Tri-functional platform for construction of modular antibody fragments for in vivo $^{18}$F-PET or NIRF molecular imaging†

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Positron emission tomography (PET) molecular imaging is a powerful tool for interrogating physiological and biochemical processes to understand the biology of disease and advance therapeutic developments. Near-infrared fluorescence (NIRF) optical imaging has become increasingly popular for intraoperative staging to enable cellular resolution imaging of tumor margins during surgical resection. In addition, engineered antibody fragments have emerged as promising molecular imaging agents given their exquisite target selectivity, rapid systemic clearance and site-selective chemical modification. We report a tri-functional platform for construction of a modular antibody fragment that can rapidly be labeled with radionuclides or fluorophores for PET or NIRF molecular imaging of prostate stem cell antigen (PSCA).

Introduction

Molecular imaging techniques such as positron emission tomography (PET) and near-infrared fluorescence (NIRF) optical imaging are highly valuable tools for diagnosis, staging and therapy monitoring of disease. These modalities offer complimentary data, with PET enabling whole-body functional information about disease localization and potential metastasis while the optical component provides fluorescent-guided intraoperative staging. A significant advantage of PET in the cancer space is the noninvasive tumor detection and localization of an expressed biomarker throughout all tissues of the body. Visualization of antigen-positive cells via optical imaging during surgery can assist surgeons to maximize resection of primary and metastatic cancer cells, minimize resection of healthy tissue, limit potential recurrence and improve overall survival rate for cancer patients.

Antibody-based PET (immunoPET) is a versatile platform for the development of molecularly targeted, sensitive and quantitative imaging of cell-surface protein expression in vivo. Advances in antibody engineering techniques to reduce immunogenicity, modify pharmacokinetics and improve clearance have facilitated the development and translation of antibody fragments to be utilized as molecular imaging probes. Recombinant antibody fragments called cys-diabodies (cysteine-modified scFv dimers; 50 kDa) contain a C-terminal cysteine that forms a disulfide bridge to stabilize dimerization which can be preferentially reduced to unveil free thiols for well-defined, site-specific conjugation. Importantly, the cysteine modification is located away from the antigen-binding site to preserve immunoreactivity of the protein conjugate. These constructs are particularly powerful imaging agents due to their small size, high tumor uptake, thiol-specific conjugation and fast blood clearance which results in high tumor to background ratios within 2 hours of probe injection. Labeled with positron-emitting radionuclides ($^{18}$F, $^{64}$Cu, $^{124}$I and $^{89}$Zr), recombinant diabody/cys-diabody constructs have been employed as immunoPET probes for a variety of tumor types including breast, pancreatic, prostate and colorectal cancers as well as for tumor-infiltrating CD8+ T cells. In addition, fluorescently labeled antibody fragments have been evaluated in vivo for their potential use as targeted optical imaging probes in xenograft models.

To provide a universal approach towards the targeted delivery of PET and optical imaging agents, we have developed a tri-functional platform (TFP) for the construction of modular cys-diabodies. This novel platform rapidly yields a site-specific bioconjugate with improved pharmacokinetic properties that can be readily labeled via a highly efficient (4+2) cycloaddition reaction to incorporate either fluorine-18 or a near-infrared fluorescent dye. This robust strategy leverages the tetrazine-trans-cyclooctene ligation which has been gaining interest for the selective introduction of fluorine-18 into biologically relevant molecules. The remarkable speed of this ligation is well suited for $^{18}$F-labeling of proteins, where the short radioactive...
half-life and stringent conditions demand the need for rapid, efficient chemistry.\textsuperscript{10} For proof-of-concept, a cys-diabody against prostate stem cell antigen (PSCA) was conjugated to the tri-functional platform and immunoPET and optical imaging were performed to access whole-body biodistribution as well as tumor targeting potential in mice carrying prostate cancer xenografts. This model demonstrates not only the capability of the platform to afford molecular imaging tracers that provide high-contrast images against PSCA-positive cells, but also reveals the potential of this approach to provide \textit{in vivo} information on cell-surface protein expression for any target-specific construct.

