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Rapid ^{18}F-radiolabeling of peptides from [^{18}F]fluoride using a single microfluidics device

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***Abstract*:**

To date the majority of ^{18}F-peptide radiolabeling approaches are multi-step, low yielding and time-consuming processes. Given the short half-life of ^{18}F (109.8 min), it is critical that methods are developed to increase the efficiency of this process with simpler, higher yielding and faster reactions that can be rapidly translated into clinical use. Here, we demonstrate the first microfluidic synthesis of the [^{18}F]F-Py-TFP prosthetic group with radiochemical yields of up to 97% and a synthesis time of 3 min. In addition, we utilized a single microfluidics device to prepare the [^{18}F]F-Py-YGGFL peptide using [^{18}F]F-Py-TFP, from [^{18}F]fluoride in a two-step, fully automated approach. The model peptide NH_2-YGGFL was radiolabeled with [^{18}F]F-Py-TFP in up to 28% overall radiochemical yield within 8 minutes starting from anhydrous [^{18}F]fluoride.

Introduction:

Synthesis of ^{18}F-peptide based radiotracers are multi-step, complex, low yielding and often require a preparation time extending beyond one hour [1]. This presents a challenge for radiolabeling with ^{18}F due to its short half-life (109.8 min) in addition to the low molarity of radioisotope (fM-nM) and the small amount of reagents used. These type of radiosyntheses benefit greatly from automation, in particular miniaturization [2] [3]. Automated devices have proved to be excellent tools for speeding up overall radiotracer synthesis times, improving synthesis reproducibility and manipulations of small volumes, as well as limiting radiation exposure to the radiochemist [4]. Examples of automated devices that are commercially available for the preparation of clinical radiotracers are the GE FASTlab® [5], the Siemens Explora® and Eckert and Ziegler's Modular-Lab® [6]. These devices have demonstrated improved synthesis of many useful radiotracers and labeling agents, including [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) [7], [^{18}F]fluoromisonidazole ([^{18}F]FMISO) [8], [^{18}F]fluorothymidine ([^{18}F]FLT) [9] , and *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) [5] .

Trends towards miniaturization of automated devices have led to the development of microfluidic technology for radiotracer syntheses [3, 10]. In particular microfluidic technology has demonstrated advantages as a platform to address some of the low yielding and time consuming syntheses challenges associated with radiotracer development [11]. The small reaction volumes and high surface area to volume ratio achievable with microfluidics result in fast and high yielding reactions [3]. Several microfluidic devices have been developed applying both chip-based and flow through chemistry reactors. Van Dam *et al* developed a chip-based microfluidic technology, in which liquids are manipulated with electric potentials through the electrowetting-on-dielectric (EWOD) mechanism [12]. This chip-based technology allows for fast and efficient chemistry on a small scale (2-12 μL), and has enabled a wide range of radiotracer synthesis with the use of one EWOD chip. Chen *et al* demonstrated the synthesis of [^{18}F]FDG, [^{18}F]FLT, [^{18}F]SFB, and [^{18}F]fallypride all on one single microfluidic chip [12]. The Advion NanoTek® is a commercially available microfluidic flow chemistry device that is specifically designed for radiochemistry, and is highly adaptable for a variety of syntheses [13]. The Advion NanoTek® is a three-pump flow chemistry system in which reactions take place in reactors composed of crystal quartz capillary tubing. The Advion NanoTek® has been used in both the optimization process and batch production of several radiotracers, including [^{18}F]fluoroazomycin arabinoside ([^{18}F]FAZA) [14], [^{18}F]altanserin [15], [^{18}F]fallypride [16] and [^{18}F]fluoroethyltyrosine [^{18}F]FET [17]. Further, the Advion NanoTek® has shown initial promise as a tool for the production of ^{18}F-radiolabeled peptides [18] [19] [20] .

