One-pot production of 18F- biotin by conjugation with 18F-FDG for pre-targeted imaging: Synthesis and radio-labelling of a PEGylated precursor

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Running title: One pot 18FDG labelling of biotin

**Abstract**

The biotin-avidin affinity system is exploited in pre-targeted imaging using avidin-conjugated antibodies.18F-FDG is available at PET centres. 18F-FDG forms oximes by reaction with oxyamine. Herein we describe the synthesis of oxyamine - funtionalised biotin, its 18F-labelling by conjugation with 18F-FDG and confirm its ability to interact with avidin.

1. **Introduction**

PET (positron emission tomography), which uses trace amounts of agents labelled with positron-emitting nuclides such as 18F or 11C to image and probe tissue metabolism, is the most sensitive technique for in-vivo imaging.

Tumour cells overexpress receptors and other cell-surface molecules compared with normal tissue and these can be imaged using radiolabelled peptides and proteins such as whole and fragment antibodies. 18F is the most ideal PET nuclide for most imaging purposes due to its moderate t1/2 of 110min. It is also a pure positron emitter so minimising scatter and gamma (non-true) coincidences. Furthermore, the distance between emission and annihilation, a further source of image degradation, is relatively short due to the low *β*+ energy (0.64 MeV) of 18F.

The imaging of cell surface molecules in-vivo can be carried out using directly labelled tracers or by using a two (or sometimes three) step procedure in which the target molecule is pretargeted with a conjugate consisting of the targeting moiety fused with a member of an affinity pair such as biotin/avidin. Biotin and avidin have a very high mutual affinity so after administration of the complementary member conjugated to a radionuclide (after a period of time to allow blood clearance of the antibody-conjugate), it will seek out and become associated with the pretargeted antibody. Compared with directly labelled tracers, the use of pretargeted imaging has been shown to produce cleaner images due to lower background activity**1**.

Radio-labeling with 18F generally requires the initial preparation of a 18F-labelled group (e.g. *p*-[18F]-fluorobenzoic acid (FBA)), involving a multi-step procedure in which the 18F- is introduced into the prosthetic component early**2**. The preparation of these prosthetic groups is generally low yielding (**3-5** refs therein), time-consuming and requires expensive specialist equipment. Also lipophilicity of the molecule and consequent bilary excretion is increased by conjugation with FBA. On the other hand, glycosylation of tracers makes them more hydrophilic so increasing renal excretion.

A number of recent studies have detailed the use of [18F]-FDG as a prosthetic group for radio-labelling specifically of peptides either after modification**6** e.g. to the thiol group-reactive prosthetic group [18F]-FDGmaleimidehexyloxime for reaction with thiol-containing peptides or directly**7-9** as the open chain aldehyde form, which reacts with an oxy-amine group on functionalised peptides to form an oxime.

To facilitate the application of 18F-FDG labelling to a broader spectrum of molecules, including antibodies and their fragments, we have investigated the labelling of biotin with 18F-FDG by functionalising biotin with an oxy-amine moiety *via* a PEG linker.

Labelling biotin with 18F-FDG rather than with 18F-fluoride has the advantages that it is universally available at all PET centres and so there is no need for a further specialist rig to produce a different prosthetic group to label 18F-FDG-biotin.

