Published in final edited form as:

Biomaterials. 2011 March ; 32(8): 2141–2148. doi:10.1016/j.biomaterials.2010.11.053.

Affibody-based nanoprobe for HER2-expressing cell and tumor imaging

Jinhao Gao^{a,b}, Kai Chen^a, Zheng Miao^a, Gang Ren^a, Xiaoyuan Chen^c, Sanjiv S. Gambhir^{a,d}, and Zhen Cheng^{a,*}

^a Molecular Imaging Program at Stanford (MIPS), Canary Center at Stanford for Cancer Early Detection, Department of Radiology and Bio-X Program, School of Medicine, Stanford University, 1201 Welch Road, Stanford, CA 94305-5484, USA

^b Department of Chemical Biology and The Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China

^c Laboratory of Molecular Imaging and Nanomedicine (LOMIN), NIBIB/CC/NIH, 10 Center Dr, Bethesda, MD 20892, USA

^d Department of Bioengineering, Stanford University, Stanford, CA 94305, USA

Abstract

This article reports the affibody-based nanoprobe specifically target and image human epidermal growth factor receptor type 2 (HER2)-expressing cells and tumors. The simple, robust, and precise structure of affibody molecules are a promising class of targeting ligands with high affinity. Using near-infrared (NIR) quantum dots (QDs) and iron oxide (IO) nanoparticles as two representative nanomaterials, we designed anti-HER2 affibody molecules with an N-terminus cysteine residue (Cysteine-Z_{HER2:342}) and precisely conjugated with maleimide-functionalized nanoparticles to make nanoparticle-affibody conjugates. The in vitro and in vivo study showed the conjugates are highly specific to target and image HER2-expressing cells and tumors. This work indicated the nanoparticle-affibody conjugates may be excellent candidates as targeting probes for molecular imaging and diagnosis.

Keywords

Affibody; bioconjugation; nanoprobe; HER2; molecular imaging

1. Introduction

This article describes the use of affibody molecules as targeting proteins to conjugate with nanoparticles (quantum dots (QDs) or iron oxide (IO) nanoparticles) for imaging of human epidermal growth factor receptor type 2 (HER2) expressing cells and tumors. Small proteins

*Corresponding author. Fax: 1-650-721-6921, zcheng@stanford.edu (Z. Cheng).

Supplementary materials

Supplementary materials associated with this article can be found in the on-line version.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

as platforms for the development of molecular imaging probes have attracted significant interest because of their favorable properties, such as high affinity and specificity, small size, facile synthesis and preparation, and rapid blood clearance [1]. Affibody molecules, the engineered small protein scaffolds with 58-amino acid residues and a three-helix bundle structure, are a promising class of disease-specific ligands with high affinity [2–4]. Different from antibodies, the key features of affibody molecules are their much smaller size, faster tumor targeting ability, and more well-defined structure which could potentially be site-specifically modified. The simple, robust structure of affibody molecules in combination with their low molecular weight (7 kDa) make them suitable for a wide variety of applications, especially in tumor-targeted imaging. For example, radiolabeled (e.g., ^{18}F , ^{64}Cu , ^{68}Ga , ^{111}In , ^{125}I , and ^{177}Lu) affibody molecules have shown great promise for tumor positron emission tomography (PET) or single photon emission computed tomography (SPECT) imaging and radiotherapy [5–12]. The near infrared (NIR) dyes labeled epidermal growth factor receptor (EGFR)-specific affibody molecules have also exhibited excellent properties for in vivo optical imaging of EGFR-overexpressing tumors [13,14].

Nanobiotechnology, the combination of nanotechnology and biomedicine, has become a flourishing research area because of their great potential to offer abundant opportunities and tools for discovering and understanding new materials, processes, and phenomena in biology and medicine. The basic rationale is that the metal, metal oxide, semiconductor, or self-assembled molecular nanostructures have novel properties and functions that are not available from bulk counterparts or individual molecules. Among the well-established nanomaterials, both QDs and IO nanoparticles have found notable and successful applications in biomedicine: the former one are receiving increased acceptance as fluorescent probes for visualizing biological processes in vitro and in vivo [15–18], while the latter one have served as magnetic resonance imaging (MRI) contrast agents for the clinical diagnosis of many diseases, including cancers [19–21]. Recently, nanoplateform-based molecular imaging has attracted more and more attentions because of the unique properties and multifunctionality in nanoplateforms [22–25]. Besides the QDs as optical probes and IO nanoparticles as MRI contrast agents, there are many other novel nanomaterials developed as excellent molecular imaging agents. For example, Rabin et al. reported the polymer-coated Bi_2S_3 nanoparticles as an injectable computed tomography (CT) contrast agent [26]. De la Zerda et al. demonstrated that carbon nanotubes can be used as targeted photoacoustic molecular imaging agents after conjugated with cyclic Arg-Gly-Asp (RGD) peptides [27].

