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A novel ^{18}F -labeled two-helix scaffold protein for PET imaging of HER2-positive tumor

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Abstract

Purpose—Two-helix scaffold proteins (~ 5 kDa) against human epidermal growth factor receptor type 2 (HER2) have been discovered in our previous work. In this research we aimed to develop an ^{18}F -labeled two-helix scaffold protein for positron emission tomography (PET) imaging of HER2-positive tumors.

Methods—An aminoxy-functionalized two-helix peptide (AO-MUT-DS) with high HER2 binding affinity was synthesized through conventional solid phase peptide synthesis. The purified linear peptide was cyclized by I_2 oxidation to form a disulfide bridge. The cyclic peptide was then conjugated with a radiofluorination synthon, 4- ^{18}F -fluorobenzyl aldehyde (^{18}F -FBA), through the aminoxy functional group at the peptide N terminus (30% yield, non-decay corrected). The binding affinities of the peptides were analyzed by Biacore analysis. Cell uptake assay of the resulting PET probe, ^{18}F -FBO-MUT-DS, was performed at 37°C . ^{18}F -FBO-MUT-DS with high specific activity (20–32 MBq/nmol, 88–140 $\mu\text{Ci}/\mu\text{g}$, end of synthesis) was injected into mice xenograft model bearing SKOV3 tumor. MicroPET and biodistribution and metabolic stability studies were then conducted.

Results—Cell uptake assays showed high and specific cell uptake (~12% applied activity at 1 h) by incubation of ^{18}F -FBO-MUT-DS with HER2 high-expressing SKOV3 ovarian cancer cells. The affinities (K_D) of AO-MUT-DS and FBO-MUT-DS as tested by Biacore analysis were 2 and 1 nM, respectively. In vivo small animal PET demonstrated fast tumor targeting, high tumor accumulation, and good tumor to normal tissue contrast of ^{18}F -FBO-MUT-DS. Biodistribution studies further revealed that the probe had excellent tumor uptake (6.9%ID/g at 1 h post-injection) and was cleared through both liver and kidneys. Co-injection of the probe with 500 μg of HER2 Affibody protein reduced the tumor uptake (6.9 vs 1.8%ID/g, $p<0.05$).

Conclusion—F-FBO-MUT-DS displays excellent HER2 targeting ability and tumor PET imaging quality. The two-helix scaffold proteins are suitable for development of ^{18}F -based PET probes.

Keywords

PET; HER2; ^{18}F ; Affibody; Scaffold protein

Introduction

Recent advancements in in vitro display technologies (phage, bacterial, yeast, ribosome, and mRNA display technology) and rational protein engineering have resulted in many novel non-immunoglobulin protein scaffolds that can specifically bind to cancer-specific biomarkers [1–5]. These scaffold proteins can be easily modified by alternation of a few amino acid residues at the binding loop or surface, generating high-affinity binders to various growth factors and signal receptors which play important roles in oncology and

other diseases. They also generally have good availability, low immunogenicity and toxicity, good in vivo stability, and fast tumor targeting ability [6, 7]. Thus, these versatile protein scaffolds became promising platforms for development of imaging probes.

Monoclonal antibodies (mAbs) and fragments such as Herceptin [human epidermal growth factor receptor type 2 (HER2) mAb] and cetuximab [epidermal growth factor receptor (EGFR) mAb] can specifically target tumor receptors and have achieved enormous success for cancer treatment in recent years [8]. Hundreds of mAbs are currently in clinical trials. More than 20 therapeutic mAbs have been approved by the US Food and Drug Administration (FDA) for use in oncology and treatment of inflammatory diseases [9]. Various radioisotopes, organic dyes, contrast reagents, and nanoparticles have been used to label mAbs such as HER2-targeted Herceptin and antibody fragments [10, 11]. However, mAbs and antibody fragments are generally optimized for therapeutic applications. They show slow accumulation in tumor, slow clearance from blood, and high nonspecific accumulation in normal tissues. Therefore, they are not ideal platforms for imaging applications. In contrast, scaffold proteins and peptides such as Affibodies have the advantages of fast accumulation and relatively quick blood and normal organ clearance, which thus could lead to better image quality at an earlier time post-administration [12–14]. In addition, scaffold proteins can bind to different tumor target epitopes than therapeutic antibody, and they may provide additional advantages or even more accurate information for monitoring treatment effects [4, 15, 16].

