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Bioorthogonal labelling of 3-nitrotyrosine in peptides and proteins through diazotisation mediated azidation†

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A new chemical derivatisation method to functionalise peptide and protein bound 3-nitrotyrosine residues is described. By transforming 3-nitrotyrosine to 3-azidotyrosine, the method enables 'click chemistry'-based labelling of the 3-nitrotyrosine residues and provides new opportunities for the study of this post-translational modification in biological samples.

Formation of 3-nitrotyrosine from tyrosine residues is a post-translational modification detected under a range of pathological conditions[1-3] and it is become increasingly clear that this modification may be capable of altering protein function and playing a role in disease[4-6]. Nevertheless, studies of protein 3-nitrotyrosine face numerous technical challenges. Concentrations of 3-nitrotyrosines are typically low, in the range of nM to low μM in biological samples[7-11]. Furthermore, they are generally found to form disproportionately to the levels of protein abundance[12]. This confounds the capacity limits and dynamic range of standard mass spectrometry (MS) analysis. To circumvent these challenges antibody based enrichment of 3-nitrotyrosines have come to the fore with significant advances, however antibody approaches possess their own limitations including difficulties in targeting buried sites in proteins, tyrosine site biases and inherent irreproducibility of different batches of antibodies. Methods in the field of chemical derivatisation have had some success in enriching and labelling 3-nitrotyrosines for MS-based protein detection. The combined fractional diagonal chromatography (COFRADIC) method identifies 3-nitrotyrosine containing peptides by taking advantage of the hydrophobic shift of nitrated peptides after treatment with a reducing agent in reverse phase chromatography[13]. A second category of methods also converts 3-nitrotyrosine into 3-aminotyrosine containing peptides but subsequently biotinylates the aromatic amine by either taking advantage of the high pI of aromatic amines[14] or by initial blocking of all other primary amines[15, 16]. Although the methods have shown to be able to identify nitrated proteins and also enable site specific identification of 3-nitrotyrosines, the methods have only had limited success for the detection of endogenously nitrated proteins.

The aromatic nitro functional group of 3-nitrotyrosine or its reduced form 3-aminotyrosine is not known to be present on other biomolecules in living cells and as such, presents an opportunity for direct bioorthogonal targeting of this post-translational modification. Diazotisation is a chemical reaction that functionalises aromatic amines. Importantly, azido groups can be derived from diazonium intermediates. X-ray crystal structure analysis indicated that the azide ion acts as a strong nucleophile to that attack the β nitrogen, to form pentazole and pentazene intermediates which ultimately undergo cleavage of nitrogen to form the final azido-aromatic structure[17].

The synthesis of the 3-azidotyrosine amino acid by azidation of 3-aminotyrosine has been previously described[18]. In the study, traditional conditions of creating the diazonium intermediate were performed (i.e. mineral acid, sodium nitrite, 0^oC) then NaN_3 was added in darkness and incubated for 1 hour at 0^oC, in aqueous solution. While, the study illustrated the feasibility of synthesising 3-azidotyrosine, derivatisation was limited to the amino acid and did not investigate the potential applicability of azidation of the tyrosine residue in the context of functionalisation of the 3-nitrotyrosine post-translational modification for protein analysis. Significantly, the transformation of the nitro-group to a 'click chemistry' compatible bioorthogonal azide group on 3-nitrotyrosine residues opens up a myriad of chemical reporters that can be used for its detection.

In this study, we demonstrate that 3-nitrotyrosine in peptides and proteins can be transformed to 3-azidotyrosine through a two-step reduction and diazotization mediated azidation reaction. A schematic diagram showing the series of reactions to derivatise 3-nitrotyrosine to 3-azidotyrosine for subsequent 'click chemistry' reactions is shown in Scheme 1.

