A protein functionalization platform based on selective reactions at methionine residues

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Nature has a remarkable ability to carry out site-selective post-translational modification of proteins, therefore enabling a marked increase in their functional diversity¹. Inspired by this, chemical tools have been developed for the synthetic manipulation of protein structure and function, and have become essential to the continued advancement of chemical biology, molecular biology and medicine. However, the number of chemical transformations that are suitable for effective protein functionalization is limited, because the stringent demands inherent to biological systems preclude the applicability of many potential processes². These chemical transformations often need to be selective at a single site on a protein, proceed with very fast reaction rates, operate under biologically ambient conditions and should provide homogeneous products with near-perfect conversion²-⁷. Although many bioconjugation methods exist at cysteine, lysine and tyrosine, a method targeting a less-explored amino acid would considerably expand the protein functionalization toolbox. Here we report the development of a multifaceted approach to protein functionalization based on chemoselective labelling at methionine residues. By exploiting the electrophilic reactivity of a bespoke hypervalent iodine reagent, the S-Me group in the side chain of methionine can be targeted. The bioconjugation reaction is fast, selective, operates at low-micromolar concentrations and is complementary to existing bioconjugation strategies. Moreover, it produces a protein conjugate that is itself a high-energy intermediate with reactive properties and can serve as a platform for the development of secondary, visible-light-mediated bioorthogonal protein functionalization processes. The merger of these approaches provides a versatile platform for the development of distinct transformations that deliver information-rich protein conjugates directly from the native biomacromolecules.

The sheer structural diversity of the proteome in any single organism means that no one protein functionalization method is likely to provide a universal solution for the preparation of protein constructs⁸-¹¹ (Fig. 1a). Although encoded by the AUG start codon at the beginning of protein synthesis, methionine is often post-translationally excised and thus has a low abundance in proteins (around 2%). It is also frequently used as a replacement for hydrocarbon-containing residues¹². The limited function of methionine, being mainly responsible for protection against oxidative stress, compared to other residues means that its functionalization is less likely to impair protein function¹³. Targeting methionine would not only provide a distinct bioconjugation approach but also, using our strategy, the resulting methionine conjugates would provide exploitable intrinsic reactivity such that they could lead to the rapid synthesis of diverse, functional protein constructs from native proteins.

So far, there has been only one effective method reported for bioconjugation at methionine, in which the oxidation of thioethers with ozonide reagents provided the basis for an elegant bio-inspired strategy to form stable protein-bound sulfoximines¹⁴. We aimed to target the polarizable thioether on the methionine side chain with a suitable electrophile to form a cationic sulfonium species, selectively installing a versatile payload and distinct functionality at a methionine residue, thereby providing a fundamentally different bioconjugation approach⁸-¹¹ (Fig. 1a). Methionine residues react with cyanogen bromide (CNBr); however, such a process cannot function as a bioconjugation method because the instability of the resulting cyanosulfonylamine cation triggers cleavage of the protein backbone¹⁵. Conversely, methionine residues undergo slow S-alkylation reactions with iodoacetamide or with other benzyl-derived electrophiles to form relatively stable trialkyl sulfonium cations¹⁵-¹⁷. The need to strike a balance between the stability of the protein-bound sulfonium cations, the compatibility of the reaction conditions, and the reaction rate of the thioether with suitable electrophiles has, so far, precluded the development of an effective alkylation-based method for bioconjugation at methionine. Guided by these limitations, we posited that a distinct class of electrophile, based on the hypervalent iodine scaffold of λ⁵-iodanes, could make for a functioning bioconjugation process at methionine. Tailoring the substituents and counteranion on the I(iii) atom should enable us to tune both the reactivity of the polarizable I(iii) nodal centre, to dovetail with the electron lone pair of the thioether, and also the stability of the resulting sulfonium conjugate, through modulation of the electronic features of the groups directly attached to the cationic sulfur motif. We noted that a structurally remarkable iodonium salt (1 in Fig. 1, R = Et and X = OTf) reacts rapidly with dimethylsulfide (the simplest possible mimic of the thioether motif in methionine) to form a sulfonium adduct. Successful reaction of this iodonium salt with methionine would not only represent a distinct method for bioconjugation, but also deliver a high-energy conjugate equipped with reactive ‘on-protein’ groups that could serve as a basis for designing new transformations towards protein constructs with diverse functionality (Fig. 1b).

We first examined the reaction between dipeptide 2a and iodonium triflate 1a (Fig. 2a). We observed the formation of the desired sulfonium conjugate 3a, although it was clear from the low yield (27%) that the iodonium salt was poorly stable in aqueous solution. By tailoring the aryl group of the iodonium salt (to the electron-deficient 2,4-difluorobenzene) and replacing the triflate counteranion with tetrafluoroborate, we found that a readily prepared reagent 1b displayed superior physical properties (half-life in water is >50 h). Treatment of reagent 1b with dipeptide 2a gave 72% of the desired product 3b accompanied by the corresponding sulfoxide (not shown) after reaction for 30 min.

