Chemical Science

EDGE ARTICLE

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Cite this: Chem. Sci., 2021, 12, 14159

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Late-stage modification of peptides and proteins at cysteine with diaryliodonium salts[†]

Stephen A. Byrne, Dab Max J. Bedding, Dab Leo Corcilius, ab Daniel J. Ford, ab Yichen Zhong,^c Charlotte Franck,^{abc} Mark Larance,^{cd} Joel P. Mackay ^{bc} and Richard J. Payne (D *ab

The modification of peptides and proteins has emerged as a powerful means to efficiently prepare high value bioconjugates for a range of applications in chemical biology and for the development of nextgeneration therapeutics. Herein, we report a novel method for the chemoselective late-stage modification of peptides and proteins at cysteine in aqueous buffer with suitably functionalised diaryliodonium salts, furnishing stable thioether-linked synthetic conjugates. The power of this new platform is showcased through the late-stage modification of the affibody zEGFR and the histone protein H2A.

Received 9th June 2021 Accepted 21st September 2021

DOI: 10.1039/d1sc03127a

rsc li/chemical-science

Introduction

Peptides and proteins occupy a central role as the functional biomolecules that drive most processes in living systems. Following ribosomal synthesis, additional molecular complexity is added to peptides and proteins through posttranslational modifications (PTMs) that serve to expand and diversify the functionality possible from the 20 proteinogenic amino acids.^{1,2} These PTMs range from the alkylations and acylations that drive gene expression, to the addition of large glycan and lipid chains - many of which underpin crucial protein-protein interactions and therefore endow additional functionality to their cognate proteins.²⁻⁴ Furthermore, a large number of therapeutics known as 'biologics',5-9 including antibody-drug conjugates (ADCs), rely on the functionalisation of peptides and proteins with distinct entities.¹⁰ The importance of protein modifications in biology and the pharmaceutical/ biotechnological industry has led to the search for new methods that can be used for efficient access to modified peptides and proteins in homogeneous form (either with PTMs or unnatural 'designer' modifications, e.g. PEGylation)11 from fully unprotected peptides and proteins.

To date, a number of so called 'bioconjugation' methods have been developed that enable site-selective functionalisation

of peptides and proteins.12 Necessitating high degrees of chemo- and regio-selectivity in order to generate homogeneous protein conjugates, the development of such methods has focussed on two overarching approaches: (1) the incorporation of non-proteinogenic amino acids or engineered peptide sequences which take advantage of unique chemical (bioorthogonal)13 or chemoenzymatic reactivity,14 and (2) the utilisation of selective chemical reactions at proteinogenic amino acids.15 While both approaches have been successfully executed for the synthesis of peptide and protein conjugates, targeting native amino acids for late-stage modification provides a more operationally simple approach. However, this remains a challenging endeavour given that the reaction must be selective in the presence of a plethora of functionality that decorate the side chains of proteins, and reaction conditions must be mild and biocompatible (i.e. aqueous solvent, pH 6-8, temperatures \leq 37 °C).

Cysteine (Cys) represents an attractive amino acid for the development of functionalisation chemistry due to the unique nucleophilicity of its thiol side chain. This provides the potential for chemoselectivity,16 while the relatively low natural abundance of Cys in proteins (ca. 1.8%)17 provides an avenue for enhanced regioselectivity.¹⁸ Maleimide reagents are frequently employed to covalently modify Cys residues both in academic and industrial settings. Notably this chemistry is employed for the functionalisation of monoclonal antibodies with cytotoxic payloads (e.g. brentuximab vedotin, also known as Adcetris®).10 However, the resulting conjugates can suffer from poor stability due to retro-Michael reactivity following conjugation.19

This drawback has prompted the exploration of alternative chemistry that could be used to generate stable Cys conjugates chemoselectively. While several elegant Cys conjugation methods have been reported recently, including a number of



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[&]quot;School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia. E-mail: richard.payne@sydney.edu.au

^bAustralian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Sydney, Sydney, NSW 2006, Australia

School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia

^dCharles Perkins Centre, The University of Sydney, NSW 2006, Australia

[†] Electronic supplementary information (ESI) available. See DOI: 10.1039/d1sc03127a

