Development and Application of a Generic Platform for Radiolabelling Affinity Peptides & Proteins with PET Isotopes

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine & Health

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Abbreviations

[¹⁸F]EYFB  Ethynyl-4-[¹⁸F]fluorobenzene
[¹⁸F]FA    [¹⁸F]Fluoroacetaldehyde
[¹⁸F]FBA   [¹⁸F]Fluorobenzaldehyde
[¹⁸F]FBEM  N-[2-(4-[¹⁸F]fluorobenzamido)ethyl]maleimide
[¹⁸F]FDG   [¹⁸F]Fluorodeoxyglucose
[¹⁸F]FEA   [¹⁸F]Fluoroethylazide
[¹⁸F]FETos  [¹⁸F]Fluoroethyltosylate
[¹⁸F]FPCA  2-[¹⁸F]Fluoro-3-pyridinecarboxaldehyde
[¹⁸F]FPyKYNE  2-[¹⁸F]Fluoro-3-pent-4-yn-1-ylloxypyridine
[¹⁸F]FPyMe  1-[3-(2-[¹⁸F]Fluoropyridin-3-yl oxy)propyl]pyrrole-2,5-dione
[¹⁸F]FTMS  [¹⁸F]Fluorotrimethylsilane
[¹⁸F]SFB   N-Succinimidyl-4-[¹⁸F]fluorobenzoate
[¹⁸F]SiFB  N-Succinimidyl 3-(di-tert-butyl-[¹⁸F]fluorosilyl)benzoate
2,4-DNPH  2,4-Dinitrophenylhydrazine
AD       Alzheimer's disease
ADME    Absorption, distribution, metabolism, excretion
Aff     Affibody
AoA     Aminooxyacetic acid
APP     Affinity peptide and protein(s)
a₅β₃   Alpha-v beta-3
Aβ      Amyloid beta
BGO     Bismuth germanate oxide
BLI     Bioluminescence imaging
CEA     Carcinoembryonic antigen
CGC     Critical gelation concentration
CT      Computed tomography
CuAAC   Copper catalysed azide alkyne cycloaddition
DBCO    Dibenzocyclooctyne
DC      Decay corrected
DCM     Dichloromethane
Df      Desferrioxamine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Df-Bz-NCS</td>
<td>p-Isothiocyanatobenzyl-desferrioxamine</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>EDG</td>
<td>Electron donating group</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>EWG</td>
<td>Electron withdrawing group</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLI</td>
<td>Fluorescence imaging</td>
</tr>
<tr>
<td>GE</td>
<td>General Electric</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IEDDA</td>
<td>Inverse electron demand Diels Alder</td>
</tr>
<tr>
<td>K_{222}</td>
<td>Kryptofix 222 (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane)</td>
</tr>
<tr>
<td>LINAC</td>
<td>Linear accelerator</td>
</tr>
<tr>
<td>LYSO</td>
<td>Lutetium yttrium oxyorthosilicate</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionisation</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MIP</td>
<td>Molecularly imprinted polymer</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>n</td>
<td>Neutron</td>
</tr>
<tr>
<td>Nb</td>
<td>Nanobody</td>
</tr>
<tr>
<td>NDC</td>
<td>Non decay corrected</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NIR</td>
<td>Near infra-red</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>NOTA</td>
<td>1,4,7-Triazacyclononane-N,N’,N”-triacetic acid</td>
</tr>
<tr>
<td>p</td>
<td>Proton</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyridinium dichromate</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCP</td>
<td>Radiochemical purity</td>
</tr>
<tr>
<td>RCY</td>
<td>Radiochemical yield(s)</td>
</tr>
<tr>
<td>rhIL1RA</td>
<td>Recombinant human interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>RI</td>
<td>Retro-inverso</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RUV</td>
<td>Relative uptake value</td>
</tr>
<tr>
<td>scFV</td>
<td>Single-chain variable fragment</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Size exclusion</td>
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<tr>
<td>SiFA</td>
<td>Silicon fluoride acceptor</td>
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<td>SPAAC</td>
<td>Strain promoted azide alkyne cycloaddition</td>
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<td>SPE</td>
<td>Solid phase extraction</td>
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<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
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<td>SPR</td>
<td>Surface plasmon resonance</td>
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<td>SUV</td>
<td>Standard uptake value</td>
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<tr>
<td>$t_{1/2}$</td>
<td>Half-life</td>
</tr>
<tr>
<td>TAC</td>
<td>Time activity curve</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivator of transcription</td>
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<td>TCO</td>
<td>Transcyclooctyne</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TFP-N-sucDf</td>
<td>Tetrafluorophenol-N-succinyldesferal</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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<tr>
<td>ThT</td>
<td>Thioflavin-T test</td>
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<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>$t_R$</td>
<td>Retention time</td>
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Table of amino acids

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<th>Amino acid</th>
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<td>Arginine</td>
<td>R</td>
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<tr>
<td>Histidine</td>
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<td>Lysine</td>
<td>K</td>
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<td>Aspartic acid</td>
<td>D</td>
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<td>Glutamic acid</td>
<td>E</td>
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<td>Serine</td>
<td>S</td>
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<td>Threonine</td>
<td>T</td>
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<td>Asparagine</td>
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<td>Cysteine</td>
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<td>Proline</td>
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<td>Alanine</td>
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<td>Isoleucine</td>
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<td>Methionine</td>
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<td>Phenylalanine</td>
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<td>1b</td>
<td>Reaction pathway for the radiolabelling of rhIL-1RA through reductive alkylation</td>
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<td>2</td>
<td>Schematic of GE TRACERlab FX-FN setup</td>
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<td>3</td>
<td>Superimposed RP-HPLC radio-chromatogram showing radiosynthesis of $[^{18}$F]FEtTos in DMSO (radio and 254 nm UV detection shown in brown and green respectively)</td>
</tr>
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<td>4</td>
<td>Superimposed RP-HPLC radio-chromatogram showing characterisation of $[^{18}$F]fluoracetalddehyde with 2,4-DNPH (radio and 254 nm UV detection shown in brown and green respectively)</td>
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<td>5</td>
<td>Superimposed SE-HPLC radio-chromatogram showing crude reaction mixture of $[^{18}$F]fluoracetalddehyde and rhIL-1RA (radio and 254 nm UV detection shown in brown and purple respectively)</td>
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<td>6</td>
<td>Superimposed SE-HPLC radio-chromatogram showing purified $[^{18}$F]rhIL-1RA (radio and 254 nm UV detection shown in brown and green respectively)</td>
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<td>7</td>
<td>Mean intensity projection images of A) mouse and B) rat after injection of $[^{18}$F]rhIL-1RA at 1, 8 and 60 minutes post-injection</td>
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<td>Time activity curve in kidney cortex (A) and heart ventricles (B) from present study (shown in black) and previous publication by Cawthorne et al. (shown in grey)</td>
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<tr>
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<td>RP-HPLC chromatogram showing radiosynthesis of $[^{18}$F]FEtTos in DMSO i) radio-chromatogram and UV chromatograms at ii) 254 nm and iii) 220 nm</td>
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**Chapter 3**

<p>| | | |</p>
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<tbody>
<tr>
<td>1</td>
<td>Schematic of the modified Advion® microfluidics</td>
<td>101</td>
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</tbody>
</table>
system setup

Supp A

Superimposed RP-HPLC radio-chromatogram of purified [\(^{18}\)F]fluoroacetaldehyde (radio and UV signal shown in brown and green respectively)

1a

Reaction pathway showing the radiosynthesis of [\(^{18}\)F]FPCA

1b

Reaction pathway showing the conjugation of [\(^{18}\)F]FPCA to N-\(\varepsilon\)-aminoxy-D-lysine (Aoa-k) modified peptide (Aoa-k-RI-OR2-TAT) through oxime bond formation

2

Schematic of customised GE TRACERlab FX-FN configuration permitting [\(^{18}\)F]FPCA radiosynthesis, purification and isolation followed by Aoa-k-RI-OR2-TAT peptide radiolabelling and purification

3

TRACERlab FX-FN radio-chromatogram trace showing crude [\(^{18}\)F]fluorination mixture of [\(^{18}\)F]FPCA (220 nm). The top and bottom spectra show the gamma and UV detector (AU) traces

4

TRACERlab FX-FN radio-chromatogram trace showing SE-HPLC purification of [\(^{18}\)F]RI-OR2-TAT

5

MALDI-MS analysis of Aoa-k-RI-OR2-TAT peptide labelling with isotopically unchanged FPCA ([\(^{19}\)F]FPCA). [\(^{19}\)F]RI-OR2-TAT is shown at 2969 m/z (M+H\(^+\))

6

Results of the Thioflavin-T assay (ThT) showing percentage inhibition of amyloid aggregation in presence of either [\(^{19}\)F]RI-OR2-TAT (red) or non-functionalised RI-OR2-TAT (blue). Amyloid aggregation in the absence of inhibitor (RI-OR2-TAT) is shown in grey

7

Representative sum images (20-60 min) of a Wistar rat after injection of [\(^{18}\)F]RI-OR2-TAT. Time activity curves in bladder, kidneys and spleen are from 1 animal. Data for bone marrow and liver are expressed as SUV (mean ± SD)
<table>
<thead>
<tr>
<th>Supp A</th>
<th>Quality control RP-HPLC radio-chromatogram of $[^{18}\text{F}]$RI-OR2-TAT</th>
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<td>Supp B</td>
<td>RP-HPLC radio-chromatogram of a plasma sample showing parent $[^{18}\text{F}]$RI-OR2-TAT (5 min time point)</td>
</tr>
</tbody>
</table>

Chapter 5

| 8 | Representative sum images (20-60 min) post-injection (insert: 13-22 sec post-injection) of $[^{18}\text{F}]$RI-OR2-TAT uptake in brain, bone and lungs (mean ± SD, n=2 for brain, n=3 for bone and lungs) |

| 1 | Reaction mechanism showing the oxidation of vicinal hydroxyl groups on a sugar monomer by sodium periodate |
| 2 | Reaction pathway of oxidised dextran and Aoa-RGD to form large molecular weight dextran-RGD complex |
| 3 | SE-HPLC UV-radiochromatogram showing crude $[^{18}\text{F}]$RGD radiolabelling reaction mixture before dextran incubation |
| 4 | SE-HPLC UV-radiochromatogram showing crude $[^{18}\text{F}]$RGD radiolabelling reaction mixture after dextran incubation |

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Abstract

The University of Manchester for the degree of PhD Imaging in the Faculty of Biology, Medicine & Health

Olivia Morris

Development & Automation of a Generic a Platform for Radiolabelling Affinity Peptides & Proteins with PET Isotopes

15th March 2017

Positron emission tomography (PET) is a quantitative and non-invasive investigative tool, which permits the identification of pivotal biomarkers and their role in disease onset, transformation and progression. Quantitative detection of these disease biomarkers has qualified PET as a pioneering approach to rapid diagnosis. The tool is a powerful approach to patient diagnosis and investigation of inter- and intra-patient disease heterogeneity thereby supporting patient stratification and forging a truly personalised medicine approach.

The sensitivity of PET is complemented by use of highly selective radiopharmaceuticals; this has fuelled the popularity of radiolabelled affinity peptides and proteins (APPs). Inspired by the high selectivity and affinity of biological protein-protein interactions, APP-based radiotracers are an increasingly popular class of radiotracer.

Monoclonal antibodies (mAbs) are an important category of APP-based radiotracer; their prevalence in the field is attributed to their high target selectivity and affinity characteristics, which, will likely, uphold their popularity in the field. Yet, advancements in protein engineering has transformed the landscape of APP-based radiotracers, indicated by a preference for small radiolabelled APPs which exhibit rapid pharmacokinetics whilst retaining high target specificity and affinity. Their fast pharmacokinetics is matched by the half-life of $^{18}$F which has been defined as an ideal PET radionuclide.

$^{18}$F APP radiolabelling hasn’t yet met its full potential due to its inherent challenges; many approaches have been adopted, ranging from non-site specific radiolabelling with amine reactive prosthetic groups to site-specific methods including oxime bond formation. The radiochemistry of $^{18}$F APP radiolabelling is diverse and evolving; yet ideal goals are apparent including site-specificity, fast reaction kinetics, mild labelling conditions and applicability to automation.

Automation is challenging for multi-step $^{18}$F APP radiolabelling methods, but is a key developmental step. Automation helps with conformance to good manufacturing practice (GMP) by enhancing process robustness, consistency and reliability. It also permits the radiosynthesis of clinically relevant radiotracer doses.

The development of an automated generic platform, or a method that adopts a general approach, for APP radiolabelling with $^{18}$F would assist in $[^{18}$F]APPs meeting their potential in PET and, importantly, aid their translation from bench-to-bedside.
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Dedications

To my mum and dad: my biggest influences. You have always been, and always will be, my compass – without you, I wouldn’t have found my way.

To my Michael, you are my rock. Where would I be without you? And to my beautiful baby girl, Thea, who is always the light at the end. You two are my reason for everything.

I feel a huge sense of achievement having come this far and I dedicate it to you all, I love you so very much.

To my Supervisors, thank you for your support and guidance. I feel very privileged to have had such a supportive (and enviably brainy) supervisory team. Also to the basement team at WMIC – you have been incredibly accommodating!

Christian, I want to make an unreserved thank you to you. You have always gone above and beyond, and in you I have made a dear friend.

And finally to the wonderful bunch at the WMIC: those who have come and gone, those who have just started and those who have become my closest friends (Bones);

I’ve had a blast.
Chapter 1, part 1: An Introduction to PET

Molecular imaging has been defined as the ‘visualisation, characterisation, and measurement of biological processes at the molecular and cellular levels in humans and other living systems’ \(^{(1)}\). Nuclear medicine is an important division of molecular imaging, with PET qualifying as a key non-invasive imaging modality that, owing to its high sensitivity, permits quantitative identification of biomarkers in healthy and diseased states.

PET is a technique that makes use of a radiolabelled probe comprising a target-specific molecular vehicle and a positron-emitting radioisotope (a radiolabel). The radiolabelled probe is often referred to as a radiotracer, used to ‘trace’ biological pathways, and is typically administered at low concentrations in order to avoid initiation of a physiological response.

1.1 PET radionuclides

Characteristically, positrons annihilate with electrons to form two coincident and anti-collinear 511 keV annihilation gamma photons, described in Equation 1, and it is upon this phenomenon that PET is established.

\[
\beta^+ + e^- \rightarrow \gamma (511 \text{ keV}) + \gamma (511 \text{ keV})
\]

*Equation 1) Annihilation of a positron and electron resulting in two anti-collinear annihilation gamma photons*

In order to radiolabel a molecular vehicle, a radioisotope must first be produced. Accelerators are a means by which positron-emitting, proton-rich isotopes can be artificially generated via acceleration of a particle, such as a proton or deuteron, to energies that allow it to penetrate the nucleus of target atoms. This results in a transient and unstable nucleus which decomposes to form a radioactive isotope.
Formation of the radioisotope is considered a two-stage process, firstly characterised by the generation of a compound nucleus comprising the nuclei of the target material and the accelerated incident particle. Second, the compound nucleus decomposes to form the corresponding radioisotope, alongside additional species \[^{2, 3}\].

The linear accelerator (LINAC) generates an accelerated incident particle by propelling a charged species through a series of linearly placed chambers of alternating electronic charge \[^{4}\]. However, the large size of the LINAC motivated development of the cyclotron, which uses a static magnetic field (B) generated by an electromagnet, to orientate the charged incident particle (q) and an oscillating electrical field (E), generated by electrodes (dees), to accelerate the particle. As the particle velocity increases, so too does the radius of its orbit in order accordance with electrodynamic theory. The theory describes the maintenance of a charged particle’s rotational frequency in a magnetic field, as a function of kinetic energy, and its independence from the radius of orbit. This forces the charged and accelerating particle in a spiral-like outward ‘cyclotron’ trajectory \[^{3, 4}\]. The force (F) on the charged incident particle is described by the Lorentz equation, given in Equation 2. A schematic of particle acceleration in a cyclotron is also depicted in Figure 1.

\[
F = qE + qvB
\]  

*Equation 2) Lorentz equation describing the force experienced by the charged particle in an electromagnetic field*

The probability of a nuclear reaction occurring is represented by the nuclear reaction cross-section which describes the requirement for an incident particle to have sufficient energy to overcome electrostatic repulsion from the target nuclei, described as the ‘Coulomb barrier’, as well as endoergic effects \[^{3}\].

Solid, liquid and gaseous target materials are used in PET radionuclide generation; target materials are chosen in order to maximise radionuclide yield whilst minimising nuclear
transformation by-products. Solid target materials are, on the one hand, preferred on account of their restricted modes of heat transfer and higher efficiencies that are afforded as a result of their density, but liquid and gaseous target materials are often used in medical cyclotrons due to the ease with which the radionuclide is recovered \[^{3}\].

Table 1 documents the commonly used radioisotopes in PET and provides the nuclear equation (most commonly used nuclear reaction) for each of the radioisotopes, half-life \(t_{\frac{1}{2}}\) together with positron energy and branching ratio of radioactive decay by positron emission. Fluorine-18, as an example, is produced via the bombardment of enriched \([^{18}\text{O}]\text{H}_2\text{O}\) (98% enrichment) with accelerated protons. The \(^{18}\text{F}\) produced has low positron energy \((0.6\ \text{MeV})\) which is an advantageous characteristic. The higher positron energy of oxygen-15, for example, results in a longer mean path length before successful electron annihilation due to the positron having to shed some of its excess energy. In the context of PET, a longer mean path length prior to annihilation translates to poorer image resolution. A high positron branching ratio is preferred for PET imaging, particularly in cases where

![Diagram](image)

*Figure 1) Schematic showing particle acceleration using a cyclotron (magnetic field is perpendicular to the plane)*
alternative radionuclide decay-modes include gamma emission that falls within the PET detection window and potentially contributes to background signal.

Radionuclide $t_{1/2}$ is defined as the time taken for the number of radioactive nuclei to halve. Radioactive decay, and positron emission, are due to instability within the proton-rich nucleus; the resulting repulsion exceeds strong nuclear forces, consequently a proton is converted to a neutron and a positron is emitted. The $t_{1/2}$ values of radioisotopes listed in Table 1 are varied, from 110 minutes for $^{18}$F and 2 minutes for $^{15}$O; the radionuclide should be selected to match the pharmacokinetics of the molecular vehicle to be studied in the PET experiment.

### 1.2 PET radiosynthesis

Radiotracer synthesis is often automated using a commercial or lab-built synthesisers, to which the cyclotron produced radioisotope can be delivered. Figure 2 shows a photograph of a commercially available synthesiser, the GE TRACERlab FX-FN. This serves as a protective measure for the radiochemist and also enhances process robustness, consistency and reliability whilst permitting the use of higher levels of radioactivity $^{[5-8]}$. Automation is a critical developmental process that helps with conformance to GMP and aids translation of the radiotracer synthesis from bench-to-bedside $^{[8]}$. This is clearly seen by the prevalence of $^{[68]}$Ga[APPs in clinical practice for which all-in-one, self-shielded radiosynthesisers are used to prepare the radiotracers using fully-automated synthesis $^{[9,10]}$.  

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Nuclear equation</th>
<th>Max. Positron energy (MeV)</th>
<th>Branching ratio</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{15}$O</td>
<td>$^{14}$N(d,n)$^{15}$O</td>
<td>1.70</td>
<td>1.00</td>
<td>2.03 min</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>$^{11}$B(p,n)$^{11}$C, $^{14}$N(p,$\alpha$)$^{11}$C</td>
<td>0.96</td>
<td>1.00</td>
<td>20.4 min</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>$^{18}$O(p,n)$^{18}$F</td>
<td>0.60</td>
<td>0.97</td>
<td>109.80 min</td>
</tr>
</tbody>
</table>

*Table 1) Summary of commonly used PET radioisotopes $^{[4]}$ where p = proton, d = deuteron, n = neutron and $\alpha$ = alpha particle $^{[4]}$*
1.3 Principles of PET

The arrangement of PET camera detectors in opposite positions permits detection of the annihilation photons from a positron-emitting source within its field of view, this is illustrated in Figure 3. By setting energy discrimination and time-coincidence windows specific (350 – 650 keV and ~ 3.5 ns respectively in typical pre-clinical PET scanners\(^\text{[11]}\)) to annihilation photons, the selectivity of detecting a true annihilation event is very high. A true annihilation event is the detection of two anti-collinear annihilation gamma photons from a single annihilation point, within the time-coincidence window, that have not previously undertaken any significant interactions\(^\text{[12]}\). Other types of coincidence events include: scattered and random, both of which must be corrected for.

PET cameras are made up of scintillation crystals that convert the energy of the annihilation photons to an interpretable photon signal, which is amplified by photomultiplier tubes (PMT). A standard block detector design contains 4 PMTs, from

---

Figure 2) Photograph of a GE TRACERlab FX-FN, a commercially available synthesiser
which a ratio of the signal is used to more accurately determine the site where the annihilation photon hits the scintillation crystal \cite{12}. High-density scintillation crystals are preferred, such as bismuth germanate (BGO) and lutetium-based (LYSO) scintillants, in order to efficiently detect 511 keV annihilation photons \cite{13, 14}.

![Figure 3) Schematic showing positron annihilation detection using a PET scanner](image)

Accumulation of the target-specific radiotracer allows its \textit{in vivo} visualisation via detection of annihilation photons emitted from the local tissue. The intensity of the signal corresponds to the radiotracer concentration in a given 3D pixel (voxel) \cite{15}. Analysis of radiotracer concentration distribution as a function of time provides dynamic data relating to the radiotracer’s pharmacokinetics. Co-registration of PET data (functional imaging modality) with an additional imaging modality, such as magnetic resonance imaging (MRI) or computed tomography (CT) provides anatomical information.

Representative quantification of radiotracer accumulation is, somewhat, dependent on its specific radioactivity. Specific radioactivity is the radioactivity per total amount of isotopes and pertains to the ratio of radioisotopically labelled species to the stable isotope labelled
(but otherwise identical) molecule and is defined as the measured radioactivity per mole of compound and often measured in GBq/μmol $^{[3]}$. 

However, the term is also used to describe the specific radioactivity of radiotracers, where the separation of both the radioisotope and stable isotope labelled tracer from the unlabelled precursor has not been possible, this is illustrated in Figure 4. In such instances, the term ‘effective specific radioactivity’ can be used. According to the Figure, the specific radioactivity of B can be interpreted as the ratio of B/C and the effective specific radioactivity as the ratio of $B/(A+C)$. 

Figure 4) Components of $^{89}$Zr radiolabelled antibody product A) unlabelled antibody, B) $^{89}$Zr radiolabelled antibody and C) stable isotope labelled antibody
Low radiotracer specific radioactivity is undesirable for PET imaging, in such cases there is competition between the radiotracer and the unlabelled precursor for the biological target. Receptor-based biological targets are particularly prone to issues of poor radiotracer specific radioactivity, due to possible saturation of the target, and in such cases poor quality images can result. Saturation of a biological target may also lead to pharmacodynamic changes, which could result in a biased interpretation of PET data, and even toxicity.

1.4 Final remarks

PET is an essential limb of nuclear medicine and a powerful tool; a culmination of improvements to cyclotron targetry, PET detectors, software and radiochemistry has contributed to its exquisite sensitivity. PET has aided the identification of pivotal biomarkers that have fundamental roles in disease onset and progression. PET can also be used as a drug-development tool, helping to profile a drug-candidate’s pharmacodynamic profile. Importantly, PET is a non-invasive method by which biomarkers can be quantitatively measured and is an attractive alternative to traditional diagnostic methods such as biopsy or post-mortem analysis, which can be prone to inter-lab variability. Rapid quantification of disease biomarkers permits prompt diagnosis, outlines an appropriate treatment plan and also supports treatment response monitoring, important hallmarks of patient stratification^{20, 22}. The technique can also provide comprehensive investigation of inter- and intra- disease heterogeneity. This, therefore, highlights the significance of PET in the future of population health and its pioneering influence on a personalised medicine approach.

The next section will review the literature of approaches to peptide and protein radiolabelling: a promising class of radiotracer.
Chapter 1 part 2: A Review of Affinity Peptide & Protein Radiolabelling

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Abstract

Affinity peptide and protein- (APP) based radiotracers are an increasingly popular class of radiotracer in positron emission tomography (PET), which was once dominated by the use of small molecule radiotracers. Radiolabelled monoclonal antibodies (mAbs) are important examples of APPs yet a preference for smaller APPs, which exhibit fast pharmacokinetics and permit rapid PET aided diagnosis, has become apparent. \[^{18}\text{F}\]Fluoride exhibits favourable physical characteristics for APP radiolabelling and has been described as an ideal PET radionuclide. Notwithstanding, \[^{18}\text{F}\] radiolabelling of APP is challenging and this is echoed in the literature where a number of diverse approaches have been adopted. This review seeks to assess and compare the approaches taken to \[^{18}\text{F}\] APP radiolabelling with the intention of highlighting trends in the momentum of this engaging field. Generic themes have emerged in the literature, namely the use of mild radiolabelling conditions, a preference of site-specific methodologies with an impetus for short, automated procedures which produce high-yielding \[^{18}\text{F}\] APPs.

Due to the continued prevalence of mAbs in this field, the review will additionally examine the current popular methods of mAb radiolabelling which makes use of \(^{89}\text{Zr}\).

1 Introduction

1.1 Affinity peptides & proteins in PET

The field of PET was dominated by the use of radiolabelled small molecules. Over recent decades, however, radiolabelled affinity peptides & proteins (APPs) have become an important class of radiotracer including radiolabelled somatostatin analogues and octreotides \(^{[8]}\).

The high selectivity and affinity of biological protein-protein interactions initially motivated their exploitation in biomedical fields. Key developments in the knowledge of APP antigen-binding, speed of generation using solid-phase and recombinant techniques,
as well as progressions in affinity selection techniques have strengthened their utility in a number of areas \[8\]. APP-based pharmaceuticals represent a rapidly growing market in pharmaceutical industry, despite some challenging absorption, distribution, metabolism and excretion (ADME) properties of APPs, such as low cell membrane permeability, short biological half-life and limited stability \[16\]. The advantages of their use including exquisitely high target binding affinity and specificity alongside low toxicity and immunogenicity have overwhelmed the challenges associated with their use \[16\].

Naturally, their utility generated attention in the field of PET, where APPs could be radiolabelled and their high target selectivity and affinity could be exploited \textit{in vivo}. The protein-nature of many disease targets and their natural binding partners, including tumour cell surface receptors and hallmarks of dementia such as tau and amyloid plaque, also reinforces the use of radiolabelled APPs in the disease diagnoses and highlights their utility in both peripheral and neurological pathologies \[8\].

Table 1 provides a list of APPs with varying molecular mass. Monoclonal antibodies (mAbs) (~150 kDa), categorised as a large APP, are an important subclass of immunoglobulin; the extensive use of mAbs is evident in the number of therapeutic antibodies that are available and used in a clinical setting, with many more in the latter

<table>
<thead>
<tr>
<th>APP</th>
<th>Molecular mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large APP</strong></td>
<td></td>
</tr>
<tr>
<td>Monoclonal antibody</td>
<td>150</td>
</tr>
<tr>
<td><strong>Small APPs</strong></td>
<td></td>
</tr>
<tr>
<td>Fab fragment</td>
<td>55</td>
</tr>
<tr>
<td>scFv</td>
<td>28</td>
</tr>
<tr>
<td>Nanobody</td>
<td>10-15</td>
</tr>
<tr>
<td>Affibody</td>
<td>6-7</td>
</tr>
</tbody>
</table>

\[16, 34\]
stages of clinical development. The popularity of mAbs is credited to their naturally-evolved target selectivity and specificity; their ubiquity in other areas of medicine led to mAbs filtering across into the field of nuclear medicine and literature as early as the 1950s can be found, documenting the use of antibodies radiolabelled with iodine-131, a therapeutic radionuclide, in oncology. An in vitro method, by Koehler et al. in 1975, reported the generation of high volumes of target-specific antibodies and described the importance of this method in both medical and industrial fields. A review by Larson et al. recognised the method as a key development in the use of radiolabelled mAbs and more recent achievements in recombinant techniques, permitting humanisation and chimerisation, have strengthened the clinical utility of radiolabelled mAbs.

Encouraging results using radiolabelled mAbs in oncological clinical trials have been reported, where their use has permitted diagnosis, patient stratification and identification of the most appropriate treatment.

The rate of uptake from the blood to the biological target is approximately inversely proportional to the molecular mass of the APP, consequently target accumulation of mAbs is relatively slow. For this reason, longer-lived radioisotopes such as zirconium-89, iodine-124 and copper-64 have been conventionally used to radiolabel mAbs. The half-lives and unfavourably low positron branching ratios (0.18-0.23) of these radioisotopes can be seen in Table 2. The slow biodistribution of radiolabelled mAbs can lead to poor target tissue to background contrast unless the mAb is given sufficient time to accumulate at its biological target. This is time and labour intensive and the use of longer lived radioisotopes contribute to a high patient radiation dose; later pre-targeting approaches to mAb radiolabelling permit the use of shorter-lived radioisotopes, however these methods are still relatively new. The slow biodistribution and use of longer-lived radioisotopes are considered to be significant limitations to the use of radiolabelled mAbs.

In addition to this, large proteins have complex tertiary structures with multi-domain
features, formed by glycosylation patterns and disulphide bridges, resulting in thermal and chemical vulnerability that can limit the scope of a radiolabelling method \cite{29, 30}. The issue of fragility is underlined in the literature, where the use of physiological temperatures and aqueous buffers for radiolabelling procedures is consistently described \cite{25, 31, 32}.