Moving to a more complex substrate, the GLP-1 receptor agonist exenatide (Byetta, 2b, a 39-residue helical polypeptide containing a single mid-chain methionine, Fig. 2b), we found that treatment of a 100 µM aqueous solution with 1b led to decomposition of the polypeptide. A key breakthrough revealed that the addition of a low concentration of thiourea (20 mM) resulted in a substantial improvement of the reaction, such that sulfonium conjugate 4a was formed with 68% conversion in less than 2 min, accompanied by non-specific oxidation and labelling. Further improvements could be made by performing the reaction in the presence of TEMPO ((2,2,6,6-tetramethylpiperidine-1-yl)oxyl, 10 mM), which minimized...
the formation of oxidative by-products, and adding aqueous formic acid solution (5 mM, approximately pH 3), which reduced the formation of non-specifically labelled by-products to trace levels. Finally, we found that the labelling process proceeded effectively when conducted in distilled water. Routine analysis of electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) fragmentation data confirmed selective reaction at methionine and, although the concentration of thiourea is well below the levels needed for protein denaturation, it was confirmed by circular dichroism spectra of exenatide conjugate 4a that the characteristic helical structure was retained (see Supplementary Fig. 10). Although we are not yet certain of the role of thiourea, it is important to note that its presence appears to be fundamental in providing a bioconjugation process at reaction rates needed for transformation on complex biomolecules.

Fig. 1 | The development of a methionine-selective protein functionalization strategy. a, Existing protein functionalization strategy, and the potential for methionine-selective bioconjugation. R = peptide or protein; R’ = various organic groups; R” = aryl or ester group. b, Functionalized hypervalent iodine reagents enable methionine-selective protein modification, leading to methionine-based bioorthogonal protein diversification. X = leaving group; R3 = functional payload.

Fig. 2 | Evolution of a methionine-selective bioconjugation strategy. a, Initial results for functionalization at methionine with hypervalent iodine reagents. b, Optimal process for the thiourea-accelerated methionine-selective bioconjugation of exenatide.
5–500 µM without compromising the conversion, and a larger, milligram-scale reaction enabled the purification of conjugate 4a by semi-preparative high-performance liquid chromatography to give a 79% yield of isolated product. Given the similarity of the exenatide conjugate to the intermediate that would arise from reaction of the polypeptide with cyanogen bromide (leading to peptide cleavage),
it is remarkable that 4a has a half-life in water of over 100 h. We believe that the observed stability of 4a is a result of the ethyl diazoacetate motif imparting a lesser electron-withdrawing effect (than the cyano group) on the sulfonium salt, which in turn leads to a species that is less reactive to attack by the proximal carbonyl group and is, therefore, a more stable conjugate.

The optimized conditions, using 1b, were used to evaluate the substrate scope (Fig. 3a). Random coil polypeptides that contain methionine, such as aviptadil and tetracosactide, are efficiently converted to the sulfonium conjugates 5 and 6 respectively. GTP-binding protein fragment Goα, which contains a free sulfhydryl group in an N-terminal cysteine residue, underwent smooth methionine labelling to 7 with concomitant oxidative disulfide formation. Teriparatide, a polypeptide containing two methionines, formed bis-sulfonium conjugate (8) with good conversions. α-Lactalbumin, a globular protein with a readily oxidized methionine residue,
undergoes bioconjugation to 9 with minimal competitive oxidation. Aprotinin (Trasylol) also forms the corresponding sulfonium conjugate 10 in good conversion to product. Particularly notable is the observation that two cysteine disulfide linkages within the structure of aprotinin (and the labelling to 7) are not affected by 1b, with a single methionine-derived conjugate obtained in high conversion. When the methionine residues are buried within the tertiary structure of the protein, for example with RNA-ase B, the bioconjugation does not occur, highlighting the inherent selectivity of the labelling process for exposed rather than inaccessible thioether groups. This feature is highlighted by the case of ubiquitin: the N-terminal methionine residue has only moderate surface exposure and can slow down the rate of functionalization to the point where oxidation becomes competitive. Accordingly, reaction under deoxygenated conditions enabled efficient conversion to the labelled product (84%), with a 10:1 label:oxidation ratio (11). The methionine-selective bioconjugation strategy effectively functionalizes a range of polypeptides and proteins in high conversion at micromolar concentrations and in very short reaction times. Given that 1b is a carbon electrophile with two of the best leaving groups known to organic chemists, it would be thought to be very reactive; it is therefore remarkable that this species selectively engages the moderately nucleophilic methionine residue in the presence of competitively nucleophilic and oxidizable amino acid residues. Bioconjugation strategies that target other amino acids should, therefore, be compatible with, and complementary to, our methionine-functionalization process. To exemplify this, we showed that the GTP-binding protein fragment Gαδ could be first labelled at cysteine, using a maleimide derivative13, and then conjugated at methionine using iodonium salt 1b to form 12. The methionine-selective process does not interfere with the cysteine–maleimide motif, which itself contains a thioether linkage, thereby highlighting possible applications towards multi-site hetrolabelling of proteins14.

