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^{68}Ga chelating bioorthogonal tetrazine polymers for the multistep labeling of cancer biomarkers†

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We have developed a ^{68}Ga metal chelating bioorthogonal tetrazine dextran probe that is highly reactive with *trans*-cyclooctene modified monoclonal antibodies for multistep imaging applications. Confocal microscopy and positron emission tomography (PET) were used to characterize the dextran probe *in vitro* and *in vivo*.

There is clinical interest in the use of readily available positron emitting isotopes to image affinity ligands *in vivo via* positron emission tomography (PET).[1,2] Though ^{18}F is readily available, the requirement of cyclotron production and harsh labeling chemistry has sparked interest in exploring alternative radionuclides. One of the most promising is ^{68}Ga, which can be conveniently produced on-site using a variety of commercially available generators.[2] Also, unlike ^{18}F, which is covalently attached by harsh substitution chemistry, ^{68}Ga can be readily incorporated into a number of standard chelating agents such as diethylene triamine pentaacetic acid (DTPA). Here we report a novel metal chelating bioorthogonal tetrazine DTPA dextran probe that is capable of being radiolabelled using the positron emitting isotope ^{68}Ga.[3,4] These probes are highly reactive with *trans*-cyclooctene (TCO) modified monoclonal antibodies (Fig. 1).[5–7] Previous work has established the use of tetrazine bioorthogonal chemistry using ^{18}F radiolabels, however ^{68}Ga offers the ability to directly label tetrazines due to the mild nature of the chelation conditions.[8–10] Additionally, ^{68}Ga can be generator produced, offering advantages over other positron emitting metal isotopes such as ^{64}Cu. However, it was unclear whether tetrazines would be compatible with ^{68}Ga coordination chemistry, as tetrazines themselves can interfere with metal binding ligands.[9] To our knowledge this is the first example of adapting tetrazine bioorthogonal chemistry for use with ^{68}Ga PET imaging. The short half-life of ^{68}Ga is poorly matched with the clearance half-life of monoclonal antibodies.[11] Bioorthogonal labeling using metal-chelating tetrazines could enable use of the generator produced ^{68}Ga radionuclide for the multistep labeling and imaging of monoclonal antibodies bound to cancer related biomarkers.

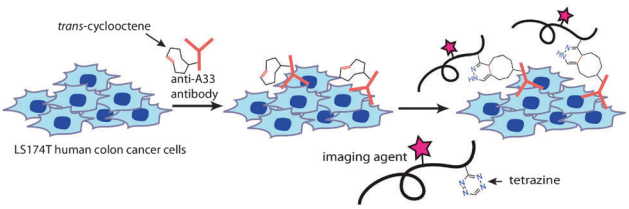

Fig. 1 Bioorthogonal inverse Diels–Alder reaction between tetrazine and *trans*-cyclooctene for multistep labeling of cancer cells with tetrazine DTPA dextran containing an imaging agent.

Previous work has indicated that polymeric scaffolds can improve the efficiency of *in vivo* reactions by tuning the blood clearance rate.[7] In choosing a polymer scaffold, we decided to explore the use of dextrans due to their well-established clinical safety record, hydrophilicity, low expense, ready availability in numerous molecular weights, and our previous experience working with dextran imaging agents.[7,12,13] Dextrans with amine leashes and radionuclide agents have been extensively studied as radionuclide imaging agents for sentinel lymph node detection.[3,14,15] In order to chelate the ^{68}Ga, we decided to utilize DTPA chelation groups based on prior clinical work with DTPA albumins and dextrans and the known stability of DTPA chelates[16,17] which exhibit adequate *in vivo* stability for gallium during the moderately short biological half-life of the dextran conjugates.[17,18] However, although the DTPA chelate is suitable for the intended proof-of-principle studies, future clinical implementation of the proposed ^{68}Ga imaging probes would likely utilize alternative chelators.[19,20]

Although a multistep PET imaging approach would have application to numerous disease models, to initially test and

