Synthesis, Biocooptimisnjugation and Stability Studies of [^{18}F]Ethenesulfonyl Fluoride

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Short title

[^{18}F]ethenesulfonyl fluoride: synthesis, conjugation and products stability

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Abstract

Fluorine-18 labelled prosthetic groups (PGs) are often necessary for radiolabelling sensitive biological molecules such as peptides and proteins. Several shortcomings, however, often diminish the final yield of radiotracer. In an attempt to provide higher yielding and operationally efficient tools for radiolabelling biological molecules, we describe herein the first radiochemical synthesis of [^{18}F]ethenesulfonylfluoride ([^{18}F]ESF) and its Michael conjugation with amino acids and proteins. The synthesis of [^{18}F]ESF was optimised using a

microfluidic reactor under both carrier-added (c.a.) and no-carrier-added (n.c.a.) conditions, affording, in a straightforward procedure, 30-50% radiochemical yield (RCY) for c.a. [^{18}F]ESF and 60-70% RCY for n.c.a. [^{18}F]ESF. The conjugation reactions were performed at room temperature using 10 mg/mL precursor in aqueous/organic solvent mixtures for 15 min. The radiochemical stability of the final conjugates was evaluated in injectable formulation and rat serum, and resulted strongly substrate dependent and generally poor in rat serum. Therefore, in this work we have optimised a straightforward synthesis of [^{18}F]ESF and its Michael conjugation with model compounds, without requiring chromatographic purification. However, given the general low stability of the final products, further studies will be required for improving conjugate stability, before assessing the use of this PG for PET imaging.

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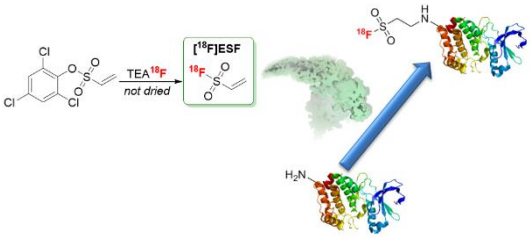

We report herein the radiosynthesis of [^{18}F]ethenesulfonyl fluoride [^{18}F](ESF), that proceeds straightforwardly without the need of chromatographic purification. We then investigated the use of [^{18}F]ESF as a new prosthetic group on amino acids, insulin and BSA. While the conjugation methodology and the purification processes were simple and efficient, the stabilities of the final products are strongly substrate-dependent, and generally low in serum.

Introduction

Positron emission tomography (PET) is a widely used molecular imaging tool that monitors the uptake and distribution of radiolabelled molecules *in vivo*.[1] It is used both in medical diagnostic procedures and as a powerful tool for investigating the fundamental mechanisms of disease pathology.[2] Fluorine-18 is one of the most commonly used PET radionuclides due to its clean decay profile, low positron energy, ideal half-life (109 minutes)[3,4] and propensity to be incorporated into target molecules either directly or indirectly. The more direct and efficient method usually involves [^{18}F]fluoride displacement of a suitable leaving group from a precursor molecule, requiring the use of harsh reaction conditions (high temperature and strong base) which are not suitable for biological molecules (proteins, peptides, antibodies).[5] The indirect method is typically a two-step process which involves the preliminary synthesis of a radiofluorinated prosthetic group (PG), followed by incorporation of the PG into the target molecule. The second step is run under mild conditions to avoid degradation of the biological vector.

Indirect fluorine-18 radiolabelling can be achieved by three methodologies. In the first approach, the radiolabelled PG is conjugated under mild conditions with an unmodified macromolecule. In this case, the radiolabelling precursor would be a bifunctional molecule, which undergoes selective radiolabelling on one site and specific bioconjugation on another site. Typical examples of these PGs are 4-nitrophenyl-2-[^{18}F]fluoropropionate and *N*-succinimidyl-4-[^{18}F]fluorobenzoate, which react with free amine functionalities naturally available within the target.[6,7] The second approach requires prior modification of the target macromolecule with a selected PG that can be radiolabelled under mild conditions using "raw" forms of fluorine-18. Typical examples are PGs that consist of B, Si or NOTA (for

[^{18}F]AlF^{2+}).[8–11] The third approach involves the use of click-chemistry reactions, and therefore requires modification of the macromolecule with an unnatural moiety (e.g. alkyne or azide) and the synthesis of a radiolabelled PG bearing the "partner" click functionality.[12] Even if the radiochemistry of the second and third approaches is relatively simple, their application might be hindered by the need to modify the target biomolecule. In general, the first approach would be preferred as it does not require modification of the target biomolecule and thus widens the applications. However, several shortcomings limit the use of this approach, such as an often complicated synthesis of the PG, the sensitivity of the biomolecule to optimised conjugation conditions, the need for HPLC pre-purification of the PG, post-purification of the conjugate, and the potential pharmacokinetic impact of adding the PG to target molecules. Given this, our goal was to develop a PG which can overcome most of these shortcomings, can be shipped to an imaging centre, and can be used in a simple "shake and bake" procedure to synthesize a wide range of radiotracers for PET imaging.

PGs that consist of radioactive sulfonyl fluorides were first reported in 2012. They have been prepared in high radiochemical yields (RCYs) even at room temperature and possess high stability in aqueous environments, although the decomposition of some of the studied analogues have been observed in mouse serum.[13,14] Among the molecules with sulfonyl fluoride moieties, ethenesulfonyl fluoride (ESF) is one of the strongest Michael acceptors reported in the literature.[15] It reacts with various soft nucleophiles, including amines, thiols, enamines, dienes, phosphines and activated methylene groups, under mild conditions with typically high yields, but is unreactive towards water and other hard nucleophiles.[16] Consequently, [^{18}F]ESF has significant potential to be used as a PG to radiolabel targets that

contain lysine or cysteine residues or other 'soft' nucleophilic groups naturally present in biomolecules. The small size and hydrophilic property of [^{18}F]ESF makes it ideal for radiolabelling oligopeptides since the resulting conjugation adducts may be less likely to adversely affect target binding and radiotracer pharmacokinetics, especially when compared to some larger and more hydrophobic PGs currently known. In this work, we aimed to systematically explore and optimise a simple and high yielding radiochemical synthesis of [^{18}F]ESF and investigate its utility for radiolabelling model structures and biological vectors.

