Chemical Science

ROYAL SOCIETY OF CHEMISTRY

EDGE ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Chem. Sci.*, 2021, **12**, 15374

All publication charges for this article have been paid for by the Royal Society of Chemistry

Luminol anchors improve the electrochemical-tyrosine-click labelling of proteins†

Sébastien Depienne,*[a] Dimitri Alvarez-Dorta,[a] Mikael Croyal,[bcd] Ranil C. T. Temgoua,[a] Cathy Charlier,[e] David Deniaud,[a] Mathieu Mével,[af] Mohammed Boujtita[a] and Sébastien G. Gouin*[a]

New methods for chemo-selective modifications of peptides and native proteins are important in chemical biology and for the development of therapeutic conjugates. Less abundant and uncharged amino-acid residues are interesting targets to form less heterogeneous conjugates and preserve biological functions. Phenylurazole (PhUr), *N*-methylphenylurazole (NMePhUr) and *N*-methylluminol (NMeLum) derivatives were described as tyrosine (Y) anchors after chemical or enzymatic oxidations. Recently, we developed the first electrochemical Y-bioconjugation method coined eY-click to activate PhUr in biocompatible media. In this work, we assessed the limitations, benefits and relative efficiencies of eY-click conjugations performed with a set of PhUr, NMePhUr and NMeLum derivatives. Results evidenced a high efficiency of NMeLum that showed a complete Y-chemoselectivity on polypeptides and biologically relevant proteins after soft electrochemical activation. Side reactions on nucleophilic or heteroaromatic amino-acids such as lysine or tryptophan were never observed during mass spectrometry analysis. Myoglobine, bovine serum albumin, a plant mannosidase, glucose oxidase and the therapeutically relevant antibody trastuzumab were efficiently labelled with a fluorescent probe in a two-step approach combining eY-click and strain-promoted azide–alkyne cyclization (SPAAC). The proteins conserved their structural integrity as observed by circular dichroism and the trastuzumab conjugate showed a similar binding affinity for the natural HER2 ligand as shown by bio-layer interferometry. Compared to our previously described protocol with PhUr, eY-click with NMeLum species showed faster reaction kinetics, higher (complete) Y-chemoselectivity and reactivity, and offers the interesting possibility of the double tagging of solvent-exposed Y.

Received 31st August 2021
Accepted 3rd November 2021

DOI: 10.1039/d1sc04809k

rsc.li/chemical-science

Introduction

Protein bioconjugation is an extensively explored and ever-growing field of research with wide-ranging applications in pharmacology, biotechnology and chemical biology.[1–3] These include the development of medically relevant conjugates such as antibody–drug conjugates (ADCs)[4] and targeted covalent inhibitors (TCIs),[5,6] the improvement of protein-based diagnostics and therapeutics with enhanced solubility and bioavailability,[7] and a better understanding of protein–protein interactions (PPIs).[5,8]

Bioorthogonal conjugations can be done with a minutely fine degree of control by site-selective incorporation of an unnatural amino acid in the polypeptidic chain with engineered aminoacyl-tRNA synthetase/tRNA (aaRS/tRNA) pairs.[9] Such genetic code expansion techniques are powerful new tools but have to be conducted in specialized laboratories. The chemical modification of native proteins, more easily produced in bulk, remains the most conventional and user-friendly labelling method. Chemical bioconjugations are still mostly performed on nucleophilic lysine (Lys) and cysteine (Cys) amino acids using electrophilic reagents such as NHS-activated esters or maleimides. Modification of the rare Cys precludes a high payload of conjugate and may also be detrimental to protein integrity. Targeting the abundant, surface-exposed and charged Lys residues generally results in the formation of highly heterogeneous mixtures and may significantly impact biological binding. Promising approaches have recently been described to overcome the initial limitations of Lys[10,11] and Cys[12,13] modifications, and much effort is now dedicated to selectively target

[a]*Université de Nantes, CNRS, CEISAM UMR 6230, F-44000 Nantes, France. E-mail: sebastien.gouin@univ-nantes.fr; sebastien.depienne@univ-nantes.fr*
[b]*Université de Nantes, CNRS, INSERM, L'institut du Thorax, F-44000 Nantes, France*
[c]*Université de Nantes, CHU Nantes, Inserm, CNRS, SFR Santé, Inserm UMS 016, CNRS UMS 3556, F-44000 Nantes, France*
[d]*CRNH-Ouest Mass Spectrometry Core Facility, F-44000 Nantes, France*
[e]*IMPACT Platform, Interactions Moléculaires Puces ACTivités, UMR CNRS 6286 UFIP, Université de Nantes, F-44000 Nantes, France*
[f]*INSERM UMR 1089, Université de Nantes, CHU de Nantes, 44200 Nantes, France*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d1sc04809k

less exploited amino acids such as methionine,[14] tryptophan,[15,16] histidine[17,18] or tyrosine (Y).[19,20]

Y usually occurs at low frequency in proteins (<5%) and is partially buried in the surface, offering unique opportunities for controlled labelling of the most reactive residues.[21,22] The phenol side chain of Y is neutral at physiological pH and experiences several biologically relevant post-translational modifications (phosphorylations, sulfations, nitrations, *O*-glycosylations, oxidations).[23] Thus, growing numbers of methods for Y-labelling have been reported over the last decade. Efficient chemical strategies include the use of three-component Mannich-type coupling,[24] transition metal complexes,[25,26] sulfur(VI) fluoride exchange chemistry (SuFEx),[27,28] aryldiazonium salts[29,30] and diazodicarboxyamides.[31–33] The last of these methods is among the most promising and exemplary. Barbas and co-workers initially showed that silent phenylurazoles (PhUrs) are readily activated to phenyltriazolinediones (PTADs) using chemical oxidizers (Method A, Fig. 1) such as dibromodimethylhydantoin (DBDMH) or *N*-bromosuccinimide (NBS). Nonetheless, PTADs are known to be unstable in aqueous environment and to decompose into an electrophilic phenylisocyanate specie responsible for undesired side reactions on amino groups.[32,34] In a previous study, we developed the first electrochemical tyrosine bioconjugation, named eY-click (Method C, Fig. 1).[35]

