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Tetrazine-*trans*-cyclooctene ligation for the rapid construction of integrin $\alpha_v\beta_3$ targeted PET tracer based on a cyclic RGD peptide†

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Abstract

Labeling biomolecules with ^{18}F is usually done through coupling with prosthetic groups, which generally requires several time-consuming radiosynthetic steps resulting in low labeling yield. Recently, the tetrazine-*trans*-cyclooctene ligation has been introduced as a method of bioconjugation that proceeds with fast reaction rates without need for catalysis. Herein, we report the development of an extremely fast and efficient method for generating ^{18}F labeled probes based on the tetrazine-*trans*-cyclooctene ligation. Starting with only 30 μg (78 μM) of a tetrazine-RGD conjugate and 2 mCi (5 μM) of ^{18}F -*trans*-cyclooctene, the ^{18}F labeled RGD peptide could be obtained in more than 90% yield within five minutes. The ^{18}F labeled RGD peptide demonstrated prominent tumor uptake *in vivo*. The receptor specificity was confirmed by blocking experiments. These results successfully demonstrate that the tetrazine-*trans*-cyclooctene ligation serves as an efficient labeling method for PET probe construction.

Keywords

Tetrazine-*trans*-cyclooctene ligation; Integrin $\alpha_v\beta_3$; Positron emission tomography (PET); RGD peptide; Fluorine-18

Positron emission tomography (PET) is a non-invasive imaging technique based on positron-emitting radionuclides including C-11, N-13, O-15 and F-18.¹ In PET, a positron (β^+) emitted from a tracer molecule travels through the medium until collision with an electron occurs, resulting the emission of two gamma-rays which can be imaged with exceptionally high spatial resolution (~ 1 mm). PET imaging is used extensively to diagnose,

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study and monitor disease *in vivo* and has applications in areas including, but not limited to, oncology, neurology and cardiology.²⁻⁹

F-18 PET has a number of attributes that make it clinically attractive. F-18 is produced as fluoride from a cyclotron from ¹⁸O enriched water, essentially free of F-19 fluoride (no carrier added).¹⁰ The resulting F-18 has a very high specific radioactivity (activity per unit mass of a pure radionuclide), which enables radiotracers to be administered with low mass dosages, thereby limiting toxic effects. The short half-life of F-18 (~110 min) also provides the advantage that radiation exposure to the patient is minimized.

While the short half-life is a clinically attractive feature of F-18 PET, the short half-life imposes severe time restrictions for the synthesis of radiolabeled molecules. This situation is exacerbated by the low nucleophilicity of fluoride, making it difficult to incorporate F-18 in complex molecules. For the field of PET, the speed and simplicity of chemistry are critical factors.¹¹ F-18 labeled acyltransfer reagents¹² and maleimides¹³⁻¹⁷ have been introduced for the labeling of amine and sulfhydryl functionalities, respectively. New methods for the introduction of F-18 labels based on Cu-catalyzed azide-alkyne 'click' chemistry have also been introduced. Impressive strides have been made with such reagents, but challenges exist for improving rates and efficiency of F-18 incorporation.¹³⁻¹⁷

The inverse-electron demand Diels-Alder reactions of tetrazines have a rich history in organic synthesis, and key developments from the labs of Boger¹⁹ and Sauer²⁰ had established that tetrazines are powerful dienes for synthetic applications. Bertozzi first demonstrated that strain-driven reactions of azides and cyclooctynes are powerful tools for bioconjugation.²¹ In 2008, our group described a general, photochemical method for preparing functionalized *trans*-cyclooctenes,²² and a method for bioconjugation based on the reaction between tetrazines and *trans*-cyclooctene that proceeds with unusually fast rates (k_2 2000 L/mol•s for *trans*-cyclooctene and 3,6-di(2-pyridyl)-*s*-tetrazine).²³ Subsequently, other groups described bioconjugations with less reactive dienophiles.²⁴ However, the use of *trans*-cyclooctenes is necessary to achieve fast rates of reactivity. A number of applications of the tetrazine-*trans*-cyclooctene ligation have been developed, including elegant work by Robillard and coworkers, who demonstrated that our system for tetrazine-*trans*-cyclooctene ligation could be utilized in ¹¹¹In-imaging of tumors in live mice.²⁵

Previously, we developed a F-18 radiolabeling method for bioconjugation based on the tetrazine-*trans*-cyclooctene ligation.²⁶ This method utilizes a novel F-18 labeled *trans*-cyclooctene probe, and the efficiency of the reaction has been demonstrated with 3,6-di(2-pyridyl)-*s*-tetrazine, which can be readily functionalized via amido derivatives.²³ The reaction proceeds with exceptionally fast rates, making it an effective conjugation method at low concentration. Herein, we report the construction of a ¹⁸F labeled PET probe for cancer imaging based on the tetrazine-*trans*-cyclooctene ligation.

The integrin $\alpha_v\beta_3$ is upregulated on the endothelial surface of tumor blood vessels, and has been linked to tumor progression and metastasis.²⁷ The use of radiolabeled synthetic RGD antagonists of the integrin $\alpha_v\beta_3$ have been shown to be effective tools for cancer imaging.^{28,29} Although strategies for F-18 incorporation into RGD mimics have been described,^{28, 30-32} many of these procedures involve lengthy synthetic procedures. Consequently, it is challenge to obtain high radiochemical yields and to develop synthetic protocols that can be automated. We hypothesized that the F-18 labeled RGD peptides could be constructed using our newly developed tetrazine-*trans*-cyclooctene ligation, which will allow cancer detection, patient stratification, and treatment monitoring through PET imaging of integrin $\alpha_v\beta_3$ expression *in vivo*.

