Luminol Anchors Improve the Electrochemical-Tyrosine-Click Labelling of Proteins

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

New methodologies for the chemo-selective modifications of peptides and native proteins are of great importance 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 oxydations. 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 offer the interesting possibility for the double tagging of solvent-exposed Y.

Introduction

Protein bioconjugation is an extensively explored and ever-growing field of research owing to the inherently 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 may be achieved with an exquisite level of control by the site-selective incorporation of an unnatural amino acid in the polypeptidic chain with engineered aminoacyl-tRNA synthetase/tRNA (aaRS/tRNA) pairs.[9] These genetic code expansion techniques are new powerful tools but should be conducted in specialized laboratories. Thus, the chemical modification of native proteins, more easily produced in bulk, remains the most conventional and friendly labelling strategy. Chemical bioconjugations are still mostly performed on the 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 the conjugate and may also be detrimental for the 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 been recently described to overcome the initial limitations of Lys[10,11] and Cys[12,13] modifications, and much efforts are now dedicated to selectively target less exploited amino acids such as methionine,[14] tryptophan,[15,16] histidine[17,18] or tyrosine (Y).[19,20]

Y usually occur with low frequency in proteins (~3-4%) and are partially buried at the surface, offering unique opportunities for a controlled labelling of the most reactive residues.[21] The phenol side chain of Y is neutral at physiological pH and experience several post-translational modifications of biological relevance (phosphorylations, sulfations, nitrations, *O*-glycosylations, oxydations).[22] Thus, methods for Y-labelling have been increasingly reported during the last decade and efficient chemical strategies include the use of three-component Mannich-type coupling,[23] transition metal

[a] *Université de Nantes, CNRS, CEISAM UMR 6230, F-44000 Nantes, France. E-mail: sebastien.gouin@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*
† Electronic supplementary information (ESI) available.

complexes,[24,25] SuFEx chemistry,[26,27] aryldiazonium salts[28,29] and diazodicarboxyamides.[30–32]. The latter methodology is among the most promising and exemplary. Barbas and co-workers initially showed that silent phenylurazoles (PhUr) are readily activated to phenyltriazolinediones (PTAD) using chemical oxidizers (Method A, Fig.1) such as dibromodimethylhydantoin (DBDMH) or *N*-bromosuccinimide (NBS). Nonetheless, PTAD is known to be unstable in aqueous environment and to decompose into an electrophilic phenylisocyanate specie responsible for undesired side reactions on amino groups.[31,33] In a previous study, we developed the first electrochemical tyrosine bioconjugation coined eY-click (Method C, Fig.1).[34] Increased protein labelling and Y-selectivity could be achieved compared to the chemical approach, in conventional buffers, without the need for an isocyanate scavenger. Thus electrochemical methodologies offer interesting perspectives for the site-selective modification of proteins.[34–37] 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 the group of Nakamura (Method B & C, Fig.1).[36,38–42] NMePhUr and NMeLum were activated using ruthenium photocatalysts,[43] hemin and H_2O_2,[39] enzymatic systems (horseradish peroxidase and H_2O_2, laccase)[36,38,44] and electrochemically.[42] In this work, we aimed to evaluate and compare the relative efficiency of new or previously reported urazoles and luminols derivatives in the electrochemical-click labelling strategy (Fig. 1). The scope and efficiency of the eY-click protocol was significantly improved with NMeLum derivatives. A complete Y-selectivity is now achieved in a fully biocompatible procedure with faster kinetics and higher payload of the conjugate.

