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Efficient radiosynthesis of 3'-deoxy-3'-[^{18}F]fluorothymidine using electrowetting-on-dielectric digital microfluidic chip

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ABSTRACT

Access to diverse PET tracers for preclinical and clinical research remains a major obstacle in cancer and other diseases research. The prohibitive cost and limited availability of tracers could be alleviated by microfluidic radiosynthesis technologies combined with high-yield microscale radiosynthetic methodology. In this report, we demonstrate the multistep synthesis of [^{18}F]FLT with high yield on an electrowetting on dielectric (EWOD) microfluidic radiosynthesizer, previously developed in our group. We have identified and established several parameters that are most critical in the microscale radiosynthesis such as the reaction time, reagent concentration, and molar ratios, to successfully synthesize 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT) with a high yield in a compact platform. **Methods:** [^{18}F]FLT was synthesized from the 3-*N*-Boc-1-[5-*O*-(4,4'-dimethoxytrityl)-3-*O*-nosyl-2-deoxy-β-D-lyxofuranosyl] thymine on the EWOD chip starting from the first solvent exchange and [^{18}F]fluoride ion activation step to the final deprotection step. The fluorination reaction was performed in a mixture of thexyl alcohol and DMSO to assist the nucleophilic substitution reaction. The crude product after deprotection was collected from the chip and purified on a custom-made solid phase extraction (SPE) cartridge. The purified [^{18}F]FLT was used for microPET studies in multiple nude mice xenografted with the A431 cell line. **Results:** [^{18}F]FLT was successfully synthesized on the EWOD microdevice coupled with an off-chip SPE purification with a decayed corrected radiochemical yield of 63±5% (n=5) and passed all of the required quality control test recommended by the United States Pharmacopeia. We have successfully demonstrated the synthesis of several batches of [^{18}F]FLT on EWOD starting with ~ 333 MBq of radioactivity and obtained up to 52 MBq (non-decay corrected) of [^{18}F]FLT upon cartridge purification. **Conclusion:** The combination of the high yielding methodology, the compact microfluidic radiosynthesizer platform with configurable syntheses steps, and the

cartridge purification module could potentially decouple PET probe production from the cyclotron and specialized radiochemistry facilities.

INTRODUCTION

Positron emission tomography (PET) is an extremely sensitive molecular imaging technique capable of measuring *in vivo* metabolism that is increasingly being used in clinical practice and research to diagnose and study a wide range of diseases including cancer, Alzheimer, and Parkinson disease.(*1-3*) However, due to the difficulties and challenges involved in PET probe production(*4*), the majority of PET imaging studies are limited to 2-fluoro-2-deoxy-D-glucose ([^{18}F]FDG), a glucose analog used to quantify glucose metabolism. Other PET probes that are currently used in clinical trials and research settings, such as 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT), [^{18}F]fluoromisonidazole, [^{18}F]fluoroethylcholine, 6-[^{18}F]fluoro-3,4-dihydroxy-L-phenylanaline, and many others, are only available at high cost and with limited availability from specialized research laboratories.(*5*) Thus, there is a critical need to develop a new, affordable radiosynthesizer technology coupled with a high-yielding radiosynthetic methodology that could empower researchers and clinicians to synthesize probes of interest on-demand (at the imaging site) at low cost to meet the diversity of biological events being studied via PET imaging. The new technology platform should produce probes such as [^{18}F]FLT with a high radiochemical yield and a final product that can be purified without the need for additional equipment, for example via a simple cartridge purification, similar to the synthesis of [^{18}F]FDG.(*6*)

Recently, our group and others (include references Gillies, Steel, Elizarov) have investigated microfluidic technology platforms as a means of achieving on-demand radiosynthesis of diverse PET probes.(*7*) Microfluidic devices that integrate many laboratory

functions on a single chip, also known as lab-on-chip, can automate repetitive laboratory tasks, and enable users to perform hazardous reactions on chip in a safer manner.(*8, 9*) Throughout this manuscript, macroscale synthesis refers to any reaction volume above 250 µL, while microscale synthesis refers to any reaction volume between 1-10 µL. Of particular importance for PET probe synthesis using short-lived radioisotopes, microfluidic reactors enable radiosynthesis to be completed in a shorter time, minimize dilution of the radioisotopes (nmol – µmole amount) to speed up reaction kinetics, simplify purification due to the increased reaction selectivity, use smaller amounts of reagents, and have the potential to eliminate the high cost of infrastructure such as hot cells needed in a typical radiopharmacy facility.(*4, 10*) Our group has developed an all-electronic (i.e., no fluidic systems external to the chip) microfluidic radiosynthesizer based on electrowetting-on-dielectric (EWOD) principle(*11*) and successfully demonstrated reliable synthesis of [^{18}F]FDG.(*7*) EWOD is an exemplary microfluidic platform for performing batch radiosynthesis, where a finite volume of liquid can be manipulated sequentially by applying electrical potential, without the need of moving parts such as pumps and valves. It could form the basis of a compact, self-shielded bench-top radiosynthesizer. This work focused on the development of a high yielding and reliable microscale radiochemistry methodology on EWOD chip. The microscale method established here will serve as the building block towards the development of a fully automated, bench-top microfluidic radiosynthesizer for diverse PET probes production at the imaging clinic. To demonstrate the capability of this platform to synthesize other radiotracer, we choose to demonstrate the two-step synthesis of 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT), a radiolabeled analog of thymidine, on the EWOD microfluidic device. (Fig. 1)

