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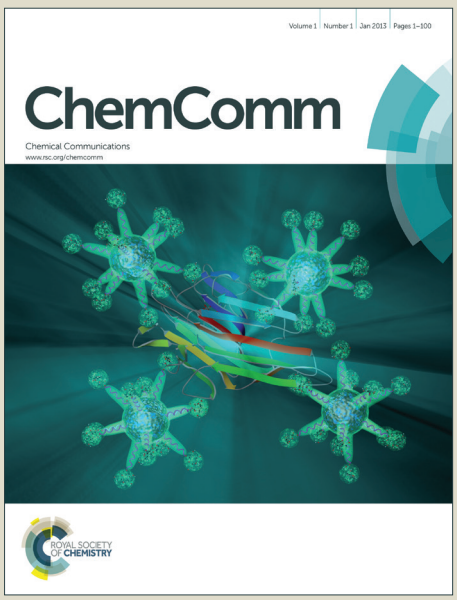

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COMMUNICATION

Sonogashira cross-coupling reaction with 4-[^{18}F]fluoroiodobenzene for rapid ^{18}F-labelling of peptides†

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The study describes the Sonogashira cross-coupling reaction with 4-[^{18}F]fluoroiodobenzene ([^{18}F]FIB) as novel and efficient method for rapid labelling of peptides with the short-lived positron emitter fluorine-18.

Radiolabelled peptides have been used for targeted molecular imaging and therapy for more than 20 years[1]. The high interest in radiolabelled peptides as targeting vectors mainly stems from the over-expression of various specific peptide-binding receptors in numerous cancers and inflammatory tissues[2].

Positron emission tomography (PET) is a non-invasive molecular imaging technique to assess physiological and biochemical processes in living organisms by the use of compounds labeled with short-lived positron emitters, also referred to as radiotracers. For PET imaging with peptides as radiotracers, the majority of peptides were labeled with positron emitting radiometals like copper-64, gallium-68, and yttrium-86[3].

Unlike radiometals, short-lived positron emitter fluorine-18 (^{18}F) offers several advantages such as high abundance of positron emission (97%), high production yields on small biomedical cyclotrons, high spatial resolution through low positron energy (0.635 MeV), and convenient half-life of 109.8 min allowing for extensive radiochemistry and molecular imaging studies[4]. However, radiofluorination of peptides still remains a special challenge, and only a few ^{18}F-labeled peptides have been used in the clinic. Commonly used methods for radiolabelling of peptides with ^{18}F can be subdivided into three general categories: (1) use of ^{18}F-labelled prosthetic groups which are activated as active esters or maleimides to undergo bioconjugation reaction with functional groups of the peptide backbone such as NH_2 and SH[5-7]; (2) exploitation of fluoride-acceptor chemistry based on the strong affinity of [^{18}F]fluoride to silicon (Si-^{18}F), boron (B-^{18}F), and aluminum (Al-^{18}F)[8-10]; and (3) application of various click chemistry concepts[11-13]. Scope and limitations of various methods for radiofluorination of peptides have been summarized in numerous excellent reviews.

Transition metal-mediated cross-coupling reactions have stimulated significant advancements in PET radiochemistry, especially with the short-lived positron emitter ^{11}C and ^{18}F. Various Pd-mediated cross-coupling reactions proved to be valuable and popular synthesis strategies for the preparation of ^{18}F-labelled radiotracers[14]. Pd-mediated cross-coupling reactions were successfully applied to the synthesis of various small molecule PET radiotracers, including ^{18}F-labelled steroids[15], nucleosides[16], and amino acids[17]. However, the potential of Pd-mediated reactions has not yet been fully recognized for the ^{18}F-labelling of higher molecular weight compounds like peptides and proteins. An exception is the recently reported synthesis of ^{18}F-labelled polypeptides using Suzuki-Miyaura cross-coupling reaction with 4-[^{18}F]fluorophenylboronic acid as the coupling partner[18]. On the other hand, there are numerous reports describing the application of the Sonogashira cross-coupling reaction for peptide and protein functionalization in aqueous media. In a first report, Dibowski*et al.*[19] described the Castro-Stephens-Sonogashira reaction for bioconjugation of peptides in water using a Pd-guanidino-phosphane catalysts. The reaction was applied to cross-coupling reactions with 4-iodo-benzoate with propargylglycine to form the desired cross-coupled product in good chemical yields of 75% after a reaction time of 3 h. Further proof-of-concept of this regioselective C-C bond formation was demonstrated by the reaction of biotinylglutamoyl-propargylamide with 4-iodo-phenyl-functionalized undecapeptide in buffer to yield the cross-coupled product in 91% after 3 h.