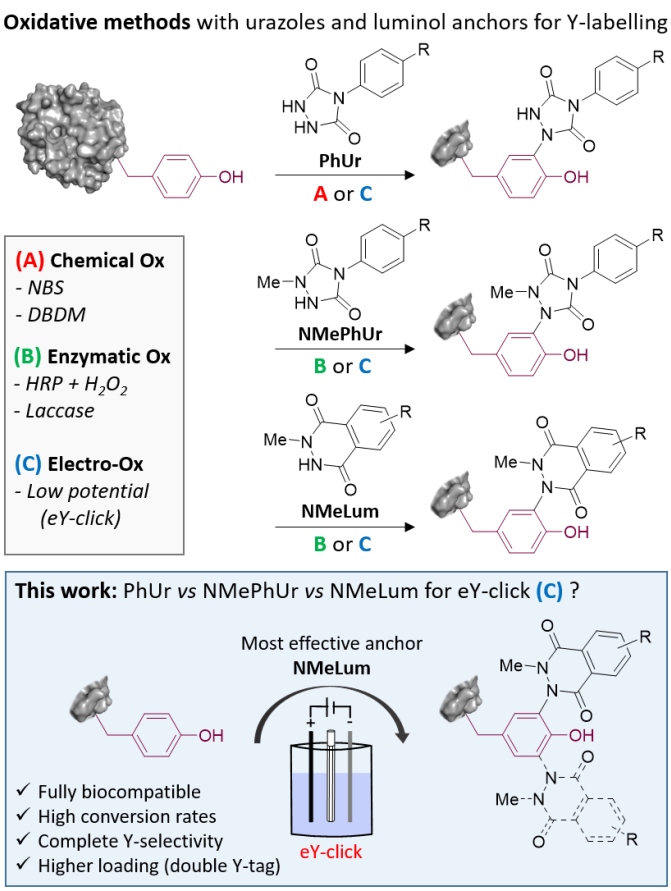


Fig. 1 PhUr, NMePhUr and NMeLum may be activated by chemical oxidation (Method A), enzymatically (Method B) or electrochemically (Method C). In this work we found that NMeLum are very efficient anchors for the soft electrochemical Y labelling of proteins.

Results and discussion

We first studied the electrochemical behaviour of PhUr **1**, new PhUr derivatives **2**-**6** (Synthesis in SI) and previously reported NMePhUr **7**-**8** and NMeLum **9**-**10** reagents (Fig. 2a).[42] The stability of their oxidized species was compared by cyclic voltammetry. Compound **1**-**6** were designed (synthesis described in Supporting Information) to assess if 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 electrodes 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**) didn't impact the oxidation potentials of the compounds ($\mathbf{O_1}$, 0.33V vs SCE) or the intensity of the reduction peak ($\mathbf{R_1}$), *i.e.* the stability of PTADs. 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. In 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 ($\mathbf{O_2}$, 0.52V vs SCE and $\mathbf{O_3}$, 0.62V 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 peaks intensities were observed over 20 cycles, confirming a high stability of the oxidized species. PhUr **1** is reported to generate PTAD *via* a two-electron chemical or electrochemical oxidation. [30,34] Interestingly, primary observations based on relative intensities of the oxidation peak of **1** and **7** ($\mathbf{O_1}$ vs $\mathbf{O_2}$), and relative stabilities of their oxidized specie ($\mathbf{R_1}$ vs $\mathbf{R_2}$) could support a single electron oxidation process for NMePhUr that may also prevail for NMeLum considering the similar *N*-methylhydrazide function.

