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REVIEW ARTICLE

Protein scaffold-based molecular probes for cancer molecular imaging

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Abstract Protein scaffold molecules are powerful reagents for targeting various cell signal receptors, enzymes, cytokines and other cancer-related molecules. They belong to the peptide and small protein platform with distinct properties. For the purpose of development of new generation molecular probes, various protein scaffold molecules have been labeled with imaging moieties and evaluated both in vitro and in vivo. Among the evaluated probes Affibody molecules and analogs, cystine knot peptides, and nanobodies have shown especially good characteristics as protein scaffold platforms for development of in vivo molecular probes. Quantitative data obtained from positron emission tomography, single photon emission computed tomography/CT, and optical imaging together with biodistribution studies have shown high tumor uptakes and high tumor-to-blood ratios for these probes. High tumor contrast imaging has been obtained within 1 h after injection. The success of those molecular probes demonstrates the adequacy of protein scaffold strategy as a general approach in molecular probe development.

Keywords Protein scaffolds · Peptide, molecular imaging · Cancer · PET

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Introduction

Molecular imaging is a relatively new emerging research field, which has demonstrated great potential for improvement of healthcare in clinic, especially in clinical oncology-from drug development and cancer early detection to cancer patient prognosis and stratification for effective therapeutic regimens. Molecular imaging brings together knowledge in imaging, oncology, immunology, biology, chemistry, engineering, computational sciences, etc., and aims at early detection and guided therapy. Molecular probes are one of the major driving force of the molecular imaging research (Massoud and Gambhir 2003; Gambhir 2002). They are the agents used to visualize, characterize and measure biological processes in living systems.

Many different types of platforms have been explored in molecular probe discovery, including ions, small molecules, peptides, proteins, aptamers, nanoparticles, etc. The size of these molecules varies from sub-nanometer (nm) as in ions to a few micrometers as in microbubbles (Fig. 1). Small proteins and peptides are low nm-sized molecules with favorable pharmacokinetics for molecular imaging (Signore et al. 2001; Ladner 1999). The small proteins and peptides typically show rapid clearance from blood, non-target tissues and the body, leading to a low background signal. Their clearance rate and route of excretion can be optimized by modification of the protein sequence and labeling strategies. The small protein and peptide-based

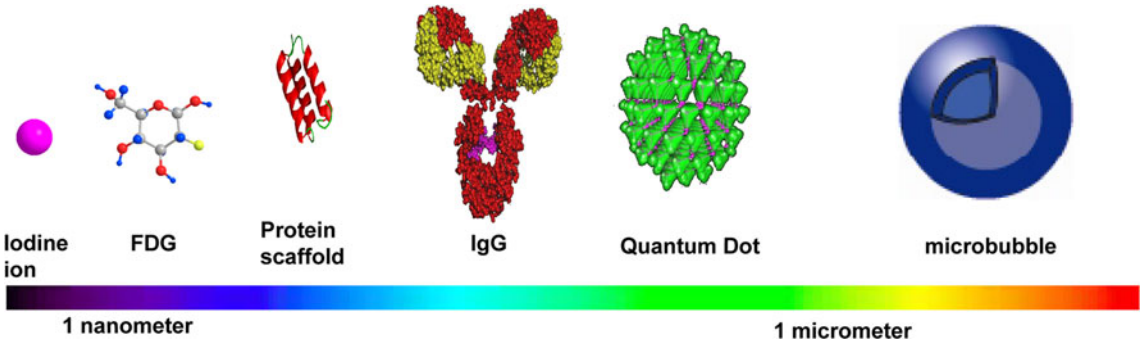

Fig. 1 Representative imaging probes with various size based on different platform (*FDG*: abbrev of fluorodeoxyglucose) (objects are not drawn in proportional size)

molecular platforms usually possess high tumor penetration ability, low toxicity, and low immunogenicity because of its relatively small size. They can be chemically or biologically synthesized and modified with different reporting moieties. With many desirable properties, small proteins and peptides represent excellent molecular platforms for molecular probe development.

Recently, significant advancement has been made in the in vitro display technologies (phage, bacterial, yeast, ribosome and mRNA displayed technology) and rational protein engineering. Many novel protein scaffolds that specifically bind to targeted biomarkers have been discovered (Rothe et al. 2006; Hosse et al. 2006; Uchiyama et al. 2005; Nygren and Skerra 2004; Binz et al. 2004). A common feature of these protein scaffolds is that they are highly structured, generally displayed fast in vivo clearance, rapid tumor accumulation, sufficient in vivo stability and good bioavailability, and low immunogenicity and toxicity (Orlova et al. 2006a; Gill and Damle 2006). Protein mutants derived from these scaffolds that recognize different biomarkers can be obtained in relatively short time, by evolution directed selection of a few amino acid residues at the binding loop or binding surface of a protein (Orlova et al. 2006a; Gill and Damle 2006).

The in vivo behavior of these mutants is likely to have many similarities with the parent protein they are derived from. The versatile production, consistent in vivo performance, and ability to recognize a variety of biomarkers highlight the importance of using protein scaffold mutants for developing probes for molecular imaging. In this article, we wish to provide a brief review of protein scaffolds as a universal platform for molecular probe development and their application in cancer imaging.

