Radiolabelling of proteins with fluorine-18 *via* click chemistry

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The study describes for the first time the application of Cu(I)-mediated 1,3-dipolar [3 + 2]cycloaddition for the labelling of proteins with the short-lived positron emitter fluorine-18 as exemplified with azide-functionalized human serum albumin (HSA).

Positron emission tomography (PET) is a powerful non-invasive molecular imaging technique which provides functional information on physiological, biochemical and pharmacological processes *in vivo*.[1–4] The steadily growing numbers of novel molecular targets for PET imaging is accompanied with a permanent demand for novel or improved radiolabelling methods, especially for higher molecular weight compounds like peptides, proteins, antibodies and antibody fragments. As key regulators of cell growth and cellular function in living organisms, radiolabelled peptides and proteins have been the subject of intense research efforts for targeted diagnostic imaging and radiotherapy in nuclear medicine over the last 30 years.

The short-lived positron emitter fluorine-18 (^{18}F, $t_{1/2}$ = 109.8 min) is the radionuclide most frequently used for the design and synthesis of PET radiotracers due to its favorable nuclear and chemical properties. However, incorporation of ^{18}F into high molecular weight compounds like proteins represents a special challenge. Proteins cannot be labelled with ^{18}F at high specific activity directly, due to the required strongly basic reaction conditions at elevated temperatures, which are not compatible with the structural and functional integrity of proteins. To circumvent this obstacle, protein labelling with ^{18}F is usually accomplished by means of prosthetic groups, also referred to as bifunctional labelling agents,[5,6] to allow ^{18}F labelling under physiological conditions. Linkage of ^{18}F-labelled prosthetic groups to proteins can be accomplished *via* various bioconjugation techniques, including acylation,[7–10] imidation,[11] photochemical conjugation,[8] and thioether formation.[12–14] However, every method has advantages and limitations, and further work on the development of rapid, clean and mild synthesis techniques for ^{18}F-labelled proteins is still needed.

In recent years click chemistry has entered into many fields of chemical sciences, including radiopharmaceutical chemistry.[15,16] The copper(I)-catalyzed 1,2,3-triazole formation involving azides and terminal acetylenes according to a 1,3-dipolar [3 + 2]cycloaddition has proved to be a particularly valuable tool for efficient ^{18}F labelling of peptides. Click chemistry was further applied for the design and synthesis of radiometal-based radiotracers. Click chemistry was used to form novel multidentate ligand scaffolds containing a triazole group for the efficient chelation of a ^{99m}Tc(CO)$_3$ core.[17]

However, to date, click chemistry has not yet been extended to the radiolabelling of proteins with the short-lived positron emitter ^{18}F. In this work, we describe for the first time the application of click chemistry for ^{18}F labelling of proteins. The reaction was accomplished through copper(I)-mediated [3 + 2]cycloaddition between azide-functionalized human serum albumin (HSA) and 4-[^{18}F]fluoro-*N*-methyl-*N*-(prop-2-ynyl)-benzenesulfonamide (*p*[^{18}F]F-SA) **[^{18}F]3** as a novel click chemistry building block with ^{18}F on an aromatic carbon to form a metabolically stable C–F bond.

Sulfonamide **3** as reference compound and sulfonamide **6** as a labelling precursor for ^{18}F incorporation were prepared starting from commercially available 4-fluorobenzene-1-sulfonyl chloride or 4-nitrobenzene-1-sulfonyl chloride, respectively, according to Fig. 1.

Acylation of sulfonyl chlorides with *N*-methylpropargyl amine gave compounds **3** and **4** in excellent yields of 99% and 96%. Reduction of nitro group in compound **4** with $SnCl_2$ afforded the corresponding amine **5** in 62% chemical yield. Treatment of amine **5** with an excess of MeOTf in the presence of 2,6-di-*tert*-butyl-4-methylpyridine as the base provided trimethyl ammonium triflate salt **6** as a labelling precursor in excellent 97% yield suitable for subsequent radiolabelling with cyclotron-produced [^{18}F]fluoride according to a nucleophilic aromatic substitution reaction. The radiosynthesis of ^{18}F-labelled click chemistry building block **[^{18}F]3** was accomplished in a single step in a remotely-controlled synthesis apparatus (GE TRACERlab® FX-FN) using the powerful nucleophilic radiofluorinating agent [^{18}F]KF (generated by treatment of cyclotron-produced [^{18}F]fluoride with Kryptofix K_{222}–potassium carbonate) in sulfolane as the solvent at 80 °C for 10 min (Fig. 2). After HPLC purification, compound **[^{18}F]3** was isolated in 32 ± 5% radiochemical yield (n = 11) at a specific activity in the range of 120–570 GBq μmol^{-1}. The

