Bioconjugation

Last-step enzymatic $[^{18}F]$-fluorination of cysteine-tethered RGD peptides using modified Barbas linkers

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Abstract: We report a last-step fluorinase catalyzed $[^{18}F]$-fluorination of a cysteine-containing RGD peptide. The peptide was attached through sulfur to a modified and more hydrophilic variant of the recently disclosed Barbas linker which was itself linked to a chloroadenosine moiety via a PEGylated chain. The fluorinase was able to use this construct as a substrate for a transhalogenation reaction to generate $[^{18}F]$-radiolabelled RGD peptides, which retained high affinity to cancer cell relevant α5β3 integrins.

Introduction

During the past decade, bioactive peptides, in particular cyclic RGD peptides, have been increasingly identified as targets for radiolabeling as positron emission tomography (PET) tracers due to their high target-selectivity, non-immunogenicity, good tissue penetration and rapid metabolic clearance. $[^{18}F]$-Fluoride is the isotope of choice for PET as it has a moderate to long half-life (109.8 min) and is amenable to relatively extended synthesis and preparative manipulations. It also has a high percentage of $\beta^-$ decay (97%) and a low $\beta^+$ energy (635 Kev) and therefore a short positron range (2.3 mm in water). This leads to increased image resolution. Also the ease of production of a short positron range (2.3 mm in water). This leads to $\beta^-$ preparative manipulations. It also has a high percentage of metabolic clearance.

$[^{18}F]$-fluorination of cysteine containing RGD peptides using modified Barbas linkers can often require harsh conditions incompatible with anhydrous solvents to ensure fluoride has been reacted in last step protocols. While the best coupling reactions between the $[^{18}F]$ prosthetic group and peptides are mild, biorthogonal and site-specific, the syntheses of the $[^{18}F]$-building blocks requires pre-drying of the $[^{18}F]$fluoride, which is generated from $[^{18}F]$H2O. Good nucleophilic reactions require anhydrous solvents to ensure fluoride nucleophilicity. This can often require harsh conditions incompatible with peptides, such as elevated temperatures. Therefore, a last-step $[^{18}F]$-fluorination of a substrate under ambient aqueous conditions is attractive for radiosynthesis and is a current research focus within the radiochemistry community. To this end $[^{18}F]$-fluoride has been reacted in last-step protocols with organosilicon decorated peptides to give $[^{18}F]$-SiFA-conjugate via $[^{18}F]$-HF isopic exchange. Similarly, arylfluoroborinate modified peptides have been induced to capture carrier-added $[^{18}F]$-fluoride under aqueous acidic condition in a single step to generate $[^{18}F]$-ArBF3-peptides. Alternatively $[^{18}F]$fluoride can be locked into chelator (NODA or NOTA) functionalized peptides by coordination chemistry with Al2+ as a last step treatment, to generate Al-$[^{18}F]$ tracers.

Our focus has been on enzymatic incorporation of $[^{18}F]$ into candidate radiotracers under ambient conditions. The fluorinase enzyme, originally isolated from Streptomyces cattleya, catalyzes the conversion of S-adenosyl-L-methionine (AdoMet) and fluoride ion to 5′-fluoro-5′-
deoxyadenosine (5'-FDA) and L-methionine (L-Met).\textsuperscript{20} Fluorinase also functions as a chlorinase, particularly in the reverse direction.\textsuperscript{21} The reversibility allows 5'-chloro-5'-deoxyadenosine (5'-CIDA) to be used as a substrate for fluorination in a one step reaction via AdoMet under ambient (pH 7.8 phosphate buffer, 37°C) aqueous conditions (Scheme 1).

