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Direct radiolabelling of proteins at cysteine using [^{18}F]-fluorosugars†

Omar Boutureira, Gonçalo J. L. Bernardes, François D'Hooge and Benjamin G. Davis*

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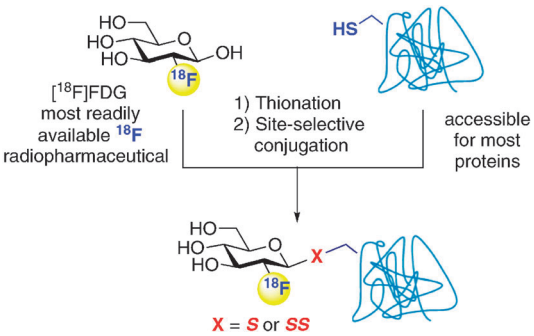
A strategy for the site-specific attachment of 2-deoxy-2-fluorosugars to cysteine and dehydroalanine tagged proteins is reported. When combined with thionation of fluorosugars, such as the widely available ^{18}F probe 2-deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG), this methodology allows fast and direct access to site-specific [^{18}F]FDG-labelled proteins.

The combined use of positron emission tomography (PET) and X-ray transmission computed tomography (CT) has emerged as an important technique for medical diagnosis and evaluation of treatment progress.[1,2] 2-Deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG) is the most extensively used PET radiotracer in nuclear medicine[3] and the most widely available organic source of ^{18}F, an issue of particular relevance given limited access to cyclotrons. Wider dissemination of PET/CT for *in vivo* imaging of important biological events at the molecular level would be enabled by more selective [^{18}F]-radiotracers that could fulfil the potential demand of disease-specific PET radiopharmaceuticals. In this context, [^{18}F]-radiolabelled proteins have emerged as a new generation of selective radiopharmaceuticals for PET imaging techniques.[4,5] Thus, although in principle almost unlimited methods and reagents may be found for attaching ^{18}F to proteins, most current strategies[6–10] have as their limiting elements the long synthesis times, poor radiochemical yield, low specific activities (mainly due to the use of prosthetic groups) and reduced site-selectivity which eventually may lead to poor structural characterization, probe purity and labelling/imaging reproducibility.[5]

An ideal system would take proteins accessible in any laboratory and combine them with a readily available ^{18}F source, such as [^{18}F]FDG (the most available organic source), in a direct manner. Strategies have been devised for [^{18}F]FDG incorporation[11,12] into peptides[4,13–15] and more rarely into proteins,[4,12,16] however these often need specialist synthesis and use either protected [^{18}F]FDG derivatives or prosthetic groups that limit their application in protein modification. To date, direct site-selective protein radiolabelling from unprotected [^{18}F]FDG is not known. We recently synthesized the first examples of chemically defined [^{18}F]fluoro-labelled glycoproteins;[12] this method required the incorporation of an unnatural amino acid 'tag' into proteins prior to modification with a bespoke sugar reagent.[12] Regardless of the utility of this procedure, we considered that a complementary and perhaps more widely applicable method would instead target a natural amino acid (cysteine, Cys) and would use readily-available $^{18/19}$FDG as a building block in one-pot protein site-specific labelling. This approach would exploit ready access to Cys protein mutants and site-specific chemistry at Cys along with availability of FDG as a fluorine source (Scheme 1).



Scheme 1 Direct site-selective radiolabelling of proteins with [^{18}F]FDG.

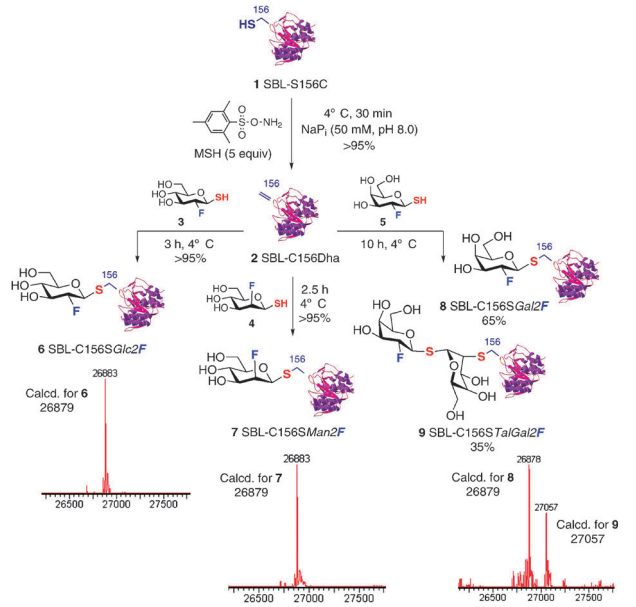
We describe here a practical and convergent approach for the site-specific conjugation of thiol fluorosugars to natural cysteine (Cys)[17] and dehydroalanine (Dha, directly accessible from Cys[18,19]) tagged proteins. The strategy uses a 'tag-and-modify' approach[20,21] coupled with the straightforward formation of glycosyl thiols from sugars without the need for any protecting group chemistry.[22]