Experimental

Materials & Instruments

All the reagents used were purchased from Sigma-Aldrich (analytical grade or higher) and used without further purification, unless stated otherwise. All solvents used for chromatography were purchased from Merck and were of HPLC grade. MP1 cartridges were purchased from either ORTG or Medchem Imaging. Solid Phase Extraction (SPE) cartridges were purchased from Waters.

HPLC for quality control of non-radioactive products consisted of a Waters 626 pump, Waters in-line degasser AF, Waters 717 plus autosampler, Waters 2996 photodiode array detector, Waters 600S controller and Alltima C18 column (3 µm pore size, 53 mm length and 7 mm internal diameter). The Radio-HPLC used for quality control of radioactive products was carried out using a Shimadzu system comprising of a CBM-20 controller, LC-20AD pump, SIL-20AHT auto injector SPD-M20A PDA and a Lablogic Posi-RAM gamma detector. The eluent and stationary phases of HPLC runs are reported in **SI (*Conditions A, B, C*)**.

A Bruker Advance DPX-400 NMR spectrometer was run at 400.13 MHz for ^{1}H NMR, 100.61 MHz for ^{13}C NMR and 376.50 MHz for ^{19}F NMR. A Waters Micromass ZQ quadrupole mass spectrometer was used to record mass data, and a Bruker Alpha Fourier Transform spectrometer was used to record IR data. A Buchi Reveleris Flash Chromatography System, with UV detection at both 254 nm and 280 nm was used to purify the synthesised compounds using a Silica column.

Aqueous [^{18}F]fluoride of 10-20 GBq was produced from an IBA Cyclone 18 Twin cyclotron by the $^{18}O(p,n)^{18}F$ nuclear reaction and delivered to a NanoTek microfluidic system from Advion.[17] Precursor solution and [^{18}F]fluoride complexes were pre-loaded onto two separate storage loops, and were delivered (10-20 µL for optimisation, 100-200 µL for production) at a set rate into a microreactor (2 m × 100 µm, 15.6 µL, fused silica) heated at predetermined temperatures. Reaction conditions which were varied during optimisation runs were: precursor to [^{18}F]fluoride ratio, [^{18}F]fluoride volume, flow rate and microreactor temperature. After the reaction mixture passed though the microreactor, the total volume of the microfluidic system tubing (400 µL) was swept with inert solvent (typically H_2O for carrier-added (c.a.) and DMSO for no-carrier-added (n.c.a.)), and the final product mixture was delivered to a collection vial. The outlet could be connected for HPLC analysis or directed towards purification to either the c.a. or the n.c.a. route in as shown **Figure 1**.

Synthesis of non-radioactive precursors and adducts

Ethenesulfonyl chloride (ESCl)

The synthesis process was an adaptation of a previously reported procedure.[18] 2-Chloroethane-1-sulfonyl chloride (5.00 g, 30.7 mmol) was dissolved in diethyl ether (20 mL) in a round bottom flask and cooled at -50 °C. A solution of 2,6-lutidine (3.94 g, 36.8 mmol) in

diethyl ether (7 mL) was slowly added dropwise, maintaining the bath temperature at -50 °C. Once the addition was complete, the mixture was stirred for 45 min and warmed to room temperature. The reaction was quenched by cooling at 0 °C and the additional of ice-cold 1% H_2SO_4 (15 mL). The organic layer was separated, washed once more with ice-cold 1% H_2SO_4 (5 mL), followed by water (5 mL) and 20% NaCl solution. This worked-up organic phase was then fractionally distilled under vacuum at 30 °C to get 1.73 g clear liquid (yield 45%).

2,4,6-Trichlorophenyl ethenesulfonate (TCPE)

The synthesis process was an adaptation of a previously reported procedure.[19] 2-Chloroethane-1-sulfonyl chloride (1.81 g, 11.1 mmol) was dissolved in dichloromethane (12.5 mL) and cooled to -50 °C. 2,4,6-trichlorophenol (2.00 g, 10.1 mmol) and triethylamine (2.35 g, 23.3 mmol) were pre-mixed in dichloromethane (2.5 mL) and added dropwise to the cooled sulfonyl chloride solution. During addition, the temperature was kept below 0 °C. Following complete addition, the reaction was allowed to warm to room temperature and stirred for additional 1 h. The reaction mixture was then diluted with diethyl ether (0.5 mL) and was washed with HCl (5 mL, 2 M) and saturated $NaHCO_3$ (5 mL) solution. The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude product was purified by flash chromatography (Silica, gradient of ether/ hexane) to give the title compound as a white solid (2.00 g, yield 69%).

Non-radioactive adducts

The synthesis procedure was adapted from the literature.[16] Ethenesulfonylfluoride (ESF) (200 mg, 1.81 mmol) was added to amino acid analogues (1 eq) in DMF (1 mL). The mixture was stirred for 1 hour at room temperature, before being quenched with water (5 mL) and

extracted with diethyl ether (5 mL). The organic layer was rotary evaporated to give the crude product. Flash chromatography (Silica, gradient of hexane/ethyl acetate) was used to purify the compounds.

Synthesis of [^{18}F]ESF

The synthesis of both c.a. and n.c.a. [^{18}F]ESF was performed in a microfluidic reactor system as described in the previous section. The purification of c.a. [^{18}F]ESF was achieved by connecting the outlet of the microreactor to a neutral alumina Light (nAl) SPE. The purification of n.c.a. [^{18}F]ESF was performed using the distillation setup in **Figure 1** (distillation time 20-30 min). Distillation progress was monitored using a radiodetector near SPE **D** and typically complete after 20-30 min. Radiochemical purity (RP) was measured by Radio-HPLC using ***Conditions B***.