The integration of affibody with nanoprobe as targeted molecular probes may be of great importance in the field of molecular imaging and cancer diagnosis. Herein, using two kinds of well-established nanomaterials (QDs with emission wavelength at about 800 nm, denoted as QD800, and IO nanoparticles) as representative examples, we conjugated an anti-HER2 affibody molecule ($Z_{\text{HER2:342}}$) and demonstrated the high specificity of affibody-based nanoprobe for HER2-expressing cell and tumor imaging. HER2 is a well-established tumor target overexpressed in a wide variety of cancers, including breast, ovarian, lung, and gastric cancers. In this study, we designed and chemically synthesized the anti-HER2 affibody molecules ($Z_{\text{HER2:342}}$) with adding a cysteine residue at the N terminus of the protein, then precisely conjugated with maleimide-functionalized nanoparticles to make nanoparticle-affibody conjugates (Scheme 1). Comparing with radiolabeled affibody molecules, the multivalent binding effect of nanoparticle-affibody conjugates may potentially enhance the targeting ability because the collective binding in a multivalent interaction is much stronger than that of the monovalent binding [28–30]. The in vitro and in vivo study further showed that the nanoparticle-affibody conjugates were highly specific to target HER2-expressing cell and tumor (e.g., SKOV3). The fluorescence imaging results indicated that QD800-

affibody displayed strong fluorescent signal in SKOV3 tumor with high specificity, cellular MRI data revealed the significant MR contrast signal in IO-affibody treated SKOV3 cancer cells over IO-PEG treated SKOV3 cancer cells.

2. Experimental sections

2.1. Materials

InAs/InP/ZnSe core/shell/shell QDs (~5 nm in diameter, denoted as QD800) [31] and magnetite nanoparticles (~15 nm in diameter, denoted as IO nanoparticles) [32] were provided by Ocean Nanotech LLC (Fayetteville, Arkansas). Affibody (Z_{HER2:342} with the amino acid sequence of VDNKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLYDDPSQSANLLAEAKKLNDQA APPK) with the N terminus cysteine residue (Cys-Z_{HER2:342}) and acetylation was produced by conventional solid phase peptide synthesis using a peptide synthesizer (CS336X, CS Biocompany).

2.2. Preparation of PEGylated nanoparticles

We used the conjugate of phospholipid and polyethylene glycol (DSPE-PEG2000 amine: 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000], Avanti Polar Lipids, Inc.) to encapsulate the nanoparticles and made them becoming water-dispersible [33,34]. Typically, the nanoparticles (10 nmol) and DSPE-PEG2000 amine (~1.8 μmol) were mixed in 200 μL of chloroform and left the container open in a fume hood to evaporate the chloroform solvent slowly at room temperature. The dry sample was pumped under vacuum for about 4 hours to remove chloroform completely and then re-dispersed in water by gentle sonication. The water-dispersible samples were purified by centrifugation using Millipore (Centrifugal filter devices, 100K) at 4000 rpm for 20 min to remove the excess amount of DSPE-PEG2000 amine. We also further purified the samples using size-exclusion chromatography (NAP-10 column, GE Healthcare Life Sciences). The final concentration of nanoparticles in PBS buffer (1X) was about 10 μM ready for further bioconjugation.

2.3. Synthesis of the conjugates QD800-affibody and IO-affibody

We used the heterobifunctional linker to conjugate the affibody with PEGylated nanoparticles. The procedure is similar as the previous report with a minor modification [35]. In detail, we mixed PEGylated nanoparticles (1 nmol) and the heterobifunctional linker, 4-maleimidobutyric acid N-succinimidyl ester (1 μmol) in borate buffer (pH ~ 8.5) and incubated the mixture at room temperature for about 2 hours with gentle shaking. After the purification by NAP-10 column to remove the excess amount of heterobifunctional linker, affibody molecules (Cys-Z_{HER2:342}, 0.1 μmol) were added into the activated nanoparticles and kept general shaking for about 2 hours at room temperature. After the further purification using size-exclusion chromatography (NAP-10 column), we stored the nanoparticle-affibody conjugates in PBS buffer with the concentration of 1 μM ready for in vitro and in vivo biological evaluation.

2.4. Gel-filtration chromatography (GFC)

We performed the GFC analysis on a Superose-6 10/300 GL column (GE Healthcare Life Sciences) using PBS (1X), pH 7.4 as the mobile phase [28]. The flow rate was 1.0 mL/min. The calibration of hydrodynamic diameter was performed by injecting 100 μL of protein standards containing Blue dextran (2,000 kDa, 29.5 nm HD), thyroglobulin (669 kDa, 18.0 nm HD), alcohol dehydrogenase (150 kDa, 10.1 nm HD), and ovalbumin (44 kDa, 6.13 nm HD). The proteins and nanoparticles were monitored by a high performance liquid chromatography (HPLC) (UltiMate 3000, Dionex) at the absorbance of 280 nm and 365 nm.

