

RAPID COMMUNICATION

Direct Site-Specific Radiolabeling of an Affibody Protein with 4-¹⁸F]Fluorobenzaldehyde via Oxime Chemistry

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Abstract

Purpose: In this study, we introduce a methodology for preparing ¹⁸F-labeled Affibody protein, specifically ¹⁸F-Anti-HER2 dimeric Affibody (14 kDa), for *in vivo* imaging of HER2neu with positron emission tomography (PET).

Procedures: We have used 4-¹⁸F]fluorobenzaldehyde as a synthon to prepare ¹⁸F-Anti-HER2 Affibody. Aminoxy-functionalized Affibody (Anti-HER2-ONH₂) was incubated with 4-¹⁸F]fluorobenzaldehyde in ammonium acetate buffer at pH 4 in the presence of methanol at 70°C for 15 min. The resulting ¹⁸F-labeled Affibody molecule was evaluated as a PET probe in xenograft models expressing HER2.

Results: We have successfully prepared ¹⁸F-Anti-HER2 dimeric Affibody (14 kDa), *N*-(4-¹⁸F]fluorobenzylidene)oxime-Anti-HER2 Affibody, [¹⁸F]FBO-Anti-HER2, in 26–30% radiochemical yields (decay corrected). High-contrast small-animal PET images with relatively moderate tumor uptake (1.79±0.40% ID/g) were observed for the ¹⁸F-Anti-HER2 Affibody.

Conclusion: Site-specific ¹⁸F-labeled Affibody against HER2 has been synthesized via chemo-selective oxime formation between an aminoxy-functionalized Affibody and ¹⁸F-fluorobenzaldehyde. The results have implications for radiolabeling of other affibodies and macromolecules and should also be important for advancing Affibody imaging with PET.

Key words: Affibody, HER2, Positron emission tomography (PET), Imaging, ¹⁸F

Introduction

Despite the high potential and interest in targeted [¹⁸F]-labeled macromolecules (e.g., proteins) for positron emission tomography (PET)-based molecular imaging of living subjects [1–2], imaging probes have been infrequent. The major reasons include (1) incompatibility of the short radioactive half-life of ¹⁸F with the pharmacokinetics of the biological molecules and (2) the difficulty of directly

radiofluorinating typically fragile biomolecules and, furthermore, site-specifically to retain affinity. Affibody molecules [3–4], a novel class of proteins with molecular weight range of 6.5 to 14 kDa, have been engineered via phage display technology and have the potential to bind a variety of targets. Affibodies, specifically targeted against human epidermal growth factor receptor 2 (HER2, also known as HER2neu) labeled primarily with single-photon isotopes, including ^{99m}Tc [5], ¹²⁵I [6], and ¹¹¹In [7], have been reported, all of which show the affibodies to have *in vivo* pharmacokinetics compatible with short-lived isotopes. The

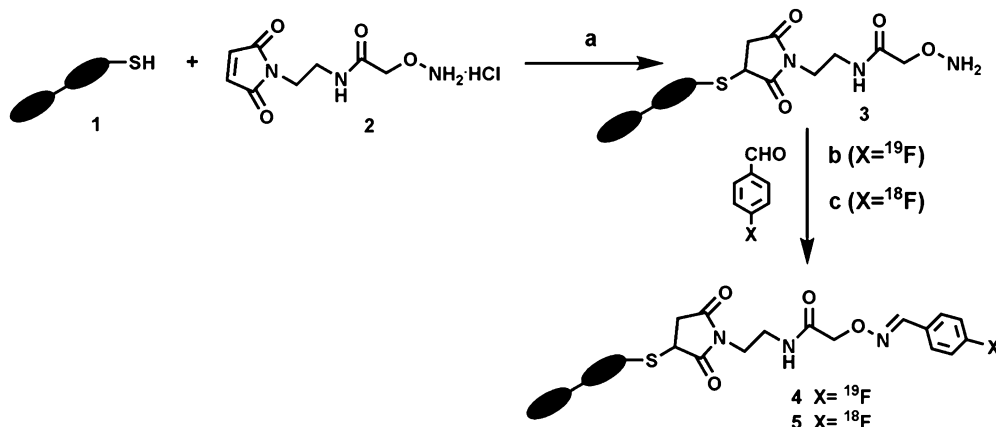
molecular size in combination with the high *in vivo* stability of the affibodies show both blood clearance and target uptake in the minutes time frame with imaging possible in an hour or less. Furthermore, their three-helical bundle structure provides very high chemical stability at various pH and temperatures that may enable more direct [¹⁸F]-labeling of the molecules. Herein, we report a direct and high-yield synthetic approach that allows for site-specific [¹⁸F]-labeling of an Affibody molecule targeted against HER2, using oxime chemistry and preservation of the Affibody's biological activity. The [¹⁸F]-labeled Affibody is also used to image tumors *in vivo* with microPET in living mice carrying tumor xenografts.