Protein scaffolds

Protein scaffold is a constrained polypeptide consisted of either α-helix or β-sheet, with a size of 3–20 kD. Different regions of protein scaffolds like α-helix (shown in red), β-sheets (shown in yellow) or loops (shown in green) have been used for engineering and in vitro display selection against molecular targets (Fig. 2). Recently, protein scaffold-based molecules against cancer biomarkers such as receptor tyrosine kinases (HER2, EGFR), carcinoembryonic antigen (CEA), Tumor Necrosis Factor Alpha (TNF), integrin $\alpha_v\beta_3$, etc., with low nanomolar and even picomolar affinity have been discovered (Table 1).

Single domain immunoglobin is a class of protein scaffold present in the human single domain antibody (sdAb) and camelidae antibody variable domain (nanobody). Antibody fragments and variants represent a new direction in molecular probe development that promises great improvement over well established antibody platform (Wu and Senter 2005). Nanobodies are small protein scaffolds which can be easily derived from the single variable domain (VHH) of camelidae antibody, because camelidae antibody lacks light chain and has only two heavy chain containing a VHH and two constant domains. Nanobody technology is basically an in vitro technology, even though it requires the immunization of a dromedary. Cloning of repertoire of the variable domain of camelid heavy chain can be conducted to construct a library for display selection (Arbabi Ghahroudi et al. 1997). High affinity nanobodies have also made directly from phage display with VHH gene repertoires without immunizing a dromedary (Goldman et al. 2006).

Nanobodies have unique structural and functional properties. They have 7-β sheets forming a sandwich structure and preserve the full antigen binding capability. Nanobodies have demonstrated high target specificity and affinity (Huang et al. 2008). Moreover, camelidae antibody variable domain has very high homology with human IgG heavy chain variable fragment, thus it has low immunogenicity (Harmsen and De Haard 2007). In addition, the single domain feature of nanobody makes further manipulation feasible, such as production of multivalent formats and bispecific nanobody.

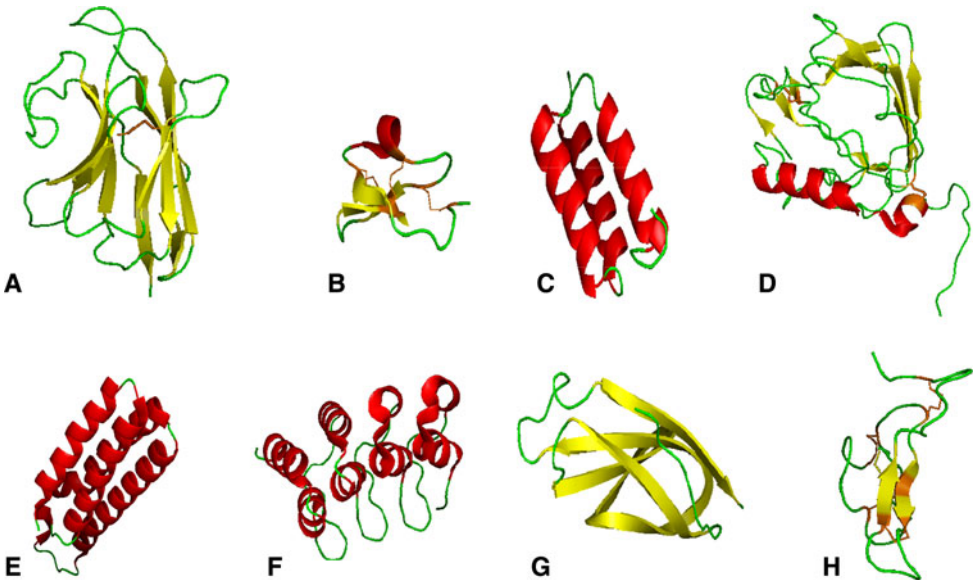

Fig. 2 Structure cartoons of representative protein scaffolds. Various parts of protein scaffolds like α-helix (*red*), β-sheets (*yellow*) or loops (*green*) can be used for in vitro display selection. Disulfide bridges are shown as *orange lines*. The PDB IDs used to generate this figure are given in *parentheses*: **a** nanobody [1QD0], **b** EETI II [2IT7], **c** Affibody [1Q2N], **d** anticalin [1T0V], **e** cytochrome *b*-562 [1M6T], **f** DARPins [1N0R], **g** 10 Fn-III [1FNA], **h** AgRP [1HYK]

Non-immunoglobin-based scaffolds are present in novel scaffold proteins such as Affibody and its analog 2-helix small protein (Orlova et al. 2006a; Webster et al. 2009), designed ankyrin repeat proteins (DARPs) (Binz et al. 2004), and 10-FnIII (Xu et al. 2002), Lipocalin (called anticalin when used for protein engineering) (Vogt and Skerra 2004; Lamla and Erdmann 2003, 2004) and TEM-1 β-lactamase (Legendre et al. 2002), etc. (Table 1). Affibody, 2-helix small protein and DARPs are made of multiple α-helixes, while 10-FnIII has a β-sandwich structure similar to immunoglobin folding. Affibody proteins are derived from one of the IgG-binding domains of staphylococcal protein A (SPA). These molecules are composed of a relatively small engineered protein scaffold with 58-amino acid residues and a three-helical bundle scaffold structure. Library of Affibody mutants was made by randomizing up to 13 surface residues on two α-helixes. First round bio-panning selection produces nanomolar affinity molecules. Maturation of moderate affinity molecules is done by construction of a narrower library with certain position bias and selection under more stringent conditions. Lower nanomolar and even picomolar affinity molecules have been made after two rounds of selections (Orlova et al. 2006a; Xu et al. 2002; Binz et al. 2004). Two-helix analogs are similar to Affibody molecules. They have similar binding surface as three-helix Affibody molecules, but the backbone of the proteins is stabilized with disulfide bond and stiffened by enhancing helix structure (Webster et al. 2009). Unlike small peptide, 10-Fn3, DARPs and Affibody protein have excellent thermo-stability and resistance to enzyme degradation, therefore there is a higher chance to obtain high affinity mutant (Xu et al. 2002; Ekblad et al. 2008).