These limitations have complicated the translation of radiolabelled mAbs to the routine clinical practice and, as a result, a change in the landscape of APP-based radiotracers is evolving. This corresponds to a preference for smaller APPs that exhibit rapid pharmacokinetics and enhanced robustness, whilst retaining high target affinity and selectivity \cite{17, 33}. Importantly, the rapid pharmacokinetics of small APPs permits the use of short lived radioisotopes. The diagnostic value of small APPs has not yet been validated; yet their exploitation in diagnostic PET has the potential to be a powerful tool helping to guide patient stratification and identify those who would benefit from mAb-based therapy, an expensive treatment option, which is a routinely employed APP-based therapeutic.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Nuclear equation</th>
<th>Max. Positron energy (MeV)</th>
<th>Branching ratio</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{68})Ga</td>
<td>(^{70})Zn(p,n)(^{68})Ga Germanium-68 generator</td>
<td>1.8</td>
<td>0.81</td>
<td>68.3 mins</td>
</tr>
<tr>
<td>(^{18})F</td>
<td>(^{18})O(p,n)(^{18})F</td>
<td>0.6</td>
<td>0.97</td>
<td>109.80 mins</td>
</tr>
<tr>
<td>(^{89})Zr</td>
<td>(^{89})Y(p,n)(^{89})Zr</td>
<td>0.8</td>
<td>0.23</td>
<td>3.30 days</td>
</tr>
<tr>
<td>(^{124})I</td>
<td>(^{124})Te(p,n)(^{124})I</td>
<td>1.5</td>
<td>0.23</td>
<td>4.2 days</td>
</tr>
<tr>
<td>(^{64})Cu</td>
<td>(^{64})Ni(p,n)(^{64})Cu</td>
<td>0.6</td>
<td>0.18</td>
<td>12.7 hrs</td>
</tr>
</tbody>
</table>

*Table 2) Commonly used radionuclides in APP radiolabelling* \cite{44}

Table 2 documents the radioisotopes that have been utilised in APP radiolabelling; the Table has been separated into short and long lived radionuclides. The majority of the radionuclides listed are most commonly produced using a cyclotron however \(^{68}\)Ga is
normally generator-produced, which is considered a significant advantage. The wide range of radionuclides available is highly advantageous in order to match the diverse biological half-lives of different APPs.

Somatostatin analogues were amongst the first small APP to be radiolabelled and approved for clinical use in oncology, in the 1980s [8]. Early APP-based radiotracers used in PET, including somatostatin analogues, typically made use of radioisotopes such as $^{68}$Ga [8]. Use of $^{18}$F radiolabelled APP in clinical trials would not be documented for another 20 years and this has been ascribed to challenges associated with the radiochemistry [8].

Given the prevalence of mAbs, unsurprisingly, the utility of antibody fragments, such as Fab (antigen-binding fragment) and scFv (single-chain variable fragment), as APP-based radiotracers has been investigated. However the target affinity of the parent antibodies was rarely retained in these fragments and issues of poor solubility and aggregation were apparent [21]. The loss of affinity observed in mAb fragments was also observed in a trastuzumab Fab fragment, suggesting a likely role of bivalency in mAb-based antigen interaction [34].

Nanobodies, which are 12-15 kDa fragments of camelid heavy chain only antibodies, are alternative immunoglobulin-based APPs. They exhibit high target affinity and selectivity together with rapid biodistribution, whilst avoiding the issues of poor solubility and aggregation observed in mAb fragments [35]. Vaneycken et al. [23] described the use of an anti-HER2 (human epidermal growth factor receptor 2) nanobody; the ease of nanobody production as well as improved affinity selection techniques permitted efficient deduction of lead nanobodies that were able to identify HER2 expressing lesions and also regions of metastases [23]. This is indicative of the work that has underlined the potential of nanobodies and they have thus been labelled the ‘magic bullet’ of molecular imaging and are predicted to have an important future role in the field [35].
Advances in APP engineering have facilitated the arrival of non-immunoglobulin based APP \[^{17}\]. Designed using combinatorial protein libraries, non-immunoglobulin based APPs have been engineered to possess characteristics, such as enhanced solubility or stability that challenge some of the limitations of immunoglobulin-based APPs \[^{17, 29}\]. The affibody molecule is a promising example of a non-immunoglobulin APP and originated from staphylococcal protein A (SPA). The affibody is derived from the B-domain: one of the five domains that make up SPA. The high stability and solubility of the domain originally highlighted its potential and key mutations were subsequently made that afforded enhanced stability of the newly named Z-domain; it is upon this protein-scaffold backbone that more than 60 target-specific affibodies have been constructed \[^{36}\] \[^{17, 29}\]. Literature cited in this review, describing the use of affibodies in oncology, unanimously described successful and rapid tumour targeting alongside fast clearance from non-target tissue \[^{36-38}\]; indeed Kramer-Marek et al. \[^{37}\] report tumour uptake as early as 20 minutes post-injection thus stressing the potential of the APP in rapid PET-guided diagnosis.

Great variety in the characteristics of APPs used in PET exists: from large immunoglobulin-based mAbs to single-polypeptide, chains such as anti-\(\alpha_v\beta_3\) RGD motif-containing APPs used as angiogenesis radiotracers. Yet further tailoring of APP characteristics is reported, where conjugation of biomolecules, such as poly-histidine and albumin, \[^{16, 38}\] or inert polymers, such as polyethylene-glycol (PEG), have been shown to manipulate the \textit{in vivo} behaviour of APP. Kalia and Raines \[^{39}\] report the enhanced water-solubility, reduced immunogenicity and enhanced biological half-life of PEGylated APPs. The described characteristics of smaller APPs are profitable attributes in PET; a key advantage is their compatibility with short lived radioisotopes, namely \(^{18}\text{F}\) which has been described as an ideal PET radionuclide \[^{8, 40}\]. However, as previously discussed, the radiochemistry of \(^{18}\text{F}\) APP radiolabelling can be challenging and this was outlined in a review article by Richter \textit{et al.} \[^{8}\] where the incompatibility of direct fluorination and lack of automation of complex multi-step methods have impeded the translation of \(^{18}\text{F}\)
radiolabelled APP to clinical practice \cite{8}. The review stresses the importance of process automation in aiding conformance to GMP but also the difficulties associated with automation of multi-step approaches to APP radiolabelling with $^{18}$F \cite{8}. The importance of process automation is reflected in the successes of $^{68}$Ga radiolabelled APPs, where all-in-one, self-shielded, fully-automated radiosynthesisers are used to prepare the radiotracers, stimulating an exponential growth in their use \cite{9,10}. Gallium-68 radiochemistry lends itself more favourably to process automation due to one-step chelation methods, unlike the current multi-step approaches to $^{18}$F radiolabelling. However, the advantageous physical characteristics of $^{18}$F (Table 2) make the endeavour, to automate $^{18}$F approaches, a profitable one.

The field of $^{18}$F APP radiochemistry is diverse and dynamic yet, despite the extensive literature surrounding $^{18}$F APP radiolabelling, very few have progressed to clinical-trials or gained widespread use. The review will summarise the literature documenting approaches to $^{18}$F APP radiolabelling, seeking to highlight the developments made to the radiochemistry and advancements towards routine clinical use.

The high target specificity and affinity of mAbs reinforces their use in many fields of medicine and biology and it can be argued that their target affinity remains a reference against which other APP can be compared. It is for this reason that mAb are likely to remain a prevalently used radiolabelled APP in PET. A recent review by van de Watering \textit{et al.} \cite{41} defined $^{89}$Zr as a ‘near ideal’ radionuclide for mAb radiolabelling, a view that is echoed in the literature by the popularity of well-established $^{89}$Zr mAb radiolabelling procedures \cite{31,32}. For this reason, a review of the literature of popular methods to radiolabel mAbs with $^{89}$Zr will also be described.
2. \textsuperscript{18}F APP radiolabelling

The biological half-life of a small or intermediately sized APP (defined in Table 1) is complemented by an \textsuperscript{18}F radiolabel as a result of the commensurate physical half-life of the radionuclide.

The introduction of fluorine into a molecule, at the outset, initiated much debate given the limited natural abundance of fluorine in biological molecules \cite{40}. It was argued that its addition may alter the physical characteristics of any tracer in view of the electronegative character of fluoride. It has, however, been found that its electronic properties can lead to improved pharmacological characteristics and the strength of the C-F bond (112 kcal/mol), in comparison to the C-H bond (98 kcal/mol), enhances its metabolic stability \cite{40}. The small size of fluorine (van der Waals radius 1.47 Å) bears similarity to hydrogen (1.2 Å) and electronic nature is comparable to a hydroxyl group \cite{40}. This, therefore, provides scope for their replacement in a whole host of molecules, where fluoride can be described as a bioisostere \cite{42}.

\textsuperscript{18}F has been referred to as an ideal PET radionuclide owing to its favourable characteristics, including its low mean positron energy and high branching ratio for positron emission and $t_{1/2}$ of which permits more complex radiochemistry and longer imaging studies; a summary of the physical characteristics of \textsuperscript{18}F can be seen in Table 2 \cite{8,40}. This is of particular importance when radiolabelling APPs, which frequently necessitates multi-step radiosynthetic strategies as direct fluorination of the APP is often not possible due to the necessary harsher radiolabelling conditions and multiple fluorination sites of the APP.

The review will separate \textsuperscript{18}F APP radiolabelling into non-site-specific and site-specific methods.
2.1 Non-site-specific radiolabelling

Prosthetic groups are small molecules that conjugate to the APP under mild conditions, they are $^{18}$F-fluorinated before conjugation to the APP thereby avoiding issues arising from the harsher reaction conditions often required for direct fluorinations \[^{43, 44}\].

Non-site-specific radiolabelling is commonly achieved using carbonyl-containing $^{18}$F labelled prosthetic groups which can target the N-terminal or ε-amine belonging to lysine residues and thus making use of the inherent nucleophilic sites of the APP. Table 3 provides a summary of commonly used fluorine-18 prosthetic groups, the Table summarises key features of prosthetic groups radiosynthesis allowing comparisons to be drawn.

Reductive alkylation, using amine-reactive prosthetic groups, is a commonly employed strategy in APP radiolabelling. It involves the formation of an imine (or Schiff base) which is subsequently reduced with an appropriate reducing agent. [$^{18}$F]fluorobenzaldehyde (FBA) is a popular example of an amine-reactive $^{18}$F prosthetic groups used in reductive alkylation \[^{45}\]. More recently [$^{18}$F]fluoroacetaldehyde ([18F]FA) and [$^{18}$F]fluoropyridinecarboxaldehyde ([18F]FPCA) were reported, as water-soluble prosthetic group alternatives \[^{6, 7, 46}\].

![Figure 1) Reaction scheme showing reductive alkylation in a) acidic and b) basic media](image)

\[ ^{18}\text{F}\text{FBA, } R = C_6H_{14}^{18}\text{F} \]

*Figure 1) Reaction scheme showing reductive alkylation in a) acidic and b) basic media*
Figure 1 gives the mechanism of reductive alkylation in both acidic and basic conditions. Reductive alkylation proceeds well in both conditions yet the literature points to more favoured use of acidic conditions \cite{6, 45, 47}. The instability of the imine and production of H$_2$O or hydroxyl group, which is thermodynamically disfavoured in aqueous media, is overcome upon addition of an appropriate reducing agent. NaCNBH$_3$ is often used as a reducing agent in APP radiolabelling on account of its mild reducing properties, stability in water and compliance with the pH range required for imine formation \cite{48}.

2.1.1 $[^{18}\text{F}]$Fluorobenzaldehyde ($[^{18}\text{F}]$FBA)

$[^{18}\text{F}]$FBA is a popular $^{18}$F prosthetic group and is generally synthesised via direct fluorination of 4-formyl-$N$-$N$-$N$-trimethylammonium-triflate commercially-available precursor, this can be seen in Figure 2.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{reaction_scheme.png}
\caption{Reaction scheme to show $[^{18}\text{F}]$FBA radiosynthesis}
\end{figure}

The presence of an ortho/para electron withdrawing group and powerful leaving group permits nucleophilic aromatic substitution of the precursor, which without these elements would not be susceptible to nucleophilic attack. Many of the arene-based $^{18}$F prosthetic groups make use of a trimethylammonium precursor owing to the solubility of ammonium salts, ease of separation of the $^{18}$F-labelled prosthetic group from the ammonium salt precursor and stability of the trimethylamine leaving group. $[^{18}\text{F}]$Fluoromethane has been reported as a possible side-product, however its generation is disfavoured when substituents with large Hammett constants are present on the arene \cite{49}. 

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Speranza et al.\textsuperscript{[50]} report the automation of $^{18}$F-FBA radiosynthesis using a GE TRACERlab FX-FN. Automated radiosynthesis of $^{18}$F-FBA was completed in 45 minutes and radiochemical yields (RCY) of 90\% decay-corrected (DC) were reported\textsuperscript{[50]}. The method made use of SPE purification, rather than HPLC, for a number of reasons including ease of automation, time-saving aspects and reproducibility\textsuperscript{[50]}. Importantly the group report comparable $^{18}$F-FBA recovery between the two methods and $^{18}$F-FBA radiochemical purity (RCP) measurements of $>99\%$ were described\textsuperscript{[50]}. The group insist on the importance of automation in order to provide a reliable tool to produce large quantities of $^{18}$F-FBA to radiolabel APPs for use in routine clinic\textsuperscript{[50]}.

Apana et al.\textsuperscript{[45]} report the radiolabelling of an APP via reductive alkylation with $^{18}$F-FBA. The APP, anginex, is a 33-residue peptide with anti-angiogenic properties and has been reported to sensitise tumours to radiotherapy and inhibit tumour growth; $^{18}$F-anginex was evaluated as an anti-angiogenic radiotracer in pre-clinical models\textsuperscript{[45]}. $^{18}$F-Anginex yields of 76\% (decay-correction status unknown) were reported alongside specific radioactivity measurements of 14.4 GBq/μmol\textsuperscript{[45]}. $^{18}$F-Anginex was evaluated in tumour bearing mice and, although high liver uptake was observed, tumour uptake of $^{18}$F-anginex was 16\% higher than the maximal liver uptake and accounted for 30\% of the total body distribution. The authors concluded that $^{18}$F-anginex had retained target selectivity, achieved high target to background ratios and is thus a potentially useful antiangiogenic diagnostic radiotracer\textsuperscript{[45]}. The yield and specific radioactivity of $^{18}$F-anginex were significantly improved by removal of polytetrafluoroethylene (PTFE) which is a material used in tubing and as a surface coating for various components of radiochemistry synthesisers. PTFE derived stable fluoride has a carrier effect and its removal yielded $\sim 25\%$ improvement in $^{18}$F-anginex yields\textsuperscript{[45]}. PTFE removal from the radiosynthesis of $^{18}$F-FBA proved to be a profitable modification; the average mass of carrier fluoride was calculated to be between 400-800 nmol, which was reduced to 25 nmol after its elimination\textsuperscript{[45]}. 

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2.1.2 $^{18}$FFluoroacetaldehyde ($^{18}$F]FA)

$^{18}$F]FA is a small, water-soluble prosthetic group reported by Prenant et al. [46] and was used to radiolabel a recombinant human interleukin-1 receptor antagonist (rhIL1RA, 17.5 kDa) via reductive alkylation [47]. A remotely-controlled semi-automated configuration was used for $^{18}$F]FA radiosynthesis and subsequent rhIL1RA radiolabelling; the method achieved $^{18}$F]FA yields of 34 ± 3 % (DC, n=3) and overall $^{18}$F]rhIL1RA RCY of 11.4 ± 4 % (DC, n=17) in 100 minutes [46, 47].

The non-site-specificity of reductive alkylation was described as a drawback of the radiolabelling approach [47]. Yet ex-vivo binding assays revealed retention of $^{18}$F]rhIL1RA binding specificity and it was thought that the small size of $^{18}$F]FA was, in part, responsible [47].

Full process automation of $^{18}$F]FA radiosynthesis and subsequent APP radiolabelling was later reported by Morris et al. [6]; rhIL1RA was radiolabelled via reductive alkylation using $^{18}$F]FA, in order to make direct comparisons between the semi- and fully-automated procedures. The automation permitted larger starting quantities of $^{18}$F]fluoride which contributed to larger quantities of $^{18}$F]FA, despite some observed loss in $^{18}$F]FA yields (~25 % loss) [6]. Overall $^{18}$F]rhIL1RA yields of 5 ± 3 % (DC, n=5) and effective specific radioactivity measurements of 13.5 GBq/μmol were reported. $^{18}$F]rhIL1RA effective specific radioactivity measurements reported by Morris et al. [6] are significantly higher than measurements described by Prenant et al. [47] (0.9 – 5.0 GBq/μmol) despite the inability of both methods to separate from unlabelled rhIL1RA. This was largely attributed to the ability to use higher starting levels (15-fold higher) of radioactivity [6, 47].

2.1.3 $^{18}$FFluoropyridinecarboxaldehyde ($^{18}$F]FPCA)

The ease with which pyridine can be fluorinated at both the C2 and C4 positions, attributable to the presence of the nitrogen 'electron-sink', has led to an increasing number of pyridine-based prosthetic groups [7, 51, 52]. One such example is $^{18}$F]FPCA, a water-
soluble $^{18}$F prosthetic group developed by Morris et al.\textsuperscript{[7]} Its radiosynthesis has been fully-automated using a customised GE Tracerlab FX-FN and $[^{18}\text{F}]$FPCA RCY of 28 ± 2\% (DC, n=10) are reported\textsuperscript{[7]}. Morris et al.\textsuperscript{[7]} describe the replacement of RP-HPLC as a means to purify $[^{18}\text{F}]$FPCA with SPE for time-saving reasons and also to simplify automation and improve reproducibility\textsuperscript{[7]}, akin to the motivations that encouraged Speranza et al.\textsuperscript{[50]} to purify $[^{18}\text{F}]$FBA using SPE. The RCP of SPE purified $[^{18}\text{F}]$FPCA was verified and comparable to the RCP of RP-HPLC purified $[^{18}\text{F}]$FPCA (data not shown)\textsuperscript{[7]}. The automation of $[^{18}\text{F}]$FPCA was extended to include the subsequent radionlabelling of an APP. Although the APP described in the work by Morris et al.\textsuperscript{[7]} was radiolabelled in a site-specific manner, the group describe the applicability of the prosthetic group to reductive alkylation\textsuperscript{[7]}.

### 2.1.4 N-Succinimidyl-4-$^{18}$Ffluorobenzoate ($[^{18}\text{F}]$SFB)

$[^{18}\text{F}]$SFB, similarly to $[^{18}\text{F}]$FPCA, radiolabels APPs through conjugation with the inherent nucleophilic sites of the APP, such as the N-terminal amine or ε-amine groups of lysine residues. Unlike the other amine-reactive prosthetic groups described, $[^{18}\text{F}]$SFB has the advantage that it does not require a reducing agent. Alkylation of APPs with $[^{18}\text{F}]$SFB is commonly carried out between pH 8 and 9 to promote deprotonation of the APP amine groups\textsuperscript{[53]}.

Automation of $[^{18}\text{F}]$SFB radiosynthesis has been described by Zijlstra et al.\textsuperscript{[53]}; in brief, $[^{18}\text{F}]$SFB was produced by first synthesising ethyl 4-$[^{18}\text{F}]$fluorobenzoate through fluorination of a trimethylammonium-precursor\textsuperscript{[53]}. 4-$[^{18}\text{F}]$Fluorobenzoic acid was then produced via base hydrolysis of the fluorinated ester\textsuperscript{[53]}. In the final step, a succinimidyl-containing synthon (O-[N-succinimidyl]-N,N,N,N-tetramethyluronium tetrafluoroborate) (TSTU) was incubated with 4-$[^{18}\text{F}]$fluorobenzoic acid to yield $[^{18}\text{F}]$SFB in yields of ≤ 35\% (DC) and RCP of 98\%\textsuperscript{[53]}. A summary of $[^{18}\text{F}]$SFB radiosynthesis can be seen in Figure 3.
High RCP values were reported despite having to reuse SPE cartridges owing to limitations of the automated radiochemistry module\textsuperscript{[53]}. \([^{18}\text{F}]\text{SFB}\) was used to radiolabel the apoptosis radiotracer, annexin-V, and RCY of \(\leq 20\%\) (DC) were reported\textsuperscript{[53]}.

\[
\begin{align*}
\text{Ph} &\quad \text{N}^+\text{N}^-\text{N}^+\text{N}^-\text{O}\quad \text{O}^+\text{O}^-\text{N}^+\text{N}^-\text{O}\quad \text{O}^+\text{O}^-\text{N}^+\text{N}^-\text{O}^-\quad \text{Ph}
\end{align*}
\]

\textit{Figure 3) Reaction scheme to show} \textit{[^{18}\text{F}]SFB radiosynthesis from [^{18}\text{F}]fluorobenzoic acid and O-[N-succinimidyl]-N,N,N,N-tetramethyluronium tetrafluoroborate}

In vitro experiments using apoptotic cells verified the selectivity of \(^{[18}\text{F}]\text{annexin-V}\) and cell uptake of the radiotracer was dose dependent in experiments where varying concentrations of unradiolabelled annexin-V were used, thereby verifying retention of the biological activity of \(^{[18}\text{F}]\text{annexin-V}\)\textsuperscript{[53]}. Conjugation of \(^{[18}\text{F}]\text{SFB}\) to an APP, such as annexin-V, can be seen in Figure 4.

\[
\begin{align*}
\text{Ph} &\quad \text{N}^+\text{N}^-\text{N}^+\text{N}^-\text{O}\quad \text{O}^+\text{O}^-\text{N}^+\text{N}^-\text{O}^-\quad \text{Ph}
\end{align*}
\]

\textit{Figure 4) Reaction scheme to show APP radiolabelling with} \textit{[^{18}\text{F}]SFB}

2.1.5 Evaluation of non-site-specific methods

The literature reviewed, hitherto, has described the use of \(^{18}\text{F}\) prosthetic groups to radiolabel APPs via a non-site-specific pathway. Apana \textit{et al.}\textsuperscript{[45]} sought to establish the
preferential site of non-site-specific radiolabelling and results indicated that the singly-
substituted labelled anginex, also the major product, was N-terminally labelled suggesting
this as the preferred site and the likely site of \(^{18}\text{F}\)FBA radiolabelling \(^{45}\). This was a
useful undertaking in order to understand the impact of labelling on the interaction of the
APP with its binding site.

Table 3 summarises the radiosyntheses of the \(^{18}\text{F}\)-prosthetic groups discussed above. The
literature shows widespread use of \(^{18}\text{F}\)FBA and \(^{18}\text{F}\)SFB: they are well-established
methods and can achieve attractive RCY \(^{50}\). The commercial availability of the \(^{18}\text{F}\)FBA
precursor augments its popularity and the additional advantage of not having to use a
reducing agents adds to the appeal of \(^{18}\text{F}\)SFB, this is indicative of an initiative towards
simplified radiolabelling procedures. The popularity of these prosthetic \(^{18}\text{F}\)FBA and
\(^{18}\text{F}\)SFB is also reflected in the commercial availability of bespoke molecularly imprinted
polymer (MIP) SPE cartridges that can be used to purify \(^{18}\text{F}\) prosthetic groups obtained
from ammonium-containing precursors by nucleophilic aromatic substitution, including
\(^{18}\text{F}\)FBA and \(^{18}\text{F}\)SFB \(^{7, 54}\).

Process automation has been reported for all the discussed prosthetic groups with
radiosyntheses not exceeding 45 minutes. This conforms to the generally held view that
radiotracer synthesis, purification, formulation and analysis should not exceed two half-
lives of the PET radionuclide used \(^{8}\). The automation of all prosthetic groups uses SPE
purification \(^{7, 50, 53}\), with exception to \(^{18}\text{F}\)fluoroacetaldehyde which uniquely uses
distillation purification \(^{6, 47}\). There are, however, significant challenges and considerations
to process automation. Morris et al. \(^{6}\) report a loss in yields as a result of process.

Additional considerations are the physical boundaries imposed by automated
radiosynthesisers; limitations to the number of valves and ports that are available can place
restrictions on process automation. Zijlstra et al. \(^{53}\) describe reusing SPE cartridges owing
to restrictions enforced by automating radiosynthesis of \(^{18}\text{F}\)SFB. Additionally, the use of
radiochemistry synthesisers can increase the exposure of \(^{18}\text{F}\) to stable fluoride owing to the
multiple reactors and tubing used to transfer a radionuclide reaction mixture in a multi-step radiosynthesis. Apana et al. [45] had recognised the impact that process automation can have on specific radioactivity and report the removal of PTFE in an effort to minimise exposure to stable fluoride.

The ability to use larger starting levels of $^{18}$F and the consistency and reliability that process automation can guarantee means that its challenges are outweighed by its advantages. This importance of process automation is reflected in the literature, where there is a trend towards prosthetic group automation yet the limitations of commercial radiosynthesizers and considerations of automation pose significant challenges.

Alongside the encouraging movement towards automated radiosynthesis is the advent of water-soluble $^{18}$F prosthetic groups, such as $[^{18}\text{F}]$FA and $[^{18}\text{F}]$FPCA. The water-solubility of the prosthetic groups is an advantage over non-water soluble prosthetic groups as it demonstrates their versatility and compliance with mild radionuclide conditions; radionuclide of APP with $[^{18}\text{F}]$FBA, for example, is often carried out in an organic solvent, such as methanol [45].

2.1.6 Summary of non-site-specific methods

Radiosynthesis simplification, process automation and compatibility of a radionuclide strategy to mild reaction conditions are key developments that have emerged from the literature surrounding methods of non-site-specific radionuclide with fluorine-18. The developments are indicative of the measures being taken to align with the generic features that are required of a versatile platform for $^{18}$F radionuclide and would facilitate the translation of $[^{18}\text{F}]$APPs.

The ability to radionuclide the native APP using its naturally-occurring nucleophilic sites, without pre-modification, is the foremost advantage of the technique and, encouragingly, the literature reports retention of the APP biological activity, despite non-site-specificity of the methods [45, 47, 53]. Despite this, there has been significant interest in the development of
site-specific APP radiolabelling methods. The appeal of these methods is not only characterised by site-selectivity but also the faster rates of reaction that can be afforded \[33, 39, 55, 56\].
<table>
<thead>
<tr>
<th>¹⁸F prosthetic group</th>
<th>RCY DC (approx. %)</th>
<th>Purification</th>
<th>No. of steps</th>
<th>Automation</th>
<th>Radiosynthesis time (min)</th>
<th>APP radiolabelling temp (°C)</th>
<th>Comments</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFB</td>
<td>35</td>
<td>SPE MIP SPE</td>
<td>3</td>
<td>Yes</td>
<td>45 (estimated from data in the paper)</td>
<td>30</td>
<td>1. No reducing agent 2. Availability of bespoke MIP SPE cartridge</td>
<td>[53, 54]</td>
</tr>
<tr>
<td>FBA</td>
<td>90</td>
<td>C18 SPE MIP SPE</td>
<td>1</td>
<td>Yes</td>
<td>45</td>
<td>60</td>
<td>1. High RCY 2. Availability of bespoke MIP SPE cartridge 3. Commercial availability of precursor</td>
<td>[50, 54]</td>
</tr>
<tr>
<td>FA</td>
<td>26</td>
<td>Distillation</td>
<td>2</td>
<td>Yes</td>
<td>45</td>
<td>40</td>
<td>1. No SPE or HPLC purification 2. Water-soluble 3. Full process automation 3. Commercial availability of precursor</td>
<td>[6, 46, 47]</td>
</tr>
</tbody>
</table>

Table 3) Summary of selected carbonyl-containing ¹⁸F prosthetic groups used in non-site-specific APP radiolabelling
2.2 Site-specific radiolabelling

The requirement for site-specific APP radiolabelling has been recognised, this was a result of concerns surrounding non-site-specific conjugation of a radiolabel located at or near the antigen binding site. By virtue of their chemistry, these methods can offer faster rates of reaction and thus are more high yielding approaches to APP radiolabelling.

By way of comparison, Table 4 documents the rates that have been reported in the literature for non-site-specific and site-specific methods of bioconjugation. Consequently site-specific approaches have become preferable means by which to radiolabel APPs and safeguard its biological activity.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Method</th>
<th>Rate (M⁻¹s⁻¹)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-site-specific</td>
<td>Reductive alkylation (imine formation)</td>
<td>$k_1 = 10^{-1}$</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Oxime &amp; Hydrazone</td>
<td>$k = 10^{-3} - 1.0$</td>
<td>[55]</td>
</tr>
<tr>
<td>Site-specific</td>
<td>Huisgen 1,3-dipolar cycloaddition</td>
<td>$k = 2.3$</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Inverse electron demand Diels-Alder</td>
<td>$k = 10^4 - 10^5$</td>
<td>[77]</td>
</tr>
</tbody>
</table>

Table 4: A comparison of rates between non-site-specific and site-specific bioconjugation methods

Rate constants for site-specific methods have been included, however overall rate constants of reductive alkylation are not readily available in the literature, for this reason, $k_1$ values of imine formation have been included.

Site-specific approaches to APP radiolabelling include $^{18}$F acceptor chemistry and click-chemistry methods.

2.2.1 $^{18}$F acceptor chemistry

Richter et al. described $^{18}$F acceptor radiolabelling synthons as attractive alternatives to the use of prosthetic groups. Two types of $^{18}$F acceptor chemistry used to radiolabel APPs
will be discussed: Al\([^{18}\text{F}]\)^{2+} complexation and isotopic exchange. A Table summarising the key features of site-specific approaches to APP radiolabelling with \(^{18}\text{F}\) has been provided in section 2.2.3.

### 2.2.1.1 \(([^{18}\text{F}]\text{AlF})^{2+}\)

\(([^{18}\text{F}]\text{AlF})^{2+}\) has been used to radiolabel a number of APPs in a one-pot, high yielding reaction characterised by Al chelation and the formation of a strong Al-F bond, first reported by McBride et al. \([61]\). Da Pieve et al. \([27]\) used the \(([^{18}\text{F}]\text{AlF})^{2+}\) method to radiolabel a HER3-specific affibody with a view to assessing its usefulness as radiotracer in oncology; the method mirrored conditions reported by Glaser et al. \([59]\). The affibody was derivatised with 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and incubated with \(\text{AlCl}_3\). Final incubation of the complex with \(^{18}\text{F}\) gave rise to yields of 38.8 ± 5.8 % non-decay-corrected (NDC, unknown \(n\) number) and specific radioactivity measurements of 6.0 – 11.9 GBq/\(\mu\)mol were reported \([27]\). A reaction by-product was observed, presumed to be an APP degradation product as a result of the radiolabelling conditions \([27, 59]\), as a result Da Pieve et al. \([27]\) report the requirement for both RP-HPLC and SPE purification to ensure final RCP of > 98 %. *In vitro* analysis using HER3 expressing cells confirmed retention of \(^{18}\text{F}\)Al-F-NOTA-affibody target specificity and affinity, indicated by preservation of the affibody’s low nanomolar binding constant \([27]\). These results were echoed by the *in vivo* analysis, using HER3 tumour-bearing mice, where successful tumour targeting and fast blood clearance of Al\(^{18}\text{F}\)NOTA-affibody was observed \([27]\).