MATERIALS AND METHODS

Reagents

Tetrabutylammonium bicarbonate ($TBAHCO_3$), 2,3-dimethyl-2-butanol, HCl, anhydrous acetonitrile (99.8%), anhydrous dimethyl sulfoxide (DMSO, 99.9%), hexanes, ethyl acetate, ethanol, and methanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 3-*N*-Boc-5′-*O*-dimethoxytrityl-3′-*O*-nosyl-thymidine (DMTr-Boc-nosyl precursor), 5′-*O*-dimethoxytrityl-3′-*O*-nosyl-thymidine (DMTr-nosyl precursor) and the 3-fluoro-3-deoxythymidine (FLT) standard compound were purchased from Advanced Biochemical Compounds (ABX) (Radeberg, Germany). Ion retardation resin (AG11 A8) and cation exchange (AG-50W-X4) were purchased from BioRad Laboratories (Hercules, CA). 2',3'-Didehydro-3'-deoxy-thymidine reference standard was purchased from Tokyo Chemical Industry (Japan) and used as received. Neutral alumina (particle size 50–300 μm), and C-18 (particle size 55–105 μm), and the Oasis Hydrophilic Lipophilic Balanced HLB resin were purchased from Waters corporation (Milford, MA).

No-carrier-added [^{18}F]fluoride ion was obtained from the UCLA Crump Institute for Molecular Imaging Cyclotron Facility by irradiation of 97% ^{18}O-enriched water with an 11 MeV proton beam using an RDS-111 cyclotron (Siemens Medical Solution, Knoxville, TN). Radioactivity was measured using a calibrated ion chamber (CRC-15R, Capintec Inc., Ramsey, NJ). A radioactive thin layer chromatography scanner (MiniGITA star, Raytest USA, Inc, Wilmington, NC) was used to analyze fluorination efficiency. Samples of the crude fluorination product were spotted on silica TLC plates and developed in a mixture of ethanol and ethyl acetate (50:50 v/v). Analytical high performance liquid chromatography (HPLC) was carried out using a Phenomenex Luna (Torrance, CA) reversed-phase C-18 column (250 × 4.6 mm). Elution was

performed at constant flow rate of 1 mL/min with water:ethanol (90:10 v/v) at and the UV detection was set at 268 nm. Apyrogeneticity test was measured using the Charles River Endosafe-PTS Portable Testing System (California, USA).

EWOD chip fabrication and operation

Similar to our previous report(*7*), the EWOD chip was constructed from two parallel plates: the base plate and the cover plate. In comparison to our previous chip design, two additional reagent loading sites and inlet pathways were added to increase the flexibility of the EWOD chip multistep radiochemistry (Fig. 2). Additional details on the EWOD chip fabrication and operation can be found in the supplemental information.

Radiosynthesis of [^{18}F]FLT on EWOD chip

Stock solution of $TBAHCO_3$ (5 µL, 75 mM) was mixed with the no-carrier-added [^{18}O]H_2O/[^{18}F]F^- (20 µL; ~740 MBq) solution to form the [^{18}F]TBAF complex. Three droplets (2 µL each) of the TBA[^{18}F]F complex were pipetted to loading site **1** and then transported to the reaction site by EWOD actuation, followed by the addition of a 3 µL droplet of MeCN. The TBA[^{18}F]F complex was heated to 105 °C and held at 105 °C for 1 min to evaporate the solvent. Subsequently, one cycle of azeotropic distillation was performed by adding a 9 uL MeCN droplet to the dried residue and heating to 105 °C for 1 min. Upon activation of the TBA[^{18}F]F complex, DMTr-Boc-nosyl precursor (4.5 mg) was dissolved in a mixture of DMSO (20 µL) and 2,3-dimethyl-2-butanol (40 µL) to achieve a final concentration of 90.4 mM. One droplet of the DMTr-Boc-nosyl precursor solution (2 µL) was pipetted to loading site **2** and transferred to the dried TBA[^{18}F] complex on the reaction site at room temperature, followed by the addition of a 3 uL droplet of MeCN. The reaction mixture was gradually heated to 120 °C and held at 120 °C

for 3 min to perform the fluorination reaction. After the fluorination reaction, a 4:1 mixture of 1N HCl/MeCN (two 3.5 µL droplets) was pipetted to loading site 3, and transported to and mixed with the crude intermediate product at the reaction site. The reaction mixture was slowly heated to 95 °C and held for 1.5 min to complete the hydrolysis reaction. The cover plate was removed and the crude product was extracted using 200 µl H_2O, 50 µL MeCN and 18 µL DMSO, sequentially to achieve an average extraction efficiency of 84±2% (n = 5). A small amount of the crude product was used for radio-thin layer chromatography (radio-TLC) and radio-HPLC analyses, while the remainder of the crude product was purified using a miniaturized cartridge as described in the following section. The total synthesis time and cartridge purification was about 63 minutes.