Affibody molecules are small engineered protein scaffolds with 58 amino acid residues and a three-helix bundle scaffold structure. They have been demonstrated to be excellent platforms for development of molecular probes [12–15]. The two-helix scaffold proteins have two thirds the size of the three-helix anti-HER2 Affibodies and preserve the high HER2 binding affinities. They were recently engineered and developed by us with an expectation of improved performance over their larger molecular counterparts [17]. One of such two-helix scaffold proteins, MUT-DS [sequence: VENK(homoC) NKEMRNRYWE AALDPNLNNQQKRAKIRSIYDDP (homoC)-NH₂, a disulfide bridge was formed between two L-homocysteines], was conjugated to DOTA and radiolabeled with a positron emission tomography (PET) radionuclide ⁶⁸Ga (*T*_{1/2}: 68 min, emits β⁺ particles at *E*_{max} = 1,899 keV; 89% abundant), and the resulting PET probe shows excellent and fast tumor imaging contrast. However, the radiometal-labeled two-helix scaffold protein displays extremely high uptake in the kidney [18]. ¹⁸F is the most commonly used PET radionuclide and has almost ideal physical properties (*T*_{1/2}: 110 min, emits β⁺ particles at *E*_{max} = 635 keV; 97% abundant) for clinical PET imaging. In our previous studies, we also found that radiolabeling of three-helix Affibody proteins with an ¹⁸F moiety could dramatically reduce the kidney uptake [19, 20]. In this study, to further evaluate the clinical translation ability of the engineered two-helix scaffold proteins, the two-helix scaffold protein MUT-DS was modified with an aminooxy group at the N-terminal of the protein to provide a site for site-specific radiolabeling. The resulting functionalized protein, AO-MUT-DS, was then conjugated with a radiofluorination synthon, 4-¹⁸F-fluorobenzyl aldehyde (¹⁸F-FBA) (Fig. 1). Finally, the resulting radiofluorinated MUT-DS, ¹⁸F-FBO-MUT-DS, was evaluated in nude mice bearing subcutaneous SKOV3 xenografts with high levels of HER2 expression.

Materials and methods

General

Triisopropylsilane (TIPS), dithiothreitol (DTT), tricine, 4-fluorobenzaldehyde (4-FBA), (Boc-aminoxy)-acetic acid, 1,2-ethanedithiol (EDT), and all other standard synthesis reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methylene chloride was from Fisher Scientific. High-performance liquid chromatography (HPLC)-grade acetonitrile (CH₃CN) and 18 mV water (Millipore) were used for peptide purification. The SKOV3 human ovarian cell line was obtained from American Type Culture Collection (Manassas, VA, USA). All instruments including electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), reverse-phase HPLC with a radioactive detector, and radioactive dose calibrator are the same as described in our previous publication [19].

Chemistry and in vitro analysis

The linear AO-VENK (homoC) NKEMRNRWYWEAALDPNLNNQQKRAKIRSI YDDP(homoC)-NH₂ peptide (Fig. 1) was synthesized by using the standard solid phase peptide synthesis technique (Fmoc chemistry with HBTU/HOBT activation) with a protocol similar to DOTA-MUT-DS [18]. Cleavage of the peptide from the resin was accomplished by using a cocktail consisting of trifluoroacetic acid (TFA)/EDT/water/TIPS (94:2.5:2.5:1, v/v/v/v) for 2 h while being stirred at room temperature. Cyclization of the linear peptides was achieved by I₂ oxidation of the two L-homocysteines to form a disulfide bridge. Both crude linear and cyclized peptides were purified by a reverse-phase preparative HPLC with a Vydac protein and peptide C4 column. The mobile phase was solvent A (0.05% TFA in water, v/v) and solvent B (0.05% TFA in acetonitrile, v/v), and different gradients were used for purification of the peptides. The flow rate was typically 25 ml/min. The desired fractions were collected, frozen immediately, and lyophilized. The identity of the target peptides was confirmed by ESI-TOF-MS.