A recent publication from the group describes full process automation of the approach, having been encouraged by the results of the method and the recognised requirement for automated processes in GMP production \([60]\). High temperatures and a water-miscible solvent were, again, used in the fully-automated procedure which resulted in efficient radiolabelling of two model APPs and required single SPE-purification of the final radiolabelled product \([60]\). A clear advantage of the approach is the simplicity of the single-
vial method and ability to use commercially available aqueous $[^{18}\text{F}]$fluoride/H$_2$O.

Application of the fully-automated approach to a NOTA-derivatised octreotide, chosen for its commercial availability, achieved RCY of $>45\%$ and RCP measurements of $>98\%$ within 26 minutes.$^{[60]}$.

In a similar vein, McBride et al.$^{[61]}$ report the development of single-vial lyophilized kits as a method to simply and rapidly label APPs with $([^{18}\text{F}]\text{AlF})^{2+}$ and although not automated, the group demonstrate the feasibility of translating the simplified single-vial method into a clinical setting, owing to the ease at which freeze-dried APPs can be radiolabelled and purified using SPE.$^{[61]}$.

The potential of the $([^{18}\text{F}]\text{AlF})^{2+}$ radiolabelling method is clear and encouraging achievements in process automation will surely stimulate the appeal of the approach and an additional advantage to the technique is the ability to exploit the myriad NOTA-derivatised APPs that are commercially available and used as precursors for $^{68}\text{Ga}$ radiolabelling. However, the electronic and/or steric influences of a chelate and metal ion complex on the $\textit{in vivo}$ performance of an APP should be considered. Strand et al.$^{[36]}$ report the difference in tumour accumulation of chelate-derivatised affibodies, which was in agreement with previous work reported by the group that exposed a significant influence of radiolabelling method on the $\textit{in vivo}$ behaviour of affibodies.

### 2.2.1.2 Isotopic exchange

Silicon-based isotopic exchange agents are attractive alternatives to $^{18}\text{F}$ radiolabelled prosthetic groups owing to the enhanced rate kinetics of fluorination at silicon relative to that at carbon and enhanced stability of the Si-F bond, as opposed to that of C-F (142.3 and 116.4 kcal/mol respectively)$^{[62, 63]}$.

Rosenthal et al.$^{[62]}$ report the development of a silicon-fluoride acceptor (SiFA), $[^{18}\text{F}]$fluorotrimethylsilane ($[^{18}\text{F}]\text{FTMS}$), by nucleophilic substitution of a chlorotrimethylsilane with $^{18}\text{F}$, achieving RCY of 80 % (DC). However, the $\textit{in vivo}$
instability of the Si-F limited its applicability but encouraged developmental of isotopic exchange agents with bulky alkoxy substituents which exhibit enhanced *in vivo* stability, whilst retaining high RCY, and thus would be more useful radiolabelling synthons.\[62, 64\]
The steric impact of bulky alkoxy substituents is in agreement with the work of Brinker *et al.*\[65\] where steric factors were shown to have the greatest impact on the hydrolytic stability of organosilanes.
Schirrmacher *et al.*\[64\] report the development of an isotopic exchange agent, \([^{18}\text{F}]\text{fluorodi-tert-butylphenylfluorosilane}\), described as a highly efficient and hydrolytically stable SiFA. The group report RCY of 80-95 % (decay-correction status unknown, unknown *n* number) and specific radioactivity between 194-230 GBq/μmol; the competitive RCY and specific radioactivity measurements were ascribed to the rapid rates of isotope exchange\[64\].
The group identified the requirement for a simplified and, if possible, single-vial method to yield \([^{18}\text{F}]\text{APPs}\) to act as an attractive alternative to multi-step radiosyntheses\[66\]. In order to achieve this, the group derivatised the SiFA with a benzaldehyde-derivative for site-specific conjugation to an aminooxy-functionalised APP\[64\]. The APP was incubated with azeotropically dried \([^{18}\text{F}]\text{fluoride/Kryptofix}222/K^+\) and RCY of 60 ± 5 % (decay-correction status unknown, unknown *n* number). were attained alongside RCP measurements of > 98 % The group describe the APP radiolabelling conditions as the mildest documented to date. This is true of the pH and room temperature conditions used, yet the use of 100 % acetonitrile as the reaction media is not a mild and generic approach that could be applied to APPs that are sensitive to use of acetonitrile. To further enhance the versatility of the technique, Schirrmacher *et al.*\[64\] evaluated the approach using commercially available aqueous \([^{18}\text{F}]\text{fluoride}\) and removal of azeotropic distillation steps; a requirement for elevated temperatures (95 °C) to achieve comparable yields to those attained using dried \(^{18}\text{F}\) was discovered, nevertheless this modification highlights the applicability of the method to a single-vial approach.
2.2.2 Click-chemistry

Click-chemistry is characterised by high-yielding and site-specific reactions that are compatible with benign solvents and do not generate offensive by-products; the author refers readers to the comprehensive list of criteria provided by Sharpless et al. [67]. Click-chemistry reactions were originally divided into two types of reactions: ring-opening and cycloaddition reactions [67], yet the literature indicate an expansion to the definition of click-chemistry to incorporate a number of other reactions, which will be discussed. Clearly, the hallmarks of click-chemistry reactions highlight its appeal in radiochemistry and in APP radiolabelling terms click-chemistry describes the site-specific conjugation of a radiolabelled synthon, containing a reactive group, to a complementary reactive group belonging to the APP.

A number of click-chemistry approaches have been adopted to radiolabel APPs with $^{18}$F; this includes oxime bond and hydrazone formation, the products of the reaction between amine-reactive prosthetic groups and aminoxy- and hydrazide- functional groups respectively. The efficiency of hydrazone and oxime bond formation can be explained by the alpha effect, where an adjacent electron lone-pairing bearing atom acts to enhance the nucleophilicity of the amine group by first, raising the energy of the highest occupied molecular orbital (HOMO) and stabilisation of the transition state [70, 30].

Another click-chemistry approach that will be discussed is the Michael-addition of thiol-containing APPs with maleimide containing prosthetic groups [68]; thiol-Michael additions are the only type of click-chemistry method by which APPs can be radiolabelled using a naturally-occurring residue: cysteine.

An additional, and very important, category of click-chemistry approaches are cycloadditions in which new $\sigma$-bonds are formed at the expense of $\pi$-bonds to form a cyclic compound [69]; examples include copper catalysed and strain-promoted azide-alkyne cycloadditions and inverse electron demand Diels-Alders cycloadditions [28, 76, 80].
2.2.2.1 Oxime bond formation

Aminooxyacetyl groups, or aminooxy (Aoa), are important functionalities in site-specific conjugation to APPs and have been used in a number of fields, including the synthesis of complex proteins from peptide fragments [70]. As such, oxime bond formation for the purpose of APP radiolabelling has become an increasingly popular approach, moreover the oxime bond has been described as hydrolytically stable thereby highlighting its suitability for in vivo studies [30, 55, 71]. Figure 5 shows the conjugation of an Aoa-functionalised APP with a carbonyl-bearing prosthetic group, such as $^{[18]}$FBA or $^{[18]}$FPCA.

Poethko et al. [70] report the development of a high-yielding and chemoselective method by which Aoa-functionalised APPs are radiolabelled with amine-reactive prosthetic groups [70]. Poethko et al. [70] sought to verify the site-specificity of the conjugation and for this $^{[18]}$FBA was incubated with a range of amino-acids both in the presence and absence of aminooxy-acetic acid. Results indicated that, in the presence of aminooxy-acetic acid, oxime bond formation accounted for 93 % of the radiolabelled product; $^{[18]}$FBA showed some reactivity with cysteine but, nevertheless, site-specificity was high [70]. $^{[18]}$FBA was subsequently used to radiolabel Aoa-derivatised model APPs: an RGD and somatostatin analogue, and overall RCY of $\leq 40$ (decay-correction status unknown, unknown n number) were reported [70].

A pH of 2-3 was reported to produce the highest yields of radiolabelled APP (60-80 %, decay-correction status unknown, unknown n number) [70]. The radiolabelled APPs were

![Figure 5) Reaction scheme to show conjugation of aminooxy-functionality to carbonyl-bearing prosthetic group](image)
evaluated *in vivo* to confirm hydrolytic stability of the oxime bond but results also showed good tumour-to-background ratios of the radiolabelled APPs, despite the APPs originally being chosen as model compounds \[70\].

Namavari *et al.* \[30\] adopted the same approach to radiolabel an Aoa-derivatised dimeric HER2 affibody with \[^{18}F\]FBA to assess its use as a radiotracer for HER2 detection: HER2 is another member of the human epidermal growth factor family and is also associated with a more aggressive tumour phenotype and poorer patient prognosis \[34\]. \[^{18}F\]HER2 RCY of 27 ± 3 % (DC, unknown *n* number) were reported; the lower yields of Aoa-functionalised APP, compared to those described by Poethko *et al.* \[70\], were ascribed to the use of a higher pH (pH 4). However, Namavari *et al.* \[30\] had wanted to assess the applicability of the method to APPs with more complex structures that might be susceptible to the use of low pH, especially in combination with elevated temperatures such as 60 °C \[30\]. Unfortunately the tumour uptake of the radiolabelled affibody was relatively moderate, ascribed to the dimeric nature of the affibody, yet the group report the success with which the APP was radiolabelled via oxime bond formation and the high *in vivo* stability of the oxime bond \[30\].

Dirksen *et al.* \[55\] report the slow kinetics of oxime bond formation at neutral pH and low pH conditions are widely reported in APP radiolabelling via oxime bond formation \[7, 70\].

Further work by the group demonstrated the utility of aniline to catalyse the reaction, even at neutral pH \[55\]; indeed, aniline catalysis at neutral pH was demonstrated in the work by Glaser *et al.* \[59\] where an Aoa-functionalised affibody was radiolabelled at neutral pH using aniline and radiolabelled affibody yields of 13 ± 3 % (NDC, *n*=5) were reported. Figure 6 gives the reaction pathway of aniline catalysis on oxime bond formation.

Full process automation of APP radiolabelling via oxime bond formation has recently been reported and underlines the developments to this prevalent type of click-chemistry \[7\]. Full process automation resulted in a 4-fold reduction in overall yields of radiolabelled APP owing to challenges of translating the radiosynthesis from a semi-manual to an automated
approach\textsuperscript{[7]}. This was recompensed by the enhanced consistency, reliability and process robustness that were afforded using the automated radiosyntheseis, alongside the ability to use larger starting levels of $^{18}$F\textsuperscript{[7]}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Reaction scheme to show the catalysis of oxime bond formation with aniline}
\end{figure}

\subsection*{2.2.2.2 Hydrazone formation}

Hydrazone formation is an additional chemoselective ligation method that permits site-specific APP radiolabelling\textsuperscript{[55]}. Hydrazide-derivatised APPs can be radiolabelled using well-established amine-reaction $^{18}$F prosthetic groups. Hydrazone formation has, arguably, had limited utility in APP radiolabelling and this can be ascribed to the limited stability of the hydrazone product. Oxime bonds have been shown to possess greater hydrolytic stability compared to hydrazones\textsuperscript{[55]}; this is supported by the work of Kalia \textit{et al.}\textsuperscript{[72]} which documents the half-lives of an oxime (25 d) and a corresponding hydrazone (2 h). Dirksen \textit{et al.}\textsuperscript{[55]} compared the rates of hydrazone and oxime bond formation and report K\textsubscript{eq} of hydrazone formation that were two orders of magnitude larger than those for oxime bond formation, despite a 20-fold smaller $k_1$ attributable to the instability of the hydrazone.
product\textsuperscript{[55]}. The work provides evidence for the limited utility of hydrazone formation in
APP radiolabelling; despite encouraging rates of hydrazone formation, the stability of the
radiolabelled product would translate to low radiolabelled APP yields and does not align
with some of the generic features of $^{18}$F APP radiolabelling, where fast rates of reaction
coupled with high yields as well as \textit{in vivo} stability are important in order to motivate the
translation of $^{18}$F radiolabelled APP into the clinic.

\textbf{2.2.2.3 Thiol-Michael addition}

\textit{N}-[2-($^{18}$F-fluorobenzamido)ethyl]maleimide ($[^{18}\text{F}]$FBEM) is a maleimide-containing $^{18}$F prosthetic group, which site-specifically radiolabels thiol-containing APPs. The relative ease with which thiol-groups can be incorporated in APPs, in comparison to Aoa-
derivatives, drove the developments in the area\textsuperscript{[30]}.

As seen in Figure 7, $[^{18}\text{F}]$FBEM can be synthesised through addition of $\text{N}$-(2-
aminoethyl)maleimide to $[^{18}$F]SFB in the presence of a non-nucleophilic base, typically $\text{N, N-diisopropylethylamine (DIPEA)}$. Conjugation of $[^{18}\text{F}]$FBEM to an $\text{N}$-terminal cysteine proceeds via a Michael addition, the mechanism of which can be seen in Figure 8. Radiolabelling mixtures containing thiol-bearing APPs require a reducing agent to prevent disulphide bridge formation between two cysteine residues, such as tris(2-
carboxyethyl)phosphine (TCEP).

Cai \textit{et al.}\textsuperscript{[57]} report using $[^{18}\text{F}]$FBEM to site-specifically radiolabel a thiolated RGD peptide; $[^{18}\text{F}]$RGD yields of 80\% (NDC, unknown \textit{n} number) and specific radioactivity measurements of 100-150 GBq/\textmu mol were reported, yet the overall RCY was impacted by the low $[^{18}\text{F}]$FBEM RCY of 5 ± 2\% (NDC, unknown \textit{n} number). \textit{In vitro} cell binding assays verified specificity of $[^{18}\text{F}]$RGD and were able to inhibit binding of the $\alpha_\text{v}\beta_3$
antagonist echistatin and \textit{in vivo} analysis showed specific accumulation of the radiotracer in xenograft tumour models alongside good metabolic stability\textsuperscript{[57]}.
Cai et al. [57] describe the method as a high-yielding approach to radiolabel thiol-containing APPs with $^{18}$F with high specific radioactivity and the mild reaction conditions for APP radiolabelling with $[^{18}\text{F}]$FBEM highlight the versatility of the technique [57]. The relevance of the technique to routine production is unclear, the multi-step radiosynthesis of $[^{18}\text{F}]$FBEM is time consuming (150 ± 20 min) and low-yielding and thus neglects some of the requirements of an efficient approach to APP $[^{18}\text{F}]$fluorination. However, Kiesewetter et al. [73] report the automation of $[^{18}\text{F}]$FBEM with RCY of 17 ± 6 % (NDC, n=21) achieved within 115 minutes.

**2.2.2.4 Cu$^{1}$ catalysed azide alkyne cycloaddition (CuAAC)**

CuAAC is a prevalent click-chemistry technique, characterised by the catalysis of azide and alkyne conjugation by copper (Cu$^{1}$) and the formation of a stable triazole [74].
chemistry of CuAAC, a type of Huisgen 1,3-dipolar cycloaddition, originally necessitated the use of high reaction temperatures or pressures, yet important methodical improvements reported by Rostovstev et al. [74] involved the use of a copper catalyst which eliminated the requirement for such harsh reaction conditions [74]. Cu\(^{1}\) is often generated \textit{in situ} by reduction of Cu\(^{II}\)SO\(_4\), sodium ascorbate is commonly used for this purpose. The theory behind the mechanism of copper catalysis has evolved, from originally hypothesising the formation of copper(I)acetylide, via coordination to the alkyne, to, more recently, the supposition that two copper atoms are involved. This is discussed in more detail in a review by Pretze et al. [75].

Glaser et al. [76] compared the CuAAC approach with oxime bond formation to radiolabel an RGD peptide. For the investigation, the azide-containing prosthetic group \(^{[18}\text{F}]\text{fluoroethylazide} (\(^{[18}\text{F}]\text{FEA}) was conjugated to an alkyne-derivatised APP, in the presence of a copper catalyst [76]. \(^{[18}\text{F}]\text{RGD RCY} of 47 \pm 8 \%, \text{(DC, n}=3)\) were reported; comparable to those attained using oxime bond formation (40 \pm 12 \%, DC, n=5).

Optimised CuAAC radiolabelling was reported in slightly basic conditions; the use of pH 8, however, led to the formation of a side-product, which was thought to be a copper catalyst adduct. Generation of the by-product was minimised by reducing the amounts of Cu\(^{II}\)SO\(_4\) and ascorbic acid [76], an adaptation that would also be of benefit to APPs that are vulnerable to reduction and susceptible to structural rearrangements to coordinate Cu\(^{II}\) [77].

**2.2.2.5 Bioorthogonality**

More recently, click-chemistry criteria have matured to include a preference for bioorthogonal methods, which are highly site-selective click-chemistry approaches that are compatible with physiological conditions and inert to biological reactions [77]. SPAAC and inverse electron demand Diels-Alder (IEDDA) are click-chemistry techniques that have seen significant attention for their bioorthogonality [77].
2.2.2.5.1 Strain promoted cycloaddition

A highly strained alkyne, characterised by constrained bond angles flanking the alkyne moiety (< 180°, linearity of sp-hybridisation), can be used to drive the cycloaddition and lower the activation barrier and thus eliminating the requirement for a catalyst [78]. Dibenzocyclooctyne (DBCO) is commonly used on account of its high ring strain alongside relative stability, owing to the presence of two phenyl rings that stabilise the triple bond [79]. A number of highly strained alkyne reagents are commercially available; those bearing electron withdrawing groups have reportedly faster kinetics due to alkyne polarisation - although a trade-off exists between alkyne stability and reactivity [79].

2.2.2.5.1.1 Strain promoted azide-alkyne cycloaddition (SPAAC)

Like CuAAC, strain-promoted azide-alkyne cycloadditions (SPAAC) involve the formation of a triazole product. Kim et al. [80] radiolabelled an RGD peptide using the SPAAC approach in order to evaluate its potential as a PET radiotracer in oncology. The APPs were functionalised with a DBCO moiety for strain-promoted click-labelling with an azide-bearing 18\text{F} prosthetic group [80]; the radiosynthesis of the prosthetic group had previously been reported on and achieved RCY of 63 % (DC) [81]. The bioorthogonality of SPAAC click-chemistry enabled use of ambient temperature and neutral pH; quantitative conjugation of the azide-bearing prosthetic group was reported and [18\text{F}]RGD RCY of 92 % (DC, unknown n number) were attained [80]. In vivo analyses of [18\text{F}]RGD, in tumour-bearing mice, showed good tumour accumulation and tumour to background ratios and thus highlighted the preservation of RGD biological activity [80].
Figure 9 shows the reaction pathway for SPAAC click-radiolabelling with $[^{18}\text{F}]$fluoroethylazide, a commonly used azide-bearing prosthetic group, and a DBCO-functionalised APP. Owing to the robustness and adaptability of the approach, the literature reports the use of both $^{18}\text{F}$ azides and DBCO prosthetic groups for use in APP radiolabelling via SPAAC $^{[77]}$.

2.2.2.5.1.2 Strain promoted alkyne-nitrone cycloadditions (SPANC)

SPANC is an additional click-chemistry approach which exploits alkynes and their reactivity with a nitrone functional group and is characterised by the formation of an oxazole product. Although the literature doesn’t report its use in PET, the enhanced rates of reaction when compared to SPAAC approaches ($\sim$50-fold) highlight the potential of the technique $^{[78]}$.

2.2.2.5.2 Inverse electron demand Diels-Alder cycloadditions (IEDDA)

The [4+2] Diels-Alder cycloaddition is the most common pericyclic reaction and can be reversible or irreversible depending on the diene and dienophile substituents. Figure 10 shows the differences between reversible and irreversible Diels-Alder reactions and, as can be seen, the inverse electron demand Diels-Alder (IEDDA) occurs between an electron deficient diene and a dienophile $^{[82]}$. 

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*Figure 9) Reaction scheme to show SPAAC between DBCO-functionalised APP and a radiolabelled azide*
The absence of charged intermediates permits a solely aqueous reaction medium and proceeds well, via a single-step, on heating due the stability of the transition state which has six delocalised π-electrons.

Tetrazine and trans-cyclooctyne (TCO) are commonly reported IEDDA synthons as a result of their fast reaction rates \[75\]. In an APP radiolabelling application, an attempt was made to conjugate an \(^{18}\)F radiolabelled tetrazine and a TCO-derivatised APP; the instability of the fluorinated tetrazine, however, limited the success of the technique \[75\]. Selvaraj et al. \[83\] adapted the approach to successfully radiolabel a tetrazine-derivatised RGD with a fluorinated TCO synthon. The mechanism of IEDDA between tetrazine and TCO can be seen in Figure 11; the endo product is formed: the kinetic product.

\[^{18}\text{F}]\text{TCO} \text{RCY} \] were not provided, but \[^{18}\text{F}]\text{RGD} yields of 90 % (NDC) were achieved within 5 minutes and at room temperature. The mild reaction conditions and compatibility of the click-chemistry to a range of solvents including cell media and buffer highlight the
bioorthogonality of the approach\textsuperscript{[75]}. These favourable characteristics promoted an investigation into their application in pre-targeting methods.

The pre-targeting labelling approach is a two-step methodology involving the labelling of a prosthetic group bearing a diene followed by reaction with an APP functionalised with a click reagent dienophile (or vice versa), which can selectively react \textit{in vivo}. Firstly, the APP is administered, and allowed to accumulate at its biological target in accordance with its relatively slow pharmacokinetics, followed by administration of the more rapid, labelled prosthetic group\textsuperscript{[79]}. Pre-targeting has been applied in clinical practice, most particularly in therapeutic oncology applications for localised radiotherapy or chemotherapy rather than imaging\textsuperscript{[84]}.

Meyer et al.\textsuperscript{[28]} notably used the IEDDA reaction between a tetrazine and TCO to radiolabel a mAb with $^{18}$F\textsuperscript{[28]}. For the investigation, an [$^{18}$F]Al-F-NOTA-radiolabelled tetrazine synthon was synthesised achieving RCY of $60 \pm 5\%$ (DC, unknown $n$ number)\textsuperscript{[28]}. The mAb, which targeted the tumour associated antigen CA19.9, was derivatised with TCO and administered to mice bearing CA19.9-expressing tumours before administration of the radiolabelled tetrazine. \textit{In vivo} evaluation of the pre-targeting method confirmed that tumour delineation was possible after 1 hour post administration of Al[$^{18}$F]NOTA-tetrazine and good tumour to background ratios were reported between 2 and 4 hours\textsuperscript{[28]}. The \textit{in vivo} results are encouraging, yet a real merit of the technique is the ability to radiolabel this influential class of APP with $^{18}$F, an ideal PET radionuclide\textsuperscript{[8, 17, 40]}.

2.2.2.5.3 Staudinger ligations

The reactivity of azides is exploited in Staudinger ligations, where an azide reacts with a phosphine functional group\textsuperscript{[39]}. A particularly attractive variant of the Staudinger ligation, the traceless Staudinger ligation, forms an amide bond between the APP and the prosthetic group whilst the phosphine group is eliminated\textsuperscript{[39]}. This is a very appealing prospect and removes concerns surrounding the impact of bulky or hydrophobic click products on the
pharmacokinetics of the APP \[^{39, 77}\]. However, the more modest \textit{in vivo} rate kinetics that have been reported for Staudinger ligations as opposed to cycloadditions \[^{39}\] alongside the vulnerability of phosphines to oxidation have limited its application to pre-targeting approaches of APP radiolabelling \[^{78}\].

### 2.2.3 Evaluation of site-specific radiolabelling with $^{18}$F

A review by Meyer \textit{et al.} \[^{77}\] describes the importance of site-specific APP radiolabelling and the superiority of the approaches over non-site-specific methods by providing a ‘homogenous product’ with better \textit{in vivo} behaviour \[^{77}\]. A number of site-specific approaches to APP radiolabelling with $^{18}$F have been adopted, Table 5 provides a summary of these methods.

Encouraging features of fluoride acceptor chemistries have emphasised their appeal and highlighted their potential as attractive alternatives to click-chemistry based approaches \[^{8}\]. Full process automation of an ($[^{18}$F]AlF)$^{2+}$ radiolabelling method \[^{60}\] and the development of single-vial methods using lyophilized APPs \[^{61}\] are key developments in the approach that enhances the ease at which the methods can be translated to GMP. The ability to use commercially available aqueous $[^{18}$F]fluoride and removal of azeotropic drying steps simplifies the overall procedure and increases the ease at which the methods can be implemented in a clinical setting. The versatility of the fluoride acceptor approaches may, however, be limited to APPs where the use of organic solvents and/or high temperatures does not lead to loss of function \[^{30}\].

Oxime bond formation and thiol-Michael additions are additional approaches to APP radiolabelling that have yielded good results. The flexibility of the methods to a variety of media, including aqueous buffers, and numerous APPs, including those with more complex structures, highlights the versatility of the approaches \[^{30, 37, 57}\]. Alongside this are the developments towards automated radiosyntheses that have improved the consistency and reliability of multi-step methods \[^{7}\] and, in the case of $[^{18}$F]FBEM, resulted in higher yields.
The compliance of the methods with mild labelling conditions also underlines their appeal (Table 6) and although neutral pH had been reported to significantly impact yields attained using oxime bond formation, the use of an aniline catalyst circumvents this issue [55, 59].

Approaches to APP radiolabelling via cycloaddition are very encouraging. The faster rates of reaction that are afforded using cycloaddition chemistries have given rise to a number of advantages (Table 4 and 5). Glaser et al. [76] report near complete consumption of crude radiolabelled synthon using CuAAC, thereby underlining the robustness and high-yielding nature of the method and highlights the feasibility of a single-vial method of APP radiolabelling. Comparable RCY using oxime bond formation required a 3-fold increase in reaction time [76]. The high-yielding nature of cycloadditions is also reflected in SPAAC approaches to APP radiolabelling. Kim et al. [80] report quantitative conjugation of a radiolabelled synthon to an APP, which enabled the use of a bespoke scavenger purification method to purify the $[^{18}\text{F}]$APP from unlabelled APP. Alongside the possibility of unwanted Cu-mediated by-products [77] and a growing impetus for an automated, site-specific and mild APP radiolabelling strategy reinforced concerns regarding the use of cytotoxic Cu(I) in CuAAC. The desire for mild reaction conditions motivated the emergence of bioorthogonality and the applicability of an APP radiolabelling method to physiological conditions. SPAAC and IEDDA are very promising methods, their use of mild reaction conditions highlights their potential in pre-targeting approaches, which promises to be a very powerful technique. However, the size and hydrophobic nature of the dibenzocyclooctatriazole SPAAC and bicyclic IEDDA products and their impact on the pharmacokinetics of the APP should be considered [77]. An additional disadvantage of SPAAC is isomerisation in the final radiolabelled APP product, which raises issues of product consistency and homogeneity [77].
The use of pre-targeting methods overthrows the convention of matching a PET radioisotope to the pharmacokinetics of an APP and enhances the versatility of the approach, removing the need for longer-lived radioisotopes, such as $^{89}$Zr, where radioactive burden to non-target tissues is a considerable limitation of the technique. Meyer et al. [28] report effective dose measurements of an $^{18}$F pre-targeting approach that were 60-fold lower than directly labelled mAb using $^{89}$Zr. The method also circumvents issues of low radiotracer effective specific radioactivity, an issue that is commonplace in [$^{18}$F]APP radiosynthesis. This is because the method involves the use of a radiolabelled small-molecule that binds to the target-bound APP click-reagent, rather than the [$^{18}$F]APP directly targeting the antigen binding site. However the translation of a pre-targeting method to clinical practice may be hindered by the inconvenience of the technique, where a lag time of 24-72 hours between administration of the APP and radiolabelled prosthetic group is not uncommon [28, 78], relative to a method that involves a single injection and rapid scanning protocol which would be more suitable.