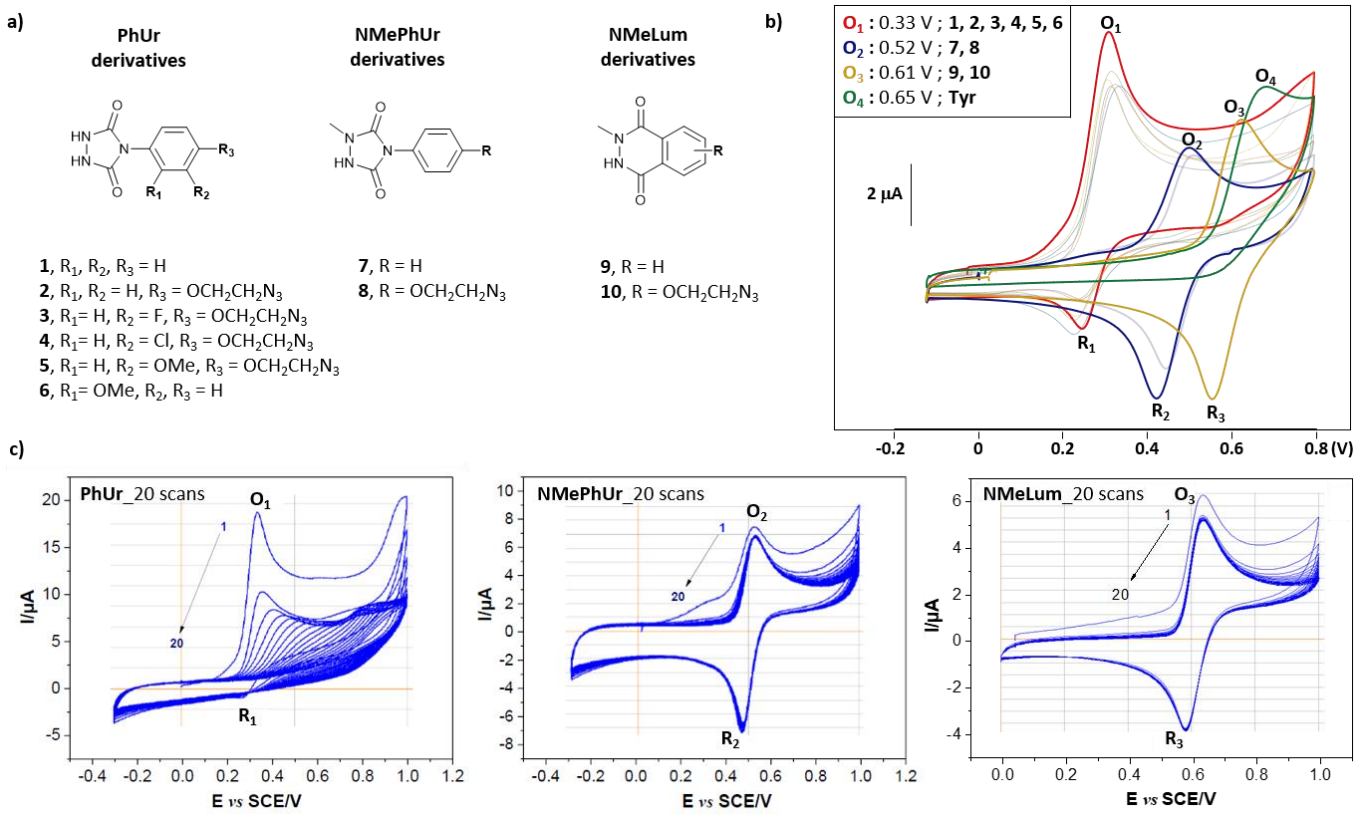
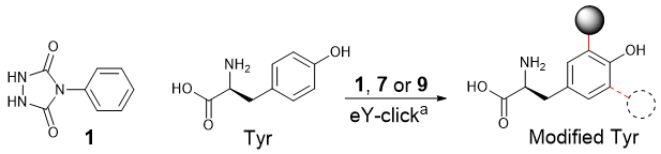


Fig. 2 Electrochemical behaviour of the studied Y anchors PhUr **1**-**6**, NMePhUr **7**, **8** and NMeLum **9**, **10**. a) Chemical structures of the compounds **1**-**10**, b) Cyclic voltammetry of **1**-**10** and Y (cathode: graphite carbon electrode 2 mm disc, anode: platinum wire, reference: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

As NMePhUr and NMeLum oxidation potentials shifted to higher values close to the one of Y (**O_4**, 0.65V vs SCE), we investigated if these two anchors could be selectively electro-oxidized in presence of Y. The Y-tagging efficiency was compared with the one observed with PhUr in eY-click conditions (Table 1). The electrolysis were conducted under stirring conditions, in phosphate buffer (PB) at pH 7.4 (100 mM). Appropriate working potentials were applied until 90% of theoretical charge given by 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 0.62V vs SCE led to a partial competitive Y oxidation. This issue was solved by switching PB for ammonium acetate (pH 7.4) as electrolyte. When two equivalents of reagents were used, Y-labelling with **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 highlight that electro-oxidized species of *N*-methyl derivatives **7** and **9** can tag Y in higher conversion than PTAD, and offer the perspective for 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 nonapeptide TAAQNLYEK bearing one Y and one lysine (K) and HAWQNLYEK bearing one Y, one K, one tryptophan (W) and one 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 for control experiment. The eY-click protocols were