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optimize our method we chose to work with a human colon cancer model and target the A33 antigen.[21,22] Initially, we were interested in determining whether the chelating tetrazine DTPA dextrans were capable of specifically targeting *trans*-cyclooctene modified monoclonal antibodies bound to the extracellular A33 marker. To verify extracellular localization, we decided to perform fluorescence microscopy studies using tetrazine DTPA dextran modified with a near-infrared emitting fluorescent probe, AlexaFluor 647 (AF647). DTPA dextran containing reactive amine ''leashes'' was synthesized as previously described and modified with approximately 1 equivalent of AF647.[4] The remaining amines were then modified with 5 equivalents of tetrazine NHS and finally capped by excess acetic anhydride creating AF647 tetrazine DTPA dextran. A33 expressing LS174T human colon cancer cells were targeted with a TCO modified anti-A33 antibody (~5.3 equivalents of TCO per antibody), washed, and subsequently reacted with 10 μM of the fluorescent dextran for 30 minutes. After washing, the cells were imaged using fluorescence microscopy. As shown in Fig. 2, the cells surfaces were brightly stained (green), indicating that the fluorescent tetrazine DTPA dextran had modified the surface bound TCO antibodies. Staining is absent inside the cells, indicating that the dextran has not internalized, as expected for the non-internalizing A33 antigen.[23] Control experiments showed minimal surface staining, indicating that the chelating tetrazine DTPA dextrans are highly reactive with cellular bound dienophiles such as TCO, similar to previously introduced tetrazine imaging agents.[24] We also note that previous work has demonstrated that a fluorescent tag does not alter the receptor affinity or *in vivo* behavior of the receptor–specific dextran conjugate, Tc-99m-labeled Cy7-tilmanocept.[12]

Having demonstrated that tetrazine DTPA dextrans are capable of multistep labeling of strained dienophile modified surface biomarkers, we proceeded to explore metalation of tetrazine DTPA dextran with the positron emitting radionuclide ^{68}Ga. In contrast to ^{18}F, ^{68}Ga is an emerging generator produced PET radionuclide that, in addition to not requiring a cyclotron, is also appended to molecules *via* non-covalent and mild chelation chemistry.[25] Thus we expected that tetrazine reactive groups would be compatible with the conditions required for ^{68}Ga chelation of pendant DTPA ligands. ^{68}Ga was chelated to tetrazine modified DTPA dextran following previously published procedures in 99% radiochemical yield (RCY) (Fig. S1a, ESI†).[3]

We next determined if the resulting ^{68}Ga tetrazine DTPA dextran was suitable for multistep cellular labeling similar to the fluorescent AF647 tetrazine DTPA dextran (Fig. S1b, ESI†). LS174T cells were labeled with *trans*-cyclooctene anti-A33 monoclonal antibodies and subsequently exposed to 40 μCi ^{68}Ga tetrazine DTPA dextran. Radiolabel uptake was quantified and compared to cells that received a control lacking dienophile. We also compared how TCO antibody loading affected the radiolabel uptake similar to the fluorescent AF647 tetrazine DTPA dextran (Fig. S1b, ESI†). Previous work has demonstrated that stoichiometric amplification of secondary imaging agents can be achieved by changing the number of TCO dienophiles on pretargeted monoclonal antibodies.[26] Indeed, decreasing the amount of TCOs that the antibodies were exposed to (30 equivalents *versus* 50) resulted in decreased ^{68}Ga uptake.

We monitored the *in vivo* pharmacokinetics and biodistribution of ^{68}Ga tetrazine DTPA dextran with PET imaging followed by sacrifice and measurement of the percent injected

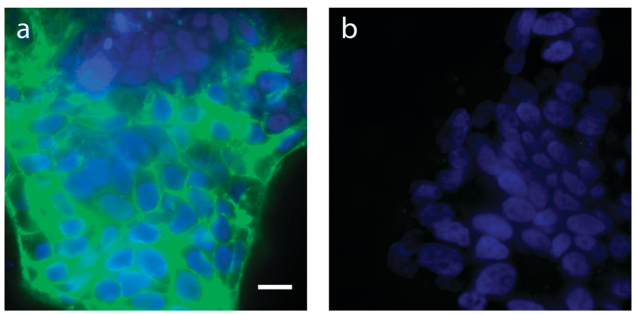

Fig. 2 Confocal images of cells treated with fluorescent AlexaFluor 647 (AF647) tetrazine DTPA dextran. (a) Cells pretargeted with *trans*-cyclooctene anti-A33 (sample). Scale bar (lower right) 15 microns. (b) Cells lacking A33/TCO (control).