2.2.4 Summary of site-specific radiolabelling with $^{18}$F

Alongside process automation and radiosynthesis simplification, a trend towards click-chemistry methods of APP radiolabelling is apparent in the literature with some momentum towards bioorthogonal approaches. The notion of a generic radiolabelling platform by which APPs can be radiolabelled with $^{18}$F appeared unfeasible due to the striking diversity of APPs yet the encouraging developments in these bioorthogonal methods, most particularly pre-targeting methods, and the flexibility and versatility that these methods can afford relieve some of the challenges of APP radiolabelling with $^{18}$F.
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| Oxime             | pH 3-4, 50-60°C     | ≤ 40 (DCU)        | 1. Good stability of oxime-product  
2. Aniline catalyst enhances rate, even at neutral pH  
3. Full process automation                                                                                                                     | [7, 55, 70] |
| Hydrazine         | -                   | -                 | 1. Instability of hydrazine product has limited the appeal of the approach, despite rapid rates of hydrazine formation  
- $k_1$ of hydrazine and oxime (aniline catalysed), respectively: $170 \pm 10$ and $8.2 \pm 1.0 \text{ M}^{-1}$ | [55]  |
| Thiol-Michael     | pH 7-7.5, room temp | ~4 (NDC, calculated) | 1. Physiological reaction conditions  
2. Low overall yield as a result of low $[^{18}\text{F}]\text{FBEM RCY}$  
3. Requirement for reducing agent for disulphide bridging (TCEP.HCl)  
4. Process automation of $[^{18}\text{F}]\text{FBEM synthesis}$                                                                 | [57, 73] |
| CuAAC             | slightly basic, 80°C| ≤ 55 (DC)         | 1. Issues of Cu(I) toxicity  
2. Higher temperatures often used  
3. Slightly basic pH enhanced yield                                                                                                                | [76]  |
| SPAAC             | Neutral, room temp | ~58 (DC, calculated) | 1. Bioorthogonality  
   a. No Cu(I) toxicity  
   b. Mild reaction conditions                                                                                                                   | [80]  |
| IEDDA             | Neutral, room temp | ~60 (DC)          | 1. Bioorthogonality  
   a. No Cu(I) toxicity  
   b. Mild reaction conditions  
2. Pre-targeting application                                                                                                                    | [28]  |
| Al$[^{18}\text{F}]^{2+}$ | pH 4,100°C        | ≤ 45 (NDC)        | 1. Water miscible solvent enhances rate  
2. Single-vial/fully-automated methods  
3. No azeotropic distillation  
4. Electronic/steric impact of macrocyclic on APP                                                                                          | [27, 60, 36, 61] |
| **Fluoride acceptor chemistry** |                  |                   |                                                                                                                                                                                                          |      |
| Isotopic exchange | pH 9, room temp    | ≤ 65 (DCU)        | 1. Mild APP radiolabelling conditions  
2. No azeotropic distillation  
3. Competition with boron exchange (BF$_3$) side reactions                                                                                   | [64]  |

Table 5) Summary of site-specific methods using $^{18}\text{F}$ (non-decay corrected (NDC), decay-corrected (DC), decay-correction status unknown (DCU))
2.3 $^{18}$F radiolabelling methods: final remarks

A number of approaches have been adopted to radiolabel small and intermediately sized APPs with $^{18}$F. Non-site-specific methods, generally achieved using amine targeting prosthetic groups, offer a rapid approach to radiolabel native APPs allowing the potential of the radiolabelled APP to be rapidly validated. The importance of this is reiterated in a publication by Wester et al. [85], where it was argued that the ability to make a ‘go’ or ‘no go’ decision as early as possible is vital in order to help focus efforts towards the most promising radiotracers. Non-site-specific radiolabelling methods can, arguably, be used to identify these strong APP candidates, yet it should be noted that different radiolabelling methods can alter a radiotracer’s pharmacokinetics, metabolism and excretion [38] [36] [86].

Site-specific methods of APP radiolabelling have become the preferred approach to APP radiolabelling [33]. Site-specific methods help safeguard the biological properties of the APP antigen-binding site, provide consistency in the final radiolabel product and also permit exploitation of the faster reaction rates (Table 5) [30, 33]. However, site-specific approaches often require APP-functionalisation and whilst functionalisation of smaller APPs is often possible during solid phase peptide synthesis, functionalisation of larger, more complex, multi-domain APPs often requires a different approach. Theile et al. [87] report an enzyme-mediated method by which APPs can be functionalised. The enzyme, sortase, is expressed by gram-positive bacteria and involved in cell-wall synthesis. It is within the pocket of the sortase enzyme that an APP and a derivatised small peptide, bearing the sortase recognition motif, are conjugated [87]. It is clear that APP-functionalisation has time and monetary implications, most particularly using multi-step, enzyme-mediated derivatisation methods. Such investments may well be worthwhile, in order to exploit the advantage of site-specific methods, if the potential of an APP as a radiotracer has already been established.
It may, therefore, be concluded that non-site-specific radiolabelling approaches serve as rapid tool to, first determine the feasibility of APP radiolabelling and second, the usefulness of the labelled APP as a PET radiotracer, before adapting the approach to permit site-specific APP radiolabelling.

3. $^{89}$Zr radiolabelling of large APPs

An important category of large APPs is the mAb, the high target selectivity and affinity of mAbs is well understood and valued in medical fields, this is reflected in the number of immunoglobulin-based therapeutics $^{[17]}$. Their prevalence in nuclear medicine is a natural extension of their popularity in other areas of medicine and thus their use as APP-based radiotracers is likely to continue. As a result of their prevalence, mAb radiolabelling methods are well-established. A popular radionuclide for mAb radiolabelling is $^{89}$Zr, which is the best of the available radioisotopes for mAb radiolabelling owing to its physical characteristics (Table 3) $^{[41]}$. There is additional 909 keV gamma emission in the decay profile of $^{89}$Zr, but this is far from the 511 keV annihilation photon energy therefore has little impact on PET count rate and quantitative bias $^{[3, 32]}$.

$^{89}$Zr requires the use of a chelate for APP radiolabelling and the hexadentate chelate desferrioxamine (Df) and its analogues have commonly been used for this purpose $^{[32]}$, Figure 12 shows two commonly used Df analogues: tetrafluorophenol-N-succinyldesferal (TFP-N-sucDf) and an isothiocyanate-bearing Df chelate (Df-Bz-NCS). Df is a bacterial siderophore and the natural binding partner of Fe$^{3+}$ and is used in medical applications to remove excess iron from the body $^{[88]}$.

Df binds $^{89}$Zr$^{4+}$ with exploitable stability, this is reflected in the high in vitro plasma stability of the [$^{89}$Zr]Df complex reported by Meijs et al. $^{[89]}$ where minimal loss of $^{89}$Zr$^{4+}$ (0.2 %) from the chelate over 24 hours was observed. More recently, Holland et al. $^{[90]}$ report less than 2 % complex demetallation after 7 days, using in vitro stability tests,
concluding that the stability of the complex is sufficient for the timescale of mAb-based radiotracers.

Holland et al. \[90\] used density functional theory (DFT) to show that the ligation of one to two water molecules to the chelate was thermodynamically more favourable, with cis-coordination of two water molecules resulting in the highest stability (95 kJ mol\(^{-1}\)) \[90\].

Figure 13 shows the chelation of \(^{89}\)Zr\(^{4+}\) by Df and two additional cis water molecules. This increased the coordination number (CN) of the complex from 6, as is favourable for Fe\(^{3+}\), to 8, pointing towards a difference in complexation patterns of the two metals. This might be explained by the smaller ionic radius of Fe\(^{3+}\) compared to Zr\(^{4+}\) (0.68 and 0.84 Å respectively) and the preference of larger metals to have larger CNs. The development of zirconium-specific chelates that fulfil the metal’s oxophilic, octadentate and size characteristics is an ongoing area of research \[88\].
3.1 $^{89}\text{Zr}]N$-Suc-Df

In 2002, Verel et al. $^{[31]}$ developed a $^{89}\text{Zr}$ radiolabelling protocol using a Df-analogue, TFP-N-sucDf (Figure 12). The method is based on the synthesis of the modified Df-chelate, reported by Herscheid et al. $^{[91]}$. The chelating agent, which is now commercially available, is prepared with a chelated Fe(III) atom in order to temporarily protect the reactive hydroxamate group. After conjugation to the mAb, Fe(III) is removed using ethylenediaminetetraacetic acid using EDTA. Usefully, the presence of the Fe(III) chromophore means that the modification of the mAb with the chelate can be monitored using HPLC $^{[31]}$.

Verel et al. $^{[31]}$ describe the application of the procedure to radiolabel U36, a mAb which binds to the cell-surface glycoprotein CD44: a marker associated with a more aggressive tumour phenotype and treatment resistivity $^{[92]}$. Pre-modification of U36 with TFP-N-Suc-Df achieved conjugation yields of 54 % (room temperature, pH 9.5-9.7), using an approximate 2:1 ratio of chelate to mAb. N-suc-Df modified U36 radiometallation yields of 80 %, according to HPLC, were reported alongside RCP of 95 % (neutral pH, room temperature) $^{[31]}$. In vitro and in vivo analyses confirmed stability of $[^{89}\text{Zr}]$U36; pre-clinical
studies showed specific and sensitive detection in tumour-bearing mice, with improving tumour to background ratios from 24 to 72 hours \[^{31}\].

### 3.2 \[^{89}\text{Zr}]\text{DF-Bz-NCS}

The multi-step procedure described by Verel \textit{et al}.\[^{31}\] using TFP-\textit{N}-sucDF was described as ‘relatively complicated’ and ‘time-consuming’ by Perk \textit{et al}.\[^{93}\], resultantly its translation to clinic was challenging. As a consequence, the methodology was later modified in 2010 by Vosjan \textit{et al}.\[^{32}\] and makes use of the \[^{89}\text{Zr}^{4+}\] chelate DF-Bz-NCS (Figure 10), which is commercially available. Modifications to the chelate improved its stability, permitting removal of \(^{2+}\text{Fe}\) protection step, and ensured shorter reaction \[^{32}\]. Between 0.3 and 0.9 chelates per antibody were reported using the method (37 °C, pH 8.9-9.1). The method was used to radiolabel U36, similarly to Verel \textit{et al}.\[^{31}\], and obtained radiometallation yields of 85 % (neutral pH, room temperature) and RCP of 95 % \[^{32}\]. Pre-clinical evaluation in tumour-bearing mice produced comparable yields to those reported in the study by Verel \textit{et al}.\[^{31}\].

### 3.3 \[^{89}\text{Zr}\] radiolabelling: final remarks

Antibodies are abundantly used APPs and their popularity as immunoglobulin-based therapeutics reflects their prevalence in many fields of medicine. Correspondingly, the radiochemistry of mAb radiolabelling is well-established; popular current methods of mAb radiolabelling make use of \[^{89}\text{Zr}\] \[^{17,32,41}\]. DF-based chelating agents are commonly used in this application and more recently, modified DF-analogues have offered greater stability and simplified methods of mAb radiolabelling, yet the development of a \[^{89}\text{Zr}\]-specific chelating agent is on-going \[^{88}\]. Other radiochemistry modifications include the work by Knight \textit{et al}.\[^{94}\] which makes used of microgram rather than milligram quantities of mAb, an important methodical modification due the high costs of mAbs.

\[^{89}\text{Zr}]\text{mAb} arguably have a limited role in diagnostic PET, which is better suited to high-throughput screening and shorter-lived radioisotopes with lower patient dose . The demand
to rapidly diagnose, stratify and monitor treatment response is growing. Nevertheless, the prevalence of mAbs is likely to ensure their sustained use in PET, but the continued convention of mAb radiolabelling approaches using long-lived radioisotopes with the advent of powerful pre-targeting methods is yet to be determined.

4. Final remarks

Although mAbs are likely to remain the prevalent APPs in PET, there are a number of very promising $^{18}$F radiolabelled APPs. The potential of $[^{18}\text{F}]$APPs hasn’t yet met its full potential due to the inherent challenges of fluoride radiolabelling and this is plain from the dearth of $[^{18}\text{F}]$APPs in routine clinical use$^{[8]}$. In response to the recognised need to harness the unmet potential of $[^{18}\text{F}]$APPs in the clinic, momentum in the field has unfolded and a diverse range of approaches have been reported. Despite the diversity of the field, a number of generic features of $^{18}$F APP radiolabelling methods have manifested, including process automation and site-specificity of methods that are high-yielding and mild. The propensity for mild APP radiolabelling conditions has given rise to an appetite for bioorthogonality. The coming together of these generic features has afforded the arrival of very promising APP radiolabelling methods that are high-yielding, site-specific and bioorthogonal. Whilst these developments are very promising, the definition of high-yielding is disputable when comparing against enzyme-mediated conjugations, where rate constants of $2.7\times10^6 \text{ M}^{-1}\text{s}^{-1}$ and yields of $>90\%$ are typical$^{[39]}$. Nevertheless, the impetus in the field of APP radiolabelling with $^{18}$F is encouraging and the concerted efforts alongside the afforded results provide assurance for the long awaited appearance of $[^{18}\text{F}]$APPs in routine clinical use.

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References


Chapter 2: Automation of $^{18}$Ffluoroacetaldehyde synthesis: application to recombinant human interleukin-1 receptor antagonist (rhIL-1RA)

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Abstract

[^18F]Fluoroacetaldehyde is a biocompatible prosthetic group that has been implemented pre-clinically using a semi-automated remotely-controlled system. Automation of radiosyntheses permits use of higher levels of ^18F whilst minimising radiochemist exposure and enhancing reproducibility. In order to achieve full-automation of[^18F]fluoroacetaldehyde peptide radiolabelling, a customised GE TRACERlab FX-FN with fully programmed automated synthesis was developed. The automated synthesis of[^18F]fluoroacetaldehyde is carried out using a commercially available precursor, with reproducible yields of 26 % ± 3 (decay-corrected, n=10) within 45 min. Fully-automated radiolabelling of a protein, recombinant human interleukin-1 receptor antagonist (rhIL-1RA), with[^18F]fluoroacetaldehyde was achieved within 2 hours. Radiolabelling efficiency of rhIL-1RA with[^18F]fluoroacetaldehyde was confirmed using HPLC and reached 20 % ± 10 (n=5). Overall RCY of[^18F]rhIL-1RA were 5 % ± 2 (decay-corrected, n=5) within 2 hours starting from 35-40 GBq of ^18F. Effective specific radioactivity measurements of 8.11-13.5 GBq/μmol were attained (n=5), a near three-fold improvement of those achieved using the semi-automated approach.

The strategy can be applied to radiolabelling a range of peptides and proteins with[^18F]fluoroacetaldehyde analogous to other aldehyde-bearing prosthetic groups, yet automation of the method provides reproducibility thereby aiding translation to Good Manufacturing Practice manufacture and the transformation from pre-clinical to clinical production.

Introduction

Positron emission tomography (PET) is an imaging modality permitting quantitative non-invasive in vivo molecular imaging; its increasing popularity in a clinical setting is attributable to its sensitivity and ability to identify unique biomarkers of disease and
capacity to classify physiological processes at the molecular level. The ability to automate radiotracer synthesis has been key in the advancement of PET. It not only improves reproducibility which eases transition to Good Manufacturing Practice (GMP) manufacture, a key objective of novel radiotracer development, but also allows GBq scale quantities of $^{18}\text{F}$ to be used with minimal radiochemist exposure. The GE TRACERlab FX series of synthesisers are commonly used as radiochemistry modules and a customised FX-FN system has been used to automate the $[^{18}\text{F}]$fluoroacetaldehyde synthesis originally described by Prenant et al. which used a semi-automated experimental setup.

$^{18}\text{F}$ is a PET radioisotope with a number of advantageous features including 97 % positron emission, low positron energy and a 109.8 minute radioactive half-life, which permits more extensive radiochemical syntheses yet also limits patient dose.

Peptides and proteins are complex and have multiple functionalities that are incompatible with direct fluorination using $^{18}\text{F}$. It is for this reason that prosthetic groups capable of being covalently linked to the peptide under mild reaction conditions are used. $[^{18}\text{F}]$Fluoroacetaldehyde is an important prosthetic group owing to its small size and, importantly, its solubility in water thereby removing the requirement for organic solvents that might denature a protein or peptide. The volatility of fluoroacetaldehyde permits purification via distillation, eliminating the requirement for solid phase extraction (SPE) or high performance liquid chromatography (HPLC) separation. To date, $[^{18}\text{F}]$fluoroacetaldehyde has been successfully implemented in a pre-clinical trial using $[^{18}\text{F}]$fluoroacetaldehyde radiolabelled recombinant human interleukin-1 receptor antagonist (rhIL-1RA), 17.5 kDa. Radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde from $^{18}\text{F}$ achieved radiochemical yields (RCY) of 31-37 % in a two-step, one-pot reaction scheme. This was achieved in a semi-automated synthesis using a remotely controlled experimental system and reached overall RCY of $[^{18}\text{F}]$rhIL-1RA ranging from 7.1 to 24.2 %.

Here, we describe the fully-automated synthesis of $[^{18}\text{F}]$fluoroacetaldehyde and subsequent radiolabelling of rhIL-1RA using a modified GE TRACERlab FX-FN. Radiolabelling of
rhIL-1RA with $[^{18}\text{F}]$fluoroacetaldehyde permits direct comparison between the methods of Prenant et al.\cite{2} and the present study.

![Reaction mechanism for the radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde](image)

**Figure 1a)** Reaction mechanism for the radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde

Figure 1a shows the reaction mechanism for the synthesis of $[^{18}\text{F}]$fluoroacetaldehyde, whilst figure 1b illustrates the mechanism of rhIL-1RA reductive alkylation with $[^{18}\text{F}]$fluoroacetaldehyde.

![Reaction pathway for the radiolabelling of rhIL-1RA through reductive alkylation](image)

**Figure 1b)** Reaction pathway for the radiolabelling of rhIL-1RA through reductive alkylation

**Experimental**

All solvents were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) and used without further purification. rhIL-1RA (100 mg/ml), anakinra (Kineret) was provided by Amgen (Thousand Oaks, CA. USA) in a formulated solution containing 100 mg anakinra, 1.29 mg sodium citrate, 5.48 mg sodium chloride, 0.12 mg disodium EDTA and 0.70 mg polysorbate 80 in 1 ml water.

$^{18}\text{F}$ was produced onsite via the $^{18}\text{O}(p, n)^{18}\text{F}$ nuclear reaction by 16.7 MeV proton bombardment of enriched $[^{18}\text{O}]\text{H}_{2}\text{O}$ using a GE PETtrace cyclotron (GE Healthcare).
Analytical HPLC was performed using a Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector) controlled by LabLogic (Sheffield, UK) Laura 3 software via a CBM-20A controller. HPLC eluate was measured for radioactivity using a Bioscan (Oxford, UK) Flowcount B-FC 3100 gamma detector. All pre-clinical PET scans were carried out using a Siemens (Oxford, UK) Inveon® PET-CT scanner.

**Radiosynthesis, GE TRACERlab FX-FN**

Figure 2 provides the schematic for the GE TRACERlab FX-FN configuration; the experimental section refers to Reactors and Vials labelled in the Figure.

**[^18F]Potassium fluoride**

Cyclotron produced ^18^F was trapped on a Sep-Pak QMA carbonated cartridge (Waters, UK) then eluted with K$_2$CO$_3$ solution from Vial 1 (0.01 M, 0.4 ml) into a 3 ml vial (Reactor 1 on Figure 2) containing Kryptofix 222 (3 mg, 8 μmol) in acetonitrile (0.7 ml). The mixture was azeotropically dried at 110 °C with 3 sequential additions of acetonitrile from Vial 2 (3 x 0.5 ml).

**[^18F]Fluoroethyltosylate (FEtTos)**

A solution of ethylene di-p-toluenesulphonate (5 mg, 13.5 μmol) in DMSO (150 μl) was added from Vial 3 (V3 on Figure 2) to dried[^18F]KF/Kryptofix complex in Reactor 1. The reactor was then heated to 80 °C and cooled to 50 °C after 10 min HPLC analysis of the crude reaction mixture was performed to measure[^18F]FEtTos RCY using a Prodigy column (10 μm, ODS (3), 100 Å. 250 x 10 mm Phenomenex UK, Macclesfield, Cheshire UK) eluted with acetonitrile:water 50:50, 6 ml/min, 254 nm, $t_R = 9$ min.

**[^18F]Fluoroacetaldehyde**

To the reactor containing[^18F]FEtTos in DMSO was added DMSO (150 μl) from Vial 4 (V4 on Figure 2). The reactor was then heated to 160 °C for 5 min and a route from
Reactor 1 to Reactor 2 was opened (through V5). [\(^{18}\text{F}\)]Fluoroacetaldehyde was distilled and collected in Reactor 2 containing pH 6 citrate buffer (150 μl, 20 mM). Distillation proceeded for 4 min to minimise DMSO carry-over. RCY 26 % ± 3 (decay-corrected, n=10).

\[^{18}\text{F}\]rhIL-1RA

\[^{18}\text{F}\]Fluoroacetaldehyde was transferred from Reactor 2 to Reactor 3 (see Figure 2) containing a solution of rhIL-1RA (30 μl, 1.7 μmol), sodium cyanoborohydride in a solution of pH 6 citrate buffer (10 μl, 1.0 M) and pH 6 citrate buffer (55 μl, 20 mM) before heating to 40 °C for 20 min. \[^{18}\text{F}\]Fluoroacetaldehyde radiolabelled rhIL-1RA was purified using SE-HPLC (GE Superdex 200 10/300 GL, PBS, 1 ml/min, 254 and 220nm, \(t_R = 15\) min) and analysed for quality control purposes using analytical SE-HPLC (GE Superdex 200 10/300 GL, PBS eluent, 0.6 ml/min, 254 and 220 nm, \(t_R = 15\) min). Overall RCY and effective specific radioactivity measurements of 5 % ± 2 and 8.11-13.5 GBq/μmol respectively (decay-corrected, n=5). Specific radioactivity measurements were calculated using a standard of rhIL-1RA as a reference for HPLC analysis.

\[^{18}\text{F}\]Fluoroacetaldehyde-2,4-DNPH

Derivatisation of \[^{18}\text{F}\]fluoroacetaldehyde was performed using the methodology reported by Prenant \textit{et al}. \[^3\]. In short, 2,4-dinitrophenylhydrazine (DNPH) (25 μl, 5 μmol) was added to \[^{18}\text{F}\]fluoroacetaldehyde (30 μl) in ethanol (60 μl). The solution was heated for 15 min at 90 °C before HPLC analysis (Phenomenex Prodigy C18, 10 μm, ODS (3), 100 Å. 250 x 10 mm, acetonitrile:water 50:50, 3 ml/min, 360nm, \(t_R = 25\) min).

Synthesis of reference compounds

Synthesis of fluoroacetaldehyde

Fluoroacetaldehyde was prepared as per the methodology reported by Prenant \textit{et al}. \[^3\]. In short, fluoroethanol (62 μl, 1 mmol) was added to pyridinium dichromate (PDC) (576 mg,
1.5 mmol) in dichloromethane (DCM) (1.5 ml) and was stirred at room temperature for 24 hours. The liquid portion was removed from the PDC precipitate and then heated to 80 °C. The distillate of which was collected in deionised H₂O (1 ml) and a sample of aqueous phase, containing the fluoroacetaldehyde, was used for HPLC analysis (Phenomenex Prodigy C18, 10 μm, ODS (3), 100 Å. 250 x 10 mm, water eluent, 2 ml/min, 277 nm, \( t_R = 8 \) min).

**Synthesis of fluoroacetaldehyde-2,4-DNPH**

To the fluoroacetaldehyde solution (0.5 ml) 2,4-DNPH phosphoric acid solution (0.8 ml, 0.2 M) was added and the mixture was heated to 90 °C for 15 min before HPLC analysis (Phenomenex Prodigy C18, 10 μm, ODS (3), 100 Å. 250 x 10 mm, acetonitrile:water 50:50, 3 ml/min, 360 nm, \( t_R = 25 \) min).

**Pre-clinical PET imaging**

All animal handling was in accordance with UK legislation under the 1986 Animals (Scientific Procedures) Act. The pre-clinical methodology followed was as described by Cawthorne *et al.*[^6] in 2011. In short, one male C57Bl6 mouse and one male Sprague-Dawley rat were anaesthetised using isoflurane (induction 4 % and maintained 1.5 %) in 70 % N₂O and 30 % O₂ mixture. \([^{18}F]rhIL-1RA\) was injected in the tail vein (17.9 MBq for the mouse and 21.4 MBq for the rat). The scans were performed on a Siemens Inveon® PET-CT scanner. The acquisition protocol consisted of the following parameters: a CT scan was performed prior to the PET acquisition to obtain the attenuation correction factors. PET data were acquired over 1 hour with the time coincidence window set to 3.432 ns and the levels of energy discrimination set to 350 keV and 650 keV. The list mode acquisition data files were histogrammed into 3D sinograms with a maximum ring difference of 79 and span 3. The list mode data of the emission scans were sorted into 16 dynamic frames (5×1 min, 5×2 min, 3×5 min, 3×10 min). Finally, the emission sinograms (each frame) were normalised, corrected for attenuation, scattering and radioactivity decay,
and reconstructed using OSEM3D (16 subsets and 4 iterations) into images of dimensions 128^3 (transaxially) × 159 (longitudinally) with 0.776×0.776×0.796 mm voxels (FOV diameter: 99.3 mm × 126.6 mm longitudinally).

**Results and discussion**

A schematic of the customised GE TRACERlab FX-FN can be seen in Figure 2. The modifications permitting automated [^{18}F]fluoroacetaldehyde synthesis and subsequent rhIL-1RA radiolabelling will now be discussed alongside details regarding process development and finally [^{18}F]rhIL-1RA pre-clinical results.

![Figure 2) Schematic of GE TRACERlab FX-FN setup](image)

**Addition of a third reactor**

A third reactor was added to the GE Tracerlab FX-FN in order to mitigate issues of detrimental peptide foaming upon prolonged exposure to a gas flow. The process of [^{18}F]fluoroacetaldehyde distillation requires constant helium flow at 15 ml/min and, in order to collect the volatile product, it was necessary to distill the [^{18}F]fluoroacetaldehyde
into a solution; it was found that this process caused excessive foaming of the peptide and prevented a high-yielding reaction. In the current setup, \([^{18}\text{F}]\text{fluoroacetaldehyde}\) is distilled into citrate buffer pH 6 (150 μl), in a second reactor, and this solution is then transferred to a third reactor. The ability to both retract and insert the needle in to and out of the solution in Reactor 3 was an important modification. In the first instance, addition of the \([^{18}\text{F}]\text{fluoroacetaldehyde}/\text{buffer solution}\) whilst the needle is in a retracted position ensures minimal exposure of the peptide to helium flow. Once the transfer of \([^{18}\text{F}]\text{fluoroacetaldehyde}\) to Reactor 2 is complete, the needle can be lowered into the reaction mixture to be transferred to the HPLC loop and injected onto the HPLC column. This, therefore, required the reactor head to be moved from its original position at Reactor 1 to Reactor 3. A buffer volume of 150 μl in Reactor 2 was required to minimise the degree of loss during the transition from Reactor 2 to 3. As a result, the peptide reaction mixture volume in Reactor 3 was kept to a minimum to maintain a high concentration for radiolabelling.

**Distillation reaction conditions**

Distillation was carried out using Reactors 1 and 2. As the reactor head had to be moved from Reactor 1 to Reactor 3, it was necessary to produce a home-made reactor, with silicon septum screw top, that fitted directly into the heating block, as can be seen in Figure 2. A temperature of 160 °C was required for distillation of \([^{18}\text{F}]\text{fluoroacetaldehyde}\) from Reactor 1 to 2. This temperature achieved reproducible RCY of the prosthetic group and minimised the degree of DMSO carry-over into the second reactor. It was established that an increase in reactor 1 temperature from 160 °C to 170 °C had minimal impact on the RCY of \([^{18}\text{F}]\text{fluoroacetaldehyde}\) and increased the amount of DMSO carry-over into Reactor 2, an undesirable result owing to the sensitivity of some peptides and proteins to DMSO.
A distillation time of 4 min was sufficient to reach a plateau in the RCY of 
\(^{18}\text{F}\)fluoroacetaldehyde. Additional time resulted in further DMSO carry-over.

Prenant et al. \cite{2} describe a flow-rate of 7-8 ml/min in the reductive alkylation of rhIL-1RA with \(^{18}\text{F}\)fluoroacetaldehyde. Therefore a flow-meter and needle-valve, connected to valve 6, were incorporated in the automated setup to control the helium flow for the distillation step between Reactor 1 and Reactor 2. A flow-rate of 15 ml/min was found to produce the optimal distillation conditions.

\(^{18}\text{F}\)Fluoroacetaldehyde syntheses reproducibly attained RCY of 26\% ± 3 (decay-corrected, n=10) within 45 min starting from 35-40 GBq of \(^{18}\text{F}\).

![Figure 3](image)

*Figure 3) Superimposed RP-HPLC radio-chromatogram showing radiosynthesis of \(^{18}\text{F}\)FeTos in DMSO (radio and 254 nm UV detection shown in brown and green respectively)*

**Synthesis of \(^{18}\text{F}\)FeTos in DMSO to remove acetonitrile evaporation step**

Block et al. \cite{7} report synthesising \(^{18}\text{F}\)FeTos in acetonitrile, achieving appreciable RCY > 90\% within 10 min. The synthesis of \(^{18}\text{F}\)fluoroacetaldehyde requires Kornblum oxidation thus requiring DMSO (Figure 1a). As a result of this and for ease of automation, the synthesis of \(^{18}\text{F}\)FeTos in DMSO was investigated. Figure 3 gives the radio-UV-chromatogram data from the synthesis of \(^{18}\text{F}\)FeTos in DMSO, achieving reproducible analytical RCY of 60\% (non-superimposed HPLC chromatograms of \(^{18}\text{F}\)FeTos
radiosynthesis can be found in supplementary figure B). As a consequence of this, DMSO was used for the synthesis of $[^{18}F]$FETos. $[^{18}F]$FETos can be seen at 9 min and the ditosylate precursor can be seen in the UV chromatogram at 25 min. It should be noted that, despite DMSO being used to carry out $[^{18}F]$FETos synthesis, an additional DMSO aliquot was required for the Kornblum oxidation and production of $[^{18}F]$fluoroacetaldehyde. This may be attributable to degradation of the initial DMSO aliquot used during $[^{18}F]$FETos synthesis.

$[^{18}F]$Fluoroacetaldehyde characterisation with 2,4-DNPH

The synthesis of $[^{18}F]$fluoroacetaldehyde was confirmed by derivatisation to the corresponding hydrazone followed by radio-HPLC analysis. 2,4-DNPH was used as the derivatising agent $[^8]$.

![Figure 4](image-url) Superimposed RP-HPLC radio-chromatogram showing characterisation of $[^{18}F]$fluoroacetaldehyde with 2,4-DNPH (radio and 254 nm UV detection shown in brown and green respectively)

Figure 4 gives the radio-UV-chromatogram data from a co-injection of isotopically unmodified-fluoroacetaldehyde-2,4-DNPH solution and $[^{18}F]$fluoroacetaldehyde-2,4-DNPH solution. 2,4-DNPH has a retention time of approximately 10 min and co-elution of
the corresponding hydrazone complex for both $[^{18}\text{F}]$fluoroacetaldehyde and isotopically unmodified fluoroacetaldehyde is seen at approximately 24 min.

**Radiolabelling of rhIL-1RA with $[^{18}\text{F}]$fluoroacetaldehyde**

Radiolabelling of rhIL-1RA with $[^{18}\text{F}]$fluoroacetaldehyde was carried out at 40 °C for 20 min. Radiolabelling efficiency of rhIL-1RA with $[^{18}\text{F}]$fluoroacetaldehyde was confirmed using HPLC and reached 20% ± 10 (n=5). The radio- and UV-chromatogram can be seen in Figure 5.