^{18}F-radiolabeled peptides have demonstrated their clinical usefulness as effective *in vivo* imaging agents in oncology [21].This is attributed to their excellent targeting ability, low immunogenic effects, small size with rapid and preferential renal excretion [1]. Peptides containing the RGD motif have been used for imaging of the $\alpha_v\beta_3$ integrin, a receptor that is over expressed in many cancers cells as well as on new blood vessels formed during tumor angiogenesis [22]. RGD peptides have been radiolabeled with several radionuclides (^{99m}Tc, ^{64}Cu, ^{68}Ga, and ^{18}F) for use in both PET and SPECT [23]. Additionally, our group has done pioneering work in developing the ^{18}F-radiolabled PEGylated version of A20FMDV for imaging of the $\alpha_v\beta_6$ integrin, a receptor that is overexpressed in many cancers including colon, breast, lung and pancreatic [24] [25] [26] . Traditionally, radiolabeling of peptides with fluorine-18 requires the synthesis of an ^{18}F- prosthetic group followed by its conjugation to the peptide using mild reaction conditions [27]. This approach can be time consuming, result in multiple labeled products, and is typically low yielding. Many different prosthetic groups have been extensively used, each with their own advantages and disadvantages [21]. The most commonly utilized prosthetic group for ^{18}F peptide radiolabeling is [^{18}F]SFB. Peptide radiolabeling with [^{18}F]SFB is a 4-step process that takes up to 3 hours to complete, and is reported in 10-24% overall yields. Attempts have been made

to improve this synthesis by decreasing reaction time and improving yields using microfluidics and microwave heating. However, a simpler, faster, automated approach would be deemed valuable in ^{18}F peptide radiolabeling. Recently, microfluidic syntheses of [^{18}F]SFB were described where they reported an overall synthesis time of 25-60 min and radiochemical yields of 33-55% [12] [28]. [^{18}F]Fluorobenzoic acid has also been used to site specifically radiolabel peptides on solid phase in high purity [29]. While successful, radiochemical yields of these reactions are highly variable depending on the peptide sequence and type of solid support used. From our studies, typical reaction conditions for solid-phase peptide radiolabeling result in yields ranging from 18-41% [30] [24] [31]. Most recently, McBride *et al* has developed a chelate approach for ^{18}F-radiolabeling, involving an NOTA- Al[^{18}F]F^{-} complex that is conjugated to the peptide [32]. This approach has many advantages in that it is simple, fast and high yielding, but sometimes suffers *in vivo* drawbacks including high renal uptake and retention [25] [33] . To address some of the challenges of multistep synthesis, Olberg *et al* [23] developed a one-step synthesis of the activated ester prosthetic group, 6-[^{18}F]fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester ([^{18}F]F-Py-TFP, Figure 1 **II**). [^{18}F]F-Py-TFP can be used to radiolabel peptides bearing free-amines. Several groups have shown the effectiveness of [^{18}F]F-Py-TFP to radiolabel peptides [34] and small molecules [35] in fast (10-20 min) and relatively high yielding (40-90%) reactions.

Here we report the first microfluidic synthesis of [^{18}F]F-Py-TFP (Figure 1 **II**) and its subsequent use in^{18}F-radiolabeling of the model peptide, NH_2-Tyr-Gly-Gly-Phe-Leu (NH_2-YGGFL) on a microfluidic platform. The process is fully automated and allows for the rapid microfluidic synthesis of ^{18}F radiolabeled peptides from [^{18}F]fluoride, on a single microfluidic device (Advion Nanotek®).