Table 1. Electrochemical modification of Y

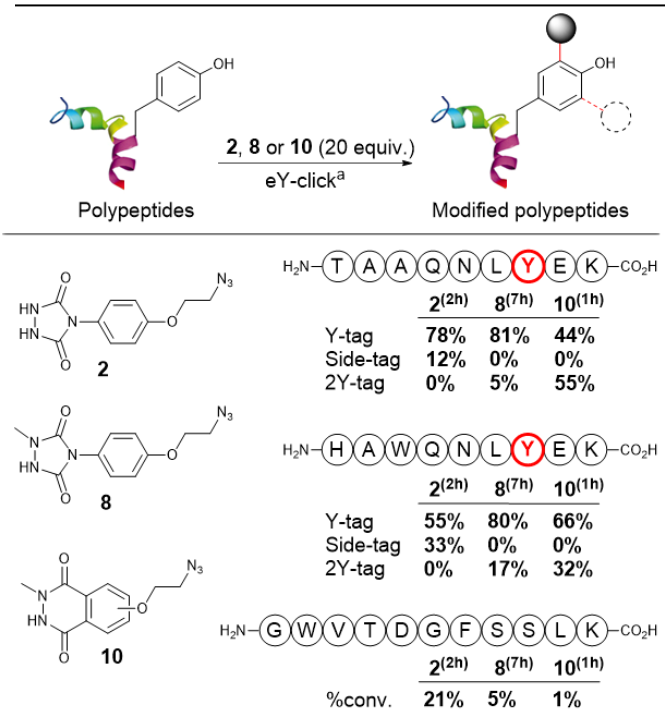


Entry	Reagent	Reagent:Y	Yield[b]
1	1	1:1	66%
2	7	1:1	84%
3	9	1:1	>91%[c,d]
4	1	2:1	94%
5	7	2:1	100%[c]
6	9	2:1	100%[c,d]

[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), 2-5h, [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.

performed with 20 equivalents of **2**, **8** and **10** (2mM), 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 2h electrolysis as determined by MS/MS fragmentation. *N*-terminus urea adducts (12%) from partial decomposition of **2** into an isocyanate was also observed. Longer electrolysis increased the urea adducts proportion (results not shown). As in previous experiments,[34] K adducts were not observed, which could be explained by a lower pKa value of the *N*-terminus amino groups, due to amide bond proximity.[45] 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 a complete Y-selectivity. The eY-click with NMeLum 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 1h. Thus, faster kinetics, complete Y selectivity, and higher level of double Y modification was 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** afforded 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.[46] A recent study showed the TAD-W instability and the kinetically favoured reactivity of

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, 2-7h. Location of conjugation determined by LC-MS/MS analysis.