(a) SnAr transformations



Scheme 1 Previously reported (a) nucleophilic aromatic substitution (SnAr) and (b) oxidative addition of transition metal complexes for Cys arylation on peptides and proteins; R = functional cargo; X = halide or pseudohalide; (c) proposed use of diaryliodonium salts for direct arylation of Cys to generate thioether-linked peptide and protein conjugates described in this work.

novel Cys arylation methodologies (Scheme 1a and b),²⁰ these approaches still suffer from modest chemoselectivity,²¹ the need for highly specific peptide sequences,²² or potential problems with biocompatibility owing to the use of transition metal reagents.^{23,24}

Hypervalent iodine chemistry has recently emerged as an attractive means to modify peptides and proteins.²⁵⁻³⁰ A report from Kirkwood et al. in 1947 that diphenyliodonium chloride reacts with Cys in boiling water to produce phenylcysteine³¹ provided an encouraging proof of principle that this class of reagents could be used for direct functionalisation of peptides and proteins through the thiol side chain of Cys residues. Furthermore the air- and moisture-stability of hypervalent iodine reagents,³² coupled with their simple access from cheap and readily available aryl iodide and aryl boronate precursors³³ led us to propose their application in the development of a platform for the chemoselective arylation of peptides and proteins at Cys (Scheme 1c). The proposed mechanism of arylation involves initial nucleophilic attack of the electrophilic iodine(III) centre by the thiolate of Cys to afford a 3-centre 4electron hypervalent bond, followed by reductive elimination to



Scheme 2 (a) Arylation of model peptide 2 at Cys with 1 to generate aryl conjugate 3; (b) monitoring of arylation reaction by analytical UPLC; (c) HR-ESI-MS of conjugate 3 and confirmation of Cys chemoselectivity through HMBC.

release an aryl iodide and generate the target thioether linkage (Scheme 1c).³⁴ Herein, we report the development of diaryliodonium salts as reagents for the late-stage modification of peptides and proteins at Cys. We demonstrate the power of the methodology *via* the chemoselective functionalisation of the mucin 1 variable number tandem repeat (MUC1 VNTR), an affibody to the epidermal growth factor receptor Ac-Cys- $Z_{EGFR;1907}$ (zEGFR),³⁵ and the protein histone 2A (H2A).³⁶

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Results and discussion

The development of the direct Cys arylation chemistry began with the synthesis of a suitable diaryliodonium salt (1) that could be used to optimise the proposed chemistry on a model peptide system (Scheme 2). To this end, we chose to adopt a one-pot procedure reported by Olofsson et al.37 that uses readily available aryl iodide and aryl boronic acid precursors to synthesise the bis(4-trifluoromethylphenyl)iodonium tetrafluoroborate salt 1 (Scheme 2, see ESI[†] for synthetic details). With the bis(4-trifluoromethylphenyl)iodonium reagent 1 in hand we next investigated conditions for the proposed direct arylation chemistry on an unprotected model peptide bearing a Cys residue, namely H-CSPGYS- NH_2 (2) that was prepared by Fmoc-strategy solid-phase peptide synthesis (SPPS) (Scheme 2a, see ESI[†] for synthetic details). Several reaction conditions and buffers were systematically screened on this model system (see ESI[†]). Optimal reaction conditions that emerged involved the use of aqueous 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 8.0, tris(2-carboxyethyl)phosphine (TCEP) to prevent cystine formation, with the addition of 10 vol% acetonitrile (MeCN) as co-solvent, which was necessary to dissolve the diaryliodonium salt 1 (Scheme 2a). Importantly, the reactions did not need to be conducted under an inert atmosphere and proceeded to 90% conversion in 90 min when performed open to air [as judged by analytical ultraperformance liquid-chromatography (UPLC) analysis] (Scheme 2b). The target arylation product 3 was isolated in 60% yield following high-performance liquid-chromatography (HPLC) purification and was characterised by nuclear

magnetic resonance (NMR) spectroscopy and high-resolution electrosprav ionisation mass-spectrometry (HR-ESI-MS) (Scheme 2c, see ESI† for details). Importantly, the chemoselectivity for the side chain of Cys was confirmed by a heteronuclear multiple bond correlation (HMBC) experiment revealing a correlation between the aromatic ipso-carbon and the methylene protons of Cys (Scheme 2c, see ESI[†]). Following the success of this initial model system, we explored the effect of aryl substituents on reactivity with Cys (see ESI[†] for details). Consistent with the previously established reactivity reported for this class of reagents,^{34,38} we observed a decrease in the rate of arylation and overall efficiency of the reaction when moving from an electron withdrawing aryl system to more electron rich systems (e.g. p-methoxyphenyl, see ESI[†]).³⁹

