**Efficient Bioconjugation of 5-Fluoro-5-Deoxy-Ribose (FDR) to RGD Peptides for Positron Emission Tomography (PET) Imaging of v3 Integrin Receptor**

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**ABSTRACT:** The utility of 5-fluoro-5-deoxyribose (FDR) as an efficient bioconjugation agent for radiolabelling of the RGD peptides c(RGDfK) and c(RGDfC) is demonstrated. The bioconjugation is significantly superior to that achieved with 2-fluoro-2-deoxyglucose (FDG) and benefits from the location of the fluorine at C-5, and that ribose is a 5-membered ring sugar rather than a 6-membered ring, which both favour the smooth formation of an oxime bond with ligands displaying a hydroxylamine function. [18F]-FDR was prepared in this study by synthesis from fluoride-18 using an automated synthesis protocol adapting that used routinely for [18F]-FDG. c(RGDfK) was functionalised with an aminooxyacetyl group (Aoa) *via* its lysine terminus, while c(RGDfC) was functionalised with an aminooxyhexanylmaleimide (Ahm) through a cysteine-maleimide conjugation. Bioconjugation of [18F]-FDR to c(RGDfC)-Ahm proved to be more efficient than c(RGDfK)-Aoa (92% versus 65%). The unlabelled (19F) bioconjugates c(RGDfK)-Aoa-FDR and c(RGDfC)-Ahm-FDR were prepared and their *in vitro* affinity to purified integrin v3 was determined. c(RGDfK)-Aoa-FDR showed the greater affinity. Purified “hot” bioconjugates c(RGDfK)-Aoa-[18F]-FDR and c(RGDfC)-Ahm-[18F]-FDR were assayed by incubation with MCF7, LNCaP and PC3 cell lines. In both cases the conjugated RGD peptides showed selectivity for PC3 cells, which express v3 integrin, with the c(RGDfK)-Aoa-[18F]-FDR demonstrating better binding, consistent with its higher *in vitro* affinity. The study demonstrates that [18F]-FDR is an efficient bioconjugation ligand for RGD bioactive peptides.

Introduction

There is a significant research focus presently aimed at tagging bioactive peptides with the fluorine-18 (18F) isotope, to monitor *in vivo* distribution by positron emission tomography (PET).1 This imaging modality has the advantage of requiring low dose administration due to the sensitivity of detection. The 18F isotope has a relative long half-life (t1/2 = 110 min) in the context of appropriate PET active nuclides (11C = 20.4 min, 13N = 10 min, 15O = 2.1 min), but the relatively short half-life demands that the synthesis of [18F]-ligands is rapid. This restricts the diversity of candidate molecules for [18F]-labelling of candidate PET tracers.2 Direct radio-fluorination of peptides, proteins and other bio-macromolecules by 18F-C bond-forming reactions is generally difficult, whereas introduction of 18F can be more conveniently achieved by the use of 18F-labelled “prosthetic groups” which can be linked to the peptide/protein by “click”-chemistry, and other “click-type” reactions (oxime-bond formation, thiol-maleimide chemistry, etc.).3 2-[18F]Fluoro-2-deoxyglucose ([18F]-FDG) is the most commonly used radio tracer synthesised routinely in the clinical environment as it is an excellent diagnostic in oncology and other pathologies.4 [18F]-FDG can be used for 18F-fluoroglucosylation of hydroxylamine-functionalised peptides *via* oxime-bond formation.5 However, in practice [18F]-FDG is a rather poor prosthetic group for this purpose, because the position of the electronegative fluorine atom at C-2 inhibits ring opening of the sugar to its aldehydic form, necessary for oxime or imine formation. To circumvent this problem other strategies have emerged for tagging the [18F]-isotope to peptides. The most common strategy has involved the preparation of [18F]-fluorobenzoate derivatives6 or [18F]-fluorobenzaldehyde-derived oximes.7 These motifs have emerged as there are suitable synthetic methods for the rapid introduction of 18F into the *para* position of the aryl moiety. However these motifs suffer from higher hydrophobicity which can alter the overall physicochemical properties of the peptide. More recently ligands such as the NOTA-aluminium ligand sequester 18F directly avoiding covalent C-18F bond formation although clearly the NOTA ligand remains attached to the peptide.8 The challenge is to tag the protein without significantly modifying its bioactivity. We have recently introduced [18F]-FDR as a bioconjugation ligand, and explored ligation to linear peptides.9