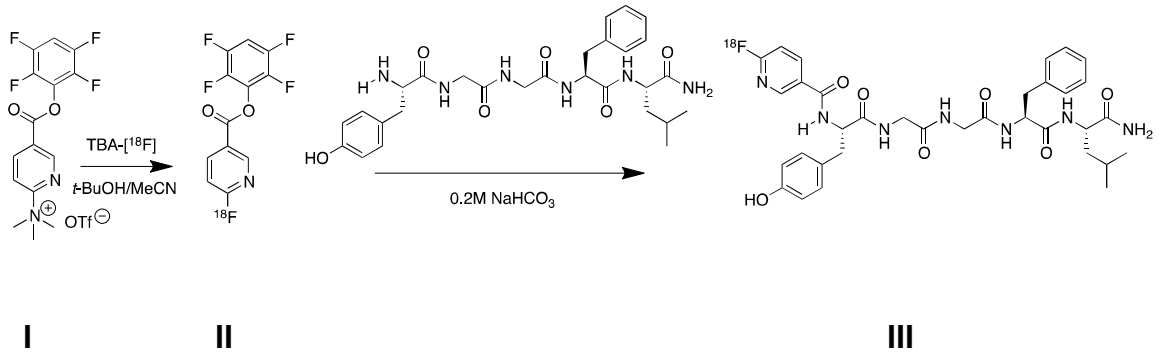

Figure 1: Reaction scheme of [^{18}F]F-Py-TFP synthesis and peptide radiolabeling

Experimental:

General

All reagents were purchased from Sigma Aldrich (Milwaukee, WI), and VWR (Radnor, PA, USA) and used without further purification, unless otherwise stated. Peptides were assembled on rink amide resin (Novabiochem, Darmstadt, Germany) using traditional solid-phase 9-Fluorenylmethoxycarbonyl (Fmoc) chemistry [36]. [^{18}F]fluoride was purchased from PETNet Solutions (Sacramento, CA, USA) and delivered on a solid ion cartridge (PS-HCO_3, ABX GmbH, Radeberg, Germany). All solvents used for microfluidic synthesis were filtered prior to use with a Millex syringe driven 0.42μm filter (Millipore, Bedford, MA, USA).

Microfluidic device description and operation

Radiolabeling reactions were performed on an Advion NanoTek® microfluidic flow chemistry device (Figure 2). The Advion NanoTek® is a three-pump microfluidic flow chemistry device that is specifically designed for radiochemistry. The Advion NanoTek® is comprised of three main modules: a concentrator module, pump module, and reactor module. The concentrator module is comprised of a syringe pump, a 6-way valve, and a heating block that is designed to hold a 3mL V-vial, and is used for [^{18}F]fluoride drying and resolubilization. The pump module consists of 2 high pressure syringe pumps with attached storage loops, pump 1 (used to hold [^{18}F]F-Py-TFP precursor, **I**) and pump 2 (used to hold peptide). The reactor module consists of 1 syringe pump, pump 3, and is used to transfer solvent through the system. Typical configuration of the device allows for a one or two-step reaction to be performed. The

three-pump device can be loaded with three different reagents into Pump 1 (P1), Pump 2 (P2), and Pump 3 (P3) (Figure 2). The first reaction consists of reagent one (from P1) and reagent 3 (from P3) combining in reactor one, and the second reaction (if applicable) involves the combination of the bolus of reaction 1 with reagent 2 (from P2) in reactor 2.

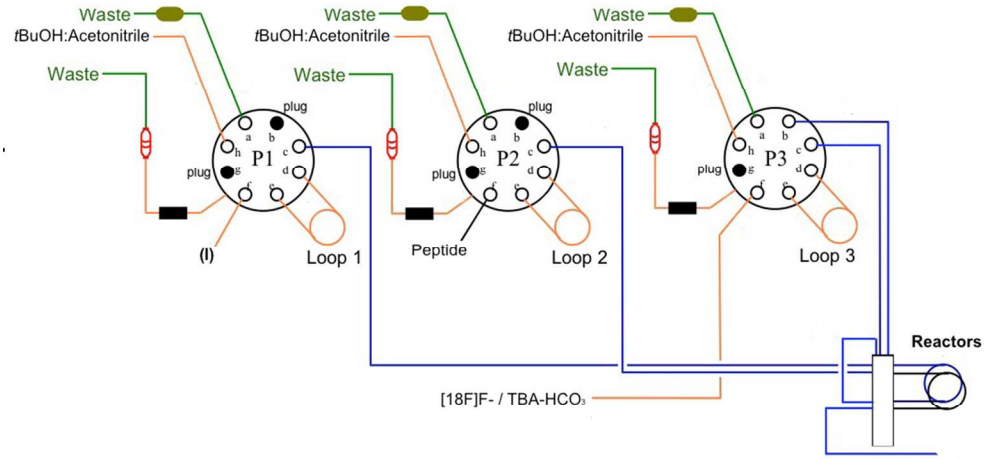

Figure 2: Schematic of Advion NanoTek® microfluidics device

The entire system is controlled by a standalone laptop computer, which allows for the precise setting of reaction parameters including reaction volume, temperature, flow rate, and reagent ratio. Reactions were done in Discovery Mode of the device. Reactions were carried out in 2 or 4-meter long reactors with 100 µm inner diameter (holding 15.7µL or 31.4 µL, respectively).