The SE-HPLC chromatogram shows the $[^{18}\text{F}]$rhIL-1RA at 9 min and $[^{18}\text{F}]$fluoroacetaldehyde at 19 min. A side-product is also observed at 23 min, thought to be as a result of a competing reaction with the reducing agent and was also seen in the original publication by Prenant et al. [2], yet the possibility of $[^{18}\text{F}]$fluoride carry-over cannot be dismissed. This would be resolved under chemical-ligation conditions on account of the enhanced stability of an oxime or hydrazide which negates the requirement of a reducing agent.

The overall RCY of $[^{18}\text{F}]$rhIL-1RA was 5% ± 2 (decay-corrected, n=5) starting from 35-40 GBq of $^{18}\text{F}$. Effective specific radioactivity measurements of 8.11-13.5 GBq/μmol were
attained (n=5) using the automated method as opposed to 0.8-5.0 GBq/μmol achieved using the semi-automated approach\cite{2}. This significant increase in specific-activity is a considerable advantage to the employ of the automated method and is a result of the ability to use higher starting levels of radioactivity (35-40 GBq compared to 1.73-3.42 GBq).

The relatively low RCY can be expected from reductive alkylation, on account of the concentration dependence of the second order rate kinetics and thus the relatively slow rates of reaction\cite{9}. The radiolabelling of a protein with $[^{18}\text{F}]$fluoroacetaldehyde is not, however, limited to reductive alkylation and the strategy can be used to site-specifically radiolabel aminooxy- or hydrazide-functionalised peptides or proteins. Additionally, the requirement to use larger reaction volumes in order to efficiently transfer reaction mixtures around the module can negatively impact the RCY. Higher RCY were attained under reductive alkylation in the original publication by Prenant et al., however the process was not compatible for translation to an automated platform\cite{2,3}.

![Figure 6](image)

*Figure 6) Superimposed SE-HPLC radio-chromatogram showing purified $[^{18}\text{F}]$rhIL-1RA (radio and 254 nm UV detection shown in brown and green respectively)*

In the semi-automated procedure, a sephadex gel was used to stabilise the rhIL-1RA which is prone to foaming, as described\cite{2}. It was not possible to stabilise the protein in this manner using the automated platform on account of the following SE-HPLC purification step. Despite the automated synthesis attaining lower overall RCY than the semi-
automated configuration, the enhanced reproducibility and considerable specific radioactivity improvements are significant advantages to use of the automated method \[^2\]. Importantly, \[^{18}\text{F}]\text{fluoroacetaldehyde}\ radiolabelling occurs in aqueous conditions and at low temperatures without the requirement for organic solvent, unlike other \(^{18}\text{F}\) prosthetic groups such as \[^{18}\text{F}]\text{fluorobenzaldehyde}\[^{10,11}\]. Such conditions are likely to be more suitable for peptides and proteins. Figure 6 shows the quality control (QC) analysis of the formulated \[^{18}\text{F}]\text{fluoroacetaldehyde}\ labelled \text{rhIL-1RA}. The SE-HPLC chromatogram shows good radiochemical purity of the final radiolabelled product, although a small shoulder can be seen on the peak attributable to a dimer of the rhIL-1RA; this was also reported by Prenant \textit{et al.} in 2010 \[^2\].

**Pre-clinical results**

\[^{18}\text{F}]\text{rhIL-1RA}\ was pre-clinically assessed using both wild type rat and mouse models and can be seen in Figure 7. According to pre-clinical analyses, the biodistribution shows no defluorination as would be evidenced by \(^{18}\text{F}\) bone uptake. Preferential distribution of \[^{18}\text{F}]\text{rhIL-1RA}\ in the kidneys and renal excretion was seen, as previously described by Cawthorne \textit{et al.} \[^6\], signifying preservation of \text{rhIL-1RA}\ integrity.

The time activity curves (TAC) (see supplementary information, Fig 1) show the concentration of \[^{18}\text{F}]\text{rhIL-1RA}\ over time in the kidneys (cortex) and heart ventricle. The TAC presented, show agreement between data acquired in the current work (shown in black) and the previous published work (shown in grey) which used a semi-automated \[^{18}\text{F}]\text{rhIL-1RA}\ radiosynthetic approach \[^6\]. The level quantified in the heart is lower than previously reported \[^6\] which is thought to be attributable to the absence of attenuation and scatter correction with the HIDAC scanner previously used when compared to the Inveon scanner used in the present study.
Summary

We report upon an automated radiosynthesis of $^{18}$F-fluoroacetaldehyde and its successful application in radiolabelling rhIL-1RA. $^{18}$F-Fluoroacetaldehyde syntheses reproducibly attained RCY of 26% ± 3 within 45 min (decay-corrected, n=10) starting from 35-40 GBq of $^{18}$F-fluoride. The automated platform additionally permitted successive radiolabelling of a protein with $^{18}$F-fluoroacetaldehyde. The 17.5 kDa protein, rhIL-1RA, was radiolabelled with $^{18}$F-fluoroacetaldehyde with labelling efficiencies of 20% ± 10 (n=5), achieving overall RCY of 5% ± 2 within 2 hours (decay-corrected, n=5) starting from 35-40 GBq of $^{18}$F-fluoride. Effective specific radioactivity measurements of 8.11-13.5 GBq/mmol.
GBq/μmol were achieved (n=5). Use of the automated radiosynthesis of $	ext{[^{18}F]}$fluoroacetaldehyde and subsequent rhIL-1RA labelling not only minimised radiochemist exposure, but importantly improved reproducibility and produced a marked improvement in specific radioactivity measurements on account of the ability to start with higher levels of radioactivity.

**Disclosure statement**

The authors declare no potential sources of conflict of interest or competing financial interest.

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**References**


Supplementary material

Figure A) Time activity curve in kidney cortex (A) and heart ventricles (B) from present study (shown in black) and previous publication by Cawthorne et al. [6] (shown in grey)
Figure B) RP-HPLC chromatogram showing radiosynthesis of $[^{18}\text{F}]$FEtTos in DMSO i) radio-chromatogram and UV chromatograms at ii) 254 nm and iii) 220 nm

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Abstract

$[^{18}\text{F}]$Fluoroacetaldehyde is a small and water-soluble prosthetic group and its radiosynthesis has been successfully automated using a TRACERlab FX-FN. The current work describes extension of the radiosynthesis to an Advion® microfluidics system. Radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde and subsequent peptide radiolabelling is a 2-part reaction; $[^{18}\text{F}]$fluoroacetaldehyde synthesis using the microfluidic module concentrating units eases translation of the prosthetic group to the microfluidic flow-reactor for peptide radiolabelling. $[^{18}\text{F}]$Fluoroacetaldehyde is produced in a one-pot, two-step reaction from a commercially available precursor, ethylene di($p$-toluenesulfonate) and makes use of distillation for purification. The process of distillation in the current work can be applied to the purification of other volatile radiolabelled prosthetic groups for subsequent use in microfluidic peptide radiolabelling.

$[^{18}\text{F}]$Fluoroacetaldehyde radiosynthesis using the Advion® microfluidics system achieved radiochemical yields of 25 ± 3 % within 45 minutes (decay-corrected, n=5).

Introduction

$[^{18}\text{F}]$Fluoroacetaldehyde is a prosthetic group suitable for use in affinity peptide and protein (APP) radiolabelling owing to its small size and water-solubility. Previously, this group reported the development of $[^{18}\text{F}]$fluoroacetaldehyde and its automation using a GE TRACERlab FX-FN \cite{1}, achieving $[^{18}\text{F}]$fluoroacetaldehyde radiochemical yields (RCY) similar to those attained using the original semi-automated setup devised by Prenant et al. \cite{2,3} (26 ± 3 % RCY, decay-corrected) . The preparation of $[^{18}\text{F}]$fluoroacetaldehyde uses distillation as a means of purification, which was successfully implemented using the TRACERlab FX-FN. Radiosynthesis automation is an important developmental step on account of enhanced reproducibility, minimised human-error and allows the radiochemist to start with a larger amount of $^{18}\text{F}$ whilst minimising radiation exposure. The current work presents the automated radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde, including distillation
purification, using an alternative radiosynthetic platform; the Advion® microfluidics system.

Microfluidic radiosynthesisers have boasted higher reaction yields achieved in shorter reaction times and permit rapid optimisation of reaction conditions \cite{4-6}. The intention of translating $^{18}$F-fluoroacetaldehyde to another radiosynthetic module was to enhance the accessibility of the prosthetic group in radiochemistry. APP radiolabelling with the $^{18}$F-prosthetic groups is a 2-part reaction, comprising the radiosynthesis of $^{18}$F-fluoroacetaldehyde and subsequent APP radiolabelling. The translation of $^{18}$F-fluoroacetaldehyde to the concentrating units of the microfluidics system facilitates facile integration of the prosthetic group to the loop and flow-reactor components of the radiosynthesisers; allowing exploitation of the reported advantages of microfluidic syntheses for APP radiolabelling \cite{7}. Automation of $^{18}$F-fluoroacetaldehyde distillation purification using the microfluidic module highlights the potential of applying the procedure to other small and volatile radiolabelled molecules; this includes $^{18}$F-fluoroethylazide employed in APP labelling via azide-alkyne cycloadditions under both strain-promoted and copper-catalysed conditions \cite{8,9}.

**Experimental**

Solvents were purchased from Sigma-Aldrich (Sigma-Aldrich, UK) and used without further purification. $^{18}$F was produced onsite via the $^{18}$O($p, n$)$^{18}$F nuclear reaction by 16.7 MeV proton bombardment of enriched $^{18}$O$\cdot$H$_2$O using a GE PETtrace cyclotron (GE-Healthcare). Analytical HPLC was performed using a Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector) controlled by LabLogic (Sheffield, UK) Laura 3 software via a CBM-20A controller. HPLC eluate was measured for radioactivity using a Bioscan (Oxford, UK) Flowcount B-FC 3100 gamma detector.
Radiosynthesis, Advion® microfluidics

Figure 1 provide a schematic of the Advion® microfluidics and provides further detail regarding the configuration of the radiosynthesiser.

[^18F]Potassium fluoride

Cyclotron produced ^18F (100-200 MBq) was trapped on a Chromafix cartridge (Hichrom, UK) then eluted with a solution containing K_{2}CO_{3} solution (0.01 M, 0.4 ml) and Kryptofix222 (3 mg, 8 μmol) into a 3 ml vial (Figure 1: Vial 1 on Advion® Microfluidic module). The mixture was azeotropically dried at 100 °C with 3 sequential additions of acetonitrile (1.5 ml total).

[^18F]Fluoroethyltosylate(FEtTos)

To the dried[^18F]KF/Kryptofix complex was added a solution of ethylene di-p-toluenesulphonate (5 mg, 13.5 μmol) in acetonitrile (400 μl) and reacted at 90 °C for 5 minutes. This was followed by acetonitrile evaporation until dryness (90 °C, under vacuum). Vial 1 was then cooled to 50 °C for addition of DMSO and subsequent Kornblum oxidation. The identity of[^18F]FEtTos was confirmed using reverse phase- (RP-) HPLC, according to conditions previously employed\[^{1,3}\], using a Prodigy C18 column (10 μm, ODS (3), 100 Å. 250 x 10 mm, Phenomenex UK, Macclesfield, Cheshire UK) and eluted with acetonitrile:water 50:50, 6 ml/min, 254 nm, t_{R} = 9 min\[^{[1]}\] (data not shown).

[^18F]Fluoroacetaldehyde

To Vial 1 containing dried[^18F]FEtTos was added DMSO (300 μl) from Concentrator 2 (Figure 1).[^18F]FEtTos oxidation with DMSO was then carried out (155 °C for 5 min) before distillation and trapping of[^18F]fluoroacetaldehyde from Vial 1 into Vial 2 containing deionised water (150 μl). For this, a route between Vials 1 and 2 was opened (Figure 1) and a N_{2} flow-rate of 40 ml/min was applied. The temperature in Vial 1 was decreased to 140 °C after a transfer of activity was observed from Vial 1 to Vial 2, followed by a further decrease in temperature to 130 °C after 2 minutes. The identify of
[\textsuperscript{18}F]fluoroacetaldehyde was confirmed using RP-HPLC with co-injection of isotopically unmodified fluoroacetaldehyde according to conditions previously employed\textsuperscript{[1, 3]} (Phenomenex Prodigy C18, 10 μm, ODS (3), 100 Å. 250 x 10 mm, water, 2 ml/min, 277 nm, \( t_R = 8.2 \) min).

**Results and discussion**

The Advion® microfluidic system consists of ‘concentrating’ units used for fluoride-18 drying, shown in Figure 1, and a ‘discovery’ module, comprising reaction loops and flow-reactor components. For the reasons already discussed, radiosynthesis of [\textsuperscript{18}F]fluoroacetaldehyde was carried out using the concentrating units of the module to allow for delivery of the prosthetic group to the discovery module for subsequent bioconjugation to APP. As a result, it was necessary to modify the concentrating units to permit [\textsuperscript{18}F]fluoroacetaldehyde synthesis. Modification to the unit as well as amendments to the published radiosynthesis of [\textsuperscript{18}F]fluoroacetaldehyde are discussed below.

The original publication reporting [\textsuperscript{18}F]FEtTos radiosynthesis made use of acetonitrile\textsuperscript{[10]}. Morris \textit{et al.}\textsuperscript{[1]} describe using DMSO for [\textsuperscript{18}F]FEtTos radiosynthesis, owing its role in the subsequent Kornblum oxidation to [\textsuperscript{18}F]fluoroacetaldehyde and in order to simplify the method for automation. In the current work, acetonitrile was found to be the most appropriate solvent for [\textsuperscript{18}F]FEtTos radiosynthesis, according to the method described by Block \textit{et al.}\textsuperscript{[10]}. This is as a result of the lower chemical purity of [\textsuperscript{18}F]fluoroacetaldehyde when DMSO was used, ascribed to DMSO carry-over with the distillate. It was, therefore, necessary to implement an additional acetonitrile evaporation step to yield dry [\textsuperscript{18}F]FEtTos that could be re-dissolved in a smaller volume of DMSO for Kornblum oxidation. The process of acetonitrile evaporation produced minimal loss in radioactivity (<10 %) and was rapidly achieved (10 minutes).
Two 3-way valves were added to the microfluidic module, this can be seen on Figure 1 and has been labelled as point 1. The purpose of the valves was three-fold: firstly, the valves permit generation of a vacuum in Vial 1 for azeotropic drying and solvent evaporation steps whilst secondly, providing a vent during addition of material into Vial 1 and finally, the valves supply a singular and direct route of $[^{18}\text{F}]$fluoroacetaldehyde distillation to Vial 2.

The azeotropic drying and subsequent solvent evaporation steps require a strong nitrogen flow, yet the process of distillation requires a steady and low flow. For this reason, it was necessary to install a 3-way Valco VICI® valve with additional flow-meter and needle-valve. This configuration can also be seen in Figure 1 and has been labelled as point 2; position A of the Valco VICI® valve permits a high flow of nitrogen to Vial 1, whilst position B restricts the flow of nitrogen through use of the flow-meter and needle valve. It was possible to monitor the distillation of $[^{18}\text{F}]$fluoroacetaldehyde through use of two point-radioactivity detectors placed on both Vial 1 and 2.

Figure 1) Schematic of the modified Advion® microfluidics system setup
A decrease in radioactivity observed in Vial 1 was paralleled by an increase in radioactivity in Vial 2. Cessation of distillation was signalled by a plateau in the amount of radioactivity seen in Vial 2. The optimal time for $[^{18}\text{F}]$fluoroacetaldehyde distillation was 8 minutes, using an N$_2$ flow of 40 ml/min; this provided reproducible RCY and also ensured minimal DMSO carry-over, which was monitored by UV absorbance at 220 nm using RP-HPLC (DMSO UV absorbance can be seen in supplementary figure). The radiochromatogram of the $[^{18}\text{F}]$fluoroacetaldehyde distillate can be seen in supplementary data and confirms good radiochemical purity (> 98 %). Automated radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde using the concentrating units of the microfluidic system achieved RCY of 25 ± 3 % (decay-corrected, n=5) within 45 minutes; comparable to the time and yields attained using automated radiosynthesis on a GE TRACERlab FX-FN (26 ± 3 %, decay-corrected, n=10).

**Summary**

We have previously reported the automation and radiosynthesis of $[^{18}\text{F}]$fluoroacetaldehyde using a GE TRACERlab FX-FN, the current work presents expansion of $[^{18}\text{F}]$fluoroacetaldehyde radiosynthesis to an Advion® microfluidics system. The customised configuration of the Advion® microfluidics system achieved comparable $[^{18}\text{F}]$fluoroacetaldehyde RCY to those attained in the automated radiosynthesis on a modified GE TRACERlab FX-FN. [1]

Although the current work describes the process of distillation as a means of product purification for $[^{18}\text{F}]$fluoroacetaldehyde, a similar approach can be adopted for the distillation of other volatile radiolabelled molecules such as $[^{18}\text{F}]$fluoroethylazide for alkyne-azide cycloadditions; the generally adopted method of which uses distillation purification [11, 12]. Radiosynthesis of such compounds permits facile translation of the prosthetic groups to the loop and flow-reactor components of the system for subsequent
APP radiolabelling; thereby presenting the microfluidic system as an attractive all-in-one APP radiolabelling platform.

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References

Supplementary material

Superimposed RP-HPLC radio-chromatogram of purified $[^{18}F]$fluoroacetaldehyde (radio and UV signal seen in brown and green respectively)
Chapter 4: Development & Automation of a novel $^{18}$F prosthetic group, $2-[^{18}$F]-fluoro-3-pyridinecarboxaldehyde, and its application to an aminooxy-functionalised Aβ peptide.

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Abstract

2-[18F]-Fluoro-3-pyridinecarboxaldehyde ([18F]FPCA) is a novel, water-soluble prosthetic group. Its radiochemistry has been developed and fully-automated for application in chemoselective radiolabelling of aminooxy-derivatised RI-OR2-TAT peptide, Aoa-k-RI-OR2-TAT, using a GE TRACERlab FX-FN. RI-OR2-TAT is a brain-penetrant, retro-inverso peptide that binds to amyloid species associated with Alzheimer’s Disease. Radiolabelled Aoa-k-RI-OR2-TAT was reproducibly synthesised and the product of the reaction with FPCA has been fully characterised. In vivo biodistribution of [18F]RI-OR2-TAT has been measured in Wistar rats.

Background

Positron emission tomography (PET) is a highly-sensitive and quantitative molecular imaging modality, enabling non-invasive investigation of physiological events at the molecular level. The advent of novel radiotracers for use in PET has facilitated the discovery of influential pathological biomarkers. Automation of radiosyntheses aids translation from pre-clinical to clinical studies by enhancing reproducibility and reliability. Automation is, therefore, an important step in radiotracer development. The GE TRACERlab FX-FN is a popular radiosynthesis module which is easily adaptable to automate a variety of radiosynthetic routes.

Taylor et al. and Parthsarathy et al. [1, 2] have reported the development of RI-OR2 (Ac-rGffvlkGr-NH₂), a retro-inverso peptide comprised of D-amino acids which confers high proteolytic stability to the peptide. RI-OR2 binds to amyloid species associated with Alzheimer’s Disease (AD) with nanomolar affinity (kd = 58-125 nM) and has been identified as an inhibitor of early-stage amyloid aggregates [2]. More recently, RI-OR2 was functionalised with a retro-inverso TAT motif to aid its crossing the blood brain barrier (BBB), resulting in the sequence Ac-rGffvlkGrrrrrqrrkkrGy-NH₂, and a molecular weight of
TAT is the HIV protein transduction domain and has been reported to enhance cell-permeability and aid transport across the BBB.\(^3\) Radiolabelling of RI-OR2-TAT would establish its usefulness as a PET radiotracer for use in AD. In order to do this, it was first necessary to devise a radiosynthetic strategy to radiolabel RI-OR2 with the \(^{18}\text{F}\), a PET isotope with an appropriate radionuclide half-life (\(t_{1/2}\) 109.8 min).

Here we report a novel, water-soluble prosthetic group, 2-[\(^{18}\text{F}\)]fluoro-3-pyridinecarboxaldehyde (\([\^{18}\text{F}]\text{FPCA}\)) which has been developed for use in peptide and protein radiolabelling. Its solubility in aqueous media makes it highly suitable for radiolabelling peptides and proteins, such as RI-OR2-TAT, that are sensitive to organic solvent. The half-life of \(^{18}\text{F}\) (\(t_{1/2}\) 109.8 min.) not only permits multi-step syntheses but is also commensurate with the biological half-life of peptides and many small proteins, such as RI-OR2-TAT. \([\^{18}\text{F}]\text{FPCA}\) was produced in a one-pot, one-step direct \([\^{18}\text{F}]\text{fluorination of a trimethylammonium precursor, 3-carboxaldehyde-N,N,N-trimethylpyridine-2-aminium bromide, that was synthesised in-house. The }^{18}\text{F}-\text{fluorination of the precursor is depicted in Figure 1a.}\]

\[
\begin{align*}
\text{N(Me)}_3^+ & \quad \text{Br}^- \\
\text{OX} & \quad \text{KF/crown} \\
\text{DMSO, 70 °C} & \quad \text{OX}^{18}\text{F} \\
\text{3-carboxaldehyde-\textit{N,N,N}-trimethylpyridine-2-aminium bromide} & \quad \text{2-[\^{18}\text{F}]fluoro-3-pyridinecarboxaldehyde (\([\^{18}\text{F}]\text{FPCA}\))}
\end{align*}
\]

\textit{Figure 1a) Reaction pathway showing the radiosynthesis of \([\^{18}\text{F}]\text{FPCA}\)}

In order to preserve the biological activity of the peptide, it was necessary to site-specifically radiolabel RI-OR2-TAT. Therefore, the peptide was further modified with an aminooxy-moiety to permit chemoselective labelling with \([\^{18}\text{F}]\text{DFPCA}\) via oxime bond formation.\(^4\) For this, an additional D-lysine residue was linked to the C-terminal of the RI-OR2-TAT sequence, to which the aminooxy-functionality was added to the \(\varepsilon\)-amine...
group, resulting in Aoa-k-RI-OR2-TAT. The radionabelling pathway can be seen in Fig. 1b. It has been reported the derivatisation of the C-terminal belonging to the TAT fragment does not impact its ability to mediate cellular uptake [6].

Oximation of aminooxy-functionalsed peptides with the aldehydic prosthetic group \([^{18}F]f\text{luorobenzaldehyde (}[^{18}F]FBA)\ has, to date, been widely reported upon [7-9]. However, the lipophilicity of \([^{18}F]FBA\) makes radionabelling of proteins sensitive to organic solvents both problematic and unreliable. This prompted the development of \([^{18}F]FP\), on account of its enhanced hydrophilicity. Diversely functionalised \(^{18}F\)-fluoropyridine-based prosthetic groups have been used for chemoselective \(^{18}F\)-labelling of macromolecules. This includes \([^{18}F]FP\text{M}e^{[10]}\), a maleimide derivative for terminal cysteine conjugation, and \([^{18}F]FP\text{KYNE}\), employed in azide-alkyne cycloaddition click chemistry [11]. Pyridine-based \(^{18}F\)-prosthetic groups are not only interesting in terms of the hydrophilic character but also the electronic properties of the ring which enables facile \(^{18}F\)-aromatic nucleophilic substitution.

This peptide radionabelling strategy presented here can be applied to both aminooxy-derivatised peptides, as has been described, as well as non-functionalised, native macromolecules via reductive alkylation of the \(N\)-terminal amine or \(\varepsilon\)-amine group of lysine residues [12, 13].

![Reaction pathway showing the conjugation of \([^{18}F]FP\) to N-\(\varepsilon\)-aminooxy-D-lysine (Aoa-k) modified peptide (Aoa-k-RI-OR2-TAT) through oxime bond formation](image)

*Figure 1b) Reaction pathway showing the conjugation of \([^{18}F]FP\) to N-\(\varepsilon\)-aminooxy-D-lysine (Aoa-k) modified peptide (Aoa-k-RI-OR2-TAT) through oxime bond formation*
Here, we describe development and automation of $^{18}$F]FPCA peptide radiolabelling with application to an Aoa-k-RI-OR2-TAT, process automation using a customised GE TRACERlab FX-FN together with \textit{in vitro} investigation and pre-clinical metabolite and biodistribution analysis in Wistar rats.

**Experimental**

All solvents were purchased from Sigma-Aldrich and used without further purification. The aminooxy-peptide was purchased from Biomatik (Ontario, Canada) in acetate salt at a purity of $>98\%$. $^{18}$F was produced onsite via the $^{18}$O($p$, $n$)$^{18}$F nuclear reaction by 16.7 MeV proton bombardment of enriched $[^{18}$O]$H_2O$ using a GE PETtrace cyclotron (GE Healthcare). Analytical HPLC was performed using a Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector) controlled by Laura 3 software (LabLogic, Sheffield, UK), Laura 3 software via a CBM-20A controller. HPLC eluate was measured for radioactivity using a Bioscan (Oxford, UK) Flowcount B-FC 3100 gamma detector. All pre-clinical PET scans were carried out using a Siemens (Oxford, UK) Inveon® PET-CT scanner. MALDI-MS was carried out using AXIMA performance MALDI-TOF MS (Shimadzu, UK). Mass spectra were acquired using a Waters SQD2 (Waters, UK) and $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer (Bruker, US) operated with TOPSPIN NMR software (version 2.0).

**Radiosynthesis, GE TRACERlab FX-FN**

Figure 2 provides the schematic for the GE TRACERlab FX-FN configuration; the experimental section refers to Reactors and Vials labelled in the Figure.
[\textsuperscript{18}F]Potassium fluoride

Cyclotron produced \textsuperscript{18}F was trapped on a Sep-Pak QMA cartridge (Waters, UK) then eluted with K\textsubscript{2}CO\textsubscript{3} solution from Vial 1 (0.01 M, 0.4 ml) into a Reactor 1 containing 18-crown-6 (8 mg, 30 \textmu mol) in acetonitrile (0.6 ml). The mixture was azeotropically dried with 3 sequential additions of acetonitrile from Vial 2 (1.6 ml total) at temperature of 90 °C.

[\textsuperscript{18}F]FPCA

To the reactor containing azeotropically dried \textsuperscript{18}F a solution containing 3-carboxaldehyde-\textit{N,N,N}-trimethylpyridine-2-aminium bromide (2 mg, 8 \textmu mol) in DMSO (200 \textmu l) from Vial 3 was added and heated to 70 °C for 10 min. \textsuperscript{18}F]FPCA was purified using AFFINIMIP\textsuperscript{\textregistered} (AFFINISEP, France) 2.0 ml SPE cartridges. Elution from the SPE cartridge was achieved using methanol from Vial 9 (3 ml) followed by solvent evaporation at 60 °C, under vacuum for 6.5 min. The chemical identity of \textsuperscript{18}F]FPCA was determined by comparing its chromatographic properties with those of the isotopically unmodified FPCA (obtained from Sigma-Aldrich, UK) using RP-HPLC using a Prodigy C18 ODS (3) column, 10 μm, 100 Å. 250 x 10 mm column (Phenomenex UK, Macclesfield, Cheshire UK) eluted with 20 % methanol with 0.1 % TFA, 5 ml/min, 254 nm, \( t_R = 11 \) min.

[\textsuperscript{18}F]RI-OR2-TAT

A solution of Aoa-k-RI-OR2-TAT (2 mg, 740 nmol), gentisic acid sodium salt (1 mg) anilinium chloride (2.6 mg) and citric acid (0.02 M, 250 \textmu l) was added to the purified and dried \textsuperscript{18}F]FPCA (pH 2.7). The reaction mixture was heated to 50 °C for 20 min and purified using SE-HPLC (Superdex peptide 10/300 GL, PBS with 1 % ascorbic acid, 1 ml/min, 280 nm, \( t_R \) 16.5 min) and analysed for quality control purposes using RP-HPLC using a Jupiter C4 column (5 μ 300 Å 250 x 10 mm, Phenomenex UK, Macclesfield, Cheshire, UK) and eluted using a water (solvent A) and acetonitrile (solvent B) solvent
system (gradient with respect to solvent B: 10 – 85 % over 25 min, 3 ml/min at 280 nm, tR 13.5 min).

**Synthesis of stable compounds**

**3-Carboxaldehyde-N,N,N-trimethylpyridine-2-aminium bromide**

The precursor was synthesised according to a procedure reported in the literature \(^{14,15}\). In short, 1.1 ml trimethylamine (1M solution in THF, Fisher Scientific, UK) was added dropwise to a solution containing 2-bromo-3-pyridinecarboxaldehyde (1 mmol) in DMF 0.5 ml). The reaction mixture was left under stirring at 70 °C for 72 hours. The white precipitate was collected by filtration, washed with Et₂O (50 ml) and cold DCM (10 ml) and dried under vacuum. The product 3-carboxaldehyde-N,N,N-trimethylpyridine-2-aminium bromide was obtained with 80 % yields. ESI-MS: [M - Br]⁺ = 165. \(^1\)H-NMR (500 Mz, DMSO-d₆): 10.32 (s, 1H, CHO), 8.86 (d, 1H, J 3.0 Hz, Ar H-6), 8.80 (d, 1H, J 7.5 Hz, Ar H-4), 8.06 (dd, 1H, J 7.5, 4.5 Hz, Ar H-5), 3.71 (s, 9H, NMe₃).