Angiogenesis, the growth of new blood vessels from pre-existing vessels, is one of the hallmarks of growing tumours. The integrin protein αvβ3 (also known as the vitronectin receptor) plays an important role in regulating angiogenesis and tumour metastasis. Indeed, there is good correlation between αvβ3 expression on tumour endothelial cells and the extent of angiogenesis.10 The Arg-Gly-Asp (RGD) tripeptide motif binds to the αvβ3 receptor, therefore the RGD sequence has been extensively used as a targeting function for αvβ3 integrin over-expressing tumours.11 Several RGD peptides have been identified that bind to αvβ3 with high affinity and specificity, including c(RGDfK) and c(RGDfC).12 RGD-peptides radio-labelled with 68Ga,13 64Cu,14 and 18F nuclides have been investigated as PET tracers in pre-clinical and clinical studies, mainly for oncological, bone and cardiovascular pathologies.15 Several strategies have been pursued for the incorporation of 18F into RGD peptides. The most important examples of [18F]-RGD PET tracers include; (1) [18F]-galacto-RGD, which is a c(RGDfK) glycopeptide bearing a [18F]-2-fluoropropionate tag;16 (2) [18F]-Fluciclatide (or 18F-AH111585) which is a rigid bicyclic RGD oligopeptide incorporating a PEG linker radiolabelled with a *para*-[18F]-benzaldehyde-derived oxime;17 (3) cyclic and linear RGD peptides bearing an oxime-bound [18F]-FDG tag;18 (4) cyclic RGD peptides displaying [18F]-silylated prosthetic groups. (5) 18F-(RGD)2 peptides which are a class of dimeric cyclic RGD peptide tracers generally labelled with *para*-[18F]-benzoate tags;19 (6) [18F]-RGD-K5 and related RGD tracers bearing a “click-type” [18F]-fluoroalkyl-triazole tag.20 Interestingly, a direct comparison between “click”-radiofluorination strategy and oxime- or amide-bond forming 18F-prosthetic groups was performed, showing that oxime-bond formation is at least as convenient as “click”-chemistry for the radiofluorination of RGD peptides.21

The oxime-bioconjugation of [18F]-FDR to two cyclic RGD peptides is reported in this paper. An Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to investigate whether FDR conjugation to these RGD peptides affected their ability to bind to immobilised αvβ3 protein. The ability of [18F]-FDR labelled RGD peptides to specifically bind its target was assessed in cells which express different levels of αvβ3 protein, in the presence and absence of an excess of non-radiolabelled RGD peptide.

**Results and discussion**

*Synthesis of RGD-FDG conjugates.*

Two cyclic RGD peptides, c(RGDfC) and c(RGDfK) were chosen for αvβ3 integrin recognition. In order to conjugate these peptides to FDR, they were each functionalised with an aminooxy functionality *via* a suitable linkage.9 For c(RGDfC) a conventional Michael addition of the cysteine residue to an aminooxyhexylmaleimide (Ahm)22 was employed (Scheme 1). The c(RGDfK) prepeptide was prepared by solid phase synthesis and functionalised by acylation of its lysine residue with Boc-protected aminooxyacetic acid (Aoa) before removal of protecting groups and cleavage from the resin under acid conditions (Scheme 2).23 Conjugation of c(RGDfC) and c(RGDfK) with FDR was then carried out in NaOAc or NH4OAc buffer (100-250 mM, pH 4.6), using the previously described optimised conditions (Scheme 1 and 2).9

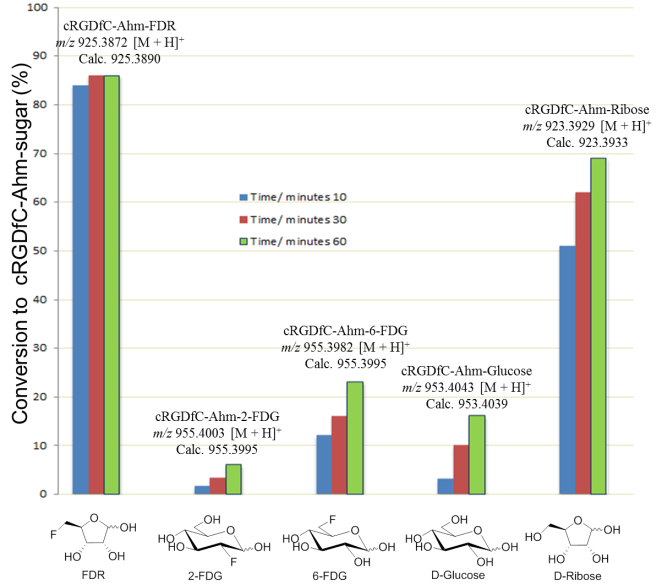


**Scheme 1**. Functionalisation of cRGDfC *via* aminooxy conjugation with various sugars: (i) H2O, RT 20 min; (ii) NH4OAc or NaOAc buffer (250 mM, pH 4.6).



**Scheme 2**. Preparation of c(RGDfK)-Aoa and conjugation with FDR: **(i)** Boc-amino-oxy-acetic acid (2eq), HATU (2eq), sym-collidine (4eq), rt, 2 h; **(ii)** TFA-CH2Cl2 (1:1), rt, 2h; **(iii)** FDR (1.3 eq) in sodium acetate buffer (0.5 M, pH 4.6), rt, 10min.