Recently, Poethko et al. [8] reported oxime formation between aminoxy-functionalized small peptides (decamers or less) and ¹⁸F-fluorobenzaldehyde as a high-yield two-step synthesis method to generate [¹⁸F]-labeled peptides that can be potentially translated to proteins. Unlike aminoxy functionalities, thiol moieties are more readily engineered into biologically generated proteins and have also been used for site-selective radiolabeling via maleimide-based ¹⁸F-synthons. Examples include *N*-[4-(4-[¹⁸F]fluorobenzylidene)aminoxybutyl]maleimide (¹⁸F-FBAM) [9], *N*-[6-(4-[¹⁸F]fluorobenzylidene)aminoxyhexyl]maleimide (¹⁸F-FBAHM) [10], and *N*-[2-(4-[¹⁸F]-fluorobenzamido)ethyl]maleimide (¹⁸F-FBEM) [11]. While both aminoxy and maleimide chemistry appear relatively chemo-selective, the latter class of synthons suffer from laborious multi-step synthetic preparations leading to low yields compared to the former, where Poethko et al. consistently obtained yields of 60–80%, under tracer chemistry conditions [8]. To achieve both site selectivity and high yield of a diagnostically relevant protein, we sought to combine the two strategies [12–16]. However, compared to small peptides, a protein with distinct tertiary structural folds and multiple reactive groups is potentially more susceptible to loss of function post high exposure to organic reagents (e.g., methanol), acidic pH (or pH close to the protein's isoelectric point), heat, or combinations thereof as used during ¹⁸F-aminoxy chemistry. Hence, an empirical

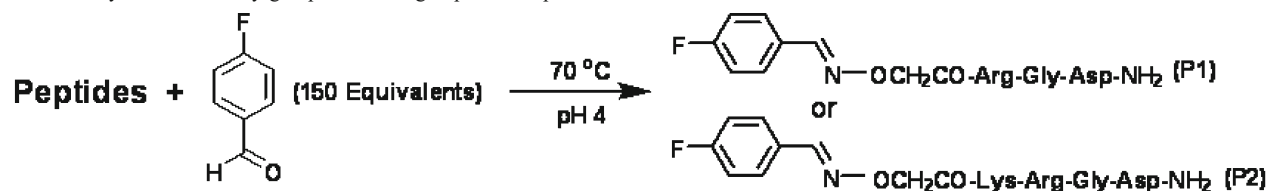
demonstration that both chemoselectivity and Affibody's target-binding ability are retained after ¹⁸F-aminoxy chemistry is warranted.