Results and discussion

Tri-functional platform design and synthesis

Exploiting strain-energy to facilitate molecular reactivity has been established as a highly effective strategy, prompting the discovery and applications of promising bioorthogonal reactions.\textsuperscript{11} The inherent nature of the inverse electron demand Diels–Alder (IEDDA) cycloaddition\textsuperscript{12} between tetrazine and \textit{trans}-cyclooctene – catalyst-free, extremely rapid, highly selective, modular, robust – make it an attractive reaction for protein labeling applications and provided the rationale to incorporate it into the tri-functional platform design and construction.\textsuperscript{11}

The maleimide synthon is a powerful tool for site-specific bioconjugation, and perhaps the most common functional group for selective modification of proteins. Fast kinetics and remarkably high chemoselectivity towards cysteine residues account for its persistent popularity in protein labeling. Despite susceptibility of the succinimimidyl thioether linkage to hydrolysis and retro-Michael reactions, the half-life of a maleimide-protein linkage is \(\approx 60\) h \textit{in vivo} which is considerably longer than the 3–7 h circulating half-life of a cys-diabody.\textsuperscript{14,15} The rapid pharmacokinetics of cys-diabodies permit bioconjugation \textit{via} maleimide-based synthons and is well established in molecular imaging applications.\textsuperscript{7c,8b,27w}

The tri-functional platform consists of three key moieties: (1) a maleimide for site-specific conjugation to the cys-diabody \textit{via} a succinimidyl thioether linkage; (2) a tetrazine for rapid labeling of the immunocomjugate \textit{via} a \((4+2)\) cycloaddition reaction; and (3) a hydrophilic side chain to improve the \textit{in vivo} biodistribution profile and favor renal clearance (Fig. 1). Traditional labeling methods require optimization of the protein conjugation, purification and characterization steps each time the labeling is conducted, which may lead to inconsistent batches or variability in purity and immunoreactivity. The modular strategy affords flexibility by providing a stock of cys-diabody, with a favorable biodistribution profile, that can readily be labeled \textit{via} rapid \((4+2)\) cycloaddition with a broad range of TCO-modified tags without the need for further optimization of reaction conditions. Ultimately, the power of this approach lies in its modularity; it enhances the versatility of a single cys-diabody, potentially of any desired specificity, without compromising affinity to its molecular target, and facilitates facile labeling with radionuclides and fluorophores.

A critical challenge in the development of molecular imaging agents is the image contrast between signal and background. Radiolabeled antibodies and their fragments can display exquisite target affinity, with predictable kinetics and clearance routes \textit{in vivo}. However, imaging is also impacted by the catabolism and the clearance/retention of subsequent radiometabolites, which are strongly influenced by properties such as lipophilicity and charge. Much has been learned from pharmacokinetics and biodistribution studies on radiolabeled peptides, which have been modulated by chemical moieties such as aspartate residues,\textsuperscript{15} histidine/glutamic conjugates,\textsuperscript{16} succinic acid,\textsuperscript{17} glycosylation\textsuperscript{18} and the metal chelator DOTA.\textsuperscript{19} Increasing negative charge or incorporating chemical groups such as these has resulted in increased plasma clearance\textsuperscript{20} and diverted tracer or metabolite clearance in favor of renal over hepatobiliary.\textsuperscript{3b,10b}

Previously, our group reported a dual-modality linker for conjugation to a cys-diabody for imaging PSCA expression.\textsuperscript{21} With a molecular weight of \(\approx 53\) kDa, below the threshold for first-pass renal clearance, the initial clearance route for the biocomjugate was \textit{via} the kidneys. Radiometabolites were reabsorbed and ultimately routed through the hepatobiliary system, resulting in extended blood pool activity and retention in the liver, kidneys and gastrointestinal tract (GI), presumably due to high lipophilicity of tracer radiometabolites.\textsuperscript{22} Size, structure and charge distribution are key properties that influence renal retention of radiolabeled metabolites.\textsuperscript{23} As a result of this study, the tri-functional platform was designed to preserve kidney retention of radiometabolites, reduce liver and GI activity and provide a clean background in the lower abdomen by incorporating an anionic moieity to decrease lipophilicity. We chose to incorporate a DOTA for its ease of synthetic installation and its potential for chelation of therapeutic radioisotopes towards the future development of theranostic tools. The rationale for our platform design is strengthened by a recent report that biodistribution of an \textsuperscript{18}F-labeled peptide tracer could be significantly modulated by installation of a metal-free DOTA appendage.\textsuperscript{23b}