Conjugation of [^{18}F]ESF

Small scale reactions

On each experimental day, all conjugation reactions were performed in parallel with all the tested substrate solutions, directly in the inserts of HPLC autosampler vials, by adding 20 µL (around 20 MBq) of [^{18}F]ESF to 80 µL of amino acid (AA) analogues solution (1 mg/mL in EtOH, DMF, DMSO, saline, water and PBS pH 7.4, with or without adding triethylamine (TEA)). After completion of [^{18}F]ESF addition, the test vials were transferred in the HPLC system and the whole series have been analysed sequentially up to 3 times (i.e. over-night analysis). Radio-HPLC: ***Conditions B*** for 10 mg/mL reaction***, Conditions C*** for 1 mg/mL reactions.

Large scale reactions

Large scale reactions were performed by adding 100 µL of [^{18}F]ESF (around 100 MBq) to 400 µL of amino acid analogue solutions in their best solvent conditions (determined from previous small scale reactions) in a 4 mL vial. After 15 min reaction time (45 min for **Ani**, 30 min for **Insulin**), the reaction mixture was purified by passing through a nAl SPE. The activity of the purified eluents and the nAl SPE was then measured, which enabled the calculation of the isolated RCY. The RP was measured by Radio-HPLC using ***Conditions B***.

Stability of [^{18}F]ESF conjugates

Stability in injectable formulation

The purified adducts from large scale reactions were diluted in saline to make injectable formulations (saline containing 10% v/v of EtOH or DMSO), and then analysed by Radio-HPLC at 0, 15, 30, 45, 60 and 120 min using ***Conditions B***.

Stability in rat serum

10 µL injectable formation of each adduct (c.a. and n.c.a. **Ani**, c.a. **Cys**, n.c.a. **Insulin**, n.c.a. Bovine serum albumin (**BSA**)) was added into 100 µL of rat serum in a shaker at 37 °C. Samples were taken at 15, 30, 45 and 60 min and then analysed by Radio-HPLC using ***Conditions B***.

Results and discussion

Non-radioactive conjugation reactions

Conjugation reactions of non-radioactive ESF with selected amino acid analogues and aniline (**Figure 2**) were performed to evaluate the conjugation ability of ESF and to synthesize the non-radioactive adduct standards for characterisation. This set of compounds was chosen

because it consisted of a range of functionalities (i.e. aliphatic amine, aromatic amine and thiol). Among the AA analogues, side chains with different electron withdrawing abilities were selected to evaluate their effects on the amine group reactivity. All analogues reacted with ESF at room temperature with satisfying yields, except for **Lys** which could not be synthesized as the reaction always provided the double conjugated **Lys-bis(ESF)**.

Synthesis of [^{18}F]ESF

The synthesis of [^{18}F]ESF was first attempted based on the reported synthesis of non-radioactive ESF using 2-chloroethanesulfonyl chloride (**ClSCl**, **Scheme 1A**) or ethenesulfonyl chloride (**ESCl**, **Scheme 1B**).[14,16] Unfortunately both routes were not successful, most likely due to the instability of **ESCl** and the degradation of intermediates under basic radiolabelling conditions. We then changed our strategy and tested a simple ^{18}F/^{19}F isotopic exchange on non-radioactive ESF to obtain carrier added (c.a.) [^{18}F]ESF (**Scheme 1C**).

The exchange reaction was performed in a microfluidic system, which enabled fast optimisation of reaction parameters (i.e. temperature and reaction time) and minimisation of operational variation. We first tested the effect of different forms of ^{18}F fluorination complex, and we obtained a maximum RCY of 63% at 160 °C using the traditional azeotropically dried $K_{2.2.2}/K_2CO_3$ [^{18}F]fluoride complex. Since we suspected that the basicity of the fluorinating complex was leading to the degradation of [^{18}F]ESF, we repeated the reaction with non-azeotropically dried (wet) tetraethylammonium bicarbonate (TEAB) [^{18}F]fluoride complex, and we were able to increase the maximum RCY to 83% at 130 °C.

Following this lead, we directly tested untreated irradiated target water as a source of [^{18}F]fluoride, and a maximum RCY of 32% was obtained at 100 °C. This source, obtained directly from cyclotron target production, although operationally simple, could be

problematic due to cross-site differences in targetry and the presence of longer-lived radioactive cationic species. Encouraged by this result, we reverted back to trapping the [^{18}F]fluoride on an anion exchange resin (MP1 or QMA) followed by elution with isotonic saline, which resulted in a maximum RCY of 90% at 100 °C.

Upon testing the effect of ESF concentration, we observed an increased RCY at higher ESF concentration, with a maximum RCY of 90% at 20 mg/mL. However, we chose 5 mg/mL as the optimised concentration because of little RCY difference between 20 mg/mL and 5 mg/mL. We also noticed the optimised temperature was 100 °C; further increasing or decreasing the temperature resulted in less RCY, probably due to decomposition of the product and incomplete reaction respectively.

Lastly, we tested the effect of residence times (equivalent to reaction time in flow chemistry, controlled by flow rate), and we obtained the best RCY of 81% with a residence time of 47 seconds. Longer and shorter residence times did not improve the RCY, most likely due to the same reason as the increase or decrease of temperature (decomposition of the product and incomplete reaction respectively).

In order to achieve the best flexibility in the subsequent conjugation step, we reduced the ionic strength in the reaction mixture by employing hypotonic saline (0.45% NaCl) as [^{18}F]fluoride eluent and 5 mg/mL ESF in water as precursor. This gave us a satisfying RCY of 64% at 130 °C with a residence time of 47 seconds.