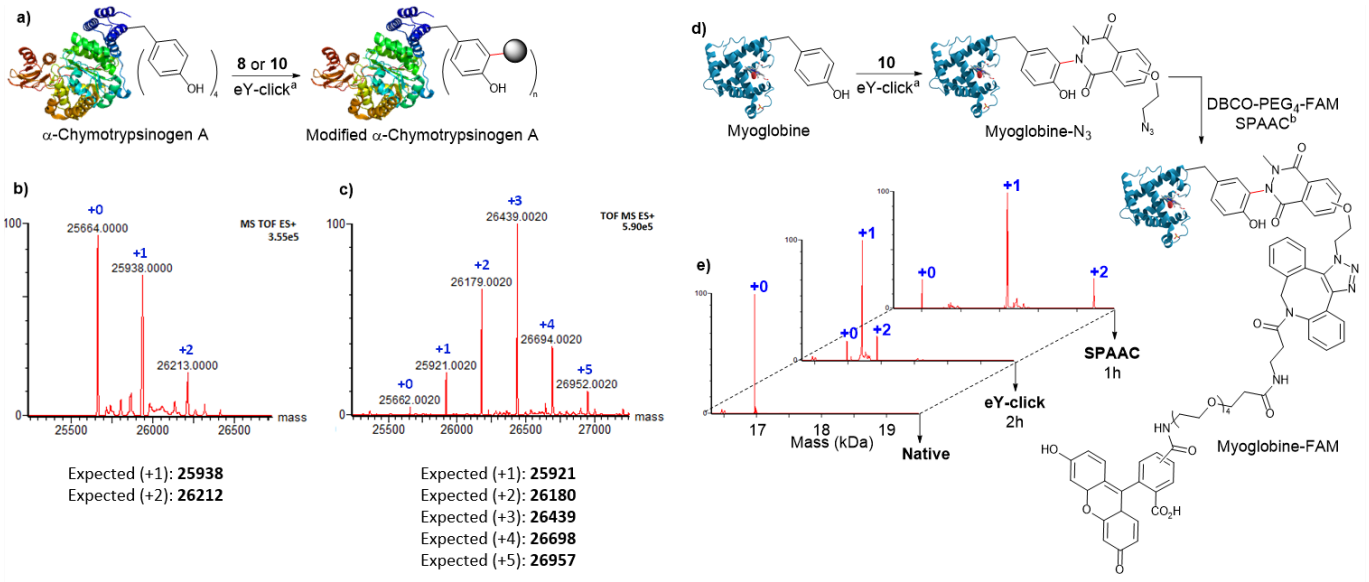


Fig. 3 eY-click protocol on proteins. a) Modification of α-Chymotrypsinogen A, [a] Reaction conditions: graphite plate anode, platinum plate cathode, constant voltage vs Ag/AgCl, modification reagent (5.0 μmol, 1.0 mM), α-Chymo (5.0 nmol, 1.0 μM), phosphate buffer (100 mM, 5.0 mL), 500 rpm, room temperature, 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, 1h, [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, 1h, e) Deconvoluted profiles (MS) evolution of Myo two-steps modification.