The tri-functional platform 3 was prepared in four steps starting from commercially available N-boc-L-lysine (Scheme 1). PEGylated succinimimidyl ester 1 was readily conjugated to \(\text{N}-\text{boc-}l-\text{lysine} to furnish the maleimide–lysine conjugate. We chose to employ the 3-benzylamino-6-methyl-1,2,4,5-tetrazaine for its improved chemical stability at the sacrifice of slightly slower cycloaddition kinetics.\textsuperscript{23} Thus, the
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Modular antibody fragment construction

Prostate stem cell antigen (PSCA) is a cell surface protein expressed by prostate, pancreatic and bladder cancers with low expression in normal tissues and no expression in bone or lymph nodes, primary sites of cancer metastasis. Nearly all prostate cancers express high levels of PSCA and higher expression levels correlate with poor prognosis and metastatic prostate and pancreatic tumors in both transgenic and xenograft mouse models. In our current study, the anti-PSCA cys-diabody (cDb) was site-specifically conjugated to the tri-functional platform (3) and utilized for PET and NIR optical imaging. The construction process starts with mild reduction of the cysteine thiol, which cleaves the disulfide bridge and provides two free thiol groups. The resulting tri-functional platform is shown in Scheme 1.

Scheme 1 Synthesis of the tri-functional platform 3. Reagents and conditions: (a) N-Boc-L-lysine, i-Pr₂NET, DMF, 23 °C, 16 h, 78%; (b) 1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester. Excess reagents were separated from the protein conjugate using gel filtration column and the modular cys-diabody TFP-cDb was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC) (Fig. S3†). To afford ¹⁸F-cDb or sCy5-cDb, purified TFP-cDb efficiently underwent a Diels-Alder cycloaddition reaction with either [¹⁸F]fluoroethoxy TCO or sulfonate Cy5 TCO, respectively (Fig. 2B). The modularity of this approach allows for the creation of a common stock of immunonjugate, TFP-cDb, that can directly be applied to either PET or optical imaging.

In vivo molecular imaging

Synthesis of [¹⁸F]fluoroethoxy TCO was automated on the ELIXYS FLEX/CHEM radiochemical synthesizer (Sofie Biosciences) as described previously. For ¹⁸F-labeling, reformulated [¹⁸F]fluoroethoxy TCO (37 MBq) was added to TFP-cDb (100 μg in 100 μL PBS, pH 7.4) and the mixture was incubated for 10 minutes at room temperature (Fig. 3A). Purification by microspin size exclusion spin column afforded ¹⁸F-cDb with a specific activity of 0.17 MBq μg⁻¹. Radiolabeling efficiency and radiochemical purity of ¹⁸F-cDb were measured by instant thin layer chromatography (ITLC) and determined to be 63% and 98% respectively. The immunoreactive fraction (63%) was well within the previously reported range for A2 cys-diabody based immunonPET tracers (40–80%) (Fig. 3B). Many PET probes are metabolized via the hepatobiliary route, resulting in high liver signal which hinders the ability to

Following reduction, site-specific conjugation to the tri-functional platform was achieved via a Michael addition between the sulfhydryl and maleimide group, producing the modular cys-diabody TFP-cDb. Excess reagents were separated from the protein conjugate using gel filtration column and the modular cys-diabody TFP-cDb was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC) (Fig. S3†). To afford ¹⁸F-cDb or sCy5-cDb, purified TFP-cDb efficiently underwent a Diels–Alder cycloaddition reaction with either [¹⁸F]fluoroethoxy TCO or sulfonate Cy5 TCO, respectively (Fig. 2B). The modularity of this approach allows for the creation of a common stock of immunonjugate, TFP-cDb, that can directly be applied to either PET or optical imaging.