To test the feasibility of generating a functional ¹⁸F-Affibody protein, an anti-HER2 aminoxy-functionalized Affibody (Anti-HER2-ONH₂, 3) was prepared from its corresponding 14 kDa, bivalent Anti-HER2 Affibody 1 construct (Scheme 1). The presence of an engineered and distal C-terminal cysteine residue of the Affibody molecule provides a thiol moiety upon which site-selectivity chemistry can be effected. In order to introduce the aminoxy group, a bifunctional linker (2) was synthesized consisting of two orthogonal groups: a thiol-reactive maleimide group for conjugation to the engineered cysteine and an ¹⁸F-aldehyde-reactive aminoxy group. This linker was prepared in one step by reacting *N*-(2-aminoethyl)maleimide with 2-(tert-butoxycarbonylaminoxy)acetic acid using carbodiimide-mediated coupling conditions. On a subsequent step, the aminoxy group was exposed by cleavage of the Boc group under acidic conditions (3 M HCl). Introduction of the linker to the Affibody with a deprotected aminoxy does not additionally expose the protein to harsh conditions used for Boc-removal or produce observed adducts to the aminoxy group when using alternate acids such as trifluoroacetic acid. The linker was selectively conjugated to the Affibody by reduction of the disulfide-bridged Affibody dimer using dithiothreitol, followed by coupling at pH 7.4. Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) detected the presence of the expected product (calculated MW, 14431.26; found, 14431.45 Da).

Subsequently, we wanted to understand the selectivity of the aldehyde towards the oxime over more predominant amine and other reactive groups on the Affibody. Aldehydes can selectively react with aminoxy groups to form an oxime bond, but amine groups can also potentially react with the aldehyde-forming Schiff bases. Given the difference in pK_a between lysines and oximes (10.8 and 5, respectively) [17], tuning the pH reaction conditions can contribute to better selectivity. In addition, the reversible nature of Schiff



Scheme 1. Reagents and conditions (yields): **a** 1% DMSO, phosphate-buffered saline, pH 7.4, 2 h (79%); **b** 1% DMSO, NH₄OAc, pH 4, 1 h, 70°C (78%); **c** 33% CH₃OH, NH₄OAc, pH 4, 15 min, 70°C (26–32% decay corrected).

Table 1. Summary of results from control experiments performed with RGD peptides used as a model system to test the selectivity of ¹⁹F-fluorobenzaldehyde for aminoxy group vs. amine groups under specified conditions

Peptides	Reaction time (min)	Product
NH ₂ OCH ₂ CO-Arg-Gly-Asp-NH ₂ ^{a,b}	60	P1
NH ₂ OCH ₂ CO-Arg-Gly-Asp-NH ₂	45	P1
NH ₂ OCH ₂ CO-Lys-Arg-Gly-Asp-NH ₂	90	P2
Lys-Arg-Gly-Asp-NH ₂	90	No product

^a Reaction was performed at pH 3. ^b 8.5 Equivalents of 4-fluorobenzaldehyde was used.

bases against the non-reversible formation of oxime bonds supported the possible success in larger proteins [12–16].

To identify conditions favoring oxime formation over imines, a simple peptide model system utilizing “cold” chemistry that could be easily characterized was prepared. Two aminoxy-functionalized Arg-Gly-Asp (RGD) peptides and a control RGD peptide were designed and synthesized: NH₂OCH₂CO-Arg-Gly-Asp-NH₂ (6) and NH₂OCH₂CO-Lys-Arg-Gly-Asp-NH₂ (7), and Lys-Arg-Gly-Asp-NH₂ (8) (Table 1), and their reactions with 4-fluorobenzaldehyde under various conditions were studied. Peptide 6 contains only a single aminoxy group, peptide 7 bears an aminoxy and an ε-amine (Lys), and 8 contains an ε-amine and two free N-terminal amines. When both 6 and 7 were reacted at the same conditions (8.5–150 equivalents, 45–60 min, 70°C, acetate buffer pH 4) with 4-fluorobenzaldehyde and studied by analytical high-performance liquid chromatography (HPLC), in each case only one product was observed. Electrospray ionization mass spectrometry (ESI-MS) detected the HPLC-corresponding expected products (calculated MH⁺ of P1 from 6, 523.21, found 523.30; and calculated MH⁺ of P2 from 7, 653.31, found 653.43; Table 1). To confirm the formation of the oxime and not a Schiff base, control peptide 8 was also reacted under the same oxime-forming conditions, and no imine product was detected under analysis conditions employed. It is also noteworthy to mention that only one product corresponding to a single addition of 4-fluorobenzaldehyde was observed when the aminoxy was in the presence of an amine even when the reaction was pushed with 150 equivalents of fluorobenzaldehyde. The fact that no imine formation was observed with control 8 even with this large excess leads us to believe that the one detectable product formed with models 6 and 7 indeed corresponds to the oxime product.

