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Radiolabeling of HER2-specific Affibody® molecule with F-18

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ABSTRACT

The presence of human epidermal growth factor type 2 (HER2) on 20–30% of human breast cancer is a prognostic indicator of more rapid disease progression and a therapeutic indicator for anti-HER2 monoclonal antibodies. Because the literature has demonstrated some discordance between primary and metastatic tumors in the same patient for expression of the HER2 marker, we set out to develop an imaging agent that could be used to assess the marker concentration in vivo in an individual patient. The pharmaceutical company Affibody® AB has optimized the specificity of Affibody® molecules for HER2. Two Affibody® molecules, a 7 kDa and an 8 kDa protein, were designed with a single carboxy terminal cysteine in order to provide a specific location for the purposes of labeling for various types of imaging. We have prepared [^{18}F]FBEM utilizing a coupling reaction between [^{18}F]fluorobenzoic acid and aminoethylmaleimide. We then optimized the conjugation of this radiolabeled maleimide to the free sulfhydryl of cysteine by incubating at pH 7.4 in phosphate buffered saline containing 0.1% sodium ascorbate. An overall uncorrected yield of radiolabeled Affibody® molecule of approximately 10% from [^{18}F]fluoride was achieved in a 2 h synthesis. These conjugated Affibody® molecules were obtained with a specific activity of 2.51 ± 0.92 MBq/μg. Characterization of the product by HPLC–MS supported the conjugation of [^{18}F]FBEM with the Affibody® molecule. The radiolabeled Affibody® molecule retained its binding specificity as demonstrated by successful imaging of xenografts expressing HER2.

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1. Introduction

Positron emission tomography (PET) is a uniquely sensitive imaging technique that can achieve specificity with the appropriate radiolabeled compounds. The technique relies on the development of specific molecules radiolabeled with positron emitting isotopes. A significant amount of early work in PET tracer development focused on small molecules for imaging metabolism and receptors for neurotransmitters. As the field expanded away from neurochemistry into the realm of cancer diagnosis and therapy, large molecules such as antibodies were radiolabeled. It soon became apparent that the large size of these molecules and the unfavorable uptake and clearance kinetics did not justify the exploration of radiolabeling techniques with the abundantly prepared short half-life positron emitting radionuclides F-18 ($t_{1/2}$ = 109.8 min) or C-11 ($t_{1/2}$ = 20.4 min).

One solution was to explore smaller fragments of antibodies with the hope that the smaller molecules would have faster distribution kinetics. Some success was achieved following this route [1]. Recently, even smaller Affibody® molecules (hereafter the registration will not be shown), based on a 58-amino acid residue derived from the B domain of immunoglobulin binding region of staphylococcal protein A, have been developed providing the smallest protein molecules designed with specificity to receptors [2]. We chose to investigate the development of a F-18 analogue of the Affibody® molecule His_6-$Z_{HER2-342}$ ($Z_{HER2:342}$) which has high affinity and specificity for HER2 (human epidermal growth factor receptor type 2) [3]. This particular receptor has prognostic and therapeutic utility for breast cancer patients [4–7]. An imaging agent that can detect and quantify this receptor would be of value to the oncologist planning and following the treatment of individual patients.

Proteins have numerous available functional groups, but we wished to achieve a specific site labeling. *N*-Hydroxysuccinimidyl-4-[^{18}F]fluorobenzoyl ([^{18}F]SFB) is the most commonly used agent for the labeling of lysine residues on proteins. A recent publication by Vaidyanathan and Zalutsky describes the most recent optimized radiosynthesis of [^{18}F]SFB and contains references to many of the applications [8]. The Affibody® molecules we had available contained six lysine residues. Thus, radiolabeling on a specific lysine residue would probably not be completed successfully. The

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Affibody® molecule $Z_{HER2:342}$, which contained no cysteine, was modified with a C-terminal cysteine to provide a unique site for attachment of a radiolabeling group. Cysteine is known to react selectively with maleimide groups in the presence of amino groups at pH < 7.5 [9]. It remained for us to choose and develop the appropriate radiolabeled maleimide group for conjugation.

The radiolabeling of C-terminal cysteine Affibody® molecules with a ^{76}Br ($t_{1/2}$ 16.2 h) labeled maleimide has been reported [10]. Other radiolabeled Affibody® molecules have been prepared for imaging with single-photon emitting radionuclides including In-111 [11] and Tc-99m [12,13]. We pursued radiolabeling with the more readily available PET radionuclide fluorine-18. A previous abstract by Shiue et al. reported two [^{18}F]labeled maleimides, 1(4-[^{18}F]fluorophenyl)pyrrole-2,5-dione ([^{18}F]FPPD) and *N*-[3-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)phenyl]-4-[^{18}F]fluorobenzamide ([^{18}F]DDPFB) [14]. de Bruin et al. reported the preparation of the heteroaromatic [^{18}F]maleimide, 1-[3-(2-[^{18}F]fluoropyridine-3-yloxy)propyl]pyrrole-2,5-dione) [15]. *N*-[4-[(4-[^{18}F]Fluorobenzylidene)aminooxy]butyl]-maleimide has also been prepared as an alternative for ^{18}F-labeling of sulfhydryls [16]. Cai et al. had prepared *N*-[2-(4-[^{18}F]fluorobenzamido)ethyl]maleimide ([^{18}F]FBEM) for the labeling of an RGD peptide-based tracer [17].