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1.3 MBq) was injected via the tail vein into male nude mice (n = 4) and microPET/CT scans were acquired. Fig. 3C shows PET imaging and ex vivo biodistribution analysis which confirm that 18F-cDb demonstrated rapid clearance from circulation within 2 h followed by renal metabolism and retention of radiometabolites in the kidneys, enabling high contrast images within a few hours after probe injection (see also Fig. S4†). In comparison to our previously reported construct,21 the observed hepatobiliary clearance of radiometabolites was considerably decreased, as reflected by 3.6% ID g⁻¹ activity in gallbladder and 0.51% ID g⁻¹ activity in liver (Fig. 3C and Table S1†). A direct comparison between the previous construct and the current tri-functional platform is depicted in Fig. 4. Different biodistribution profiles are clearly defined within 1 h post tracer injection and distinct improvements with the DOTA-containing platform are further emphasized by the ROI analysis (Fig. 4). For the previous construct, there is initial renal clearance (evident in the kidney ROI on the left), followed by release and subsequent hepatobiliary clearance of radiometabolites, resulting in high liver uptake and retention of radioactivity in the GI tract, which can obscure visualization of metastases. The charged, hydrophilic side chain of the tri-functional platform ensures that, following initial renal clearance, there is retention of radiometabolites as indicated by significantly reduced liver signal in the PET/CT image (Fig. 4, right panel) as well as the consistent 40% ID g⁻¹ kidney uptake in the ROI analysis. This resulted in lower non-specific background and an unobstructed view of the pelvic region that would allow for detection of prostate cancer. Importantly, a 22-fold reduction of radiometabolite retention in the GI was observed and the absence of bone uptake confirmed metabolic stability of 18F-cDb towards [18F]fluoride hydrolysis and release.

In order to evaluate the tumor targeting capability of 18F-cDb, an imaging study was performed in a mouse model of prostate cancer. Male nude mice (n = 4) bearing PSCA-positive (right shoulder) and PSCA-negative (left shoulder) subcutaneous xenografts were intravenously injected with 18F-cDb (10 μg, 1.85 MBq) and imaged at 2 and 4 h post injection (p.i.) (Fig. 5). At 4 h, the PET/CT images and ex vivo biodistribution analysis showed ~8-fold higher probe uptake in the antigen-expressing tumor compared to the control tumor (Fig. 5 and Table S2†). Representative transverse and coronal PET/CT images provided clear tumor visualization (Fig. 5B).

These results independently confirm the influence of a DOTA moiety to reduce GI tract accumulation and to redirect clearance to the kidneys over liver, which was recently demonstrated by Roxin et al. using a VLA-4 radiopeptide.100 Likewise, in the case of the radiopeptide study, a DOTA moiety reduced GI accumulation by 10-fold to 4.5% ID g⁻¹ in tumor-bearing mice.100 Analogous findings for both peptide and small protein-based imaging tracers reinforces the critical influence of hydrophilic, anionic chemical moieties, such as DOTA, on tracer biodistribution and performance. Additionally, in a radionuclide therapy application of our construct, long retention times of radiometabolites causing renal toxicity could be counteracted by infusion of basic amino acids (arginine, lysine) or polygelines (e.g. Gelofusine®) to block tubular reabsorption.25,26,28

Fluorescence image-guided surgery allows for real-time tumor delineation. Optical imaging agents that use fluorescence to identify cancerous cells offers the potential advantage of intraoperative imaging, ultimately improving surgical margins and reducing incidence of cancer recurrence. To

![Fig. 4](image_url) ImmunoPET imaging and ROI analysis comparison between the dual-modality linker and tri-functional platform. Representative PET/CT MIP images in nude mice at 1 h p.i. of 18F-DML-A2cDb or 18F-cDb and quantitative ROI analysis of heart, liver and kidney. B, bladder; GB, gallbladder; GI, gastrointestinal tract. Adapted from research originally published in JNM. Zettlitz KA, Waldmann CM et al. A dual-modality linker enables site-specific conjugation of antibody fragments for 18F-immunoPET and fluorescence imaging. J Nucl Med., 2019, DOI: 10.2967/jnumed.118.223560. ©SNMMI.