More importantly, it strongly suggests that in a scenario where multiple amines are present with respect to aminoxy groups and tracer level chemistry, the formation of the oxime will still be the preferred product under these conditions. Similar results were observed by Poethko et al. [8], where they studied the specificity of amino acids argenine, histidine, serine, and lysine toward 4-¹⁸F]fluorobenzaldehyde both in the presence and the absence of 2-aminoxyacetic acid.

We subsequently demonstrated the predominant selectivity of 4-fluorobenzaldehyde for aminoxy over the other groups in an Affibody under the identified conditions.

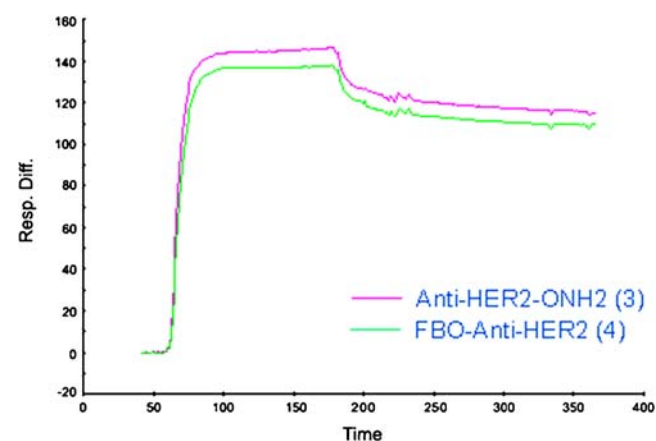


Fig. 1. Relative binding sensorgram of Biacore surface plasmon resonance analysis of 100 nM Anti-HER2-ONH₂ 3 and 100 nM FBO-Anti-HER2 4 against chimeric HER2/Fc protein used as a ligand. Full kinetic analysis was performed on both conjugates at concentrations ranging from 0 nM to 100 nM to determine effect of modification of the conjugate upon modification on binding affinity.

Table 2. Biodistribution of [¹⁸F]Anti-HER2-Affibody 5 in mice bearing SKOV3 xenografts

Organ (% ID/g)	0.5 h	1 h	3 h
Blood	3.81±0.5	1.56±0.29	0.36±0.03
Liver	12.02±1.57	4.28±0.55	1.35±0.19
Muscle	1.01±0.9	0.54±0.14	0.19±0.06
Kidney	20.90±3.37	7.41±0.92	2.08±0.45
Intestine	8.61±11.54	9.75±12.10	13.87±10.80
Tumor	2.03±0.31	2.23±0.51	1.08±0.15

Data are expressed as percentage of injected radioactivity per gram of organ or tissue (% ID/g) after intravenous injection of 5 (20–45 µCi) at different time post injection (*N*=3 for each group).

Conjugations of Anti-HER2-ONH₂ Affibody 3 with 4-fluorobenzaldehyde at different concentration (1, 2.5, 100, and 1,000 equivalents) at 25–70°C from 30 min to 1 h were performed in 1% dimethyl sulfoxide (DMSO) in acetate buffer pH 4 (Scheme 1b). We found that under these conditions, conjugate 4 formed only by addition of a single molecule of 4-fluorobenzaldehyde to the Affibody molecule. No multiple fluorobenzaldehyde-labeled affibodies were observed as characterized by high-resolution MALDI-MS as well as ESI-MS, where in both techniques at least the qualitative detection of various Affibody adducts has been found in our lab to be similar. Given the results obtained with the previous model peptides, it is reasonable to believe that the one predominant product observed is the desired oxime product at the known position.