Additional reports further demonstrated chemoselective Sonogashira cross-coupling reaction of peptides in water. The reaction proceeded best at pH 5.5 to provide high yields[20]. Recently, the use of 4-fluoroiodobenzene in Sonogashira cross-coupling reactions with alkyne-encoded proteins in aqueous medium was reported by Li *et al.*[21]. These reports clearly demonstrated the suitability of Sonogashira cross-coupling reactions in bioorthogonal bioconjugations for the introduction of fluorophenyl groups into peptides and proteins in aqueous solvents under mild conditions.

However, to the best of our knowledge the Sonogashira cross-coupling reaction with 4-[^{18}F]fluoroiodobenzene ([^{18}F]FIB) has not yet been reported for peptide labelling in ^{18}F- radiochemistry. We have recently reported on the synthesis of various metabolically stabilized ^{18}F-labeled bombesin derivatives for targeting gastrin-releasing peptide receptors in prostate cancer.[5] Radiolabelling was accomplished through classical bioconjugation using acylation reaction with succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) or oxime formation with 2-[^{18}F]fluoro-2-deoxy-glucose ([^{18}F]FDG).

Application of Sonogashira reaction with [^{18}F]FIB requires presence of a terminal alkyne group in the peptide backbone. Introduction of a terminal alkyne group was achieved through coupling L-propargylglycine to the N-terminal end of bombesin derivative.

The synthesis of propargylglycine-containing bombesin derivative**1** as labelling precursor was performed using solid-phase peptide synthesis. Peptide **1** was isolated in 20% yield after HPLC purification and subsequent lyophilisation. Reference compound **[^{19}F]2** was prepared using Sonogashira reaction conditions with 4-fluoroiodobenzene in solution and on resin in 9% and 15% yield, respectively. Higher chemical yields of 15% favour on-resin Sonogashira cross-coupling compared with the reaction in solution.

Structures of peptides **1** and **[^{19}F]2** are displayed in Fig. 1.

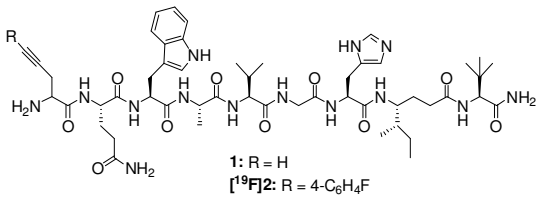

Fig. 1 Structures of peptide **1** and peptide **[^{19}F]2**.

Reaction conditions for Sonogashira cross-coupling between [^{18}F]FIB and alkyne-functionalized bombesin derivative **1** for the radiosynthesis of **[^{18}F]2** were optimized by screening several Pd-complexes, solvents, reaction temperatures, reaction times, and concentrations of peptide **1**. The general outline of the radiolabelling reaction according to Sonogashira reaction conditions is given in Fig. 2.

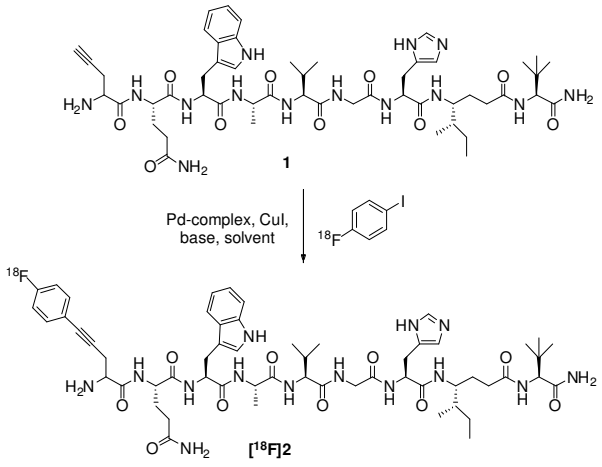

Fig. 1 Sonogashira reaction between peptide **1** and [^{18}F]FIB.

All reactions were processed by acidification of the reaction mixture with 1N HCl (0.7 mL), followed by centrifugation for 5 min, and transfer of the reaction mixture from the Eppendorf tube into a glass vial. Both the Eppendorf tube and the glass vial were measured for radioactivity amount, and the reaction mixture was analyzed using radio-TLC. Radiochemical yields represented show the percentage of product present in the reaction mixture. The results are summarized in Table 1.