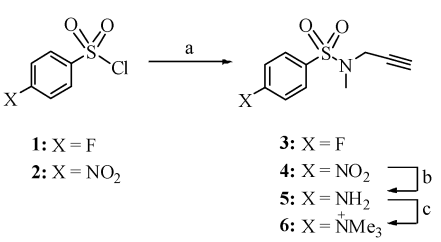

Fig. 1 Synthesis of reference compound **3** and labelling precursor **6**. (a) *N*-Methylpropargyl amine, CH_2Cl_2, 99% (**3**), 96% (**4**); (b) $SnCl_2$, EtOH, 62%; (c) MeOTf, 2,6-di-*tert*-butyl-4-methylpyridine, 97%.

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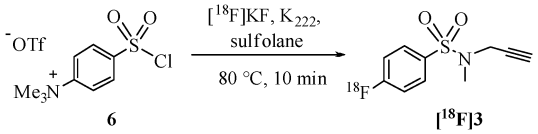

Fig. 2 Radiosynthesis of click chemistry building block **[^{18}F]3**.

radiochemical purity exceeded 95%. The use of other aprotic polar solvents like DMF or DMSO gave lower radiochemical yields.

In a typical experiment, starting from 16 GBq of [^{18}F]fluoride, 6.5 GBq (dc) of click chemistry building block **[^{18}F]3** could be obtained in a total synthesis time of 80 min, including HPLC purification.

The convenient radiosynthesis of compound **[^{18}F]3** as a novel ^{18}F-labelled sulfonamide-based click chemistry building block in a remotely-controlled synthesis unit allows its wide application for a broad range of click chemistry reactions. Moreover, the experimentally determined favorable lipophilicity (log P = 1.7) of sulfonamide **[^{18}F]3** allows subsequent click chemistry in aqueous media as relevant for proteins.

Human serum albumin (HSA) is the most abundant protein in human blood plasma. HSA is composed of 585 amino acids, and it has a molecular mass of 67 kDa. The amino acid sequence contains 59 lysine residues which can be used for the introduction of azide groups into HSA *via* reaction of ε-amino groups in the protein with an excess of 1-succinimidyl-5-azidopentanoate.[18,19] The modified protein[20] was purified with size-exclusion chromatography (SEC) to give 29% of azide-containing HSA after lyophilization. MALDI-TOF MS analysis of HSA and azide-functionalised HSA is given in Fig. 3.

The number of introduced azide residues was determined by enzymatic digest of the azide-functionalized HSA followed by MALDI-TOF MS analysis of the protein fragments according to literature procedure.[21] The enzymes trypsin, endoproteinase Lys-C and endoproteinase Glu-C were used for enzymatic digest. Each enzyme was used for a single digest of azide-modified HSA and native HSA as a control. The resulting solutions were processed according to the delayed extraction procedure[22] prior to MALDI-TOF MS analysis. Seven modified lysine residues could be detected after enzymatic digest with trypsin and Glu-C, respectively, whereas 22 modified lysine residues were determined after enzymatic digest with Lys-C. Taking into account multiple detections of modified lysine residues in the different enzymatic digests, the total number of azide-modified lysine residues was determined to be 27.

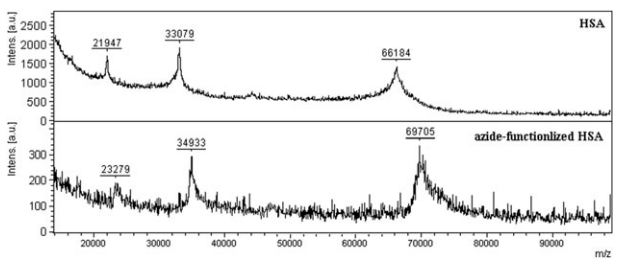

Fig. 3 MALDI-TOF MS spectra of HSA (m/z = 66 184) and azide-functionalised HSA (m/z = 69 705).

The 27 azide group-containing HSA was used for click chemistry according to a 1,3-dipolar [3 + 2]cycloaddition with ^{18}F-labelled alkyne **[^{18}F]3**. Application of *in situ* reduction of Cu(II) salts like $CuSO_4$ into Cu(I) as required reactive copper species within the copper-mediated [3 + 2] cycloaddition by means of sodium ascorbate led to significant degradation of HSA presumably due to partly or complete cleavage of the 17 disulfide bridges abundant in the protein.