A series of experiments was carried out with c(RGDfC), comparing the conjugation ability of several sugars, and in particular comparing 5- and 6- membered ring sugars. Sugars exist in solution as an equilibrium mixture of both their cyclic and acyclic aldehydic forms. The less favoured aldehydic form is required for reaction with the aminooxy group to form the conjugated oxime. It is emerging as a feature of FDR that it conjugates rapidly, and in this study conjugations were explored with c(RGDfC)-Ahm in both NH4OAc and NaOAc (250 mM) solutions at room temperature. Figure 1 illustrates the relative conversions of oxime product formation of c(RGDfC)-Ahm with FDR, D-ribose, 2-FDG, 6-FDG and D-Glucose. All sugars were incubated in 1 : 1 molar ratio with c(RGDfC)-Ahm and each at a final concentration of 22mM. NH4OAc solution was used as it offers a suitable buffer solution for direct MS analysis. FDR was the most efficient sugar explored with conjugation conversions of up to 84% in 10 min. This is followed by D-ribose (51% in 10 min), a comparison which illustrates the influence of the fluorine in place of the OH at C-5. All of the pyranoses (6-membered sugars) reacted considerably slower than the ribose sugars, in the order 6-FDG > D-Glucose > 2-FDG consistent with the thermodynamically more stable 6-membered rings.9 A positive fluorine effect is observed comparing 6-FDG to D-glucose, while the fluorine in 2-FDG will disfavour oxocarbenium ion formation and inhibits ring opening to the aldehydic form. Conjugation with 2-FDG is very sluggish under the conditions generating negligible conjugation product (ca. 2-5%) over a 1h reaction. The reactions of FDR benefits from an increased temperature (40°C, 1:1 molar ratio, 90% conversion, 10 min) and an excess of FDR (1.5 equiv., 25°C), resulted in a conversion up to 93% in 10 min. It is clear from the histogram (Figure 1) that FDR is the most efficient bioconjugation reagent of those explored.



**Figure 1** Comparison of the reaction conversions (HPLC) of cRGDfC-Ahm with various sugars (1 : 1 molar ratio) in NH4OAc buffer (200 mM, pH 4.6) at 25°C.

Isotopically labelled (18F) conjugations of the RGD peptides were carried out with an excess of both c(RGDfC)-Ahm and c(RGDfK)-Aoa (ca. 2 mg) using [18F]-FDR (15-75 MBq) at pH 4.6 (NaAc buffer) at room temperature. C(RGDfC)-Ahm (92% conversion, n=2) reacted more rapidly than c(RGDfK)-Aoa (65% conversion, n=2) at 15 min under the same conditions. This is consistent with the more nucleophilic aminooxy group of c(RGDfC)-Ahm relative to c(RGDfK)-Aoa, whose aminooxy group bears an electron-withdrawing carbonyl in -position. The reaction mixtures were purified by semi-prep RP-HPLC and the radioactive fractions were collected by eluting the radioactivity with a 1:1 water/ethanol solution (1.5mL) from an Oasis HLB cartridge. The solution was reanalysed by analytical radio-HPLC, which demonstrated that each product was radiochemically pure as illustrated in Figure 2. The conjugation and purification of the [18F]-FDR-RGD adducts took ~50 min.



**Figure 2**. Analytical radio-RP-HPLC traces of c(RDGfK)-Aoa-[18F]FDR (blue trace) and c(RDGfC)-Ahm-[18F]-FDR (red trace) products.

*αvβ3 Integrin affinity evaluation.*

The cyclic RGD peptide conjugates were submitted to assays to measure their affinity for the αvβ3 receptor. Competitive affinities were calculated analyzing the interactions between immobilized αvβ3 and the commercially available c[RGDfK(Biotin-PEG-PEG)] peptide in the presence of the RGD conjugates. The linear heptapeptide GRGDSPK (Sigma Chem Co. Ltd)) was used as a reference competitive peptide and IC50 values for each compound were normalised with respect to this peptide IC50 to allow comparisons between different experiments. Competitive inhibition data in Table 1 show that all of the conjugated c(RGD) peptides have affinities for αvβ3 in the nanomolar range. The highest affinity (68 nM) was displayed by c(RGDfK)-Aoa-FDR, slightly higher than that of the non-conjugated peptide c(RGDfK). Overall, the αvβ3 binding affinity data in Table 1 indicate that FDR is an efficient prosthetic group that does not affect the αvβ3 targeting capacity of RGD peptides. The relatively low affinity of c(RGDfC)-Ahm-FDR compared to c(RGDfK)-Aoa-FDR might be the result of increased hydrophobicity introduced by the relatively longer aliphatic linkage.