Some small protein scaffolds have rigid structural frameworks and stable cores, and they tolerate grafting of small bioactive motifs in loops. The flanking sequence of the loop can be used for further affinity and selectivity maturation. The examples of such scaffolds are the cystine knot peptides (knottin) derived from Ecballium elaterium trypsin inhibitor II (EETI II) and agouti-related protein (AgRP) (Kimura et al. 2009a, b, c; Silverman et al. 2009), α-amylase inhibitor tendamistat (Li et al. 2003), etc. (Table 1). Cystine knot has some advantages over tendamistat for peptide scaffold molecules production, such as easier chemically synthesis due to the smaller size (3–4 vs. 9 kD) and toleration of mutations at multiple loops. High affinity scaffold molecules (integrin $\alpha_v\beta_3$ specific, $K_D = 0.7$ nM) have been achieved using the AgRP scaffolds (Silverman et al. 2009) with yeast display technology, while phage display of tendamistat scaffold only produced moderate affinity integrin targeting molecules with poor selectivity of integrin subtypes (Li et al. 2003). Other small protein scaffolds such as cyclotides MCoTI-I/II also have stable core structure and tolerate mutations for construction of combinatorial libraries. Cyclotides have been widely used for selection of inhibitors of inflammatory diseases. But they have not yet been directly used for cancer-targeted applications (Austin et al. 2009; Thongyoo et al. 2009). It is worthy of further studies of this interesting protein scaffold for molecular probe.

Production of protein scaffolds

Protein scaffolds can be produced by either chemical or biological approaches. For some protein scaffolds with

Table 1 Display selected and rational design engineered protein scaffold molecules

Protein scaffold	Core structure	Variable regions	Targeting molecule	Highest affinity	Selected references
Affibody	3-α helixes	13 Residues on first and second helix surface	EGFR, HER2, TNF-alpha, amyloid, HSA, fibrinogen, etc.	50 fM	Jonsson et al. (2008, 2009), Orlova et al. (2006a)
2-Helix small protein	2-α helixes	13 Residues on 2-α helixes surface	HER2	5 nM	Webster et al. (2009)
Nanobody (camelid heavy variable domain)	β-Sheet sandwich	CDR 1, 2, 3	EGFR, CEA, etc.	100 pM	Huang et al. (2008)
dAbs (human VH or VL single domain)	β-Sheet sandwich	CDR 1, 2, 3	Viral antigen B5R	~1 nM	Chen et al. (2008)
Cystine knot, squirting cucumber Ecballium elaterium (EETI II)	A triple-stranded β sheet fold and three disulfide bond	1–2 Loops	Integrin αvβ3, αvβ5, α5β1	13 nM	Kimura et al. (2009a)
Cystine knot, agouti-related protein (AgRP)	A double-stranded β sheet and four disulfide bond	1–2 Loops	Integrin αvβ3	0.7 nM	Silverman et al. (2009)
Cystine knot, cellulose binding domain	A triple-stranded β-sheet	11 Residues distributed over β-sheets and loops	α-Amylase	15 μM	Lehtio et al. (2000)
Cytochrome b-562	4-α helixes	2 Loops	BSA	5 μM	Ku and Schultz (1995)
Designed ankyrin repeat proteins (DARPins)	β-Hairpin-helix-loop-helix (repeat)	β-Turn, 1 α-helix and loop	Intracellular kinase	0.5 nM	Amstutz et al. (2005)
Kunitz domain inhibitors, BPTI/APPI	α-Helixes, β-sheets	1–2 Loops	Neutrophil elastase	1 pM	Roberts et al. (1992)
PDZ domain, ras-binding protein AF-6	α-Helixes, β-sheets	Entire domain	C-terminal peptide	160 nM	Schneider et al. (1999)
10-FnIII	β-Sheet sandwich	2–3 Loops	TNF-alpha, VEGFR, etc.	20 pM	Xu et al. (2002); Getmanova et al. (2006)
Neocarzinostatin	β-Sheet sandwich	Up to 13 residues pointing toward the binding crevice	Enediyne	20 nM	Heyd et al. (2003)
Anticalins, Apolipoprotein D	β-Barrel and loops	24 Residues in 4 loops	Hemoglobin	2.1 μM	Vogt and Skerra (2004)
Anticalins, FABP	β-Barrel and loops	N terminus	Streptavidin	4 nM	Lamla and Erdmann (2003, 2004)
α-Amylase inhibitor tendamistat	β-Sheet sandwich	Loop 1	Integrin	N/A	Li et al. (2003)
TEM β-lactamase	α-Helixes, β-sheets	1–2 Loops	Ferritin and β-galactosidase	10 nM	Legendre et al. (2002)