2.5. Cell culture

SKOV3 cells were cultured in McCoy 5 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin. All the cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37 °C with the medium changed every other day. A confluent monolayer was detached with trypsin and dissociated into a single cell suspension for further cell culture.

2.6. Animal model

Animal experiments were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee. We established the xenografted tumor models by subcutaneous injection of SKOV3 or PC-3 cells ($\sim 5 \times 10^6$ in 50 μ L of PBS) into the front flank of female or male athymic nude mice (Harlan), respectively. The mice were subjected to imaging studies when the tumor volume reached 200–500 mm³ (about 4 weeks after inoculation).

2.7. In vivo and ex vivo fluorescence imaging

After the tail vein injection of QD800-affibody and QD800-PEG, respectively (~ 200 pmol each mouse), the mice were imaged at multiple time points (e.g., 0.5, 1, 4, 6, and 24 h) using an IVIS Imaging System (IVIS[®] 200 Series; Cy5.5 excitation filter: 605–665 nm, ICG emission filter 800–875 nm). For ex vivo imaging, after the tumors and major organs were harvested, the tissues were subjected to fluorescence imaging using the IVIS Imaging System immediately.

2.8. Immunofluorescence staining

The tumor frozen tissue slices were fixed in cold acetone for about 10 min and dried in air for about 30 min. The sections were blocked with 10% donkey serum for about 10 min and then incubated with a rat anti-mouse CD31 monoclonal antibody (1:50; BD BioSciences) for 30 min at room temperature. After incubation with a Cy3-conjugated donkey anti-rat secondary antibody (1:100; Jackson ImmunoResearch Laboratories, Inc.) for another 30 min, the tumor sections were examined under the fluorescence microscope. The fluorescent staining agent DAPI (Vector Laboratories, Inc., Burlingame) was used to label the location of nucleus in tissue slides.

2.9. MRI scanning

We incubated 1×10^6 SKOV3 and PC-3 cells (5 mL of medium) with different concentrations (200, 400, and 800 μ M of Fe, respectively) of two types of IO nanoparticles at 37 °C for 30 min. The cells were harvested and washed with PBS buffer three times to remove the free IO nanoparticles. Then we embedded the cells in 1% agarose gel (300 μ L) in multi-well plates for MRI scanning. The MRI experiment was performed on a 7 Tesla MRI scanner (PharmaScan, Bruker Biospin GmbH, Germany). These cell plates were scanned using a multi-echo T_2 -weighted fast spin echo imaging sequence to collect a series of echo time (TE) dependent data points simultaneously.

2.10. Statistical Method

Statistical analysis was performed using the Student's t-test for unpaired data. A 95% confidence level was chosen to determine the significance between groups, with $P < 0.05$ being designated as significantly different.

3. Results and Discussion

3.1. Characterization of nanoparticle-affibody conjugates

The as-produced QD800 and IO nanoparticles only dispersed in non-polar organic solvents, such as hexane and chloroform. Poly(ethylene glycol) phosphatidylethanolamine (e.g., DSPE-PEG2000 amine) is one kind of micelle-forming hydrophilic polymer-grafted lipids [33,36,37]. This well-established micelle strategy indeed provides a general route to accomplish the transformation and modification of hydrophobic nanoparticles [34]. We thus used DSPE-PEG2000 amine to coat the nanoparticles by hydrophobic-hydrophobic interaction, make the nanoparticles become water-dispersible and improve their biocompatibility. The procedure of coating is very facile and highly repeatable. During the evaporation, the complete removal of chloroform is necessary to prevent the formation of free micelle by excess amount of DSPE-PEG2000 amine in water. The PEGylated nanoparticles maintain their own features and show ultrastable in aqueous solution. Moreover, the water-dispersible nanoparticles with amine terminal group render the further modification and conjugation.

To precisely conjugate the affibody molecules with PEGylated nanoparticles, we designed to synthesize affibody molecules (Z_{HER2:342}) with adding a cysteine residue to the N terminus of the protein (Cys-Z_{HER2:342}). As shown in Scheme 1, The thiolated affibody molecules provide the well-identified conjugation position when we use 4-maleimidobutyric acid N-succinimidyl ester as a heterodimeric cross-linker [35], that is, affibody molecules are specifically linked to the nanoparticles' surface through their N terminus cysteine residue. Comparing with antibody conjugates [38], the structurally well-defined nanoparticle-affibody conjugates are ideal candidates as targeting probes. We then used gel-filtration chromatography (GFC) to determine the change of hydrodynamic diameters (HDs) before and after bioconjugation. The core sizes of QD800 and IO nanoparticles are about 5 and 15 nm, respectively [31,32,39,40]. After the PEGylation, the HDs of QD800-PEG and IO-PEG nanoparticles are about 18 and 27 nm, respectively (Fig. S1, see Supporting Information), and the HDs become slightly bigger (about 19 and 28 nm, respectively) when they were conjugated with the affibody molecules. The small size of affibody molecules is negligible to the HDs of nanoparticles, which ensure the conjugates are small enough for targeting and imaging *in vivo*. Based on the size of these PEGylated nanoparticles, the number of amine group on the surface of each nanoparticle, and the maximum ligand conjugation efficiency (40–50%) [29], we estimated the number of affibody molecules on QD800-PEG and IO-PEG nanoparticles were about 12 and 20, respectively.