Cartridge Preparation

A miniaturized purification cartridge was designed based on our previous report (*7*) to achieve a high purification efficiency from the microliter-scale volume of reaction product synthesized on the EWOD chip. (Fig. 3A) The custom-made cartridges consisted of 5 mg of ion retardation resin, 5 mg of cation exchange, 30 mg of neutral alumina (50–100 mesh size), and 150 mg of Oasis HLB resins packed within a 1-mL syringe barrel (Becton, Dickinson and Company, New Jersey). To prevent the formation of air bubbles, two polyethylene frits (20 µm pore size) were used to sandwich the resins. The cartridge was conditioned with methanol (1 mL) and water (2 mL) before use.

Cartridge purification of [^{18}F]FLT

Using a 1-mL syringe, the collected crude reaction mixture was passed through the conditioned miniature cartridge to trap the desired product, followed by sequential elution with 1% ethanol in water (9 mL) and 5% ethanol in water (6 mL) to release the side products to waste. After

removal of all the side products (analyzed by HPLC), the final [^{18}F]FLT product was eluted from the cartridge using 500 µL of ethanol and was collected into a sterile empty vial. The ethanol was evaporated by heating and blowing nitrogen for 5 minutes into the vial and the dried [^{18}F]FLT residue was redissolved in 200 µL of saline prior to administration into the mice. The residual solvents (ethanol, acetonitrile, DMSO, thexyl alcohol) in the formulated [^{18}F]FLT were analyzed via gas chromatography following the method reported in our previous work [REF] (details in Supporting Information). The chemical and radiochemical purity of the formulated [^{18}F[FLT was analyzed using an analytical HPLC equipped using the UV detection at 254 nm and a radio-detector. The product and impurities in the final sample were identified by comparing their retention to the known standards. A calibration curve for FLT and several chemical impurities, such as 2',3'-didehydro-3'-deoxy-thymidine, thymine, 3'-deoxy-3'-chlorothymidine, and furfuryl alcohol, were performed and was used to quantitate the amount of impurities present in the final product.

Specific activity analyses

Four batches of [^{18}F]FLT was prepared sequentially on the Teflon-glass substrates on the same day to obtain sufficient mass for UV detection on the HPLC. The synthesis procedure on the Teflon-glass substrates was described in detail in the Supporting Information. Briefly, the crude product from each batch of the reaction was combined, purified via the analytical HPLC and the fraction containing the [^{18}F]FLT product was collected. The collected product in a mixture of water and ethanol was further diluted in water and passed through the custom-made purification cartridge. The cartridge purification procedure was similar to the single batch experiment. Upon evaporation of the ethanol, the concentrated [^{18}F]FLT (~20 µL) from four batches of [^{18}F]FLT synthesis was injected into the analytical HPLC for quantification of the cold mass of FLT. The

cold mass of FLT was quantified based on the standard calibration curve performed on the same analytical HPLC used for specific activity analysis using UV detection at 268 nm. The average decay corrected radioactivity of the purified [^{18}F]FLT obtained from four batches of synthesis was used for specific activity analysis. The specific activity reported here was calculated by averaging the decay corrected radioactivity of the crude [^{18}F]FLT obtained after each synthesis on the Teflon-glass substrate and divided by the average cold mass measured from the analytical HPLC.

Apyrogeneticity test

An aliquot of the final [^{18}F]FLT product in 500 µL of saline was tested for the presence of bacterial endotoxin utilizing a Limulus Amebocyte Lysate (LAL) Test. The sample was further diluted by 20 times before the LAL analysis. The test was performed using a Charles River Endosafe-PTS Portable Testing System, which is based on a kinetic chromogenic BET.

RESULTS

The multistep on-chip reaction begins with the [^{18}F]fluoride ion activation step, followed by the radiofluorination of DMTr-Boc-nosylate FLT precursor, and finally the deprotection of the tert-butyloxycarbonyl (Boc) and the 4,4'-dimethoxytriphenylmethyl (DMTr) groups via acid hydrolysis. A systematic optimization of various reaction parameters including reagent concentration, precursor, precursor to base ratio, reaction temperature, reaction time, and phase transfer catalyst was performed on the Teflon coated glass substrates, which mimics the microdroplet reaction on the EWOD chip. In the first phase of the method development, we found that the optimal fluorination condition used the DMTr-Boc-nosylate FLT precursor and the $TBAHCO_3$ as the phase transfer catalyst in 2:1 molar ratio to achieve 80±7% (n=10) fluorination efficiency. Fluorination efficiency and yield were reported with a standard deviation based on n number of experiments. Upon further investigation, we found that a