small size, both methods can be used. Because the display selection provides the genetic information of the DNA template, the high affinity scaffold molecules identified can usually be produced in large quantity by regular recombinant techniques. Many scaffold proteins such as Affibody, Nanobodies, DARPins can be generated in *E. coli*, which makes the production relatively easy and cheap compared with complicated antibody production. Usually a His-6 tag was added to the sequence of scaffold proteins for nickel column purification under denatured conditions. The added His-6 can also serve as a site-specific labeling moiety. Besides in *E. coli*, nanobodies can also be expressed in yeast *Saccharomyces cerevisiae* (Harmsen and De Haard 2007). More complicated post-transcriptional modification in yeast could be a disadvantage and maybe some times an advantage such as glycosylation. Since the folding of a protein is determined by the primary sequence, chemical synthesis of some of scaffold proteins is also feasible. Chemically synthesized Affibody and cystine knot peptides have retained binding affinity and specificity (Kimura et al. 2009a, b, c, Tran et al. 2007a, b). Orthogonally protected amino acids within the scaffold's

Table 2 Radio-scintigraphy imaging using protein scaffold-based probe

Radio-isotopes	Protein scaffold molecules	Tag	Targeting biomarker	Imaging modality	Reference
^{99m}Tc	$Z_{HER2:342}$	maEEE, CGG, CGGG	HER2	Gamma camera	Tran et al. (2007a, b)
	$Z_{HER2:342}$	His-6 tag	HER2	Gamma camera	Orlova et al. (2006b)
	$Z_{HER2:2395\text{-}Cys}$	GGC	HER2	Gamma camera	Ahlgren et al. (2009)
	EGFR Nanobody 8B6, 7D, 7C	His-6 tag	EGFR	SPECT, SPECT/CT	Huang et al. (2008), Gainkam et al. (2008)
	CEA1 Nanobody	His-6 tag	CEA	SPECT	Cortez-Retamozo et al. (2008)
^{111}In	$Z_{HER2:342}$	DOTA and DTPA	HER2	SPECT, gamma camera	Baum et al. (2006), Orlova et al. (2006a), Tolmachev et al. (2006)
	$Z_{HER2:2395\text{-}Cys}$	Maleimide derivative of DTPA and DOTA	HER2	Gamma camera	Tolmachev et al. (2008), Ahlgren et al. (2008)
	$Z_{EGFR:1907}$, $(Z_{EGFR:1907})_2$	DTPA	EGFR	Gamma camera	Tolmachev et al. (2009)
^{18}F	$Z_{HER2:477}$, $(Z_{HER2:477})_2$	4-Fluorobenzaldehyde	HER2	PET	Cheng et al. (2008)
	$Z_{HER2:342}$	4-FBEM	HER2	PET	Kramer-Marek et al. (2008)
^{124}I	$Z_{HER2:342}$	PIB	HER2	PET	Orlova et al. (2009)
^{68}Ga	$Z_{HER2:342}$	DOTA	HER2	PET/CT	Baum et al. (2006)
	2-Helix small protein (MUT-DS)	DOTA	HER2	PET	Ren et al. (2009)
^{64}Cu	Cystine knot 2.5D, 2.5F	DOTA	Integrin $\alpha_v\beta_3$	PET	Kimura et al. (2009a)
	Cystine knot, AgRP 7C	DOTA	Integrin $\alpha_v\beta_3$	PET	Jiang et al. (2010)
	$Z_{HER2:477}$, $(ZH_{ER2:477})_2$	Maleimide derivative of DOTA	HER2	PET	Cheng et al. (2009)
	$Z_{EGFR:1907}$	Maleimide derivative of DOTA	EGFR	PET	Miao et al. (2009b)