Synthesis of 4-FBA conjugated AO-MUT-DS: To 92 µl of an aqueous solution of ammonium acetate (50 mM, pH 4) was added a 7-µl aliquot of a 0.5 mM stock solution of AO-MUT-DS in water. A 1-µl aliquot of a stock solution of 3.4 M 4-FBA in dimethyl sulfoxide (DMSO) was then added. The resulting reaction mixture was thoroughly mixed and incubated overnight at room temperature. The reaction mixture was then purified by HPLC. Characterization of the resulting purified FBO-MUT-DS was achieved using LC-ESI-MS. The purity of the bioconjugate was confirmed by HPLC.

The HER2 binding affinities of proteins were measured in vitro by using surface plasmon resonance (SPR) detection with a Biacore 3000 instrument (GE Healthcare) according to the previously reported method [19, 20]. Briefly, the Fc-HER2 (R&D Systems, Minneapolis, MN, USA) was covalently attached to a CM-5 dextran-functionalized sensor chip (GE Healthcare) by using EDC and *N*-hydroxysuccinimide (NHS). A second flow cell on the same sensor chip without Fc-HER2 immobilization was used as a control. Prior to the kinetic study, binding of the target analyte was tested on both surfaces and a surface stability experiment was performed to ensure adequate removal of the bound analyte and

regeneration of the sensor chip following treatment with NaCl (2.5 M) and NaOH (50 mM). SPR sensorgrams were analyzed by using the BIAevaluation software (GE Healthcare). SPR measurements were collected at eight analyte concentrations (0–100 nM protein) and the resulting sensorgrams were fitted to a 1:1 Langmuir binding model.

Radiosynthesis of ^{18}F -FBO-MUT-DS

In order to minimize the radiation exposure to personnel, the aminoxy-functionalized two-helix scaffold protein AO-MUT-DS was radiofluorinated with an ^{18}F -labeled prosthetic group (4- ^{18}F -FBA) prepared by use of a GE TRACERlab FX F-N synthetic module (GE Healthcare) [20]. In brief, non-carrier-added $^{18}\text{F-F}^-$ trapped on a QMA cartridge [11.1–37 GBq (300–1,000 mCi)] was washed with a solution of K_2CO_3 and Kryptofix 2.2.2 in water and acetonitrile. After evaporation of the solution and azeotropic drying of the residue, a solution of a 4-formyl-*N,N,N*-trimethylanilinium triflate precursor (4–6 mg) in DMSO was added to the reactor, and the mixture was heated for 10 min at 85°C. Purification of the reaction mixture with a C18 cartridge system afforded the product 4- ^{18}F -FBA at a 50–70% radiochemical yield (decay corrected) and an overall synthesis time of about 40 min [specific activity: 592–1,480 MBq/ μg (16–40 mCi/ μg)]. Subsequently, AO-MUT-DS [0.5 mg in 100 μl of ammonium acetate (pH 4)] (Fig. 1) was incubated with 4- ^{18}F -FBA [0.185–0.259 GBq (5–7 mCi) in 50 μl of methanol] at 70°C for 15 min. The reaction solution was cooled and the product ^{18}F -FBO-MUT-DS was purified by use of an analytic HPLC with C4 column. The flow rate was 1 ml/min, with the mobile phase being 80% solvent A and 20% solvent B from 0 to 3 min, 55% solvent A and 45% solvent B at 33 min, and 15% solvent A and 85% solvent B from 33 to 36 min. This solvent composition was then maintained for another 3 min (36–39 min), and the initial solvent composition was resumed by 42 min. The HPLC fractions containing ^{18}F -FBO-MUT-DS were collected, combined, and then evaporated with a rotary evaporator to dry the product. ^{18}F -FBO-MUT-DS was reconstituted in phosphate-buffered saline (PBS) and passed through a 0.22- μm Millipore filter into a sterile vial for in vitro and animal experiments.

Cell assays

SKOV3 cells were cultured in McCoy's 5 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was changed every other day. A confluent monolayer was detached with trypsin and dissociated into a single-cell suspension for further cell culturing. In vitro cell uptake assays for ^{18}F -FBO-MUT-DS were performed with SKOV3 cells as previously described [21]. Briefly, the SKOV3 cells were washed three times with PBS, and dissociated with 0.25% trypsin-ethylenediaminetetraacetate (EDTA). McCoy's 5 medium was then added to neutralize trypsin-EDTA. Cells were spun down and resuspended with serum-free McCoy's 5 medium. The cells (5×10^5) were then incubated at 37°C for 0.25–1 h with the PET probe (18.5 kBq, ~5 ng of ^{18}F -FBO-MUT-DS, final concentration 10 ng/ml) in 0.5 ml of serum-free McCoy's 5 medium. The nonspecific binding of the probes with SKOV3 cells was determined by co-incubation with non-radiolabeled Z_{HER2:342} (final concentration 4 $\mu\text{g/ml}$). The cells were washed three times with chilled PBS (pH 7.4, 0.01 M) and spun down at a speed of 7,000–8,000 rpm. The radioactivity of the pellets was measured using a gamma