\(^{13}\)C-NMR (125 Mz, DMSO-d₆): 190.8 (C=O), 151.8 (Ar C-3), 150.6 (Ar C-6), 147.2 (Ar C-4), 126.6 (Ar C-5), 124.6 (Ar C-2), 54.0 (NMe₃).

\[^{19}\text{F}]\text{RI-OR2-TAT}\)

To a solution of Aoa-k-RI-OR2-TAT (2.9 mg, 1.01 µmol) and anilinium chloride (1.3 mg, 10 µmol) in acetate buffer (500 µl, pH 4.7) was added 13 µl of a 0.1M solution of 2-fluoro-3-pyridinecarboxaldehyde (Sigma Aldrich, UK) in ethanol. The mixture was incubated at 50 °C for 4 hours before being purified by SE-HPLC (GE Superdex peptide 10/300 GL, PBS, 1 ml/min, 280 nm, tR 16.5 min). The collected fraction was loaded onto an Oasis HLB cartridge (Waters) and the product was eluted with ethanol. Evaporation of the solvent and drying of the product in a vacuum desiccator yielded 1.9 mg of \[^{19}\text{F}]\text{RI-OR2}\) (yield = 64 %). Labelling of Aoa-k-RI-OR2-TAT was verified using MALDI-MS using the following procedure. The sample (1 µl) was spotted onto a MALDI steel target plate, followed immediately by an equivalent volume of matrix (10 mg/ml α-cyano-4-
hydroxycinnamic acid in 50 % acetonitrile). $[^{19}\text{F}]$RI-OR2-TAT was analysed in reflectron mode.

$[^{19}\text{F}]$RI-OR2-TAT thioflavin-T assay (ThT)

Thioflavin-T (ThT) assays were carried out at Lancaster University as previously described by Taylor et al. $[^1]$. In short, Aβ peptides (25 μM) were incubated with ThT (15 μM) at 25 °C in 96-well clear-bottom microtiter plates (NUNC). Inhibitor (RI-OR2-TAT or $[^{19}\text{F}]$RI-OR2-TAT) was added in molar ratios of 1:1, 1:2, 1:10 relative to Aβ. Plates were read every 10 min. ($\lambda_{\text{ex}}$ 442 nm and $\lambda_{\text{em}}$ 483 nm) using a BioTek Synergy plate reader (Swindon, UK) for a total of 48 hours.

Pre-clinical PET analyses

All animal handling was in accordance with UK legislation under the 1986 Animals (Scientific Procedures) Act.

Pre-clinical PET

Wistar rats (n = 3) were anaesthetised using isoflurane (induction 4 % and maintained 1.5 %) in 70 % N₂O and 30 % O₂ mixture. 44-55 MBq of $[^{18}\text{F}]$RI-OR2-TAT was injected in the tail vein. All scans were carried out using a Siemens Inveon® PET-CT scanner. The acquisition protocol parameters consisted of a preliminary CT scan to attain attenuation correction factors followed by a 1 hour PET acquisition with time coincidence window of 3.432 ns and levels of energy discrimination set between 350 keV and 650 keV. Images were reconstructed and analysed as described elsewhere $[^{16}]$.

Metabolite analysis

For metabolite analysis, Sprague-Dawley rats (n=4) were used and analysis carried out as described by Cawthorne et al. $[^{17}]$. In short, brain, liver and blood samples (250 μl) were taken 5 (n = 2) and 20 minutes (n = 2) post injection of $[^{18}\text{F}]$RI-OR2-TAT. Liver and brain were quickly removed and homogenised (Ultra-turrax®, Sigma-Aldrich) in 2 ml of either
ice-cold acetonitrile or PBS. The samples were centrifuged (3 min, 9000 g, 4 °C, Thermo ALC multispeed refrigerated centrifuge PK121R (Thermo Fisher Scientific, UK) and the supernatant was separated from the pellet. For each homogenate and supernatant, an aliquot was counted using a γ-counter. Blood samples were centrifuged using a Thermo ALC multispeed refrigerated centrifuge (5 min, 2200 g, 4 °C) and the plasma was collected and added to either PBS or acetonitrile. Brain, liver and plasma samples in both acetonitrile and PBS were then analysed using RP-HPLC to determine the non-polar and polar metabolites respectively (Phenomenex Jupiter C4, 5 μ 300 Å 250 x 10 mm, acetonitrile gradient from 10 – 85 % over 25 min).

Results and discussion

Radiochemistry development & automation

Figure 2 shows the customised configuration of the GE TRACERlab FX-FN permitting [18F]FPCA radiosynthesis, subsequent peptide labelling and product purification. In initial productions [18F]FPCA was purified by means of RP-HPLC, followed by SPE concentration, Figure 3 shows the chromatogram of the crude fluorination reaction mixture.

According to HPLC data seen in Figure 3, the incorporation of 18F is higher than 85 %, (n=20) and remained reproducible. [18F]FPCA can be seen with a retention time of 9.5 min with residual 18F seen at 3.5 min.

Replacement of RP-HPLC purification with a SPE method was implemented in the interest of time reduction but also to simplify automation and improve reproducibility. Due to the polar nature of the prosthetic group, it was not suitable for use with RP-SPE cartridges and use of a hydrophobic-lipophilic balance (HLB) cartridge resulted in unwanted water content in the eluted fraction of [18F]FPCA. Subsequent evaporation of elution solvent proved very difficult and problematic for automation, owing to varying volumes between
productions. The variable volumes of co-eluted water meant that evaporation times and aqueous methanol volumes were inconsistent between reactions. As a result, a vigorous drying step was required which caused a significant loss in radioactivity and caused a marked reduction in yield. Under other conditions, excess water remaining after incomplete evaporation acted to dilute the peptide labelling reaction mixture and, again, resulted in lower radiochemical yields.

For this reason, the AFFINIMIP® SPE $^{18}$F-Aromatic Nucleophilic Substitution 2.0 ml cartridge was assessed and found to achieve both good retention and resolution of $[^{18}\text{F}]$FPCA from the trimethylammonium precursor. AFFINIMIP® SPE $^{18}$F-Aromatic Nucleophilic Substitution 2.0 ml cartridges comprise a molecular imprinted polymer that has been validated for use with the radiotracers $[^{18}\text{F}]$fluorobenzaldehyde (FBA) and $[^{18}\text{F}]$ethyl 4-fluorobenzoate. The prosthetic groups are produced from an aromatic nucleophilic substitution with the corresponding ammonium-based precursor. Good separation of the $^{18}$F-prosthetic groups, from the ammonium precursor as well as the
phenol- and dialkylamino-based impurities, is achieved using the cartridge. The radiochemical purity (RCP) and chemical purity of SPE-purified \([^{18}\text{F}]\text{FPCA}\) was verified using RP-HPLC (data not shown). Reactor 1 was cleaned by addition of ethanol (2 ml) from Vial 5 before re-addition of \([^{18}\text{F}]\text{FPCA}\) from the SPE cartridge for subsequent conjugation to Aoa-k-RI-OR2-TAT. This ensured that impurities from the fluorination of FPCA, that could impair peptide radiolabelling, were minimised.

An AFFINIMIP® SPE \(^{18}\text{F}\)-Aromatic Nucleophilic Substitution 0.7 ml cartridge was additionally assessed in the application of \([^{18}\text{F}]\text{FPCA}\) purification. It was found that elution from the 0.7 ml cartridge was more efficient and RCY of \([^{18}\text{F}]\text{FPCA}\) of 43 % were attained, however resolution from the precursor was poor which rendered the cartridge unsuitable in this application. The loss in RCY of \([^{18}\text{F}]\text{FPCA}\) using a 2.0 ml
AFFINIMIP® cartridge was preferable owing to the high chemical purity that was achieved.

Despite high analytical yields of $^{[18}\text{F}]$FPCA, as observed in Fig. 3, RCY of $28 \pm 2\%$, (decay corrected, n=10) were obtained attributable to loss during SPE purification and solvent evaporation steps. However, the AFFINIMIP® SPE $^{18}\text{F}$-Aromatic Nucleophilic Substitution cartridge has not been produced specifically for in FPCA purification resulting in low recovery of $^{[18}\text{F}]$FPCA. This would be markedly improved by using a bespoke FPCA specific molecular imprinted polymer cartridge. Despite the loss of $^{[18}\text{F}]$FPCA, owing to purification and solvent evaporation, it presents as an alternative water-soluble $^{18}\text{F}$ prosthetic group for use with peptides and proteins that are sensitive to organic solvents.

Figure 4) TRACERlab FX-FN radio-chromatogram trace showing SE-HPLC purification of $^{[18}\text{F}]$RI-OR2-TAT

Figure 4 shows the SE-HPLC radio-chromatogram of the crude $^{[18}\text{F}]$RI-OR2-TAT radiolabelling mixture. SE-HPLC was used to both purify and formulate the final
radiolabelled peptide product through use of PBS eluent. $^{18}$FRI-OR2-TAT and $^{18}$FPC can be seen with a retention times of 16.5 min. and at 27 min respectively.

A negative UV peak is observed for both the unlabelled Aoa-k-RI-OR2-TAT and FPCA precursor attributable to the high absorbance density of the ascorbate-containing mobile phase. RP-SPE cartridges were also assessed for their suitability in purifying $^{18}$FRI-OR2-TAT. The radiolabelled peptide was strongly retained and required a large volume of organic eluent, additionally co-elution of more lipophilic components and unreacted $^{18}$FPC was observed resulting in sub-standard radiochemical purity. Furthermore, $^{18}$FPC radiosynthesis was developed and automated with a view to radiolabelling further peptides and proteins, in this regard SE-HPLC serves as a method by which larger or organic-sensitive proteins can be purified and formulated.

RP-HPLC was used to assess the RCP of the final formulated $^{18}$FRI-OR2-TAT product for quality control purposes. The UV-radiochromatogram can be seen in the supplementary material (Figure a). The Figure shows a large initial peak attributable to ascorbate and two peaks at 12.75 and 13.5 min, identified as Aoa-k-RI-OR2-TAT and $^{18/19}$FRI-OR2-TAT respectively with a RCP > 90%.

The overall $^{18}$FRI-OR2-TAT RCY achieved using the automated platform was 12 ± 2% within 2 hours from end of bombardment, starting from 25-30 GBq of $^{18}$F (decay corrected, n=5). The effective specific radioactivity of $^{18}$FRI-OR2-TAT was calculated to be 3.2 ± 1.3 GBq/μmol (at end of synthesis).

Despite the appreciable radiolabelling efficiencies of $^{18}$FPC and $^{18}$FRI-OR2-TAT, the overall yield using the fully-automated platform was lower than expected and largely attributable to suboptimal elution of $^{18}$FPC from the AFFINIMIP® SPE cartridge. Overall radiolabelling yields using a semi-automated synthesis, used during early developmental stages, achieved 20 ± 5% (decay corrected, n = 5) starting with 100 MBq
of $[^{18}F]$FPCA. In which, $[^{18}F]$FPCA synthesis and SPE purification was achieved using the automated platform and $[^{18}F]$FPCA evaporation and peptide radiolabelling was completed manually (data not shown).

Figure 5 shows the MALDI-MS analysis of (Aoa-k)-RI-OR2-TAT peptide labelling with isotopically unchanged FPCA ($[^{19}F]$FPCA). $[^{19}F]$RI-OR2-TAT is shown at 2969 m/z (M+H$^+$)

Figure 5 shows the MALDI-MS analysis of $[^{19}F]$RI-OR2-TAT peptide. The $^{19}$F-labelled peptide can be seen at 2969 m/z (M+H$^+$). The results of the MALDI-MS confirm labelling of Aoa-k-RI-OR2-TAT with FPCA.

$[^{18}F]$RI-OR2-TAT in vitro analysis

ThT assay

A ThT assay was used in the original publications to assess the potency of RI-OR2 and RI-OR2-TAT against amyloid (Aβ) plaque aggregation $^{[1,2]}$. ThT has affinity for amyloid plaque, but not for unaggregated amyloid species, therefore the fluorescent intensity of a ThT corresponds to the degree of amyloid aggregation.

Although the ThT assay does not provide evidence for specific vs non-specific binding of the RI-OR2-TAT analogues, it does confirm their reactivity towards amyloid species and
permits a direct comparison of RI-OR2-TAT and [\(^{19}\text{F}\)]RI-OR2-TAT. Results of the ThT assay can be used to verify that the radiolabelling method has not modified the characteristics of the peptide.

The results of the ThT assay can be seen in Figure 6. The data indicate the percentage inhibition of amyloid aggregation in the presence of RI-OR2-TAT or cold labelled [\(^{19}\text{F}\)]RI-OR2-TAT, shown in blue and red respectively, at different inhibitor:Aβ molar ratios. Aβ aggregation, in the absence of inhibitor, can be seen in grey. The chart shows that the inhibition efficiency of [\(^{19}\text{F}\)]RI-OR2-TAT is reduced 3-fold at an inhibitor:Aβ molar ratio of 1:2. Although the ability of the modified peptide is lowered, this assay suggests [\(^{19}\text{F}\)]RI-OR2-TAT still binds, demonstrating the potential of the \(^{18}\text{F}\)-analogue as a PET tracer.

![Figure 6](image)

*Figure 6) Results of the Thioflavin-T assay (ThT) showing percentage inhibition of amyloid aggregation in presence of either [\(^{19}\text{F}\)]RI-OR2-TAT (red) or non-functionalised RI-OR2-TAT (blue). Amyloid aggregation in the absence of inhibitor (RI-OR2-TAT) is shown in grey*

**Pre-clinical results**

*In vivo* biodistribution of [\(^{18}\text{F}\)]RI-OR2-TAT was performed in Wistar rats and results of which can be seen in Figure 7 and Table 1. Overall, the highest uptake was observed in liver, with a rapid uptake reaching a plateau within 10 min post-injection, followed by
bladder (urine), kidneys, spleen, bone marrow, lungs, bone and brain (Figures 7 & 8). Some uptake could be observed in bone marrow but was distinctively higher than the low uptake observed in bone (3.188 ± 0.912 SUV and 0.380 ± 0.237 SUV respectively 60 min post-injection) (Figures 7D and 8B). Our measures by γ-counting of liver and brain samples were in good agreement with our PET quantification (Table 1). The nature of the retro-inverso peptide is such that hepatobiliary clearance is a main route of excretion instead of renal excretion, as is often observed with small peptides. The high proteolytic stability of peptide owes to the absence of blood proteases able to recognise its D-amino acid peptide bonds. It is hypothesised that the peptide accumulates in the liver before being excreted through the intestinal tract via the bile.

Figure 7) Representative sum images (20-60 min) of a Wistar rat after injection of $[^{18}F]$RI-OR2-TAT. Time activity curves in bladder, kidneys and spleen are from 1 animal. Data for bone marrow and liver are expressed as SUV (mean ± SD)
Defluorination is a key metabolic process that will limit the utility of any $^{18}$F-labelled radiotracer and is detected when accumulation of radioactivity is observed within the bone. However Figures 7 and 8 show relatively little bone uptake thereby demonstrating stability of $[^{18}\text{F}]{\text{FPCA}}$ to defluorination. An evaluation of $[^{18}\text{F}]{\text{fluoropyridines}}$ by Dolle remarks on the metabolic resistance of the $^{18}$F radiolabel in the ortho-position of pyridine-based radiotracers.

Figures 8A & C show the distribution of $[^{18}\text{F}]\text{RI-OR2-TAT}$ in rat brain. As can be seen, the brain pharmacokinetic follows a rapid peak (within 30 s post-injection) of uptake of $[^{18}\text{F}]\text{RI-OR2-TAT}$ corresponding to the first pass of the peptide with the blood flow followed by a rapid washout (Figure 8C). After 10 min, very little brain retention of $[^{18}\text{F}]\text{RI-OR2-TAT}$ is observed. This was as expected due to absence of AD pathology in the brains of Wistar rats.

Figure 8) Representative sum images (20-60 min post-injection (insert: 13-22 sec post-injection) of $[^{18}\text{F}]\text{RI-OR2-TAT}$ uptake in brain, bone and lungs (mean ± SD, $n=2$ for brain, $n=3$ for bone and lungs)
The specific radioactivity of the $[^{18}\text{F}]\text{RI-OR2-TAT}$ is currently low, however RI-OR2-TAT binds to Aβ plaques but also to Aβ oligomers. Binding to such a complex is not readily saturable, as is expected with receptor-based binding (i.e. there is no 1-1 stoichiometry but multiple binding sites per plaques or oligomers). Consequently, it is important to qualify that the specific radioactivity of $[^{18}\text{F}]\text{RI-OR2-TAT}$ does not contribute to poor brain signal. Despite the presence of multiple binding sites, transportation of $[^{18}\text{F}]\text{RI-OR2}$ across the BBB maybe hindered by competition of $[^{18}\text{F}]\text{RI-OR2-TAT}$ with unlabelled Aoa-k-RI-OR2-TAT. Therefore, an improvement in specific radioactivity by separation of labelled and unlabelled peptide would safeguard against issues of radiotracer concentration affecting its pre-clinical performance.

Table 1) Biodistribution of $\gamma$-counting of $[^{18}\text{F}]\text{RI-OR2-TAT}$ in brain, liver, whole-blood and plasma and plasma to blood ratio 5 and 20 min post-injection (n=2 per time-point, data expressed as SUV, mean ± SD)

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Table 2 shows the analysis of the polar and non-polar fractions in the brain, liver and plasma samples collected in PBS and acetonitrile portions respectively at 5 and 20 minutes post-injection.

In liver and plasma, the radioactivity detected is almost exclusively a polar species that has been identified using RP-HPLC as parent $[^{18}\text{F}]\text{RI-OR2-TAT}$; no other radiolabelled component was identified using RP-HPLC (Supplementary Figure b). In the brain, some non-polar species were detected in higher proportions than in the plasma or liver. This suggests the presence more lipophilic compounds which traverse the BBB more easily.
Considering the extremely low level of radioactivity detected in the brain (0.017 ± 0.002 SUV 60 min post-injection), further evaluation is needed to deduce the impact of such compounds on the signal to noise ratio in models of AD with detectable β-amyloid. The results of this analysis, however, confirm the in vivo stability of both the peptide and the \([^{18}F]\text{FPCA}\) radiolabel.

**Conclusion**

We report the radiosynthesis of a novel, water-soluble prosthetic group, \([^{18}F]\text{FPCA}\), and its automation using a GE TRACERlab FX-FN. \(^{18}\text{F}\)-radiolabelling efficiency of \([^{18}F]\text{FPCA}\) achieved yields > 85 % (n=20). Purified and dried \([^{18}F]\text{FPCA}\) was obtained within 45 minutes from end of bombardment with RCP of approx. 95 % and RCY of 28 % ± 2 (decay-corrected, n=10). Overall RCY of the \([^{18}F]\text{RI-OR2-TAT}\) achieved were 12 ± 2 % within 2 hours, starting from 25-30 GBq of \(^{18}\text{F}\) (decay corrected, n=5).

The pre-clinical study of \([^{18}F]\text{RI-OR2-TAT}\) demonstrates in vivo stability of the tracer and primarily hepatobiliary excretion of the intact peptide owing to its retro-inverso amino-acid sequence. Very low brain penetration was observed conceivably resulting from the low specific radioactivity. Further work is in progress to improve the specific radioactivity, by means of separation of the labelled and unlabelled peptide, and assess the tracer in animal models of AD.
Development and automation of $^{[18}\text{F}]$FPCA radiochemistry has produced reproducible radiolabelling of Aoa-k-RI-OR2-TAT. This presents $^{[18}\text{F}]$FPCA automated platform as a useful and generic $^{18}\text{F}$-prosthetic group for use in peptide and protein radiolabelling via both oxime bond formation, as has been described in this article, or reductive alkylation.

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Competing interests

Lancaster University, David Allsop and Mark Taylor have RI-OR2-liposome patent applications (WO2013/054110, US2014/356418 and EP2766094). No other authors have any competing interests to declare.

References


Supplementary material

Figure A) Quality control RP-HPLC radio-chromatogram of $[^{18}F]$RI-OR2-TAT

Figure B) RP-HPLC radio-chromatogram of a plasma sample showing parent $[^{18}F]$RI-OR2-TAT (5 min time point)
Chapter 5: Enrichment of $^{18}$F-labelled peptide by separation of labelled and unlabelled peptide using oxidised dextran.

Jessica Coco Day$^a$, Adam McMahon$^{b,c}$, Christian Prenant$^{b,c}$, Olivia Morris$^{b,c}$

§ both authors contributed equally to this work
Abstract

The amino(oxy)-functional group is often utilised in site-specific bioconjugation of peptides, including $^{18}$F-radiolabelling, due to its chemoselective reaction with aldehydes. The current work reports a method of increasing the concentration of $^{18}$F-labelled peptide in a crude reaction mixture, with excess unlabelled peptide, by derivatisation of the latter with an oxidised polysaccharide (dextran). Application of the method to an $[^{18}F]RGD$ peptide, a model compound, after radiolabelling of an aminooxy-functionalised precursor (Aoa-KRGDY) by oxime ligation with an $^{18}$F-prosthetic group, 2-$[^{18}F]$fluoro-3-pyridinecarboxaldehyde, is described. Incubation conditions of the crude $[^{18}F]RGD$ reaction mixture with oxidised dextran of 50 °C, pH 3 for 15 minutes, yielding an improvement in $[^{18}F]RGD$ specific radioactivity measurements of ca. 50 % (n= 5).

Introduction

The radiolabelling of affinity peptides and proteins (APP) is an expanding area of radiochemistry owing to their high target affinity and specificity [1-5]. Site-specific radiolabelling of peptides is a useful tool which helps to safeguard the biological activity of the peptide. Aminooxy-functionalised peptides are widely used for this purpose as they undergo a highly chemoselective reaction with $^{18}$F-aldehydic prosthetic groups to form strong and hydrolytically stable oxime bonds [4, 6-8]. 2-$[^{18}F]$Fluoro-3-pyridinecarboxaldehyde ($[^{18}F]FPCA$) is an aldehyde-containing prosthetic group. Its development and automation has recently been reported [9] for use in aminooxy-peptide radiolabelling and is the prosthetic group used in the current work.

The relative ratio of labelled vs. unlabelled peptide in any radiolabelling reaction mixture is typically very low and difficulties in their separation result in a mixture of the two species in the final product. Separation from the unlabelled precursor using HPLC is more difficult for peptides (M, > 1 kDa) using reverse phase- (RP-) HPLC and can require high flow-
rates and a mobile phase with a high organic content; unfavourable for use with proteins and peptides that might be sensitive to the organic components. Size exclusion- (SE-) HPLC serves as a more generic approach for radiolabelled APP purification which allows separation and then formulation of the radiolabelled APP with a buffered aqueous eluent. However, separation of labelled from unlabelled APP is not often possible using SE-HPLC as the method is unable to differentiate the molecular size change on radiolabelling. Excess unlabelled peptide can be problematic due to saturation of the biological target for which both labelled and unlabelled peptide compete for when the mixture is injected for positron emission tomography (PET) studies. This can result in poor quality images and the higher than ‘tracer concentration’ administration can alter pharmacodynamics and may even lead to toxicity. Increasing the ratio of labelled to unlabelled peptide has a similar affect as increasing the specific radioactivity of the tracer, or effective specific radioactivity. Therefore, a method by which the two species can be resolved using SE-HPLC would serve as a very useful tool and ultimately improve the quality of radiolabelled APP.

Enrichment is a well-established technique within the field of chromatography; assisting in concentration bolstering of a compound of interest within a complex mixture. The method is of particular value as a means to simplify complex proteins mixtures for analysis \[^{10}\]. The biotin streptavidin system is a recognised method by which proteins are enriched via protein-biotinylation and its interaction with a streptavidin solid support \[^{11}\]. However, the method has been criticised for the steric and electronic impact of biotinylation and also the harsh reaction requirements to cleave a biotinylated compound from the avidin solid support \[^{11}\].

Here, we describe the separation of an \(^{18}\)F-labelled from unlabelled peptide by addition of a large molecular weight species bearing multiple aldehyde functionalities, able to selectively react with unlabelled peptide thereby acting to enrich the concentration of \(^{18}\)F-labelled peptide. This, therefore, provides a method of enrichment that has no steric or
electronic impact on the $^{18}$F-labelled peptide and neither requires harsh cleavage conditions nor additional purification steps.

The method makes use of oxidised dextran ($M_r$ 12 kDa), a large molecule weight polysaccharide formed of glucose monomers. The widely-known oxidation of dextran with sodium periodate (NaIO$_4$) involves mild oxidation of two vicinal hydroxyl groups on the glucose monomer unit to produce two aldehyde groups$^{[12, 13]}$. The mechanism of sugar oxidation with NaIO$_4$ can be seen in Figure 1.

![Figure 1] Reaction mechanism showing the oxidation of vicinal hydroxyl groups on a sugar monomer by sodium periodate

A 5 amino-acid peptide containing the RGD motif was derivatised with an aminooxy-functionality at the $\varepsilon$-amine group of a terminal lysine moiety. This afforded the sequence Aoa-KRGDY ($Mr$ 710.70 Da), referred to in the text as Aoa-RGD. An RGD bearing a tyrosine fluorophore was chosen as, unlike dextran, it absorbs efficiently at 254 nm allowing its conjugation with dextran to be monitored by HPLC, as seen by a reduction in Aoa-RGD UV absorbance. An increase in the associated absorbance of the dextran-RGD complex was also observed; the increase in dextran-RGD and decrease in Aoa-RGD absorbance were, however, not comparable due to additional absorption of the oxime bond formed in the dextran complex.

Aoa-RGD was used as a model peptide, however the enrichment technique was developed with a view to its application to any aminooxy-functionalised APP. The method is most pertinent for APP where purification by means of RP-HPLC or solid phase extraction (SPE) is not possible on account of size or organic solvent-sensitive properties; where
purification via size-exclusion (SE-HPLC or SE-cartridge based methods) is most appropriate.

The peptide was radiolabelled using the $[^{18}F]$FPCA platform, as described by Morris et al.$^{[9]}$ and the crude reaction mixture was incubated with oxidised dextran using optimised conditions that were mindful of time restrictions associated with working with $^{18}F$ ($t_{1/2}$ 110 min) and constrains in the reaction conditions of some APP, such as temperature and pH. Figure 2 shows the conjugation of oxidised dextran to the aminooxy-bearing peptide via oxime bond formation. Formation of the large molecule weight peptide-dextran complex permitted separation of $[^{18}F]$RGD from unlabelled Aoa-RGD using SE-HPLC. Consequently, a simple step is added to the radiosynthesis procedure leaving the purification conditions unchanged.

![Figure 2) Reaction pathway of oxidised dextran and Aoa-RGD to form large molecular weight dextran-RGD complex](image)

**Experimental**

All solvents and chemicals used were purchased from Sigma-Aldrich (Sigma-Aldrich, UK). The Aoa-RGD peptide was purchased from Biomatik (Cambridge, Ontario, Canada) as an acetate salt, 98 % purity. $^{18}F$ was produced onsite via the $^{18}O(p, n)^{18}F$ nuclear reaction by 16.7 MeV proton bombardment of enriched $[^{18}O]H_2O$ using a GE PETtrace cyclotron (GE Healthcare). The HPLC system used for all analysis was a Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector). LabLogic (Sheffield, UK) Laura 4 software was
used via a CBM-20A controller and a Bioscan (Oxford, UK) Flowcount B-FC 3100 gamma detector was used to measure radioactivity.

**NaIO₄ oxidation of dextran**

Dextran (Mₖ 12,000 Da) was oxidised with NaIO₄ according to procedures reported in the literature \(^{12,13}\). In short, to NaIO₄ (2 mg, 9.3 μmol) was added a solution of dextran (12 mg, 1.0 μmol) in acetate buffer (0.1 M, pH 5.5, 500 μl) and incubated in the dark at room temperature for 90 min. The reaction mixture was then purified using Zeba-spin desalting columns (7 kDa MWCO, Life Technologies Thermo-scientific) to yield a solution of aqueous oxidised dextran. The pH of oxidised dextran was adjusted to pH 3.

**Optimisation of NaIO₄ concentration**

A ~ 10, 20 and 45 mM solution of NaIO₄ was prepared using NaIO₄ (1, 2 and 5 mg respectively) in acetate buffer (0.1M, pH 5.5, 500 μl) and added to dextran (5, 12, 26 mg respectively) to maintain a ~ 1:10 molar ratio. The solutions were incubated at room temperature for 30 minutes before purification using Zeba-spin desalting columns, as above, yielding oxidised dextran solutions A, B and C. To a solution of A, B or C (500, 200 and 100 μl respectively; 0.4 μmol) was added 50 uL (0.2 μmol) of a stock Aoa-RGD solution (2.8 mg/ml); total volume was adjusted to 550 μl with water (if necessary). The reaction mixture was incubated at 50 °C for 15 minutes. A sample was taken for analysis of the yield using SE-HPLC (GE Superdex 200 10/300 GL, PBS, 1 ml/min, 254 nm, Aoa-RGD \( t_R = 18.0 \) min, dextran-RGD \( t_R = 10.0 \) min).

**Radiosynthesis, GE TRACERlab FX-FN**

Figure A in supplementary data provides the schematic for the GE TRACERlab FX-FN configuration; the experimental section refers to Reactors and Vials labelled in the Figure.
[**18**F]Potassium fluoride

Cyclotron produced **18**F (3 ± 0.5 GBq) was delivered to a GE TRACERlab FX-FN radiochemistry system and trapped on a Sep-Pak QMA carb light cartridge (Waters, UK) then eluted with K$_2$CO$_3$ aqueous solution from Vial 1 (0.01 M, 0.4 ml) into a reactor containing 18-crown-6 (8 mg, 30 μmol). The mixture was azeotropically dried with 3 sequential additions of acetonitrile from Vial 2 (1.6 ml total) at an initial temperature of 90 °C before rising to 100 °C.

[**18**F]FPCA

As previously described [9], to the reactor containing azeotropically dried **18**F a solution containing 3-carboxaldehyde-**N**,**N**,**N**-trimethylpyridine-2-aminium bromide (2 mg, 8 μmol) in DMSO from Vial 3 (200 μl) was added and heated to 70 °C for 10 min. [**18**F]FPCA was purified using AFFINIMIP® (AFFINISEP, France) 1.5 ml reversible SPE cartridges. Elution from the SPE-cartridge was achieved using methanol from Vial 9 (3 ml) followed by solvent evaporation at 60 °C.