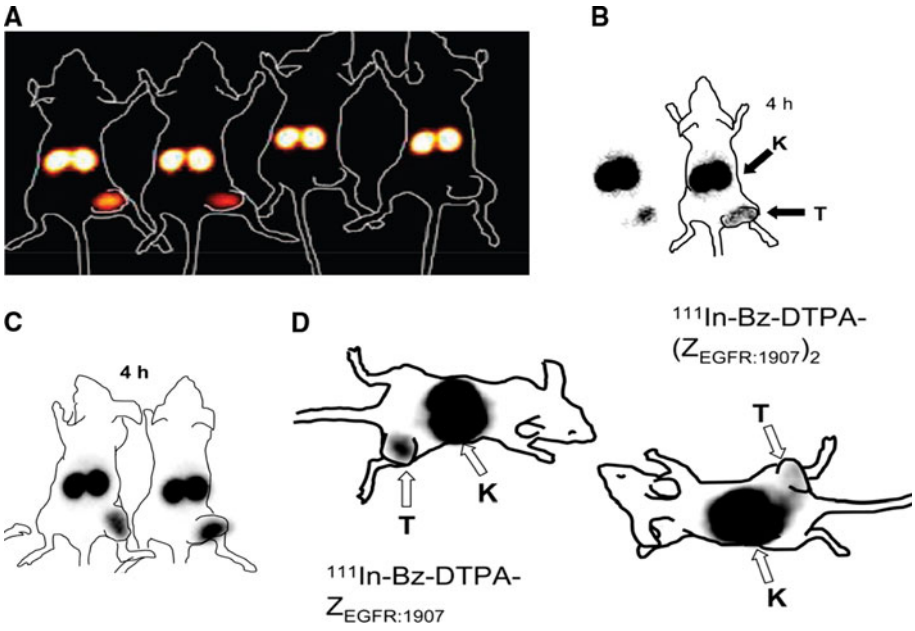

Fig. 3 Gamma-camera imaging with Affibody molecules based probes. **a** Imaging of HER2 expression in SKOV-3 xenograft in BALB/c nu/nu mice with ^{99m}Tc-maEEE-$Z_{HER2:342}$. **b**, **c** Imaging of HER2 expression in LS174T and SKOV-3 xenografts in BALB/c nu/nu mice with ^{99m}Tc-$Z_{HER2:2395\text{-}C}$. **d** Imaging of EGFR expression in A431 xenografts in BALB/c nude mice using ^{111}In-Bz-DTPA-$Z_{EGFR:1907}$ (*left*) and ^{111}In-Bz-DTPA-$(Z_{EGFR:1907})_2$ (*right*) (Tran et al. 2007a; Ahlgren et al. 2009; Tolmachev et al. 2009)

sequence can be used for site-specific modifications of protein scaffold molecule with multiple imaging moieties or with fluorescence resonance energy transfer (FRET) acceptor and donor. Thus they are potentially useful for multimodality imaging and smart probe development (Engfeldt et al. 2005).

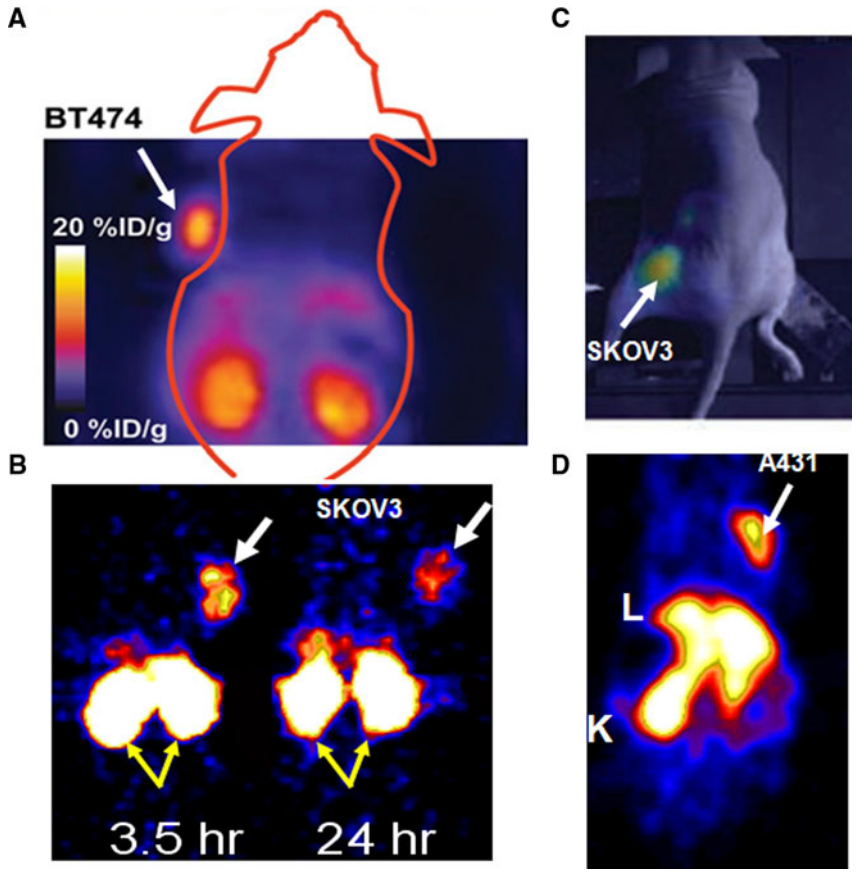

Fig. 4 PET and optical imaging with Affibody molecule-based probes. Imaging of HER2 expression in BT474 xenografts in BALB/c nu/nu mice with **a** ^{18}F-FBEM-$Z_{HER2:342}$. Imaging of HER2 expression in SKOV-3 xenografts in BALB/c nu/nu mice with **b** ^{64}Cu-DOTA-$Z_{HER2:477}$ and **c** ABD-$(Z_{HER2:342})_2$-AlexaFluo750. **d** Imaging EGFR expression in A431 xenografts in BALB/c nu/nu mice with ^{64}Cu-DOTA-$Z_{EGFR:1907}$. *Arrows* are pointed at tumors (Kramer-Marek et al. 2009; Cheng et al. 2009; Lee et al. 2008; Miao et al. 2009b)