Angiotensin I (DRVYIHPFHL) was used as a model peptide to demonstrate the derivatisation method. Angiotensin I is a well characterised and stable peptide containing a single tyrosine (Y4), arginine (R2), phenylalanine (F8), two histidine residues (H6/H9), and an N-terminal amine. Together it allows the examination of the

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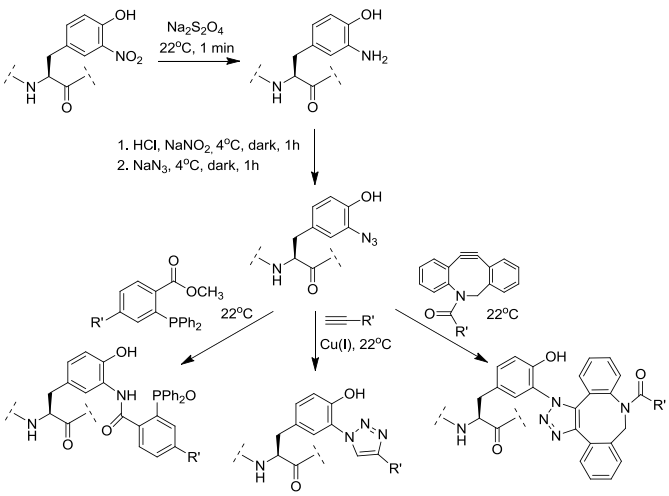

Scheme 1 Conversion of 3-nitrotyrosine to 3-azidotyrosine and subsequent labelling by 'click chemistry' type reactions.

specificity of the derivatisation reaction to tyrosine as well as observation of any reactivity towards other reactive side chains.

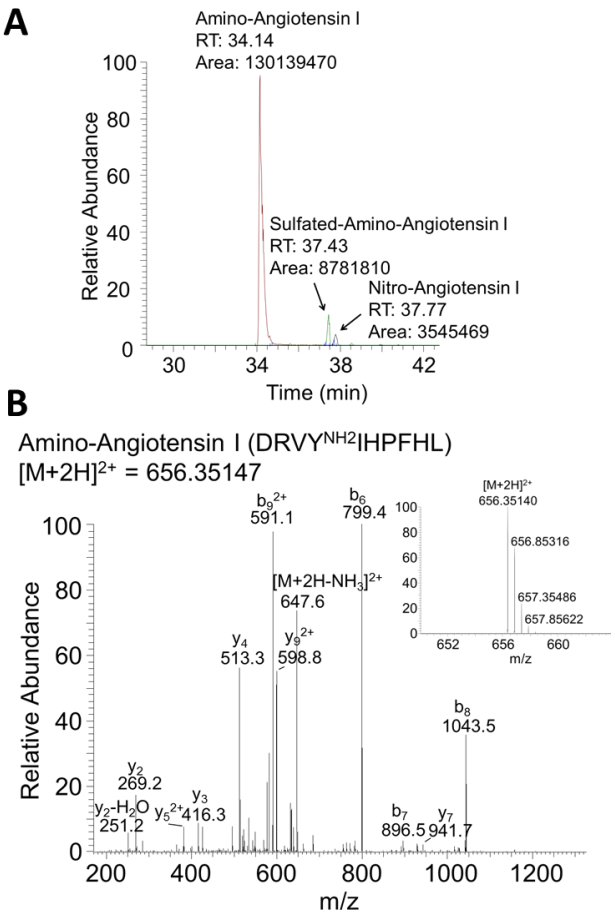

Fig 1 (A) Extracted ion chromatograms showing amino-angiotensin I (red), sulphated amino-angiotensin I (green) and nitro-angiotensin I (black) with retention time and area under chromatograms for each peptide. (B) Tandem mass spectrum for amino-angiotensin I. The accurate mass spectrum of the doubly charge ion of the intact peptide is shown in the inset.

Detail description of the experimental procedure is described in the ESI†. Briefly, nitrated angiotensin I was first generated using the heme-catalysed nitration method as previously described[19]. The nitrated sample was analysed using LC-MS/MS to validate that nitro-angiotensin I has been successfully synthesised. To estimate the reaction yield, the area under the extracted ion chromatograms of unmodified angiotensin I and nitrated angiotensin I was used and the yield of nitrated peptide was estimated to be 33% (Suppl Fig 1A). Even though the ionisation efficiency of the different modified forms of angiotensin I would vary, comparing extracted ion chromatograms should still provide a reasonable estimate of the yield since the basic N-terminal amine, arginine and histidine are common between all peptides would contribute substantially to the ionisation efficiency of each peptide. The identity of nitrated angiotensin I was confirmed by accurate mass and MS/MS and compared with unmodified angiotensin I (Suppl Fig 1B).