significant percentage of radioactivities were lost during the fluorination and hydrolysis step. In our attempt to minimize the loss by reducing the reaction time, the fluorination efficiency decreased from 80±7% (n=10) to 51±7% (n=3). To increase the reaction kinetics, the overall reagent concentration was increased by two-folds with concurrently increased in the reaction temperature (from 110 °C to 120 °C). Under a higher reagent concentration and a slightly higher temperature, the fluorination efficiency increased from 51±7% to 79±6% (n=2) after 3 minutes of fluorination reaction. The fluorination efficiency was further increased to 94±3% (n=9) when the reaction droplet volume was reduced from 4 μL to 2 μL, while maintaining the 2-fold increase in the reagents concentration. Finally, the protected [^{18}F]FLT intermediate was hydrolyzed in a mixture of HCl and MeCN at 95 °C for 1.5 minutes to obtain [^{18}F]FLT in 82±10% (n=10) crude radiochemical yield [defined by the (radioactivity remained on-chip * radio-TLC conversion)/estimated radioactivity loaded onto chip]. In comparison to the previous method, the crude radiochemical yield obtained from the optimized methodology improved from 49±11% (n=10) to 82±10% (n=10). After the radiosynthesis of [^{18}F]FLT on the EWOD chip, the crude mixture was first collected using a mixture of DMSO, MeCN and water with 84±2% (n=5) collection efficiency. The collected crude reaction mixture (~270 μL) was then purified on a custom-made miniaturized cartridge to obtain [^{18}F]FLT in >99% radiochemical purity and an overall decay-corrected radiochemical yield of 63±5% (n=5). The cartridge purification efficiency was 89±2% (n=5). The cartridge purification efficiency is defined by the total [^{18}F]FLT collected from the EWOD chip (crude radioactivity * radio-TLC conversion) to the purified [^{18}F]FLT obtained after the cartridge purification. Starting with ~ 333 MBq of radioactivity ([^{18}F]fluoride ion) on EWOD chip, we have successfully prepared 52 MBq (non-decay corrected) of [^{18}F]FLT upon cartridge purification and formulation. The purified [^{18}F]FLT

sample was subjected to a set of quality control procedure recommended for testing purity and safety before administering into humans. The final product solution was observed to be clear and free of particulates. The pH was measured to be between 6.5 and 7 using a pH paper. The GC analysis showed that the final product contained <20 ppm of MeCN, DMSO, ethanol and thexyl alcohol. The allowable limit for the residual organic solvents are as the following: MeCN (400 ppm), DMSO (5000 ppm), ethanol (5000 ppm), and thexyl alcohol (5000 ppm). The chemical purity of the formulate product was analyzed using an analytical HPLC. Based on the standard UV calibration curve for FLT and the other known impurities, we found that the final sample contained 0.2 ppm of stavudine, 0.01 ppm thymine and 0.09 ppm of thymidine. Other UV active peaks that eluted between 1 and 4 minutes were unidentified and does not match the retention times of any of the known impurities found in a typical [^{18}F]FLT synthesis. The LAL test detected less than 1 EU/mL in concentration of the final sample, which is lower than the established USP endotoxin limit of 175 EU/mL per dose for radiopharmaceuticals. The specific activity of [^{18}F]FLT synthesized on the EWOD chips and Teflon glass substrate was measured to be between 52-138 Ci/μmole based on 10 batches of [^{18}F]FLT syntheses.

DISCUSSION

Since the first radiosynthesis of [^{18}F]FLT reported by Grierson and coworkers(*12*), there have been a multitude of methods developed to achieve higher and more reliable yield.(*13*) Notably, Eisenhut and co-workers developed a new FLT precursor, DMTr-Boc-nosyl to facilitate synthesis automation and it has become the precursor of choice to achieve high radiosynthesis yield.(*14*) Since then, multiple research groups have reported the radiosynthesis of [^{18}F]FLT using this DMTr-Boc-nosyl FLT precursor based on the conventional no-carrier-added (n.c.a.) radiofluorination methodology with radiochemical yield ranges from 23-50%.(*13, 15-18*)

Particularly, the use of a bulky alcohol as co-solvent in assisting the n.c.a radiofluorination is attractive and has been reported to attain [^{18}F]FLT in a high radiochemical yield (60-65%)(*19-22*) Taking advantage of the high yielding and the high selectivity of the protic solvent chemistry, we adapted this methodology to the microdroplet radiosynthesis of [^{18}F]FLT on the EWOD microdevice, followed by the development of a miniature cartridge purification method to eliminate the expensive and complicated HPLC purification.

During this developmental process, the majority of the processes are performed manually, such as reagent loading, product collection and cartridge purification, which limits the amount of radioactivity that was used in this work. While this report focused on the development of a reliable microscale radiochemistry on the EWOD chip, we are also currently developing an automated reagent delivery, product collection and purification.(*23-26*) We anticipated that high activity production within microliter volume can be achieved based on the recent demonstration of the clinical dose production of [^{18}F]FDG and [^{18}F]fallypride on a batch microfluidic device using a miniaturized anion exchange cartridge to concentrate ~32 GBq of [^{18}F]fluoride/[^{18}O]H_2O in 5 µL volume.(*27, 28*) Such volume is commensurate with the design of EWOD chip reaction site, which can accommodate up to 17 µL of droplet.

