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# Synthesis and Evaluation Of F-18 Labeled Mono- And Di-crgd Peptides *Via* Strain-Promoted Click Chemistry For Micro PET Imaging Study

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## 1. Introduction

Over the past decade, many F-18 radiolabeled cyclic RGD peptides have been evaluated as radiotracers for imaging tumors by SPECT or PET. $^{18}F$ is the most widely used positron-emitting radioisotope for PET imaging due to the short half-life of fluorine-18 ($t_{1/2}$ = 109.8 min), and its physical properties and nuclear characteristics are ideally can be incorporated into cyclic RGD peptide via a covalent bond without the need of bifunctional chelator (BFC). We developed $^{18}F$ labeled mono-, and di-cRGD Peptides using Strain-promoted azide-alkyne cycloadditon reaction for micro PET imaging.

## 2. Method:

In this method, the strain-promoted azide and alkynes cycloaddition reaction using the mono-and di-cRGD-ADIBO peptide precursors with the $^{18}F$-PEG-azide and subsequent chemo-orthogonal purification reaction with azide resin proceeded fast and selectively under physiologically friendly reaction condition (i.e., toxic chemical reagents-free, aqueous medium, room temperature, pH≈7), and provided $^{18}F$-labeled mono- and di- cRGD Peptides. In addition, microPET images were acquired using a microPET/CT scanner.

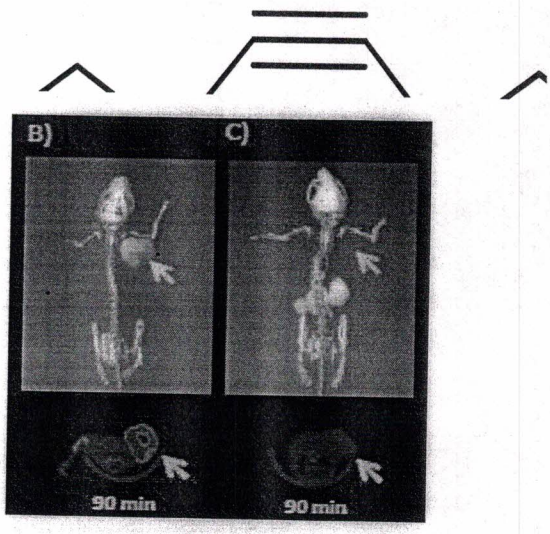


**Figure 1.** A) Synthesis of F-18 labelled (cRGD)n based on chemo-orthogonal SPAAC reaction protocol; microPET-CT images of U87MG tumor bearing mice at 90 min post-injection of 1.8 MBq of cRGD2-PEG4-ADIBOT-$^{18}F$ without (B) and with (C) (denoted as "Blocking") a co-injection of nonradioactive cRGD2-PEG4-ADIBOT-F. Tumors are indicated by white arrows.

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**Results and Discussion:**

We synthesized mono-, and di- $^{18}$F-labelled tumor targetable bioactive peptides

such as cRGD1-ADIBOT-$^{18}$F, cRGD1-PEG4-ADIBOT-$^{18}$F, cRGD2-ADIBOT-$^{18}$F, and cRGD2-PEG4-ADIBOT-$^{18}$F in excellent dcRCYs (90-92%) and radiochemical purities (> 98%) within only a 35 min total reaction time with high specific activity and *in vivo* PET molecular imaging study using the $^{18}$F-labelled cRGD peptides also demonstrated successful application of our $^{18}$F-labeling protocol.

**Conclusion:**

In summary, this contribution described the advantage of strain-promoted alkyne-azide 1,3-dipolar cycloaddition in comparison to copper-catalyzed version for selective $^{18}$F-radiolabeling of mono-, and di-cRGD peptides without apparent physiological harm. This compound showed rapid and higher tracer uptake in U87MG tumors and relatively good metabolic stability, as well as favorable in vivo pharmacokinetics. We expect that the reaction condition presented here will widen the application of the click reaction for the preparation of $^{18}$F-labeled peptides to various types of biomolecules for microPET imaging study.

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# $^{18}$F-Labeled Mono- and Di- Crgd Peptides Basedon SPAAC under Physiologically Friendly Reaction Conditions for Molecular Imaging Study.

