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Solid Phase Synthesis of [^{18}F]Labelled Peptides for Positron Emission Tomography

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Abstract—A strategy for the solid phase synthesis of [^{18}F]labelled peptides has been developed. The peptides were prepared on solid support and acylated with 4-[^{18}F]fluorobenzoic acid using HATU within 3 min and the labelled peptide was released from the solid support within 7 min. The [^{18}F]labelled peptides were produced in good purity with a specific activity of 20–25 GBq/μmol. © 2000 Elsevier Science Ltd. All rights reserved.

At present [^{18}F] has been incorporated into many biological molecules including peptides,[1–4] proteins[5,6] and nucleotides[7] using a prosthetic group approach for imaging studies. Peptides are usually labelled in solution by treatment with an activated [^{18}F]fluorobenzoate derivative.[8–11] With peptides containing more than one basic group, either these basic groups are replaced[4] or the difference in pK_a between the α-amino and the ε-amino is utilised to produce the [^{18}F]labelled peptide.[12] In these procedures, the reaction results in mixtures of labelled, unlabelled and multiply labelled compounds, along with other impurities such as excess reagents and by-products and requires extensive purification.

Owing to these difficulties with solution phase synthesis, we decided to develop a new route to the synthesis of [^{18}F]labelled peptides using a solid phase approach. Model peptides were designed to investigate the solid phase strategy for the [^{18}F]labelling of peptides. In the design criterion cysteine and tryptophan were both omitted since they are susceptible to oxidation. The peptide sequence contained most other naturally occurring amino acids and contained a RGD motif, H-KPQVTRGDVFTM-NH_2. At the onset, we decided to use hyperacid labile protection where possible, since these groups would be rapidly deprotected. The side chains of the amino acids were protected with trityl based protecting groups[13] where possible, the guanidine group of arginine was protected with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl (Pbf) group[14,15] and the ϵ-amino group of lysine protected with a 4-methyltrityl (Mtt) group.[16]

The synthesis of the peptides was achieved by Fmoc-based solid phase techniques from C→N; stepwise synthesis was carried out on a polyethylene glycol-polystyrene (PEG-PS) support derivatised with a xanthen-2-oxovalerate (XAL) linker.[17] Fmoc deprotection was achieved by the treatment with 20% piperidine/DMF. Fmoc amino acids were used in 4-fold excess and activation was carried out using [(1H-benzotriazol-1-yl)(dimethylamino)methylene] - *N* - methylmethanaminium tetrafluoroborate-*N*-oxide (TBTU)[18] in the presence of diisopropylethylamine (DIPEA). At the completion of the synthesis, a small sample of the peptide was cleaved from the support by incubation with 95% trifluoroacetic acid (TFA) containing water, phenol and triisopropylsilane (TIPS) as carbocation scavengers.[19] The cleavage product was assessed to be greater than 95% pure by analytical RP-HPLC and covalent structure was confirmed by FAB and MALDI-TOF MS.

Before proceeding with radiotracer synthesis, 'cold' reactions of 4-fluorobenzoic acid with the H-K(Mtt)PQ (Trt) VT(Trt)R(Pbf)GD(tBu)VFT(Trt)M-XAL-PEG-PS (peptide-XAL-PEG-PS) were investigated in order to establish the optimal reaction conditions for the radiotracer synthesis. Experiments to optimise product yields were carried out on the reaction of 4-fluorobenzoic acid and the peptide-XAL-PEG-PS using a range of activating agents (Table 1).

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Table 1. Percentage yields for the coupling of the 4-fluorobenzoylpeptide[a]

Coupling	Time (min)			
	2	3	10	20
DIPCDI/HOBt	16	24	44	66
TBTU	82	87	>95	>95
DIPCDI/HOAt	33	37	57	74
HATU	87	>95	>95	>95

[a]Calculated by integration of the peaks corresponding to the 4-fluorobenzoyl peptide and the parent peptide in HPLC of the crude peptides directly after extraction with water and ether of the cleavage reagent TFA: H_2O: Phenol: TIPS (90:4:5:1).