Synthesis Method 1

Starting from the original report for the radiosynthesis of [^{18}F]FLT in protic solvent(*20*), we first performed systematic screening of various reaction parameters including reagent concentrations, precursors, precursor to base ratios, and phase transfer catalysts on the microscale using the Teflon coated glass substrates. In contrast to the macroscale methodology, we chose to use a mixture of DMSO and thexyl alcohol to improve the solubility of the precursor throughout the entire fluorination reaction, which is critical for synthesis automation and to increase the reaction

reliability. While macroscale synthesis generally avoids the use of DMSO (bp: 182 °C) and thexyl alcohol (bp: 120 °C) due to the difficulty in removing these solvents after the synthesis, the much smaller volume used in the microscale synthesis (2 μL versus 500 μL – 1000 μL) facilitates its rapid removal. At the end of the synthesis, the droplet volume has already shrunk to ~0.2 μL due to solvent evaporation during the fluorination reaction (Fig. S-1). Without further evaporation, we have confirmed that the level of residual solvents of the final purified product is below the recommended limit standardized by the USP pharmacopeia. A summary of result from the optimization studies performed on Teflon-coated glass is presented in Table 1. We found that the precursor to base concentration ratio is one of the critical parameters affecting the fluorination efficiency, which is consistent with the work by Suehiro et al.(*29*) In addition to the precursor to base ratio, we have also investigated the use of the cryptand complex as the phase transfer catalyst. As shown in Table 1, the fluorination yield using the cryptand complex is lower (58%; Table 1, condition 2) in comparison to the $TBAHCO_3$ (80%; Table 1, condition 4) when performed under similar condition (i.e.: precursor to base ratio ~ 2), which is also consistent with the report by Kim et al.(*30*) To confirm the catalytic effect of the alcohol in the radiosynthesis, we have also performed a control experiment by replacing the DMSO/thexyl alcohol with an aprotic solvent mixture of DMSO and MeCN. The DMSO/MeCN solvent ratio was determined empirically such that the droplet size at the end of fluorination reaction was a similar size. We observed significant reduction in the fluorination efficiency of 38±4% (n=2) when DMSO/MeCN was used versus 80±7% (n=10) when thexyl alcohol was used in the fluorination reaction (Table 1, Conditions 4 and 5), which confirmed the role of thexyl alcohol in assisting the nucleophilic substitution reaction. In our attempt of using the DMTr-nosyl FLT precursor, we found that the fluorination yield was lower and unreliable in comparison to the

DMTr-Boc-nosylate FLT precursor under similar condition (Table 1, condition 1 versus 3). The final hydrolysis step was performed at 95 °C for 5 minutes. The completion of the hydrolysis reaction was tentatively confirmed by the emergence of a single radio peak at around 15 minutes, which is corresponded to [^{18}F]FLT in the radio-HPLC. (in supporting information;Fig. S-2) This first phase of optimized methodology is referred to as Method 1 in Table 2.

Synthesis Method 2

Though Method 1 exhibited a high fluorination and hydrolysis efficiencies, we found that the crude radiochemical yield of [^{18}F]FLT at the end of the synthesis is relatively low (47±19%; n=7). To understand the discrepancy between overall efficiency and yields of individual steps, we measured the radioactivity losses after each step of the synthesis on a Teflon-coated glass substrate (inexpensive substitute for EWOD chip). The sandwiched Teflon-glass substrate was removed from the Peltier heater and the radioactivity on the substrate was measured using the dose calibrator after each step of the Method I synthesis. (Table 2A) Based on this study, we found that the major loss occurred during both the fluorination and hydrolysis steps. While the exact mechanism of the radioactivity loss is yet to be determined, our initial approach was to reduce the reaction times for both the fluorination and hydrolysis reaction to reduce the overall radioactivity losses, while ideally not diminishing the yields.

In order to do so, we first investigated the reaction kinetics on Teflon-glass substrate and found that a reduction of the fluorination reaction time from 5 minutes to 3 minutes resulted in a significant decrease in the fluorination efficiency. Through a series of optimization reaction condition, we found that the fluorination reaction kinetics can be improved by increasing the reagent concentration and the reaction temperature. Interestingly, we also found that the

fluorination yield was further increased (from 79% to 94%) by simply reducing the droplet size from 4 μL to 2 μL, while maintaining the two-fold increase in the concentration of the reagent. The enhanced fluorination yield could be speculated by the increase in the [^{18}F]fluoride ion concentration as the reaction volume was decreased by two-folds. While the increased in the radioactivity concentration, and thus the fluorination kinetics, have been speculated by miniaturizing radiochemical reaction on microfluidic platforms (*10*), we are yet to fully investigate this effect experimentally.

Based on the new optimized synthesis Method 2 (Table 2B), we indeed observed a reduction in radioactivity loss from 33% loss to only about 15% at the end of synthesis on EWOD chip (Table 2). The new microscale [^{18}]FLT conditions also yielded higher fluorination efficiency (91±4% (n=10), which resulted in an overall increase in the crude radiochemical yield of [^{18}F]FLT from 49±11% (Table 2(A)) to 82±10% (Table 2 (B)).