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**Abstract**

*A series of $^{18}$F-labeled cRGD peptides have been developed based on chemo-orthogonal strain-promoted cycloaddition using aza-dibenzocyclootyne-substitutedmono- and di- cRGD peptides as precursors with $^{18}$F-azide synthon for positron emission tomography (PET) imaging of tumor $\alpha_v\beta_3$ integrin expression. In this study, the SPAAC reaction and subsequent chemo-orthogonal purification reaction with azide resin proceeded quickly and selectively under physiologically friendly reaction condition (i.e., toxic chemical reagents-free, aqueous medium, room temperature, pH≈7), provided $^{18}$F-labelled tumor targetable bioactive peptides in high radiochemical yield and high specific activity. In vitro binding assay and in vivo PET molecular imaging study using the $^{18}$F-labelled mono-, di- cRGDpeptides also demonstrated successful application of our $^{18}$F-labeling protocol.*

## INTRODUCTION:

Radio labelled cyclic RGD peptides have the potential for early detection of rapidly growing tumors and non invasive visualization of tumor metastasis and therapeutic response in cancer patients (1). Over the past decade, significant progress has been made in the development of $\alpha_v\beta_3$-targeting radiotracers for the visualization of $\alpha_v\beta_3$ expression in tumors using Cyclic RGD peptides with various radioisotopes such as $^{99m}$Tc and $^{111}$In for SPECT imaging and labeled with $^{18}$F, $^{64}$Cu, $^{68}$Ga, and $^{89}$Zr for PET imaging. Among them, $^{18}$F is the most widely used positron-emitting radioisotope for PET imaging due to the short half-life of fluorine-18 ($t_{1/2}$ = 109.8 min), and its physical properties and nuclear characteristics are ideally can be incorporated into cyclic RGD peptide via a covalent bond without the need of bifunctional chelator (BFC). For example, [$^{18}$F]-galacto-RGD (*2*) and to improve integrin $\alpha_v\beta_3$ binding affinity, multimeric RGD peptides such as cyclic RGD dimer ([$^{18}$F]FPPRGD2) (*3*), and cyclic RGD tetramer ([$^{18}$F-EPRGD4) (*4*) has been developed for PET imaging of $\alpha_v\beta_3$ integrin in the last few years. Radiofluorination of peptides thus generally uses $^{18}$F-prosthetic groups such as *N*-succinimidyl-4-$^{18}$F fluorobenzoate ($^{18}$F-SFB) (*5*), 4-$^{18}$Ffluorobenzaldehyde ($^{18}$F-FBA)(*6*), 3-$^{18}$F-fluoro-5-nitrobenzimidate ($^{18}$FFNB) (*7*), 4-azidophenacyl $^{18}$F-fluoride ($^{18}$F-APF) (*8*), and 4-$^{18}$Ffluorophenacyl bromide ($^{18}$F-FPB) (*7*). However, most of these procedures suffer from lengthy and tedious multistep synthetic procedures. As a result, these long, difficult processes make them a challenge to automate and adversely decrease the overall radiolabeling yield.

The Cu(I)-catalyzed [3 + 2] azide-alkyne cycloaddition successfully introduced into organic PET chemistry with the short-lived positron emitter $^{18}$F as a azide with alkynes (or vice versa) providing 1,2,3-triazole formed in a high yield under mild condition (*9*). Therefore the usefulness of the 1,3-dipolar Huisgen cycloaddition, a numerous $^{18}$F-labeled RGD peptides have been recently reported (*10*). However, in spite of its vast successes, this reaction is not ideal for bioorthogonal chemistry; Cu(I) catalyst can bind to biomolecules, blocking or reducing the biological activity and copper ion contamination of the final product can culminate in issues of cytotoxicity using the CuAAC conjugation method (*11-13*). At present the foremost significant method is copper-free "click chemistry" based on strain-promoted alkyne azide cycloaddition (SPAAC) has been developed as a fast and bioorthogonal conjugation protocol for biological application in live-cell imaging, radioisotope labeling and surface modification (*13-17*).In particular, dibenzocyclooctyne (DIBO) or aza-dibenzocyclooctyne (ADIBO) derivatives have showed good performance in this SPAAC reaction for these purposes (*18-20*).

## MATERIALS AND METHODS:

### A. Synthesis of ADIBO substituted mono- and di-cRGD peptide precursors

Succinimidyl ester **9** was therefore conjugated to cyclic RGD monomer cRGDyK(denoted as cRGD1) and cyclic RGD dimer H-E[c(RGDyK)]$_2$ (denoted as cRGD2) under basic conditions (Scheme S4). Full conversion to the product cRGD1-ADIBOand cRGD2-ADIBO was achieved after 12 h as determined by reverse-phase (RP) HPLC, and subsequently it was purified by RP-HPLC and characterized by mass spectrometry. In addition, because a poly(ethylene glycol) (PEG) linker can fine-tune the *invivo* pharmacokinetics of imaging probes (*21*, *22*), our aimed to incorporate a PEG linker between cyclic RGD peptide and dibenzocyclooctyne. Our synthesis started from commercially available dibenzocyclooctyne, which was coupled with a PEG linker (denoted as PEG4-

ADIBO) was therefore conjugated to cRGD1 and cRGD2 to produce cRGD1-PEG4-ADIBO and cRGD2-PEG4-ADIBO as described in the experimental section (Scheme S5).