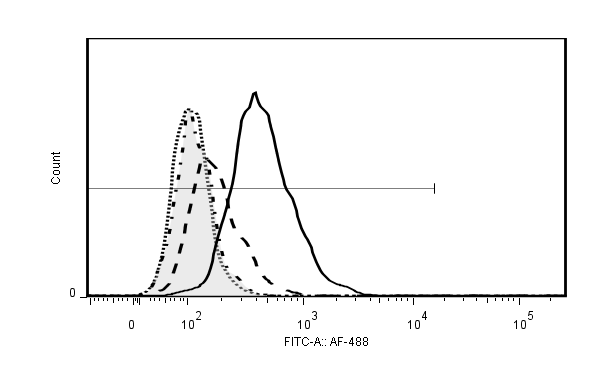
|  |  |  |
| --- | --- | --- |
| **Compound** | **IC50 (M)** | **Q** |
| Ref peptide (GRGDSPK) | 2.27 | 1 |
| C(RGDfC) | 0.085 | 0.037 |
| C(RGDfC)-Ahm | 0.222 | 0.098 |
| C(RGDfC)-Ahm-FDR | 0.403 | 0.178 |
| C(RGDfK) | 0.138 | 0.061 |
| C(RGDfK)-Aoa-FDR | 0.068 | 0.030 |

**Table 1:** Inhibition of cyclo[RGDfK(Biotin-PEG-PEG)] binding to immobilised αvβ3. Data are the average of two independent experiments performed in triplicate. Q = normalized activities as ratio IC50(peptide)/IC50(GRGDSPK).

*Cell incubation experiments*

PC3, LNCaP and MCF7 cells were chosen for the binding experiments as they are known to express high, low and no αvβ3 protein, respectively.24 Flow cytometry analysis (Figure 3) confirmed that our cell line clones expressed αvβ3 levels consistent with published findings.

Binding of [18F]-labelled RGD peptides to αvβ3 was assessed for each of these cell lines. Binding of c(RGDfK)-Aoa-[18F]-FDR and c(RGDfC)-Ahm-[18F]-FDR was higher in PC3 cells compared to the other cells (Figure 4), consistent with the αvβ3 expression levels (Figure 3). Inclusion of cold c(RGDfK) (10 M) to competitively block specific binding to αvβ3 decreased [18F]-RGD binding to PC3 cells by approximately 60-70%, but had no effect in the other two cell lines. These findings indicate that the majority of bound radioactivity in PC3 cells can be attributed to specific binding to αvβ3, whereas the low level binding to LNCaP and MCF7 is non-specific. Interestingly, in PC3 cells c(RGDfK)-Aoa-[18F]-FDR exhibited a higher level of binding compared to c(RGDfC)-Ahm-[18F]-FDR. This increase is consistent with the higher *in vitro* affinity (Table 1), and suggests that c(RGDfK)-Aoa-[18F]-FDR is the better prospect for imaging angiogenesis. It is concluded that these [18F]-RGD conjugates bind to αvβ3 in cells against a background of some non-specific binding.



**Figure 3.** *Characterisation of αvβ3 expression on MCF7, LNCaP and MCF7 cells.* PC3 (solid line), LNCaP (dashed line) and MCF7 (alternating dotted/dashed line) cells were incubated with an anti-αvβ3 receptor antibody and an Alexa-fluor488 secondary antibody. Cells were also incubated in the presence of secondary antibody only (dotted line). The relative level of αvβ3 expression was determined by flow cytometry analysis as described in the General Experimental section. For each treatment the number of cells (counts) was plotted against fluorescence intensity at A488. Result is representative of duplicate samples.



**Figure 4.** *Differential binding of 18F-labelled FDR-conjugated RGD peptides to cultured cells that express different αvβ3 levels****.*** MCF7, LNCaP and PC3 cells were incubated with 18F-labelled c(RGDfK)-Aoa-FDR (Fig. 4a) or 18F-c(RGDfC)-Ahm-FDR (Fig. 4b) in the presence (+) or absence (-) of 10 uM c(RGDfK) peptide for 1h, as described in methods and materials. 18F-RGD binding is expressed as counts per minutes (cpm), as a function of the radioactivity added/plate and normalised for protein content. Each datapoint is the average ± standard deviation of at least two independent data points.

**Conclusions**

[18F]-FDR proved to be the most efficient bioconjugation agent of several monosaccharides explored for conjugation to the RGD peptides. The combination of the electronegative fluorine located at the 5-position and 5 rather than a 6- membered ring monosaccharide, promotes ring opening to the reactive aldehyde form. Thus efficient conjugations occur in a few minutes at ambient temperature and at pH 4.6 to generate oximes. The conjugates c(RGDfK)-Aoa-FDR and c(RGDfC)-Ahm-FDR were assessed for their affinity to purified integrin αvβ3. In the event c(RGDfK)-Aoa-FDR showed the greater affinity. In each case c(RGDfK)-Aoa-[18F]-FDR and c(RGDfC)-Ahm-[18F]-FDR were prepared by conjugation with [18F]-FDR.9

Cellular uptake of c(RGDfK)-Aoa-[18F]FDR and c(RGDfC)-Ahm-[18F]FDR was assessed in MCF7, LNCaP and PC3 cell lines. In both cases the conjugated RGD peptides showed selectivity for PC3 cells, which highly express αvβ3 integrin. The c(RGDfC)-Aoa-[18F]-FDR gave the better uptake. In conclusion, the study demonstrates that [18F]FDR is an easily accessible and efficient conjugation ligand for RGD peptides. Its role could clearly be extended to PET radiolabelling studies with other bespoke bioactive peptides.