In a previous manuscript [18], we briefly described our approach to the radiochemical synthesis of [^{18}F]FBEM-$Z_{HER2:342}$ and the in vitro and in vivo characterization of the protein preparation, including it pharmacokinetics and application to PET imaging of HER2-positive tumors. In this manuscript, we report in more detail our studies to improve the yield of the coupling of [^{18}F]FBEM to the C-terminal cysteine of two HER2-specific Affibody® molecules, $Z_{HER2:342}$-cys and $Z_{HER2:2395}$-cys. In addition we describe improved chromatographic and mass spectral characterization methods and application of these Affibody® molecules for in vivo imaging of HER2-positive breast cancers.

2. Results and discussion

2.1. [^{18}F]FBEM

Malemide chemistry was used for site-specific labeling of $Z_{HER2:342}$-cys and $Z_{HER2:2395}$-cys Affibody® molecules with [^{18}F]fluoride. The conjugation group *N*-[2-(4-fluorobenzamide)ethyl]maleimide ([^{18}F]FBEM) has been reported [17]. We utilized a different radio-synthetic approach for the preparation of [^{18}F]FBEM by first preparing [^{18}F]fluorobenzoic acid (**3**) [19] and coupling this to 2-aminoethylmaleimide using diethyl cyanophosphonate (Fig. 1). This coupling reagent was previously applied to the preparation of [^{18}F]paclitaxel [20] and was applied in our previous communication on the biological selectivity of [^{18}F]$Z_{HER2:342}$-cys [18]. This procedure was very reproducible and provided adequate yields of [^{18}F]**4** (22.0 ± 4.7% uncorrected). Further optimization of the preparation of [^{18}F]FBEM has not been explored.

2.2. Conjugation

The $Z_{HER2:342}$-cys Affibody® molecule was demonstrated (see below) to exist as a mixture of monomeric and dimeric forms due to intermolecular disulfide bond formation. In our initial report on the radiolabeling of $Z_{HER2:342}$-cys [18], the Affibody® molecule was first treated with dithiothreitol (DTT) to reduce the disulfide bond. The excess DTT was removed using size exclusion on NAP-5 column eluted with 100 mM sodium acetate. The most concentrated fraction was mixed with [^{18}F]FBEM for the radiolabeling reaction (Fig. 2). This purification resulted in the loss of some protein and a more dilute protein solution for the radiolabeling reaction. Using this conjugation procedure, the overall radiochemical yield based on initial [^{18}F]fluoride was 6.5 ± 2.2% (n = 26, uncorrected for decay).

We explored conjugation conditions to improve the radiochemical yield and to provide a more reliable radiochemical synthesis. Literature and product specifications suggested that the protein could be converted to all monomeric free sulfhydryl form by treatment with excess tris(2-carboxyethyl)-phosphine hydrochloride (TCEP). This procedure would not require protein purification with the subsequent loss of protein and dilution of concentration. However, the conjugation reaction between [^{18}F]FBEM and $Z_{HER2:342}$-cys failed in the presence of excess TCEP. Addition of [^{18}F]FBEM to an excess of TCEP led to rapid formation of a new, slightly earlier eluting radioactive peak from the HPLC column. In an NMR tube experiment, in which equimolar quantities of TCEP and FBEM were mixed, the vinylic proton signals of the maleimide disappeared quickly (Fig. 3).

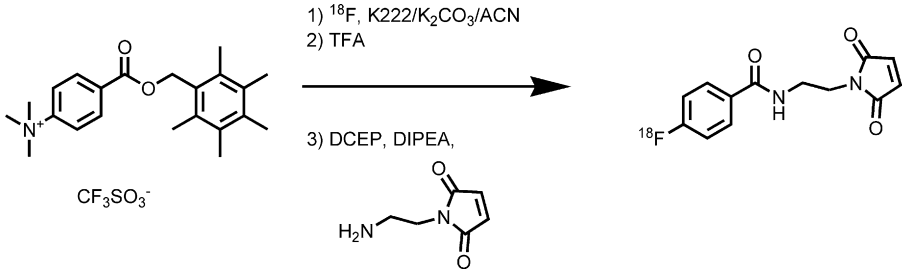

Fig. 1. Synthesis of *N*-[2-(4-[^{18}F]fluorobenzamide)ethyl]maleimide ([^{18}F]FBEM) (**4**).

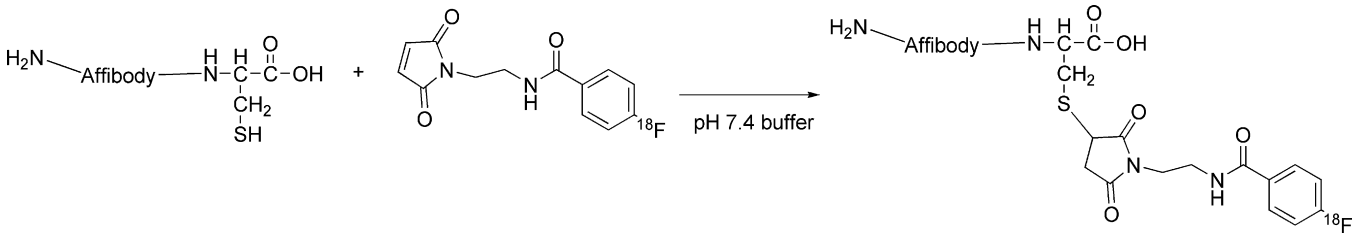

Fig. 2. . Conjugation of Affibody® molecule.