[^{18}F]fluoride drying procedure

Azeotropic drying of the [^{18}F]fluoride was done using the concentrator module of the microfluidics device. [^{18}F]fluoride was delivered on a PS-HCO_3 cartridge and placed into the microfluidics device. [^{18}F]fluoride was eluted from the cartridge with 450 µL of a tetrabutylammonium bicarbonate solution (30 µL 0.2M TBA-HCO_3 in 600 µL 1:1 acetonitrile:water). [^{18}F]fluoride/TBA-HCO_3 solution was then azeotropically dried with 3x1mL anhydrous acetonitrile. [^{18}F]fluoride/TBA-HCO_3 was then resolubilized in anhydrous acetonitrile.

Analysis:

Radiochemical reactions were monitored using radio thin layer chromatography (radioTLC) and radio high performance liquid chromatography (radioHPLC) and identity of radiolabeled product was confirmed by co-elution with non-radioactive standards. RadioTLC was performed on a Bioscan 3000 TLC (Washington DC, USA) with a 1:1 ethyl acetate:hexanes mobile phase on silica TLC plates (EMD, Darmstadt, Germany). RadioHPLC was performed using a Phenomenex Jupiter Proteo 90 Å column (250×4.6 mm, 4 µm) with HPLC mobile phase solvents 0.05 % trifluoroacetic acid in water (v/v; solvent A) and 100 % acetonitrile (solvent B). Mobile phase gradient was 9 % solvent B isocratic for 2 min, then a linear gradient from 9 % to 81 % over 30 min with a flow rate of 1.5 mL/min. All peptides were analyzed by mass spectrometry using an ABI 4700 matrix-assisted laser desorption-ionization time of flight/time of flight (MALDI TOF/TOF) spectrometer (Applied Biosystems, Foster City, CA, USA).

[^{19}F]-reference standards and precursor synthesis:

N,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)-carbonyl)pyridine-2-aminium trifluoromethanesulfonate (**I**) and [^{19}F]F-Py-TFP reference standard were synthesized as described by Olberg *et al* [27]. For [^{19}F]F-Py-YGGFL reference standard synthesis, 50 mg of NH_2-YGGFL on TGR resin was combined with 3 equivalents of 6-fluoronicotinic acid, 3 equivalents of 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU), and 6 equivalents of *N*-diisopropylethylamine (DIPEA) and rotated at room temperature for

1.5 hours. After completed reaction, peptide was cleaved from resin with 95:2.5:2.5:1 v:v:v:v trifluoroacetic acid (TFA): triisopropylsilane (TIPS): water: ethanedithiol (EDT) and purified by semi-preparative HPLC. [^{19}F]F-Py-TFP reference standard was characterized by HPLC (retention time 8.5 min, supplemental Figure 1) and ESI MS (m/z=288.2). NH_2-YGGFL peptide was cleaved from resin, purified by semi-preparative HPLC, and characterized by HPLC (retention time 4.5 min) and MALDI-TOF-MS (*m/z+H*= 555.3). [^{19}F]F-Py-YGGFL reference standard was characterized by HPLC (retention time 5.5min, Supplemental Figure 2) and MALDI-TOF MS (*m/z+H* =677.4).