Having demonstrated the direct arylation of a simple model system, we next sought to synthesise a diaryliodonium salt equipped with an alkyne handle that would enable late-stage Cu(1)-catalysed azide–alkyne cycloaddition (CuAAC)^{40,41} chemistry on peptide and protein substrates, taking advantage of the variety of azides that can be obtained commercially or through simple synthetic procedures. Attempts to prepare the tetra-fluoroborate analogue of compound 4 (Scheme 3a) *via* the same procedure used to prepare 1 provided the product in extremely low yield and purity. Therefore, compound 4 was synthesised *via* an alternative route with a modified version of a protocol reported by DiMagno *et al.* (see ESI† for synthetic details).⁴²

With compound 4 in hand we aimed to apply our methodology to a larger peptide substrate containing 11 of the 20 proteinogenic amino acids, namely, MUC1 VNTR (5).⁴³ The peptide was synthesised by Fmoc-strategy SPPS with a Cys



Scheme 3 (a) Arylation of peptide MUC1 VNTR 5 with diaryliodonium salt 4 to afford 6 and subsequent functionalisation by CuAAC in one-pot to furnish 7, 8, 9, and 10; (b) characterisation of MUC1 VNTR conjugates 7 (PEG₁₀), 8 (GlcNAc), 9 (biotin), and 10 (TAMRA) by ESI-MS and analytical UPLC.



Scheme 4 Arylation of zEGFR affibody 11 with 4 to afford the intermediate 12, which was converted to biotin conjugate 13 in one-pot using the CuAAC reaction. Structure of zEGFR from PDB (ID: 2KZI).

residue installed internally (in place of a serine) to eliminate steric bias at position 11 (Scheme 3a, see ESI† for synthetic details). Pleasingly, the arylation of 5 with diaryliodonium salt 4 under the optimised conditions proceeded to 91% conversion within 120 min (as indicated by UPLC-MS analysis), and the product MUC1-alkyne (6) peptide conjugate could be isolated in 52% yield following HPLC purification (see ESI† for synthetic details). Importantly, the thioether linkage in 6 was stable for 24 h in plasma, and phosphate buffered saline containing 100 mM glutathione, dithiothreitol or TCEP (see ESI† for details). Diaryliodonium reagent 4 was itself stable to storage in solid form at room temperature for 18 months.

Alkyne-containing peptide **6** was subsequently reacted with azides bearing a PEG₁₀, *N*-acetyl glucosamine (GlcNAc), biotin or fluorescent TAMRA functionality under CuAAC conditions^{40,41} to afford peptide conjugates 7–10 in good yields over the two-step process (see ESI† for details). However, arylation of peptide **5** with **4** and CuAAC could also be performed in a one-pot manner without any purification of the MUC1-alkyne intermediate **6** (Scheme 3a). This one-pot arylation-CuAAC chemistry on **5** was used to generate four peptide conjugates bearing PEG₁₀ (7), GlcNAc (**8**), biotin (**9**) and TAMRA (**10**) functionalities. Each of the reactions proceeded smoothly to

generate the conjugates with 91% conversion over the two steps (as judged by UPLC-MS analysis). These conjugates were then purified by HPLC to afford **7–10** in 28–57% isolated yield (Scheme 3b). We note that the modest yields in some cases were a result of poor recoveries from HPLC rather than inefficient reactions. The peptide conjugates were characterised by electrospray ionisation mass-spectrometry (ESI-MS) and analytical UPLC.