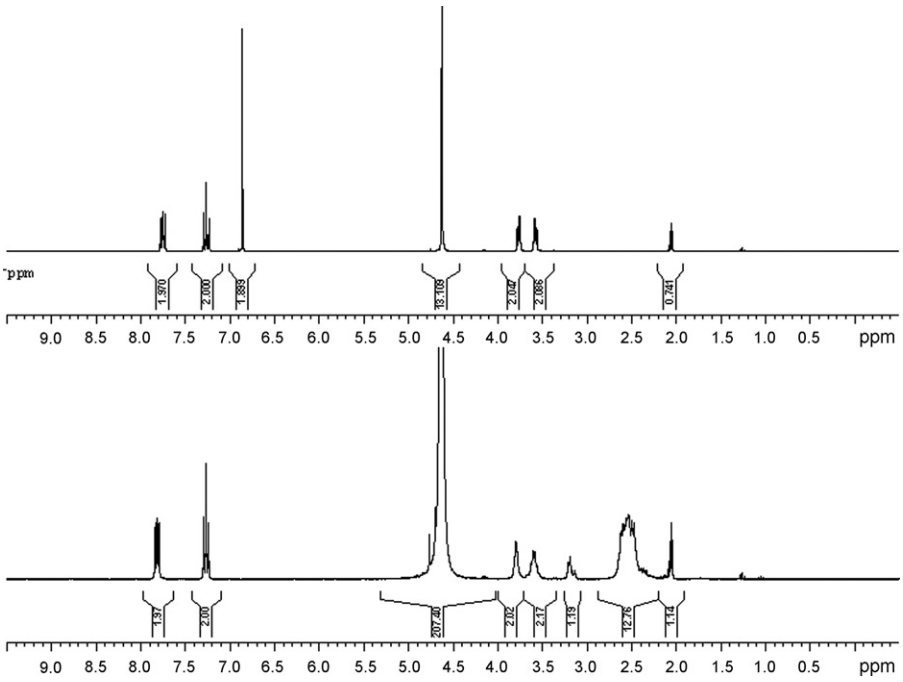

Fig. 3. ^{1}H NMR spectra of FBEM (upper panel) and FBEM treated with 1 equiv. of TCEP (lower panel) in D_2O/CD_3CN.

Re-examination of earlier literature revealed reports on the formation of phosphorous ylides from reaction of phosphines with maleimides [21]. Other publications have demonstrated the reaction of TCEP with maleimide without determining the product formed [22,23]. Thus, our observation of a reaction between TCEP and the maleimide was not unexpected. In standard usage of TCEP for disulfide reduction, large excess of maleimide over both protein and TCEP are utilized, which explains the claim that TCEP does not interfere with maleimide chemistry. In our radiosynthesis the [^{18}F]FBEM is the limiting reagent and excess TCEP prevents the desired reaction.

We were able to successfully label the protein by treating the stock Affibody® molecule with 0.5 equiv. of TCEP for 40 min followed by addition of the [^{18}F]FBEM. This procedure, we

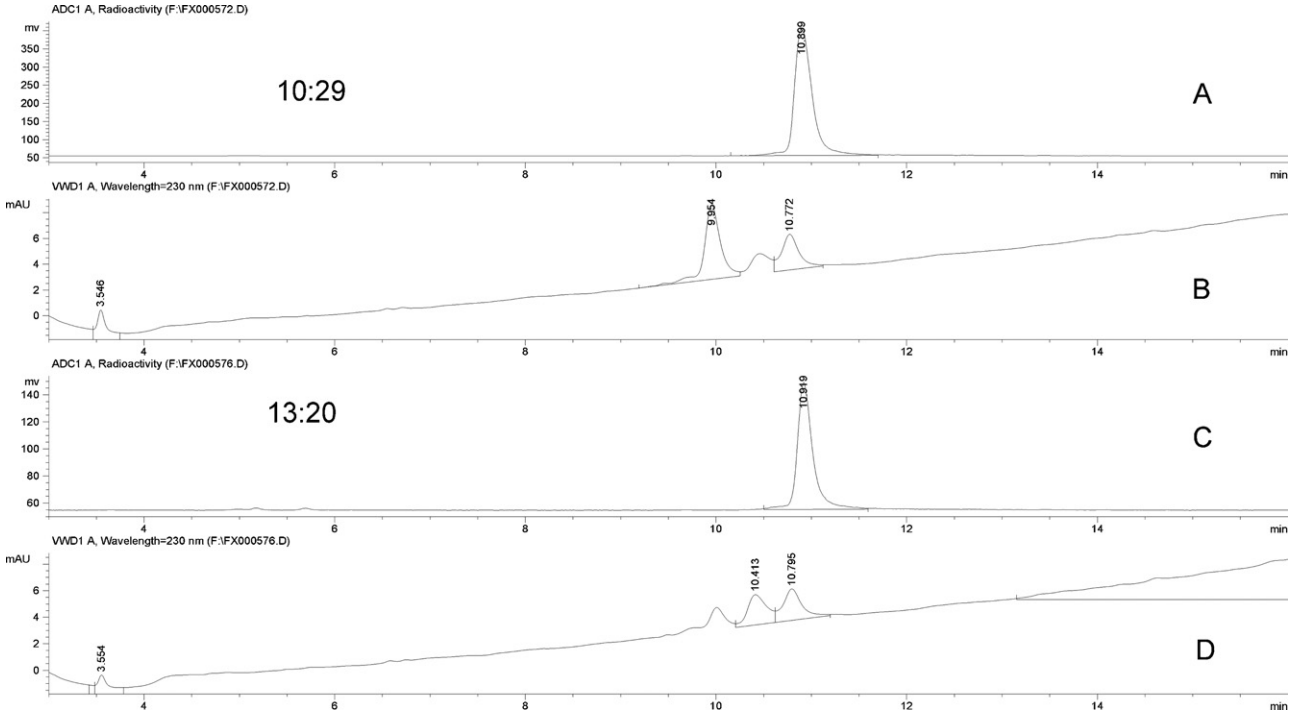

Fig. 4. HPLC (UV at 230 nm) and radioactivity of $Z_{HER2:342}$-cys–[^{18}F]FBEM showing resolution of monomeric (9.9 min), dimeric (10.4 min), and conjugated species (10.8 min). The radiochemical stability was demonstrated by re-assay after 3 h. Time 0 is shown in panels A (radioactivity) and B (UV); panels C (radioactivity) and D (UV) show the 3 h time point.