Collection of crude product and cartridge purification

With the exception of [^{18}F]FDG and [^{18}F]NaF, other PET probes generally require final purification via HPLC to remove excess precursor or other radiolabelled or toxic side products that cannot be easily removed via solid phase extraction (SPE). However, this technique is not easily miniaturized as would be needed for a benchtop radiosynthesis platform. These shortcomings of HPLC have led to the emergence of several HPLC-free radiochemistry methods.(*31-33*)

From the point of view of the cartridge purification, the most desirable solvent to collect the product from the chip is water. However, in the microscale, where the surface to volume ratio is large, we found that the collection efficiency (ratio of radioactivity recovered from the chip versus the total radioactivity that was measured on-chip after the synthesis) of the final

crude [^{18}F]FLT from the EWOD chip was below 15% when only water was used as the extraction solvent. In the presence of 50:50 v/v MeCN/H_2O as the extraction solvent, the collection efficiency improved to 47±10% (n=5). Finally, we attempted a mixture of DMSO, MeCN and water (18 µL, 50 µL and 200 µL, respectively) and obtained 84±2% (n=5) collection efficiency. However, in this solvent mixture, we found that the [^{18}F]FLT was not able to be separated from the other side products when the conventional reversed phase C18 resins were used in the cartridge purification. This observation can be explained by the increasing composition of non-polar solvent during the cartridge purification, which is sufficient to disrupt the van der Waals forces between the [^{18}F]FLT analyte and the reversed phase resin.(*34*) To address this issue, we investigated a new type of sorbent known as the Oasis **H**ydrophilic-**L**ipophilic-**B**alanced resins (HLB). Based on our systematic cartridge purification studies, we determined that the optimal HLB resin to be 150 mg to efficiently retain [^{18}F]FLT from a solvent mixture of MeCN, water and DMSO, while enabling elution of the other side products from the cartridge. Upon the cartridge purification of [^{18}F]FLT as described in the materials and methods section, the final purified [^{18}F]FLT product was collected in 500 µL of 100% ethanol. Upon evaporation and reformulation in saline, 52 MBq of [^{18}F]FLT was obtained for micro-PET imaging studies of several A431 tumor bearing mice. The micro-PET images showed a high accumulation of [^{18}F]FLT in the tumor due to the high level of expression of the thymidine kinase-1 (TK-1) enzyme in rapidly proliferating cells. (Fig. S-5)

Quality control

Based on the several [^{18}F]FLT samples that were subjected to a standard quality control procedure a typical chemical purity of the final [^{18}F]FLT solution was found to have negligible amount of impurities upon formulation in ~ 0.5 mL of saline. The only radioactive component

present in the final compound was [^{18}F]FLT upon successful removal of the unreacted [^{18}F]fluoride ion using the custom-made cartridge (Fig. 3B and radio-TLC in Fig. S-4)). Similarly, the residual solvents found in the final compound were below the USP allowable limit for administering into human. We have also confirmed that the final formulated product have less than 1 EU/mL of bacterial endotoxins, which is significantly lower than the 175 EU/mL limitations that was recommended by the US Pharmacopeia. Due to the minute amount of reagent used on the EWOD microfluidic device for radiosynthesis, the absolute amount of impurities and residual solvent reported here and in our previous report [PNAS REF] was extremely small. Additionally, the level of impurities reported here will be ~ 50-folds lower upon diluting the single dose of [^{18}F]FLT from the EWOD chip in 10 mL of saline for clinical PET imaging. For clinical production, we anticipate to only increase the amount of radioactivity loaded onto the EWOD chip while keeping the amount of reagent and reaction condition the same. Therefore there will be a significant reduction in the level of impurities, bacterial endotoxins , and residual solvents that are present in a single dose of PET radiopharmaceuticals for clinical studies.

The specific activity of [^{18}F]FLT synthesized on the EWOD chip was measured to be more than 50-folds higher than literature reports using conventional macroscale radiosynthesizer. This result is consistent with the recent report by Rensch et al. [REF] on the reduction of radiolysis on microfluidic devices due to the geometric confinement. The simulation and experimental studies conducted by Rensch suggested that the majority of the high energy positron was deposited onto the glass layer of the chip when the dimension of the microfluidic channel or gap is smaller than the positron range (~ 400 μm). Based on this report and the high specific activity of [^{18}F]FLT synthesized on the EWOD chip suggested that the Teflon layer on

the chip produced negligible amount of carrier fluoride ion. This preliminary result also suggested the advantages of microfluidic platforms for the production of high specific radiopharmaceuticals for imaging low abundance receptors. We are currently investigating key contributing factors that lead to an increase of specific activities when miniaturizing the reaction volume from one milliter to several microliters.