Surface Plasmon Resonance analysis using Biacore (GEHC) confirmed that the binding affinity of Anti-HER2-ONH₂ 3 is not significantly affected upon introduction of the fluorobenzaldehyde (FBO-Anti-HER2, 4). Addition of the bifunctional linker 2 is expected to have minimal and comparable results to blocking the thiol group using, for example, *N*-methyl-maleimide to prevent dimerization during Biacore analysis. Figure 1 shows sensorgrams for the

relative binding against HER2 at 100 nM concentration using chimeric HER2/Fc protein as a ligand. The HER2 binding affinities (K_{off}/K_{on}) of the conjugates were analyzed and determined from the kinetic analysis using BIAevaluation (GEHC) software. The fast on rate (7.9×10^5 and 9.3×10^5) and slow off rate (1.4×10^{-4} and 1.9×10^{-4}) of conjugates 3 and 4, respectively, exhibit a high and comparable binding affinity of ~200 pM. These results suggest that conditions (acetate buffer pH 4 at 70°C) used for preparation of conjugate 4 did not affect its binding affinity. Stable ¹¹¹In-labeled Affibody with high binding affinity has been prepared at high temperature (up to 90°C) [7].

The aminoxy-functionalized Affibody 3 was subsequently radiolabeled with 4-¹⁸F]fluorobenzaldehyde (¹⁸F]FBA). No carrier-added [¹⁸F] fluoride was prepared by the ¹⁸O(p,n)¹⁸F nuclear reaction on a GE PETtrace cyclotron. [¹⁸F]Fluoride processing and synthesis of 4-¹⁸F]fluorobenzaldehyde were completed in the GE TRACERlab FX-FN synthesis module. No carrier-added [¹⁸F] fluoride trapped on a QMA cartridge (300–1,000 mCi) was washed with a solution of K₂CO₃ (3.5 mg) and kryptofix 2.2.2 (15 mg) in water (0.9 ml) and acetonitrile (0.1 ml; concentrations of K₂CO₃ and kryptofix were 25.3 mM and 39.8 mM, respectively). The solvent was removed under vacuum, and to the anhydrous residue was added a solution of 4-formyl-*N,N,N*-trimethylanilinium triflate precursor (4–6 mg) in DMSO (0.5 ml). The mixture was heated for 10 min at 85°C, and after cooling to room temperature, the reaction mixture was diluted with 5 ml of water. The mixture was loaded onto a preconditioned C18 cartridge. The cartridge was washed with 10 ml of 0.1 N HCl, and 4-¹⁸F]fluorobenzaldehyde was eluted with 2 ml of methanol into a vial. The decay-corrected radiochemical yield was 50–70% (actual end of synthesis yields, 116–540 mCi), and the synthesis was completed in 40 min. To generate the radiolabeled probe, aminoxy-functionalized Anti-HER2-