3.2. Fluorescence tumor imaging studies using QD800-affibody

After the preparation of nanoparticle-affibody conjugates in phosphate buffered saline (PBS) buffer, we first tested the *in vivo* fluorescence targeted imaging using QD800-affibody conjugates (1 μ M). We chose two tumor models with different HER2 expression levels as examples: SKOV3 human ovarian cancer (high HER2 expression) [11] and PC-3 human prostate carcinoma (low HER2 expression) [41]. We did the intravenous injection (200 μ L per mouse) into the athymic nude mice bearing subcutaneous tumors and used IVIS 200 instrument to take the fluorescence imaging studies at multiple time points (0.5, 1, 4, 6, and 24 h). We set the Cy5.5 excitation filter (615–665 nm) and the ICG emission filter (800–875 nm) to obtain the fluorescence images with the strong fluorescent signal and low background signal. As shown in Fig. 1A, as early as 0.5 h postinjection (p.i.), the fluorescence signal derived from QD800-affibody appeared in the SKOV3 tumor. After about 4 hours, the tumor was very distinguishable from other tissues with good fluorescence contrast (arrows) in the SKOV3 tumor-bearing mice injected with QD800-affibody, indicating the highly specific targeting to SKOV3 tumor. As expected, we did not observe

the tumor contrast through the period in the SKOV3 tumor mice injected with QD800-PEG or the PC-3 tumor-bearing mice injected with QD800-affibody (Fig. 1A). Furthermore, it was found that the tumor contrast was still prominent even after 24 h p.i. in the SKOV3 tumor-bearing mice injected with QD800-affibody, suggesting the high HER2 targeting induces the retention of QDs in tumor site, which was further confirmed by the analysis of tumor-to-background ratios (Fig. 1B). For the SKOV3 tumor-bearing mice injected with QD800-affibody, the fluorescence signal in tumor reached maximum at 4 h p.i. and then slightly decreased over the time (tumor-to-background ratios were 2.09 ± 0.11 , 2.67 ± 0.29 , 3.93 ± 0.54 , 2.89 ± 0.16 , and 1.87 ± 0.17 at 0.5, 1, 4, 6, and 24 h p.i., respectively, $n = 3/\text{group}$), while there was no obvious tumor contrast in the mice injected with QD800-PEG (tumor-to-background ratios were 1.12 ± 0.09 , 0.94 ± 0.10 , 0.99 ± 0.05 , 0.93 ± 0.09 , and 1.00 ± 0.11 at 0.5, 1, 4, 6, and 24 h p.i., respectively, $n = 3/\text{group}$) or PC-3 tumor-bearing mice injected with QD800-affibody (tumor-to-background ratios were 1.17 ± 0.10 , 1.12 ± 0.13 , 1.06 ± 0.10 , 1.03 ± 0.04 , and 0.94 ± 0.02 at 0.5, 1, 4, 6, and 24 h p.i., respectively, $n = 3/\text{group}$). Notably, because of the robust binding of QD800-affibody to HER2-expressing tumor by active targeting, the tumor-to-background ratio still maintained considerable value even after 24 h p. i., which was different from the passive targeting of ultrasmall nanoprobe due to the enhanced permeability and retention (EPR) effect [29]. Because of the possibility of rapid renal clearance of ultrasmall nanoprobe after administration [42–44], the tumor uptake of ultrasmall nanoprobe may decrease dramatically after a certain length of time (e.g., 6 h p. i.) [39].

To confirm the in vivo fluorescence imaging, we further investigated the specificity of QD800-affibody by ex vivo fluorescence imaging. The fluorescence signal intensity of ex vivo imaging is a real reflection of the QDs retained inside the organs because of the less or even no autofluorescence in ex vivo images [40]. Because the good tumor contrast appeared at 4 h p.i. of QD800-affibody, the mice were sacrificed at this time point. We collected the tumors and major organs to acquire fluorescence images under the same conditions as in vivo imaging immediately. As shown in Fig. 2A, ex vivo imaging further confirmed the strong fluorescence signal in SKOV3 tumor of mice injected with QD800-affibody, whereas virtually no fluorescence signal in the SKOV3 tumor of mice injected with QD800-PEG or PC-3 tumor of mice injected with QD800-affibody. Then we performed the region of interest (ROI) analysis on the ex vivo fluorescence images to semi-quantitatively study the uptake ratio of QDs in each organ (Fig. 2B). The ROI analysis showed high tumor uptake of 14.9 ± 0.8 percentage of injected dose per gram (%ID/g) in the SKOV3 tumor-bearing mice injected with QD800-affibody, while the tumor uptake of QD800-PEG and PC-3 tumor uptake of QD800-affibody under the same condition were only 4.3 ± 0.6 %ID/g and 5.7 ± 1.8 %ID/g, respectively. The semi-quantitative ROI analysis further confirmed QD800-affibody could be an excellent optical probe for tumor-targeted fluorescence imaging. Although the fluorescence intensity of liver was highest among all of the organs, the difference between tumor fluorescence signals indicated QD800-affibody has the capability to specifically target and detect HER2-overexpressing tumors.