Protein scaffold-based molecular probes

Affibody molecules and analogs

Protein scaffold as a platform for molecular probe development has been tested with various imaging modalities, especially for single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging (Table 2). Currently, Affibody molecules are the dominant protein scaffold used in imaging applications. The Affibody scaffold typically shows high in vivo stability, good and fast tumor targeting ability, high kidney uptake and low uptake in other normal tissues. HER2 Affibody molecules labeled with ^{99m}Tc and ^{111}In have been successfully used for planar scintigraphy and SPECT imaging (Tran et al. 2007a, b; Ahlgren et al. 2009; Tolmachev et al. 2009) (Fig. 3a–c). Several radiolabeled Affibody proteins such as ^{99m}Tc-maEEE-$Z_{HER2:342}$, ^{99m}Tc-$Z_{HER2:2395\text{-}C}$, ^{111}In-CHX-A″-DTPA-$Z_{HER22395\text{-}C}$ all give high imaging contrast at as early as 1 h post-injection (p.i.) in SKOV3 tumor model. Among them, ^{99m}Tc-$Z_{HER2:2395\text{-}Cys}$ has the highest tumor-to-blood ratio (121 ± 24 at 4 h p.i.). It also shows high kidney accumulation, moderate liver uptake and low uptakes in all other organs. In LS174T colon cancer tumor model with moderate HER2 expression, ^{99m}Tc-$Z_{HER2:2395\text{-}Cys}$ also shows good imaging with tumor-to-blood ratio of 88 ± 24 (Ahlgren et al. 2009) (Fig. 3c).

^{18}F-labeled anti-HER2 Affibody molecules ^{18}F-FBO-$Z_{HER2:477}$ and ^{18}F-FBEM-$Z_{HER2:342}$ have also been prepared using site-specific labeling techniques (Cheng et al. 2008; Kramer-Marek et al. 2009) (Fig. 4a). Biodistribution and microPET imaging studies further show that these probes have rapid, high and specific accumulation in HER2 over-expressing SKOV3 tumor. Good tumor-to-blood and tumor-to-muscle ratios, while relatively fast clearance through kidney have also been observed for these probes, demonstrating that ^{18}F-labeled anti-HER2 Affibody molecules are a promising new class of HER2 PET probes. More importantly, changes in HER2 expression have been quantified by PET using the ^{18}F-FBZM-$Z_{HER2:342}$ (Kramer-Marek et al. 2009). Quantitative PET imaging analysis of 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG)-treated mice-bearing MCF7/clone18 and BT474 xenograft shows significantly reduced tumor uptake compared with tumor uptake of untreated mice. In vitro analysis including western blot and ELISA confirms the reduced HER2 expression (Kramer-Marek et al. 2009). Other HER2 targeting probes such as ^{124}I-, ^{64}Cu-labeled anti-HER2 Affibody (Z_{HER2}) and Alexa-750 labeled Z_{HER2}-ABD fusion

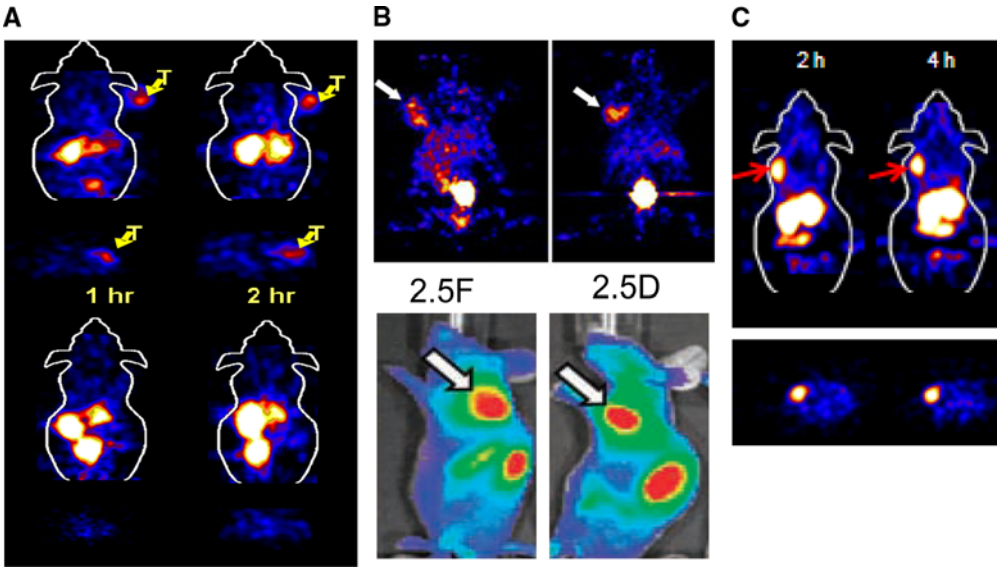

Fig. 5 PET and optical imaging with 2-helix small proteins, cystine knot EETI and AgRPs based probes. **a** Monitoring Her2 expression reduction in SKOV3 xenografts in BALB/c nu/nu mice treated with (*bottom*) or without (*top*) 17-DMAG. Imaging of integrins expression in U87MG xenografts in BALB/c nu/nu mice with (**b**) ^{64}Cu-DOTA-2.5F, ^{64}Cu-DOTA-2.5D (*top*), Cy5.5-2.5F, Cy5.5-2.5D (*bottom*) and **c** ^{64}Cu-DOTA-AgRP-7C. *Arrows* are pointed at tumors (Ren et al. 2009; Kimura et al. 2009a; Jiang et al. 2010)

proteins have also been evaluated for microPET imaging in a BT474 tumor model and optical imaging in SKOV3 tumor model, respectively (Orlova et al. 2009; Cheng et al. 2009; Lee et al. 2008) (Fig. 4b, c). Excellent tumor imaging contrast is observed at a later time point (6–24 h), while most of the probe was cleared out from normal tissues at 24 h p.i. (except kidney) (Lee et al. 2008) (Fig. 4c).