However, direct application of Cu(I) salts such as CuBr or CuI would avoid the use of detrimental reducing agents like sodium ascorbate. Various multidentate triazoles are known as excellent chelates for stable complex formation of Cu(I) preventing re-oxidation of Cu(I) into Cu(II).[23,24] Therefore, we used oligotriazole tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)-methyl]-amine (TBTA) as a suitable chelator for stabilizing Cu(I). Cu(I)–TBTA complex was applied to the click chemisty reaction between azide-functionalized HSA and ^{18}F-labelled alkyne **[^{18}F]3**. The reaction was performed in phosphate buffer (pH 7.5) at 30 °C for 20 min. The product was purified by SEC to give the desired ^{18}F-labelled HSA[25] in 55–60% decay-corrected radiochemical yield based upon **[^{18}F]3**. Fig. 4 shows the results of SEC and subsequent polyacrylamide gel electrophoresis with sodium dodecylsulfate (SDS-PAGE) for analysis of the reaction mixture and purified fractions. Up to 240 MBq of the final product were obtained within a total synthesis time of 120 min starting from [^{18}F]fluoride.

Small animal PET imaging with radiolabeled HSA in mice was performed using a microPET_ P4 primate model scanner (CTI Concorde Microsystems Inc., Knoxville, TN).[26] Fig. 5 shows small animal PET images at 5 min, 60 min and 120 min after radiotracer injection. After 120 min, most of the activity was found in the gall bladder and intestines, and in the bladder. The half-life of blood activity of the radiotracer was calculated to be 31 min, which is faster compared with other ^{18}F-labelled HSA derivatives reported in the literature.[27] Hence, introduction of azide residues into HSA and subsequent radiolabeling *via* click chemistry has significantly altered structural and functional integrity of HSA.

In summary, for the first time, click chemistry could successfully be applied to the ^{18}F labelling of proteins as exemplified for azide-modified HSA. The reaction occurred in a short reaction time compatible with the short half-life of ^{18}F, and under mild reaction conditions compatible with the

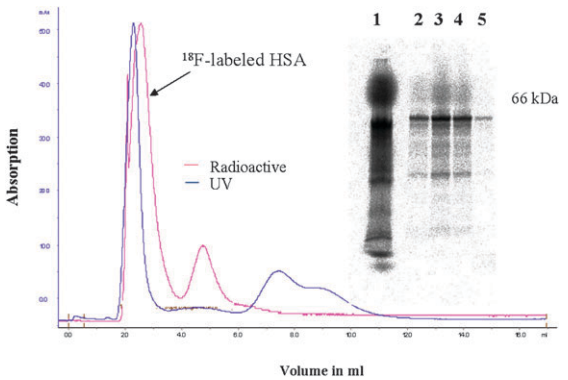

Fig. 4 SEC profile of the reaction mixture and SDS-PAGE analysis of reaction mixture (lane 1) and purified fractions (lanes 2–5).

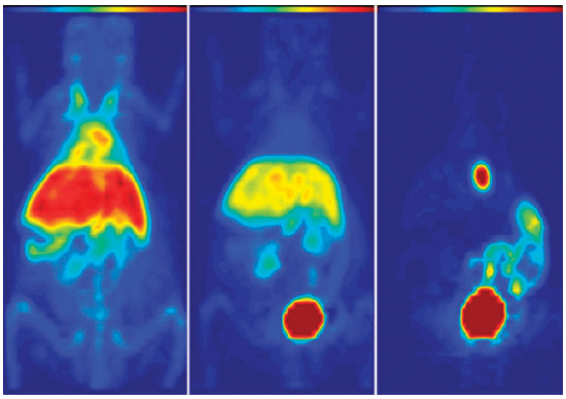

Fig. 5 Small animal PET images of nude mice after intravenous injection of ^{18}F-labelled HSA at 5 min (left), 60 min (middle) and 120 min.

structural and functional integrity of proteins. The application of Cu(I)–TBTA complex provides a reliable and stable source of Cu(I) for the click chemistry, and it circumvents *in situ* reduction of Cu(II) salts as typically employed for Cu(I)-mediated 1,3-dipolar [3 + 2]cycloaddition reaction. The successful labelling of azide-functionalized HSA with the short-lived positron emitter ^{18}F according to Cu(I)-mediated 1,3-dipolar [3 + 2]cycloaddition reaction further expands the scope of click chemistry as a versatile tool for a broad array of radiolabelling reactions.

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