To investigate the microscopic histology of the QDs after the tail vein injection, the tumors and major organs at 4 h p.i. of the nanoparticles were harvested and frozen in OCT medium (Sakura Finetek) immediately. The tissues were cut into 5 μm -thick slices using a microtome for fluorescence microscopy studies. We used fluorescent staining agent 4',6-diamidino-2-phenylindole (DAPI) to label the location of nucleus in tissue slides. As shown in Fig. 3, we found minimum QD fluorescence signal in SKOV3 tumor slide of mice injected with QD800-PEG or the PC-3 tumor slide of mice injected with QD800-affibody, on the contrary, there was obvious QD fluorescence signal in the SKOV3 tumor slide of mice injected with QD800-affibody. We also found the presence of QD signals in liver and kidney slides because of the reticuloendothelial system (RES) uptake. CD31

immunostaining of tumor tissue slides would allow the visualization of tumor vasculature. Interestingly, we found the QD fluorescence signal in the SKOV3 tumor cells of mice injected with QD800-affibody (Fig. S2) after CD31 staining, suggesting QD800-affibody has the ability to extravasate from the tumor vessels and specifically binds to HER2-overexpressing tumor cells.

3.3. In vitro and in vivo MRI studies using IO-affibody

We next investigated IO-affibody nanoparticles as the HER2-targeted probe for MR imaging. The IO-PEG and IO-affibody dispersed in water very well even after the storage of 6 months. We tested the contrast enhancement effect of IO-PEG and the commercial sample ferumoxide (Feridex). Under the same iron concentration ranging from 270 to 4.2 μM , T_2 -weighted images indicated IO-PEG exhibit slightly stronger MR relaxation enhancement than that of Feridex (Fig. 4A). The relaxivity values (r_2) of IO-PEG and Feridex are 172.34 and 152.86 $\text{mM}^{-1}\text{s}^{-1}$, respectively (Fig. 4B). Comparing with the commercial contrast agent Feridex, IO-PEG indeed showed higher MR contrast enhancement effect, suggesting that IO-PEG should serve as an efficient MR contrast agent.

To test the ability of IO-affibody to target and image cancer cells, we incubated SKOV3 human cancer cells with different concentrations (200, 400, and 800 μM of Fe, respectively) of IO-PEG and IO-affibody, respectively. Then we embedded the cells in 1% agarose gel (300 μL) in multi-well plates for MRI scanning. As shown in Fig. 5A, comparing with the cells without Fe, T_2 -weighted MR images indicated the strong MR signal in the cells incubated with IO-affibody, while the very weak MR signal in the cells incubated with IO-PEG. Moreover, the intensity of MR signal increased along with the augment of concentration of IO-affibody. We converted the signal intensity values to show the quantitative measurement. The relaxation rates (R_2) of cells incubated with IO-PEG were closed to that of cells without IO nanoparticles, indicating almost no uptake of IO-PEG in SKOV3 cells. The relaxation rates of cells incubated with IO-affibody significantly increased when the raise of concentration of nanoprobe (Fig. 5B), suggesting the high cellular uptake. In order to further evaluate the result of MR images, we conducted the elemental analysis using inductively coupled plasma (ICP) spectrometry. The ICP analysis of Fe content (Fig. 5C) indicated the cellular uptakes of Fe were only about 2.6×10^{-3} , 3.4×10^{-3} , and 9.8×10^{-3} μmol at the concentrations of 200, 400, and 800 μM of IO-PEG, respectively. Significantly, the cellular uptakes of Fe were 0.068 ± 0.022 , 0.13 ± 0.02 , and 0.27 ± 0.03 μmol at the concentrations of 200, 400, and 800 μM of IO-affibody, respectively. The ICP results further confirmed almost no uptake of Fe in SKOV3 cells incubated with IO-PEG. While the cellular uptake of IO-affibody was about 6.8% of applied nanoparticles after 0.5 h incubation at 37 $^{\circ}\text{C}$, indicating the highly specific targeting to SKOV3 cancer cells. The parallel experiments using PC-3 cell lines showed ultralow cellular uptake of both IO-PEG and IO-affibody (Fig. S3 and Table S1), further confirmed affibody-based nanoprobe only specifically bind to HER2-expressing cells. However, the in vivo T_2 -weighted MR imaging indicated only the slight decrease of MRI signal in SKOV3 tumor area before and after intravenous injection of IO-affibody (8 nmol/kg, Fig. S4). The indistinctive signal change of MR contrast in tumor maybe due to the relative low tumor uptake of IO-affibody, the negative MR imaging (T_2 -weighted signal decrease [45]), and low sensitivity of MRI modality which requires high amount of probe accumulation in the targeted organs, the further investigation is ongoing.