CONCLUSION

In this report, we have developed an optimal two-step, one-pot procedure using the bulky alcohol as a co-solvent on the EWOD radiosynthesizer to achieve a high and reliable radiochemical yield of 63±7% (n=5) (decay-corrected) for the radiosynthesis of [^{18}F]FLT with a synthesis time (including cartridge purification) of 63 mins. The optimized microscale radiofluorination strategy yielded up to 52 MBq (non-decay corrected) [^{18}F]FLT. The final product passed the typical quality control analyses, which include, pH, chemical purity, residual solvent analyses and pyrogenecity test that are required before administering into humans. With increasing degree of automation of the EWOD microfluidic platform, such as radioactivity concentration, reagent delivery, cartridge purification and an integrated quality control module, this technological platform could emerge as a compact and robust radiosynthesizer towards rapid, on-demand production of individual doses of PET probes at the imaging clinic by laboratory technicians to increase the diversity of PET probes needed to elucidate biological events in vivo. We have demonstrated the versatility of an all-electronic EWOD microfluidic chip for the multistep radiosynthesis of PET probes. As demonstrated in this report and in our previous publication, the same EWOD chip design can be used to synthesize different PET tracers. To date, we have demonstrated the syntheses of [^{18}F]FDG, [^{18}F]FLT and others PET probes (manuscript in preparation) by simply changing the reagents and reaction condition for PET

tracers of interest. Currently, the EWOD chip is designed to be one-time used, similarly to the disposable reagent cassettes used in the conventional macroscale radiosynthesizer. Secondly, the overall shielding around the compact footprint of the microfluidic platform is anticipated to be significantly reduced in comparison to the size of current hot cells and mini-cells. The reduction in the overall size and shielding enable the synthesizer to be self-shielded and placed on a standard laboratory bench-top, thus eliminating the need of specialized radiopharmacy infrastructure.

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Figure legend

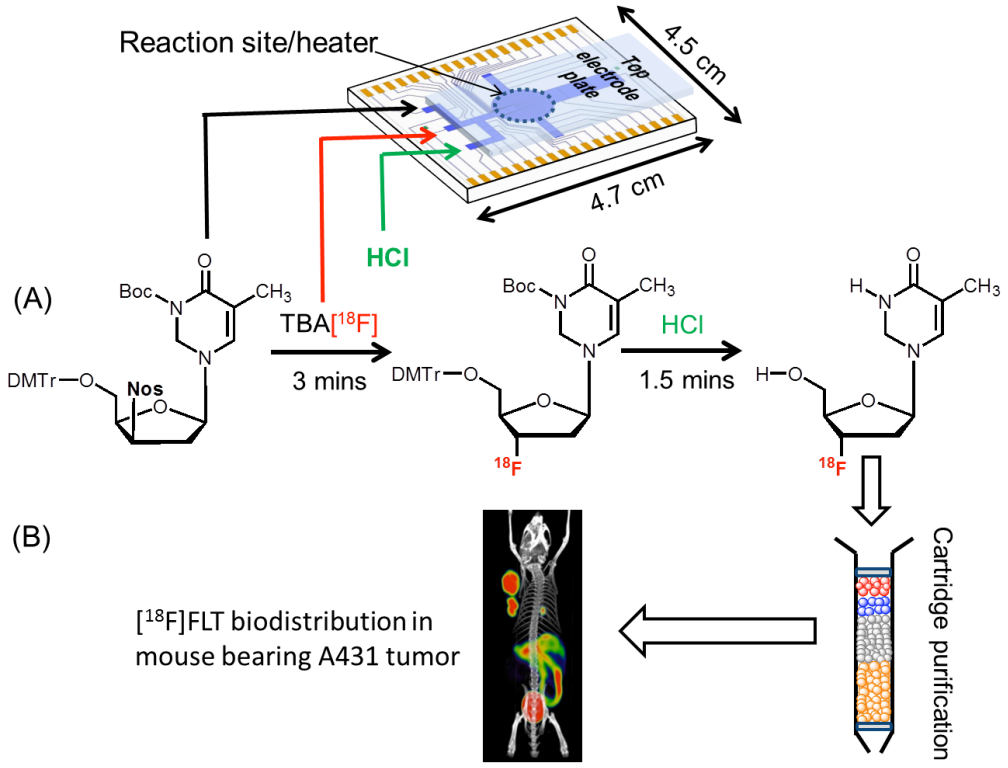

Figure 1: Overall workflow of radiosynthesis of [^{18}F]FLT on EWOD chip followed by cartridge purification to produce an injectable dose of [^{18}F]FLT for mice imaging. (A) Synthetic scheme of the radiosynthesis of [^{18}F]FLT using a mixture of thexyl alcohol and DMSO in the fluorination reaction. (B) The crude [^{18}F]FLT product was extracted and purified via a simple cartridge purification to yield ~ 52 MBq (non-decay corrected) of [^{18}F]FLT.

Figure 2: (A) EWOD chip base plate design with a reaction site and multiple loading sites. A reagent droplet (blue circle), sandwiched between the EWOD device plate and the cover plate, is shown at loading site 3. (B) Detail of multifunctional electrowetting, heating, and temperature sensing electrodes. (C) Cross-sectional view of EWOD chip with a sandwiched droplet.