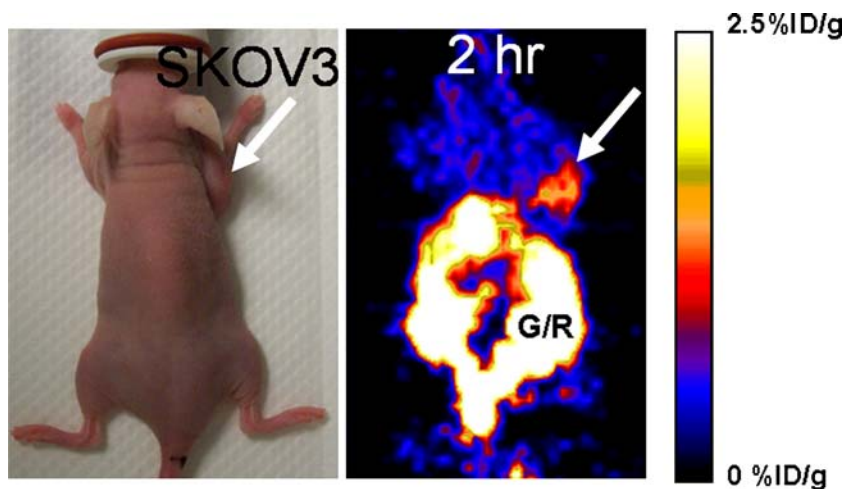


Fig. 2. Decay-corrected coronal microPET images of a nude mouse (photograph shown on left) bearing SKOV3 xenograft at 2 h after tail injection of 50 µCi of [¹⁸F]Anti-HER2-Affibody 5. Arrow indicates location of tumor, and G/R is gastrointestinal/renal activity. Color bar represents % ID/g.

ONH₂ Affibody 3 (14 kDa, 0.5 mg) in 100 µl of ammonium acetate at pH 4 (Scheme 1c) was incubated with 4-¹⁸F]fluorobenzaldehyde (5–7 mCi in 50 µl of methanol) at 70°C for 15 min to afford ¹⁸F]FBO-Anti-HER2, 14 kDa (5) in 26–30% non-optimized decay-corrected radiochemical yield (actual end of synthesis yields, 1.0–1.6 mCi). Our radiochemical yield of ¹⁸F] Anti-HER2 Affibody (5) is lower than that of the ¹⁸F]-labeled peptides (60–80%) reported by Poethko et al. [8], despite our use of approximately 1.4 times the nanomole amount of Affibody. Likely explanations include (a) the concentration of aminoxy precursor (Anti-HER2-ONH₂ Affibody 3, 0.23 mmol/l) that we used is less than the amount used for aminoxy-peptide precursor (0.5 mmol/l) or (b) the pH was higher (pH 4) in the case of ¹⁸F]-Anti-HER2 Affibody compared to those found to be optimal in the ¹⁸F]-peptides case (pH 2–3).

In order to gain initial insight into the *in vivo* imaging performance of ¹⁸F]-labeled Affibody molecules, we evaluated 5 in xenograft models expressing HER2. *In vivo* biological studies of ¹⁸F]-Anti-HER2 Affibody in SKOV3 xenograft-bearing nude mice (*N*=9) were investigated. Human ovarian cancer cells (SKOV3) have been well documented to express HER2 [18]. Table 2 shows the biodistribution of 5 in SKOV3 xenograft-bearing nude mice. The ratio of tumor to blood was 3.00±0.48 at 3 h of post-injection. High-contrast small animal PET images, with relatively moderate tumor uptake (1.79±0.40% ID/g) and low muscle uptake (0.44±0.01% ID/g) after 2 h of injection of 5, were observed (Fig. 2). However, heptabiliary excretion was high and produced high accumulation of radioactivity in the abdomen. Although the cause of this observation is not clear, use of ¹⁸F]-labeled smaller monovalent Affibody constructs (7 kDa) or reducing the lipophilicity of radiolabeled Affibody conjugates should be explored as supported by Affibody-related literature [6]. It was found that substitution of glycyl residues in the chelating sequence with more hydrophilic seryl residues is a promising method to alter the biodistribution of ^{99m}Tc-labelled Affibody molecules [19].

In conclusion, site-specific ¹⁸F]-labeled Affibody, ¹⁸F]-Anti-HER2 Affibody 5, 14 kDa, has been successfully generated utilizing oxime chemistry and used to image tumors *in vivo*. While our non-optimized yields were somewhat lower than previously demonstrated using similar chemistry for small peptides, a number of parameters exist for further exploration, e.g., smaller monovalent Affibody constructs, concentrations, *etc.* The combination of the oxime-based radiochemistry and highly stable, both chemically and *in vivo*, Affibody molecules suggests potential use of this platform to rapidly generate targeted ¹⁸F]-labeled molecular imaging probes for PET imaging.

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