[**18**F]RGD peptide

Amino(oxy)-RGD peptide (0.3 mg, 400 nmol) in pH 3.0 citrate buffer (0.1 M, 100 μl) containing aniline (5 μl, 2M) was added to the purified and dried [**18**F]FPCA (800 ± 130 MBq) and heated to 50 °C for 10 min. [**18**F]RGD was purified and formulated using SE-HPLC (Superdex peptide 10/300 GL, PBS, 1 ml/min, 254 nm, $t_R$ 18.0 min).

Incorporation yields of [**18**F]FPCA of 85 ± 5 % were obtained, according to HPLC, and overall radiochemical yields of 18 ± 1 % were calculated (decay-corrected, n=5). Despite high incorporation yields of [**18**F]FPCA, the overall RCY was limited by an observed loss of radioactivity. This was discussed in the recent publication by Morris *et al* [9].
**Dextran enrichment**

Purified oxidised dextran solution (500 μl, 12 mg, 1.0 μmol) was added to a crude reaction mixture of $[^{18}\text{F}]$RGD peptide. The reaction was incubated at 50 °C for 15 min. $[^{18}\text{F}]$RGD peptide was purified using SE-HPLC (GE Superdex 200 10/300 GL, PBS, 1 ml/min, 254 nm, $t_R = 18.0$ min).

**Results and discussion**

SE-HPLC of the crude Aoa-RGD radiolabelling mixture was unable to resolve $^{18}$F-labelled and unlabelled peptide ($t_R$ 18 min) on account of the small mass increment on conjugation with $[^{18}\text{F}]$FPCA. As a result of their co-elution, it was not possible to measure true specific radioactivity of the labelled peptide. However, calibration of the UV detector response of Aoa-RGD and subsequent HPLC analysis of $[^{18}\text{F}]$RGD provided of approximate specific radioactivity (effective specific radioactivity) measurements of $1.25 \pm 0.1$ GBq/μmol.

Figure 3 gives the radio-chromatogram (SE-HPLC) showing the crude Aoa-RGD radiolabelling mixture.

*Figure 3) SE-HPLC UV- radiochromatogram showing crude $[^{18}\text{F}]$RGD radiolabelling reaction mixture before dextran incubation*
Following the conjugation of $[^{18}\text{F}]$FPCA to Aoa-RGD, the reaction mixture was incubated with purified oxidised dextran. Whilst developing reaction conditions for incubation, it was necessary to be aware of the reaction requirements of oxime bond formation but also of constraints in the reaction conditions of some APP, in an effort to develop a generic method for APP enrichment, and also radionuclide half-life.

The preferred NaIO$_4$ concentration for oxidation was determined by SE-HPLC to be 20 mM (maintaining a 1:10 molar ratio of dextran to NaIO$_4$).

Masarova et al. $^{[14]}$ report on sugar degradation when using NaIO$_4$ concentrations greater than 50 mM which could result in lower molecular weight polysaccharide chains; conjugation of the lower molecular weight units with unlabelled Aoa-RGD could then impact the resolution of the adduct from $[^{18}\text{F}]$RGD by SE-HPLC.

Table 1) Showing percentage reduction in AoaRGD UV peak response after dextran incubation

<table>
<thead>
<tr>
<th>NaIO$_4$ concentration</th>
<th>Percentage reduction in Aoa-RGD UV peak response</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>20 mM</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>45 mM</td>
<td>46 ± 7</td>
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</tbody>
</table>

Conjugation of unlabelled Aoa-RGD to oxidised dextran proceeds via oxime bond formation; previous literature has reported optimal pH conditions between 2-3 for this chemistry $^{[8]}$ and previous work by this group has described the use of pH 3 to radiolabel an aminooxy-functionalised peptide with $[^{18}\text{F}]$FPCA $^{[9]}$. Accordingly, a pH of 3 was adopted in the current for the conjugation of oxidised dextran to unlabelled Aoa-RGD. It was hoped that maintaining the same pH conditions, would enhance the applicability of the enrichment technique to other peptides and also simplify the addition of the incubation step.
The time for which the oxidised dextran was incubated with the crude peptide radiolabelling mixture was chosen as a compromise between dextran conjugation efficiency and length of reaction in order to minimise its impact on overall length of synthesis. An incubation time of 15 minutes was found to be most profitable achieving yields, according to SE-HPLC, of 45 ± 5 %. Radiotracer synthesis should not exceed two half-lives \(^{15}\); the addition of a 15 minute dextran incubation permitted total synthesis time, including radiosynthesis, purification, formulation and analysis, to sit comfortably within this time-frame.

Figure 4 shows the crude \(^{18}\text{F}\)RGD radiolabelling mixture after incubation with oxidised dextran. The large molecular weight dextran-RGD complex can be seen \(t_R \sim 10\) min and is well resolved from \(^{18}\text{F}\)RGD at 18 minutes, permitting separation of the two entities by SE-HPLC. The new effective specific radioactivity was calculated to be 2.46 ± 0.3 GBq/μmol (specific radioactivity improvement of 45 ± 5 %, \(n=5\)), according to the UV peak response, as seen by a ~ 50 % reduction in Aoa-RGD peak response which infers a ~ 50 % conjugation yield with dextran.

![Figure 4] SE-HPLC UV- radiochromatogram showing crude \(^{18}\text{F}\)RGD radiolabelling reaction mixture after dextran incubation
The scope of the enrichment technique is encouraging, however, significant advancement on the 45 ± 5 % dextran-RGD conjugation yield was not observed despite an excess of oxidised dextran being used. We speculate that this might, in part, be attributed to the differences in optimal pH ranges for oxime bond formation in radiochemistry and conventional organic syntheses (pH 4-7.5)\(^{16}\).

**Summary**

We report an enrichment technique to improve overall \(^{18}\text{F}\)-radiolabelled APP concentrations thereby improving effective specific radioactivity measurements.

Application to a model Aoa-functionalised RGD peptide yielded a ca. 50 % increase in effective specific radioactivity by partial separation of unlabelled from \(^{18}\text{F}\)-labelled peptide. This has been achieved by incubation of a crude \[^{18}\text{F}\]RGD peptide radiolabelling mixture with oxidised dextran (Mr 12000 Da) to form a high molecular weight dextran-RGD complex. The complex was then separated using conventional SE-HPLC from \[^{18}\text{F}\]RGD, resulting in an enriched \(^{18}\text{F}\)-labeled product. The procedure involved derivatisation of the unlabelled peptide therefore has no impact on the radiolabelled APP, unlike conventional protein enrichment techniques. Additionally, there is no requirement for unduly harsh conditions or additional purification steps; therefore presenting oxidised dextran enrichment as a modest yet facile method for improving effective specific radioactivity measurements of radiolabelled APP.

**Acknowledgements**

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Imaging Centre and members of the radiochemistry team at Wolfson Molecular Imaging Centre. OM would additionally like to thank AM, JG and CP for critically reviewing this manuscript.

References

Figure A) Schematic of customised GE TRACERlab FX-FN configuration permitting $[^{18}\text{F}]$FPCA radiosynthesis, purification and isolation followed by Aoa-RGD peptide radiolabelling, dextran incubation and purification
Chapter 6: *In vivo* Characterisation of a therapeutically relevant self-assembling $^{18}$F-labelled β-sheet forming peptide and its hydrogel using positron emission tomography

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Abstract

Positron emission tomography (PET) and fluorescence labelling have been used to assess the pharmacokinetics, biodistribution and eventual fate of a hydrogel-forming nonapeptide, FEFKFEFKK (F9) in healthy mice, using $^{18}$F-labelled and fluorescein isothiocyanate (FITC) - labelled F9 analogues. F9 was site-specifically radiolabelled with 2-[$^{18}$F]fluoro-3-pyridinecarboxaldehyde ($[^{18}$F]FPCA) via oxime bond formation using fully-automated radiosynthesis. $[^{18}$F]FPCA-F9 in vivo fate was evaluated both as a solution, following intravenous administration, and as a hydrogel when subcutaneously injected. The behaviour of FITC-F9 hydrogel was assessed following subcutaneous injection. $[^{18}$F]FPCA-F9 demonstrated high plasma stability and primarily renal excretion; $[^{18}$F]FPCA-F9 when in solution and injected into the bloodstream displayed prompt bladder uptake (53.4 ± 16.6 SUV at 20 minutes post injection) and rapid renal excretion, whereas $[^{18}$F]FPCA-F9 hydrogel, formed by co-assembly of $[^{18}$F]FPCA-F9 monomer with unfunctionalised F9 peptide and injected subcutaneously, showed gradual bladder accumulation of hydrogel fragments (3.8 ± 0.4 SUV at 20 minutes post injection), resulting in slower renal excretion. Gradual disaggregation of the F9 hydrogel from the site of injection was monitored using FITC-F9 hydrogel in healthy mice (60 ± 3 over 96 hours), indicating a biological half-life between 1-4 days. The in vivo characterisation of F9, both as a gel and a solution highlights its potential as a biomaterial.

Introduction

The potential of peptide hydrogels as biomaterials is vast on account of their in vivo and in vitro potential uses in a variety of biomedical applications. These include the use of peptide hydrogels as scaffolding for 3D cell cultures, tissues engineering and regenerative medicine as well as their use as vehicle for localised drug delivery $^{[1-6]}$. Peptide hydrogels are made of short synthetic peptides composed of either natural or non-natural amino acids that are designed to self-assemble into higher biomimetic structures. This is achieved via
non-covalent interactions in response to external triggers, such as pH, ionic strength, enzymes and light, to form three-dimensional networks of entangled fibrous structures\textsuperscript{[7, 8]}.

In the present study, the \textit{in vivo} behaviour of a β–sheet forming peptide FEFKFEFKK (F9) (F: phenylalanine; K: Lysine; E: glutamic acid) and its hydrogel was characterised. F9 is one of a family of amphipathic peptides with alternating hydrophobic and hydrophilic residues that self-assembles into β–sheet fibres that above a critical gelation concentration (CGC) associates to form a hydrogel. The properties of the hydrogels can be tailored through peptide design, concentration, media pH and ionic strength\textsuperscript{[9]}; as such they have been shown to hold promise for application in the biomedical field as 3D cell culture matrices\textsuperscript{[10]} and/or drug delivery vehicles\textsuperscript{[5]}.

Positron emission tomography (PET) is a quantitative imaging modality boasting high levels of sensitivity. The tool was used to assess the behaviour of radiolabelled F9 ([\textsuperscript{18}F]FPCA-F9) \textit{in vivo} using C3H mice. [\textsuperscript{18}F]FPCA-F9 was first delivered as a solution (concentration below CGC, pH 5.6) into the tail-vein to study the fate of peptide monomer in the blood stream. Subsequently [\textsuperscript{18}F]FPCA-F9 was delivered as part of an F9-hydrogel (concentration above CGC, pH 5.6) by co-assembly with unfunctionalised F9 peptide, before subcutaneous injection into the mouse flank.

Site-specific radiolabelling of F9 was selected to safeguard the β-sheet forming and therefore the gel-forming capability of the peptide. To achieve this, the N-terminal amine was functionalised with an aminooxy-group to give Aoa-F9 (Mr 1338.59 Da). Functionalisation of the peptide with the aminooxy-moiety permits its radiolabelling with the \textsuperscript{18}F-prosthetic group 2-[\textsuperscript{18}F]fluoro-3-pyridinecarboxaldehyde (FPCA) via oxime-bond formation, as has previously been described\textsuperscript{[11]}. The radiosynthesis pathway of [\textsuperscript{18}F]FPCA-F9 by radiolabelling of Aoa-F9 with [\textsuperscript{18}F]FPCA is shown in Figure 1.
PET studies were complemented by in vivo fluorescence imaging, making use of a fluorescein isothiocyanate N-terminally labelled F9 peptide (FITC-F9). Fluorescence data provided information regarding the long term stability of the hydrogel, in healthy mice, over 5 days; a time-frame outside the window of the radionuclidic half-life of $^{18}\text{F}$ (110 minutes).

**Experimental**

All solvents were purchased from Sigma-Aldrich and used without further purification. Aoa-F9 was purchased from Biomatik (Ontario, Canada) in trifluoroacetic acid (TFA) salt, purity >98 %. FITC-F9 was purchased from Biomatik as a TFA salt (purity >98 %) and converted into the HCl salt of the peptide. $^{18}\text{F}$Fluoride was produced onsite via the $^{18}\text{O}(p, n)^{18}\text{F}$ nuclear reaction by 16.4 MeV proton bombardment of enriched $[^{18}\text{O}]\text{H}_2\text{O}$ using a GE PETtrace cyclotron (GE Healthcare, Sweden). Analytical HPLC was performed using a

Figure 1: Reaction pathway showing Aoa-F9 radiolabelling with $^{18}\text{F}$FPCA
Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector) controlled by LabLogic (Sheffield, UK), Laura 3 software via a CBM-20A controller. HPLC eluate was measured for radioactivity using a Bioscan (Oxford, UK) Flow-count B-FC 3100 gamma detector. All pre-clinical PET scans were carried out using a Siemens (Oxford, UK) Inveon® PET-CT scanner. Fluorescence in vivo imaging was carried out using a Biospace Lab Photon Imager Optima (Nesles-la-Valee, France) controlled by Biospace Lab Photo Acquisition software v3.4 and images analysed using Biospace Lab M3 Vision software.

**Radiosynthesis, GE TRACERlab FX-FN**

Figure D in supplementary data provides the schematic for the GE TRACERlab FX-FN configuration; the experimental section refers to Reactors and Vials labelled in the Figure.

**[^18F]Potassium fluoride**

Cyclotron produced[^18F]fluoride was delivered to a TRACERlab FX-FN radiochemistry system (GE Healthcare, UK) and trapped on a Sep-Pak QMA cartridge (Oasis, Waters, UK). The cartridge was then eluted with a K$_2$CO$_3$ solution from Vial 1 (0.01 M, 0.4 ml) into a reactor containing 18-crown-6 (8 mg, 30 μmol) in acetonitrile (0.6 ml). The mixture was azeotropically dried with 3 sequential additions of acetonitrile from Vial 2 (1.6 ml total) at 90 °C under nitrogen.

**[^18F]FPCA**

[^18F]FPCA was produced as per the methodology described by Morris *et al*[^11]. In short, 3-carboxaldehyde-\(N,N,N\)-trimethylpyridine-2-aminium bromide (2 mg, 8 μmol) in DMSO from Vial 3 (200 μl) was added to the reactor containing azeotropically dried[^18F]fluoride and heated to 70°C for 10 min. [^18F]FPCA was trapped and washed on an AFFINIMIP® (AFFINISEP, France) 2.0 ml solid phase extraction (SPE) cartridge. Elution of [^18F]FPCA was achieved using methanol from Vial 9 (3 ml) into the reactor followed by solvent
evaporation under vacuum at 60°C for 6.5 min. Synthesis of the 3-carboxaldehyde-\(N,N,N\)-trimethylpyridine-2-aminium bromide precursor is described elsewhere\[^{11}\].

**\[^{18}\text{F}\]**FPCA-F9 solution

A mixture of Aoa-F9 (1.5 mg, ~880 nmol), anilinium hydrochloride (2.6mg, 20 μl) and gentisic acid (sodium salt hydrate, 1 mg) in citric acid (0.02 M, 250 μl) was added to purified and dried \[^{18}\text{F}\]FPCA (pH 2.7). The reaction mixture was heated to 70 °C for 30 minutes and purified using a TSK-gel® SE-HPLC column (7.5mm ID x 30 cm, 10 μm, Hichrom Tosoh Bioscience, Berkshire UK) eluted with PBS with 1 % ascorbic acid / 1ml/min, \(t_R = 10\) minutes and analysed for quality control purposes using size exclusion-HPLC (SE-HPLC) (Superdex peptide 10/300 GL, PBS, 1 ml/min, 220 nm, \(t_R = 18.05\) minutes). The final concentration of the solution was 0.3 mM.

**\[^{18}\text{F}\]**FPCA-F9 hydrogel

\[^{18}\text{F}\]**FPCA-F9 was prepared as described above. \[^{18}\text{F}\]**FPCA-F9 hydrogel was prepared by adding a small amount of \[^{18}\text{F}\]**FPCA-F9 to an unlabelled, unfunctionalised F9 hydrogel. In short, unfunctionalised F9 (11.6 mg) in \(\text{H}_2\text{O}\) (440 μl) was sonicated for 15 minutes before addition of 120 μl of purified \[^{18}\text{F}\]**FPCA-F9 in PBS solution. The mixture was vortexed and the pH adjusted to 5.5 using NaOH (0.5 M) and the final volume increased to 600 μl using \(\text{H}_2\text{O}\) giving a final hydrogel concentration 0.01M.

**Fluorescence preparation**

**FITC-F9 hydrogel**

FITC-F9 hydrogel was prepared using the same procedure as described above. Unfunctionalised F9 (10.5 mg) in \(\text{H}_2\text{O}\) (440 μl) was sonicated for 15 minutes before addition of FITC-labelled F9 (1.2 mg) in DMSO (5 μl). The mixture was sonicated for a further 15 minutes and the pH adjusted to 5.5 using NaOH (0.5 M). The final volume was
increased to 491.5 μl using deionised H₂O giving, once again, a final hydrogel concentration of 0.01 M.

**Pre-clinical analyses**

All animal handling was in accordance with UK legislation under the 1986 Animals (Scientific Procedures) Act.

**Pre-clinical evaluation**

**[^18F]FPCA-F9 solution & hydrogel**

C3H mice were anaesthetised using isoflurane induction 4 % and maintained 1.5 % in 70 % N₂O and 30 % O₂ mixture. [^18F]FPCA-F9 solution was injected in the tail vein (13 MBq, n=4) of healthy mice or [^18F]FPCA-F9 hydrogel was injected subcutaneously into the right flank of different healthy mice (2-5 MBq, n=3). The acquisition protocol parameters consisted of a preliminary CT scan to determine attenuation correction factors followed by a 1 and 4 hour PET acquisition time for [^18F]FPCA-F9 solution and hydrogel respectively. Major excretory organs, including liver, kidney and bladder as well as bone and hydrogel region (in [^18F]FPCA-F9 hydrogel scans only), were delineated on CT and uptake was quantified as SUV.

**[^18F]FPCA-F9 pre-clinical metabolite analysis**

Whole blood (250 μl), kidney, liver and urine samples were taken post-sacrifice at 5 (n=2) and 20 (n=2) minutes post injection of [^18F]FPCA-F9 monomer. Plasma was extracted from whole blood samples using a Thermo ALC multispeed refrigerated centrifuge PK121R (8720 g, 4 °C for 3 minutes) and added to PBS (2 ml). Kidney and liver samples were homogenised in PBS (2 ml) and centrifuged (8720 g, 4 °C for 3 minutes). Urine samples were directly injected onto HPLC without sample preparation. All samples were analysed using SE-HPLC using a GE Superdex peptide column 10/300 GL (GE Healthcare, UK) eluted with PBS (1 ml/min, 220 nm).
**In vivo fluorescence analysis**

C3H mice (n = 2) were anaesthetised using isoflurane (induction 4 % and maintained 1.5 %) in 70 % N₂O and 30 % O₂ mixture. FITC-F9 gel (200 μl) was injected subcutaneously into the right flank (C3H mice) and fluorescence data attained over 5 days using a 30 second acquisition time. Relative bioluminescent counts were normalised to acquisition time and quantified in units of counts. Excitation and emission wavelengths 487 and 547 nm respectively, FLI integration at 400 ms per frame, BVR pixel 171 μm with a pixel size of 170 x 170 μm. Hydrogel site was delineated and emission was quantified as counts per minute.

**Results and Discussion**

**[^18F]FPCA-F9 radiochemistry**

The radiochemical conversion of Aoa-F9 to [^18F]FPCA-F9 by reaction with [^18F]FPCA was 63 ± 7 % and 400 ± 200 MBq of [^18F]FPCA-F9 were produced (decay-corrected from end of bombardment) starting with 25-30 GBq of [^18F]fluoride (n=6). Radiosynthesis of [^18F]FPCA-F9 as a solution and a hydrogel was completed within 2 hours from end of bombardment.

Figure A of supplementary material provides GE TRACERlab FX-FN trace for crude reaction mixture. Radiochemical purity of [^18F]FPCA-F9 >98 % was attained, as can be seen in Figure B of supplementary material. [^18F]FPCA-F9 radiochemical yields (RCY) were not reflective of the high radiolabelling efficiency of Aoa-F9 with [^18F]FPCA, due to significant product loss during the automated radiosynthesis. This was attributable to the agglutinative nature of the peptide and issues of poor solubility and high viscosity of Aoa-F9, despite using low concentrations; this caused handling difficulties for the automated platform due to the platform’s narrow-bore tubing. Notwithstanding, [^18F]FPCA-F9 was produced in sufficient quantities for use in PET analysis. It is anticipated that use of larger-
bore tubing and the ability to transfer the reaction mixture across the platform using a syringe drive, as opposed to a helium flow, which is slow and controlled might improve recovery of \[^{18}\text{F}]\text{FPCA-F9}. However, the uniquely aggregative nature of the hydrogel-forming peptide precludes its comparison with other \[^{18}\text{F}]\text{FPCA} peptide radiolabelling.

\[^{18}\text{F}]\text{F9 solution biodistribution - PET}\n
The PET images in Figure 2 A, B and C depict \[^{18}\text{F}]\text{FPCA-F9} biodistribution at 60 seconds, 15 minutes and 1 hour post injection respectively. Figure 2D shows the uptake of \[^{18}\text{F}]\text{FPCA-F9} over 60 minutes in key organs. Kidney uptake was observed within 5 minutes post injection (5.9 ± 1.9 SUV).

This was followed by significant bladder accumulation, as clearly seen in Figures 2B and C, which begins to plateau after 20 minutes (53.4 ± 16.6 SUV). Liver uptake remained low over the time course of the scan (1.4 ± 0.1 and 0.7 ± 0.1 SUV at 5 and 20 minutes post injection respectively). The concentrations observed within the liver were consistently lower than those seen in the kidney whilst the highest concentration were seen in the bladder; confirming that the kidney is the major excretory organ for \[^{18}\text{F}]\text{FPCA-F9} and potential radiometabolite(s). Rapid renal excretion is expected of unmodified, small peptides \[^{12, 13}\].

Gradual bone accumulation can also be seen (7.8 ± 2.5 SUV 1 hour post injection), although levels of radioactivity are overshadowed by much higher concentrations in the bladder (47.4 ± 7.2 SUV 1 hour post injection); bone uptake was most notable at regions of the joint. Accumulation of radioactivity in the bone can be ascribed either to defluorination of \[^{18}\text{F}]\text{FPCA-F9} or to the interaction of the peptide with the bone tissue. Bone uptake followed kidney uptake, an organ which has been previously implicated in defluorination \[^{14, 15}\]; however no free \[^{18}\text{F}]\text{fluoride} was observed in metabolic analyses (discussed later) we cannot, therefore, exclude the possibility of an interaction between a \[^{18}\text{F}]\text{FPCA-F9} and the protein rich cartilage tissue.
This potential interaction is currently being investigated in our laboratory and is outside the scope of this article.

Liver, kidney, urine and plasma samples were analysed using SE-HPLC to quantify the degree of metabolic degradation of the peptide. The analysis identified the major peak as $[^{18}\text{F}]\text{FPCA-F9}$ with a retention time of ~18 minutes; none of the samples analysed showed SUV $>4.0$.

Figure 2: $[^{18}\text{F}]\text{FPCA-F9}$ solution at A) 60 seconds, B) 15 minutes and C) 1 hour post injection and D) TAC $[^{18}\text{F}]\text{FPCA-F9}$ biodistribution in bone and excretory organs (C3H mice, n=4)

$[^{18}\text{F}]\text{FPCA-F9}$ solution metabolite analysis

Liver, kidney, urine and plasma samples were analysed using SE-HPLC to quantify the degree of metabolic degradation of the peptide. The analysis identified the major peak as $[^{18}\text{F}]\text{FPCA-F9}$ with a retention time of ~18 minutes; none of the samples analysed showed
any metabolite of $^{[18\text{F}]\text{FPCA-F9}}$ (see Supplementary Material Figure C). Table 1 shows the percentage of parent compound ($^{[18\text{F}]\text{FPCA-F9}}$) identified in each of the samples after 60 minutes. The high percentage of parent compound in all samples demonstrates the high stability of $^{[18\text{F}]\text{FPCA-F9}}$.

Liver and kidney samples, though, showed a second peak at ~8 minutes accounting for ~5% of the radioactivity (see Supplementary Material Figure Cii). Urine samples showed a peak with a retention time of ~12 minutes, accounting for 5% of the radioactivity (see supplementary Figure Ciii) alongside the $^{[18\text{F}]\text{FPCA-F9}}$ peak. A shorter retention time is suggestive of a larger species than $^{[18\text{F}]\text{FPCA-F9}}$, conceivably a result of $^{[18\text{F}]\text{FPCA-F9}}$ aggregation or interaction with a large molecular weight protein. As discussed above, $^{[18\text{F}]\text{FPCA-F9}}$ carries a positive charge under physiological conditions and can therefore interact with negatively charged proteins. The large molecular weight radiolabelled species seen in both liver and kidney samples ($t_R$ ~8 minutes), falls outside the exclusion limit of the column ($V_0$ ~9 minutes) therefore suggestive of molecular mass >12 kDa. The species in the urine sample ($t_R$ 12.5 minutes) is likely, according to molecular weight standard analysis, to have a molecular mass between 8 and 10 kDa.

Table 1) Quantification of parent ($^{[18\text{F}]\text{F9}}$) fraction after 60 minutes (data expressed as mean ± SD, n=4)

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
<th>Urine</th>
<th>Plasma</th>
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<tr>
<td></td>
<td>94 ±0 %</td>
<td>95 ±1 %</td>
<td>95 ±1 %</td>
<td>100</td>
</tr>
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</table>

The absence of radiolabelled species with a molecular mass smaller than that of $^{[18\text{F}]\text{FPCA-F9}}$ and within the 22.5 minute column exclusion limit is suggestive of the absence of $^{[18\text{F}]\text{FPCA-F9}}$ radiometabolites, including $^{[18\text{F}]\text{fluoride}}$. The absence of a $^{[18\text{F}]\text{fluoride}}$ radiometabolite provides evidence against $^{[18\text{F}]\text{FPCA-F9}}$ defluorination and further supports the notions of an interaction between F9 and the cartilage.
If \([^{18}\text{F}]\text{FPCA-F9}\) is aggregating with circulating proteins, similar aggregation is expected to be observed in plasma sample, which is not the case. The TAC shows that F9 is rapidly transported to the kidneys, thereby minimising the time for which F9 can interact with circulating proteins such as albumin in the plasma.

\textbf{\([^{18}\text{F}]\text{FPCA-F9 hydrogel biodistribution - PET}\)}

\([^{18}\text{F}]\text{FPCA-F9}\) hydrogel was injected subcutaneously into the flank of C3H mice; its \textit{in vivo} behaviour over a 4 hour time-course can be interpreted from Figure 3. Figures 3 A and B (60 seconds and 4 hours post injection) clearly show the radiolabelled hydrogel. Figure 3C provides the biodistribution of \([^{18}\text{F}]\text{FPCA-F9}\) peptide in the bone and excretory organs. The increasing concentrations of \([^{18}\text{F}]\text{FPCA-F9}\) in the kidneys and bladder clearly show that the peptide is gradually being eluted from the hydrogel as the latter physically degrades / disagggregates.

A progressive increase in radioactivity over 150 minutes is observed in the bladder, before plateauing (17.4 ± 1.3 SUV). Although this is reminiscent of the excretion profile of the peptide solution, albeit over a different time period, there is a two-step profile for bladder accumulation that was observed in all mice. This is anticipated to be as a result of the staged non-uniform disaggregation of the hydrogel; smaller and more vulnerable hydrogel fragments at the periphery are expected to disaggregate first and rapidly before the bulk of the hydrogel.

Bone uptake of \([^{18}\text{F}]\text{FPA-F9}\) was also observed (3.8 ± 1.3 SUV 1 hour post injection) in this case and can be seen in Figure 3; most predominantly in cartilage rich regions such as the joints and snout. Uptake of \([^{18}\text{F}]\text{FPCA-F9}\) in the kidney, bladder and liver, major excretory organs, (1.15 ± 0.2, 5.9 ± 3.8 and 0.4 ± 0.1 SUV respectively, 20 minutes post injection) and also shown in Figure 3C. Akin to when \([^{18}\text{F}]\text{FPCA-F9}\) was administered as a solution, liver uptake remained below the levels observed in the kidney and significantly
below bladder levels and this was true over the duration of the experiment (0.7 ± 0.1 SUV at 20 minutes post injection).

![Image]

*Figure 3* [18F]FPCA-F9 hydrogel at A) 60 seconds and B) 4 hours post injection C) TAC showing [18F]FPCA-F9 biodistribution in bone and excretory organs (C3H mice, n=3)

The work confirms that the peptide exhibited the projected biodistribution with no significant changes in its *in vivo* behaviour, on account of its gelation.
FITC-F9 hydrogel - fluorescence imaging

Despite the inability to detect FITC-F9 penetration into the tissue, as a consequence of the limitations imposed by FITC emission, shallow injection of FITC-F9 produced fluorescence data that was a good indicator of the hydrogel biological half-life. The intention of using bioluminescence imaging (BLI) as a complementary technique was to help visualise the process of hydrogel expansion and disaggregation; this data was used to augment PET data which was used to provide information regarding biodistribution and route of F9 metabolism.

Fluorescence data is presented in Figure 4, the data shows FITC-F9 hydrogel 60 seconds, 48 and 96 hours post injection (Figure 4 A, B and C respectively) in the flank of C3H mice (n=2).

![Figure 4](image)

Figure 4) Fluorescence imaging of FITC-F9 hydrogel injected subcutaneously into the right flank of C3H mice (n=2) at A) 60 seconds, B) 48 hours and C) 96 hours post injection

Qualitative analysis of the preliminary data permits a first appreciation of the results. Signal intensity, corresponding to FITC-F9, was measured over the course of 5 days, beginning at the time of injection. Steady depletion in signal intensity was observed, with a 43 ± 8 % reduction over the first 24 hours and 60 ± 3 % over 96 hours, attributable to disaggregation of the hydrogel over time. Results of the fluorescence work, therefore, suggest an F9 hydrogel biological half-life between 1 and 4 days.
An expansion of ~10% was observed over the first 24 hours, which dropped to ~3% over 48 hours. These results suggest that, post injection, there is further penetration of the hydrogel in the surrounding tissues. These hydrogels are known to be shear thinning and animal movement will promote gel tissue penetration. This is then followed by the slow disaggregation of the hydrogel.