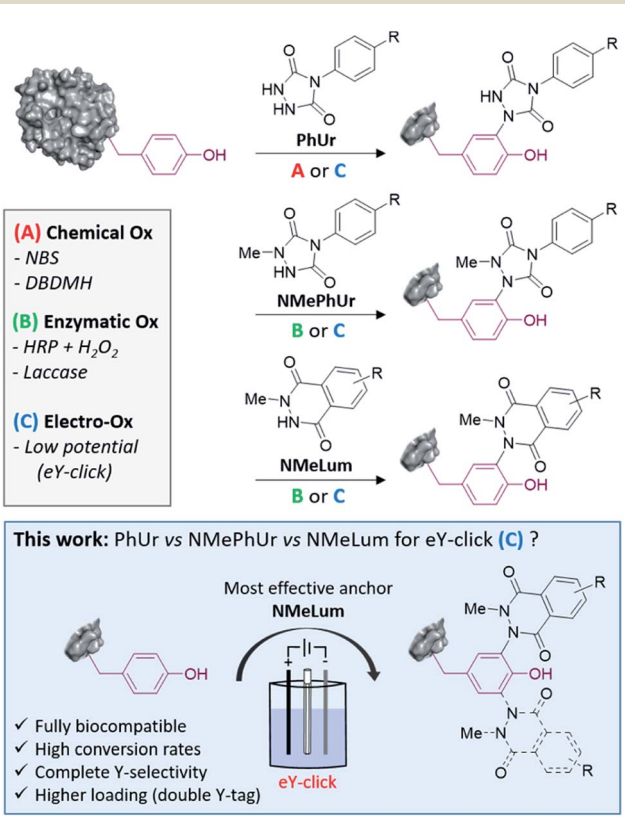


Fig. 1 PhUr, NMePhUr and NMeLum may be activated by chemical oxidation (Method A), enzymatically (Method B) or electrochemically (Method C). In this work we found that NMeLum are very efficient anchors for the soft electrochemical Y labelling of proteins.

Greater protein labelling and Y-selectivity could be achieved this way than by using the chemical approach, in conventional buffers, without the need for an isocyanate scavenger. Thus, electrochemical methods offer interesting perspectives for site-selective modification of proteins.[35–38] Y anchors based on *N*-methylphenylurazole (NMePhUr) or *N*-methylluminol (NMeLum) derivatives may also be successfully activated under single-electron transfer (SET) reactions as shown by Nakamura's research group (Method B & C, Fig. 1).[37,39–43] NMePhUr and NMeLum were activated using ruthenium photocatalysts,[44] hemin and H_2O_2,[40] enzymatic systems (horseradish peroxidase and H_2O_2, laccase)[37,39,45] and electrochemically.[43] In the present study, we aimed to evaluate and compare the relative efficiency of new and previously reported urazoles and luminols derivatives in the electrochemical-tyrosine-click labelling strategy (Fig. 1). The scope and efficiency of the eY-click protocol was significantly improved with NMeLum derivatives. Complete Y-selectivity has now been achieved in a fully biocompatible procedure with faster kinetics and a higher conjugate payload.

Results and discussion

We first studied the electrochemical behaviour of new PhUr derivatives **1–6** and previously reported NMePhUr **7-8** and NMeLum **9-10** reagents (Fig. 2a).[43] The stability of their oxidized species was compared by cyclic voltammetry. Compounds **1–6** were designed (synthesis described in ESI†) to assess whether electro-donating or electro-withdrawing substituents on the aromatic ring could impact the stability of the electro-generated PTADs, and therefore limit the formation of corresponding isocyanate by-products. The potential impact of a bioorthogonal handle (azidoethyl group) was also investigated. Experiments were performed in Tris/MeCN buffer (pH 7.4) as the electrolyte, with a three-electrode system using a graphite working electrode, a platinum wire as counter electrode, and a saturated calomel electrode (SCE) for reference. The voltammograms (Fig. 2b) showed that substitution on the aromatic ring of PhUr (**2–6**) did not impact its oxidation potential ($\mathbf{O_1}$, 0.33 V *vs.* SCE) or the intensity of the reduction peak ($\mathbf{R_1}$), *i.e.* the PTADs stability. Thus, aryl substituents do not impact the electro-oxidation process, outlining that a wide range of biorthogonal handles or ligands may be appended to the PhUr anchor. On the contrary, *N*-methyl substitution of the hydrazide function in **7**, **8** (NMePhUr) and **9**, **10** (NMeLum) resulted in a shift of the oxidation peak to higher potentials ($\mathbf{O_2}$, 0.52 V *vs.* SCE and $\mathbf{O_3}$, 0.62 V *vs.* SCE) and in substantially higher reduction intensities ($\mathbf{R_2}$ and $\mathbf{R_3}$) suggesting the formation of much more stable oxidized species. We further compared stabilities of PhUr, NMePhUr and NMeLum oxidized species by examining both oxidation and reduction peaks obtained from 20 repetitive cyclic voltammetry measurements (Fig. 2c). A significant gradual decrease in the peak intensity of **1** highlighted the well-known instability of PTAD, as the oxidation peak of the second cycle (9.5 μA) already lost half the initial intensity (19 μA). This contrasts with the results obtained for **7** and **9** where constant oxidation and reduction peak intensities are observed over 20 cycles, confirming a high stability of the oxidized species.