A vessel reaction was also performed under similar conditions (5 mg/mL ESF in water, [^{18}F]fluoride in isotonic saline, 130 °C for 15 min, 1 mL total volume), providing a lower RCY of 57%. Since the only radioactive impurity in this reaction was unreacted [^{18}F]fluoride,

purification was straightforwardly performed by connecting the microreactor outlet to a nAl SPE. An isolated RCY of 30-50% was obtained by measuring the activity of the [^{18}F]ESF product and the nAl SPE. This gave a final product with >95% RP assessed by Radio-HPLC (Radio-TLC could not be used as [^{18}F]ESF adsorbs strongly to Silica), and was stable for transportation over 4 h in the reaction media.

Since ESF has a very low molar extinction coefficient and is not detectable by the UV component of HPLC, GC-MS was instead used to identify the presence of ESF. We injected the mixture of radioactive [^{18}F]ESF spiked with an excess amount of non-radioactive ESF into the HPLC, and collected the radioactive peak suspected to be [^{18}F]ESF. After decay, we then analysed the collected peak by GC-MS and the results confirmed the expected identity (**SI 3.3**).

Having successfully produced c.a. [^{18}F]ESF and verified its potential as a PG, we investigated the development of a method to produce no-carrier-added (n.c.a.) [^{18}F]ESF. Examination of the literature showed that radiofluorination on the sulfur atom of tosylate esters can be achieved.[20,21] However, this reaction has traditionally been regarded as an undesired side process, as the tosylate group is typically used on alkyl chains as a leaving moiety to be displaced by the [^{18}F]fluoride. We therefore envisaged exploiting this possibility by building a precursor in which the only site of likely fluoride attack would be the sulfur atom. Our n.c.a. radiolabelling precursor is a stable white solid 2,4,6-trichlorophenylethenesulfonate (**TCPE**) which is synthesized by reaction of **ClSCl** with the anion generated by base treatment of 2,4,6-trichlorophenol. The **TCPE** precursor was then subjected to nucleophilic radiofluorinations to produce n.c.a. [^{18}F]ESF (**Scheme 1D**).

The synthesis of n.c.a. [^{18}F]ESF was performed on the microfluidic system using the same strategy for optimisation, and we found that use of wet TEAB/[^{18}F]fluoride complex in CH_3CN and **TCPE** precusor concentration of 20 mg/mL gave a maximum RCY of 86% at 110 °C, irrespective to residence time and solvent. We picked 24 seconds as our optimised residence time as it was operationally straightforward and time saving.

A vessel reaction was also performed under similar conditions (0.5 mL of both 20 mg/mL **TCPE** and TEAB/[^{18}F]fluoride in DMSO heated at 80 °C for 30 min), however this resulted in a reduced RCY of 35%. Exploiting the fact that ESF is volatile while **TCPE** is non-volatile, we employed a distillation approach to purify the n.c.a. [^{18}F]ESF from a high-boiling solvent (DMSO). This was achieved by connecting the microreactor to a closed vial which was heated at 80 °C and pressurized with N_2 gas. The gaseous effluents from this vial were then directed through a Silica SPE, which trapped the [^{18}F]ESF gas. At this point, [^{18}F]ESF could be eluted from this Silica SPE by using either aqueous (water, saline, PBS pH7.4) or organic solvents (EtOH, DMSO, CH_3CN). However, we noticed that when using higher activities (>500 MBq), only EtOH and CH_3CN could be used for elution, as [^{18}F]ESF was not stable in other solvents, most likely due to radiolysis. A RCY of 60-70% was achievable by this distillation method and the final product was >95% RP with no UV impurities detectable. We also verified the RP of [^{18}F]ESF in the cartridge was still >95% after 4 h. This means we can transport the cartridge to other sites where [^{18}F]ESF can be eluted with desired solvent and conjugated with molecules of interest.

Conjugation of [^{18}F]ESF

Having optimised a simple way of producing c.a. [^{18}F]ESF, we studied its utilization in conjugation reactions with selected AA analogues and aniline (**Figure 2**) in various solvents.

Radio-TLC and Radio-HPLC were both used to measure conjugation RCY and the results were comparable;[22] however, we chose Radio-HPLC to assess conjugation efficiency as it allows the measurement of both unreacted [^{18}F]ESF and [^{18}F]fluoride. In a first set of reactions utilizing AA at 1 mg/mL concentration, all the HPLC vials were queued for overnight analysis to get quick proof of principle results; in this case, the reaction time was not controlled and could be up to 8 h (complete data reported in **SI**). In this initial screening, all analogues tested in organic solvents (i.e. EtOH, DMSO, DMF) reacted with [^{18}F]ESF and gave RCYs over 35%, while the use of aqueous solvents did not provide any conjugate, except for **Pro** and **Ani**. This is most likely due to the use of the hydrochloride salts of the AA substrates, which may not have sufficient availability of the free amine form for conjugation in aqueous environment. **Pro** and **Ani** were exceptions, which may be due to their different basic characters (secondary and aromatic amines respectively).

Due to the short half-life of fluorine-18, we modified our procedure to obtain satisfactory yields in shorter reaction times. We discovered that increasing the concentration of substrates to 10 mg/mL gave improved conjugation yields for a 15 min reaction time. **Table 1** reports the example of **Trp** which gave a 64% RCY under these new conditions (entry 2), compared to 41% in 6 h for the 1 mg/mL case (entry 1). However, in most reactions, we noticed the presence of a small amount of unreacted [^{18}F]ESF (**Figure 3A**). In order to drive the reaction to completion, we tested the addition of triethylamine (TEA) to transiently convert the amine salt to its free base form. We first started adding 1 equivalent (eq) of TEA with respect to AA, and obtained significantly higher RCYs with no unreacted [^{18}F]ESF left except for **Ani**. As for **Ani**, increasing the reaction time to 45 min allowed us to increase RCY to 85% with no unreacted [^{18}F]ESF. As for the other AA, we gradually decreased the amount

of TEA and we found that 0.25 eq of TEA was sufficient to consume all the unreacted [^{18}F]ESF and gave **Trp** conjugate a higher RCY of 91% (**Table 1**, entry 3; **Figure 3B**). We also verified that this amount of TEA did not affect the stability of the conjugate by re-analysing the conjugate over time.