Having demonstrated the efficiency of the arylation chemistry on the MUC1 VNTR peptide, we next envisaged applying our novel protein conjugation platform to a more challenging system, namely the affibody zEGFR (11) equipped with an Nterminal Cys residue (Scheme 4, see ESI† for synthetic details). Pleasingly, arylation with diaryliodonium salt 4 proceeded to 95% conversion in 240 min (as judged by UPLC-MS analysis). In this case TCEP was necessary to prevent the formation of unreactive cystine during the reaction. The protein conjugate 12 was isolated in 45% yield following HPLC purification and characterised by ESI-MS, analytical UPLC, and matrix-assisted laser desorption ionisation-time of flight massspectrometry (MALDI-ToF MS) (Scheme 4). Importantly, the Cys-selectivity of the arylation reaction was confirmed by LC-MS/MS analysis of 12 following trypsin digestion (see ESI[†] for details). As with the MUC1 VNTR, we found that the arylation chemistry and CuAAC could be performed in one-pot without purification of intermediate 12. Specifically, arylation followed by CuAAC with azido-PEG₃-biotin in the same pot furnished the affibody conjugate 13 (94% conversion over the two steps as judged by UPLC-MS), which was purified by HPLC and characterised by ESI-MS, analytical UPLC, and MALDI-ToF MS (Scheme 4, see ESI[†] for synthetic details). Circular dichroism (CD) spectroscopy of functionalised zEGFR affibodies 11-13 showed that the characteristic α -helical secondary structure of the proteins was maintained following conjugation (see ESI⁺).



Scheme 5 (a) Arylation of histone protein 15 to afford 16 for subsequent oxime-ligation reactions to furnish 17, 18, and 19; (b) characterisation of the oxime ligated H2A conjugates 17, 18, and 19 by MALDI-ToF MS. Structure of H2A from PDB (ID: 4QYL).



Scheme 6 Arylation of affibody 11 with PEGylated diaryliodonium salts 20 and 21 to yield PEGylated proteins 22 and 23, respectively; MALDI-ToF MS spectra of purified 22 and 23. Structure of zEGFR from PDB (ID: 2KZI).

Having demonstrated that alkyne-derived diaryliodonium salts can be used for the efficient functionalisation of peptides and proteins via CuAAC chemistry, we next sought to generate a ketone-containing diaryliodonium salt to access single-sitefunctionalised proteins primed for diversification with an alternative reaction manifold, namely the oxime-ligation.44 To this end, we synthesised diaryliodonium salt 14 (ref. 45) (see ESI[†]) and moved to assess the efficiency of oxime ligation chemistry on a more complex protein system, namely the histone H2A, one of the four proteins found in the octameric complex that make nucleosomes (Scheme 5a).46 The 129 residue and 14 kDa protein was expressed as the T120C mutant 15 by site-directed mutagenesis in Escherichia coli and purified by HPLC (see ESI[†] for details).³⁶ It should be noted that the aqueous buffer used in these reactions was changed from HEPES to phosphate due to side-product formation when arylation of 15 was performed in HEPES (see ESI[†] for details). Gratifyingly, treatment of H2A 15 with diaryliodonium salt 14 in phosphate buffer led to the generation of the desired arylated conjugate 16 in just 60 min with 98% conversion. Purification by HPLC afforded 16 in 54% isolated yield (Scheme 5a, see ESI† for ESI-MS and analytical HPLC data). We also confirmed the Cys-selectivity of the arylation reaction by LC-MS/MS analysis of trypsin-digested 16 (see ESI† for details). The ketone-containing protein conjugate 16 was successfully functionalised with a variety of hydroxylamines using 5-methoxyanthranilic acid (5MA) as a nucleophilic catalyst^{47,48} (Scheme 5a). The resulting histone protein conjugates, functionalised with biotin (17), TAMRA (18), and the cell-penetrating peptide 'penetratin' (19),⁴⁹ were each generated cleanly following oxime ligation in 1–20 h (83–93% conversions). Each of the constructs was purified by HPLC to afford the oxime conjugates 17–19 in 82–83% isolated yields (Scheme 5b, see ESI† for characterisation data).