In the first set of reactions (entries 1 to 4), the influence of different palladium catalysts was tested.

Two Pd(II) catalysts ($PdCl_2(PPh_3)_2$ and $Pd(OAc)_2$), and two Pd(0) catalysts ($Pd(PPh_3)_4$ and $Pd_2(dba)_3$) were used. The rest of the reaction mixture contained CuI (1 mg), TEA (50 µL), and peptide **1** (85 µg/mL) in PBS (0.5 mL) with [^{18}F]FIB in CH_3CN (100 µL). Pd-complexes containing a triphenylphosphine ligand (entry 1 and 2) seemed to be more sufficient regardless the oxidation state of the metal center, and comparable radiochemical yields of about 10% were obtained with $PdCl_2(PPh_3)_2$ and $Pd(PPh_3)_4$ (entry 1 and 2).

Further optimization studies were carried out with $PdCl_2(PPh_3)_2$ as the Pd-complex. In the next series of experiments, the amount of peptide on the radiochemical yield was studied (entries 5 to 8). The amount of peptide in solution varied from 1.3 mg/mL down to 1.3 µg/mL. Results clearly demonstrated the importance of peptide amount on the radiochemical yield. High peptide concentration of 1.3 mg/mL provided highest radiochemical yield of 39%, whereas no product was formed when very low peptide concentration of 1.3 µg/mL was used (entry 5 vs. entry 8). We decided to continue optimization experiments with a peptide concentration of 85 µg/mL. In the next series of experiments, temperature and solvent were varied using DMF and CH_3CN, using a reaction temperature of 25 °C, 45 °C, and 65 °C, respectively (entries 9 to 14). Experiments displayed in entries 9 to 14 suggest that reactions using DMF as the co-solvent proceeded in higher radiochemical yield at higher reaction temperature (entries 9, 11, and 13). In the case of CH_3CN as the co-solvent, best radiochemical yields of 24% were achieved at 45 °C (entry 12), whereas lower radiochemical yields were observed when lower temperature (25 °C, entry 10) or higher temperature (65 °C, entry 14) were applied.

In course of all experiments described in entries 1 to 14, we realized that a major limitation of the Sonogashira cross-coupling reaction with peptides in aqueous solutions is the solubility of the Pd-complex. As a consequence, we tested various water soluble Pd-complexes in the next set of reactions, and water soluble complexes $Pd(tppts)_4$ and $Pd(NO_3)_2$ were used. $Pd(tppts)_4$ was prepared starting from $Pd(OAc)_2$(1.0 mg) by ligand exchange with 3,3′,3″-phosphanetriyltris(benzenesulfonic acid) trisodium salt (tppts) (10.5 mg) within 30 min through gentle vortexing[22]. Ligand exchange from $Pd(OAc)_2$to $Pd(tppts)_4$was easily visible by color change (supplementary data, Figure S1). Color of the reaction changed from yellow ($Pd(OAc)_2$), to light orange (1st ligand substitution), to green (2nd ligand substitution), to dark orange (3rd ligand substitution), to ruby red (4th ligand substitution).

Upon completion of ligand exchange reaction, complexes $Pd(tppts)_4$and $Pd(NO_3)_2$wereused for the cross-coupling reactions with peptide**1** (entries 15 and 16). Higher peptide concentration of 250 µg/mL was used. Pd-complex $Pd(NO_3)_2$ showed very poor cross-coupling potential compared to $Pd(tppts)_4$ as reflected by the very low radiochemical yield of 3% (entry 15) compared to 25% obtained with $Pd(tppts)_4$ (entry 16).Therefore, further optimization experiments were performed using $Pd(tppts)_4$ as a water soluble Pd-complex. Influence of the reaction time upon the radiochemical yield was studies in the following series of experiments (entries 17 to 20). Best radiochemical yields of about 70% were obtained after a reaction time of 10 min at 25 °C (entry 19).