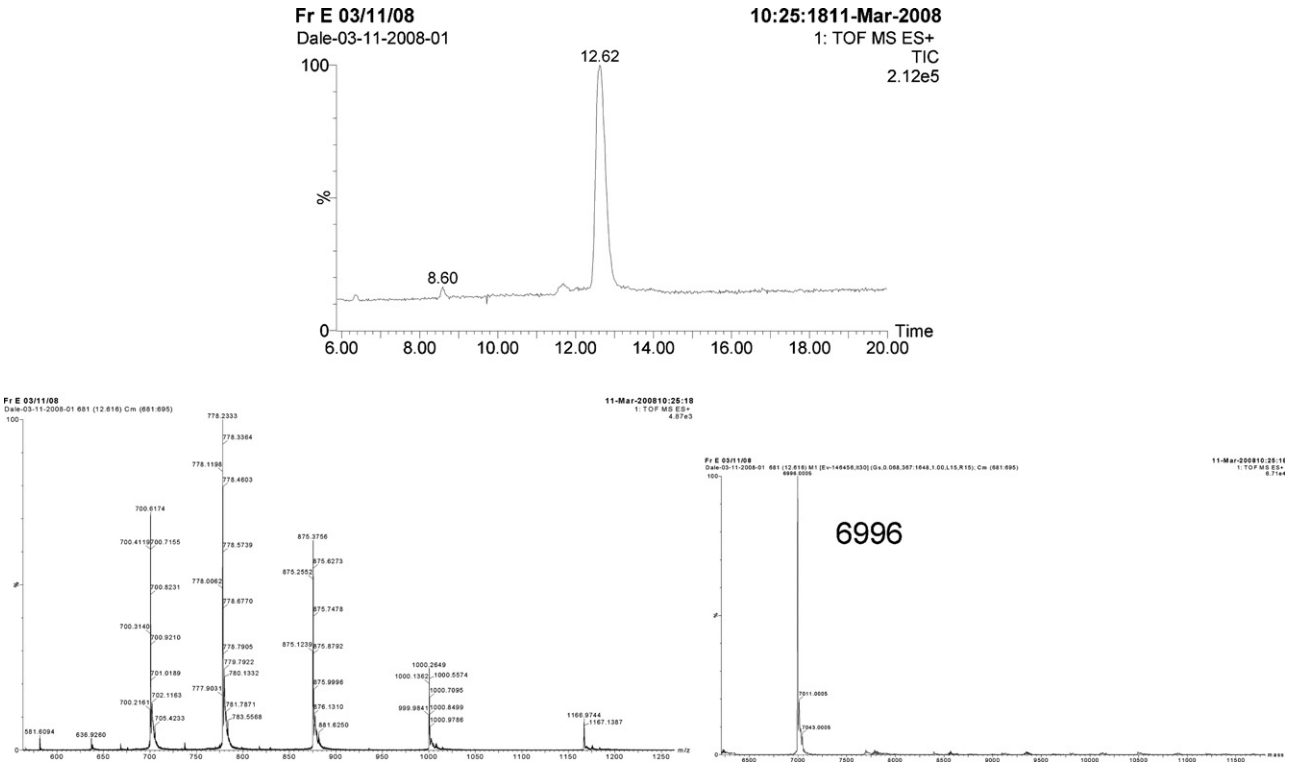

Fig. 5. UPLC–MS of $Z_{HER2:2395}$-cys following treatment with DTT and purification by NAP5 column. Monomeric protein demonstrated a deconvoluted FW of 6996 (theoretical 6993).

hypothesized, would result in complete oxidation of TCEP so that it would not react with the stoichiometrically deficient [^{18}F]FBEM. However, a significant amount of the protein could remain as a disulfide dimer. Although radiolabeling was achieved, the conditions were not robust to changes in lot of precursor protein and did not provide improvement in radiochemical yield.

We returned to the DTT conditions to optimize reaction parameters. To test the hypothesis that the pH of the conjugation reaction was inconsistent due to lack of buffering ability of acetate at pH 7, we changed to phosphate buffered saline (pH 7.4). The resulting conjugation reaction proceeded more quickly, but was accompanied by a small amount of radiochemical impurities. The addition of 0.1% sodium ascorbate to the conjugation buffer effectively eliminated these impurities. We do not routinely measure the radiochemical purity or specific activity of the [^{18}F]FBEM. The few measurements that we have indicate a specific activity of 37–74 GBq (1–2 Ci)/μmol at end of synthesis. Thus 370 MBq (10 mCi) of [^{18}F]FBEM would be 5–10 nmol. Since we utilize about 12 nmol of protein at the beginning of the process, we are approaching equal stoichiometry between substrate and reagent for the conjugation reaction. The conjugation reaction is allowed to proceed for 30 min. The average yield of the conjugation reaction based on [^{18}F]FBEM, independent of Affibody® molecule precursor, was 56.2 ± 3.9% uncorrected. With these finalized reaction conditions, we have conducted 39 radiochemical syntheses of $Z_{HER2:342}$-cys–[^{18}F]FBEM and achieved a final uncorrected radiochemical yield of 10.5 ± 1.9% in an average time of 117 min. $Z_{HER2:2395}$-cys–[^{18}F]FBEM has been prepared five times with a radiochemical yield of 11.6 ± 0.9% in an average time of 110 min. Thus beginning with about 60–68 mCi of [^{18}F]fluoride, 6.5–8.5 mCi of $Z_{HER2:2395}$-cys–[^{18}F]FBEM was obtained in two fractions totaling 0.5 mL. This improved procedure has resulted in higher overall radiochemical yields with lower deviation between runs.