Time-course observations of the product after cleavage from the resin showed that more than 65% of the reaction occurred during the first 20 min and the reaction had reached greater than 95% using the aminium activating species within 10 min. *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5,*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate-*N*-oxide (HATU)[20] was found to be particularly effective and the reaction had reached 95% within 3 min. HATU and diisopropylcarbodiimide (DIPCDI)/1-hydroxy-7-azabenzotriazole (HOAt)[21] were respectively more effective than TBTU and DIPCDI/1-hydroxybenzotriazole (HOBt) because they incorporate into their structure a nitrogen atom strategically placed at the 7 position of the aromatic ring.[20,21] Incorporation of this nitrogen in the benzene ring has two consequences. First, the electron withdrawing influence of the nitrogen effects the stabilisation of the leaving group, leading to greater reactivity. Second, placement specifically at the seven position results in classic neighbouring participation. Although HATU was initially assigned a uronium-type structure, more recently, the structure has been determined by X-ray analysis and HATU crystallises as an aminium salt rather than the corresponding uronium salt.[22]

Experiments to optimise cleavage of the 4-FC_6H_4CO-KPQVTRGDVFTM-NH_2 (4-fluorobenzoylpeptide) from the solid support were also carried out. Time course studies on the cleavage of the fluorobenzoylpeptide indicated that the cleavage of peptide was relatively slow (ca. 4h) and this was attributed to the difficult removal of the trityl side chain protection of glutamine as determined by MS. Since the Gln (Trt) containing peptides are sometimes cleaved slowly[23] the peptide sequence was re-synthesised without protection of the glutamine side chain.

The carboxyl group was preactivated as the pentafluorophenyl ester (Fmoc-Gln-OPfp)[24] since the action of activating agents on unprotected glutamine results in dehydration. Using this modification the peptide was cleaved from the resin more efficiently, however the cleavage time remained high (Table 2). The use of different scavengers and temperatures were investigated and optimal cleavage of the fluorobenzoylpeptide as determined by HPLC was achieved within 7 min at 37 °C. Over the short time period, the use of elevated temperatures did not appear to have a detrimental effect on the integrity of the peptide.

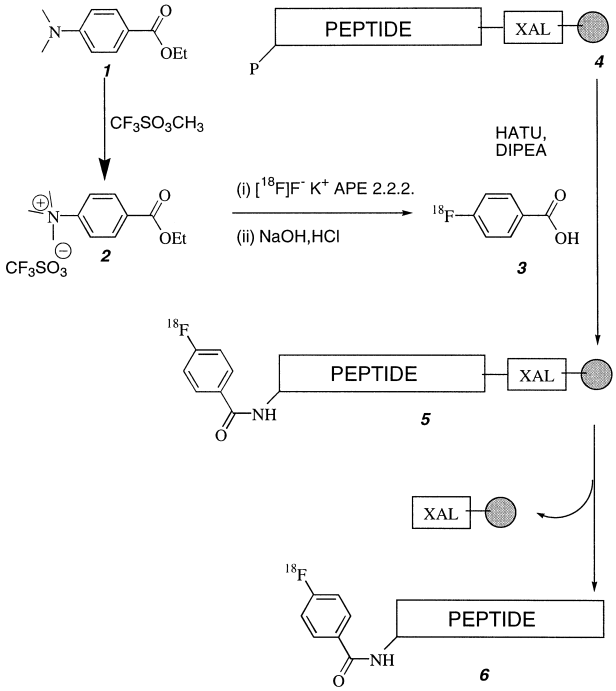
Table 2. Percentage yields for the cleavage of the 4-fluorobenzoylpeptide[a]

Cleavage Conditions	Time (min)			
	2	5	10	20
TFA:Phenol:H_2O: TIPS, RT	—	—	33	65
TFA:Phenol:H_2O:TIPS, 37 °C	24	59	>95	>95

[a]Calculated by integration of the peaks corresponding to the 4-fluorobenzoyl peptide and the parent peptide in HPLC of the crude peptides directly after extraction with ether and water of the cleavage reagent TFA:H_2O:Phenol:TIPS (90:4:5:1).