![Fig. 5](image_url) In vivo targeting of PSCA-positive tumors. (A) Male nude mice (n = 4) bearing PSCA-positive (right shoulder) and PSCA-negative (left shoulder) subcutaneous xenografts were administered 18F-cDb and microPET/CT images were acquired at 2 and 4 h p.i. Representative images in one mouse are shown as MIP/CT overlays. (B) Representative images in one mouse at 4 h p.i. are shown as 3 mm sections. (C) Ex vivo biodistribution at 4 h p.i. of 18F-cDb.
evaluate our platform for utility in image-guided surgery, the modular cys-diabody was labeled with a near infrared fluorescent dye, sulfo-cyanine 5. SulfoCy5 TCO (20 mM in DMF) was added to TFP-cDb (150 μg in 100 μL PBS, pH 7.4) at room temperature and, after 10 minutes, the (4+2) cycloadduct scy5-cDb was obtained (Fig. 6A). SDS-PAGE and SEC confirmed the purity and integrity of the labeled protein (Fig. S5†).

Male nude mice (n = 3) bearing human prostate cancer xenografts, each with PSCA-positive cells implanted in the right shoulder and PSCA-negative cells implanted in the left shoulder, were used for the in vivo optical imaging experiment. Reformulated scy5-cDb (20 μg) was injected via tail vein and mice were sacrificed 4 h after probe injection. Optical imaging performed post mortem with skin removed around the tumors allowed ready visualization of the antigen-positive tumors (Fig. 6B). Considerable autofluorescence from the skin results in high signal from the head (Fig. 6B). Ex vivo analysis of major organs revealed strong autofluorescence from the stomach and intestines and to a lesser extent the liver and spleen (Fig. 6C). As expected, renal clearance of scy5-cDb resulted in high fluorescence signal in the kidneys. Notably, strong fluorescence signal was detected in the PSCA-positive tumors with negligible nonspecific uptake in PSCA-negative tumors (Fig. 6D). scy5-cDb demonstrated excellent specificity for PSCA-expressing cells, providing high positive to negative signal contrast in the tumors and validating its potential utility for intraoperative staging. Alternative NIR fluorescent dyes, such as IRDye800CW or ICG, have been explored due to low autofluorescence, high spatial resolution and initial clinical success, and can be adapted to the current modular platform to enable rapid optimization of fluorescent dyes to identify the ideal fluorophore to utilize, which may be dependent on multiple variables. Taken together, this study demonstrates that the tri-functional platform can efficiently afford modular immunoconjugates for PET and optical imaging that maintain favorable in vivo biodistribution profiles and high affinity to the cell-surface protein of interest.

Conclusions

In summary, we have synthesized a tri-functional platform and constructed a modular antibody fragment that targets PSCA, a well-established prostate and pancreatic cancer biomarker. Using the robust and rapid inverse electron demand Diels-Alder reaction between tetrazine and trans-cyclooctene, site-specific protein labeling was successfully accomplished to rapidly afford two molecular imaging constructs. ImmunoPET and NIRF optical imaging in mice bearing PSCA-positive subcutaneous tumors confirmed in vivo targeting and revealed specific tracer uptake and remarkable contrast in antigen-positive tumors. As envisioned, the DOTA moiety of the tri-functional platform favorably influenced 18F-cDb biodistribution and metabolite clearance to significantly diminish non-specific GI uptake. We anticipate that our approach will provide a straightforward path towards the development and clinical translation of immunoPET and optical imaging tracers based on antibody fragments of desired specificity.

Conflicts of interest

A. M. Wu is a shareholder and consultant to ImaginAb, Inc.

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