2.3. Analysis of the radiolabeled product

We developed an analytical HPLC method that allowed the separation of the monomeric $Z_{HER2\ 2:342}$-cys from the dimeric form. The same system effectively separated the monomeric precursor from the radiolabeled conjugate (Fig. 4). Unfortunately the dimeric precursor was not completely separated from the radiolabeled conjugate. These chromatographic conditions are also effective for separating the monomer and dimer of $Z_{HER2:2395}$-cys, however the radiolabeled conjugate co-eluted with the dimer. The system utilized a Zorbax 300SB C-18 column and gradient elution that maintained 0.1% trifluoroacetic acid (TFA). Analytical HPLC showed the presence of a single radiolabeled peak after the final isolation from the conjugation solution. The presence of sodium ascorbate in the final solution was important to reduce the decomposition rate of the conjugated protein. Although we believe non-conjugated dimer continues to be formed from the unconjugated monomer, the radiolabeled conjugate has higher stability (Fig. 4). The radiochemical purity remains > 90% for at least 3 h.

The protein concentration was measured by UV at 280 nm using the conversion factor determined by Affibody® AB ($Z_{HER2:342}$, 1 mg/mL = 0.99 AU; $Z_{HER2:2395}$, 1 mg/mL = 0.836 AU). This measurement had to be made prior to addition of sodium ascorbate. The average specific activity for 14 batches (including measurements from both proteins) was 2.51 ± 0.92 MBq (93.0 ± 34.2 μCi)/μg.

We have recently developed conditions for LC–MS that have allowed further characterization of the radiolabeled product. This method has only been available since we began studies on the radiochemical synthesis of $Z_{HER2:2395}$-cys–[^{18}F]FBEM. Unfortunately, the TFA that is so essential to improved chromatographic resolution inhibits ionization and thus results in poor mass spectral sensitivity. We were able to substitute with HOAc but at the expense of resolution. In spite of the loss of resolution, the