**2 Materials and Methods**

**2.1 Synthesis of (2-{2-[2-(2-{2-[2-(2-{2-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester (1)**

A solution of biotin *N*-hydroxysuccinimide ester (25 mg, 73.2 *μ*mol), *O*-(2-aminoethyl)-*O*'-[2-(Boc-amino)ethyl]-hexaethylene glycol (34 mg, 72.6 *μ*mol) and triethylamine (8.0 mg, 11 *μ*L, 78.9 *μ*mol) was stirred in CHCl3 (2 mL) at room temperature under Ar for 15 hours. The reaction mixture was washed three times with H2O then dried (MgSO4­), filtered and evaporated to leave the product as a white solid; 50 mg (100 %); *δ*H(250 MHz; CDCl3) 1.40-1.77 (15 H, m), 2.18 (2 H, t, *J* 7.3), 2.70 (1 H, d, *J* 12.8), 2.85 (1 H, dd, *J* 12.5, 4.6), 3.06-3.12 (1 H, m), 3.25-3.60 (32 H, m), 4.23-4.28 (1 H, m), 4.43-4.48 (1 H, m), 5.09 (1 H, bs), 5.93 (1 H, bs), 6.80 (1 H, bs), 6.93 (1 H, bt); *δ*C(62.5 MHz; CDCl3) 25.5, 28.0, 28.2, 28.3, 35.8, 39.0, 40.2, 40.4, 55.6, 60.1, 61.6, 69.8, 69.9, 70.1, 70.2, 70.3, 70.4, 79.0, 155.9, 164.2, 173.3; m/z (ESI) 695.3892 ([M+H]+. C31H59N4O11S requires 695.3901).

**2.2 Synthesis of 5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid [2-(2-{2-[2-(2-{2-[2-(2-amino-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl]-amide dihydrochloride (2)**

A 4 M solution of HCl in dioxane (1.6 mL, 6.40 mmol) was added to a solution of **1** (88 mg, 0.127 mmol) in MeOH (1.6 mL) and the mixture was stirred for 90 minutes at room temperature under Ar. The mixture was concentrated *in vacuo* to leave the product as a colourless oil; 84 mg (99 %); *δ*H(250 MHz; CD3OD) 1.37-1.42 (2 H, q, *J* 6.1), 1.52-1.67 (4 H, m), 2.26 (2 H, t, *J* 6.7), 2.76 (1 H, d, *J* 13.1), 2.97 (1 H, dd, *J* 12.5, 3.1), 3.18-3.23 (1 H, m), 3.32-3.37 (4 H, m), 3.60-3.69 (28 H, m), 4.38-4.43 (1 H, m), 4.57-4.62 (1 H, m).

**2.3 Synthesis of (Boc-aminooxy)acetic acid pentafluorophenyl ester (3)**

EDC hydrochloride (761 mg, 3.97 mmol) and pentafluorophenol (560 mg, 3.04 mmol) were added to a solution of (Boc-aminooxy)acetic acid (580 mg, 3.03 mmol) in DCM (30 mL) and the mixture was stirred at room temperature under Ar for 3 hours. Silica gel (7.6 g) was added to the reaction mixture and stirring continued for a further 10 minutes. The mixture was filtered and the residue washed thoroughly with DCM. The DCM was evaporated to leave the product as a white solid; 874 mg (81 %); *δ*H(250 MHz; CDCl3) 1.50 (9 H, s), 4.80 (2 H, s), 7.64 (1 H, bs).

**2.4 Synthesis of [(2-{2-[2-(2-{2-[2-(2-{2-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethylcarbamoyl)-methyloxy]-carbamic acid *tert*-butyl ester (4)**

Compound **2** (58 mg, 86.9 *μ*mol) and Hünig’s base (24 mg, 32 *μ*L, 184 *μ*mol) were stirred in CHCl3 (4 mL) before compound **3** (32 mg, 89.6 *μ*mol) was added. The mixture was stirred under Ar at room temperature for 15 hours, then washed with H2O. The organic layer was dried (MgSO4), filtered and evaporated to leave the crude product, which was purified by washing with petroleum ether 40/60. This left a pale yellow oil; 65 mg (97 %); *δ*H(250 MHz; CDCl3) 1.43-1.78 (15 H, m), 2.20 (2 H, bt), 2.71 (1 H, d, *J* 12.2), 2.87 (1 H, d, *J* 12.2), 3.08-3.12 (1 H, m), 3.39-3.60 (32 H, m), 4.28-4.32 (3 H, m), 4.49-4.54 (1 H, m), 5.70 (1 H, bs), 6.58 (1 H, bs), 7.20-7.26 (2 H, m), 8.10 (1 H, bs); *δ*C(62.5 MHz; CDCl3) 25.5, 27.9, 28.1, 29.6, 35.8, 38.8, 39.1, 40.5, 55.5, 60.1, 61.7, 69.5, 69.9, 70.0, 70.2, 70.4, 71.9, 75.6, 82.2, 157.4, 164.0, 169.2, 173.4.