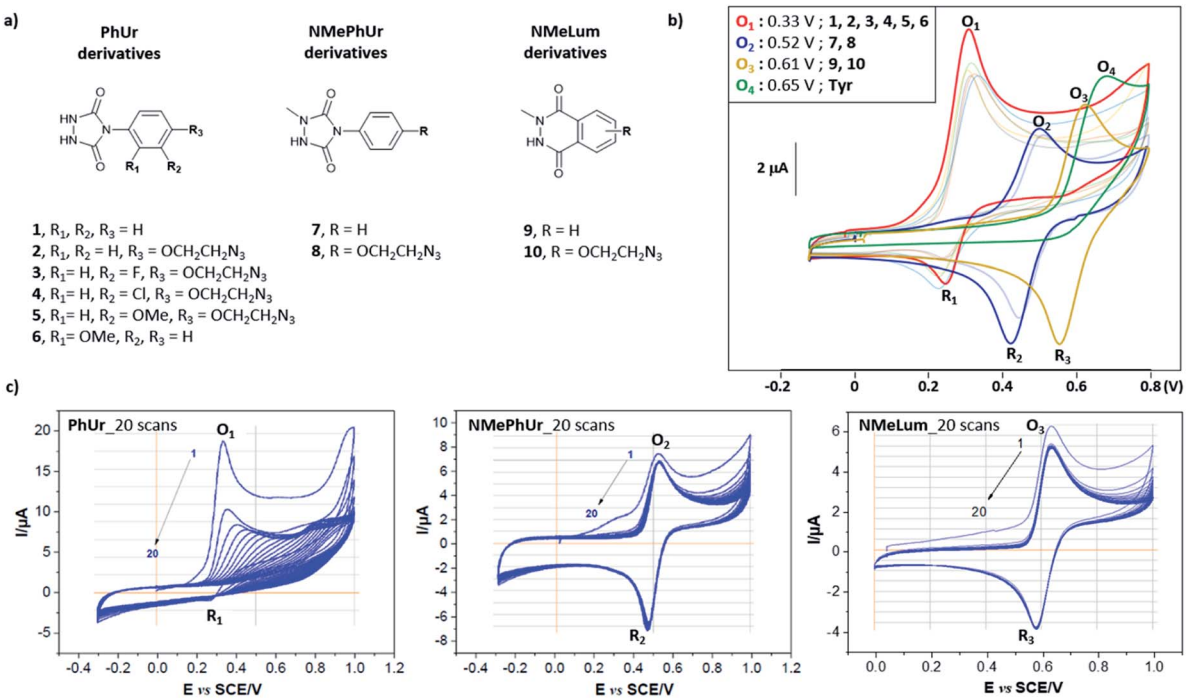
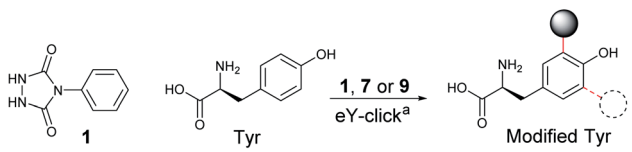


Fig. 2 Electrochemical behaviour of the studied Y anchors PhUr **1–6**, NMePhUr **7**, **8** and NMeLum **9**, **10**. (a) Chemical structures of the compounds **1–10**, (b) Cyclic voltammetry of **1–10** and Y (cathode: graphite carbon electrode 2 mm disc, anode: platinum wire, reference: saturated calomel electrode, 100 mV s^{-1}, reagent 1 mM, 1 : 1 MeCN/Tris 50 mM pH 7.4), (c) Multicyclic voltammetry of **1**, **7** and **9** (cathode: graphite carbon electrode 2 mm disc, anode: platinum wire, reference: saturated calomel electrode, 100 mV s^{-1}, reagent 1 mM, 1 : 1 MeCN/NH_4OAc 100 mM pH 7.4).

Of note, PhUr **1** is reported to be chemically or electrochemically two-electron oxidized to generate PTAD for aza–ene reaction with the phenol of Y.[31,35] Even though a similar two-electron process cannot be excluded for **7** and **9**, relative intensities of the oxidation peak of **1** ($\mathbf{O_1}$) *vs.* **7** ($\mathbf{O_2}$) support a possible single-electron electro-oxidation process for NMePhUr. The formation of a stable nitrogen-centred radical may also prevail for NMeLum considering the similar *N*-methylhydrazide function.[37,43] Cyclic voltammetry covering higher voltage up to 2 V showed a second non-reversible anodic event at 1.5–1.6 V for **7** and **9** (see ESI†), which could correspond to the second electron oxidation (diazenium formation), as recently proposed.[43] Thus, electro-activation of NMePhUr and NMeLum derivatives at low potential could promote radical coupling with Y in an analogous manner as the enzymatic and photo activation protocols.[37,44]

As NMePhUr and NMeLum oxidation potentials shifted to higher values close to that of Y ($\mathbf{O_4}$, 0.65 V *vs.* SCE), we investigated whether these two anchors could be selectively electro-oxidized in the presence of Y. The Y-tagging efficiency was compared with that observed with PhUr in eY-click conditions (Table 1). The electrolysis assays were conducted under stirring conditions, in phosphate buffer (PB) at pH 7.4 (100 mM). Appropriate working potentials were applied until 90% of the theoretical charge given by the Faraday law was generated. For a stoichiometric reagent/Y ratio, overall higher Y conversion was observed with **7** (84%) or **9** (91%) than with **1** (66%). However, the first electrolysis assay with **9** performed in PB at 0.62 V *vs.* SCE led to partial competitive Y oxidation. This issue was solved by replacing PB with ammonium acetate buffer (pH 7.4). The latter doesn't result in an oxidation potential shift of **9** or Y as confirmed by cyclic voltammetry. We suppose that NMeLum reagent has a better diffusion coefficient in this buffer of different ionic strength, favouring its oxidation in presence of Y. When two equivalents of reagents were used, Y-labelling with

Table 1 Electrochemical modification of Y



Entry	Reagent	Reagent:Y	Yield[b]
1	**1**	1:1	66%
2	**7**	1:1	84%
3	**9**	1:1	>91%[c,d]
4	**1**	2:1	94%
5	**7**	2:1	100%[c]
6	**9**	2:1	100%[c,d]

[a] Reaction conditions: carbon crucible anode, platinum wire cathode, constant voltage *vs.* SCE, reagent (0.05–0.10 mmol, 1–2 mM), Tyr (0.05 mmol, 1 mM), phosphate buffer pH 7.4 (50 mL), 2–5 h. [b] % conv. of Y determined by ^1H NMR. [c] Double modification of Y was detected by MS. [d] Ammonium acetate pH 7.4 buffer (50 mL) was used.

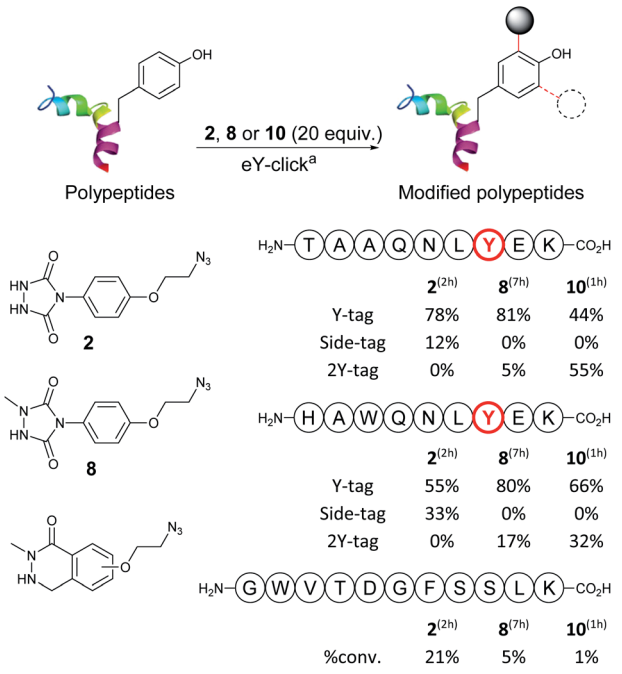
1 reached 94% (entry 4) and a complete conversion was observed with **7** and **9**. Interestingly, a double addition adduct was detected by mass spectrometry (MS) for **7**(entry 5) and **9** (entry 6). These results show that electro-oxidized species of *N*-methyl derivatives **7** and **9** can tag Y in higher conversion than PTAD and offer possibility of Y double tagging.