**Experimental**

**General**

All reagents and solvents were of highest grade from commercial sources, unless otherwise specified. Peptide c(RGDfC) was purchased from Peptides International, USA and ChinaPeptides. FDR and *N*-(6-aminoxyhexyl)maleimide hydrochloride (Ahm) were prepared as previously described.9 6-Deoxy-6-fluoro-glucopyranose (6-FDG) was prepared by hydrolysis of the fully acetylated precursor. Normal phase column chromatography was performed using silica gel 60 (40–63 µm). NMR spectra were recorded on Bruker Advance 300, 400 or 500 instruments. 1H and 13C NMR spectra were recorded using deuterated solvent as the lock and residual solvent as the internal standard. 19F NMR spectra were referenced to CFCl3 as the external standard. Chemical shifts are reported in parts per million (ppm) and coupling constants (*J*) are given in Hertz (Hz).The abbreviations for the multiplicity of the proton, carbon and fluorine signals are as follows: s singlet, d doublet, dd doublet of doublets, ddd doublet of doublet of doublets, triplet, dt double triplets, q quartet, m multiplet, br s broad singlet. When necessary, resonances were assigned using two-dimensional experiments (COSY, TOCSY, HMBC and HSQC). Mass analyses were recorded on a Micromass LCT TOF mass spectrometer using ESI in positive mode. HPLC analyses/semi-preparations were performed using a Prostar 325 or Shimadzu prominence or Agilent 1200 HPLC system with reverse phase column as indicated in individual experiment. HPLC analyses of radioactive compounds where performed using a Shimadzu Co. (Kyoto, Japan) HPLC system equipped with a SPD-M20A Prominence DAD UV detector (Shimadzu, Japan) and NaI radio-detector (Berthold Technologies, Bad Wildbad, Germany) using a Phenomenex Luna C-18(2) analytical column (250 x 4.6 mm, 5 μm, 100 A) equipped with the corresponding guard column, 0.1% TFA in ACN (solvent A) and 0.1% TFA in water (solvent B), flow rate of 1 mL/min and the following linear gradient: 100% solvent B for 4 min, then from 0% to 60% of solvent A in 10 min and finally changing to 95% solvent A to flush the column. Semi-prep HPLC purification of radioactive compounds were performed using a Gynkotek HPLC system consisting of a gradient pump (P580), column oven (STHS8S) and variable UV detector (UVD340S) coupled in series with a BIOSCAN NaI detector (B-FC-3200) and equipped with a Phenomenex Luna C-18(2) semi-prep column (250 x 100 mm, 5 μm, 100 A). The dose calibrators used to measure doses were CAPINTEC CRC 15R and CAPINTEC CRC 15PET.

*Binding affinity tests.* Binding affinity of cold bioconjugates to purified αvβ3 integrin was determined by ELISA as described previously.25

*Cell experiments*

MCF7, LNCaP and PC3 cells were purchased from ATCC and confirmed as authentic and contamination-free. Cell characterisation for αvβ3 expression on MCF7, LNCaP and PC3 was determined by flow cytometry analysis essentially as previously described.24 An anti-αvβ3 primary antibody (LM609, Millipore) was used at 1:100 dilution and detected with an Alexa-fluor 488 labelled goat anti-mouse secondary antibody (ThermoFisher scientific) at 1:500 dilution. Stained cells were detected on a FACS calibur flow cytometer (Becton Dickinson). Alexa-fluor secondary antibody was incubated with cells in the absence of primary antibody to determine background cell fluorescence.

For radiotracer binding experiments, cells were seeded at 0.5 x 105 cells in 60mm plates and cultured for 2-3 days to approximately 90% confluency. Medium was aspirated and replace with 4ml of fresh RPMI supplemented with 1mM MnCl2. [18F]-labelled RGD peptides (approximately 0.5MBq) was added/plate and incubated at 37ºC for 1h in the presence or absence of 10µM cold cRGDfK peptide. Cells were washed 5 times with ice-cold PBS, to remove unincorporated radioactivity, then detached from plates with 0.5ml Trypsin/EDTA, prior to addition of 0.5mlmedium and transfer into microfuge tubes. [18F]RGD binding to cells was measured using a well counter with a Nuclear Instruments (Oakland UK) interface until at least 1000 counts were accumulated. Cells were then pelleted by centrifugation (200g for 5min), and protein content measured using the BCA protein assay. [18F]RGD binding in each plate was expressed as a function of the radioactivity added/plate and normalised for protein content.