Microfluidic synthesis of [^{18}F]F-Py-TFP :

N,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)-carbonyl)pyridine-2-aminium trifluoromethanesulfonate (**I**) was dissolved in 1:1 *t*BuOH:MeCN (3 mg/mL) and loaded into Pump 1 (P1,Figure 2) and dried [^{18}F]F$^-$ /tetrabutylammonium bicarbonate (TBA-HCO_3) (~5-30 mCi) in 1:1 *t*BuOH:MeCN was loaded into Pump 3 (P3, Figure 2). Pumps 1 and 3 were combined in reactor 1 (2 meter reactor) for the production of **II**. Temperature (40-110°C), flow rates (15-60 μL min^{-1}), and reagent volume ratios (0.2-2, P1:P3) were varied. Pump 3 volume was set at 20 μL for each optimization reaction. Radioactivity ranged from 5-400 μCi for each reaction.

Microfluidic synthesis of [^{18}F]F-Py-YGGFL:

[^{18}F]F-Py-YGGFL synthesis was done in a two-step process. Following [^{18}F]F-Py-TFP production on the microfluidics, the [^{18}F]F-Py-TFP was transferred into the second microfluidic reactor (4 meter reactor) and reacted with NH_2-YGGFL (3 mg/mL), in 0.2M $NaHCO_3$ buffer at pH 8.5. Temperature (30-80°C), flow rate (10-30 μL min^{-1}) and reagent ratio (0.5-3 P2:Rxn1) were varied. Optimization reactions were done with P3 pump volume of 20 μL. After optimized conditions were found, a larger 50 μL P3 volume reaction was done for scale up purposes.

Results and Discussion:

Microfluidic synthesis of [^{18}F]F-Py-TFP :

Previous work in the one-step synthesis and application of [^{18}F]F-Py-TFP has shown the utility of this amine reactive prosthetic group as an alternative to the three step [^{18}F]SFB approach. The sensitivity of [^{18}F]SFB to changes in pH can have an detrimental effect on radiochemical yield even with very small shifts in pH [37]. In contrast, [^{18}F]F-Py-TFP tends to be less affected by pH, while retaining the same reactivity as [^{18}F]SFB. Currently [^{18}F]F-Py-TFP is synthesized manually with radiochemical yields of 40-90% in 10-20 minutes [27] [35] [34].

Here, we present a fast, high yielding method for the synthesis of [^{18}F]F-Py-TFP using microfluidics. For the microfluidic synthesis of [^{18}F]F-Py-TFP, temperature, flow rate, and reagent ratio by volume were separately varied in order to maximize radiochemical yield. As seen in Figure 3, for [^{18}F]F-Py-TFP synthesis, temperature was varied from 40-110°C, with highest radiochemical incorporation yield observed at 80°C. Following temperature optimization, flow rate variation was investigated while other parameters were kept constant (temperature at 80°C, reagent ratio at 0.5). Flow rate was varied from 20-40 μL min^{-1}, and highest radiochemical incorporation yield was achieved at 30 μl min^{-1}. After temperature and flow rate ratios were optimized, reagent ratio was varied and optimized for best results, keeping temperature (80°C) and flow rate (30μl min^{-1}) fixed. Reagent ratio was varied from 0.2-3 (40-600 μg of **I**) P1:P3 by volume and highest radiochemical incorporation yield of 93 ± 4% was found at a reagent ratio of 0.5 (Figure 3, n=3-5 for each condition).

Overall radiochemical incorporation yields of [^{18}F]F-Py-TFP as high as 97% were achieved within 3 min. Unlike conventional approaches using [^{18}F]F-Py-TFP, no purification of the reaction mixture was performed prior to peptide coupling. Our approach improves yields and significantly reduces synthesis time of [^{18}F]F-Py-TFP, and establishes an automated approach for its production.