The reaction benefits from the fact that the equilibrium favors 5'-FDA in fluorination but AdoMet in chlorination. In addition, the use of L-selenomethione (L-SeMet) in place of L-Met increases the reaction rate and improves the efficiency of the transhalogenation of (5'-ClDA) to FDA.\textsuperscript{22} Fluorinase displays a high substrate specificity, however, we have recently demonstrated that the introduction of an acetylene group at C-2 of the adenine base of 5'-ClDA is tolerated by the enzyme.\textsuperscript{22} In addition this acetylene can be extended at its terminus to carry a pegylated cargo. Decoration of 5'-ClDA in this way has allowed bioconjugation through “click” chemistry with azido functionalized peptides. In this context we have reported a ‘last-step’ enzymatic $^{18}$F fluorination of a 5'-CIDA- RGD peptide conjugate with the fluorinase.\textsuperscript{22} This approach was good for lysine modified azido containing RGD peptides. It became an objective to develop a protocol compatible with cysteine ligation due to the general importance of cysteine in bioconjugation, often through an engineered cysteine thiol. A successful strategy would significantly broaden the utility of the enzymatic methodology for radiolabeling peptides and proteins. Conventional site-specific conjugates of cysteine with maleimide suffer from degradation via retro-Michael reaction, and competition from thio-containing peptides or proteins such as glutathione or serum albumin present in vivo.\textsuperscript{23}

Barbas and coworkers recently introduced methylsulfonyl oxadiazole for bioconjigation through nucleophilic aromatic substitution by cysteine residues to generate a relatively stable oxadiazole-protein thiolate.\textsuperscript{24,25} We saw an opportunity for combining this Barbas linker with our fluorinase technology in order to generate a thiol ligated peptide for last step enzymatic $^{18}$F-fluorination under ambient aqueous condition (Scheme 2).

![Scheme 1](image1.png)

**Scheme 1.** Fluorinase catalyzed fluorination and chlorination of AdoMet as well as the conversion of 5'-CIDA to 5'-FDA.

![Scheme 2](image2.png)

**Scheme 2.** Design of three components for last-step $^{18}$F labelling of cysteine containing peptide: fluorinase substrate, Barbas linker and the cysteine-containing peptide.

**Results and Discussion**

**RGD conjugation to a chlorodeoxyadenosine through modified Barbas linkers**

To obtain the conjugate, we attempted to click CIDA-TEG and 2-methylsulfanyl 5-(4-azidotetraethylene glyoxylonyphenyl) oxadiazole 1a\textsuperscript{23} in the first step, but the free methylsulfanyl moiety was gradually hydrolysed during work up and this was not progressed. However when the sequence was reversed (Scheme 3) by first reacting the methylsulfonate 1a with the RGD, then the azido phenyl oxadiazole thiolate (APOT-RGD) 3a was obtained in excellent yield.

Conjugate 3a is very insoluble in common solvents such as MeOH, CH$_3$CN and H$_2$O and was only partially soluble in DMSO. A dilute solution of conjugate 3a in DMSO/H$_2$O with CIDA-TEG-acetylene 2\textsuperscript{22} under Cu catalyzed “click” conditions generated CIDA-APOT-RGD (4a), which was purified by semi-preparative HPLC. Again, CIDA-APOT-RGD (4a) is insoluble in common solvents with only partial solubility in DMSO.

To improve the solubility, a second PEGylated chain was introduced at the 3-position on the phenyl ring of the Barbas linker. This involved the selective decacylation and Williamson etherification at C-4 of the precursor benzoate
Scheme 3. Successive conjugation of methylsulfonyl azido phenyloxadiazole, RGD and CIDA-TEG or FDA-TEG and fluorinase catalyzed transhalogenation. Regents and conditions: a) c(RGDfC), PBS buffer (pH 7.4), 2 h; b) Compound 2, CuTBTA in 55% DMSO, sodium ascorbate, H₂O, overnight; c) KF, L-SeMet, fluorinase, phosphate buffer (pH 7.8), in a one pot protocol to give 7b (SI Scheme S1).

Further etherification of 3-OH with tosylate 13 afforded 8b. Product 8b was condensed with hydrazine and then cyclized with carbon disulfide in the presence of KOH to generate oxadiazole 10b. Finally thiol methylation and then oxidation with m-CPBA gave bisPEGlated azido phenyloxadiazole 1b (SI Scheme S1).

Conjugation of 1b with c(RGDfC) as shown in Scheme 3 afforded 3b (MAPOT-RGD), and this construct was “clicked” with 2 affording (CIDA-MAPOT-RGD) 4b. The solubility of 4b was not significantly improved relative to 4a particularly in acetonitrile, methanol, ethanol and water.