*Cold bioconjugation of c(RGDfC)-Ahm with various sugars*

A fresh solution of Ahm (179 µL, 44 mM, 7.88 µmol) indegassed waterwas added to an Eppendorf tube containing c(RGDfC) (5.0 mg, 7.84 µmol). The mixture was mixed well with the pipette tip and incubated at 25°C for 20 min. HPLC monitoring indicated that the reaction reached to completion. An aliquot of the resultant solution of c(RGDfC)-Ahm (20 µL, 44 mM, 0.88 µmol) was then mixed with a sugar (FDR, 2-FDG, 6-FDG, D-glucose or *D*-ribose) (20 µL, 44 mM, 0.88 µmol) in NH4OAc buffer (pH = 4.6, 500 mM). The mixture was incubated at 25°C and an aliquot of the sample (2 µL) was taken at the interval of 10 min, 30 min and 60 min respectively and diluted with water (78 µL) for HPLC analysis. Conversion rate to c(RGDfC)-Ahm-sugar was calculated based on the integration of the peak areas of the starting c(RGDfC)-Ahm and the product c(RGDfC)-Ahm-sugar. For the more sluggish reactions at room temperature, the temperature was raised up to 100°C and again the mixture was monitored by HPLC. HPLC was performed on a Prostar 325 HPLC system with HiChrom C18column (250 × 4.6 mm, 10µ). Mobile phase consisted of 5% acetonitile water with 0.1% formic acid (A) and 95% acetonitrile with 0.1% formic acid (B). The program ran a linear gradient from 0% B to 60% B in 20 min followed by returning to initial to equilibrate the column. Peaks were detected at 220 nm with a flow rate of 1.0 mL/min.

*Preparative scale synthesis of c(RGDfC)-Ahm*

A fresh solution of Ahm (783 µL, 40 mM, 31.3 µmol) indegassed waterwas added to a screw cap glass vial containing c(RGDfC) (20.0 mg, 31.3 µmol). The mixture was incubated at 25°C for 30 min and then diluted with water. The resultant c(RGDfC)-Ahm was purified by reverse phase semi-prep HPLC using a Phenomenex Kingsorb C18 column (250 × 10 mm, 5 µ). Mobile phase consisted of A (H2O + 0.1% FA or 0.05% TFA) and B (Acetonitrile + 0.1% FA or 0.05 TFA). The program ran a linear gradient from 10% B to 40% B in 20 min, 40% B to 90% B in 30 min and returning to initial condition at 3.5 mL/min. The fraction (tR 16.8-17.2 min) was concentrated under reduced pressure and lyophilized to give the product as an amorphous solid (80-85%).1H NMR (D2O, 400MHz) δ 7.36-7.24 (m, 5H), 4.69 (t, 1H, *J=* 7.2 Hz), 4.65 (dd, 0.5 H, *J* = 6.3, 9.9 Hz), 4.56 (dd, 0.5 H, *J* = 6.0, 10.0 Hz, 9.9 Hz), 4.45 (dd, 0.5 H, *J* = 7.6, 4.7 Hz), 4.42 (dd, 0.5 H, *J* 8.7, 6.0 Hz), 4.36 (dd, 0.5 H, *J* = 8.7, 6.0 Hz), 4.30 (dd, 0.5 H, *J* = 9.5, 4.7 Hz), 4.22 (dd, 1H, *J* = 14.8, 2.8 Hz), 3.94 (t, 1H, *J* = 6.6 Hz,), 3.92 (t, 1H, *J* = 6.6 Hz), 3.80 (dd, 0.5 H, *J* = 8.9, 3.6 Hz), 3.57 (dd, 0.5 H, *J =* 8.9, 3.9 Hz), 3.52 -3.49 (m, 2H), 3.49 (dd, 1H, *J* = 14.8, 6.8 Hz), 3.26-2.90 (m, 4.5 H), 2.91-2.86 (m, 1H), 2.70 (dd, 1H), 2.57 (dd, 1H), 2.51 (dd, 0.5 H, *J =* 19.0, 4.2 Hz), 2.44 (dd, 0.5 H, *J =* 19.0, 4.2 Hz), 1.93-1.85 (m, 1H), 1.74-1.67 (m, 1H), 1.66-1.60 (quintet, 2H, *J* = 7.0 Hz), 1.59-1.50 (m, 4H), 1.38 (quintet, 2H, *J* = 7.0 Hz), 1.30 (m, 2H). 13C NMR (D2O, 125 MHz, the number of signals are not the same as the number of carbons due to the overlaps and splits between the two diastereomers) δ 179.2, 178.1, 177.0, 173.2, 172.6, 172.5, 172.3, 171.8, 171.2, 156.4, 136.4, 129.3, 129.2, 128.8, 128.7, 127.1, 75.6, 55.7, 55.2, 54.7, 54.3, 52.7, 52.6, 50.6, 43.6, 40.7, 40.5, 39.5, 39.8, 37.2, 36.4, 36.2, 35.6, 26.9, 26.5, 25.6, 24.4; *m/z* (ESI+) 791.3512 [M+H]+, C34H51N10O10S requires 791.3510.