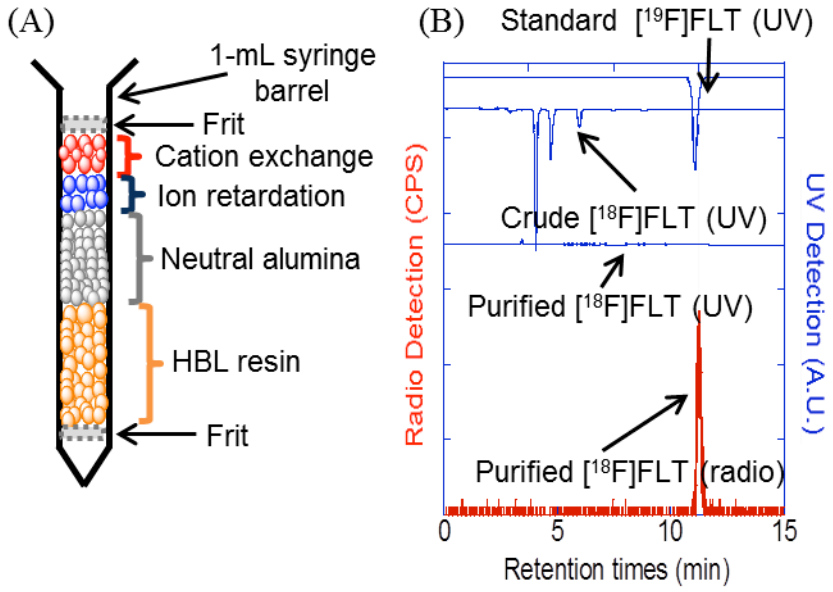

Figure 3: (A) Custom-made purification cartridge used to purify [^{18}F]FLT that was synthesized on EWOD chip. (B) HPLC stacked chromatogram of the crude, cartridge purified and standard FLT. The blue chromatogram represented the absorbance in the UV and the red chromatogram represented the radio detection.

Condition	Precursor (mM)	PTC/Base (mM)	Solvents (v/v) µL	Temp. (°C)	Time (mins)	Average Fluorination efficiency (%)
1	Non-Boc (40)	TBAHCO3 (39)	DMSO/TA (0.8/3.2)	100	5	47±18 (n=8)
2	Boc (40)	K222/K2CO3 (36/18)	DMSO/TA (0.8/3.2)	100	5	58±6 (n=2)
3	Boc (40)	TBAHCO3 (39)	DMSO/TA (0.8/3.2)	100	5	55±19 (n=3)
4	Boc (45)	TBAHCO3 (22.5)	DMSO/TA (0.8/3.2)	110	5	80±7 (n=10)
5	Boc (45)	TBAHCO3 (22.5)	DMSO/MeCN (1/3)	110	5	38±4 (n=2)

TABLE 1: Optimization of the radiofluorination with varying parameters and precursors on Teflon-glass substrate. Abbreviations: Boc: 3-N-Boc-5′-O-dimethoxytrityl-3′-O-nosyl-thymidine, non-Boc: 5′-O-Dimethoxytrityl-3′-O-nosyl-thymidine, DMSO: dimethylsulfoxide; TA: thexyl alcohol; MeCN: acetonitrile

(A) Method 1

Process	Temp. (°C)	Reaction time (mins)	Reagent	Conc. (mM)	Droplet volume (µL)	Fluorination efficiency (%)	Radioactivity remained on chip (%)	Crude Radiochemical yield (%)
(1) Load [18F]TBAF	RT		TBAHCO3	22.5	6	n/a		n/a
(2) MeCN azeotropic distillation	105	2	MeCN	n/a	9	n/a		n/a
(3)	120	5	FLT	45	4	74±6 (n=8)	80 (n=4)	n/a

Fluorination			precursor					
(4) Hydrolysis	95	5	HCl	0.75	7	n/a	67 (n=4)	49±11 (n=10)

(B) Method 2

Process	**Temp. (°C)**	**Reaction time (mins)**	**Reagent**	**Conc. (mM)**	**Droplet volume (µL)**	**Fluorination efficiency (%)**	**Radioactivity remained on chip (%)**	**Crude Radiochemical yield (%)**
(1) Load [18F]TBAF	RT		TBAHCO3	45	6	n/a		n/a
(2) MeCN azeotropic distillation	105	2	MeCN	n/a	9	n/a		n/a
(3) Fluorination	120	3	FLT precursor	90	2	91±4 (n=10)	94 (n=4)	n/a
(4) Hydrolysis	95	1.5	HCl	0.75	7	n/a	85 (n=4)	82±10 (n=11)

TABLE 2: Summary of the reaction conditions for the radiosynthesis of [^{18}F]FLT on EWOD chip based on (A) Method 1 and (B) Method 2, with the corresponding reagent concentration, reaction time, reaction volume and the percentage of radioactivities remaining on the chip. For the radioactivity measurement, the chip was removed after each step and the radioactivity on the entire chip was measured using a dose calibrator.