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Citation

Published Version
doi:10.1021/acscentsci.5b00121

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Accessibility
Use of $^{18}$F-2-Fluorodeoxyglucose to Label Antibody Fragments for Immuno-Positron Emission Tomography of Pancreatic Cancer

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ABSTRACT: We generated $^{18}$F-labeled antibody fragments for positron emission tomography (PET) imaging using a sortase-mediated reaction to install a trans-cyclooctene-functionalized short peptide onto proteins of interest, followed by reaction with a tetrazine-labeled-$^{18}$F-2-deoxygluoroglucose (FDG). The method is rapid, robust, and site-specific (radiochemical yields > 25%, not decay corrected). The availability of $^{18}$F-2-deoxygluoroglucose avoids the need for more complicated chemistries used to generate carbon-fluorine bonds. We demonstrate the utility of the method by detecting heterotopic pancreatic tumors in mice by PET, using anti-Class II MHC single domain antibodies. We correlate macroscopic PET images with microscopic two-photon visualization of the tumor. Our approach provides easy access to $^{18}$F-labeled antibodies and their fragments at a level of molecular specificity that complements conventional $^{18}$F-FDG imaging.

INTRODUCTION

Imaging of medically relevant specimens by positron emission tomography (PET) using $^{18}$F-labeled biomolecules is increasingly important for both clinical diagnosis and in biomedical research. By exploiting differences in the rate of glucose uptake and its metabolism, $^{18}$F-labeled 2-deoxy-2-$^{18}$F-fluoroglucose ($^{18}$F-FDG)-PET imaging can distinguish many tumors with increased metabolic activity from surrounding normal tissue. $^{18}$F-Labeled ligands can also be used to track expression of the receptors to which they bind. While $^{18}$F-FDG is readily available in most radiopharmacies, the generation of other $^{18}$F-labeled bioactive molecules of interest can require elaborate synthetic strategies. A further challenge is the short half-life of $^{18}$F ($t_{1/2} = 110$ min), which requires use within hours of production. In terms of radiation exposure, the use of $^{18}$F-fluorine has advantages over longer-lived isotopes such as $^{89}$Zr ($t_{1/2} = 3.27$ days) and $^{124}$I ($t_{1/2} = 4.18$ days). Use of $^{18}$F-FDG is potentially more practical in a clinical setting than are methods using elemental $^{18}$F.

Although antibodies are endowed with exquisite specificity and are of considerable therapeutic value, the use of $^{18}$F-labeled antibody fragments has yet to see widespread application for imaging purposes. Our approach enables the use of $^{18}$F-FDG to achieve efficient labeling of proteins and does so in a manner that is reproducible and site-specific, leaving intact the antibody fragment’s antigen binding site. The method also could be applicable to other suitably modified biologicals, such as cytokines and chemokines. The ability to determine the biodistribution of therapeutically useful antibodies or their fragments and a comparison of these measurements with clinical outcomes can thus expand the repertoire of diagnostic tools.

RESULTS AND DISCUSSION

Our strategy relies on a two-step process for labeling proteins equipped with a sortase recognition motif. Sortases are bacterial transpeptidases that are finding increasing use as tools for protein engineering. Sortases stand out for their ease of production, high degree of specificity, fast and efficient conversion of the appropriately modified protein substrate, and are of considerable therapeutic value, the use of $^{18}$F-labeled antibody fragments has yet to see widespread application for imaging purposes. Our approach enables the use of $^{18}$F-FDG to achieve efficient labeling of proteins and does so in a manner that is reproducible and site-specific, leaving intact the antibody fragment’s antigen binding site. The method also could be applicable to other suitably modified biologicals, such as cytokines and chemokines. The ability to determine the biodistribution of therapeutically useful antibodies or their fragments and a comparison of these measurements with clinical outcomes can thus expand the repertoire of diagnostic tools.

Received: March 30, 2015
Published: June 3, 2015
A tetrazine-aminooxy and 18F-FDG were combined in the presence of p-phenylenediamine to produce 18F-tetrazine. The TCO-tetrazine reaction is fast, allowing capture of the FDG into a tetrazine molecule via an oxime ligation; the 18F-tetrazine product is purified via HPLC. A single domain antibody (VHH) equipped at its C-terminus with the LPXTG sortase-recognition motif is site-specifically modified with a (Gly)₃-trans-cyclooctene (TCO), as confirmed by LC-MS (Supporting Information). 18F-Tetrazine was added to the TCO-modified VHH, and after ~20 min the labeled VHH was retrieved by rapid size exclusion chromatography. PET images of a representative C57BL/6 mouse 2 h postinjection of 18F-VHH7; numbers indicate (i) lymph nodes: 1, 2, 3, 4, 7, 8, 9; (ii) thymus: 5; (iii) spleen: 6. (E) PET-CT images of C57BL/6 mouse imaged with 18F-VHH7 from two different viewpoints (top and bottom panels); clearly lymph nodes and thymus are visible. See movie 01 in Supporting Information for a 3D visualization of lymphoid organs. (F) PET signals in vivo in different organs. Experiments are representative of three mice with similar results.