Having synthesised nitrated angiotensin I, the nitrated peptide was subsequently reduced to amino-angiotensin I using $Na_2S_2O_4$. The reduction is necessary to generate an aromatic amine for diazotisation. Using LC-MS/MS, the amount of reduction was quantified based on the extracted ion chromatograms of nitro-angiotensin I, amino-angiotensin I and sulphated amino-angiotensin

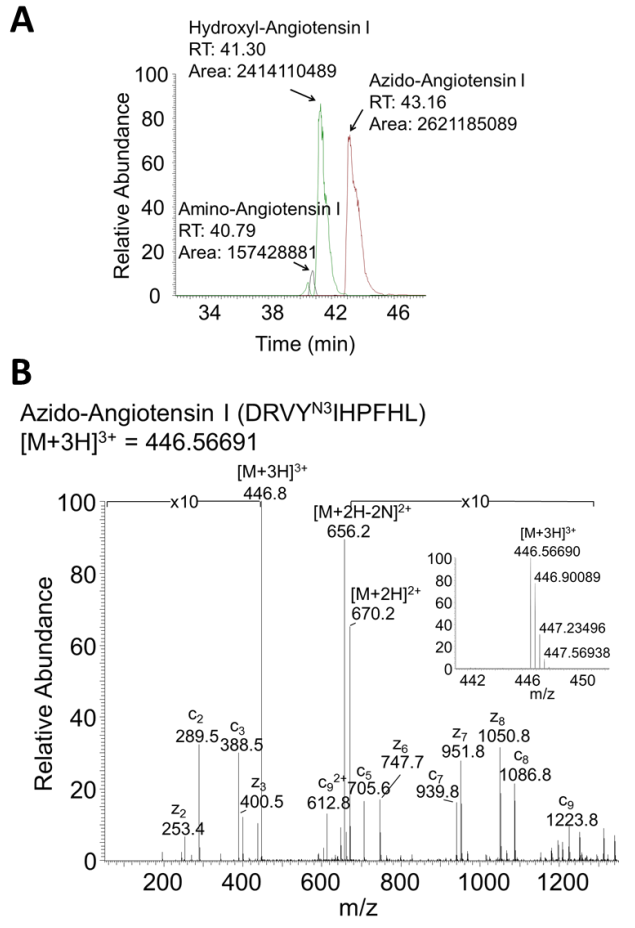

Fig 2 (A) Extracted ion chromatograms showing amino-angiotensin I (black), hydroxyl-angiotensin I (green) and azido-angiotensin I (red) with retention time and area under chromatograms for each peptide. (B) Tandem mass spectrum for amino-angiotensin I generated using electron transfer dissociation (ETD). The accurate mass spectrum of the triply charge ion of the intact peptide is shown in the inset.

I (Fig 1A). Sulfation of tyrosine is a common side product of nitrotyrosine reduction by $Na_2S_2O_4$[13]. The yield of amino-angiotensin I was estimated as the area under chromatogram for amino-angiotensin I over the sum of nitro-angiotensin I, amino-angiotensin I and sulphated amino-angiotensin I and was found to be 91.3%. The tandem mass spectrum for confirming the identity of amino-angiotensin I is shown in Fig 1B.

Once amino-angiotensin I has been synthesised, the peptide was diazotised in darkness under cold acidic conditions in the presence of $NaNO_2$. To synthesis azido-angiotensin I, NaN_3 was added and the solution was kept at on ice for 1 hour before allowing to come to room temperature. The products of the azidation of amino-angiotensin I was again examined by LC-MS/MS. Quantification of the final yield for azido-angiotensin I was estimated by dividing the abundance of azido-angiotensin I by the sum of azido-, amino- and hydroxyl-angiotensin I and was found to be 50.5% (Fig 2A). Hydroxylation is a common side reaction of the diazotisation reactions as a result of the spontaneous decomposition of the diazonium at elevated temperatures. Using the aminotyrosine amino acid, it was found that hydroxylation could be reduced by increasing acid concentrations (Suppl Fig 2). However, as the high concentrations of mineral acids can lead to protein precipitation, therefore lower acid concentration was preferred. An alternative method may be to carry out the reaction within a continuous flow micro-reactor which has been shown to significantly reduce hydroxylation of diazonium ions during azo-coupling[20, 21].