First, the feasibility of the approach was tested using 2-deoxy-2-fluorosugars with *gluco* (Glc), *manno* (Man) and *galacto* (Gal) configurations (see ESI† for synthetic details). As a model protein we used a mutant of serine protease subtilisin from *Bacillus lentus* (SBL) S156C **1** that displays a single, exposed Cys residue that can be readily converted to Dha using *O*-mesitylenesulfonylhydroxylamine (MSH)[18] (Scheme 2).

Addition of Glc2F-β-SH **3** and Man2F-β-SH **4** to SBL-C156Dha **2** proceeded successfully with complete conversion to the corresponding thioether-linked 2Fglycoproteins SBL-C156S*Glc2F* **6** and SBL-C156S*Man2F* **7** after 3 and 2.5 h at 4 °C, respectively. Interestingly, however, when similar conditions were applied (10 h at 4 °C), for the

Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford OX1 3TA, UK. E-mail: Ben.Davis@chem.ox.ac.uk; Fax: + 44 (0)1865 285002; Tel: + 44 (0) 1865 275 652

† Electronic supplementary information (ESI) available: Full experimental details, including ^{1}H, ^{13}C and ^{19}F NMR spectra for all new compounds, ESI–MS for all protein samples along with tryptic digest, SDS–PAGE analysis and radio-RP-HPLC traces. See DOI: 10.1039/c1cc13524d

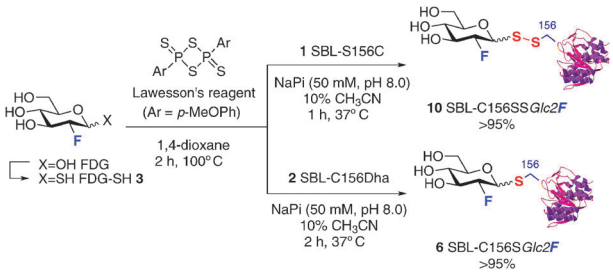


Scheme 2 Synthesis of *S*-linked 2-fluoroglycoproteins; reactions performed at 4 °C in NaPi buffer (50 mM, pH 8.0).

conjugate addition of Gal2F-β-SH **5** to SBL-156Dha **2**, only 65% conversion to the expected product **8** was observed together with an unwanted by-product (27057 Da, 35% conv.) (Scheme 2). Peptide mapping (tryptic digest and MALDI–TOF MS) of this by-product confirmed the incorporation of Gal2F into SBL-C156S*Gal2F* **8** and a thio-linked disaccharide into **9** (assumed to be 2FGalTal unit–see ESI† for details and mechanistic considerations for the formation of such a disaccharide). These results highlighted the suitability of **3** (and so FDG as a starting building block) and **4** as reagents and also revealed the unsuitability of **5** for precise labelling.

As well as being thoroughly structurally characterized, the functional integrity (and therefore biochemical utility) of thioether-linked 2Fglycoproteins **6** and **7** was also confirmed. All the modified proteins retained their inherent peptidase activity, as indicated by liberation of *p*-nitroaniline upon treatment with the chromogenic peptide suc-AAPF-*p*NA (see ESI†). In addition, **6** and **7** both proved to be active as reaction partners as acceptors during enzyme (endoglycosidase-A)-mediated glycosylations that allowed elaboration of the fluoro-sugar attachments into more complex oligosaccharides.[23]

Having gained key insight into the reaction conditions and preferred reagents necessary for the preparation of well-defined thioether-linked 2Fglycoproteins, we next turned our attention to the development of straightforward glycosylation strategies that would use $^{18/19}$FDG as a starting material and therefore, be relevant for a direct (radio)labelling approach. In this context, we considered the use of Lawesson's reagent (LR) for the thionation of 2-deoxy-2-fluoroglycopyranoses into their glycosyl thiol counterparts.[22] When [^{19}F]FDG was treated with LR for 2 h in 1,4-dioxane at 100 °C, conversion into the corresponding [^{19}F]FDG-SH **3** was observed; **3** was then reacted directly under optimized conditions (time, temperature, equivalents and co-solvent) with both SBL-S156C **1** and SBL-C156Dha **2**. Much to our delight,



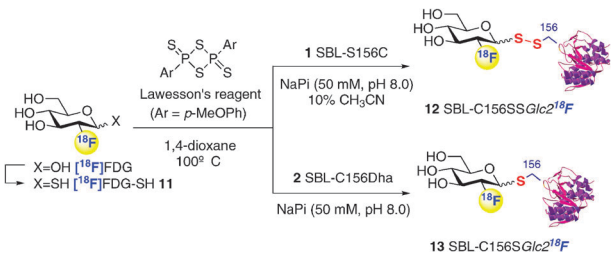
Scheme 3 Direct construction of *SS*- and *S*-linked 2-fluoroglycoproteins consisting of direct thionation of ^{19}FDG followed by site-specific conjugation to SBL-156Cys **1** and SBL-156Dha **2**, respectively.