counter (PerkinElmer 1470, Waltham, MA, USA). The uptake (counts/min) was normalized to percentage of applied radioactivity for analysis using Excel (Microsoft, Redmond, WA, USA).

Biodistribution studies

All animal studies were performed in compliance with federal and local institutional rules for animal experimentation. Approximately 5×10^6 SKOV3 cells suspended in Matrigel (BD Biosciences, San Jose, CA, USA) were implanted subcutaneously in the flanks of nude mice. Tumors were allowed to grow to a size of 200–400 mg (2–3 weeks), and then the tumor-bearing mice were subjected to in vivo biodistribution and imaging studies. For biodistribution studies, SKOV3 tumor-bearing mice ($n=3$ for each group) were injected with ^{18}F -FBO-MUT-DS [1.11–1.85 MBq (30–50 μCi); ca. 0.3 μg] via the tail vein and sacrificed at 1 and 3 h post-injection (p.i.). Tumor and normal tissues of interest were removed and weighed, and their radioactivity was measured with a gamma counter. The radioactivity uptake in the tumor and normal tissues was expressed as a percentage of the injected radioactive dose per gram of tissue (%ID/g). For testing of the in vivo HER2 targeting specificity of ^{18}F -FBO-MUT-DS, nude mice bearing SKOV3 tumors ($n=3$ for each group) were co-injected with 500 μg of Z_{HER2:342} and ^{18}F -FBO-MUT-DS (tail vein injection). Biodistribution at 1 h p.i. was examined.

In vivo metabolic stability analysis

The in vivo metabolic fate of ^{18}F -FBO-MUT-DS was determined from samples recovered from the tumor and urine of xenograft mice models using the procedure described previously [22]. The probe was injected into SKOV3 tumor-bearing mice via the tail vein, and the mice were sacrificed at 1 h p.i. Tumor and liver tissues were homogenized and extracted with dimethylformamide (DMF) and PBS sequentially (500 μl each). The urine sample was also extracted with DMF and PBS sequentially (500 μl each). After centrifugation by Costar nylon filter tube, samples were injected into the radio-HPLC for analysis. For tumor and liver extract, HPLC fractions at 0.5-min intervals were collected and counted by gamma counter.

MicroPET imaging

PET imaging of tumor-bearing mice was performed by use of a microPET R4 rodent model scanner (Siemens Medical Solutions USA, Inc., Knoxville, TN, USA). Mice bearing SKOV3 tumors ($n=3$) were injected with ^{18}F -FBO-MUT-DS [2.59–3.70 MBq (70–100 μCi); ca. 0.5–1 μg] via the tail vein. At various times p.i. (1, 2, and 3 h), the mice were anesthetized with 2% isoflurane and placed in the prone position and near the center of the field of view of the scanner. Static scans (5 min) were obtained, and the images were reconstructed by use of a two-dimensional ordered subsets expectation maximization (OSEM) algorithm. No background correction was performed. Regions of interest (ROIs; > 5 pixels for coronal and transaxial slices) were drawn over the tumors on decay-corrected whole-body coronal images. The average counts per pixel per minute were obtained from the ROIs and converted to counts per milliliter per minute by use of a calibration constant. The calibration constant for ^{18}F was determined using a tube containing a known amount of

radioactive ^{18}F for micro-PET imaging. The calibration factors for ^{18}F have been measured routinely and are around 1,300 $\mu\text{Ci}/\text{ml}$ for our scanner. On the basis of the assumption of a tissue density of 1 g/ml, the ROIs were converted to counts per gram per minute. Image ROI-derived %ID/g values were determined by dividing counts per gram per minute by injected dose. No attenuation correction was performed.