*Cold preparative scale bioconjugation of c(RGDfC)-Ahm with FDR*

FDR (1.8 mg, 11.8 µmol) in NH4OAc buffer (295 µL, 250 mM, pH 4.6) was added to a screw cap glass vial containing c(RGDfC)-Ahm (5 mg, 5.9 µmol)*.* The mixture was incubated at 25°C for 30 min and then purified by reverse phase semi-prep HPLC using a Phenomenex Kingsorb C18 column (250 × 10 mm, 5 µ) with an isocratic condition of 25% of acetonitrile in water with 0.1% formic acid at 3.5 mL/min. The fraction (tR 14.5-15.5 min) was concentrated under reduced pressure and lyophilized to give the product as an amorphous solid (4.9 mg, 86%, E:Z = 84:16). Minor resonances are denoted by an asterisk. 1H NMR (D2O, 500MHz) δ 7.55 (d, 0.8 H, *J* = 6.7 Hz), 7.37-7.25 (m, 5H, PhH), 6.89\* (d, 0.2 H, *J* = 6.0 Hz), 5.05\* (dd, 0.2 H, *J* = 6.0, 3.2 Hz), 4.70-4.63 (m, 2.5 H), 4.58-4.53 (m 1.5 H), 4.48- 4.42 (m, 1.8 H), 4.39 (dd, 0.5 H, *J* = 7.8, 6.0 Hz), 4.30 (dd, 0.5 H, *J* = 10.0, 4.8 Hz), 4.22 (dd, 1H, *J* = 14.3, 3.2 Hz), 4.09 (t, 1H, *J* = 6.3 Hz), 3.92-3.77 (m, 3 H), 3.52 (t, 2H, *J =* 6.6 Hz), 3.48 (dd, 1H, *J* = 14.7, 2.8 Hz), 3.26-3.08 (m, 5 H), 3.03 and 3.01 (2 × d, 1 H, *J* = 12.0 Hz), 2.90-2.84 (m, 1 H), 2.53 (dd, 1H, *J* = 16.0, 6.9 Hz), 2.57 (dd, 1H, *J* = 16.0, 6.9 Hz), 2.51-2.41 (m, 1 H), 1.91-1.86 (m, 1H), 1.75-1.68 (m, 1H), 1.68-1.62 (m, 2H), 1.62-1.50 (m, 4H), 1.42-1.35 (m, 4H), 1.35-1.27 (m, 2H). 13C NMR (D2O, 125 MHz, number of signals are not the same as the number of carbons due to the overlaps and splits between the two diastereoisomers) 179.7, 179.0, 178.1, 177.9, 178.0, 173.3, 172.8, 172.6, 172.4, 171.7, 171.3, 156.6, \*151.5 (*E*), 150.4 (*Z*), 135.8, 128.8, 128.7, 127.0, 84.4 (d, 1*J*CF = 161 Hz), 73.9, 71.9, 69.9 (d, 2*J*CF 14 Hz), 69.3, 55.7, 43.8, 43.6, 40.4, 38.9, 37.9, 36.8, 35.8, 35.5, 33.1, 33.9, 28.0, 27.0, 25.7, 24.5, 24.3. 19F{1H} NMR (470 MHz, D2O), -234.6, \*-235.3; *m/z* (ESI+) 925.3872 [M+H]+, C39H58N10O13FS requires 925.3890.

*Preparation of c(RGDfK)-Aoa*

The synthesis of c(RGDfK)-Aoa was accomplished following the procedure reported by McCusker *et al*.23 starting from Fmoc-Asp(NovaSyn® TGA)-OAll resin (1 g, 0.2 mmol/g loading) (Scheme 2). After removal of the (4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) (Dde) protecting group by hydrazine monohydrate-DMF (2:98) for 3 min, the lysine amino function was coupled with (Boc-aminooxy)acetic acid, using HATU (2 eq) and sym-Collidine (4 eq), RT for 2 h. A solution of TFA-CH2Cl2 (1:1) was added to allow the cleavage of the peptide from the resin and the removal of pentamethyl-dihydrobenzofuran-5-sulfonyl, (Pbf) and tert-Butyloxycarbonyl (Boc) protecting groups, at RT for 2 h. The solution was concentrated and precipitated with Et2O. The precipitate was purified by a Phenomenex Luna C18, (250 × 10.00 mm, 5µ); Mobile Phase: A (H2O, TFA 0.1%) B (MeCN); gradient: from 15%B to 30%B in 15 min; flow: 5 mL/min; tR: 6.7min) and lyophilised to give 30 mg of a white, fluffy solid (22% yield based on initial loading resin). *m/z* (ESI)+: 677.2 [M+H]+, C29H45N10O9 requires 677.3.