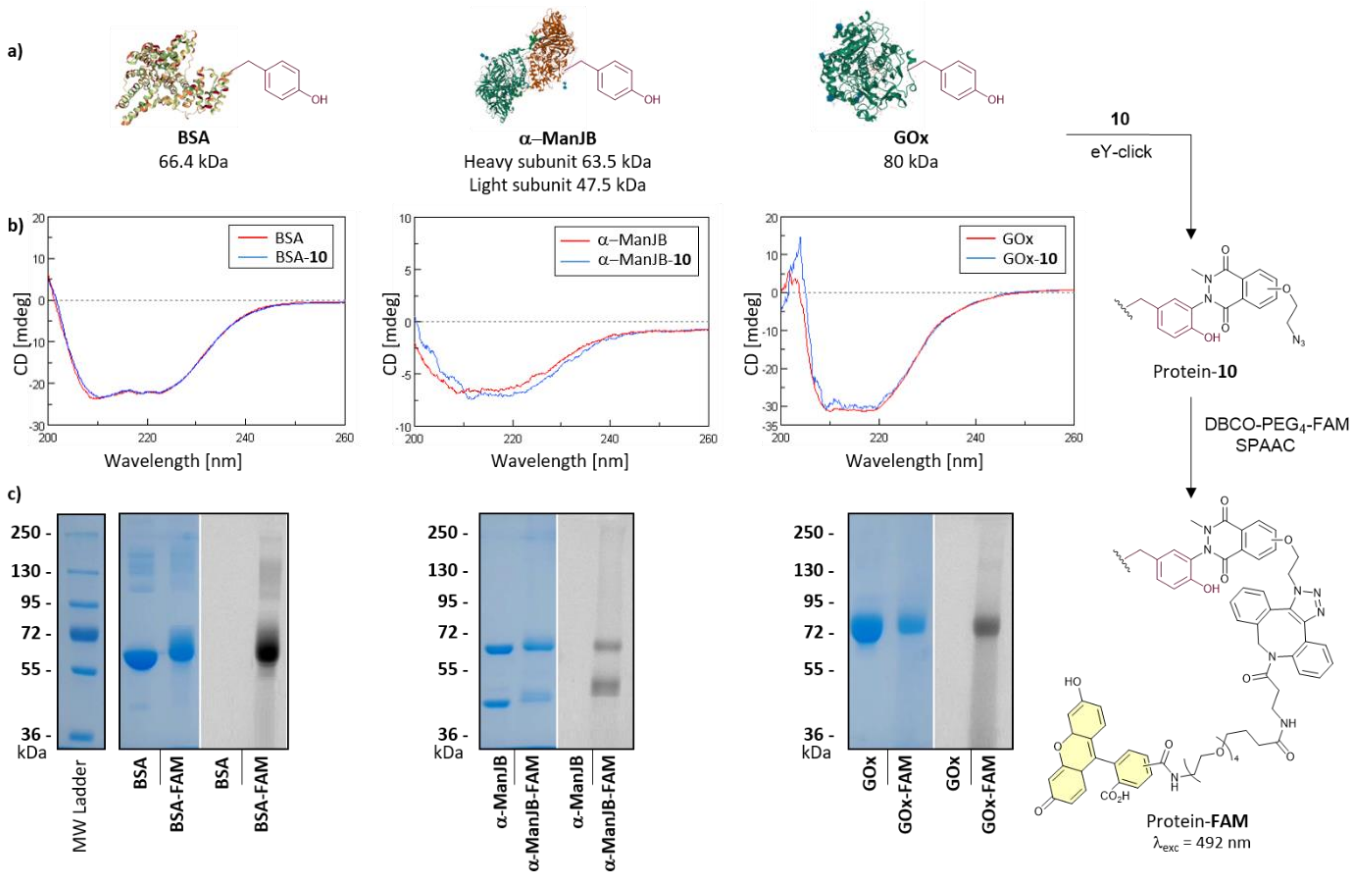
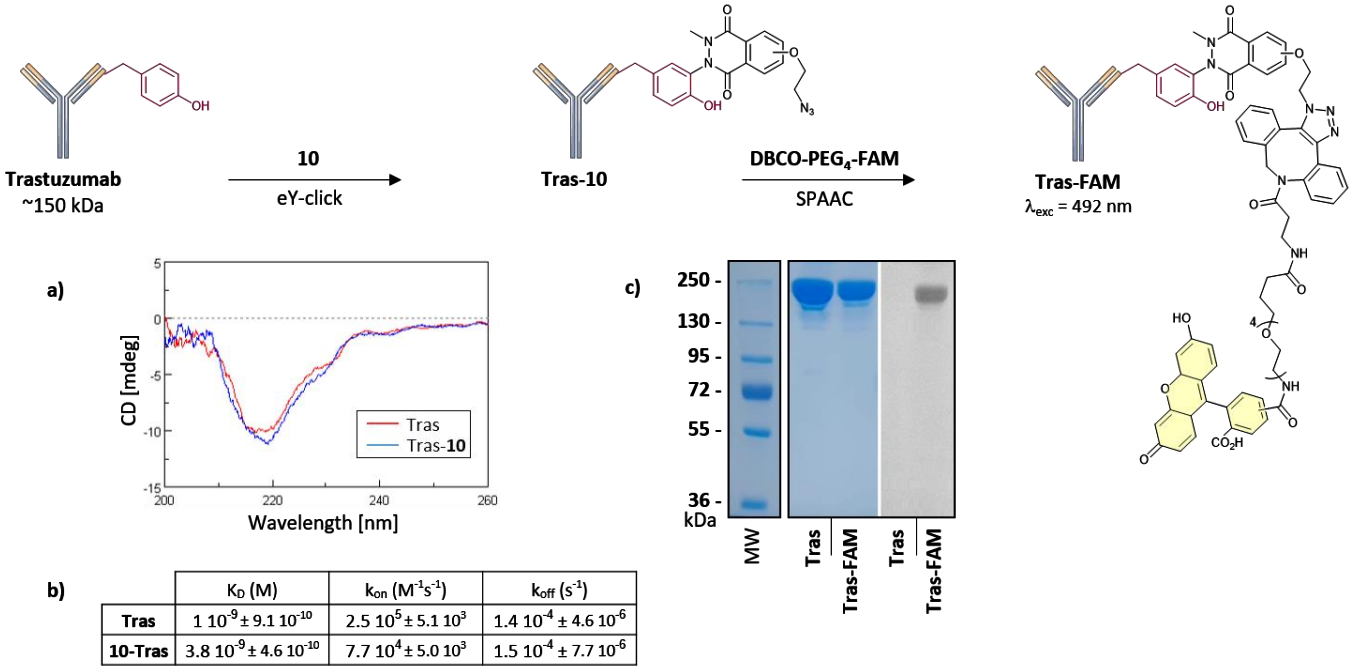