4. Conclusion

In summary, the small, simple, precise, and robust structure of affibody molecules with high affinity and specificity provide promising advantages as targeting entities. The general procedure of bioconjugation and the negligible influence on the size of nanoparticles after

conjugation warranted the ability of affibody-based nanoprobe for highly specific targeting and imaging. We demonstrated the high specificity of nanoparticle-affibody to HER2-overexpressing cancer cells using QD800 and IO nanoparticles as two representative nanomaterials. These specific nanoparticle-based probes ensure that affibody molecules are excellent candidates as targeting molecules to modify and functionalize various nanomaterials. In addition, the affibody-based nanoprobe may open up new strategies for the development of novel molecular imaging probes in cancer early detection and diagnosis applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by NCI/NIH R21 CA121842 (to Z.C.) and NCI of Cancer Nanotechnology Excellence Grant U54 CA119367 (to S.S.G.).

References

1. Miao Z, Levi J, Cheng Z. Protein scaffold-based molecular probes for cancer molecular imaging. *Amino Acids*. 2010;1007/s00726-010-0503-9
2. Nilsson FY, Tolmachev V. Affibody molecules: new protein domains for molecular imaging and targeted tumor therapy. *Current opinion in drug discovery & development* 2007;10:167–75.
3. Binz HK, Amstutz P, Pluckthun A. Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol* 2005;23:1257–68. [PubMed: 16211069]
4. Alexis F, Basto P, Levy-Nissenbaum E, Radovic-Moreno AF, Zhang L, Pridgen E, et al. HER-2-targeted nanoparticle-Affibody bioconjugates for cancer therapy. *ChemMedChem* 2008;3:1839–43. [PubMed: 19012296]
5. Cheng Z, De Jesus OP, Namavari M, De A, Levi J, Webster JM, et al. Small-animal PET imaging of human epidermal growth factor receptor type 2 expression with site-specific 18F-labeled protein scaffold molecules. *J Nucl Med* 2008;49:804–13. [PubMed: 18413392]
6. Tolmachev V, Nilsson FY, Widstrom C, Andersson K, Rosik D, Gedda L, et al. ¹¹¹In-benzyl-DTPA-Z_{HER2}:342, an affibody-based conjugate for in vivo imaging of HER2 expression in malignant tumors. *J Nucl Med* 2006;47:846–53. [PubMed: 16644755]
7. Orlova A, Nilsson FY, Wikman M, Widstrom C, Stahl S, Carlsson J, et al. Comparative in vivo evaluation of technetium and iodine labels on an anti-HER2 affibody for single-photon imaging of HER2 expression in tumors. *J Nucl Med* 2006;47:512–9. [PubMed: 16513621]
8. Orlova A, Magnusson M, Eriksson TL, Nilsson M, Larsson B, Hoiden-Guthenberg I, et al. Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Res* 2006;66:4339–48. [PubMed: 16618759]
9. Tolmachev V, Orlova A, Pehrson R, Galli J, Baastrup B, Andersson K, et al. Radionuclide therapy of HER2-positive microxenografts using a ¹⁷⁷Lu-labeled HER2-specific Affibody molecule. *Cancer Res* 2007;67:2773–82. [PubMed: 17363599]
10. Kramer-Marek G, Kiesewetter DO, Martiniova L, Jagoda E, Lee SB, Capala J. [F-18]FBEM-Z(HER2: 342) - Affibody molecule - a new molecular tracer for in vivo monitoring of HER2 expression by positron emission tomography. *Eur J Nucl Med Mol Imaging* 2008;35:1008–18. [PubMed: 18157531]
11. Cheng Z, De Jesus OP, Kramer DJ, De A, Webster JM, Gheysens O, et al. (64)Cu-labeled Affibody molecules for imaging of HER2 expressing tumors. *Mol Imaging Biol* 2010;12:316–24. [PubMed: 19779897]
12. Ren G, Zhang R, Liu Z, Webster JM, Miao Z, Gambhir SS, et al. A 2-helix small protein labeled with 68Ga for PET imaging of HER2 expression. *J Nucl Med* 2009;50:1492–9. [PubMed: 19690041]