There was the option of introducing a second PEGylated chain at C-5 of the phenyl ring, however the molecular weight of the construct is getting large, and therefore it seemed more appropriate to explore the free diphenol 4c as a potentially more soluble construct. The settled route to the analogous precursor 1c is shown in supporting information (SI Scheme S2). Thiol conjugation of 1c with peptide c(RGDfC) in THF/phosphate buffer afforded 3c, which was then “clicked” with 2 under standard Cu catalyzed conditions to give 4c (Scheme 3). It is immediately apparent that 3c and 4c have increased solubility in MeOH and CH₃CN relative to 3a/b and 4a/b. They have moderate water solubility (up to 0.1 mM for 4c). The increased solubility of 3c/4c makes them easier to handle and they do not require DMSO-d₆ for characterization.

The increased polarity and hydrophilicity of 4c is also reflected in reverse phase HPLC profiles. While 4a and 4b have almost the same retention time, 4c elutes over 1 min earlier than 4a and 4b under the conditions (Figure 1).

Simultaneously a variant of the Barbas linker was prepared without an aryl ring to explore solubility. Thus methylsulfonyl oxadiazole (1d) became a synthesis target (SI Scheme S3). A key reaction involved nucleophilic substitution of the methylsulfonate 1d with the thiol of (RGDFC) and this led to azido oxadiazole thiolate (AOT-RGD) 3d. ‘Click’ reaction of 3d with 2 gave conjugate CIDA-AOT-RGD (4d) (Scheme 4).

The solubility of 4d in water is significantly increased (2.3 mM) relative to 4c (0.1 mM). Compounds 4a and 4b, are essentially insoluble. Reverse phase HPLC retention times were used as a proxy measure of solubility/polarity and 4d and then 4c eluted most rapidly as illustrated Figure 1.

The corresponding fluorinated conjugates 5a/b/c/d were prepared from reactions of 3a/b/c/d and FDA-TEG 15 (SI Schemes S4 and S5).
**Scheme 4.** Preparation of water soluble fluorinase substrate CID-AOT-RGD. Reagents and conditions: a) c(RGDfC), PBS buffer (pH 7.4), 2 h; b) Compound 2, CuTBTA in 55% DMSO, sodium ascorbate, H₂O, 12 h; c) KF, L-SeMet, fluorinase, phosphate buffer (pH 7.8).

‘Cold’ enzymatic fluorination of 4a/b/c/d to 5a/b/c/d

Non-radiolabelled fluorinase reactions (last step in Scheme 3 and 4) were generally performed in phosphate buffer (pH 7.8, 20 to 50 mM) at 37°C with the appropriate 5′-chloro-5′-deoxygenosine substrate (0.1 mM), L-SeMet (0.1 mM), fluorinase (1 mg/mL) and potassium fluoride (75 mM). For 4a, DMSO 5% v/v was added for solubility, although DMSO is detrimental to enzyme activity and promotes gelling on heat denaturation of the enzyme on workup. The enzymatic conversion of 4a proved sluggish (SI Figure S1) and did not improve for 4b with the added PEG chain (SI Figure S2).

Constructs 4c (up to 0.1 mM) and 4d (up to 2.3 mM) had improved water solubility, and reactions were carried out in phosphate buffer (pH 7.8, 50 mM) without added DMSO. Workup of the neat aqueous reaction mixture was more straightforward than that for 4a and 4b which contained DMSO. The fluorinase enzyme was cleanly precipitated on heating at 95°C for 5 min and could be readily centrifuged (13000 rpm/5 min).

The biotransformations of 4c to 5c and 4d to 5d (Figure 2) are more efficient than that of 4a and 4b (SI Figure S1 and S2). Substrate 4d was processed most efficiently and at about twice the rate of substrates 4a/b/c. An additional HPLC peak (at 16.1 min in Figure 2A and 14.9 min in Figure 2B) was attributed to a decomposition product associated with the AdoSeMet intermediate of the transhalogenation reactions based on MS analysis (exampled by SI Figure S3).