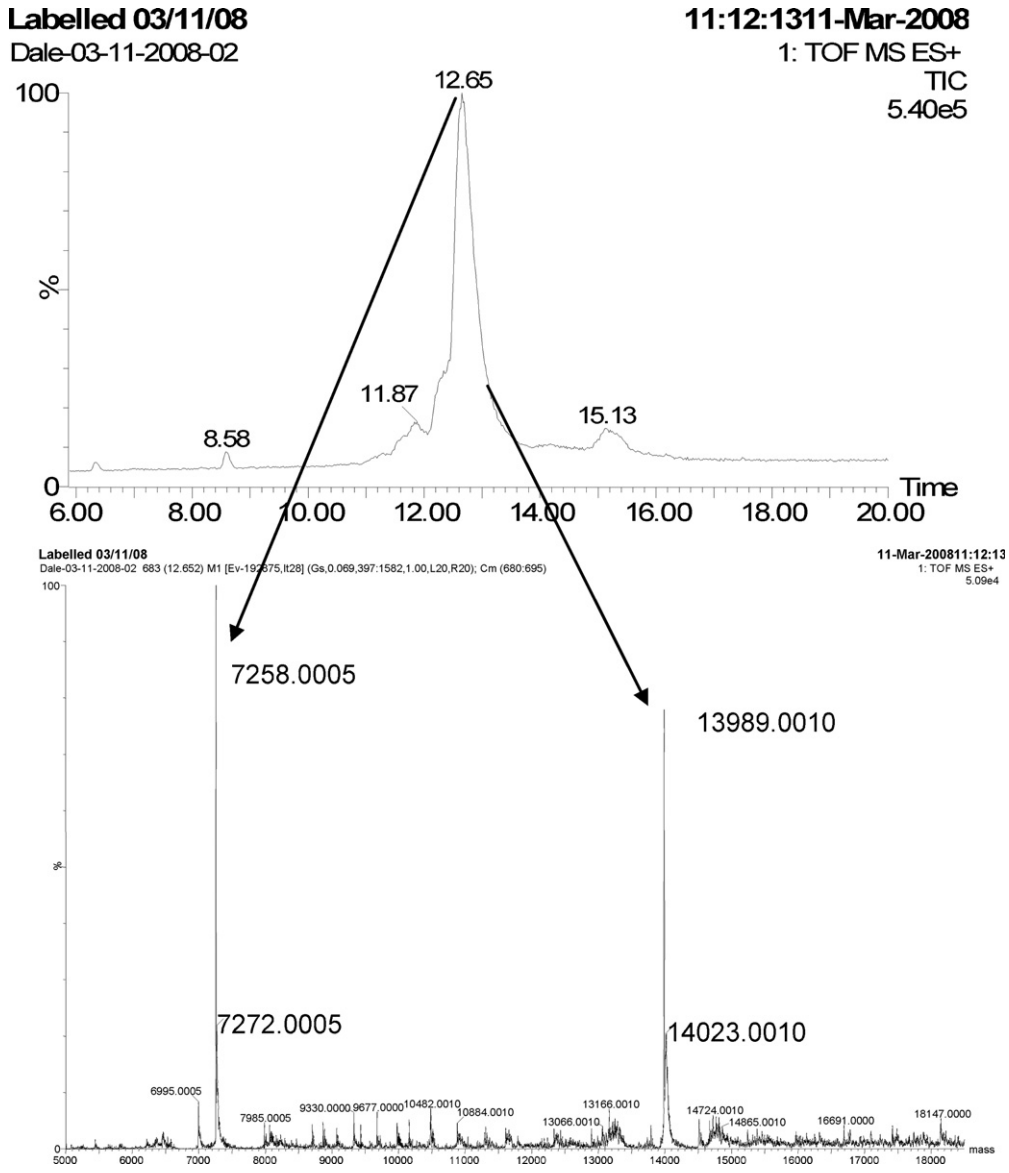

Fig. 6. UPLC–MS of $Z_{HER2:2395}$-cys-[^{18}F]FBEM. Major peak consistent with [^{18}F]FBEM conjugated protein (FW = 7258). The tail of the major peak had mass spectral peaks consistent with the non-conjugated dimer.

major protein components could be evaluated by their mass spectral deconvolution. The analyses conducted of Affibody® $Z_{HER2:2395}$-cys and its radiolabeled conjugate are shown in Figs. 5 and 6. Fig. 6 shows a mixture of dimeric precursor (calculated m/z 13984; observed 13989) and [^{18}F]FBEM conjugated Affibody® molecule (calculated m/z 7255: observed 7258).

Polyacrylamide gel electrophoresis (PAGE) of the radiochemical product from $Z_{HER2:342}$-cys indicated that > 90% of the radio-

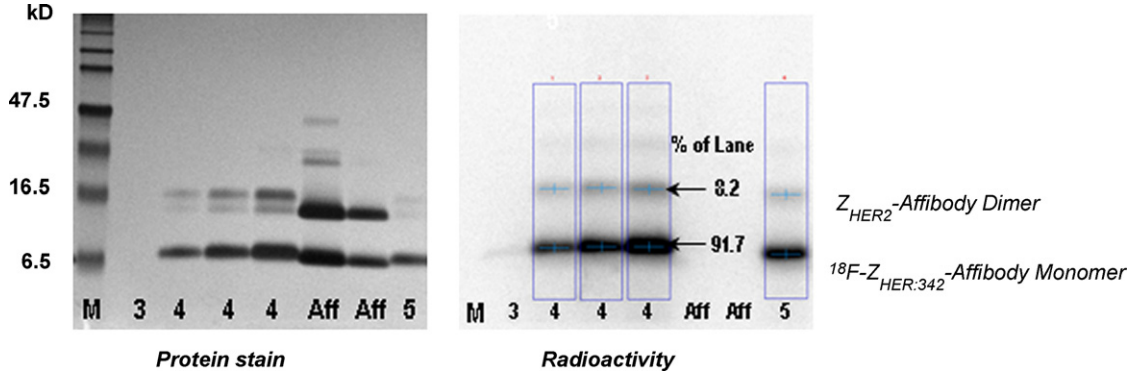

Fig. 7. Electrophoresis of NAP-5 fractions from the radiochemical synthesis of $Z_{HER2:342}$-cys-[^{18}F]FBEM.

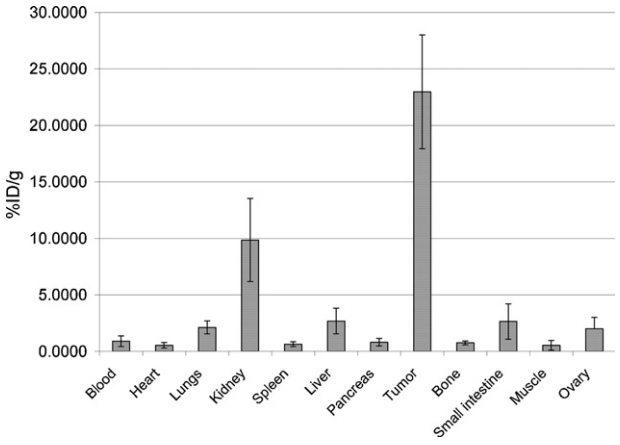

Fig. 8. Biodistribution at 2 h post i.v. injection of $Z_{HER2:342}$-cys–[^{18}F]FBEM. Each bar represents an average ±S.D. from n = 4–5 animals.

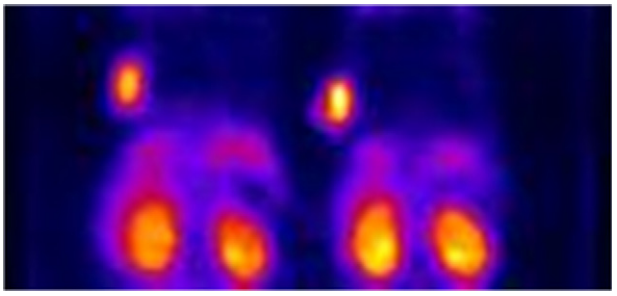

Fig. 9. Whole-body image of two mice bearing BT474 tumor located on the shoulder 2 h after i.v. injection of $Z_{HER2:342}$-cys–[^{18}F]FBEM.

activity migrated with the 8 kDa protein band (Fig. 7). Silver staining of the protein bands showed a mixture primarily of dimeric precursor and radiolabeled conjugate; small amounts of higher molecular weight proteins were observed.