13. Gong HB, Kovar J, Little G, Chen HX, Olive DM. In vivo imaging of xenograft tumors using an Epidermal Growth Factor Receptor-specific affibody molecule labeled with a near-infrared fluorophore. *Neoplasia* 2010;12:139–49. [PubMed: 20126472]
14. Miao Z, Ren G, Liu HG, Jiang L, Cheng Z. Cy5.5 labeled affibody molecule for near-infrared optical imaging of Epidermal Growth Factor Receptor1 expression. *J Biomed Opt* 2010;15:036007. [PubMed: 20615009]
15. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 2005;307:538–44. [PubMed: 15681376]
16. Gill R, Zayats M, Willner I. Semiconductor quantum dots for bioanalysis. *Angew Chem, Int Edit* 2008;47:7602–25.
17. Smith AM, Duan HW, Mohs AM, Nie SM. Bioconjugated quantum dots for in vivo molecular and cellular imaging. *Adv Drug Deliv Rev* 2008;60:1226–40. [PubMed: 18495291]
18. Yong KT, Roy I, Swihart MT, Prasad PN. Multifunctional nanoparticles as biocompatible targeted probes for human cancer diagnosis and therapy. *J Mater Chem* 2009;19:4655–72. [PubMed: 20305738]
19. Corot C, Robert P, Idee JM, Port M. Recent advances in iron oxide nanocrystal technology for medical imaging. *Adv Drug Deliv Rev* 2006;58:1471–504. [PubMed: 17116343]
20. McCarthy JR, Weissleder R. Multifunctional magnetic nanoparticles for targeted imaging and therapy. *Adv Drug Deliv Rev* 2008;60:1241–51. [PubMed: 18508157]
21. Gao JH, Gu HW, Xu B. Multifunctional magnetic nanoparticles: design, synthesis, and biomedical applications. *Acc Chem Res* 2009;42:1097–107. [PubMed: 19476332]
22. Cai WB, Chen XY. Nanoplatforams for targeted molecular imaging in living subjects. *Small* 2007;3:1840–54. [PubMed: 17943716]
23. Douma K, Prinzen L, Slaaf DW, Reutelingsperger CPM, Biessen EAL, Hackeng TM, et al. Nanoparticles for optical molecular imaging of atherosclerosis. *Small* 2009;5:544–57. [PubMed: 19226595]
24. Gao JH, Xu B. Applications of nanomaterials inside cells. *Nano Today* 2009;4:37–51.
25. Mulder WJM, Strijkers GJ, Van Tilborg GAF, Cormode DP, Fayad ZA, Nicolay K. Nanoparticulate assemblies of amphiphiles and diagnostically active materials for multimodality imaging. *Acc Chem Res* 2009;42:904–14. [PubMed: 19435319]
26. Rabin O, Perez JM, Grimm J, Wojtkiewicz G, Weissleder R. An X-ray computed tomography imaging agent based on long-circulating bismuth sulphide nanoparticles. *Nat Mater* 2006;5:118–22. [PubMed: 16444262]
27. De la Zerda A, Zavaleta C, Keren S, Vaithilingam S, Bodapati S, Liu Z, et al. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. *Nat Nanotechnol* 2008;3:557–62. [PubMed: 18772918]
28. Choi HS, Liu W, Liu F, Nasr K, Misra P, Bawendi MG, et al. Design considerations for tumour-targeted nanoparticles. *Nat Nanotechnol* 2010;5:42–7. [PubMed: 19893516]
29. Gao XH, Cui YY, Levenson RM, Chung LWK, Nie SM. In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat Biotechnol* 2004;22:969–76. [PubMed: 15258594]
30. Weissleder R, Kelly K, Sun EY, Shtatland T, Josephson L. Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. *Nat Biotechnol* 2005;23:1418–23. [PubMed: 16244656]
31. Xie RG, Chen K, Chen XY, Peng XG. InAs/InP/ZnSe core/shell/shell quantum dots as near-infrared emitters: bright, narrow-band, non-cadmium containing, and biocompatible. *Nano Res* 2008;1:457–64. [PubMed: 20631914]
32. Xie J, Chen K, Huang J, Lee S, Wang JH, Gao JH, et al. PET/NIRF/MRI triple functional iron oxide nanoparticles. *Biomaterials* 2010;31:3016–22. [PubMed: 20092887]
33. Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A. In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science* 2002;298:1759–62. [PubMed: 12459582]
34. Carion O, Mahler B, Pons T, Dubertret B. Synthesis, encapsulation, purification and coupling of single quantum dots in phospholipid micelles for their use in cellular and in vivo imaging. *Nat Protoc* 2007;2:2383–90. [PubMed: 17947980]