Results

Chemistry and radiochemistry

The linear peptide was quantitatively oxidized to the cyclized AO-MUT-DS. ESI-MS analysis of the final products (purity of >95%, as determined by HPLC analysis) confirmed the absence of starting material and only the product expected, with molecular mass of 4,823.9 [calculated molecular weight (MW) for AO-MUT-DS is 4,823.4], was obtained. The aminoxy-functionalized two-helix protein was then reacted with 4-FBA to prepare nonradioactive FBO-MUT-DS as a standard compound (Fig. 1a, b). Recovery yield was 70–90%, and coupling yields were quantitative as determined by MALDI-TOF-MS characterization (i.e., no detected starting cyclized peptide). The measured MW (4,926.9) of the purified FBO-MUT-DS was consistent with the expected MW (4,928.4) for FBO-MUT-DS.

Similarly, 4- ^{18}F -FBA was prepared and successfully conjugated to the AO-MUT-DS in moderate yield (~30%, non-decay corrected). Under the HPLC condition used, the retention time of ^{18}F -FBO-MUT-DS was found to be 23.5 min with purity greater than 95%. The overall radiochemical yield ranged from 13 to 18% at the end of synthesis (EOS; non-decay corrected), and the specific activities were 20–32 MBq/nmol (88–140 $\mu\text{Ci}/\mu\text{g}$). The total time needed for the radiosynthesis of the ^{18}F -FBO-MUT-DS was about 100 min.

HER2 binding affinities of two-helix scaffold proteins

The binding affinities of the AO-MUT-DS and FBA-MUT-DS were evaluated in vitro by SPR. The sensorgrams for measurement are shown in Fig. 2. Sensorgrams were obtained after injection of various concentrations of purified peptides onto a sensor chip containing amine-coupled Fc-HER2 chimeric protein. Binding affinity of AO-MUT-DS and FBO-MUT-DS as determined by SPR were 2 and 1 nM, respectively. The binding affinity of FBO-MUT-DS was slightly higher than that of AO-MUT-DS. The “off rate” of FBO-MUT-DS was found to be similar as the AO-MUT-DS with a dissociation constant of 7×10^{-4} 1/s. The “on rate” of the FBO-MUT-DS was only about onefold higher than that of AO-MUT-DS with association constants of 7.5×10^6 1 M/s (association time is over 2 min).

In vitro cell assays of ^{18}F -FBO-MUT-DS

Tumor cell line SKOV3, with a high level of HER2 expression, was used for evaluation of the HER2-binding ability and specificity of ^{18}F -FBO-MUT-DS. Levels of cell uptake of ^{18}F -FBO-MUT-DS at 37°C over a 1-h incubation period are shown in Fig. 3. ^{18}F -FBO-MUT-DS quickly accumulated in SKOV3 cells and reached a value of 14% of applied activity at 0.25 h. The uptake was maintained at almost the same level until 1 h. When the probe was incubated with large excesses of nonradioactive Affibody molecules, their level

of uptakes in SKOV3 cells dropped to only ~5% of applied activity after 0.25 h incubation at 37°C.

In vivo biodistribution and microPET imaging studies

The in vivo biodistribution of ^{18}F -FBO-MUT-DS was examined by use of a SKOV3 human ovarian tumor-bearing mouse model. The biodistribution of ^{18}F -FBO-MUT-DS and blocking group and tumor to normal tissue ratios at 1 h are shown in Fig. 4. For ^{18}F -FBO-MUT-DS, rapid and high levels of radioactivity accumulation in the HER2-overexpressing SKOV3 tumors ($6.9 \pm 3.8\%$ ID/g) were observed at 1 h p.i. The uptake of ^{18}F -FBO-MUT-DS in the blocking group was only $1.8 \pm 1.1\%$ ID/g at 1 h p.i. ^{18}F -FBO-MUT-DS also displayed relatively rapid blood clearance, and a blood uptake value of $2.4 \pm 0.8\%$ ID/g was observed at 1 h p.i. The kidney and liver uptake was around 7% ID/g at 1 h p.i., which suggested that ^{18}F -FBO-MUT-DS was cleared through the hepatobiliary and renal systems. The tumor targeting and imaging ability was further confirmed by microPET imaging (Fig. 5a). Good tumor imaging contrast was observed at 1 and 2 h. Moreover, PET quantification analysis showed the slow tumor washout rate of the probe. Even after 3 h, high tumor accumulation could be seen in the PET images, while the uptakes from normal organs such as liver and kidney declined dramatically (Fig. 5b). Statistical analysis confirmed that there is no significant difference for the tumor uptake at 1 and 2 h p.i. ($p > 0.05$), and the liver uptakes at 1 and 2 h are indeed significantly different ($p < 0.05$). This result highlights the good tumor retention and fast normal organ clearance ability of the probe.