**2.5 Synthesis of 5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid {2-[2-(2-{2-[2-(2-{2-[2-(2-aminooxy-acetylamino)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl}-amide dihydrochloride (5)**

A 4 M solution of HCl in dioxane (1.0 cm3, 4.00 mmol) was added to a solution of **4** (60 mg, 78.1 *μ*mol) in MeOH (1 cm3) and the mixture was stirred for 90 minutes at room temperature under Ar. The mixture was concentrated *in vacuo* to leave the product as a colourless oil; 54 mg (93 %); *δ*H(400 MHz; CD3OD) 1.46-1.77 (6 H, m), 2.26 (2 H, bt), 2.76 (1 H, d, *J* 12.2), 2.98 (1 H, d, *J* 11.3), 3.27-3.31 (1 H, m), 3.38-3.46 (4 H, m), 3.56-3.70 (28 H, m), 4.40-4.44 (1 H, m), 4.56-4.61 (3 H, m) ; m/z (ESI) 668.3568 ([M–H]+. C28H54N5O11S requires 668.3541).

**2.6 Synthesis of 5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (2-{2-[2-(2-{2-[2-(2-{2-[2-(2-fluoro-3,4,5,6-tetrahydroxy-hexylideneaminooxy)-acetylamino]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl)-amide (19F-FDG-5)**

A solution of 2-fluoro-2-deoxy-D-glucose (4.9 mg, 26.9 *μ*mol) in 10 mM phosphate-buffered saline (PBS, 1 cm3) was added to a solution of **5** (20 mg, 27.0 *μ*mol) in MeOH (1 cm3). Glacial acetic acid (700 μL) was added and the resultant mixture was stirred at 80 °C for 1 hour, then cooled to room temperature. The solvent was evaporated and the residue partitioned between H2O and CHCl3. The chloroform was dried (MgSO4), filtered and evaporated to leave the product; 11.2 mg (50 %).

**2.7 Radiolabelling of 5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid {2-[2-(2-{2-[2-(2-{2-[2-(2-aminooxy-acetylamino)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl}-amide dihydrochloride by conjugation with 18F-FDG (18F-FDG-5)**

To a solution of 1 mg of **5** in 10 *μ*L of MeOH was added 5 *μ*L of glacial acetic acid and 10 MBq of 18F-FDG (produced for clinical use by the John Mallard PET Centre, Aberdeen UK) in 10 *μ*L of 10 mM PBS. The mixture was heated without stirring for up to 90 min at 85 °C. The reaction was followed by thin layer chromatography (TLC) on silica plates with ethanol:ethylacetate (50:50) as mobile phase (under these conditions FDG moves with the solvent but the product remains at the origin) and the activity location determined on a MiniGita (Raytest) TLC plate reader. The final products were also analysed using HPLC after dilution in PBS and neutralisation with 0.1 M NaOH.

**2.8 HPLC conditions**

HPLC analysis was carried out using a Jupitor 5u C5 silica-based reversed phase 300A column (250×4.6 mm) (Phenomenex, Macclesfield UK) using a 0-50 % acetonitrile gradient (balance water) containing 0.15 % trifluoroacetic acid at a flow rate of 1 mL per min. The HPLC system consisted of a Perkin-Elmer series 200 quaternary pump, series 200 autosampler, series 200 UV/Vis detector (set to 210 nm) and 5 channel vacuum degasser. The radioactive detector used was a Berthold Radioflow Detector LB509. The autosampler was programmed to deliver 50 *μ*L of sample which had been prepared by adding 5 *μ*L of reaction mixture to 200 *μ*L of PBS and neutralised with 0.1 M NaOH. To test the stability of 18F-FDG-**5**, an HPLC analysis was carried out on a sample incubated in PBS for 6 h.