**Figure 1.** (A–C) Site-specific 18F-labeling of proteins using 18F-FDG and sortase. (A) A tetrazine-aminooxy and 18F-FDG were combined in the presence of p-phenylenediamine to produce 18F-tetrazine. Dynamic equilibrium between hemiacetal and linear forms of the aldohexose allows the installation of 18F-FDG on an aldehyde form (the reactive molecular species) and its cyclical transacylation reaction. 18F-FDG is extremely low (<nM). High-performance liquid chromatography (HPLC) showed that the coupling reaction with 18F-FDG proceeded rapidly, yielding >90% oxime 18F-FDG-tetrazine in ~10 min. Radio-HPLC showed that the coupling reaction with 18F-FDG proceeded rapidly, yielding >90% oxime 18F-FDG-tetrazine in ~5–10 min (Figure 1). We separated the 18F-labeled protein ready for imaging via HPLC. (B) A single domain antibody was prepared previously using sortase, was then added to the purified oxime 18F-FDG-tetrazine. The reaction was allowed to proceed for ~15–20 min at 25 °C with constant agitation. The 18F-labeled protein was purified by size exclusion in phosphate buffer, providing the final 18F-labeled protein ready for injection.

In view of the t₁/₂ of 18F ≈ 110 min, any synthetic process using 18F as a substrate and the necessary downstream purification steps must be rapid. Thus, we first optimized reaction conditions using nonradioactive FDG and characterized the reaction products by liquid chromatography–mass spectrometry (LC–MS) (Supporting Information). Several different catalysts have been reported for the oxime ligation reaction, of which the phenylenediamines are among the most efficient. While p-phenylenediamine is a more efficient catalyst than m-phenylenediamine (pPDA), its Schiff base is more stable and can block oxime formation if its concentration relative to the aminooxy or aldehyde is high. In our case, the concentration of aldehyde (18F-FDG) is extremely low (<nM). We therefore used pPDA as the catalyst at ∼0.4–0.6 M and tetrazine-aminooxy in the ∼0.2–0.3 M range. We incubated the aminooxy-tetrazine with fluorodeoxyglucose in the presence of the catalyst, pPDA, with constant agitation at 75 °C for ~5–10 min. High-performance liquid chromatography (HPLC) of the reaction mixture showed (near)-complete consumption of FDG (Supporting Information). To produce the radioactive aminooxy-tetrazine derivative, we performed the incubation with 18F-FDG in the presence of the catalyst, pPDA, with constant agitation at 75 °C for ~5–10 min. Radio-HPLC showed that the coupling reaction with 18F-FDG proceeded rapidly, yielding >90% oxime 18F-FDG-tetrazine in ~5–10 min (Figure 1). We separated the 18F-oxime product by HPLC, followed by capture of the product via a Sep-pak C18 column. A solution containing the TCO-labeled protein of interest, prepared previously using sortase, was then added to the purified oxime 18F-FDG-tetrazine. The reaction was allowed to proceed for ~15–20 min at 25 °C with constant agitation. The 18F-labeled protein was purified by size exclusion in phosphate buffer, providing the final 18F-labeled protein ready for injection.

We previously used a 18F-TCO-tetrazine to label proteins with 18F to image lymphoid organs using an anti-Class II MHC single domain antibody, VHH7. We evaluated the present labeling method to confirm that the binding site of the nanobody remained intact. 18F-VHH7, produced as described above, detected secondary lymphoid organs exactly as reported (Figure 1 and movie 01 in the Supporting Information).