**Enzymatic hot [¹⁸F]fluorination of 4a/b/c to 5a/b/c**

For hot labelling, the [¹⁸F]fluoride is generated in GBq and used in MBq aliquots for reactions. The actual [¹⁸F]fluoride concentration is very low in the picomolar range but the enzyme is in excess in the micromolar range and therefore the reactions are no longer catalytic.

**Figure 2.** HPLC traces monitoring fluorinase catalyzed conversions (%) of A; 4c (tR 15.7 min) to 5c (tR 14.9 min) via AdoSeMet intermediate (tR 16.1 min). B; 4d (tR 14.4 min) to 5d (tR 13.4 min) via AdoSeMet intermediate (tR 14.9 min). For full conditions see Experimental and SI Figure 1S and 2S.

This stoichiometry is dramatically reversed relative to the cold reactions which use a large molar excess of fluoride over enzyme. The very low [¹⁸F] concentration requires a
high substrate concentration, and the low solubility of 4a 0.15 mM and 0.48 mM respectively. The conversion of $^{18}$F$^-$ into 5a and 5b was only modest (SI Figure S4 and S5, based on the integration of the peaks of product and unreacted $^{18}$F$^-$ ion by HPLC). Substrate 4d, has good solubility but proved to be hydrolytically unstable (Figure 1, bottom HPLC trace shows a degraded product at 13.2 min) relative to 4a/b/c, and 5d visibly decomposed on work up, therefore radiochemical trials were not explored further with this substrate.

Substrate 4c has good solubility in water/DMSO and an efficient transhalogenation of 4c (reaction conc. ≤0.66 mM) to $[^{18}$F]$^5$c (final conc. of DMSO 2%, SI Figure S6) was accomplished (87% by HPLC)\(^5\). At 2% DMSO, the enzyme precipitated cleanly after heat denature without gel formation. The semi-prep HPLC purified [${}^{18}$F]5c was diluted with water and then secured on a C18 reverse phase cartridge. The product was finally eluted with EtOH (Figure 3, SI Figure S7) for bioaffinity experiment. The total procedure from [${}^{18}$F]fluoride (422 MBq) to EtOH elution of [${}^{18}$F]5c (22.5 MBq) took 1.5 h, and with a radiochemical yield of 5% (decay uncorrected).

![Figure 3](image.png)

**Figure 3.** Analytical HPLC radio trace of purified [${}^{18}$F]5c. For full conditions see experimental and SI Figure S7

**Bioactivity of FDA-AOT-RGD conjugate 5a/b/c**

Bioaffinity assays were conducted on cell lines expressing $\alpha$$\beta_3$ integrins. The inhibitory concentations (IC$_{50}$'s) for 5a-c, competing with a biotinylated peptide binding to immobilized $\alpha$$\beta_3$ integrins were explored. This is a proxy measure of binding affinity to $\alpha$$\beta_3$ integrins on the surface of cancer cells. The IC$_{50}$'s were compared with that of the cyclic peptide, cRGDfC (85.0 nM). The values, 5a (IC$_{50}$ 85.9 ± 50.2 nM), 5b (IC$_{50}$ 80.1 ± 14.2 nM) and 5c (IC$_{50}$ 177 ± 23 nM) proved to be similar, indicating that modification of RGD peptide with the fluoroadenosine/Barbas linker construct does not significantly affect its binding affinity.

Binding of [${}^{18}$F]5c to $\alpha$$\beta_3$ integrins in cancer cells was evaluated in U87MG and PC3 cells, which are known to express high and medium levels of receptor.\(^5\) Binding of [${}^{18}$F]5c to U87MG cells was higher than to PC3 cells. Cold c(RGDfK) (10 μM) was included in the binding assay to permit identification of specific radiotracer binding to $\alpha$$\beta_3$ and 4b in 5% DMSO limited their reaction concentrations to integrin (decreased by c(RGDfK)). Inclusion of cold peptide decreased [${}^{18}$F]5c binding to both U87MG and PC3 cells by approximately 75% and 50% respectively (Figure 4). These data are consistent with the literature\(^5\) and flow cytometry analysis which demonstrated that our U87MG cells contained 9 times more $\alpha$$\beta_3$ than PC3 (data not shown). This data provide strong evidence that [${}^{18}$F]5c is binding selectively to $\alpha$$\beta_3$ integrin in cancer cells.