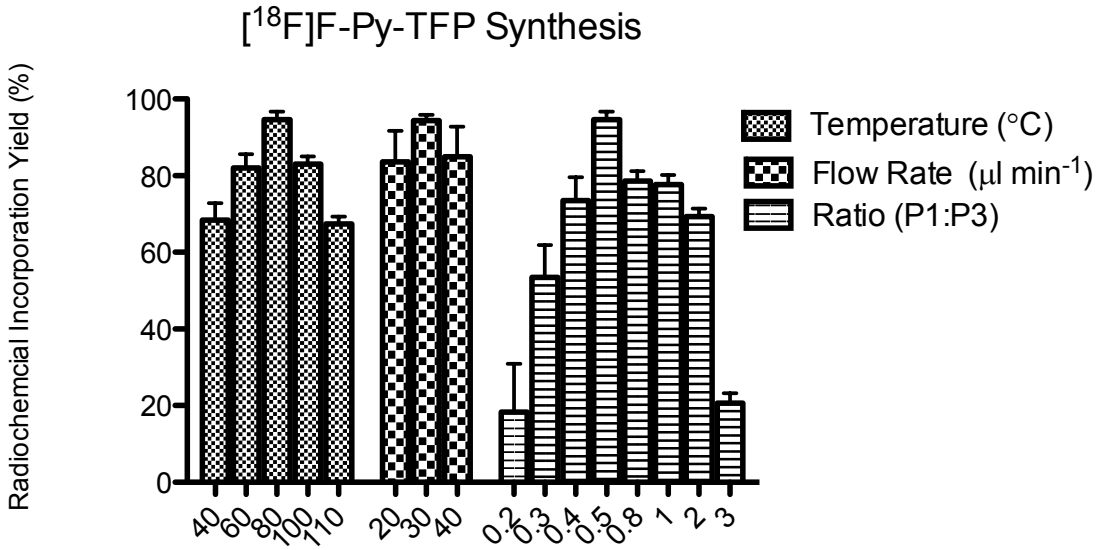

Figure 3: Temperature, flow rate, and reagent ratio variation for **II** synthesis (each n=3-5)

Microfluidic synthesis of [^{18}F]F-Py-YGGFL:

Previously, two groups have described the synthesis of ^{18}F-radiolabeled peptides using the Advion NanoTek® device. Bouvet et al [20] demonstrated effective microfluidic radiolabeling of peptides using ^{18}F-fluorodeoxyglucose ([^{18}F]FDG). In this approach, [^{18}F]FDG was used to radiolabel two different peptides, an aminooxy functionalized TATE peptide was radiolabeled in a one-step reaction via chemoselective oxime formation, and the glutathione (GSH) peptide was radiolabeled in a two-step reaction using a modified [^{18}F]FDG-maleimide-hexyloxime(MHO) as a prosthetic group. This approach required the separate production of [^{18}F]FDG on another platform prior to loading onto the microfluidics device and Bouvet et al concluded that their results varied from batch to batch, and were greatly dependent on the quality of the starting [^{18}F]FDG. Richter et al [18] demonstrated a similar approach as Bouvet et al, using [^{18}F]SFB as a prosthetic group for radiolabeling of ^{18}F-labeled phosphopeptide-cell-penetrating peptide dimers. Here, the [^{18}F]SFB was produced using a different platform and loaded onto the microfluidics device. This work showed an improvement of ^{18}F-peptide radiolabeling from 2-4% using a traditional approach to up to 26% using the microfluidic approach. Selivanova et al [19] demonstrated direct ^{18}F-radiolabeling of three modified bombesin peptides bearing either a trimethylammonium leaving group or a triarylsulfonium moiety. Direct ^{18}F labeling of peptides required modification with a leaving group prior to loading onto the microfluidic device and high temperatures were required for the fluorination.

While all of these approaches have shown success, further investigation of microfluidic radiolabeling of peptides with other ^{18}F-labeled prosthetic groups using mild reaction conditions are warranted. The goal of this work was therefore to radiolabel the NH_2-YGGFL peptide with the [^{18}F]F-Py-TFP prosthetic group on a single microfluidic device, from [^{18}F]fluoride. For peptide radiolabeling using [^{18}F]F-Py-TFP synthesis (Figure 4) temperature was varied from 30-100°C, flow rate was varied from 8-18 µl min^{-1} and reagent ratio was varied from 1-3:1 P2:Reaction 1 bolus by volume. Following a systematic approach for best conjugation of [^{18}F]F-Py-TFP, optimal conditions were found to be at 50°C, with a flow rate of 12µl min^{-1}and a P2:Reaction 1 bolus volume ratio of 2. Overall these conditions gave peptide radiolabeling yields up to 28%. Specific activity was calculated to be to 1-50mCi/µmol and can be readily improved by the addition of higher amount of starting radioactivity.

