect of intracellular expressed scFvs [27,28] or nanobodies (V_HH fragments) [29,30] on their target structure in living cells.

Live cell imaging

Live cell fluorescence imaging using fluorescent protein-tagged reporters is widely used to study protein localization and has provided unique information on protein dynamics in living cells [31,32]. However, only artificially introduced chimeric proteins are visualized, whereas endogenous proteins, their post-translational modifications, and non-protein components of the cell remain invisible.

Expression of antibody fragments in mammalian cells coupled to fluorescent reporters now opens entirely new possibilities for imaging dynamic changes of intracellular targets in real time. In a proof of principle study, intracellular expressed V_HH fragments fused to fluorescent proteins, named chromobodies, were used to visualize endogenous proteins without affecting the cell per se [33]. Because of this good biocompatibility cell lines stably expressing chromobodies, like a lamin-specific chromobody, could be generated. On the Basis

of the unique property to trace changes of the nuclear morphology for more than 72 h, a cell based apoptosis assay was developed visualizing the characteristic degradation of the nuclear lamina during apoptosis in real time [34]. The development of stable chromobody expressing cell lines allows visualization of a wide variety of intracellular parameters, including essential post-translational modifications, making chromobodies ideal tools for high-content analysis for compound screening and target validation during the early steps of drug discovery.

New strategies for selection and monitoring of biomarkers

In the light of a more personalized medicine, the detection of low abundant cellular factors including low expressed proteins, post-translational modifications and transient protein–protein interactions has a significant potential for early diagnostics and monitoring response to treatment. A promising approach herein is the proximity ligation assay (PLA) developed by Landegren and co-workers [35]. The PLA is based on the principle that a target protein or a protein–protein interaction is recognized by two or more affinity probes (typically antibodies) coupled to short stretches of DNA. Upon binding of the affinity probes the DNA strands come into proximity and participate in ligation reactions, which then can be amplified by PCR. The specific amplification of DNA provides a highly sensitive read-out to detect proteins in complex biological material like serum or tissue samples. It was demonstrated that applying the PLA, the cytokine platelet-derived growth factor (PDGF)-BB in human serum can be detected with a 1000 fold higher sensitivity compared to a conventional ELISA assay [35]. For imaging purposes, an *in situ* approach of the PLA has been developed. The binding of two affinity probes guides the formation of a circular DNA strand which then can be replicated by rolling circle amplification into a bundle of DNA capable to bind fluorescently labelled oligonucleotides complementary to the replicated DNA strand. In a proof of principle study the influence of interferon- γ (INF- γ) on the interaction of endogenous transcription factors c-Myc and Max was visualized in fixed cultured cells [36]. The combination of specific antibody binding and DNA amplification can now be used to measure new classes of plasma biomarkers and to image the distribution of low abundant proteins in cell lines and tissues for diagnostic purposes [37].

Conclusion

Recent progress in genome and proteome technologies has provided and will provide a plethora of data on molecular changes in diseased cells, tissues, and organisms. This progress is particularly evident in cancer biology, where thousands of whole cancer cell genomes with complete gene expression profiles are being compiled and compared. Leads from these genome and proteome screens are currently validated to determine their suitability as biomarker for specific pathological changes.

The challenge is now to generate optimized reagents that in combination with new imaging modalities are able to reliably discriminate between normal physiological variations and pathological changes in cells and tissues. On the imaging side, impressive progress has been made from whole body scans down to super-resolutions microscopy of subcellular structures [38]. On the reagent side, the antibody IgG format was long considered as the versatile key for *in vivo* imaging. However, low binding specificity, insufficient blood clearance, impaired tissue penetration and immunogenicity often turned out to be problematic. Recombinant protein engineering has led to the development of smaller antibody fragments and novel types of versatile binding molecules and has opened new possibilities. Specific binding proteins can be identified from recombinant libraries by efficient display and selection technologies, easily produced in microbial expression systems like bacteria or yeast, and directly coupled to detectable markers including radionuclides, dyes or fluorescent proteins.

The studies presented here impressively demonstrate that critical biochemical properties of protein reagents like affinity, specificity, immunogenicity, and blood clearance can be improved with current protein engineering technologies. However, safety issues remain formidable challenges delaying a broad application in human medicine. Especially off target effects are notoriously hard to predict and cannot simply be extrapolated from animal studies as painfully learnt from therapeutic antibodies applications. However, the benefits of early detection and accurate classification of pathological changes enabling targeted and minimal invasive therapies clearly justify the necessary research and development efforts and will undoubtedly lead to a broader application of molecular imaging in human healthcare.

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