Table 1 Summary of results for the Sonogashira reaction of [^{18}F]FIB with peptide **1**

Entry	Pd complex	Peptide concentration	Time	Temperature	Solvent	Radiochemical yield[a,b,c] (n=3)
1	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	45 ºC	H_2O/CH_3CN	8 ± 1
2	$Pd(PPh_3)_4$	85 μg/mL	45 min	45 ºC	H_2O/CH_3CN	10 ± 5
3	$Pd(OAc)_2$	85 μg/mL	45 min	45 ºC	H_2O/CH_3CN	2 ± 0
4	$Pd_2(dba)_3$	85 μg/mL	45 min	45 ºC	H_2O/CH_3CN	2 ± 1
5	$PdCl_2(PPh_3)_2$	1.3 mg/mL	45 min	45 ºC	H_2O/CH_3CN	39 ± 11
6	$PdCl_2(PPh_3)_2$	130 μg/mL	45 min	45 ºC	H_2O/CH_3CN	18 ± 6
7	$PdCl_2(PPh_3)_2$	13 μg/mL	45 min	45 ºC	H_2O/CH_3CN	7 ± 3
8	$PdCl_2(PPh_3)_2$	1.3 μg/mL	45 min	45 ºC	H_2O/CH_3CN	0 ± 0
9	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	25 ºC	H_2O/DMF	11 ± 2
10	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	25 ºC	H_2O/CH_3CN	10 ± 2
11	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	45 ºC	H_2O/DMF	13 ± 7
12	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	45 ºC	H_2O/CH_3CN	24 ± 8
13	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	65 ºC	H_2O/DMF	19 ± 6
14	$PdCl_2(PPh_3)_2$	85 μg/mL	45 min	65 ºC	H_2O/CH_3CN	8 ± 3
15	$Pd(NO_3)_2$	250 μg/mL	45 min	45 ºC	H_2O/CH_3CN	3 ± 0
16	$Pd(tppts)_4$	250 μg/mL	45 min	45 ºC	H_2O/CH_3CN	25 ± 8
17	$Pd(tppts)_4$	85 μg/mL	1 min	25 ºC	H_2O/CH_3CN	60 ± 5
18	$Pd(tppts)_4$	85 μg/mL	5 min	25 ºC	H_2O/CH_3CN	51 ± 4
19	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	71 ± 4
20	$Pd(tppts)_4$	85 μg/mL	15 min	25 ºC	H_2O/CH_3CN	73 ± 2
21	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	37 ± 5[d]
22	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	32 ± 6[e]
23	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	40 ± 5[f]
24	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	33 ± 2[g]
25	$Pd(tppts)_4$	85 μg/mL	10 min	25 ºC	H_2O/CH_3CN	35 ± 13[h]

[a] Radiochemical yields were determined by radio-TLC representing percentage of product present in the reaction mixture.
[b] 1 mg of CuI was used
[c] 50 μL of TEA was used
[d] 5 mg of NaOH was used instead of 50 μL of TEA
[e] 50 μL of DIPEA was used instead of 50 μL of TEA
[f] 10 mg of K_3PO_4 was used instead of 50 μL of TEA
[g] 10 mg of K_2CO_3 was used instead of 50 μL of TEA
[f] 10 mg of $NaHCO_3$ was used instead of 50 μL of TEA

The last set of experiments studied the influence of different bases upon the radiochemical yield of Sonogashira cross-coupling reaction with [^{18}F]FIB and peptide **1**. All reactions in entries 21 to 25 provided comparable radiochemical yields between 32 to 40% regardless the base used. Alternative bases NaOH (entry 21), DIPEA (entry 22), K_3PO_4 (entry 23), K_2CO_3 (entry 24), and $NaHCO_3$ (entry 25) gave lower radiochemical yields compared to reaction with TEA under comparable conditions (entry 19).

Based on optimization experiments summarized in Table 1, the following optimized reaction conditions were selected for radiosynthesis of **[^{18}F]2** according to a Sonogashira cross-coupling reaction between peptide **1** and [^{18}F]FIB: 0.1 mg of $Pd(tppts)_4$, 1.0 mg of CuI, 85 μg of peptide **1**, and 50 μL of TEA in 1 mL of CH_3CN:PBS (1:9) at 25°C for 10 min.

Application of this optimized reaction conditions gave peptide **[^{18}F]2** in 71 ± 4% radiochemical yield (decay-corrected, based on [^{18}F]FIB) after a reaction time of 35 min, including HPLC purification. Specific activity of **[^{18}F]2** was calculated to be 625 ± 334 GBq/μmol (n=3). Identity of purified **[^{18}F]2** was confirmed through radio-HPLC by co-injection of reference compound **[^{19}F]2** (Supplementary data, Figure S2).