In vivo metabolic stability analysis

The in vivo metabolic fate of ^{18}F -FBO-MUT-DS in the SKOV3 tumor mice was determined and the results are shown in Fig. 6b–d. At 1 h p.i., 80% of the probe remained intact in tumor, while a small percentage of the intact probe was observed from urine samples obtained at 1 h p.i., indicating relatively rapid degradation of the probe in the kidney-urinary system. The HPLC result also showed that a small fraction of probe was metabolized to a more lipophilic fragment in liver.

Discussion

PET has been demonstrated to be a powerful noninvasive tool to address many important clinical questions in oncology. It has the advantages of high sensitivity, high spatial resolution, and strong quantification ability. PET probes could have higher clinical translation potential than many other imaging modalities. Molecular probes for HER2 imaging could be used for early detection of HER2-positive tumor recurrence, stratification of cancer patients and dose optimization for HER2-targeted therapy, and monitoring the efficacy of tumor treatment [23, 24]. The HER2-targeted PET probes are expected to provide a sensitive and accurate method for real-time assay of HER2 expression in all tumor sites (primary and metastatic lesions) in living subjects. However, until now an ^{18}F PET probe for HER2 imaging has not been developed and approved in clinical use yet.

Affibody proteins have fast tumor targeting, efficient tumor extravasation, good serum stability, and fast clearance from non-targeted organs [4, 7]. In this study, we site-

specifically conjugated the engineered two-helix scaffold protein MUT-DS with $^{18/19}\text{F}$ -FBA and measured its binding specificity and affinity to HER2. The conjugated ^{19}F -FBO-MUT-DS has good binding affinity with K_D of 1 nM. The in vitro cell uptake experiment shows that the radioactive counterpart ^{18}F -FBO-MUT-DS has rapid accumulation in the SKOV3 cells. The uptake reaches a plateau in 15 min at 37°C . Furthermore, the accumulation is HER2 specific since blocking with $Z_{\text{HER2}:342}$ can significantly reduce the cell uptakes of the probe (12.1–5.0% of applied activity at 1 h at 37°C , $p < 0.05$) (Fig. 3). More importantly, ^{18}F -FBO-MUT-DS exhibits favorable in vivo pharmacokinetics. Good tumor imaging contrast can be obtained at even 1 h p.i. of the probe. The biodistribution study shows high tumor uptake ($\sim 7\% \text{ID/g}$) at 1 h p.i., which confirms the fast tumor targeting ability of the probe. Liver and kidney also have similar uptakes (Fig. 4a). Since neither of these normal organs has high HER2 expression, this result may be attributed to the fact that ^{18}F -FBO moiety is lipophilic and a significant amount of the radiometabolites could be produced and cleared by the liver (Fig. 4a). Because AO-MUT-DS itself is hydrophilic, most of the intact probes are rapidly cleared through the kidney-urinary system. It is also interesting to find out that the tumor uptake of the ^{18}F -FBO-MUT-DS is slightly higher than the radiometal ^{68}Ga -labeled MUT-DS, while the kidney uptake of the probe is much lower compared to ^{68}Ga -labeled MUT-DS, suggesting the advantages of using ^{18}F as a PET label [19]. It should be noted that because of the higher lipophilicity of ^{18}F -FBO-MUT-DS than that of ^{68}Ga -DOTA-MUT-DS, it displays relatively higher nonspecific uptakes in tumor cells (Fig. 3) and lower tumor to blood (T/B) and tumor to liver ratios than that of ^{68}Ga -DOTA-MUT-DS [18]. Using low lipophilic ^{18}F labels may help to solve this problem. The in vivo tumor targeting specificity of the probe has also been verified by the blocking study. When $500 \mu\text{g}$ of $Z_{\text{HER2}:342}$ was co-injected, both tumor uptakes and tumor to normal organ ratios decreased significantly ($p < 0.05$) (Fig. 4a, b).