Fig. 4 a) Two-steps modification of BSA, α-ManJB and GOx. b) CD analysis of the native (red) and eY-click modified (blue) proteins with **10**. c) SDS-PAGE analysis of native proteins (from left to right: BSA, α-ManJB and GOx) and their FAM conjugates after eY-click with **10** followed by SPAAC with 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).

TAD for exposed W over Y, allowing a selective W modification on proteins.[47] Reagents **8** and **10** efficiently labelled (97-98% overall yields) the peptide with a complete Y-selectivity (K, H, W tagging never observed) and a Y-tag/2Y-tag ratios of 80:17 and 66:32, respectively. Again, a much faster kinetic was observed with **10**. Labelling experiments performed on control peptide GWVTDGFSSLK, missing a Y residue, confirmed the good to excellent inertness of electroactivated compounds **2**, **8**, **10** for other amino-acids with 21%, 5% and 1% of parent peptide conversion, respectively. Thus, peptide experiments evidenced a much higher eY-click labelling efficiency with NMeLum **10** and to a lesser extend NMePhUr **8** compared to the original methodology 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 bearing a limited number of four accessible Y, to easily track by MS analysis the Y adducts. The eY-click conjugations were performed on a low α-Chymo concentration (1 μM) during a fixed time of 4 and 1 h for **8** and **10**, respectively (Fig. 3a), according to the relative compound 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 evidenced by MS after 1 h with a nearly 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.[31] 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 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 (results in SI), 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 at 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.



b)

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

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 methodology (Fig. 4b). The obtained azido-armed proteins were further functionalized by SPAAC with DBCO-PEG_4-5/6-FAM during 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 proved unambiguously the formation of protein-FAM adducts without protein degradation (Fig. 4c).

Convenient bioconjugation techniques for antibody-conjugates development are still extensively explored and required in vectorised immunotherapies or cancer diagnostics.[48–50] We finally evaluated eY-click with **10** for antibody labelling. Trastuzumab (Tras, ~150kDa) 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 **10**-Tras conjugate was confirmed by circular dichroism (Fig. 5a), and SDS-PAGE evidenced successful subsequent FAM labelling with DBCO-PEG_4-5/6-FAM (Fig. 5c). The functional activity of the **10**-Tras conjugate for HER2 receptor was then 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 **10**-Tras. In these experimental conditions, Tras showed a nanomolar affinity for HER2 (dissociation constant K_D = 1.0 nM), and a high affinity for HER2 was measured for **10**-Tras 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 represent a soft and effective methodology for antibody labelling.

Conclusions

Y-labelling methodologies have been extensively explored to study Y post-translational modifications, protein conformations,[51] 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 the soft and time-controlled Y-labelling of native protein, not requiring chemical oxidant or enzymatic catalysis. Nevertheless, reaction kinetics was slower compared to chemical approaches and the present work evidenced that a complete Y-selectivity may not be fully achieved depending on peptide substrates. Even though the well-known PhUr degradation into electrophilic PhNCO is considerably limited during electrochemical activation, preventing lysine modification, a partial side-tagging of the more nucleophilic *N*-terminal amine of peptide was observed here with PhUr **7**. The aromatic amino acid side-chain of tryptophan was also not fully inert towards electrogenerated PTAD, as previously showed after chemical activation.[47]

In this work, we showed that a complete Y-chemoselectivity is now 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 coefficient diffusion 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.

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. and R. C. T. T. (chemistry & electrochemistry), M.C. (MS analysis), C.C. (DC & BLItz).

Conflicts of interest

There are no conflicts to declare.

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