and ready access to a wide variety of nucleophiles in the transacylation reaction. As a first step, we generated a short synthetic peptide, (Gly)₃-R, where R contains a trans-cyclooctene (TCO) functionality that enables a TCO-tetrazine ligation reaction with a 18F-tetrazine. The TCO-tetrazine reaction is fast, with an estimated second order rate constant of 2×10⁻⁹ M⁻¹ s⁻¹. We established a method for 18F labeling using commercially available 18F-FDG, the principal source of 18F in clinical use. While 18F-FDG is readily available, the phenylenediamines are among the most versatile catalysts and can block oxime formation if its concentration relative to the aminooxy or aldehyde is high. In our case, the concentration of aldehyde (18F-FDG) is extremely low (<nM). We therefore used pPDA as the catalyst at ∼0.4–0.6 M and tetrazine-aminooxy in the ∼0.2–0.3 M range. We incubated the aminooxy-tetrazine with fluorodeoxyglucose in the presence of the catalyst, pPDA, with constant agitation at 75 °C for ~5–10 min. High-performance liquid chromatography (HPLC) of the reaction mixture showed (near)-complete consumption of FDG (Supporting Information). To produce the radioactive aminooxy-tetrazine derivative, we performed the incubation with 18F-FDG in the presence of the catalyst, pPDA, with constant agitation at 75 °C for ~5–10 min. Radio-HPLC showed that the coupling reaction with 18F-FDG proceeded rapidly, yielding >90% oxime 18F-FDG-tetrazine in ~5–10 min (Figure 1). We separated the 18F-oxime product by HPLC, followed by capture of the product via a Sep-pak C18 column. A solution containing the TCO-labeled protein of interest, prepared previously using sortase, was then added to the purified oxime 18F-FDG-tetrazine. The reaction was allowed to proceed for ~15–20 min at 25 °C with constant agitation. The 18F-labeled protein was purified by size exclusion in phosphate buffer, providing the final 18F-labeled protein ready for injection.

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Figure 2. (A) DC8 and DC15 specifically recognize the mouse Class II MHC complex: $10^6$ splenocytes isolated from C57BL/6 Class II-GFP knock-in and Class II knockout mice were stained with labeled VHHs as indicated. Plots are gated on live, CD19+ cells. VHH7 has been previously demonstrated to recognize murine Class II MHC. DC8 and DC15 are novel VHHs isolated through staining of dendritic cells. VHH4 is specific for human Class II MHC and does not recognize the murine homologue. (B) DC8 and DC15 are able to stain murine B cells at concentrations too low for VHH7 staining: $10^6$ splenocytes isolated from WT C57BL/6 mice were stained with the indicated concentrations of Alexa647-labeled VHHs. Populations were gated on live, CD19+ cells, and the mean Alexa647 fluorescence of each population is plotted. (C) DC8 and DC15 outcompete VHH7 for an overlapping epitope: $10^6$ splenocytes isolated from WT C57BL/6 mice were costained with TAMRA-labeled VHH and a variable concentration of unlabeled VHH. The costained splenocytes (dark gray peak) were compared to splenocytes stained only with the TAMRA-labeled nanobody (light gray peak). VHH73 does not bind to class II MHC molecules and is used as a control. (D) $^{18}$F-DC8 (anti-mouse class II MHC), produced using $^{18}$F-FDG and sortagging, detects secondary lymphoid organs. PET (left) and PET-CT (right-top and bottom) images of a representative C57BL/6 mouse 2 h postinjection of $^{18}$F-DC8; clearly lymph nodes, spleen, and thymus are visible. Numbers indicate (i) lymph nodes: 1, 2, 3, 4, 7, 8, 9; (ii) thymus: 5; (iii) spleen: 6. See movie 02 in Supporting Information for a 3D visualization of lymphoid organs. (E) PET signals in vivo in all organs. (F, G) DC8 and VHH7 (both anti-mouse class II MHC) stain secondary lymphoid organs with different affinities. Images were acquired using two-photon microscopy. VHHs were site-specifically labeled with Texas Red via sortagging. F and G are images of spleen of C57BL/6 mice injected with 10 μg of DC8-Texas Red (F) or VHH7-Texas Red (G) 90 min prior to imaging. Clearly DC8-Texas Red stains Class II positive cells with higher affinities compared to VHH7. Experiments are representative of three mice with similar results.
The half-maximal binding of VHH7 for Class II MHC+ cells on splenocytes is in the ~55–60 nM range (Figure 2). Possible in vivo applications might benefit from single domain antibodies (VHVs) with improved affinities for their targets. To that end, we identified higher affinity anti-Class II VHVs in a phage display library generated from an alpaca immunized with murine splenocytes. Specificity of the anti Class II VHVs was ascertained by the absence of staining of splenocytes from class II MHC knockout mice, and perfect costaining with GFP-positive cells from class II MHC-GFP knock-in mice using fluorescently labeled VHH derivatives. The affinity of newly identified class II MHC-specific VHVs was compared to that of VHH7. VHVDCH8 and VHVDCH15 bind ~3–4 fold better to Class II MHC molecules (Figure 2) than does VHH7. In competition experiments both VHVDCH8 and VHVDCH15 interfered with each other’s ability to bind spleen cells and inhibited binding of VHH7; similarly, an excess of VHH7 inhibited binding of VHVDCH8 and VHVDCH15 (Figure 2). These findings imply that these different Class II MHC-specific VHVs recognize a closely related epitope.