*Cold bioconjugation of c(RGDfK)-Aoa with FDR*

To a solution of c(RGDfK)-Aoa(3.5 mg, 5 µmol) in 100 µl of water was added FDR (1 mg, 6.5 µmol) in sodium acetate buffer (110 µl, 0.5 M, pH 4.6) (Figure 2). The mixture was allowed to react at 25ºC for 10 min, then was diluted with water (1 mL) and purified by a Phenomenex Luna C18 (250 × 10.00 mm, 5µ); Mobile Phase: A (H2O + 0.1% TFA), B (MeCN); gradient: from 15%B to 30%B in 15 min; flow: 5 mL/min; tR: 9.2 min). The purified peptide was lyophilized affording 4 mg of a white solid (99% yield) containing a mixture of E/Z isomers (4:1). Minor resonances are denoted by an asterisk. 1H NMR (400 MHz, D2O) δ 7.63 (d, 0.8H，*J* = 6.5 Hz) 7.32-7.17 (m, 5H), 6.96\* (d, 0.2H， *J* = 6.2 Hz), 5.06\* (dd, 0.2H，*J* = 6.1, 3.5 Hz), 4.63-4.60 (m, 1H), 4.57-4.49 (m, 1H), 4.43 (dd, 0.8H， *J* = 6.5, 3.8 Hz), 4.28 (dd, 1H， *J* = 8.9, 5.6 Hz), 4.13 (d, 1H， *J* = 15.0 Hz), 3.89-3.73 (m, 3H), 3.42 (d, 1H， *J* = 15.0 Hz), 3.17-3.05 (m, 4H), 2.99 (dd, 1H， *J* = 13.2, 6.2 Hz), 2.92-2.79 (m, 2H), 2.64 (dd, 1H， *J* = 16.7, 6.5 Hz), 1.83-1.74 (m, 1H), 1.62-1.52 (m, 2H), 1.48-1.38 (m, 3H), 1.34-1.27 (m, 2H), 0.92-0.84 (m, 2H), 13C NMR (101 MHz, D2O) δ 174.6, 174.6, 173.1, 172.7, 171.8, 171.4, 171.3, 156.7, 153.7\* , 152.8, 136.0, 129.1, 128.8, 127.3, 84.6 (d, *J* = 165.4 Hz), 72.1 (d, *J* = 6.8 Hz), 71.9, 70.0, 69.8\*, 69.3, 65.9\*, 55.4, 55.1, 52.4, 49.7, 43.5, 40.5, 38.6, 37.0, 34.5, 29.9, 27.5, 27.3, 24.4, 22.5, 19F{1H} NMR (376 MHz, D2O) δ -233.99, -234.18\*. *m/z* (ESI)+: 811.4 [M+H]+, C34H52FN10O12 requires 811.3.

*Hot bioconjugation of c(RGDfC)-Ahm with [18F]-FDR*

18F-FDR was produced at the John Mallard Scottish PET centre according to the procedure previously reported,9 in a final concentration of 300 MBq/mL solution and radiochemical purity greater than 98%. To 2 mg of *c(RGDfC)-Ahm* peptide dissolved in 20 µL of sodium acetate buffer (1 M, pH 4.6) in an eppendorf vial were added 200 µL of [18F]FDR (40-75 MBq). The mixture is allowed to react at 25 °C for 15 minutes. Then the buffered solution was injected in the semi-prep HPLC system and purified using a isocratic method (15% ACN in water with 0.1% TFA, 4.6 mL/min). Decay corrected radiochemical yield, calculated considering the activity added to the peptide, was 80% (55% non-decay corrected). The collected fraction was diluted with 10 mL of water and loaded into a Waters Oasis® HLB Cartridge (conditioning 2 mL EtOH, 5 mL water). The cartridge was washed with 20 mL of water. The desired product was collected eluting with 1.5 mL of a solution 1:1 ethanol/water. This not optimised procedure allowed the recovery of 75% of the loaded activity. Radio HPLC analysis of the formulated product showed >98% radiochemical and chemical purity. Identity of the product was confirmed by comparison with the cold reference. It was not possible determine the specific activity since no UV signal of the purified product was detectable from the HPLC system.

*Hot bioconjugation of c(RGDfK)-Aoa with [18F]-FDR*

To 2 mg of *c(RGDfK)-Aoa* peptide dissolved in 20 µL of sodium acetate buffer (1 M, pH 4.6) in a eppendorf vial were added 200 µL of [18F]FDR (15-47 MBq). The mixture is allowed to react at 25 °C for 15 minutes. Then the buffered solution was injected in the semi-prep HPLC system and purified using a linear gradient from 15% ACN in water + 0.1% TFA to 20% ACN in water + 0.1% TFA in 15 minutes. Decay corrected radiochemical yield, calculated considering the activity added to the peptide, was 60% (46% non-decay corrected). The collected fraction was diluted with 10 mL of water and loaded into a Waters Oasis® HLB Cartridge (conditioning 2 mL EtOH, 5 mL water). The cartridge was washed with 20 mL of water. The desired product was collected eluting with 1.5 mL of a solution 1:1 ethanol/water. This not optimised procedure allowed the recovery of 80% of the loaded activity. Radio HPLC analysis of the formulated product showed >98% radiochemical and chemical purity. It was not possible determine the specific activity since no UV signal of the purified product was detectable from the HPLC system.

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