The identity of azido-angiotensin I was further confirmed by MS/MS, however it was found that collision induced dissociation (CID) was ineffective in completely fragmenting azide-containing peptides and resulted in only the loss of two nitrogens in a peptide mass, likely from the azido group (Suppl Fig 3). To overcome the limitation of CID, electron transfer dissociation (ETD) was applied to the same sample. ETD works by transferring electrons on to peptides which typically induces cleavage along the peptide backbone (resulting in c and z-ions) while leaving side-chains and modifications intact[22]. Based on the tandem mass spectrum, it is evident that ETD effectively fragments azide containing peptides enabling the azido-angiotensin I to be conclusively identified (Fig 2B).

Since the diazotisation of peptides have not been thoroughly analysed previously, in order to determined other possible side products from the azidation reaction on the angiotensin I peptide, we extracted ion chromatograms for all other well established reactions that can result from diazotisation including reactions with primary and secondary amines present on angiotensin I (Suppl Table 1 for a list of products examined). Of all possible products, there was only evidence for the nitrosamine formation from the secondary amino from arginine (Suppl Fig 4). Even so, the abundance of this product was very low (Suppl Fig 4A) and could only be identified by accurate intact peptide mass as the peptide ion was not selected for MS/MS analysis by the instrument. In fact the abundance of the peptide was less than 0.1% of that of the desired azido-angiotensin I product. This suggests that the diazotisation reaction is highly specific for aromatic amines even in the presence of primary and secondary amines.

The transformation of 3-nitrotyrosine to 3-azidotyrosine opens up the possibility for nitrated peptides/proteins to be conjugated to a wide range of compounds. To demonstrate that 'click chemistry' can be applied to azido-angiotensin I, the azide-alkyne Huisgen cycloaddition[23, 24] was performed to conjugate alkyne biotin (PEG4 carboxamide-Propargyl Biotin) (Suppl Fig 5A) to azido-angiotensin I. LC-MS/MS was used to analyse the products of the reaction. Extracted ion chromatograms were calculated for the azido-angiotensin I and biotin-angiotensin I (Suppl Fig 5B). A peak was found for biotin-angiotensin I (red line), but azido-angiotensin I could not be detected (black line), suggesting that virtually all azido-angiotensin I had been biotinylated. This is consistent with the high yields known of 'click chemistry' reactions. Furthermore, the tandem mass spectrum of biotin-angiotensin I was analysed which validated that biotin-angiotensin I had indeed been synthesised (Suppl Fig 5C).

Since nitrotyrosine naturally occurs at low μM to nM concentrations in mammalian samples, to ensure that the reaction can feasibly be performed at such concentrations, a sample of nitro-angiotensin I was prepared at ~1:200 abundance ratio relative to unmodified angiotensin I based on LC-MS analysis. As the total angiotensin I concentration was 1 mg/mL, the concentration of nitro-angiotensin I would be ~4 uM (Suppl Figure 6A). The series of reactions was performed as described above except that copper-free 'click' reagent Dibenzylcyclooctyne-SS-PEG11-biotin (DBCO-biotin) was used in place of alkyne biotin. The products at each reaction step was analysed by LC-MS (Suppl Figure 6B-D). In each case, amino-, azido- and DBCO-S-angiotensin I could be clearly detected. Even though it is not possible to quantify reaction yield due to the different ionisation efficiencies of the reaction products, this nevertheless demonstrates that the derivatisation procedure is in theory applicable to low μM nitrotyrosine concentrations necessary