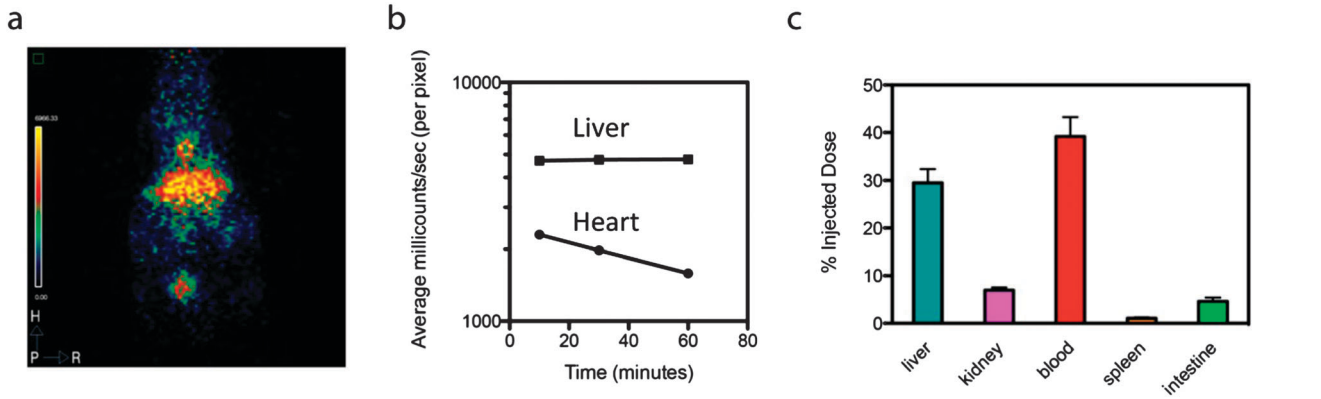

Fig. 3 (a) PET reconstruction (coronal slice) after injection of 50 μCi of ^{68}Ga dextran tetrazine. (b) Time–activity curves of liver (squares) and heart (circles). (c) Biodistribution of tetrazine dextran after sacrifice.

dose of ^{68}Ga probe in various tissues of interest. Fig. 3a depicts a typical PET image of a mouse 60 minutes after receiving 50 μCi of ^{68}Ga tetrazine dextran. Imaging for mice (n = 3) indicated that the tetrazine probe showed moderate clearance and the expected uptake pattern for a DTPA dextran imaging agent in the blood pool. Mice were sacrificed after the 60 minute PET scan, and key organs and tissues were dissected, weighed, and the radioactivity counted to determine the percent injected dose (Fig. 3b). We estimate that the blood half-life of the ^{68}Ga tetrazine dextran to be slightly less than one hour. Thus, this agent should be compatible with the 68 minute decay half-life of ^{68}Ga. Blood stability tests were performed in human plasma with ^{68}Ga DTPA Dextran. It was found that, after a 3 hour incubation period, no free ^{68}Ga was present in the plasma. Thus, the stability is compatible with the blood clearance times and tetrazine modification does not have a significant effect on ^{68}Ga DTPA dextran distribution *in vivo*.

Preliminary PET studies demonstrated the ability of ^{68}Ga tetrazine dextrans to target A33 biomarkers in *in vivo* subcutaneously implanted LS174T xenografts. Xenograft bearing mice were injected with TCO modified anti-A33 bearing a near-IR fluorescent dye. After 24 hours, the ^{68}Ga tetrazine DTPA probe was injected, followed by PET imaging, sacrifice, and fluorescence imaging of relevant tissue samples. A tumor to muscle ratio (%injected dose/gram) of 3.9 ± 1.8 was obtained. Our proposed multistep approach is highly modular, and it is conceivable that alternative tetrazines, chelators, polymers, and dienophiles may be utilized to improve the signal to background ratio. Indeed, although DTPA chelates are adequate for these initial proof-of-principle studies, clinical implementation would likely make us of more stable gallium chelators such as NOTA.[19,20] We believe that tetrazine dextrans may eventually enable the multistep labeling of a broad array of surface biomarkers using the convenient short-lived PET radioisotope ^{68}Ga.

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