Having thus far used our arylation chemistry to install a reactive handle for subsequent functionalisation, we next sought to employ the method to directly functionalise proteins (Scheme 6). PEGylation of proteins is known to improve their efficacy and half-life, and we therefore selected this system to test direct functionalisation chemistry.11 Towards this end, we first synthesised diaryliodonium salts equipped with mPEG₄ (20) and mPEG₈ (21) chains (Scheme 6, see ESI^{\dagger} for synthetic details). With the PEGylated diaryliodonium salts in hand we next investigated their efficiency in the late-stage modification of the affibody 11 (Scheme 6). Arylation of the affibody 11 with 20 proceeded smoothly to 90% conversion in 180 min in phosphate buffer. The PEGylated protein was subsequently purified by HPLC to afford 22 in 32% yield (Scheme 6, see ESI† for details). Likewise, we were delighted to find that treatment of 11 with 21 in phosphate buffer delivered the protein conjugate 23 with 90% conversion in 120 min and could be isolated by HPLC in 21% yield (Scheme 6, see ESI[†] for details). The functionalised affibodies were characterised by ESI-MS, MALDI-ToF MS, analytical UPLC, and CD spectroscopy (see ESI[†] for data). The Cys-selectivity of the arylation reactions was verified by LC-MS/MS analysis of the trypsin digested proteins (see ESI† for details).



Scheme 7 Arylation of histone protein 15 with 20 and 21 to yield protein conjugates 24 and 25, respectively; accompanied by MALDI-ToF MS spectra of crude 24 and 25 before HPLC purification. Structure of H2A from PDB (ID: 4QYL).

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Having validated our direct arylation chemistry on the affibody **11** we next moved to the histone protein **15**. Gratifyingly, arylation of **15** with **20** progressed to 94% conversion in 180 min and purification by HPLC provided **24** in 53% isolated yield (Scheme 7, see ESI† for ESI-MS and analytical UPLC data). Likewise, we were pleased to find that treatment of the protein **15** with the diaryliodonium salt **21** led to 99% conversion to **25** in 120 min as judged by MALDI-ToF MS analysis, and subsequent HPLC purification provided the PEGylated protein **25** in 77% isolated yield (Scheme 7, see ESI† for ESI-MS and analytical UPLC data). The Cys-selectivity of each of the arylation reactions were unequivocally demonstrated by tryptic digestion and LC-MS/MS analysis of the proteolysed fragments (see ESI†).

Conclusions

In summary, we have shown that diaryliodonium salts are both mild and robust Cys selective reagents for the late-stage synthesis of a variety of stable peptide and protein conjugates. The power of this chemistry was highlighted through the installation of a variety of different functionalities in aqueous buffer that is compatible with proteins. The chemoselectivity of the methodology was also exemplified through the functionalisation of the affibody zEGFR and protein H2A that possessed each of the 20 proteinogenic amino acids. Given the overall simplicity and efficiency of the chemistry, we anticipate that the Cys functionalisation methodology reported here will find widespread application in the synthesis of high value peptide and protein conjugates in the future.

Data availability

All experimental procedures, analytical, and spectroscopic data are provided in the ESI.[†] The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD028382, username: reviewer_pxd028382@ebi.ac.uk, password: 3UCckTu3.

Author contributions

SB and RJP designed the project, SB and LC synthesised the diaryliodonium salts, SB synthesised the arylated model peptide, SB synthesised the MUC1 VNTR conjugates, SB synthesised the zEGFR conjugates, SB and DF synthesised the H2A conjugates, MJB synthesised azido-PEG₃-biotin and azido-PEG₃-TAMRA, MJB synthesised zEGFR, LC conducted the NMR analysis on the arylated model peptide, LC and DJF synthesised the alkoxyamines, YZ and JPM produced the H2A protein, CF conducted the CD spectroscopy, ML conducted LC-MS/MS experiments and data analysis, SB and RJP prepared the manuscript with assistance from all authors. RJP obtained funding for the project.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was funded through the Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science (CE200100012). We would like to thank Dr Nicholas Proschogo (The University of Sydney) for assistance with mass spectrometry and Dr Ian Luck (Sydney Analytical, The University of Sydney) for assistance with NMR spectroscopy. This work was supported by The University of Sydney Dean's International Postgraduate Research Scholarship (S. A. B.) and the Australian Government Research Training Program scholarship (M. J. B. and Y. Z.). Protein structures were created with http://BioRender.com.

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