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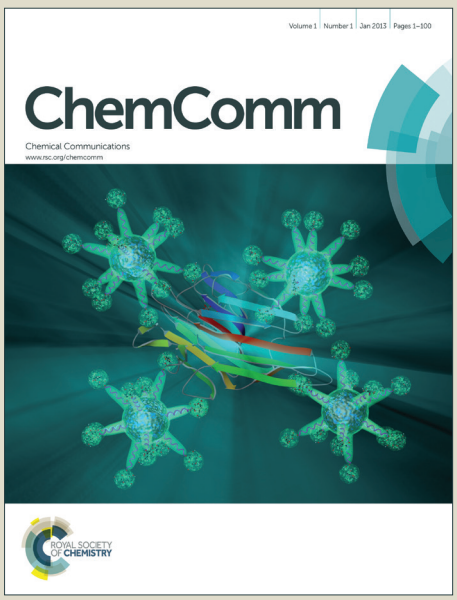

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COMMUNICATION

Synthesis of 3-chloro-6-((4-(di-tert-butyl[^{18}F]fluorosilyl)-benzyl)oxy)-1,2,4,5-tetrazine ([^{18}F]SiFA-OTz) for Rapid Tetrazine-Based ^{18}F-Radiolabeling

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An efficient method to prepare the ^{18}F-labeled tetrazine-derivative ^{18}F-SiFA-OTz for bioorthogonal radiochemistry was developed. ^{18}F-SiFA-OTz can be synthesized with a radiochemical yield of 78 ± 5 % within 25 min and can quantitatively react with a model strained dienophile, trans-cyclooctenol.

Positron emission tomography (PET) imaging has been extensively utilized in the fields of diagnostic oncology, neurology, and cardiology due to its non-invasive features derived from the radioactive decay of positron-emitting radionuclides such as ^{11}C, ^{13}N, ^{15}O, and ^{18}F.[1] ^{18}F provides ideal nuclear physical characteristics. A β^+ branch of 97% and its low β^+ energy of 635 keV result in high spatial resolution of PET images.1 However, the required short reaction times and the generally harsh labeling conditions (particularly elevated temperatures) that required for formation of a carbon-^{18}F bond suggest that improved ^{18}F labelling strategies are needed. The situation is exacerbated when dealing with complex biomolecules such as peptides and proteins. The critical requirements for the development of new ^{18}F labelling techniques are fast reaction rates and a simple chemistry, ideally under ambient conditions. The development of non-canonical ^{18}F labeling chemistries based on boron-^{18}F, aluminium-^{18}F, and silicon-^{18}F bond formation has attracted a great deal of interest and constitute viable alternatives to conventional carbon-^{18}F based radiochemistry.[2] Among those new radiochemistries, the silicon-fluoride acceptor (SiFA) labeling approach based on simple isotopic exchange (IE) on a silicon-fluorine scaffold is one of the most convenient labeling methodologies available and should be well suited for tetrazine pre-labeling with ^{18}F.[3] The ^{18}F-SiFA-tetrazine synthon can thus finally serve as a bioconjugation agent exploiting tetrazine based click chemistry.

The wide application of bioorthogonal chemical reactions in chemical biology and biomolecule synthesis, including Staudinger ligation and the 1,3-dipolar azide-alkyne cycloaddition, have attracted considerable interest.[4] However, their relatively slow kinetics (k_2 < 5 $M^{-1}s^{-1}$, in acetonitrile) present a limiting factor for application of these bioconjugation reactions. The Fox and Weissleder groups independently introduced inverse electron demand Diels-Alder reactions (IEDDA) between a 1,2,4.5-tetrazine and strained dieneophiles as a highly efficient bioorthogonal ligation for bioconjugation.[5] Due to its fast reaction kinetics (k_2 = 2,000 – 22,000 $M^{-1}s^{-1}$) and catalysis-free coupling, application of IEDDA to bioorthogonal coupling has expanded quickly.[6] Generally, radionuclides such as ^{18}F can be introduced into either the tetrazine derivative or the dienophile. However, because of the low stability of tetrazine,[6] most reports have focused on using an ^{18}F-labelled dienophile such as trans-cyclooctene and norbornene for the follow-up IEDDA conjugation.[7] For example, the Fox group has reported the synthesis of ^{18}F-labelled trans-cyclooctene for the IEDDA with tetrazine-derivatized peptides.[8] A disadvantage of this strategy is that it first requires the attachment of tetrazine to the pre-targeting probe. This not only requires extra steps involving pre-coupling of the tetrazine to a vector, followed by tedious separation, but also suffers from the low stability of tetrazine derivatives. In contrast, facile modification of biomolecules with trans-cyclooctene or norbornene is readily available and the conjugates are generally stable. As a result of those constraints, a simple radiolabelling procedure under ambient conditions for tetrazine derivatives is desirable and would facilitate IEDDA applications in radiolabeling.