For in vivo analysis, we prepared Texas Red-conjugated VHH7 for comparison with similarly labeled VHVs. We injected mice with 10 μg of the Texas Red-conjugated VHVs. Ninety minutes postinjection we excised spleen and lymph nodes for analysis by two-photon microscopy. The signal obtained from VHVDCH8-stained lymphoid organs was substantially stronger than that seen for VHH7, indicating that higher affinity for the target improved image intensity (Figure 2). Having established the utility of the new anti-Class II MHC VHH for in vivo staining, we used it for PET imaging.

\(^{18}\)F-VHVDCH8 prepared as described above detected secondary lymphoid organs (Figure 2 and movie 02 in the Supporting Information) in a manner comparable to \(^{18}\)F-VHH7 (Figure 1). Compared to VHH7, we observed stronger binding of VHVDCH8 to spleen relative to lymph nodes (compare SUVs in Figures 1 and 2). The higher affinity of VHVDCH8 and its short circulatory half-life, typical of a VHH, might lead to its more efficient capture upon passage through the spleen, leaving comparatively less available for exit from the bloodstream and staining of lymph nodes.

Pancreatic tumors are often poorly infiltrated with immune cells and develop a dense stroma, implicated in the resistance to standard chemotherapy and immunomodulatory antitumor treatments. We used the pancreatic cancer cell line Panc02 as a model for pancreatic cancer and explored the possibility of imaging its presence by tracking the arrival of Class II MHC-positive cells (activated host macrophages, dendritic cells) using \(^{18}\)F-VHVDCH8. Panc02 itself does not express Class II MHC products. Mice injected subcutaneously with \(1 \times 10^6\) Panc02 cancer cells were imaged with \(^{18}\)F-VHVDCH8 2 weeks after injection of the tumor. Although the tumors were not palpable at the time of imaging (tumor size estimated at ~1.2 mm in diameter), PET images clearly showed their presence (Figure 3 and movie 03, Supporting Information). PET imaging using \(^{18}\)F-FDG failed to detect the tumor, likely due to its small size and/or low metabolic activity (Figure 3 and movie 04 in the Supporting Information). To correlate the results obtained by PET with microscopy, we injected tumor-bearing mice with 20 μg of Texas Red-VHVDCH8. Two hours postinjection, the
tumor was excised and imaged by two-photon microscopy. The tumor was infiltrated with or surrounded by Class II MHC+ cells, consistent with the PET imaging result (Figure 3; see image 01 in the Supporting Information for high resolution visualization).

The short half-life of VHHs (~10–20 min) likely requires compensation in terms of affinity of the VHH for its target to ensure retention by the tumor. An important limitation of the use of VHHs for immuno-PET is their accumulation in the kidneys and intestine. The use of a longer-lived isotope such as 18F, starting from a widely available precursor, 18F-FDG, may allow longer retention by the tumor. An important limitation of the use of VHHs for immuno-PET is their accumulation in the kidneys and intestine without compromising imaging quality, but this remains to be explored experimentally.

In conclusion, we have site-specifically labeled biomolecules with 18F, starting from a widely available precursor, 18F-FDG. The method avoids the far more demanding generation of carbon−18F bonds and thus facilitates access to 18F-labeled biomolecules, provided these tolerate the presence of a sortase recognition motif, for example, as shown for 4-helix bundle cytokines.20 We successfully applied immuno-PET to the detection of small heterotopic pancreatic tumor transplants, using high affinity anti-Class II MHC VHHs to decorate the tumor-surrounding immune cells.

The VHH-PET method provides information on the tumor immune microenvironment, while the use of 18F-FDG-PET can identify tumors based on their increased metabolic activity compared to surrounding normal tissue. Both approaches can be applied to the same specimen repeatedly to obtain information on tumor growth and regression, for example, in response to therapy. Immunogenicity of VHHs remains an issue of concern in the case of repeated administration, but approaches for humanization of camelid-derived VHHs have been described35 to address this issue. The small size of VHHs and their ease of enzymatic modification relative to other formats commonly applied to antibody fragments present a powerful addition to the radiodiagnostic toolbox.36−38

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