2.4. In vivo biodistribution and imaging

In our previous publication, we demonstrated the selectivity of uptake to those tumors that present HER2 receptor and that the biodistribution of the [^{18}F]radiolabeled conjugate showed promising results for use as an in vivo imaging agent [18]. With this improved radiochemical synthesis, we conducted biodistribution and imaging studies in the HER2 abundant BT474 tumor xenograft model. The biodistribution studies (Fig. 8) showed high accumulation in the tumor (23% ID (injected dose)/g) and with significant, although somewhat lower, uptake in the kidney (10% ID/g). An imaging study (Fig. 9) shows the highly visible tumor and kidney at 2 h post injection. Region-of-interest (ROI) analysis showed tumor to muscle ratio was as high as 22. Very low background activity was observed even at earlier times. In fact, specific accumulation of radioactivity in HER2 expressing tumors was clearly visible 20 min post radiotracer injection (data not presented). Thus the $Z_{HER2:342}$-cys–[^{18}F]FBEM produced by the improved synthesis conditions has not altered the in vivo properties. This radiotracer has high receptor affinity and selectivity for HER2 and favorable pharmacokinetics for clinical application in tumor imaging.

3. Conclusion

We had developed a routine manual procedure for the radiosynthesis of [^{18}F]FBEM that allows selective conjugation of free cysteine residues on proteins. The [^{18}F]FBEM was prepared in 22.0 ± 4.7% yield (uncorrected). Because the protein contained a single cysteine residue, the stock protein contained a mixture of monomer and disulfide dimer. Successful conjugation required treatment of the stock protein with DTT, in order to maximize the amount of free thiol, and size exclusion to remove excess DTT. Utilization of PBS with 0.1% sodium ascorbate for the conjugation reaction proved to be the key modification in order to provide improved yield, improved product stability, and provide a reliable, robust radiosynthesis. Incubation of [^{18}F]FBEM with the reduced protein in PBS with 0.1% sodium ascorbate for 30 min followed by size exclusion on a NAP5 column provided the desired radiolabeled protein. The $Z_{HER2:342}$-cys–[^{18}F]FBEM and $Z_{HER2:2395}$-cys–[^{18}F]FBEM were obtained in 10.5 ± 1.9% and 11.6 ± 0.9% based on initial [^{18}F]fluoride, respectively, in a synthesis time of less than 2 h. Identity was confirmed by HPLC–MS-ESI. Radiochemical purity was high as shown by HPLC with radioactivity detection and PAGE. The biodistribution properties of the radiochemical product obtained by this modified synthesis route are unaltered from our original report. Thus we are still optimistic that this radiotracer will have clinical application for tumor imaging.

4. Experimental

4.1. General experimental procedures

Unless otherwise specified, all reagents were of analytical grade and were obtained from commercial sources. The Affibody® molecule His_6-$Z_{HER2-342}$-Cys and other closely related Affibody® molecules were provided by our Cooperative Research and Development Agreement partner Affibody® AB in Sweden (http://www.affibody.com). [^{18}F]Fluoride was obtained from the NIH/CC cyclotron facility from a proton irradiation of O-18 enriched water. Non-radiolabeled *N*-[2-(4-fluorobenzamide)ethyl]maleimide (FBEM) was prepared as previously described [17]. *N*-[2-(4-[^{18}F]fluorobenzamide)ethyl]maleimide ([^{18}F]FBEM) was prepared as previously described by us [18]. Protein concentration of the radiolabeled preparation was determined by measuring UV absorbance at 280 nm on NanoDrop spectrophotometer (ND-1100, Grace Scientific) using coefficient 0.99 mg/mL. Saline and phosphate buffered saline with or without 0.1% ascorbate were degassed for 20 min in an ultrasonic bath within 2 h of use for elution of protein from the NAP5 columns.

4.2. Reaction between FBEM and TCEP

FBEM (2.2 mg, 8.4 μmol) was dissolved in 200 μL CD_3CN and 600 μL D_2O. An NMR spectrum was recorded. Then TCEP (16.8 μl of a 0.5-M solution in water) was added at ambient temperature. An NMR spectrum was recorded 5 min after addition. Spectra are shown in Fig. 3.

4.3. Reduction of Affibody® molecule disulfide

A NAP-5 column (Amersham Bioscience, cut-off 5 kDa) was prepared by washing with 10 mL of phosphate buffered saline (PBS, pH 7.4) containing 0.1% sodium ascorbate. Stock Affibody® molecule $Z_{HER2:342}$-Cys or $Z_{HER2:2395}$-Cys (100 μg, 12 nmol, in 50 μL PBS) was treated with 10 μL of 1 M DTT for 40 min at 37 °C. The solution was loaded onto a NAP-5 column and then eluted, in 0.25 mL fractions of PBS (0.1% sodium ascorbate). Fraction 4 from this column was added to the ethanolic solution of *N*-[2-(4-[^{18}F]fluorobenzamide)ethyl]maleimide within 10 min from elution.

4.4. Protein conjugation

For these reactions, the solution of *N*-[2-(4-[^{18}F]fluorobenzamide)ethyl]maleimide in CH_2Cl_2, which had been eluted from a C-18 SPE column (Varian C-18 Bondelut, 500 mg) was evaporated in a 1.5-mL eppendorf tube. A drop of water (10–20 μL) was observed to remain in the tube. Ethanol (10 μL) was added to the tube followed by addition of the reduced Affibody® molecule solution. This solution was incubated for 30 min at room temperature. The reaction solution was then loaded onto and eluted from a second NAP-5 column (this column had previously been washed with 10 mL of eluent) using 250 μL portions of saline or saline containing 0.1% sodium ascorbate. The presence of sodium ascorbate prevents direct measurement of protein by UV. In order to measure protein by direct UV, saline was used as eluant, the UV was measured, and then sodium ascorbate (5 μL of 5% in saline) was added to the fractions to give a final ascorbate concentration of 0.1%. The most concentrated fraction containing the radiolabeled protein (fraction 4 in all cases) was collected and used for the biological experiments. Fraction 5 was generally the second most concentrated fraction and was also used for biological experiments. The HPLC analysis of the two fractions showed similar radiochemical purity. The radiochemical yield of ^{18}F labeled Affibody® conjugate contained in the fractions 4 and 5 was calculated based on starting ^{18}F-fluoride and not corrected for decay. The total procedure requires about 2 h.