35. Cai WB, Shin DW, Chen K, Gheysens O, Cao QZ, Wang SX, et al. Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. *Nano Lett* 2006;6:669–76. [PubMed: 16608262]
36. Ito A, Ino K, Kobayashi T, Honda H. The effect of RGD peptide-conjugated magnetite cationic liposomes on cell growth and cell sheet harvesting. *Biomaterials* 2005;26:6185–93. [PubMed: 15899515]
37. Verma A, Stellacci F. Effect of surface properties on nanoparticle-cell interactions. *Small* 2010;6:12–21. [PubMed: 19844908]
38. Yang LL, Mao H, Wang YA, Cao ZH, Peng XH, Wang XX, et al. Single chain Epidermal Growth Factor Receptor antibody conjugated nanoparticles for in vivo tumor targeting and imaging. *Small* 2009;5:235–43. [PubMed: 19089838]
39. Gao JH, Chen K, Xie RG, Xie J, Lee S, Cheng Z, et al. Ultrasmall near-infrared non-cadmium quantum dots for in vivo tumor imaging. *Small* 2010;6:256–61. [PubMed: 19911392]
40. Gao JH, Chen K, Xie RG, Xie J, Yan YJ, Cheng Z, et al. In vivo Tumor-targeted fluorescence imaging using near-infrared non-cadmium quantum dots. *Bioconjugate Chem* 2010;21:604–9.
41. Ullen A, Lennartsson L, Harmenberg U, Lennernas B, Majumder K, Holmberg AR, et al. Prostate cancer cell lines lack amplification: Overexpression of HER2. *Acta Oncol* 2005;44:490–5. [PubMed: 16118083]
42. Choi HS, Liu WH, Misra P, Tanaka E, Zimmer JP, Ipe BI, et al. Renal clearance of quantum dots. *Nat Biotechnol* 2007;25:1165–70. [PubMed: 17891134]
43. Law WC, Yong KT, Roy I, Ding H, Hu R, Zhao WW, et al. Aqueous-phase synthesis of highly luminescent CdTe/ZnTe core/shell quantum dots optimized for targeted bioimaging. *Small* 2009;5:1302–10. [PubMed: 19242947]
44. Yong KT, Roy I, Ding H, Bergey EJ, Prasad PN. Biocompatible near-infrared quantum dots as ultrasensitive probes for long-term in vivo imaging applications. *Small* 2009;5:1997–2004. [PubMed: 19466710]
45. Ferrucci JT, Stark DD. Iron-oxide enhanced MR-imaging of the liver and spleen - Review of the 1st-5 years. *Am J Roentgenol* 1990;155:943–50. [PubMed: 2120963]

Fig. 1. (A) In vivo NIR fluorescence imaging of tumor-bearing mice (arrows) injected with QD800-PEG or QD800-affibody at 0.5, 1, 4, 6, and 24 h. (B) Tumor-to-background ratio of mice injected with QD800-PEG (blue: SKOV3 tumor) or QD800-affibody (red: SKOV3 tumor; black: PC-3 tumor). The data was represented as mean $\pm$ standard deviation (SD), $n = 3$ /group. Significant higher tumor-to-background ratio for the SKOV3 mice injected with QD800-affibody than that of QD800-PEG and PC-3 mice injected with QD800-affibody was observed (* $P < 0.05$).

Fig. 2.

(A) Ex vivo NIR fluorescence imaging after 4 h p.i. of QD800-PEG or QD800-affibody. In each image: 1, tumor; 2, muscle; 3, heart; 4, lung; 5, liver; 6, spleen; 7, kidney (left); 8, kidney (right); 9, intestine; 10, bone. (B) ROI analysis of major organs in ex vivo fluorescence imaging after 4 h p.i. of QD800-PEG (blue: SKOV3 tumor) or QD800-affibody (red: SKOV3 tumor; black: PC-3 tumor). * $P < 0.05$ being significantly different compared with the mice injected with QD800-PEG.

Fig. 3. Fluorescence microscopy images and overlay of frozen SKOV3 tumor, PC-3 tumor, liver, and kidney tissue slices from mice injected with QD800-PEG or QD800-affibody (4 h p.i.). DAPI stained the nucleus in tissue slices. Magnification: 400 $\times$.

Fig. 4.

(A) T_2 -weighted MR images of IO-PEG and ferumoxide (Feridex IV) from a 7.0 T MRI system at different iron concentrations. (B) The analysis of relaxation rate R_2 ($1/T_2$) vs. Fe concentration for IO-PEG and Feridex, respectively. The relaxivity values r_2 were obtained from the slopes of linear fits of experimental data.

Fig. 5. SKOV3 cell uptake analysis of IO-PEG and IO-affibody. (A) T_2 -weighted MR images of SKOV3 cells before (right) and after (left) incubation with IO-PEG or IO-affibody at 800, 400, and 200 μM iron concentration, respectively. (B) The R_2 analysis of MR signal and (C) the ICP quantitative analysis of cell uptake after incubation with IO-PEG or IO-affibody at 200, 400, and 800 μM iron concentration, respectively. * $P < 0.05$ being significantly different compared with the cells incubated with IO-PEG.

Scheme 1.

The structure and synthesis of QD800-affibody and IO-affibody conjugates.