Next, we evaluated and compared the labelling efficiency of azido-armed analogues **2**, **8** and **10** (yields, chemo-selectivity and kinetics) on unprotected polypeptides (Table 2). We selected the synthetic nonapeptides TAAQNLYEK, bearing one Y and one lysine (K) and HAWQNLYEK, bearing one Y, one K, a tryptophan (W) and a histidine (H) to evaluate potential side-reactivity on nucleophilic amines and heteroaromatic amino-acids. GWVTDGFSSLK was also selected, as a 11-mer peptide lacking Y, to do a control experiment. The eY-click protocols were performed with 20 equivalents of **2**, **8** and **10** (2 mM), as reagent excess are generally needed for protein labelling, and to better assess potential selectivity issues. Samples were periodically collected and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). eY-click performed with **2** on TAAQNLYEK afforded mainly the expected Y-tag conjugate (78%) after 2 h electrolysis as determined by MS/MS fragmentation. N-Terminus urea adducts (12%) from partial decomposition of PTAD into an isocyanate were also observed. Longer electrolysis increased the urea adducts proportion (results not shown). As in previous experiments,[35] K adducts were not observed, which could be explained by a lower pK_a value of the N-terminus amino groups, due to amide bond proximity.[46] NMePhUr derivative **8** required a long reaction time (7 h) but converted the native peptide with high tagging yields (81% Y-tag and 5% 2Y-tag adducts). Side products were not observed, demonstrating complete Y-selectivity. The eY-click with NMe-Lum derivative **10** was also conducted in PB even though Y oxidation was observed on the free amino acid due to overlapping oxidation potentials (Table 1). Strikingly, by-products from Y oxidation were never observed and the peptide was fully converted into the Y adducts (44% Y-tag and 55% 2Y-tag) after only 1 h.

Thus, faster kinetics, complete Y selectivity, and higher level of double Y modification were observed with **10**. Despite the higher oxidation potential to generate the activated specie (620 mV), the lower diffusion coefficient of the peptide at the anode surface and its lower concentration in solution compared to **10** prevented Y oxidation. A similar trend was observed with HAWQNLYEK. eY-click with **2** led to 55% of the Y-tag peptide after 2 h, with 33% side reactions characterized by MS/MS due to N-terminus modification and W addition. The latter side-reaction has rarely been reported in literature probably because of the low W abundance/accessibility and thermoreversible indole-TAD adduct.[47] A recent study showed the TAD-W instability and the kinetically favoured reactivity of TAD for exposed W over Y, allowing a selective W modification on proteins.[48] Reagents **8** and **10** efficiently labelled (97–98% overall yields) the peptide with a complete Y-selectivity (K, H, W tagging never observed) and Y-tag/2Y-tag ratios of 80 : 17 and 66 : 32, respectively. Again, much faster kinetics were observed with **10**. Labelling experiments performed on control peptide GWVTDGFSSLK, which is missing a Y residue, confirmed the good to excellent inertness of electro-activated compounds **2**, **8**, **10** for other amino-acids with 21%, 5% and 1% of parent peptide conversion, respectively. Thus, peptide experiments revealed a much higher eY-click labelling efficiency with NMe-Lum **10** and, to a lesser extent, NMePhUr **8** compared with the original method using PhUr **2**.

Next, **8** and **10** were investigated for protein labelling. α-Chymotrypsinogen A (α-Chymo), a 25.6 kDa pre-digestive enzyme, was selected as a first model. As α-Chymo has a limited number of four accessible Y, it enabled us to easily track and analyse by mass spectrometry the Y adducts. The eY-click conjugations were performed on a low concentration of α-Chymo (1 μM) during fixed times of 4 and 1 h for **8** and **10**, respectively (Fig. 3a), according to the relative compounds reactivity on peptides (Table 2). Mass profiles showed a clean labelling distribution of protein adducts after deconvolution. As observed with peptides, **10** exhibited a high tagging potency as +1 to +5 adducts of **10**-Chymo could be revealed by MS after 1 h with near complete disappearance of the unmodified protein peak (Fig. 3c). In stark contrast, **8** only partially labelled the protein batch up to +2-tags in a 4 h electrolysis (Fig. 3b). Of note, a poor labelling efficiency of α-Chymo was reported with PhUr species after chemical activation.[32] Interestingly, formation of the +5 tag adduct with **10** supports a partial double tagging of a single Y, as observed on polypeptides (Table 2). We further studied the **10**-Chymo sample and performed enzymatic

Table 2 Electrochemical modification of peptides



[a] Reaction conditions: graphite plate anode, platinum wire cathode, constant voltage *vs.* SCE, modification reagent (25 mmol, 2 mM, 20.0 equiv.), polypeptide (1.25 mmol, 0.1 mM, 1.0 equiv.), phosphate buffer (100 mM, 12.5 mL), 700 rpm, room temperature, 2–7 h. Location of conjugation determined by LC-MS/MS analysis.