Larger scale conjugation reactions for each substrate under optimised conditions were also performed. Since all [^{18}F]ESF was consumed in these conditions, final passage of the product mixture through a nAl SPE removed the only radioactive impurity, [^{18}F]fluoride, providing conjugation products with >95% RP (**Table 1**, entry 4). Since we wanted to assure the highest concentration of the final product, we did not rinse the cartridge with additional solvent; as consequence of this, the isolated RCYs (**Table 2**) were slightly lower than the small scale reactions. However, we noticed that it was possible to obtain similar RCYs by rinsing the cartridge with further 1 mL of solvent. The identity of conjugate products was confirmed through HPLC retention time (R_t) comparison with the non-radioactive standards (see **Figure 4** for example of **[^{18}F]Pro-ESF**). The identification of **[^{18}F]Lys-ESF** was tentative since we did not have the non-radioactive standard and could only speculate from its R_t which was between **Lys** and **Lys-bis(ESF)**. These purified products were assessed for stability in their production solvent mixture (i.e. 80% organic), and were >90% RP after 4 h, except for **[^{18}F]Lys-ESF** which resulted in complete defluorination.

The conjugation reactions of n.c.a. [^{18}F]ESF were conducted using a similar strategy as for the c.a. experiments, with the additional tests of **BSA** and **Insulin**. Also in this case, we first tried the longer time reactions with AA concentrations of 1 mg/mL, and then shortened the reaction time to 15 min while increasing the AA concentrations to 10 mg/mL (**SI** and **Table 1**, entries 6-7). In general, most AA analogues afforded higher RCY with n.c.a. [^{18}F]ESF than

with c.a. [^{18}F]ESF, probably due to the increased stoichiometric ratio of precursor towards the PG. Since the amount of unreacted [^{18}F]ESF was less, we re-evaluated the amount of TEA required and found that 0.1 eq of TEA relative to AA was enough to drive the conjugation reaction to completion (**Table 1**, entries 7-8). Surprisingly, DMF gave a dramatically lower RCY (**Table 1**, entry 5), which is most likely due to the presence of small amounts of dimethylamine impurity in the solvent. We verified this hypothesis by testing the stability of n.c.a. [^{18}F]ESF in 0.04% aqueous dimethylamine and confirmed its degradation to [^{18}F]fluoride within 15 min. This different behaviour between n.c.a. and c.a. [^{18}F]ESF is a textbook example of the effect of carrier amount in radiochemistry.[23] The two additional protein candidates, **BSA** and **Insulin**, were tested in saline and H_2O (at 10 mg/mL), and showed satisfying RCYs of 34% and 70%, respectively, after 15 min.

Larger scale reactions of n.c.a. [^{18}F]ESF on each substrate in their optimised conditions were then performed. Products were obtained with >95% RP (**Table 1**, entry 9) and the purity remained unchanged for 4 h in the conjugation media, except for **[^{18}F]Lys-ESF**. The molar activity (M_A) of n.c.a. PG conjugates could only be estimated as a lower bound, as the mass amount of adduct in the HPLC injection was below the detection limit; despite this, n.c.a. M_A were at least >1000 times higher than the c.a. ones (**Table 2**). Since the [^{18}F]ESF conjugates of **BSA** and **Insulin** could not be separated from the unreacted substrates, the effective M_A (Activity/Protein amount) was instead calculated.

Stability of [^{18}F]ESF conjugates

Further stability tests were conducted on all of the purified conjugates (i.e. both c.a. and n.c.a.) in injectable formulations (saline containing 10% v/v EtOH or DMSO) over a 2 h time frame. Our aim was to understand the time needed to reduce the RP of the conjugates to a

threshold level of 10%. **Figure 5** shows a selection of the results from n.c.a. conjugates. In this case, we found that after 2 h **[^{18}F]Ani-ESF** was the most stable candidate with 92% RP, followed by **[^{18}F]Cys-ESF** with 43%; all the other AA conjugates degraded (i.e. <10% RP) within 1 h through defluorination. **BSA** and **Insulin** conjugates were both 54% pure after 2 h. Of the c.a. adducts, **[^{18}F]Ani-ESF** and **[^{18}F]Cys-ESF** proved to be the better candidates, with purities of 85% and 12% respectively after 2 h.

Having evaluated the stability of all conjugates in injectable formulations, we selected the best candidates and tested their stabilities in rat serum. **[^{18}F]Ani-ESF** approached the test threshold after one hour in both c.a. and n.c.a. cases (16% and 10% RP, respectively), whereas **[^{18}F]Cys-ESF** completely degraded within the first 15 mins. For the two proteins, the **Insulin** conjugate reached 13% RP after 15 min, while the **BSA** conjugate was completely degraded within the same time.

Conclusions

In this work, we have developed the first radiosynthesis of [^{18}F]ESF, under both c.a. and n.c.a. conditions that do not require chromatographic purification and are operationally straightforward. We have also successfully tested the conjugation of [^{18}F]ESF with selected analogues at room temperature, and developed a simple cartridge-based method for purification. Importantly, the conjugation does not require any modification of the targets, but employs naturally occurring “soft” nucleophilic functional groups (i.e. SH, NH_2). Whilst the conjugates are stable in their preparation mixture, their stabilities in injectable formulation and rat serum are generally poor and strongly substrate dependent. Further work on improving conjugate stability is needed to allow use of this PG in PET imaging. We

are currently investigating other strategies to decrease S-F bond reactivity by inhibiting hydrogen bond activation and using different formulation strategies.[14,24]

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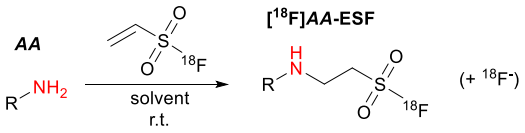
Tables