Further experiments included reactions under optimized conditions in the presence of sulfhydryl groups, and in the absence of Cu(I). Sulfhydryl groups are known to prevent Pd-mediated cross-coupling reactions. Many peptides and proteins contain cysteine residues which may interfere with Sonogashira cross-coupling reaction conditions. To test this assumption, we prepared cysteine-containing peptide derivative **3**. Synthesis of cysteine-containing peptide **3** and corresponding 4-fluorophenyl-containing reference compound **[^{19}F]4** was performed according to solid-phase-peptide synthesis protocols (Supplementary data, Figure S3). Application of optimized reaction Sonogashira cross-coupling conditions with [^{18}F]FIB and cysteine-containing peptide **3** did not result in the formation of radiolabelled peptide. This finding confirms the detrimental effect of free sulfhydryl groups on Sonogashira cross-coupling reaction with [^{18}F]FIB.

Although only little Cu(I) amounts are required to perform the reaction, we explored the reaction in the complete absence of Cu(I) to address potential toxicity concerns.

Application of optimized reaction conditions, except the lack of Cu(I), gave not product formation when peptide **1** was reacted with [^{18}F]FIB under Cu(I)-free Sonogashira reaction conditions. The observed lack of product formation in the absence of Cu(I) and the presence of free sulfhydryl groups are important limitations of the presented Sonogashira cross-coupling reaction using optimized reaction conditions.

However, in this work we have described the first example of a Sonogahira cross-coupling reaction with [^{18}F]FIB as novel radiolabelling method for the site-specific incorporation of short-lived positron emitter ^{18}F into peptides. The reaction conditions were carefully optimized through screening of different Pd complexes, solvents, and bases. Optimization also included amount of peptide, reaction time and temperature. Optimization of Sonogashira cross-coupling reaction conditions revealed importance of the used Pd complex. Pd complexes containing triphenylphosphine ligands seemed to provide highest radiochemical yields. This observation was also made with water soluble Pd-complexes containing phosphine ligand tppts. This trend was also confirmed in various non-radioactive Sonogashira reactions, in which aromatic-substituted palladium complexes were used in aqueous media for the synthesis of peptides and proteins[19-21].

The amount of peptide as labelling precursor was found to be another important reaction parameter. Peptide amounts of at least 85 μg/mL afforded good radiochemical yields. This rather low peptide amount was also beneficial to obtain reasonably high specific activities. Moreover, the use of only small amount of peptide is extremely promising if the peptide (1) very costly or (2) difficult to synthesize. In comparison, most of the previously reported peptide syntheses with ^{18}F typically required between 0.1 to 2 mg of peptide to provide reasonable radiochemical yields[5-13]. In the case of non-water soluble Pd-complexes, the reaction was optimally performed at 45 °C in CH_3CN over 45 min. In the case of water soluble complexes, the reaction proceeded more favourably, and good results were obtained when the reaction was performed at 25 °C in CH_3CN/PBS as the solvent within a short reaction time of 10 min. This ability for the reaction to proceed at room temperature in mostly PBS buffer is very beneficial to an extension of this approach to other compounds that are either more sensitive to higher temperature reactions or to organic solvents. Another interesting aspect of our approach is the possibility to incorporate alkyne-containing amino acids like homopropargylglycine in a directed way into the structure of proteins[23]. This allows for a site-specific radiolabelling reaction exploiting the Sonogashira cross-couplings with [^{18}F]FIB compared to the random incorporation using prosthetic groups like [^{18}F]SFB into the peptide or protein backbone. This site-directed radiolabelling provides radiolabelled compounds without the potential loss of biological activity resulting from non-specific bioconjugation reactions.

Overall, we have developed a novel radiolabelling method for site-specific incorporation of short-lived positron emitter ^{18}F into peptides under mild and physiological conditions. Important limitations of our synthesis approach are that the reaction does not proceed in the presence of free sulfhydryl groups as typical found in cysteine residues, and that the reaction requires even though very little, but still noticeable amounts of Cu(I).

Further optimization toward a Cu-free Sonogashira cross-coupling reaction with [^{18}F]FIB has the potential to include other biomacro-molecules such as proteins and oligonucleotides into this novel radiolabelling concept. This will further expand the arsenal of innovative ^{18}F-labelled radiotracers for targeted molecular imaging with PET.

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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