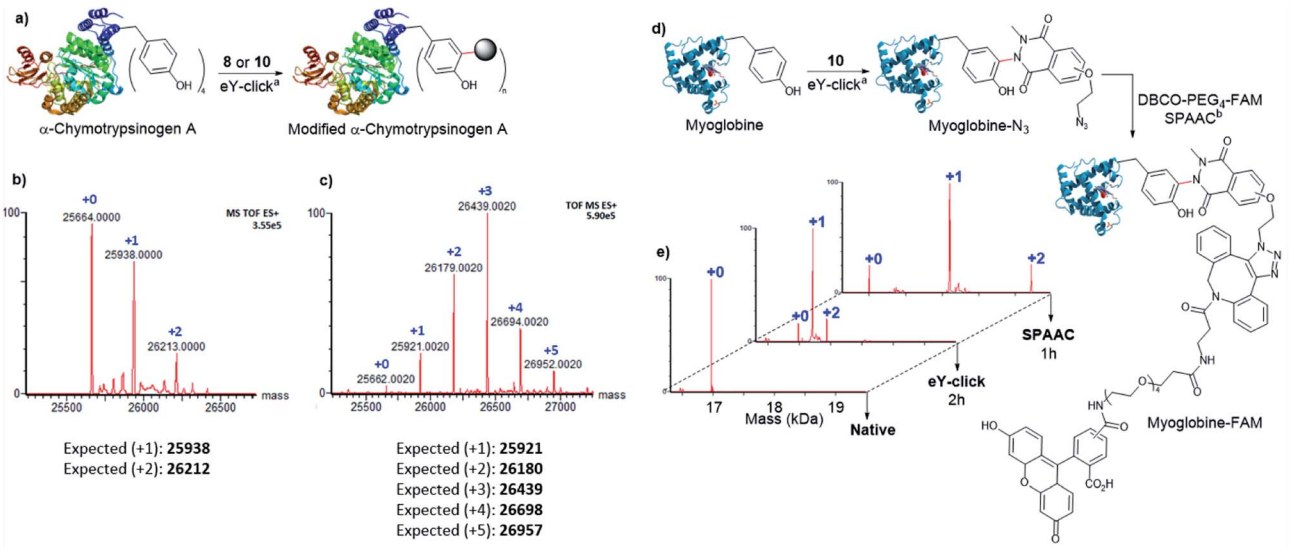


Fig. 3 eY-click protocol on proteins. (a) Modification of α-chymotrypsinogen A, [a]reaction conditions: graphite plate anode, platinum plate cathode, constant voltage *vs.* Ag/AgCl, modification reagent (5.0 μmol, 1.0 mM), α-Chymo (5.0 nmol, 1.0 μM), phosphate buffer (100 mM, 5.0 mL), 500 rpm, room temperature, 4 h (**8**) or 1 h (**10**), (b) Deconvoluted profile (MS) for α-Chymo modification by **8** after 4 h eY-click, (c) deconvoluted profile (MS) for α-Chymo modification by **10** after 1 h eY-click, (d) two-steps modification of myoglobin, [a]reaction conditions: graphite plate anode, platinum plate cathode, constant voltage *vs.* Ag/AgCl, **10** (5.0 μmol, 1.0 mM), Myo (5.0 nmol, 1.0 μM), phosphate buffer (100 mM, 5.0 mL), 500 rpm, room temperature, 1 h, [b]reaction conditions: Myo-N_3 (2.0 nmol, 1.0 equiv.), DBCO-PEG4-5/6-FAM (100.0 nmol, 50.0 equiv.), distillated water (0.5 mL), DMF (3 μL), 37 °C, 1 h, (e) deconvoluted profiles (MS) evolution of Myo two-steps modification.

proteolysis with pepsin (pH 3) to detect peptide fragments and identify location and nature of the modifications. Satisfyingly, both single and double Y-modification of the proteolytic peptide TRYTNA containing ^{146}Y could be evidenced by LC-MS analysis and MS/MS fragmentation, which confirmed the ability of **10** to double-tag solvent-exposed Y at protein scale.

Next, the capacity of **10** to label Y with a low accessibility (partially buried in the protein surface) was assessed on myoglobin (Myo), a 17 kDa oxygen-binding protein with two Y, one being inaccessible (deeply buried) and the other only poorly exposed to the solvent (Fig. 3d). A high conversion was observed after 2 h with the formation of the +1 and +2 tags protein adducts (Fig. 3e). To evaluate if both +1 and +2 **10**-Myo adducts were indeed functional for bioconjugation, the samples were engaged in a strain promoted azide–alkyne cycloaddition (SPAAC) with the fluorescent probe DBCO-PEG$_4$-5/6-FAM during 1 h at 37 °C. To our delight, the deconvoluted MS profile showcased the two functionalized adducts with the expected +1 (+880 Da) and +2 (+1760 Da) mass shift (Fig. 3e).

The softness of the eY-click protocol with **10** was next evaluated on a set of proteins including Bovine Serum Albumin (BSA), Jack bean α-mannosidase (α-ManJB), and Glucose Oxidase enzyme (GOx) which is used for blood glucose monitoring and in cancer diagnosis and treatment (Fig. 4a). Structural integrity of the proteins after eY-click conjugations of **10** was confirmed by circular dichroism analysis, where conjugated proteins showed no alteration in secondary structural contents, highlighting the softness of the method (Fig. 4b). The obtained azido-armed proteins were further functionalized by SPAAC with DBCO-PEG$_4$-5/6-FAM for 1 h at 37 °C and analyzed by SDS-PAGE. Brilliant Blue Coomassie detection of native proteins and FAM-protein adducts showed a unique band at the expected molecular weight for BSA and BSA-FAM (~65–70 kDa), GOx and GOx-FAM (~80–85 kDa) and two bands for the heterodimeric α-ManJB and α-ManJB-FAM (~45–50 kDa and ~60–65 kDa). Fluorescent detection at 492 nm unambiguously proved the formation of protein-FAM adducts without protein degradation (Fig. 4c).

Suitable bioconjugation techniques for antibody-conjugates development are still being extensively explored and are required for vectorized immunotherapies or cancer diagnostics.[49–51] We finally evaluated eY-click with **10** for antibody labelling. Trastuzumab (Tras, ~150 kDa) was selected as a model monoclonal antibody referenced for breast cancer therapies due to its high affinity for the overexpressed Human Epidermal Growth Factor Receptor-2 (HER2). Structural integrity of the Tras-**10** conjugate was confirmed by circular dichroism (Fig. 5a), and SDS-PAGE revealed successful subsequent FAM labelling with DBCO-PEG$_4$-5/6-FAM (Fig. 5c). The functional activity of the Tras-**10** conjugate for HER2 receptor was also investigated by bio-layer interferometry (BLI). HER2 was immobilized on the biosensor tip surface and the binding affinity was measured with solutions of Tras and Tras-**10**. Tras showed a nanomolar affinity for HER2 (dissociation constant K_D = 1.2 nM). A high affinity for HER2 was also measured for Tras-**10** which showed a K_D in the same order as Tras (K_D = 3.8 nM). Thus, eY-click with **10** did not compromise HER2 binding and represents a soft and effective methodology for antibody labelling.