Table 1. Examples of [^{18}F]ESF conjugation reactions



	AA[a]	Solvent[b]	[^{18}F]ESF[c]	Time (min)	RCY (%)[d]
1	Trp (1)	DMSO (80)	20	360	41
2	Trp (10)	DMSO (80)	20	15	64
3	Trp (10)	DMSO (80)	20 + 0.25 eq TEA	15	91
4	Trp (10)	DMSO (400)	100 + 0.25 eq TEA	15	97[d]
5	Phe (1)	DMF (80)	20	120	2
6	Phe (1)	DMSO (80)	20	300	80
7	Phe (10)	DMSO (80)	20	15	77
8	Phe (10)	DMSO (80)	20 + 0.1 eq TEA	15	90
9	Phe (10)	DMSO (400)	20 + 0.1 eq TEA	15	97[d]

[a] Concentrations in parentheses, expressed in mg/mL; [b] Volumes in parentheses, expressed in µL; [c] Volumes expressed in µL, c.a. for Trp, n.c.a. for Phe; [d] Assayed by RadioHPLC; [d] RP reported.

Table 2. Summary of optimised large scale conjugation reactions.

	Substrates (solvent)	RCY (%)[c]	Activity (MBq)[d]	M_A (MBq/nmol)[e]
c.a.	Ani (EtOH)	57	4.9	0.03
	Cys (EtOH)	61	34	0.05
	Gln (DMSO)[a]	61	10	0.03
	Lys (DMSO)[a]	35	5.2	n/a
	Pro (DMSO)	65	8.5	0.04
	Phe (DMSO)[a]	67	7	0.04
	Ser (DMSO)[a]	64	6.4	0.03
	Trp (DMSO)[a]	63	5.1	0.02
	Tyr (EtOH)	51	2.8	0.06
n.c.a.	Ani (EtOH)	39	19.8	>11.23
	Cys (DMSO)	73	24.9	>6.86
	Gln (DMSO)	71	21.2	>1.59
	Lys (DMSO)[b]	59	14.2	n/a
	Pro (DMSO)	71	16	>14.88
	Phe (DMSO)[b]	71	15.1	>32.04
	Ser (DMSO)[b]	72	14.9	>7.91
	Trp (DMSO)[b]	67	12.4	>15.73
	Tyr (EtOH) [b]	62	10.9	>21.38
	BSA (saline)	55	9.6	0.66[f]
	Insulin (H_2O)	29	4	0.03[f]

[a] 0.25 eq of TEA added; [b] 0.1 eq of TEA added; [c] Isolated RCY calculated for single conjugation reaction as: (Product activity)/(Starting activity), all RP were >95%; [d] Isolated activity of final product; [e] Corrected to end of [^{18}F]ESF synthesis; [f] effective M_A calculated as: (Activity)/(Protein amount**)**.

Figure captions

Figure 1. Schematics of microfluidic system used for [^{18}F]ESF production. **c.a. [^{18}F]ESF:** The outlet of the microfluidic system is connected to a neutral alumina SPE which removes the unreacted [^{18}F]fluoride. **n.c.a. [^{18}F]ESF:** The outlet of the microfluidic system is connected to a closed vial (**A**) heated at 80 °C and pressurized with N_2 gas (0.1-0.2 L/min). The gaseous effluents from this vial are then directed through silica light SPE (**C**) into a second vial (**B**). The outlet of vial **B** is connected a Silica plus SPE (**D**) to trap the [^{18}F]ESF.

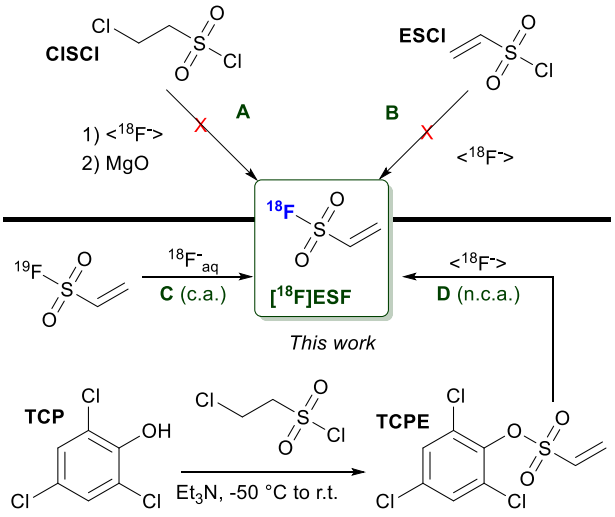
Figure 2. Aniline and amino acid analogues radiolabelled in this work. The conjugation site with [^{18}F]ESF are highlighted in italic (red).

Scheme 1. Routes to the radiosynthesis of [^{18}F]ESF. <$^{18}F^-$> represents a form of radiofluorinating complex.

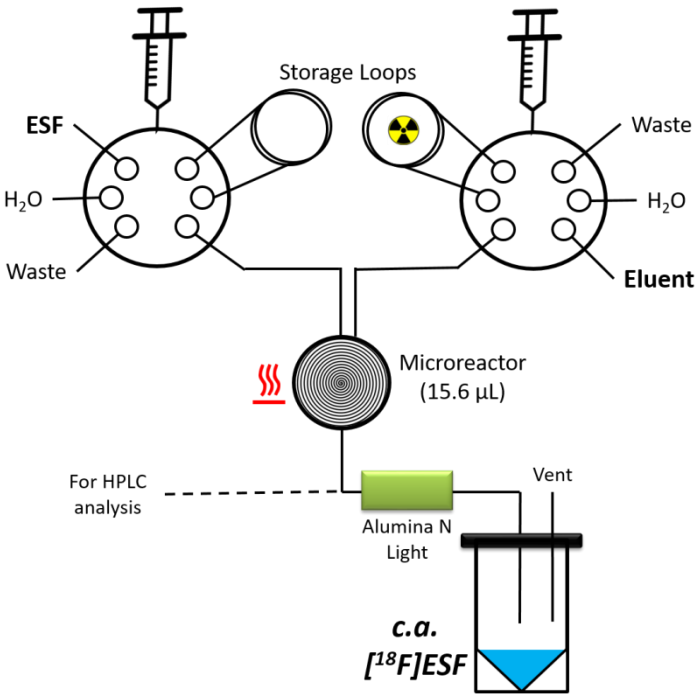
Figure 3. RadioHPLC profiles of c.a. [^{18}F]ESF conjugation with Trp (**A**), and effect of TEA addition (**B**)

Figure 4. Identification of **[^{18}F]Pro-ESF**. The UV R_t of radioactive **[^{18}F]Pro-ESF** (4.9 min, blue solid line, inverted scale) is identical to the UV R_t of non-radioactive Pro-ESF standard (purple dash line, inverted scale).