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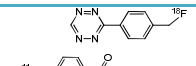
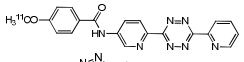
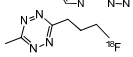
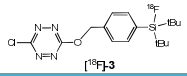
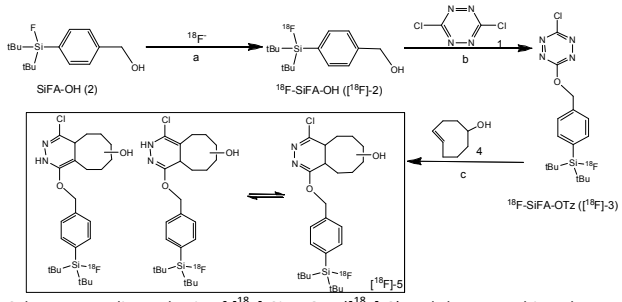
Table 1. Summary of ^{18}F- and ^{11}C-Labeled Tetrazine Derivatives

Ref.	Radiolabeled Tetrazine	RCY [a]	Time
7a Fox *et al*		< 1 %	-
10 Herth *et al*		33 %	50-60 min
11 Denk *et al*		16 ± 2 %	-
This work	 [^{18}F]-**3**	78 ± 5 %	20-25 min

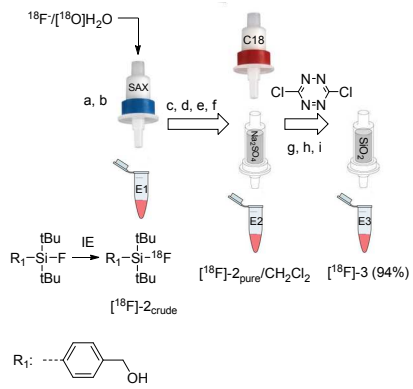
Radiochemical yield (RCY) after radioactive decay correction

Devaraj *et al* reported the preparation of an ^{18}F-labelled polymer-modified tetrazine compound by partially reacting portions of the tetrazine from the tetrazine-modified dextran with ^{18}F-labeled trans-cyclooctene. This strategy can be used in an *in vivo* click reaction involving a trans-cyclooctene-modified antibody.[9] Because the tetrazine moiety is involved in two conjugation processes, a large excess of the compounds has to be injected for *in vivo* PET imaging. Recent efforts to label tetrazines with short half-life radionuclides such as ^{18}F (half life = 109.8 min) and ^{11}C (half life = 20 min) are summarized in Table **1**. Fox *et al* reported the first attempt to synthesize ^{18}F-labeled tetrazine with a radiochemical yield (RCY) of < 1%.7[a] Herth *et al* reported the preparation of a ^{11}C-labelled N-methyl-6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine with a total synthesis time of 50-60 min (about 2.5 – 3 half lives of ^{11}C) and a RCY of 33%.[10] Denk *et al* recently reported the preparation of a [^{18}F]-3-(3-fluoropropyl)-6-methyl-1,2,4,5-tetrazine via a direct fluorination of the tosylated intermediate with a RCY of up to 18%.[11] Both the Herth and the Denk approaches require heating the tetrazine compounds at 80 – 90 ºC, followed by HPLC separation. Despite the progress made in radiolabeling of small tetrazine derivatives, the development of a simple and efficient radiolabeling methodology that does not require HPLC purification is still required. Here we introduce a facile strategy yielding ^{18}F-labeled tetrazines (Tz) by derivatizing 1,4-dichlorotetrazine (**1**) with the [^{18}F]SiFA-OH ([^{18}F]-**2**) labeling building block (Scheme 1). [^{18}F]-**2** can effectively react with **1** to generate [^{18}F]SiFA-OTz ([^{18}F]-**3**). [^{18}F]-**3** can subsequently undergo an IEDDA reaction with the model compound trans-cyclooctenol (**4**) providing [^{18}F]-**5** at 78 ± 5% total yield (n = 3). The two step conjugation process which produces [^{18}F]-**2** was carried out under ambient conditions and high overall radiochemical yields are achieved. This methodology can also be extended to the preparation of other radio-labelled tetrazine derivatives because the radiolabelled precursor is pre-formed before it reacts with 1,4-dichlorotetrazine.