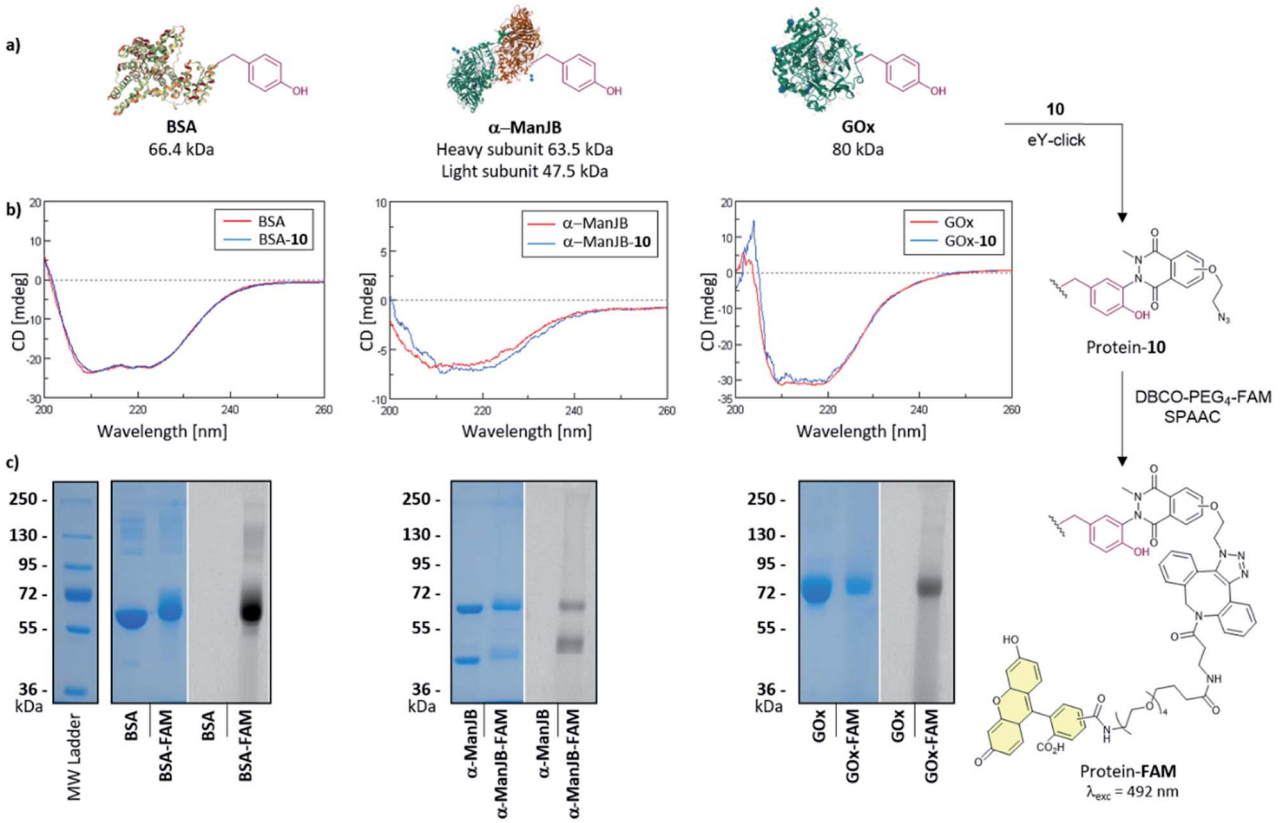
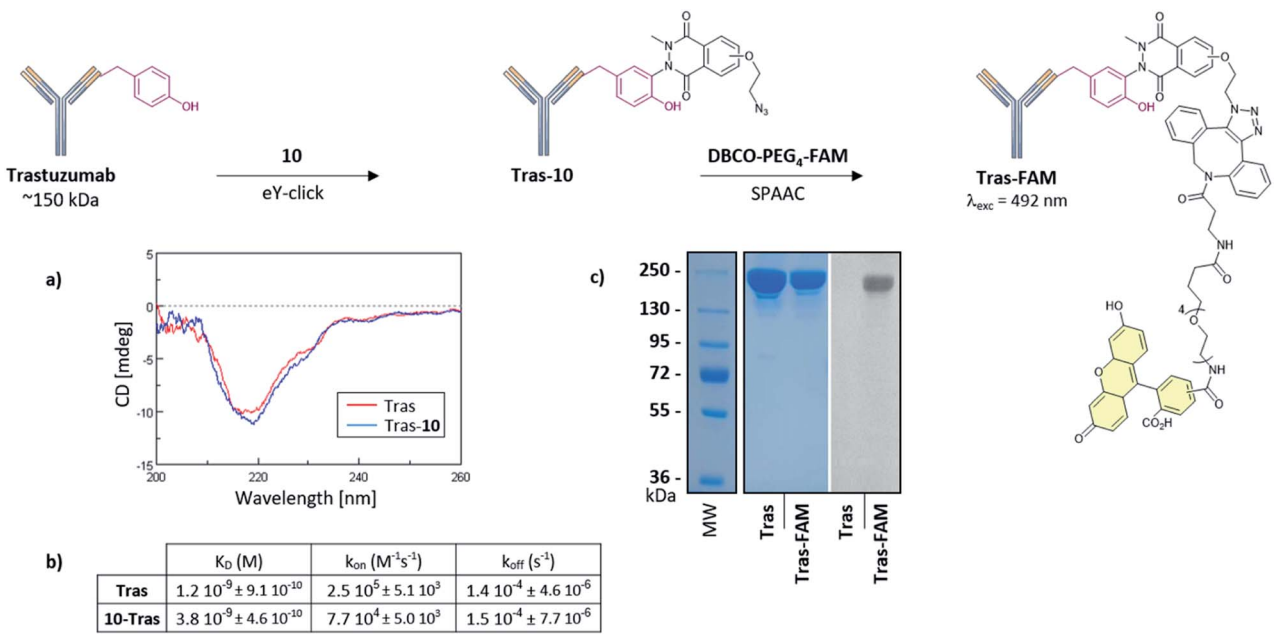


Fig. 4 (a) Two-steps modification of BSA, α-ManJB and GOx. (b) CD analysis of the native (red) and eY-click modified (blue) proteins with **10**. (c) SDS-PAGE analysis of native proteins (from left to right: BSA, α-ManJB and GOx) and their FAM conjugates after eY-click with **10** followed by SPAAC with DBCO-PEG_4-5/6-FAM. Native and conjugated proteins were revealed by coomassie brilliant blue (left side) and fluorescence was detected at 492 nm (right side).



b)