As shown in Scheme 1, tetrazine-RGD conjugate **3** was readily prepared by coupling NHS-ester **2** and the peptide c(RGDyK) (**1**). To provide an LC-standard, the tetrazine-RGD was then combined with ^{19}F -TCO (**4**)³³ to provide ^{19}F -**5** as a mixture of isomers.³⁴

The cell binding affinity studies of c(RGDyK) (**1**) and ^{19}F -**5** were conducted (Figure 1). Both peptides inhibited the binding of ^{125}I -echistatine to U87MG cells in a dose dependent manner. The IC_{50} values for c(RGDyK) and ^{19}F -tetrazine-RGD were $(1.16 \pm 0.35) \times 10^{-7}$ and $(1.93 \pm 0.27) \times 10^{-7}$ M, respectively, indicating that the fluorinated label had only a minimal effect on the integrin binding affinity of the RGD moiety of **5**.

We next tested the efficiency of **F-4** for labeling tetrazine-RGD peptide **3**. ^{18}F -**4** was synthesized following a previously reported procedure.²¹ The conjugation of ^{18}F -**4** and tetrazine-RGD was performed at room temperature for 5 min. As shown in Figure 2, the conjugation was efficient and high yielding: starting with only 30 μg (78 μM) of tetrazine-RGD conjugate **3** and 2 mCi (5 μM) of ^{18}F -*trans*-cyclooctene, the labeling yield was 90% after 5 min by HPLC analysis. The yield of this reaction compares favorably to our prior studies at higher concentration using *N*-succinimidyl-4- ^{18}F -fluorobenzoate (where the labeling yield was only 30% with 300 μg (0.94 mM solution) of an RGD conjugate) and to our studies on the Cu-catalyzed azide/alkyne cycloaddition (where the labeling yield was 70% with 1 mg (1.8 mM solution) of an RGD derivative).²⁶⁻²⁸ We also tested the stability of ^{18}F -tetrazine-RGD conjugate **3** in PBS in the presence 5% EtOH. The stability was excellent: >95% of the tracer remained after incubation for 6 h as judged by radio-HPLC analysis.

Static microPET scans were performed for ^{18}F -tetrazine-RGD conjugate **5**. Selected coronal images at different time points are shown in Figure 3. High tumor accumulation was observed as early as 30 min time point. The tumor uptake was 4.6 ± 0.2 , 4.4 ± 0.6 , 4.2 ± 0.6 , and 2.7 ± 0.5 %ID/g at 0.5, 1, 2, 4 h post injection, respectively. ^{18}F -**5** was cleared through both liver and kidney. Up to 4 h post injection, there is still a fair amount of activity accumulated in the abdomen, which may be a result of the hydrophobicity of this first generation system.

Blocking experiments were also performed by co-injecting 10 mg/kg of c(RGDyK) along with ^{18}F -**5**. The tracer uptake in U87MG tumor dropped to 1.4 ± 0.2 , 1.0 ± 0.3 , 0.6 ± 0.2 , and 0.4 ± 0.1 %ID/g at 0.5, 1, 2, 4 h post injection respectively. The successful blocking confirmed the integrin $\alpha_v\beta_3$ specific binding of the tracer ^{18}F -**5**.

In conclusion, it is demonstrated that the tetrazine-*trans*-cyclooctene ligation is an efficient labeling method for PET probe construction. A major advantage of this method lies in ability to achieve fast and efficient conjugation at low concentration. It is expected that this method of bioconjugation will become a general method for labeling peptides, proteins, antibodies, antibody fragments, and modified oligonucleotides in high radiochemical yield and high specific activity for successful PET applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- As described previously,^{23,26} the formed 4,5-dihydropyridazine products of tetrazine-*trans*-cyclooctene ligations isomerize to 1,4-dihydropyridazines via amination intermediates.

Figure 1. Cell-binding assay of c(RGDyK) (1) and ^{19}F -5 using U87MG cells (integrin $\alpha_v\beta_3$ -positive human glioblastoma). The cell-binding affinity of the peptides was determined by performing competitive displacement studies with ^{125}I -echistatin.

Figure 2.
Radio-HPLC analysis of the crude reaction between ^{18}F -*trans*-cyclooctene **4** and tetrazine-RGD conjugate **3**.

Figure 3.

(A) Decay-corrected whole body coronal images of athymic female nude mice bearing an U87MG tumor at 0.5, 1, 2, and 4 h after injection of 3.7 MBq of ^{18}F -5. (B) Coronal images of U87MG tumor bearing mice at 0.5, 1, 2, and 4 h after injection of ^{18}F -5 with coinjection of 10 mg of c(RGDyK) per kilogram of mouse body weight (denoted as “ ^{18}F -5 blocking”). In all images, the locations of tumors are indicated with arrows. Quantitative analyses of tracer uptake in U87MG tumor, liver, kidney, and muscle were performed based on microPET imaging results.

Scheme 1.

Synthesis of a tetrazine-RGD conjugate, and ligation with a fluorinated *trans*-cyclooctene to create a fluorinated probe for cancer detection.