>95% conversion to disulfide-linked 2Fglycoprotein **10** and thioether-linked 2Fglycoprotein **6** was observed by LC–MS (Scheme 3).

With a method in hand compatible with direct conversion of [^{19}F]FDG to [^{19}F]FDG-SH that can be used in a one-pot procedure to create disulfide- and thioether-linked 2Fglycoproteins, we decided to evaluate its feasibility for the radiolabelling of proteins. Radioactive [^{18}F]FDG-SH **11** was prepared directly from [^{18}F]FDG in >98% radiochemical conversion using Lawesson's reagent during 45 min (see ESI†).

Subsequent mixed disulfide formation with SBL-S156C **1** or conjugate addition to SBL-C156Dha **2** resulted in the first examples of proteins that have been directly site-specifically labelled with [^{18}F]FDG (Table 1). Overall this one-pot radiosynthetic procedure, including the preparation of the [^{18}F]-glycosylating reagent [^{18}F]FGlc-SH **11** and protein ligation, provided a radiochemical conversion of 55–60% (over two steps) after a total synthesis time of 90 min, which is suitable for effective use in PET imaging (^{18}F $t_{1/2}$ ~ 110 min).

Table 1 Construction of *SS*- and *S*-linked [^{18}F]-(fluoroglyco)-proteins[a]



Entry	Protein (tag)	Activity (MBq)	t^b/min	T/°C	Product	Conv.[c] (%)
1[d]	**1** (Cys)	5.0	90	37	**12**	60
2[d]	**1** (Cys)	5.0	15	37	**12**	43
3[d]	**1** (Cys)	3.8	15	RT	**12**	55
4[d]	**1** (Cys)	3.8	90	RT	**12**	48
5	**2** (Dha)	10.0	20	37	**13**	50
6	**2** (Dha)	10.0	105	37	**13**	57
7	**2** (Dha)	9.2	15	RT	**13**	60

[a] General conditions: crude **11** (3.8–10.0 MBq) from LR reaction (see ESI† for full details), proteins **1** and **2** in NaPi (50 mM, pH 8.2) unless otherwise indicated. [b] Protein reaction time. [c] Calculated for the one-pot procedure over two steps; determined by radio-RP-HPLC analysis and not corrected for decay. [d] 10% CH_3CN used as a co-solvent.

In summary, the use of 2-fluoro-1-thio-glycopyranoses with Cys and Dha-tagged proteins yielded the first examples of chemically defined disulfide- and thioether-linked (2-fluoroglyco)-proteins. This prompted us to develop a one-pot method for site-specific protein labelling at Cys and Dha consisting of direct thionation of [$^{18/19}$F]FDG followed by protein glyco-conjugation. While ^{19}F is an interesting label in its own right,[12,24] the introduction of ^{18}F is of high importance in nuclear medicine. Indeed, the conditions developed were fully translated to *hot* conditions and enabled the first examples of direct site-specific protein radiolabelling using [^{18}F]FDG as a building block. Given the ease of access to Cys-containing wildtype proteins or mutants in many systems coupled with the efficiency and reduced global time of synthesis this is a general, practical and useful strategy for producing ^{18}F-labelled proteins.

Here [^{18}F]FDG has effectively been used as a ready organic surrogate source of ^{18}F. Radiolabelled synthetic (glyco)-proteins are particularly rare[12,25–27] and represent potentially powerful tools for *in vivo* glycobiology. Thus, methods for the site-specific incorporation of sugars into proteins[28–30] might also provide a near unique opportunity to also introduce $^{18/19}$F labels at a defined amino acid residue that can be used to obtain unique information regarding the molecular basis underlying important carbohydrate-protein interactions by NMR, MRI and PET.[24,31–35] For example, the ability to introduce sugars to create radiolabelled (Fglyco)proteins[12] can be potentially coupled with the ability to readily transform such fluoroglycoproteins into more elaborate glycans.[23] This in turn might yield radiolabelled glycoproteins in which the glycan is both label and determinant of biological function.

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