**B. Synthesis of F-18 labeled mono- and di-cRGD peptides based onchemo-orthogonal SPAAC reaction protocol**

The SPAAC reaction of cRGD1-ADIBO and cRGD1-PEG4-ADIBO precursor (0.77 μmol) with [$^{18}$F]**13** in ethanol/water (1/1) was completed within 15 min, subsequently, treatment of this crude solution with the azide resin **14** for 20 min could remove the non-reacted cRGD1-ADIBO and cRGD1-PEG4-ADIBO precursor as shown in Figure 1. The HPLC analysis of the reaction mixture before and after treatment with the scavenger resin **14**. After filtration and washing of the resin **14** with PBS solution, this F-18 labeling protocol allowed us to produce cRGD1-ADIBOT-$^{18}$F and cRGD1-PEG4-ADIBOT-$^{18}$F within approximately 35 min total reaction time in a 92% decay-corrected RCY (dcRCY) with > 98% of radiochemical purity as a direct injectable solution for an animal PET image study without any HPLC purification and formulation process.

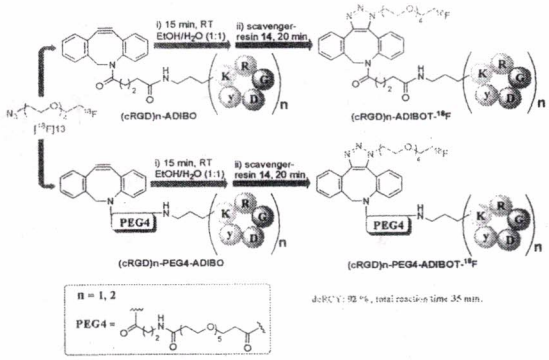


**Figure 1:**A)Schematic procedure for the preparation of Radiosynthesis of $^{18}$F-Labeled mono- and di- CyclicRGD Peptides with [$^{18}$F]**13** and polystyrene-supported azide resin **14** as a ADIBO-precursor-scavenger.

**C. In vitro cell Integrin Receptor Binding Assay**

The receptor-binding affinity studies of cRGD derivatives for $\alpha_v\beta_3$integrin positive U87MG cells. We compared the receptor-binding affinity of cRGDyk, cRGD1-ADIBOT-F, cRGD1-PEG4-ADIBOT-F, cRGD2-ADIBOT-F, and cRGD2-PEG4-ADIBOT-F by performing competitive displacement studies with $^{125}$I-echitin. All peptides inhibited the binding of $^{125}$I-echistatin to $\alpha_v\beta_3$ integrin positive U87MG cells. The $IC_{50}$ values for cRGDyk, cRGD1-ADIBOT-F, cRGD1-PEG4-ADIBOT-F, cRGD2-ADIBOT-F, and cRGD2-PEG4-ADIBOT-F were 5.5±4 μmol/L, 17.8±9.7 μmol/L, 19.8±11 μmol/L, 8.2±6 μmol/L, 3.2±2.2 μmol/Lrespectively. The comparable $IC_{50}$ values of the compounds suggest that the dimeric cRGD was highly binding to $\alpha_v\beta_3$integrin and insertion of the PEG4 spacer is effective to increase the receptor-binding affinity.

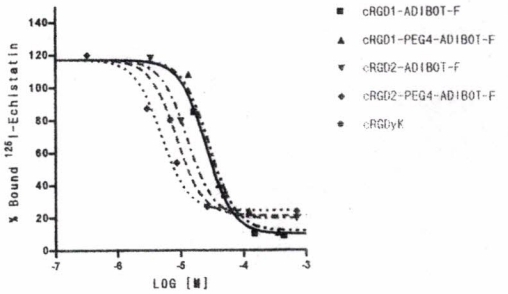


**Figure 2:**In vitro inhibition of $^{125}$I-echistatin binding to $\alpha_v\beta_3$integrin on human glioblastoma cell line U87MG by cRGDyk, cRGD1-ADIBOT-F, cRGD1-PEG4-ADIBOT-F, and cRGD2-ADIBOT-FandcRGD2-PEG4-ADIBOT-F (n=3, mean±SD).

Provided with quickly and selectively under physiologically friendly reaction condition, cRGD2-PEG4-ADIBOT-$^{18}$F was shown to bind with high affinity and specificity with integrin-positive U87MG glioma cells in vitro and proper features as a PET imaging agent.

**CONCLUSION:**

In summary, this contribution described the selective $^{18}$F-radiolabeling of cRGD peptides without apparent physiological harm. We have demonstrated that copper-free click reaction can be carried out between a short-lived $^{18}$F-labelled azide synthon and various cycloctynes conjugated mono- and di- cRGD peptides and these reaction achieved $^{18}$F-labeled tumor targetable bioactive mono-, and di- cRGD peptides in excellent dcRCYs (92%) and radiochemical purities (> 98%) within only a 35 min total reaction time with high specific activity. In the noninvasive small-animal studies, shows bind with high affinity and specificity with integrin-positive U87MG glioma cells. I expect that the reaction condition presented here will widen the application of the click reaction for the preparation of $^{18}$F-labeled peptides to various types of biomolecules for molecular imaging study.

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