Affibody-based molecular probes for EGFR imaging have also been developed. Planar scintigraphy studies of anti-EGFR Affibody-based probes, ^{111}In-Bz-DTPA-$Z_{EGFR:1907}$ and ^{111}In-Bz-DTPA-$(Z_{EGFR:1907})_2$, have shown that they can be used for imaging EGFR positive tumors (Tolmachev et al. 2009) (Fig. 3d). The ^{111}In-Bz-DTPA-$Z_{EGFR:1907}$ displays similar in vivo behaviors as radiolabeled anti-HER2 Affibody-based probes such as quick tumor targeting and high kidney accumulations (Tolmachev et al. 2008). Anti-EGFR Affibody molecule tagged with long-lived radionuclide ^{64}Cu has also been evaluated with microPET in an A431 tumor model. This probe shows good tumor contrast and fast clearance through liver and kidney at early time point (Miao et al. 2009b) (Fig. 4d).

It has been found in several studies that monomeric Affibody protein constructs show high tumor uptake in addition to faster clearance from normal tissues and thus exhibit better in vivo performance than their dimeric counterparts (Cheng et al. 2008, 2009; Tolmachev et al. 2009). For an example, compared with monomeric ^{18}F-FBO-$Z_{HER2:477}$, dimeric ^{18}F-FBO-$(Z_{HER2:477})_2$ has much lower tumor contrast. Using dimeric construct tumor was barely visible at an early time point and biodistribution study showed that tumor uptake is much lower than in monomeric ^{18}F-FBO-$Z_{HER2:477}$ (2.03 ± 0.31 vs. 4.77 ± 0.78%ID/g, 0.5 h p.i) (Cheng et al. 2008). In an effort to create even smaller versions of Affibody construct, the α-helix not responsible for receptor recognition has been truncated. Only the binding domain, composed of surface-exposed amino acid residues in the two α-helix bundles of Affibody is preserved. A number of both sequence mutations and synthetic strategies have been developed to optimize the affinity of the 2-helix small protein against HER2. Several constrained 2-helix constructs with high (low nM) HER2 affinity were successfully identified (Webster et al. 2009). Small animal PET imaging of HER2 expression has been achieved with a two-helix probe ^{68}Ga-DOTA-MUT-DS (Ren et al. 2009). Two-helix small protein has showed some favorable properties. At 1 h p.i., the tumor and liver uptake for ^{68}Ga-DOTA-MUT-DS are 4.04 ± 0.24 and 1.08 ± 0.08%ID/g, respectively. Both biodistribution data and western blot confirmed PET imaging quantification of HER2 expression (Ren et al. 2009) (Fig. 5a). Further exploration of this novel scaffold is underway.

Cystine knot proteins

Cystine knot protein mutants from Ecballium elaterium trypsin inhibitor II (EETI II) have three disulfide bonds and have excellent thermo-stability. Integrin targeting cystine

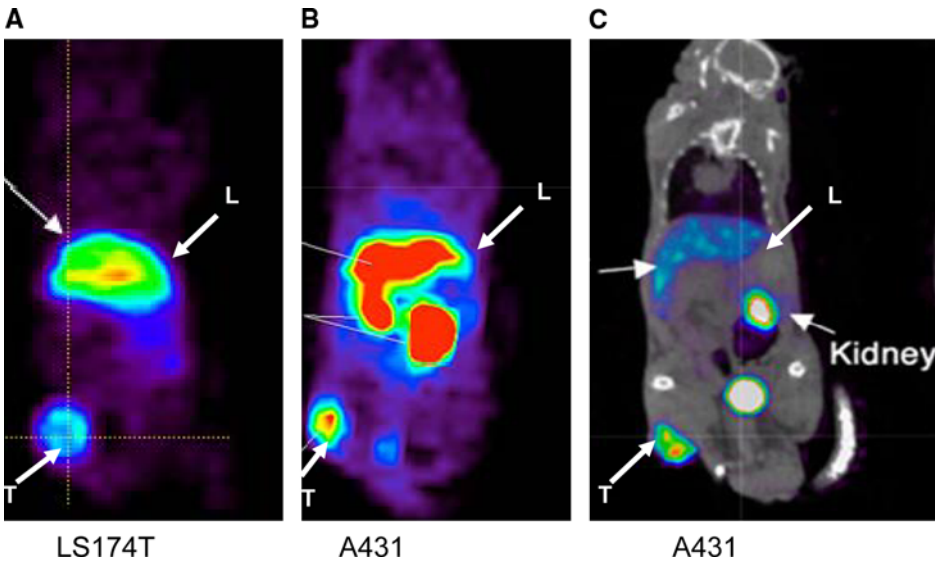

Fig. 6 SPECT and SPECT/CT imaging with nanobody based probes. **a** Imaging of carcinoembryonic (CEA) expression in LS174T xenografts in BALB/c nu/nu mice with ^{99m}Tc-His$_6$-CEA-nanobody. **b** Imaging of EGFR expression in A431 xenografts in BALB/c nu/nu mice with ^{99m}Tc-His$_6$-Nanobody-8B6. **c** Fused SPECT/CT imaging of EGFR expression in A431 xenografts in BALB/c nu/nu mice with ^{99m}Tc-His$_6$-nanobody-7C (*L* liver, *T* tumor) (Huang et al. 2008; Gainkam et al. 2008)