The previously reported low RCYs of radiolabeled Tz derivatives (cf. Table 1) likely result from the low stability of the tetrazine compounds under the relatively harsh labeling conditions that are used.7[a,10,11] In a previous study, we prepared various stable alkoxyl-chlorotetrazine derivatives from dichlorotetrazine.[12]These Tz derivatives maintained a high reactivity toward a follow-up IEDDA reaction, inspiring us to study this system for application to rapid bioorthogonal ^{18}F-labeling. The labeling procedure can be divided into three steps. First, a hydroxyl-terminated radiolabeled compound [^{18}F]-SiFA-OH ([^{18}F]-**2**) is synthesized from **2** *via* a simple isotope exchange (^{18}F for ^{19}F) reaction with a RCY of > 97%. [^{18}F]-**2** is then reacted with 1,4-dichlorotetrazine (**1**) to generate [^{18}F]-SiFA-OTz ([^{18}F]-**3**) with a coupling reaction yield of 92 ± 2%. Finally the IEDDA reaction is carried out by reacting [^{18}F]-**3** with trans-cyclooctenol (**4**), a strained dieneophile, yielding [^{18}F]-**5** in quantitative yield.



Scheme 1. Radiosynthesis of [^{18}F]-SiFA-OTz ([^{18}F]-**3**) and the IEDDA bioorthogonal reaction with trans-cyclooctenol (**4**) resulting in the cycloadducts [^{18}F]-**5**. Total preparation and purification time is 20-25 min. (a) ^{18}F$^-$/K2.2.2/$K_2C_2O_4$, $H_2C_2O_4$, MeCN, rt; (b) dichlorotetrazine, 2,4,6-collidine, DCM, r.t; (c) trans-cyclooctenol (**4**), DCM or DMSO, rt.



Scheme 2. Syntheses of [^{18}F]-**2** and [^{18}F]-**3** for IEDDA reactions. A) Synthesis of [^{18}F]-**2**: a) loading of SAX cartridge with ^{18}F$^-$/[^{18}O]H_2O; b)elution of the QMA cartridge bound ^{18}F$^-$ using a solution of lyophilised K2.2.2/KOH in CH_3CN into a small Eppendorf vessel (E1) containing **2** and $H_2C_2O_4$ (1-2 min reaction time at rt for IE); c) diluting the reaction mixture with water and loading onto a C18 cartridge; d) washing the C18 with water and subsequent air drying; e) attachment of a Na_2SO_4 cartridge to the C18 cartridge; f) elution of pure [^{18}F]-**2** with anhydrous CH_2Cl_2 into E2. B) Synthesis of [^{18}F]-**3**: g) addition of 1,4-dichlorotetrazine (**1**) and 2,4,6-collidine to E2 (reaction time 15 min at rt); h) loading onto a SiO_2 cartridge; i) elution of the SiO_2 cartridge with CH_2Cl_2 into E3 to obtain [^{18}F]-**3** in RCYs of 78 ± 5 % (n = 3).

The labeling conditions for [^{18}F]-**2** preparations were optimized and are summarized in supporting information (Supporting Materials, Table S1). The Munich method was applied to the preparation of highly nucleophilic ^{18}F$^-$ in anhydrous acetonitrile (Steps a,b; Scheme 2).[13,14] Oxalic acid was added (provided in vessel E1, Scheme 2) to partially neutralize KOH from the cocktails used to elute the ^{18}F$^-$ from the QMA cartridge. The highest RCY of the IE reaction was achieved under neutral or weak basic conditions (KOH:oxalic acid = 4:1 or 2:1, RCY [^{18}F]-**2** > 97%) within 5 min using 25-200 µg of SiFA-OH (**2**). Addition of additional oxalic acid resulted in the formation of H^{18}F and lower RCY. For a typical IE reaction, the lowest amount of SiFA-OH (**2**), 25 µg (0.093 µmol), was used yielding maximum RCYs (> 97%). A further decrease of **2**, e.g. 10 µg, resulted in a lower RCY of 69 ± 3 % after 5 min reaction time. For optimized IE reaction time, it is noteworthy that the IE reaction was finished within the first 1-2 min under these labeling conditions (RCYs > 97%), as detected by radio-TLC, when > 25 µg of precursor **2** were used.