Figure 5. Stability results of selected n.c.a. [^{18}F]ESF adducts in injectable formulations.


ClSCl
ESCl
A
B
1) <18F->
2) MgO
<18F->
19F
18F-aq
C (c.a.)
[18F]ESF
D (n.c.a.)
This work
TCP
TCPE
Et3N, -50 °C to r.t.

Scheme 1


Storage Loops
ESF
H2O
Waste
Waste
H2O
Eluent
Microreactor
(15.6 µL)
For HPLC
analysis
Alumina N
Light
Vent
c.a.
[18F]ESF

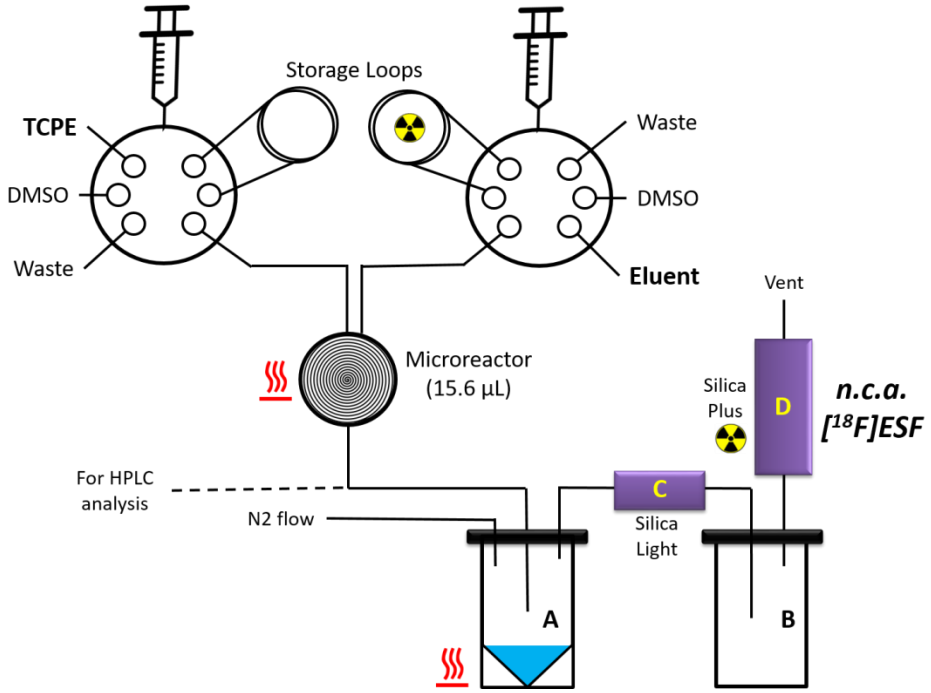


Figure 1

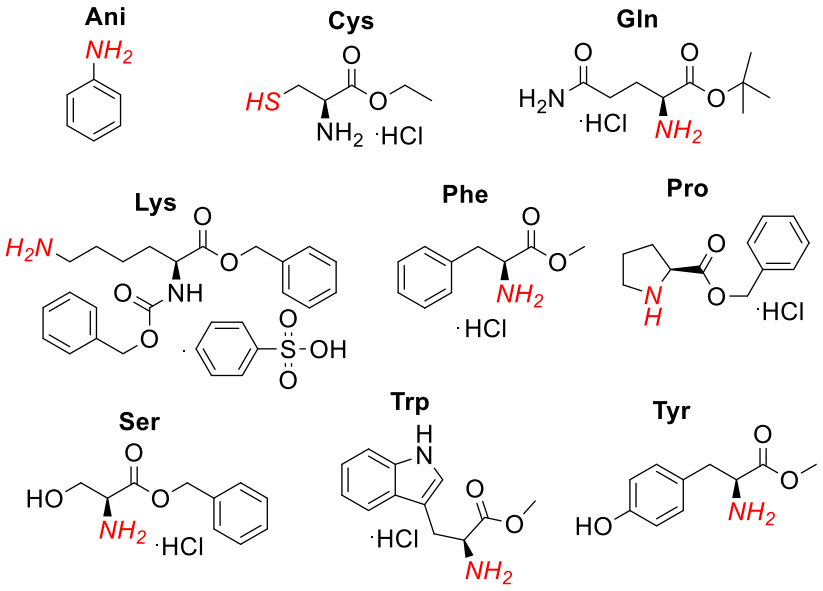


Figure 2

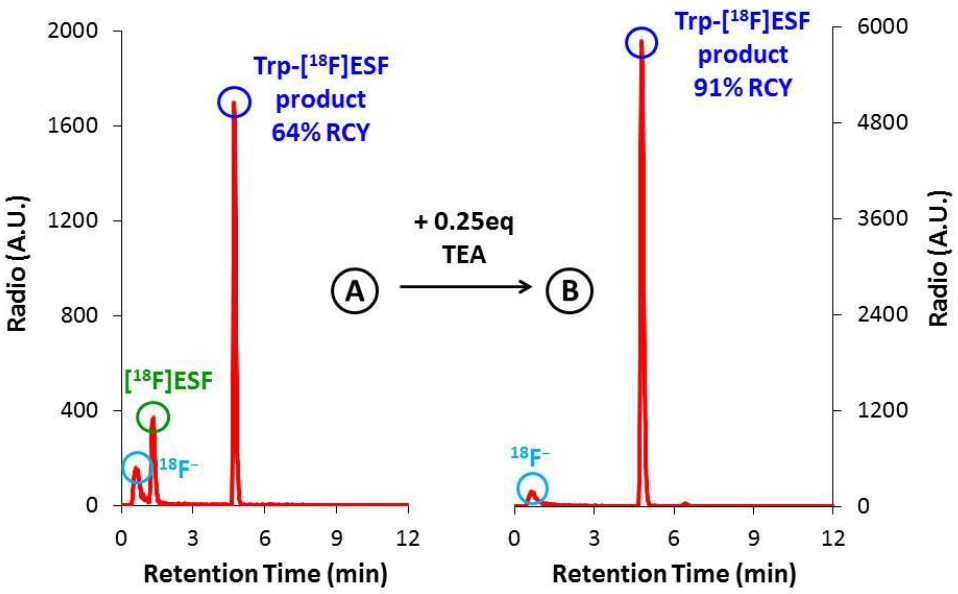


Figure 3

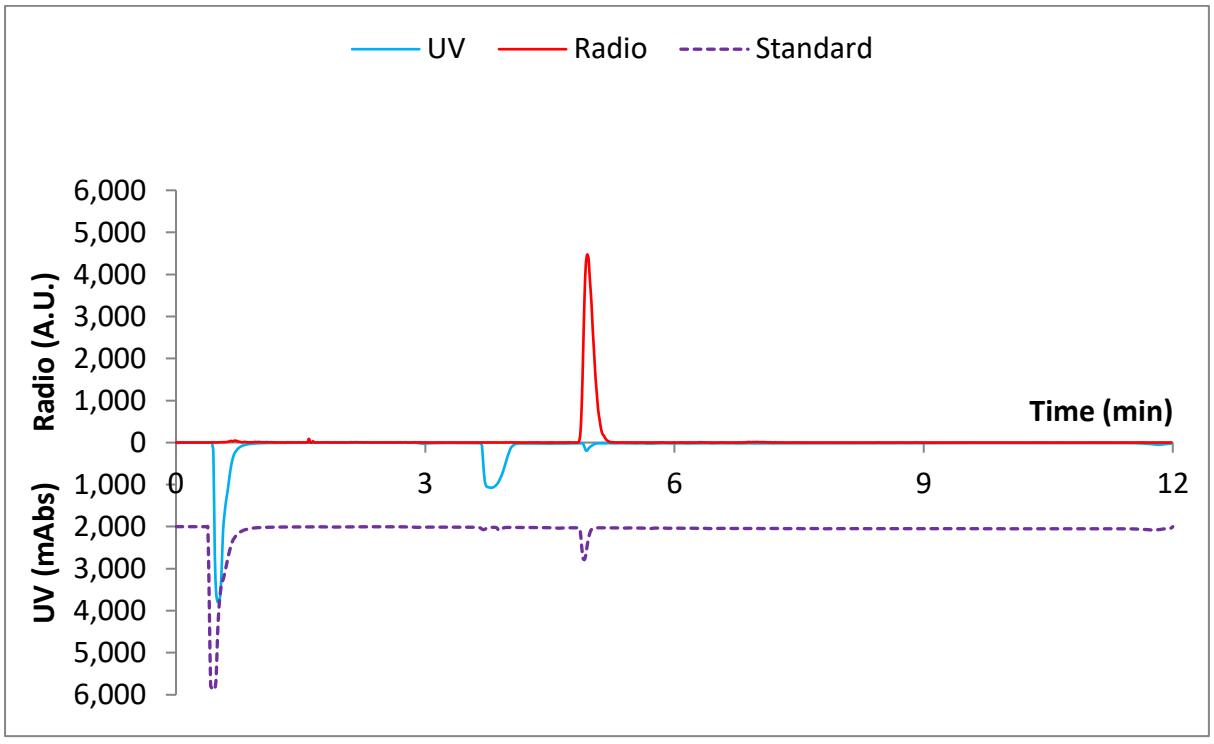


Figure 4

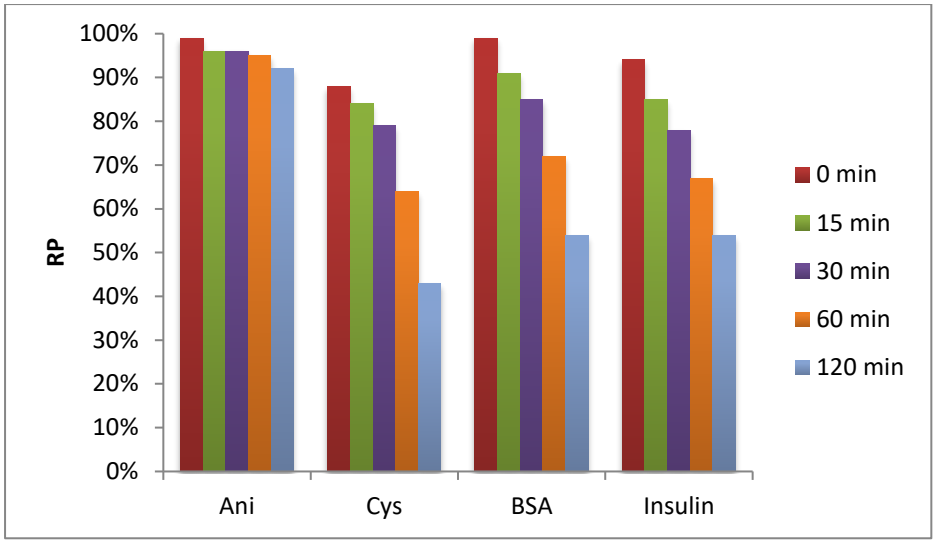

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
RP
Ani
Cys
BSA
Insulin
0 min
15 min
30 min
60 min
120 min

Figure 5