4-[^{18}F]Fluorobenzoic acid **3** was synthesised using a modification of published procedures[25] in a relatively simple three step procedure (Scheme 1) involving methylation of ethyl 4-(dimethylamino) benzoate **1**, nucleophilic displacements of the ammonium group **2** at 90 °C within 3 min using the powerful nucleophilic radiofluorinating agent, [^{18}F] F^- K^+ 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane (APE 2.2.2) and saponification of the ester moiety at 90°C within 3 min followed by SPE purification. The [^{18}F]fluoride was produced using a RDS 112 negative ion cyclotron by the $^{18}O(p, n)^{18}F$ reaction on ^{18}O-enriched water held in a silver target with 11.2 MeV proton beam. The [^{18}F]flouride was treated with APE 2.2.2 and potassium carbonate in acetonitrile and then azeotropically dried at 120°C using three aliquots of acetonitrile.

The synthesis of the radiotracer was accomplished in two rapid steps involving the labelling of the peptide-XAL-PEG-PS with 4-[^{18}F]fluorobenzoic acid using HATU within 3 min followed by cleavage of the radiotracer peptide **5** from the solid support within 7 min. The [^{18}F]labelled peptide **6** was analysed by radio HPLC. In the labelling reaction the reagents and byproducts were



Scheme 1. Solid phase synthesis of [^{18}F] labelled peptides.

removed by simple filtration, and in the cleavage the radiotracer was obtained by evaporation of the acid. In this case, the radiochemical purity of the peptide was >95% however; in some cases, purification may be required.

In an attempt to find a more efficient, selective and versatile approach for the [^{18}F]labelling of synthetic peptides a solid phase strategy was developed. This strategy offers a number of advantages over the present peptide labelling methods in the solution phase. The peptide-XAL-PEG-PS has been efficiently labelled with 4-[^{18}F]fluorobenzoic acid within 3 min using HATU as the activating agent and the [^{18}F]labelled peptide was released from the solid support within 7 min with TFA with an overall radiochemical labelling yield of (70–80%). This percetage yield is based on the [^{18}F] 4-flourobenzoic acid and it is at the end of the synthesis, not decay corrected, $n=20$, SEM 10%. During the release of the peptide from the solid support, scrambling of [^{18}F] with TFA was not observed. The solid phase strategy is very efficient and the [^{18}F]labelling was complete within 20 min, excluding the synthesis of the [^{18}F]F^- K^+ APE 2.2.2. In the solid phase approach the peptide chemistry was optimised and all reactions are expected to proceed in 99.99% yields. Prior to the labelling with the 4-fluorobenzoic acid, only one reactive site was made available and hence the process is completely specific and highly reproducible. In this case, the α-amino group was deprotected, similarly the ϵ-amino group of lysine can be deprotected using allyl chemistry and other groups such as carboxyl or hydroxyl could be deprotected as required. This approach is readily adapted to the synthesis of cyclic peptides and peptides containing disulphide bridges and hence this approach is extremely versatile. This methodology can also be adapted to Boc-benzyl chemistry although some of the protecting groups will have to be changed. Whilst solution phase chemistry requires time consuming extractions and purification, in the solid phase approach the reagents and byproducts are removed by simple filtration. In this case, the peptide was very clean and did not require purification, it is possible to release the peptide from the solid support, evaporate the TFA and inject the crude peptide directly into a HPLC column for purification. 10–50MBq of [^{18}F] flouride was used and the specific activity of the peptide was 20–25 GBq/μmol. As with solid phase peptide synthesis and solid phase combinatorial organic synthesis, this process may be readily automated and many instruments are commercially available.

In conclusion, the current study has demonstrated that the solid phase strategy presented has a number of advantages in the labelling of peptides with [^{18}F]. It is fast, efficient, clean, selective, highly versatile and may be readily automated. The [^{18}F]labelled peptides were synthesised within 20 min with a high specific activity.

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