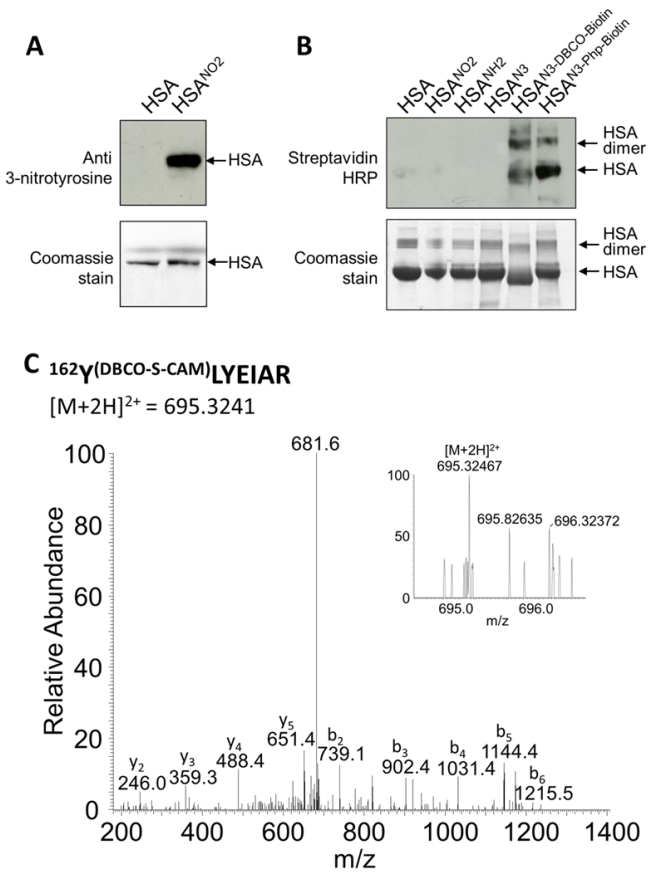

Fig 3 (A) Western blot and coomassie stained gel showing nitration of human serum albumin (HSA). (B) Western blot and coomassie stained gel showing the biotinylation of nitrated HSA using Phosphine-PEG3-Biotin (PhP-Biotin) and Dibenzylcyclooctyne-SS-PEG11-Biotin (DBCO-Biotin). (C) MS/MS of HSA peptide ^{162}YLYEIAR with evidence for DBCO-Biotinylation at Y162. The accurate MS1 spectrum is shown in the inset. CAM, carbamidomethyl.

in biological samples.

Having demonstrated the derivatisation procedure at the peptide level, the method was then applied to study a full length protein. The derivatization conditions were largely unchanged from that used for angiotensin I (see Supporting Information for specific details). The major difference is that instead of using solid phase extraction for buffer exchange between reactions, dialysis of the sample was necessary to reduce protein loss. Furthermore, the two alternative 'click'-based biotinylation agents, phosphine-PEG3-biotin (Php-biotin) and DBCO-biotin were used as they are more convenient than copper-dependent alkyne-azide coupling and also have larger linkers to reduce steric hindrance in biological systems (Suppl Fig 6).

The derivatisation was performed on purified human serum albumin (HSA). HSA is a 66 kDa protein with the mature form containing 18 tyrosine residues. HSA was first nitrated using the heme-dependent nitration method. Having confirmed that nitrated HSA^{NO2} is present (Fig 3A), the derivatisation procedure was performed on the HSA^{NO2}. At each step, the product was resolved by SDS-PAGE and stained by coomassie or transferred for western-blot analysis against streptavidin horse-radish peroxidase (HRP). It is evident that azido-HSA (HSA^{N3}) when incubated with either DBCO-biotin or Php-biotin gave a strong band at the expected mass for HSA (Fig 3B). Importantly, based on the coomassie stained gel, HSA did not appear to be significantly aggregated or degrade throughout the derivatisation procedure (Fig 3B).

To further validate that that nitrated HSA has been biotinylated at nitrated residues, mass spectrometry analysis was performed on a tryptic digest of the nitrated HSA sample derivatised and biotinylated using DBCO-biotin. The DBCO-biotin contains a disulphide linker that is cleaved and alkylated by iodoacetamide during the protein preparation procedure for tryptic digestion (see Suppl methods). The release of the linker and biotin reduces the size of the modification, making the peptide with the modification more readily detectable by MS. In total, 45 unique peptides were identified covering 68% of the protein (Suppl Table 2). Of these peptides, a single tyrosine (Y162) was found labelled with the reduced and alkylated form of DBCO-biotin (DBCO-S-CAM).

To our knowledge, this is the first time that the transformation of 3-nitrotyrosine to 3-azidotyrosine has been described at the peptide and protein level. In the context of proteins, diazotisation has previously been used to label aromatic amino acids including tyrosine through azo-coupling with aromatic amine containing small molecules[25, 26]. Together with our study, these studies demonstrate the feasibility of using diazotisation to derivatise proteins. The novel aspect of this study is that the diazotisation reaction itself occurs on an endogenous peptide/protein side-chain. This provides a way to introduce the bioorthogonal azide functional group into 3-nitrotyrosine containing peptides and proteins. The method described potentially opens up the analysis of the 3-nitrotyrosine post-translational modification by enabling its study by a myriad of click-chemistry based probes. For instance, with further development within complex biological mixtures, the technique has the potential to offer a more direct and specific method for enrichment-based characterisation of the 3-nitrotyrosine-proteome by MS analysis.

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