knot proteins have been discovered using yeast display technology (Kimura et al. 2009a). They have been labeled with ^{64}Cu-DOTA and Cy5.5 for microPET and optical imaging, respectively (Kimura et al. 2009a) (Fig. 5b). The labeling was done at the N-terminal amine of peptide which did not affect the binding affinity. This was further confirmed in a recent study of ^{18}F-labeled cystine knot peptide and dual-labeled cystine knot peptide (Miao et al. 2009a; Kimura et al. 2009c). High contrast imaging was observed by in vivo optical imaging and microPET imaging with two cystine knot EETI II mutants 2.5D- and 2.5F-based probes. Interestingly, 2.5D had a quick wash out from the body while 2.5F showed good tumor retention (Kimura et al. 2009a) because 2.5D and 2.5F have different flanking sequence in the binding loops. ^{18}F-labeled cysteine knot peptide 2.5D was washed out even faster, with only ~2%ID/g in tumor at 0.5 h p.i. (Miao et al. 2009a). Dynamic scanning of ^{18}F-2.5D showed it filtrate through kidney to urine very quickly. Correlation study of Cy5.5 and ^{64}Cu dual-labeled cystine knot peptide showed good correlation of PET and optical imaging result in tumor and normal tissues, suggesting potential translational evaluation and clinical application of dual-labeled probe for PET imaging and optical imaging guided surgery. It also showed that the lipophilicity of Cy5.5 can dominate the biodistribution of labeled cystine knot peptide. Another cystine knot protein scaffold, agouti-related protein (AgRP), showed similar pharmacokinetics. AgRP mutant 7C with high affinity and selectivity to $\alpha_v\beta_3$ was labeled with ^{64}Cu and evaluated by microPET (Jiang et al. 2010) (Fig. 5c). Excellent tumor imaging could also be obtained using this AgRP based probe. It is interesting to notice that AgRP-based probe shows significantly higher kidney uptake than that of EETI-based probe (Jiang et al. 2010). However, other properties of cystine knot peptide AgRP-7C, such as more selective binding to integrin $\alpha_v\beta_3$ and better retention in the tumor than cystine knot peptide 2.5D is making it a favorable probe for imaging purpose.

Nanobodies

Nanobodies have only recently caught attention for imaging probe development. A few nanobodies have shown good in vivo characteristics. Site-specific labeling of nanobodies was achieved by labeling a His-tag with $[^{99m}Tc(OH_2)_3(CO_3)_3]^+$. Imaging probes targeting cancer cell carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR, ErbB1) have been made with ^{99m}Tc-labeled nanobodies (Huang et al. 2008) (Fig. 6a, b). High liver uptake and high blood radioactivity was observed, which showed instability of both ^{99m}Tc complex of an EGFR targeting nanobody 8B6 and ^{99m}Tc complex of a CEA targeting nanobody mutant. A dual modality, pinhole SPECT/CT imaging with ^{99m}Tc-labeled EGFR nanobody mutant 7D and 7C showed improved imaging contrast (Gainkam et al. 2008) (Fig. 6c) compared to 8B6. Superior imaging results were obtained as early as 1 h after injection, and much lower background was observed in liver. Quantification of tumor-to-normal tissue ratio using pinhole SPECT/microCT is consistent with ex vivo biodistribution study. Good correlation was also observed for the tumor uptake and the tumor size. (Gainkam et al. 2008). In vivo molecular imaging could also be a more specific and sensitive diagnostic tool for CEA studies as an important biomarker compared with in vitro serum CEA expression analysis. For example, CEA is expressed at different level in hepatocellular carcinomas and in liver metastases (Di Carlo et al. 2001). Thus, molecular imaging of CEA expression with SPECT and SPECT/CT can help to identify both primary and metastasis tumor. It can also

help to select the proper strategy of therapy based on the precise measurement of CEA expression level because of the good quantification of SPECT and SPECT/CT.

Conclusion

In vitro display techniques have produced many high affinity, high selectivity protein scaffold molecules for a variety of biomarkers as alternative to monoclonal antibodies. A lot of efforts have been made for efficient and stable labeling of those small proteins with important radionuclides (^{99m}Tc, ^{111}In, ^{18}F, ^{124}I, ^{64}Cu, ^{68}Ga, etc.) and NIR dyes. Both synthetic and recombinant versions of protein scaffold-based molecules have shown advantages and much promise. All of the four types of protein scaffold, namely Affibody molecules, 2-helix small molecules, cystine knot proteins, and nanobodies have shown good imaging properties in vivo. Based on the data from the studies done so far, protein scaffold-based approach could become a generalizable strategy for facilitating the molecular probe development. These protein scaffolds could potentially be used to develop imaging agents that bind to a great variety of important molecular targets associated with cancers. The protein scaffold-based probes have great potential in diagnosis of disease, in monitoring therapeutic efficacy of new drugs, in the discovery of novel biomarkers and in the clinical translation studies.

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