The fluorescence data, despite not being fully quantitative, are in agreement with PET data providing evidence for the exploitable in vivo life-time of F9 hydrogels as a biomaterial for either tissue engineering or drug delivery applications.

**Conclusion**

We report the automated synthesis of a radiolabelled gel-forming peptide, $[^{18}\text{F}]\text{FPCA-F9}$, and its pre-clinical PET analysis alongside preliminary fluorescence imaging data; this permitted in vivo characterisation of its behaviour. Pre-clinical results demonstrated renal excretion as the major elimination route for labelled-F9 monomer, both after administration as a solution and a hydrogel. Results also showed the high plasma stability of the labelled-F9 monomers and established a favourable biological half-life of labelled-F9 hydrogel. The current work has confirmed that the behaviour exhibited by F9 is typical of natural, small peptides $^{[12]}$ and its in vivo biodistribution is not altered on account of its gel-form.

Collectively, the fluorescence and PET data provide insight into a possible in vivo pathway for F9 hydrogel; firstly characterised by hydrogel tissue penetration followed by disaggregation and final elimination via renal excretion. Due to the natural amino acid composition of F9 alongside its in vivo metabolic profile, F9 is not expected to have issues of toxicity $^{[16]}$. Such characteristics would highlight F9 hydrogel as ideal biomaterial that could be used as a silent carrier for localised drug-delivery. This is of particular importance
in oncological applications wherein targeted drug delivery helps to ensure maximal drug
dose at the tumour whilst minimising drug delivery to non-target sites.

Disclosure statement
The authors declare no potential sources of conflict of interest or competing financial
interest.

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All research data supporting this publication are directly available within this publication
and its associated supplementary information.

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Supplementary material

Figure A) TRACERlab FX-FN gamma trace of crude $[^{18}F]$FPCA-F9 purification using SE-HPLC

Figure Bi and ii: SE-HPLC UV and radiochromatogram of $[^{18}F]$FPCA-F9, respectively
**Figure Ci:** SE-HPLC radiochromatogram showing plasma metabolite sample

**Figure Cii:** SE-HPLC radiochromatogram showing liver metabolite sample
Figure Ciii: SE-HPLC radiochromatogram showing urine metabolite sample

Figure D) Schematic of customised GE TRACERlab FX-FN configuration permitting $^{18}$F FPCA radiosynthesis, purification and isolation followed by Aoa-F9 peptide radiolabelling and purification
Chapter 7: Application of the automate $^{[18}\text{F}]$FPCA radiolabelling platform to a HER2-affibody and HER2-nanobody and comparison with $^{[89}\text{Zr}]$trastuzumab

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Abstract

Aim: To assess the applicability of the $^{18}$F-fluoro-3-pyridinecarboxaldehyde ($^{18}$F-FPCA) radiolabelling platform to the labelling of an anti-HER2- affibody and nanobody and to evaluate the method using pre-clinical analysis in HER2 tumour-bearing mice.

Methods: The affibody and nanobody were functionalised with an aminooxy-derivative to permit fully-automated and site-specific radiolabelling with $^{18}$F-FPCA via oxime bond formation, using a GE TRACERlab FX-FN.

Results: Radiochemical yields of the $^{18}$F radiolabelled affibody and nanobody were 13 and 4 %, respectively, with radiochemical purity measurements greater than 95 %. Pre-clinically relevant doses of $^{18}$F-affibody permitted in vivo evaluation of the radiotracer, which confirmed HER2 target specificity and delineation of HER2-positive tumours.

Summary: The automated $^{18}$F-FPCA radiolabelling platform has been used to radiolabel an affibody and nanobody via oxime bond formation and is highlighted as a useful platform, with generic features, for peptide radiolabelling with $^{18}$F.

Introduction

There is a shortage of $^{18}$F radiolabelled affinity peptides and proteins (APPs) that are used in routine clinic; this is attributed to the associated challenges of $^{18}$F-radiolabelling and thus, despite the radioisotope being described as an ideal radionuclide for this purpose, the translation of $^{18}$F-APPs from bench-to-bedside has been limited [1].

A number of challenges have been highlighted in this regard: incompatibility of direct fluorination with APPs and the laborious nature of current multi-step approaches to indirect APP radiolabelling [1]. Encouragingly, some key developmental steps have been reported in the literature and a number of generic features to APP radiolabelling with $^{18}$F,
have emerged. This includes the development of site-specific radiolabelling methods, that help safeguard the antigen binding site [2], and a preference for mild reaction conditions that are biocompatible, particularly for APPs with more complex tertiary structure where the use of excessive pH and/or temperature could cause loss of function [3].

Automation is another key generic desirable for APP radiolabelling [1] permitting the use of higher amounts of radioactivity, enabling the routine radiosynthesis of clinically relevant doses, whilst minimising radiochemist exposure [4] [5]. Automation of an APP radiolabelling method also aids conformance to good manufacturing practice (GMP) and thus helps the translation to routine clinic.

Morris et al. [4] report the development and automation of a radiolabelling platform with which an aminooxy-functionalised (Aoa) peptide was radiolabelled, site-specifically, using the prosthetic group 2-[18F]fluoro-3-pyridinecarboxaldehyde ([18F]FPCA) via oxime bond formation. Importantly, both the radiosynthesis of the [18F] prosthetic group and subsequent peptide radiolabelling was fully-automated; the [18F]APP was reliably and reproducibly synthesised using the platform [4]. This automated synthesis of the aqueous-soluble [18F]FPCA, represents a generic approach to APP radiolabelling that promises to allow [18F] peptides realise their full potential as a routine clinical tool.

![Reaction pathway showing the conjugation of [18F]FPCA to Aoa-modified APP through oxime bond formation](image)

**Figure 1** Reaction pathway showing the conjugation of [18F]FPCA to Aoa-modified APP through oxime bond formation

The conjugation of [18F]FPCA to Aoa-derivatised APP via oxime bond formation can be seen in Figure 1.
In the current work, the applicability of the automated $[^{18}\text{F}]$FPCA APP radiolabelling platform to two Aoa-derivatised peptides: an affibody ($Z_{\text{HER2:342}}$ referred to as $\alpha\text{HER2Aff}$) and nanobody (referred to as $\alpha\text{HER2Nb}$) directed against human epidermal growth factor-2 (HER2) was evaluated. Although the two peptides were chosen as model compounds, their selection took into consideration the ultimate goal of synthesising clinically relevant $[^{18}\text{F}]$APPs. Affibodies and nanobodies have gained significant interest in PET owing to their desirable characteristics and promising results $^{[6,7]}$ and the HER2 target is an important biomarker in oncology: its expression has been associated with a more aggressive tumour phenotype and poorer patient prognosis $^{[8]}$.

The utility of the radiolabelling platform to APP radiolabelling was further evaluated in HER2 tumour-bearing mice. These results were compared to $^{89}\text{Zr}$ radiolabelled trastuzumab (Herceptin), the therapeutic HER2 antibody (mAb). The high affinity and selectivity of antibodies and the well-established methods of mAb labelling with $^{89}\text{Zr}$, recently defined as an ideal radioisotope for this purpose, offers $[^{89}\text{Zr}]\text{mAb}$ as a reference comparator of APP-based radiotracers $^{[9,10]}$.

**Experimental**

All solvents were purchased from Sigma-Aldrich and used without further purification. Aoa-functionalised $\alpha\text{HER2Aff}$ was provided by GE Healthcare (Oslo, Norway), the affibody sequence and further details of structural analysis can be found in a publication by Eigenbrot et al. $^{[11]}$. Trastuzumab was procured from Roche (Roche Products Limited, UK) as a freeze dried powder. $\alpha\text{HER2Nb}$ was provided and Aoa-functionalised by *in vivo* cellular & molecular imaging (Vrije Universiteit, Brussel BE). The sequence of the nanobody was not disclosed. $^{18}\text{F}$ was produced onsite via the $^{18}\text{O}(p, n)^{18}\text{F}$ nuclear reaction by 16.7 MeV proton bombardment of enriched $[^{18}\text{O}]\text{H}_2\text{O}$ using a GE PETtrace cyclotron (GE Healthcare). $^{89}$-Zirconium was purchased from BV Cyclotron (Vrije Universiteit,
Amsterdam). Analytical HPLC was performed using a Shimadzu (Milton Keynes, UK) Prominence system (LC-20AB solvent delivery system, SPD-20A dual wavelength absorbance detector) controlled by Laura 3 software (LabLogic, Sheffield, UK), Laura 3 software via a CBM-20A controller. The HPLC radiodetector was a Bioscan (Oxford, UK) Flowcount B-FC 3100 gamma detector. All pre-clinical PET scans were carried out using a Siemens (Oxford, UK) Inveon® PET-CT scanner.

**Radiosynthesis, GE TRACERlab FX-FN**

Figure A in supplementary data provides the schematic for the GE TRACERlab FX-FN configuration; the experimental section refers to Reactors and Vials labelled in the Figure.

[^18F]FPCA

Radiosynthesis of the prosthetic group was modified according to a method recently published by Basuli et al.[^12]. Cyclotron produced ^18^F was delivered to a TRACERlab FX-FN radiochemistry system (GE Healthcare, UK) and trapped on a Chromabond shorty PS-HCO₃ (45 mg) (Hichrom Ltd. UK) and dried with anhydrous acetonitrile from Vial 1 (3 ml). ^18^F was eluted by reaction with 3-carboxaldehyde-\(N,N,N\)-trimethylpyridine-2-aminium bromide (10 mg, 40 μmol) in a solution of DMSO from Vial 2 (300 μl), t-butanol 700 (μl) and acetonitrile (500 μl).[^18F]FPCA was diluted in water from Vial 3 (8 ml) before purification using AFFINIMIP® (AFFINISEP, France) 2.0 ml SPE cartridges. Elution from the SPE cartridge was achieved using methanol from Vial 9 (3 ml) followed by solvent evaporation at 60 °C, under vacuum for 6.5 min.

[^18F]αHER2Nb

To the purified and dried[^18F]FPCA was added a solution containing Aoa- αHER2Nb (200 μl, 0.4mg/ml, 5 nmol), gentisic acid sodium salt (1mg, 5.7 μmol) and anilinium hydrochloride (20 μmol, 2M), the pH was adjusted to 3.0 using TFA (1M, 19 μl). The reaction mixture was heated to 50 °C for 30 minutes and purified using SE-HPLC (Superdex peptide 10/300 GL, PBS with 1 % ascorbic acid, 1 ml/min, 280 nm, \(t_R 20.0\) min)
and analysed for quality control purposes using RP-HPLC with a PLRP-S column (5 μm, 300 Å, 250 x 4 mm, Agilent UK) and eluted using a gradient method with the eluents (A) water (0.1 % TFA) and (B) acetonitrile (0.1 % TFA) (Gradient method with respect to eluent B: 0-5 min, 25 %; 5-7 min, 25-34 %; 7-10 min, 34-100 %; 10-25 min, 100 %; 1.1 ml/min at 220 and 280 nm t_R 12.0 min)

[^18F]αHER2Aff

To the purified and dried[^18F]FPCA was added Aoa-αHER2aff (5.8 mg, 790 nmol) in a solution containing gentisic acid sodium salt (1 mg, 5.7 μmol), anilinium hydrochloride (20 μmol, 2M) in citric acid (5.7 μM, 250 μl, pH 2.7). The reaction mixture was heated at 50 °C for 15 minutes and purified using a tC2 plus-short SPE cartridge (Waters, UK).

[^18F]αHER2Aff was eluted with water-ethanol (20:80, 1 ml) and analysed for quality control purposes using a Jupiter C4 column (5 μ 300 Å 250 x 10 mm, Phenomenex UK, Macclesfield, Cheshire, UK) eluted using a gradient method with the eluents (A) water (0.1% TFA) and (B) acetonitrile (0.1% TFA) (Gradient method with respect to eluent B: 0-25 min,10-35 %; 25-26 min, 35-90%; 26-31 min, 90-10%; 5 ml/min at 220 and 280 nm, t_R 22.24).

[^89Zr]Trastuzumab

Df-Bz-NCS modified trastuzumab was radiolabelled with ^89Zr according to a procedure reported in the literature, with minor amendments[^9]. In short, to[^89Zr]oxalate (80-100 MBq) was added oxalic acid (1 M) to a volume of 200 μl. The reaction mixture was gently agitated and Na₂CO₃ (2M, 90 μl) was added before addition of HEPES buffer (0.5 M, pH 7.4). To this was added Df-Bz-NCS-trastuzumab (710 μl). The reaction mixture was left under gentle agitation at room temperature for 1 hour.[^89Zr]Trastuzumab was purified using a PD-10 pre-equilibrated with phosphate buffered saline (PBS) and eluted in PBS (2 ml). A sample was analysed using HPLC for quality control purposes (Superdex 200
Increase, 0.4 ml/min, PBS, 280 nm, t_R 23.0). Overall $^{89}$Zr-trastuzumab RCY of 59 ± 2 %
and RCP of > 98 % (n=8) were attained.

**Synthesis of stable compounds**

3-carboxaldehyde-$N,N,N$-trimethylpyridine-2-aminium bromide

The precursor was synthesised according to a procedure reported in the literature by Morris
*et al.* [4].

**Df-Bz-NCS modification of trastuzumab**

Chelation of Trastuzumab was carried out according to a procedure reported in the
literature with some modifications [9]. In short, Trastuzumab (5 mg) in water (1 ml) was
added to a PD-10 desalting column (GE Healthcare, UK), equilibrated with borate buffer
(pH 9, 20 ml), and eluted in pH 9 borate buffer (1.5 ml). 20 μl of a Df-Bz-NCS solution
(2.5 mg, 66.6 nmol) in DMSO (1 ml) was added to the Trastuzumab solution (1 ml, 22.2
nmol). The reaction mixture was incubated at 37°C for 1 hour before purification using a
PD-10 column, equilibrated with a sodium acetate buffer (0.25 M, pH 5.4-5.6) containing
gentisic acid (5 mg/ml).

**Pre-clinical PET analyses**

All animal handling was in accordance with UK legislation under the 1986 Animals
(Scientific Procedures) Act.

**Pre-clinical PET**

Mice bearing HER2-expressing tumours (n = 6) were anaesthetised using isoflurane
(induction 4 % and maintained 1.5 %) in 70 % N₂O and 30 % O₂ mixture. Mice were
injected with either $^{18}$FαHER2Aff (21 ± 0.3 MBq, n=2) or $^{89}$Zr-trastuzumab (4 ± 0.9
MBq, n=4) in the tail vein. HER2 positive tumours were initiated via the injection of
3x10⁶ U87 SKOV-3cells in a 0.1 ml PBS:matrigel (1:1) into the flank of adult, female cd1
immunocompromised mice. All scans were carried out using a Siemens Inveon® PET-CT
scanner. The acquisition protocol parameters consisted of a preliminary CT scan to attain attenuation correction factors, followed by PET acquisition. A two hour dynamic PET acquisition of $[^{18}\text{F}]\alpha$HER2Aff was performed. Static PET images were acquired of $[^{89}\text{Zr}]$trastuzumab 4 days post injection. Tumour region, reference tissue and excretory organs were delineated on the CT and uptake was quantified as SUV.

**Results & discussion**

The radiolabelling platform described by Morris *et al.*\(^4\) was used to radiolabel the Aoa-derivatised peptides with some modifications. The modifications were made in an effort to simplify the procedure by reducing overall radiosynthesis time and to enhance reliability and consistency. The modification has been labelled A in the supplementary Figure A.

The first modification adapted the fluorination of the $[^{18}\text{F}]$FPCA precursor according to a method reported by Basuli *et al.*\(^{12}\). The modified approach to $[^{18}\text{F}]$FPCA radiosynthesis used solid-support mediated fluorination of its precursor and removed $^{18}\text{F}$ azeotropic drying steps. The technique achieved $[^{18}\text{F}]$FPCA radiochemical yields (RCY) of $35 \pm 3\%$ (decay-corrected) and reduced $[^{18}\text{F}]$FPCA radiosynthesis time 3-fold, from 30 minutes to 10 minutes.

The Aoa-derivatised HER2 affibody (Aoa-αHER2Aff) was radiolabelled according to the conditions described in the original publication using the automated $[^{18}\text{F}]$FPCA radiolabelling platform\(^4\).

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*Figure 2) Schematic showing C-terminal modification of αHER2Aff with Aoa-derivative*
Derivatisation of αHER2Aff was achieved via modification of the C-terminal cysteine via Michael addition with a maleimide containing aminooxyacetyl synthon, according to the scheme in Figure 2.

Modifications were made to the purification of the \([^{18}\text{F}]\alpha\text{HER2Aff}\), where size exclusion high pressure liquid chromatography (SE-HPLC) was replaced with solid phase extraction (SPE). The methodological development was made to remove the time-consuming SE-HPLC step and also provide more consistency in the radiolabelled product. This modification has been denoted in supplementary Figure A with a B, which shows the GE TRACERlab FX-FN configuration using HPLC purification. In the radiosynthesis of \([^{18}\text{F}]\alpha\text{HER2Aff}\) the HPLC purification was replaced with a SPE cartridge.

Unoptimised \([^{18}\text{F}]\alpha\text{HER2Aff}\) RCY, using the fully-automated radiolabelling platform including \([^{18}\text{F}]\text{FPCA}\) radiosynthesis and peptide radiolabelling, achieved 12.7 ± 0.3 % (decay-corrected), alongside radiochemical purity (RCP) measurements of >95 % (n=3). Radiosynthesis of the \([^{18}\text{F}]\alpha\text{HER2Aff}\) took 75 minutes, from end of bombardment, which sits very comfortably within the general guidelines stipulating that total radiosynthesis should not exceed two radionuclide half-lives \([1]\).

The literature reports other approaches to affibody radiolabelling via oxime bond chemistry \([13, 14]\); however the methods, unlike the current work, were not fully-automated. \([^{18}\text{F}]\alpha\text{HER2Aff}\) yields attained using the radiolabelling platform are comparable to those reported in the literature, however the current work made use of larger quantities of Aoa-affibody precursor. There are challenges associated with the automation of complex multi-step radiosyntheses and reductions in yield on account of automation have been reported \([4, 5]\). Larger precursor quantities can be used in order to circumnavigate some of the imposed limitations of automation, helping to satisfy the pseudo-first order conditions of APP radiolabelling in larger reaction volumes. The inability of some purification methods, including SE-HPLC and SPE, to separate radiolabelled and unlabelled APP and the
significance this can have on radiotracer performance is more acute when using larger quantities of APP precursor.

It is recognised that administration of larger than ‘tracer’ concentrations can result in poor quality images and altered pharmacodynamics on account of biological target saturation; issues of toxicity may also arise. $[^{18}\text{F}]\alpha\text{HER2Aff}$ evaluation in HER2 tumour-bearing mice and its comparison with our reference, $[^{89}\text{Zr}]\text{trastuzumab}$, was carried out to help verify the suitability of the radiolabelling platform in this application and assess the impact, if any, of larger APP precursor quantities on radiotracer performance. For this, mice bearing HER2 positive tumours, derived from SKOV-3 cell lines, were used.

*Figure 2: In vivo evaluation of A) $[^{18}\text{F}]\alpha\text{HER2Aff}$, 2 hours post injection B)$[^{89}\text{Zr}]\text{trastuzumab}$, 4 days post injection and TAC of $[^{18}\text{F}]\alpha\text{HER2Aff}$ accumulation in C) tumour and D) excretory organs*
Figure 3 shows the results of the *in vivo* investigations; Figures A and B show \[^{18}F\]αHER2Aff and \[^{89}Zr\]trastuzumab at 2 hours and 4 days post-injection respectively and Figures C and D show the time activity curves (TAC) of \[^{18}F\]αHER2Aff accumulation in SKOV-3 tumours and excretory organs, respectively.

The Figure shows affinity of \[^{18}F\]αHER2Aff for HER2, indicated by tumour accumulation of the radiotracer; the TAC shown in Figure 1C shows rapid tumour uptake, starting as early as 5 minutes post-injection, followed by more steady accumulation from 1 hour. The TAC shown in Figure 1D shows significant bladder accumulation of \[^{18}F\]αHER2Aff in comparison to levels seen in the liver; by way of comparison the SUV at 95 minutes for liver and bladder were 1.2 ± 0.1 and 42.2 ± 12.6, respectively. This is indicative of renal excretion, which is in support of previous literature by Troussil *et al.*\(^\text{[15]}\), where an Aoa-derivatised affibody was radiolabelled with an \(^{15}\)F prosthetic group.

The standard uptake values (SUV) for \[^{18}F\]αHER2Aff and \[^{89}Zr\]trastuzumab in the tumour were ~1.40 and 2.44 respectively and tumour to muscle ratios of 4.68 and 8.18 were calculated. The tumour ratio of \[^{89}Zr\]trastuzumab is in close agreement with those reported by Wang *et al.*\(^\text{[16]}\) where, after 48 hours, tumour to muscle ratios of 9.96 were reported. The tumour to muscle ratios of \[^{18}F\]αHER2Aff are also in close agreement with literature values, where Ren *et al.*\(^\text{[17]}\) report ratios between 2.09-3.48 at one hour post injection.

Despite the tumour accumulation and tumour to muscle ratios of \[^{18}F\]αHER2Aff being lower than those of \[^{89}Zr\]trastuzumab, the pre-clinical results are encouraging and indicate the successful application of the \[^{18}F\]FPCA radiolabelling platform to the Aoa-αHER2aff.

The automated platform was also used to radiolabel the Aoa-derivatised HER2 nanobody (Aoa-αHER2Nb); solid-phase mediated fluorination of the \[^{18}F\]FPCA precursor was used but other conditions, including SE-HPLC purification, according to the published procedure were retained\(^\text{[4]}\). Unoptimised \[^{18}F\]αHER2Nb RCY and RCP measurements of
4.0 ± 0.4 % (decay-corrected) and RCP of ≥ 95 % were attained. Radiosynthesis of $[^{18}\text{F}]\alpha\text{HER2Nb}$ took 2 hours, from end of bombardment; longer reaction times and time-consuming SE-HPLC steps contributed to the increase in overall radiosynthesis time. SE-HPLC was used in accordance with literature methods of nanobody radiolabelling, where SE-disposable cartridges are used to purify and formulate the radiolabelled nanobody$^{[18, 19]}$. We postulate that the low $[^{18}\text{F}]\alpha\text{HER2Nb}$ RCY were a result of low concentration of Aoa-αHER2Nb precursor (2.5x10$^{-5}$ M). A difference in Aoa-αHER2Aff and Aoa-αHER2Nb concentrations by two-orders of magnitude will, we predict, be largely responsible for the three-fold difference in overall RCY. Although the nanobody radiolabelling reaction time was extended, in order to help compensate for the lower precursor concentration, this proved inadequate. The low nanobody concentration was a result of enzyme-mediated Aoa-functionalisation using Sortase: based on a method described by Theile et al.$^{[19]}$, which permitted site-specific radiolabelling of the Aoa-αHER2Nb. Xavier et al.$^{[18]}$ report non-site-specific $[^{18}\text{F}]\text{SFB}$ radiolabelling of a HER2 nanobody using between 17-19 nmol of nanobody precursor, overall RCY of 10 ± 5 % were reported which permitted pre-clinical evaluation of the radiotracer$^{[18]}$. The matter of low starting APP precursor concentration is further perpetuated by the generally low recovery yields using SE methods of purification. This was reported by Flavell et al.$^{[21]}$ where 50 % loss of radiolabelled APP was described, using a SE-cartridge, ascribed to protein precipitation at low concentrations$^{[19]}$. Due to the low overall RCY, $^{18}\text{F}$ labelled nanobody was not produced in pre-clinically relevant quantities and thus in vivo evaluation was not performed.

The results of this investigation have demonstrated the successful application of the $[^{18}\text{F}]\text{FPCA}$ radiolabelling platform to an Aoa-APP, yet limitations of process-automation have imposed a lower-limit to the APP precursor concentration which may limit the
applicability of the platform as a truly generic approach to APP radiolabelling via oxime bond formation.

Conclusions

The $[^{18}\text{F}]$FPCA radiolabelling platform has been successfully applied to the site-specific radiolabelling of an Aoa-αHER2Aff. $[^{18}\text{F}]$αHER2Aff RCY were comparable to literature reported yields, despite radiolabelling conditions being unoptimised. Larger quantities of precursor were used, yet in vivo analysis showed radiotracer tumour accumulation and tumour to muscle contrast. Application of the radiolabelling platform to an Aoa-αHER2Nb did not produce large enough quantities of $[^{18}\text{F}]$αHER2Nb for in vivo evaluation and thus underlines a lower limit to APP precursor concentration. The approach, therefore, cannot be regarded as a truly generic radiolabelling platform to APP radiolabelling via oxime bond formation but does possess a number of key generic features, alongside automation, such as site-specificity of the radiolabel and mild reaction conditions.

Encouragingly, the radiolabelling platform demonstrated flexibility and can accommodate methodological changes according to the APP, such as HPLC replacement with SPE purification. The platform could also be applied to the radiolabelling of unfunctionalised APPs via a reductive alkylation radiolabelling pathway $^{[4]}$, further demonstrating its versatility.

The current work is illustrative of the important developmental steps, such as process automation, that are being taken to promote the use of $[^{18}\text{F}]$APPs and further support their translation from bench-to-bedside.

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References

Figure A) Schematic of customised GE TRACERlab FX-FN configuration permitting $[^{18}F]$FPCA radiosynthesis, purification and isolation followed by Aoa-functionalised peptide radiolabelling and purification
Chapter 8: Summary & conclusions

\(^{18}\text{F}\) has been described as an ideal PET radionuclide owing to its favourable physical characteristics \(^1, 2\). The number of \(^{18}\text{F}\)-radiolabelled APPs in routine clinic is limited which is attributed, in part, to the challenges of \(^{18}\text{F}\)-radiolabelling methods \(^1\). The advent of automated approaches by which APPs can be site-specifically radiolabelled with the radioisotope would help stimulate their translation from bench-to-bedside.

The work discussed in this thesis has described the process automation of \([^{18}\text{F}]\text{FPCA}\) and \([^{18}\text{F}]\text{FA}\) using a GE TRACERlab FX-FN \(^3-5\). Process automation of \([^{18}\text{F}]\text{FA}\) radiosynthesis, a prosthetic group originally devised by Prenant et al. \(^4\), and subsequent APP radiolabelling (rhIL-1RA) has been described alongside pre-clinical assessment of the radiolabelled APP \(^5\). Automated \([^{18}\text{F}]\text{FA}\) radiosynthesis using a microfluidic radiosynthesiser was additionally reported, thereby expanding the applicability of the prosthetic group to two radiochemistry modules.

Automation of the prosthetic groups, using a GE TRACERlab FX-FN, and application to a number of APPs is a progressive step towards a generic platform for APP radiolabelling: a general approach to APP radiolabelling with \([^{18}\text{F}]\text{fluoride}\) using fully-automated radiosynthesis.

The development of the novel prosthetic group \([^{18}\text{F}]\text{FPCA}\), from conception to full process automation including \([^{18}\text{F}]\text{FPCA}\) radiosynthesis and subsequent APP radiolabelling (RIOR2), has also been described \(^3\). The \([^{18}\text{F}]\text{FPCA}\) radiolabelling platform is an attractive, fully-automated approach to APP radiolabelling with \(^{18}\text{F}\) and an encouraging development in the field; to the author’s knowledge only one other approach to APP radiolabelling using a fully-automated radiosynthesis, exploiting fluoride-acceptor chemistry, has been described. The \([^{18}\text{F}]\text{FPCA}\) platform has been applied to the site-
specific radiolabelling of 5 APPs via oxime bond formation: anti-Aβ, RGD and hydrogel-forming peptides, an anti-HER2 affibody and anti-HER2 nanobody. The successes of the \([^{18}\text{F}]FPCA\) radiolabelling platform to the anti-Aβ and hydrogel-forming peptides permitted \textit{in vivo} evaluation; the usefulness of \([^{18}\text{F}]\text{RI-OR-TAT}\) as a radiotracer for detecting early-stage AD \cite{3} was assessed and the \textit{in vivo} pathway of a hydrogel-forming pathway was elucidated. Application of the platform to the anti-HER2 affibody and nanobody measured the versatility of the radiolabelling approach to larger, more complex APPs and highlighted the flexibility of the platform, but also underlined some of its limitations.

The utility of the platform in site-specific radiolabelling has been demonstrated alongside its proposed use as a method to non-site-specifically radiolabel APPs via reductive alkylation \cite{3}. Thus highlighting the platform’s potential for radiolabelling native APPs and helping guide pertinent ‘go’ and ‘no go’ decisions \cite{7}.

Development of the radiolabelling platform was motivated by the generic requirements of APP radiolabelling; this is reflected in the aqueous-solubility of the prosthetic group, use of mild APP radiolabelling conditions and suitability for use with SE-HPLC. It was, however, recognised that SE-HPLC can lead to issues of low radiotracer specific radioactivity and for this reason a facile enrichment method using oxidised dextran was devised. Although modest, the results afforded using the novel technique resulted in ~ 50% improvements in effective specific radioactivity measurements, a healthy improvement in the context, whilst only moderately impacting overall radiosynthesis.

Upholding generic requirements of APP radiolabelling, the \([^{18}\text{F}]FPCA\) platform has demonstrated its applicability to a number of APPs. The field of APP radiolabelling with \(^{18}\text{F}\) is a dynamic one - it is very active and encouragingly productive, characteristics that are likely to continue. But process automation of APP radiolabelling methods is important, it is a prerequisite to routine clinical applications and the aim of the current work was to develop such a platform and it is hoped that developments, like this, contribute to the
concerted efforts to harness the unmet potential of $[^{18}\text{F}]$APPs and encourage their use in routine-clinic.

References