One major advantage of using [^{18}F]-**2** as a radio-labeling synthon is the simple purification procedure following synthesis.

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[^{18}F]-**2** can be purified from the reaction mixture via solid phase extraction using a C18 cartridge, obviating the need for HPLC separation (Steps c-f, Scheme 2). The labelling conditions are mild and only unreacted $^{18}F^-$ (which has no retention on the C18 cartridge) has to be separated from the final product. After diluting the crude reaction mixture with water (10X of volume of the reactant mixture) and passing it through a C18 cartridge (Step c, Scheme 2), [^{18}F]-**2** was quantitatively retained on the cartridge. Kyrptofix 2.2.2 (from the labeling cocktail), free $^{18}F^-$, excess KOH and potassium oxalate were removed from the cartridge by further washing with 10 mL of water and drying the C18 cartridge with air (Step d, Scheme 2). A Na_2SO_4 cartridge was attached to the C18 cartridge (Step e, Scheme 2) and [^{18}F]-**2** was eluted from the C18/Na_2SO_4 cartridge combination with 2 mL of anhydrous dichloromethane (Step f, Scheme 2). Because 1,4-dicholorotetrazine is a hydroscopic compound and was used in the following reaction (Step g, Scheme 2), a Na_2SO_4 drying cartridge was tandem-connected to the C18 cartridge before eluting [^{18}F]-**2** to remove all traces of water. The complete IE reaction including purification can thus be achieved within 5 min.

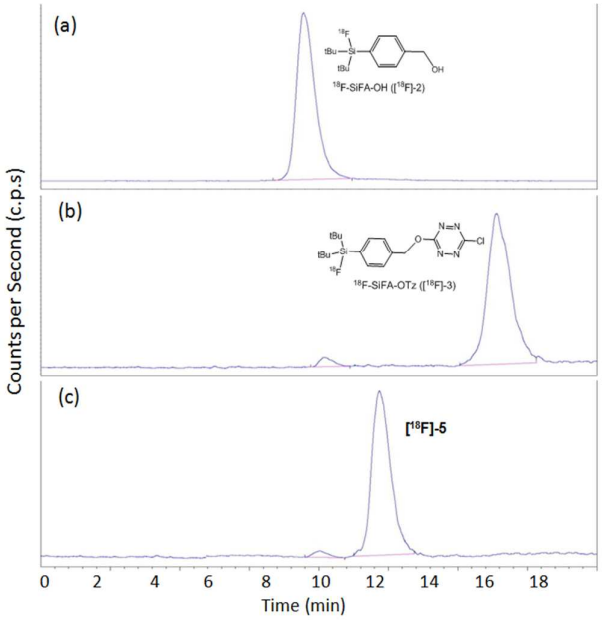

Figure 1. Radio-HPLC chromatogram of (a) [^{18}F]-SiFA-OH ([^{18}F]-**2**), (b) [^{18}F]-SiFA-OTz ([^{18}F]-**3**), and (c) the IEDDA product from [^{18}F]-**3** and trans-cyclooctenol (**4**). (a) [^{19}F]-SiFA-OH was collected after passing the labeling mixture through a C18 cartridge and dried with a Na_2SO_4 cartridge, resulting in a RCY of > 97%. [^{18}F]-**3** was synthesized with a conjugation yield of 92 ± 2 % using a molar ratio of [^{18}F]-SiFA-OH:dichlorotetrazine:2,4,6-collidine = 1:10:2 for 15 min. Radio-HPLC chromatogram of (b) [^{18}F]-**3** collected after passing the reaction mixture through a silica cartridge with dichloromethane, with a separated RCY of 78 ± 5 %. [^{18}F]-**3** was reacted with trans-cyclooctenol (**4**) with a molar ratio of 1:1 (calculated from the staring SiFA-OH used) yielding [^{18}F]-**5**. The radio-HPLC diagram of crude [^{18}F]-**5** after 1 min reaction time(c).