	K_D (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
Tras	$1.2\ 10^{-9} \pm 9.1\ 10^{-10}$	$2.5\ 10^{5} \pm 5.1\ 10^{3}$	$1.4\ 10^{-4} \pm 4.6\ 10^{-6}$
10-Tras	$3.8\ 10^{-9} \pm 4.6\ 10^{-10}$	$7.7\ 10^{4} \pm 5.0\ 10^{3}$	$1.5\ 10^{-4} \pm 7.7\ 10^{-6}$

Fig. 5 Two-steps modification of Tras. (a) CD analysis of the native (red) and eY-click modified (blue) Tras with **10**. (b) Affinities of Tras and Tras-**10** for HER2. Dissociation constants (K_D) and binding kinetic parameters (k_{on}, k_{off}) were measured and plotted by bio-layer interferometry. (c) SDS-PAGE analysis of native Tras and its FAM conjugate after eY-click with **10** followed by SPAAC with DBCO-PEG_4-5/6-FAM. Native and conjugated Tras were revealed by coomassie brilliant blue (left side) and fluorescence was detected at 492 nm (right side).

Conclusions

Y-labelling methods have been extensively explored to study Y post-translational modifications and protein conformations,[52] and to design more defined protein conjugates. Chemical, enzymatic and electrochemical activations of Y anchors are complementary tools with specific advantages and limitations. Our initial electrochemical protocol for the activation of PhUr anchors is an efficient strategy for soft, time-controlled Y-labelling of native protein, not requiring chemical oxidant or enzymatic catalysis. Nevertheless, reaction kinetics were slower than with chemical approaches and the present work revealed that complete Y-selectivity may not be fully achieved depending on peptide substrates. Even though the well-known PhUr degradation into electrophilic phenylisocyanate is considerably limited during electrochemical activation, preventing lysine modification, a partial side-tagging of the N-terminus amine of peptide was observed here with PhUr **2**. The heteroaromatic amino acid side-chain of tryptophan was also not fully inert towards electrogenerated PTAD, as previously shown after chemical activation.[48]

In this work, we showed that complete Y-chemoselectivity can now be achieved by electrochemical activation of NMeLum derivatives such as **10**. Although a higher oxidation potential is required for electro-activation of NMeLum compared to PhUr species, this has virtually no impact on peptides and proteins due to their lower diffusion coefficient at the electrode surface. NMeLum derivatives provide complete Y-selectivity, faster reaction kinetics, and display a higher reactivity for less surface-exposed Y, with possible double Y modification for a higher payload. Altogether, these results expand the scope of eY-click as a chemoselective, soft and user-friendly method for peptides and proteins bioconjugations.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.†

Author contributions

Conceptualization, supervision and writing: S. D., M. B. and S. G. Revising: D. D, Acquisition and formal analysis: S. D.; D. A-D.; M. M. and R. C. T. T. (chemistry & electrochemistry), M. C. (MS analysis), C. C. (DC & BLItz).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was carried out with financial support from the Centre National de la Recherche Scientifique (CNRS), the Ministère de l'Enseignement Supérieur et de la Recherche in France and the National Agency for Research (ANR project ECLICK).

Notes and references

1 O. Boutureira and G. J. L. Bernardes, *Chem. Rev.*, 2015, **115**, 2174–2195.
2 C. D. Spicer and B. G. Davis, *Nat. Commun.*, 2014, **5**, 4740.
3 Q.-Y. Hu, F. Berti and R. Adamo, *Chem. Soc. Rev.*, 2016, **45**, 1691–1719.
4 V. Chudasama, A. Maruani and S. Caddick, *Nat. Chem.*, 2016, **8**, 114–119.
5 T. Ueda, T. Tamura, M. Kawano, K. Shiono, F. Hobor, A. J. Wilson and I. Hamachi, *J. Am. Chem. Soc.*, 2021, **143**, 4766–4774.
6 T. Tamura, T. Ueda, T. Goto, T. Tsukidate, Y. Shapira, Y. Nishikawa, A. Fujisawa and I. Hamachi, *Nat. Commun.*, 2018, **9**, 1870.
7 F. M. Veronese and A. Mero, *BioDrugs*, 2008, **22**, 315–329.
8 M. A. Trakselis, S. C. Alley and F. T. Ishmael, *Bioconjugate Chem.*, 2005, **16**, 741–750.
9 J. C. M. van Hest, K. L. Kiick and D. A. Tirrell, *J. Am. Chem. Soc.*, 2000, **122**, 1282–1288.
10 A.-D. Guo, D. Wei, H.-J. Nie, H. Hu, C. Peng, S.-T. Li, K.-N. Yan, B.-S. Zhou, L. Feng, C. Fang, M. Tan, R. Huang and X.-H. Chen, *Nat. Commun.*, 2020, **11**, 5472.
11 M. J. Matos, B. L. Oliveira, N. Martínez-Sáez, A. Guerreiro, P. M. S. D. Cal, J. Bertoldo, M. Maneiro, E. Perkins, J. Howard, M. J. Deery, J. M. Chalker, F. Corzana, G. Jiménez-Osés and G. J. L. Bernardes, *J. Am. Chem. Soc.*, 2018, **140**, 4004–4017.
12 V. F. C. Ferreira, B. L. Oliveira, A. D'Onofrio, C. M. Farinha, L. Gano, A. Paulo, G. J. L. Bernardes and F. Mendes, *Bioconjugate Chem.*, 2021, **32**, 121–132.
13 B. Bernardim, M. J. Matos, X. Ferhati, I. Compañón, A. Guerreiro, P. Akkapeddi, A. C. B. Burtoloso, G. Jiménez-Osés, F. Corzana and G. J. L. Bernardes, *Nat. Protoc.*, 2019, **14**, 86–99.
14 A. H. Christian, S. Jia, W. Cao, P. Zhang, A. T. Meza, M. S. Sigman, C. J. Chang and F. D. Toste, *J. Am. Chem. Soc.*, 2019, **141**, 12657–12662.
15 Y. Seki, T. Ishiyama, D. Sasaki, J. Abe, Y. Sohma, K. Oisaki and M. Kanai, *J. Am. Chem. Soc.*, 2016, **138**, 10798–10801.
16 J. M. Antos and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 10256–10257.
17 X. Chen, F. Ye, X. Luo, X. Liu, J. Zhao, S. Wang, Q. Zhou, G. Chen and P. Wang, *J. Am. Chem. Soc.*, 2019, **141**, 18230–18237.
18 K. Nakane, S. Sato, T. Niwa, M. Tsushima, S. Tomoshige, H. Taguchi, M. Ishikawa and H. Nakamura, *J. Am. Chem. Soc.*, 2021, **143**, 7726–7731.
19 P. A. Szijj, K. A. Kostadinova, R. J. Spears and V. Chudasama, *Org. Biomol. Chem.*, 2020, **18**, 9018–9028.
20 D. Alvarez Dorta, D. Deniaud, M. Mével and S. G. Gouin, *Chem.–Eur. J.*, 2020, **26**, 14257–14269.
21 H. Ischiropoulos, *Arch. Biochem. Biophys.*, 1998, **356**, 1–11.
22 S. Miller, J. Janin, A. M. Lesk and C. Chothia, *J. Mol. Biol.*, 1987, **196**, 641–656.