The modular synthesis of the hypervalent iodine reagents enables facile incorporation of different acyl groups attached to the diazo motif, allowing the transfer of a range of functional payloads to proteins (Fig. 3b). Functional groups relevant to other bioorthogonal reactions are readily tolerated in both the reagent synthesis and the methionine labelling, smoothly affording sulfonium conjugates 4b and 4c with 95% and 85% conversion to product, respectively. Biochemical reporter groups, such as myristyl- and fluorescein-derived esters 4d and 4e, are also readily transferred to exenatide. Notably, we found that sulfonium conjugates of exenatide (such as 4f) underwent reaction with the tertiary phosphate TCEP (tri(2-carboxyethyl)phosphine), a standard biochemical reagent, resulting in the cleavage of the labelling group and return of the parent exenatide 2b in >90% conversion25 (Fig. 3c); the cleavage reaction also works for conjugates 5, 6 and 8 in comparable conversions and provides a stimulus-responsive means of removing the methionine label.

Next, we turned our attention to exploring the multifaceted reactivity that we anticipated would be intrinsic to the high-energy methionine-derived conjugate. The electrophilicity of the diazo sulfonium conjugate 4 prompted us to investigate the single-electron transfer chemistry of this reactive motif enabled by visible-light photocatalysis26,27. The addition of a single electron to the diazo sulfonium conjugate 4 could result in intermediate 15, which, upon cleavage of the C–Nδ bond, would form a putative radical ylide synthon 15* (Fig. 4a). We visualized two pathways through which we could exploit the reactivity of the previously unexplored radical ylide. First, combining the radical ylide with Hantzsch ester 13* (from 13) may lead to a reduction process resulting in the generation of a trialkylsulfonium motif, which would impart enhanced chemical stability to the protein conjugate. Furthermore, the use of a C–4 benzylated Hantzsch ester derivative (14, an established precursor for a benzyl radical)28 to intercept the radical ylide species would lead to a C-benzylation product that could be used to introduce functional diversity to the protein conjugate. We screened a range of photocatalysts under visible-light conditions. When 3c was irradiated with a 30 W lamp in the presence of fac-1r(ppy)2 (ppy, 2-phenylpyridinato) and the Hantzsch ester 1329,30, we observed the formation of the reduced trialkysulfonium product 17a in 95% yield (determined by 1H NMR, Fig. 4b). Using these conditions, we showed that a range of sulfonium–protein conjugates, including exenatide, glucagon and thiorodoxin derivatives, are reduced to stable trialkysulfonium species with excellent conversions (17b–e, see Supplementary Information). Notably, reduction of a thiorodoxin derivative31 to its trialkysulfonium–protein congener 17e proceeds in high conversion without affecting its labile disulfide linkage, which serves to highlight the mild nature of this protocol. Additionally, the methionine bioconjugation and photoreduction steps can be carried out in a one-pot operation, which considerably simplifies the overall process without compromising the yield or purity of the trialkysulfonium product.

In testing the viability of the proposed C-benzylation using a modified Hantzsch ester derivative29, we found that treatment of the exenatide–sulfonium conjugate 4b with o-tolyl Hantzsch ester derivative 14a, under slightly modified photocatalytic conditions, led to cross-coupling and the formation of the C-ligation product 18a in high conversion. This unique bioorthogonal protein functionalization reaction not only represents a synthetic radical–radical cross-coupling using a polypeptide scaffold, but also provides a platform for bioorthogonal protein diversification wherein two distinct functionalities could be introduced sequentially at the same amino acid residue.

In summary, through the merger of methionine-selective bioconjugation and a new visible-light-mediated photocatalytic reaction platform, information-rich synthetic constructs can be rapidly assembled by a two-step protocol directly from native proteins. The reactivity inherent to the methionine conjugate distinguishes the bioconjugation process from other methods. Moreover, the capacity for functional diversification, by tailoring the hypervalent iodine reagent and modified Hantzsch-ester derivative, means that highly functional protein conjugates could be made readily available directly from native proteins.

Data availability
The data that support the findings of this study are available within the paper and its Supplementary Information. Raw data are available from the corresponding author on reasonable request.

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