We further optimized the nucleophilic substitution reaction conditions involving 1,4-dichlorotetrazine (**1**) and [^{18}F]-**2** (Supporting Materials, Table 2). [^{18}F]-**3** was synthesized after mixing 1,4-dichlorotetrazine (**1**) and [^{18}F]-**2** in the presence of 1,3,5-collidine. A large excess of 1,4-dichlorotetrazine (10X mole ratio) was applied to push the reaction to completion (Step g, Scheme 2). After reacting for 15 min, the reactant mixture was loaded onto a silica cartridge (Step h, Scheme 2) and eluted with anhydrous dichloromethane (into E3, Step i, Scheme 2). The excess 1,4-dichlorotetrazine (**1**) was retained on the silica cartridge and a narrow pink band of [^{18}F]-**3** was collected. The highest RCY for [^{18}F]-**3** synthesis was achieved when 25µg of [^{18}F]-**2** (0.093 µmol), 149 µg of dichlorotetrazine (10 X, 0.931 µmol), and 22.5 µg of 1,3,5-collodine (2X, 0.186 µmol) were used. Due to the limitations of manual handling of radioactivity, we started with 27.4 - 35.0 mCi $^{18}F^-$; yielding 17.8 - 21.6 mCi of [^{18}F]-**3** after a total synthesis time of 25 min with a specific activity (SA) of 7.1 - 8.6 GBq/µmol. Higher SA can be achieved when starting with a larger radioactivity amount of $^{18}F^-$. In addition to the short synthesis time, this methodology benefits from the fact that the described Tz derivative chemistry is not limited to the usage of SiFA-OH (**2**) compounds as it can be easily adapted to other hydroxyl- or thiol- containing commercially available radio-tags (e.g. [^{3}H]methanol) for the preparation of radio-labelled Tz for bioorthogonal labelling.

A model reaction of non-radioactive SiFA-OTz (**3**) with the commonly used strained dienophile trans-cyclooctenol (**4**) was carried out. The reaction was very rapid as monitored by the solution color change from pink to pale yellow. ^{1}H NMR reports no proton signals from the double bond of trans-cyclooctenol, suggesting that the reaction is quantitative (Supporting Materials, Figure S3). Complex signals from the aliphatic region suggested that several isomers result from the IEDDA and retro-DA (rDA) reaction. However, characterization of each isomer is impractical due to the difficulty of separation of these isomers. The formation of product **5** was confirmed by HPLC-MS (ESI, showed one single peak at 11.6 min, m/z 481.36 $[M+H]^+$ at 254 nm, supporting materials, Figure S4) and HRMS. The mixture of isomers served as the HPLC standards for determination of the radiolabeled product from [^{18}F]-SiFA-OTz ([^{18}F]-**3**) and trans-cyclooctenol (**4**). The IEDDA reaction of [^{18}F]-**3** with trans-cyclooctenol (**4**) occurred on mixing with a molar ratio of 1:1 (calculated from the starting SiFA-OH used) followed by an rDA reaction to generate [^{18}F]-**5**. As trans-cyclooctenol (**4**) has poor solubility in dichloromethane, a stock solution of 2 mg/mL trans-cyclooctenol (**4**) in DMSO was prepared. Dichloromethane and DMSO could be simply mixed without affecting RCYs of [^{18}F]-**5**. As shown in Figure 1c, [^{18}F]-**5** was quantitatively generated within 1 min (starting with 17.8 - 21.6 mCi [^{18}F]-**3** (0.093 µmol) and 11.7 µg trans-cyclooctenol (**4**) (0.093 µmol). [^{18}F]-**5** was prepared with a specific activity of 7.0 - 8.5 GBq/µmol and [^{18}F]-**3** was quantitatively consumed. It is important to note that [^{18}F]-**3** is stable under these reaction conditions. No noticeable dissociation of [^{18}F]-**3** was detected within 2 h (one half life of ^{18}F). A long term stability test of [^{18}F]-**3** was not performed because of this short three step labelling procedure.

In conclusion, the development of a fast and reliable methodology for the synthesis of ^{18}F-radiolabled tetrazine is reported. The [^{18}F]-SiFA-OTz ([^{18}F]-**3**) can be prepared, isolated and conjugated with the model strained dieneophile trans-cyclooctenol (**4**), with a final RCY of 78 ± 5 % within 20-25 min. Importantly, only simple cartridge-based separations are required for the purification of the [^{18}F]-SiFA-OTz ([^{18}F]-**3**) labeling synthon. Because SiFA-OH (**2**) was pre-radiolabeled before incorporation into the tetrazine, this methodology provides a facile and effective route to many hydroxyl- or mercapto- tethered radioactive tags.

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Notes and references

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