23 L. H. Jones, A. Narayanan and E. C. Hett, *Mol. BioSyst.*, 2014, **10**, 952–969.
24 N. S. Joshi, L. R. Whitaker and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 15942–15943.
25 S. D. Tilley and M. B. Francis, *J. Am. Chem. Soc.*, 2006, **128**, 1080–1081.
26 J. Ohata, M. K. Miller, C. M. Mountain, F. Vohidov and Z. T. Ball, *Angew. Chem., Int. Ed.*, 2018, **57**, 2827–2830.
27 N. P. Grimster, S. Connelly, A. Baranczak, J. Dong, L. B. Krasnova, K. B. Sharpless, E. T. Powers, I. A. Wilson and J. W. Kelly, *J. Am. Chem. Soc.*, 2013, **135**, 5656–5668.
28 E. C. Hett, H. Xu, K. F. Geoghegan, A. Gopalsamy, R. E. Kyne, C. A. Menard, A. Narayanan, M. D. Parikh, S. Liu, L. Roberts, R. P. Robinson, M. A. Tones and L. H. Jones, *ACS Chem. Biol.*, 2015, **10**, 1094–1098.
29 J. M. Hooker, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 3718–3719.
30 F. W. Kimani and J. C. Jewett, *Angew. Chem., Int. Ed.*, 2015, **54**, 4051–4054.
31 H. Ban, J. Gavrilyuk and C. F. Barbas, *J. Am. Chem. Soc.*, 2010, **132**, 1523–1525.
32 H. Ban, M. Nagano, J. Gavrilyuk, W. Hakamata, T. Inokuma and C. F. Barbas, *Bioconjugate Chem.*, 2013, **24**, 520–532.
33 D. M. Bauer, I. Ahmed, A. Vigovskaya and L. Fruk, *Bioconjugate Chem.*, 2013, **24**, 1094–1101.
34 Q.-Y. Hu, M. Allan, R. Adamo, D. Quinn, H. Zhai, G. Wu, K. Clark, J. Zhou, S. Ortiz, B. Wang, E. Danieli, S. Crotti, M. Tontini, G. Brogioni and F. Berti, *Chem. Sci.*, 2013, **4**, 3827.
35 D. Alvarez-Dorta, C. Thobie-Gautier, M. Croyal, M. Bouzelha, M. Mével, D. Deniaud, M. Boujtita and S. G. Gouin, *J. Am. Chem. Soc.*, 2018, **140**, 17120–17126.
36 C. Song, K. Liu, Z. Wang, B. Ding, S. Wang, Y. Weng, C.-W. Chiang and A. Lei, *Chem. Sci.*, 2019, **10**, 7982–7987.
37 S. Sato, K. Nakane and H. Nakamura, *Org. Biomol. Chem.*, 2020, **18**, 3664–3668.
38 L. Cui, Y. Ma, M. Li, Z. Wei, Y. Huan, H. Li, Q. Fei and L. Zheng, *Anal. Chem.*, 2021, **93**, 4434–4440.
39 S. Sato, K. Nakamura and H. Nakamura, *ChemBioChem*, 2017, **18**, 475–478.
40 S. Sato, K. Nakamura and H. Nakamura, *ACS Chem. Biol.*, 2015, **10**, 2633–2640.
41 J. Ertl, M. E. Ortiz-Soto, T. A. Le, J. Bechold, J. Shan, J. Teßmar, B. Engels and J. Seibel, *Chem.–Eur. J.*, 2019, **25**, 6533–6541.
42 M. E. Ortiz-Soto, J. Ertl, J. Mut, J. Adelmann, T. A. Le, J. Shan, J. Teßmar, A. Schlosser, B. Engels and J. Seibel, *Chem. Sci.*, 2018, **9**, 5312–5321.
43 S. Sato, M. Matsumura, T. Kadonosono, S. Abe, T. Ueno, H. Ueda and H. Nakamura, *Bioconjugate Chem.*, 2020, **31**, 1417–1424.
44 S. Sato, K. Hatano, M. Tsushima and H. Nakamura, *Chem. Commun.*, 2018, **54**, 5871–5874.
45 T. A. Wang, R. Wu, Y. Hong, Z. Wang, T. Li, J. Shie and C. Hsu, *ChemBioChem*, 2021, **22**, 2415–2419.
46 C. B. Rosen and M. B. Francis, *Nat. Chem. Biol.*, 2017, **13**, 697–705.
47 S. Billiet, K. De Bruycker, F. Driessen, H. Goossens, V. Van Speybroeck, J. M. Winne and F. E. Du Prez, *Nat. Chem.*, 2014, **6**, 815–821.
48 K. Decoene, K. Unal, A. Staes, K. Gevaert, J. M. Winne and A. Madder, DOI: 10.26434/chemrxiv.13739320.v1.
49 C. Sornay, S. Hessmann, S. Erb, I. Dovgan, A. Ehkirch, T. Botzanowski, S. Cianférani, A. Wagner and G. Chaubet, *Chem.–Eur. J.*, 2020, **26**, 13797–13805.
50 S. J. Walsh, S. Omarjee, W. R. J. D. Galloway, T. T.-L. Kwan, H. F. Sore, J. S. Parker, M. Hyvönen, J. S. Carroll and D. R. Spring, *Chem. Sci.*, 2019, **10**, 694–700.
51 J. J. Bruins, A. H. Westphal, B. Albada, K. Wagner, L. Bartels, H. Spits, W. J. H. van Berkel and F. L. van Delft, *Bioconjugate Chem.*, 2017, **28**, 1189–1193.
52 M. Moinpour, N. K. Barker, L. E. Guzman, J. C. Jewett, P. R. Langlais and J. C. Schwartz, *Protein Sci.*, 2020, **29**, 1784–1793.