4.5. HPLC and UPLC–MS-ESI of isolated $Z_{HER2:342}$-cys–[^{18}F]FBEM

Analytical HPLC–UV was performed on an Agilent HP1100 system with online radioactivity and UV detection. Chromatography employed a Zorbax 300SB C-18 column and a gradient elution profile of 20% CH_3CN (0.1% TFA) and 80% water (0.1% TFA) to 50% CH_3CN (0.1% TFA) over 20 min then ramping to 75% CH_3CN to flush out the column. UV was monitored at 250 nm. Retention times were about 8 min for [^{18}F]FBEM and 10.5 min for $Z_{HER2:342}$-cys–[^{18}F]FBEM.

A Waters Acquity HPLC and Waters Q-Tof Premier high resolution mass spectrometer were employed for UPLC–MS-ESI of $Z_{HER2:2395}$. The analysis utilized an Acquity BEH 130 C18 column (1.7 μm, 150 mm × 2.1 mm). The elution profile had four stages; initial conditions 100% H_2O (0.2% HOAc); linear gradient 0–15% CH_3CN (0.2% HOAc) over 3 min; linear gradient 15–30% CH_3CN (0.2% HOAc) for an additional 15 min; a linear gradient 30–80% CH_3CN (0.2% HOAc) for 2 min; and re-equilibrated with 100% aqueous for additional 1 min. The retention time for monomer, conjugate, and dimer were 11.8, 12.7 and 12.8 min, respectively. Deconvolution of the raw mass spectral data utilized the software program MaxEnt1 (Waters, Milford, MA). The mass spectral deconvolutions for $Z_{HER2:2395}$ (Figs. 5 and 6) show m/z for monomer 6996 (calculated 6993), conjugate 7258 (calculated 7255), and dimer 13989 (calculated 13984).

4.6. Protein assay by electrophoresis

Samples (5–15 μL) were diluted in SDS protein sample buffer (Invitrogen, Carlsbad, CA, USA) and then applied to 16% Novex® Tris–glycine gel. The electrophoresis was performed in Novex® Tris–glycine running buffer (Invitrogen, Carlsbad, CA, USA). Page Ruler Prestained Protein Ladder was used as the molecular weight standard (Fermentas Inc., Glen Burnie, MD, USA). The protein bands were visualized by silver staining (BioRad, Hercules, CA, USA).

4.7. Cell line and animal model

A parental human breast tumor cell line (BT-474) with high expression of HER2 receptors was obtained from American Type Culture Collection (Rockville, MD, USA) and cultivated at standard conditions (37 °C, 5% CO_2) in RPMI1640 medium with GlutaMAX (Invitrogen Corp.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY, USA).

The BT474 tumor xenograft model was generated by subcutaneous injection of 5.5×10^6 cells suspended in Matrigel (BD Bioscience, San Jose, CA, USA) into the right flank of the shoulder of 5–6-week-old female athymic nude mice. Before tumor cell inoculation, 0.72 mg/dose 17β-estradiol pellets (Innovative Research of America, FL, USA) were placed subcutaneously. Tumors (100–250 mg) developed after 4–5 weeks. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals on approved studies from the National Institutes of Health Institutional Animal Care and Use Committee.

4.8. Biodistribution studies

Mice bearing BT474 tumors (n = 4–5) were anesthetized with isoflurane/O_2 (1.5–5%, v/v) and injected with 5.5–6.3 MBq (3–4 μg protein, 100 μL) of $Z_{HER2:342}$-cys–[^{18}F]FBEM via the tail vein. Mice were sacrificed 2 h post-injection by cervical dislocation, and their organs dissected. Then, blood, tumor, and major organs were collected and weighed. The radioactivity in the tissues was measured along with a diluted standard (1:10) of the injected dose using γ-counter. The results were calculated as percentage injected dose per gram of tissue (% ID/g).

4.9. Small-animal PET studies

Mice (n = 5) were anesthetized and injected with $Z_{HER2:342}$-cys–[^{18}F]FBEM as done for the biodistribution studies. PET scans were performed using the Advanced Technology Laboratory Animal Scanner (ATLAS) PET scanner [24]. Whole-body scans (four bed positions, each 15 min) were started 60 min after radiotracer injection and recorded with a 100–700-keV energy window.

The images were reconstructed by a two-dimensional ordered subsets expectation maximum (2D-OSEM) algorithm, and no correction was applied for attenuation or scatter. For each scan, regions of interest were drawn over the tumor, normal tissue, and major organs. The maximum radioactivity accumulation within the tumor or organs was obtained from maximum pixel values within the multiple ROI. The results were calculated as a percentage injected dose per gram (% ID/g). At the end of the study, an ^{18}F source of known activity was imaged to obtain MBq of ^{18}F per counts per second for the imaging system (calibration factor). Then, every ROI (counts per second per cubic centimeter) was multiplied by this factor and divided by injected activity.

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