**2.9 Reaction with avidin**

A 1 *μ*L sample of 18F-FDG-**5** at the end of synthesis was added to 200 *μ*L of medium and neutralised with NaOH (0.1 M), then added to 0.5 mg of avidin (Mwt 60,000 g). The mixture was incubated for 30 min at 37 °C and then applied to a 50,000 g mwt cut-off centrifugal filter (millipore UK) and centrifuged at 1500g for 30 min. The retentate was washed by addition of 1 mL of PBS and a further 30 min centrifugation at 1500g. The activity retained by the filter (18F-FDG-biotin conjugated to avidin (Mwt 60,000)) relative to total activity was determined.

**3 Results**

**3.1 Synthesis of labelling precursor**

The synthesis of the oxyaminePEG biotin hydrochloride salt (**5**) is shown in scheme 1.

**3.2 Synthesis of 19F-FDG-5**

Reaction of **5** with 19F-FDG in a mixture of methanol, acetic acid and 10 mM PBS produced 19F-FDG-**5** in a 50 % isolated yield. It is worth noting that despite the moderate isolated yield, the product was obtained pure using a simple extractive work-up: after concentration of the reaction mixture *in vacuo*, the residue was partitioned between chloroform and water. After separation and evaporation of the chloroform, 19F-FDG-**5** was obtained, with no evidence of any unreacted 19F-FDG. The UV210 HPLC chromatogram of 19F-FDG-**5** is shown in figure 2

**3.3 Synthesis of 18F-FDG-oxyaminePEG biotin**

Figure 1 shows the time course for the formation of 18F-FDG-**5** using freshly prepared clinical grade 18F-FDG. A radiochemical yield of 100 % was achieved with this method. The 100% yield was confirmed by HPLC (Figure 2). Figure 1 also shows the rate of formation of 18F-FDG-**5** using 1 and 4 h-old FDG preparations. Yields of 100% were obtained in both cases after 70 or 80 min incubations.

The formation of 18F-FDG-**5** after a 1 h incubation period using lower amounts of precursor was investigated. Using 0.25 mg of precursor the yield was >90 % but decreased to 55 % using 0.1 mg and 13 % with 0.02 mg of precursor. The specific activity of the tracer using 0.25mg is 10MBq/mmol.

**3.4 Stability of 18F-FDG-5**

Figure 2 shows chromatograms from samples of 18F-FDG-**5** near the end (B), showing the presence of some non-conjugated 18F-FDG, at the end of the 18F-FDG conjugation period (C) and after 6 h incubation in PBS (D). Chromatograms C and D show only a radioactive peak at about 31 min indicating no loss of 18F-FDG or breakdown to 18F-fluoride (neither of which are retained by the column so appear at 5 min).

**3.5 Interaction with avidin**

As the purpose of labeling biotin with 18F-FDG was to produce a tracer that could bind to avidin-conjugated molecules, we tested the ability of 18F-FDG-biotin (18F-FDG-**5**) to interact with avidin. 85 (±7) % of activity was retained by a 50 KDa molecular weight (Mwt) cut-off filter, indicating that most of the 18F-FDG-biotin product had bound to avidin during the 30 min incubation with 10 nmoles of avidin.

**4 Discussion**

In contrast to 18F-fluoride, FDG is available at all PET centres as it is the most universally utilised PET tracer and is shipped out to centres without cyclotrons. We have produced a functionalised PEGylated biotin precursor that can be labelled with 18F-FDG producing radiochemical yields of 100% even with 4 h-old FDG, facilitating the synthesis of 18F-FDG-biotin in a one-pot reaction. The moderately high temperature and acidity of the labelling process and the presence of an 18F-FDG moiety on the biotin did not disable its interaction with avidin.

**5 References**

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Scheme 1) Synthesis of the oxyaminePEG biotin hydrochloride salt **5**

Figure 1) Formation of 18F-FDG-oxy-amine-PEG-biotin using fresh (diamond), 1 h- (square) and 4 h-old (triangle) 18F-FDG

Figure 2) HPLC UV (210nm) chromatogram of 19F-FDG-biotin conjugate standard (A) (units: absorbance units) and radio-chromatograms of 18F-FDG-**5** near the end of synthesis (B) at the end of synthesis (C) and after incubating in PBS for 6 h (D) (units: mV).



Scheme 1)



Figure1

Figure 2)