![Figure 4](image.png)

**Figure 4.** Bound [${}^{18}$F]5c (cpm/mg) protein/MBq radiotracer

**Conclusions**

we have modified a cysteine-containing RGD peptide with a fluorinase-recognised chloroadenosine for fluorinase mediated radiolabelling with the fluorine-18 isotope. The recently introduced Barbas bioconjugation strategy was variously modified to improve solubility. The diphenol 4c, which derived from the sulfonl oxadiazole 1c, proved to be the most soluble construct and performed best for enzymatic mediated transhalogenation to generate radiolabelled [${}^{18}$F]5c. Sulfonl oxadiazole 1c may find more general utility due to its relatively high aqueous solubility.

**Experimental Section**

**Bioconjugation of Barbas linker with c(RGDFC)**

Compound 1a-d (1.5-4 eq.) in THF (400 ul) was added to a suspension of c(RGDFC) (1 eq) in PBS buffer (200 μL, 100 mM, pH 7.4). The mixture was stirred at rt for 0.5-2 h and then concentrated under reduced pressure to remove the organic solvent, leaving an aqueous suspension containing product and excess of starting material. The excess 1a-d was removed effectively by extraction with diethyl ether; the aqueous suspension was dissolved / diluted with H$_2$O/DMSO and purified by semi-preparative HPLC to give 3a-d.

‘Click’ reaction of 3a-d with 2 or 15 for 4a-d or 5a-d

CIDA-TEG 2 or FDA-TEG 15 (1.2-2.0 eq) in suitable amount of water was added to 3a-d (1 eq) in suitable amount of DMSO. Sodium ascorbate (fresh aqueous degassed solution 100 mM or solid, 2-5 eq) was added to the
mixture. The mixture was degassed with argon before CuTBTBA (10 mM in 55% DMSO, 0.2-0.5 eq) was added. The mixture was heated at 60°C for 10 min and stirred at RT overnight. After dilution with suitable amount of DMSO/H$_2$O, the mixture was centrifuged at 13000 rpm for 10 min to remove the Cu(I) precipitate. The supernatant was isolated by semi-preparative HPLC to give the product 4a-d or 5a-d after concentration and freeze drying.

**Fluorinase assay of 4a-c to 5a-c**

In a total reaction volume of 200 µL, recombinant fluorinase (1 mg/mL in phosphate buffer) was incubated with L-SeMet (0.1 mM), KF (75 mM) and 4a (0.1 mM) or 4b (0.1 mM) in 5% DMSO/phosphate buffer (pH 7.8, 50 mM) or 4c or 4d (0.1 mM) in phosphate buffer (pH 7.8, 50 mM) at 37 °C. Samples (20 µL) were periodically removed, denatured by heating at 95°C for 5 min, diluted with phosphate buffer (80 µL, 20 mM) before being clarified by centrifugation (13 000 rpm, 10 min). Samples of the supernatant (80 µL) were removed for analysis by HPLC. HPLC analysis (Figure 2 and SI Figure S2-3) was performed on a Shimadzu Prominence HPLC system fitted with a SIL-20A HT autosampler, LC-20 AT solvent delivery system, SPD-20 UV/vis detector using a Phenomenex Luna 5 µm, C-18 100A (250 × 4.6 mm) column and a guard cartridge. Mobile phase: A (H$_2$O + 0.05% TFA), B (MeCN + 0.05% TFA); Linear gradient: 0-4 min 0% B, 4-10 min 0-30%B, 10-25 min 30-33%B, 25-30 min 33-90%B, 30-35 min 0%B, 40 min stop. Flow rate: 1.0 mL/min.

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**Keywords:** $^{18}$F Labelling • RGD Peptide • Fluorinase • Barbas linker • Bioconjugation

**References**

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FULL PAPER

We reported a last-step $^{18}\text{F}$ labelling of chloroadenosine and oxadiazole modified cysteine-containing peptide (CIDA-AOT-RGD) by fluorinase under mild aqueous condition.

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Last-step enzymatic $^{18}\text{F}$-fluorination of cysteine-tethered RGD peptides using modified Barbas linkers