Based on the biodistribution and microPET results, it has been found that the two-helix scaffold protein-based probe, ^{18}F -FBO-MUT-DS (5 kDa), shows better tumor imaging properties for high HER2 expression tumors than that of ^{18}F -trastuzumab-Fab (50 kDa, tumor uptake $1.31\% \text{ID/g}$, T/B ratio 2) [25] and ^{18}F -FBO- $Z_{(\text{HER2}:477)_2}$ (15 kDa, tumor uptake $\sim 2\% \text{ID/g}$, T/B ratio 1.5) [20]. It has similar tumor uptake and T/B ratio than that of ^{18}F -FBO- $Z_{\text{HER2}:477}$ (8 kDa, tumor uptake $\sim 5\% \text{ID/g}$, T/B ratio 3–10) [19], but worse T/B ratio than that of ^{18}F -FBEM- $Z_{\text{HER2}:342}$ (8 kDa, tumor uptake $\sim 10\% \text{ID/g}$, T/B ratio 7.5–20) [26]. These data suggest that the ^{18}F -labeled two-helix small protein with high specific radioactivity and good tumor targeting ability has potential applications for diagnosis of HER2-positive cancer in the clinical setting. Further optimization of the radiofluorination strategy may further improve the in vivo performance of the two-helix protein-based PET probe. In a recent patient study, a low dose ($\sim 100 \mu\text{g}$, 10 nmol) of ^{68}Ga - or ^{111}In -labeled Affibody showed good imaging contrast at all metastasis sites except in liver [27]. This result highlights the potential use of ^{18}F -labeled MUT-DS which has similar hydrophilicity as radiometal-labeled Affibodies for HER2 imaging.

In conclusion, ^{18}F -FBO-MUT-DS was successfully radio-synthesized, and it demonstrates good stability. More importantly, ^{18}F -FBO-MUT-DS displays excellent HER2 targeting ability and tumor PET imaging quality. The favorable in vivo property of the two-helix

scaffold protein makes it suitable for development of ^{18}F -based PET probes for tumor imaging.

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Abbreviations

HER2	Epidermal growth factor receptor type 2
FBO	Fluorobenzyl oxime
PET	Positron emission tomography
HPLC	High-performance liquid chromatography
p.i	Post-injection

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a Two-helix scaffold protein, AO-MUT-DS (a disulfide bridge was formed between two homocysteine residues), was rationally designed and synthesized by solid phase peptide synthesis. **b** Synthetic scheme of ^{18}F -fluorination of engineered two-helix protein AO-MUT-DS

Fig. 2.

Biosensor binding studies of unmodified and modified MUT-DS. Sensorgrams were obtained after injection of various concentrations of purified AO-MUT-DS (**a**) and FBO-MUT-DS (**b**) onto sensor chip flow cell surface containing amine-coupled Fc-HER2 chimeric protein. *Resp. Diff.* respective difference

Fig. 3.
Cell uptakes of ^{18}F -FBO-MUT-DS in SKOV3 cells over time at 37°C with or without the presence of nonradioactive Affibody molecules $\text{Z}_{\text{HER2:342}}$. All results expressed as mean of triplicate measurement $\pm$ standard deviation

Fig. 4.

a In vivo HER2 tumor targeting specificity of ^{18}F -FBO-MUT-DS: 1 h blocking, 1 h and 3 h biodistribution results of ^{18}F -FBO-MUT-DS are shown for with or without co-injection of 500 μg of ^{18}F -FBO-MUT-DS ($n=3$). **b** Tumor to normal organ ratios of ^{18}F -FBO-MUT-DS in mice bearing SKOV3 tumor at 1 h p.i. and 3 h p.i. without or 1 h p.i. with co-injection of 500 μg of Affibody $Z_{\text{HER2}:342}$ ($n=3$)

Fig. 5.

a Representative coronal and transaxial images of micro-PET of a nude mouse bearing SKOV3 tumor on right shoulder at 1, 2, and 3 h after tail vein injection of ^{18}F -FBO-MUT-DS (3.0–3.7 MBq, 80–100 μCi) ($n=3$). *T* tumor, *GB* gallbladder. **b** PET quantification of ^{18}F -FBO-MUT-DS uptake in tumor, liver, and kidney. * $p<0.05$

Fig. 6.

HPLC radiochromatograms of purified radiolabeled probe ^{18}F -FBO-MUT-DS in PBS (a). In vivo metabolic stability study of ^{18}F -FBO-MUT-DS. Samples are taken from tumor (b), urine (c), and liver (d)