After optimized conditions were found, a larger scale reaction was performed to assess scale up capabilities. P3 was set at 50 µL, and all other reaction parameters were kept the same. Product was purified by HPLC to give an overall non-decay corrected yield of 9%, in an overall time of 32 min. Additionally, to compare the microfluidic vs the traditional labeling of NH_2-YGGFL in our hands, a test vial reaction was done using [^{18}F]F-Py-TFP prosthetic group in a manual synthesis technique, according to literature [27]. Here, manual synthesis resulted in a 6.2% radiochemical incorporation yield in a total time of 60 min.

In comparison to previously reported microfluidic peptide radiolabeling using either [^{18}F]FDG or leaving group- modified peptides, our approach demonstrates improvement by utilizing only one automated device, and can be used to radiolabel any peptide bearing a free-amine functional group.

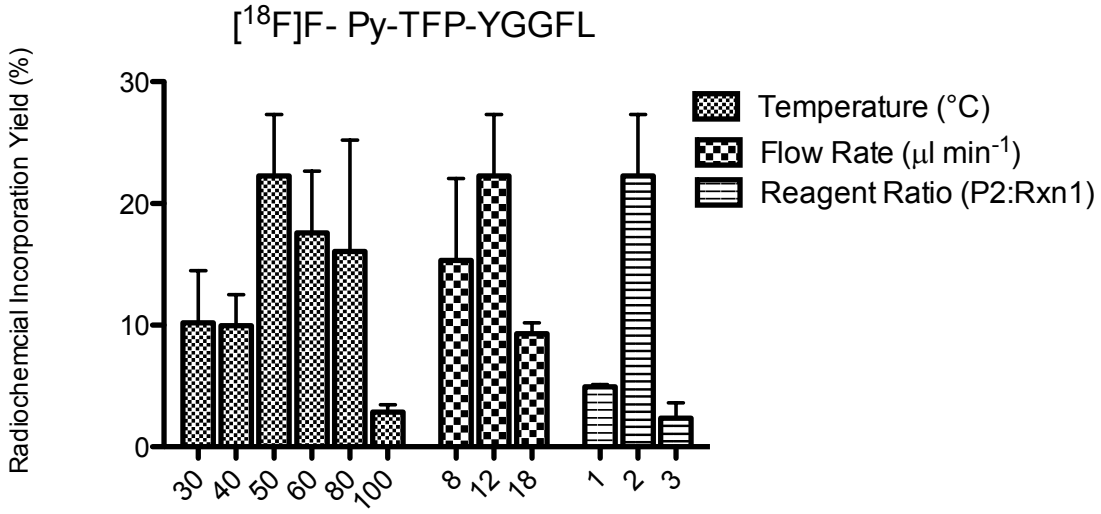

Figure 4 Temperature, flow rate, and reagent ratio variation for synthesis of radiolabeled peptide. (each n=3-5).

Conclusion:

We have demonstrated the first microfluidic synthesis and peptide radiolabeling with the [^{18}F]F-Py-TFP prosthetic group. Our approach establishes a simple, overall 2-step process to radiolabel a peptide on one microfluidic device from fluoride. To our knowledge, this is the first report of a 2-step continuous microfluidic ^{18}F-peptide radiolabeling using a prosthetic group, from [^{18}F]fluoride. This approach offers an improvement in radiochemical yield, decreased reaction time from 1 hour to 8 min from anhydrous [^{18}F]fluoride, and implements an attractive automated approach for ^{18}F radiolabeling of peptides. This can be seen as a method for ^{18}F-radiolabeling of amine-bearing peptides serving as a platform for radiolabeling of clinically relevant peptides in a rapid and automated fashion. Radiolabeling of other peptides is currently underway. Future work will focus on the radiolabeling of multiple peptides simultaneously, with the ultimate goal of developing a high-throughput, automated synthesis method for radiolabeling peptides. Additionally, we will focus on incorporation of solid-phase purification into the microfluidic platform, to further expedite the overall production of ^{18}F-radiolabeled peptides for molecular imaging.

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