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Electrochemically promoted tyrosine-click-chemistry for protein labelling.

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ABSTRACT: The development of new bioorthogonal ligation methods for the conjugation of native proteins is of particular importance in the field of chemical biology and biotherapies. In this work, we developed a traceless electrochemical method for protein bioconjugation. The electrochemically promoted tyrosine-click (e-Y-CLICK) allowed the chemoselective Y-modification of peptides and proteins with labeled urazols. A low potential is applied in an electrochemical cell to activate PTAD anchors *in situ* and on demand, without affecting the electroactive amino-acids from the protein. The versatility of the electrosynthetic approach was shown on biologically relevant peptides and proteins such as oxytocin, angiotensin 2, serum bovine albumin and epratuzumab. The fully conserved enzymatic activity of a glucose oxidase observed after e-Y-CLICK further highlights the softness of the method. The e-Y-CLICK protocols were successfully performed in pure aqueous buffers, without the need for co-solvents, scavenger or oxidizing chemicals, and should therefore significantly broaden the scope of bioconjugation.

INTRODUCTION

Bioorthogonal ligation (BL) methods are extensively explored for the development of protein conjugates,[1,2] which are of considerable importance in the therapeutic fields and in the biotechnology industry. Direct protein modifications are generally performed onto amino group of the abundant lysine amino-acid with *N*-hydroxysuccinimide-activated esters, sulfonyl chlorides or iso(thio)cyanates. Alternatively, the relatively rare cysteine residues can also be modified for single-site functionalization through disulfide exchange and Michael addition.[3] In comparison, the remaining 18 amino-acid have been much less exploited.[4]

Recently, click-like reactions specifically targeting the electron-rich tyrosine (Y) residues on native proteins have been developed. Y-labelling selectivity was achieved with π-allyl palladium complexes,[5,6] Mannich-type reactions,[7] metal-catalyzed oxidations,[8] rhodium salts and boronic acids,[9] single-electron-transfer catalysts,[10] ruthenium photocatalysts,[11] and small benzenediazonium species.[12,13,14]

Ene-like Y-modifications with cyclic diazodicarboxyamide[15,16] anchors such as *N*-methylluminol[17,18] or phenyl-urazol (Ph-Ur) derivatives[19,20] were also investigated by the groups of Nakamura and Barbas, respectively. While these approaches for Y-labeling are efficient and very promising, there is still a need to extend their potential scope. The use of chemical oxidants to generate the reactive intermediates may not be compatible with sensitive conjugates or proteins. 4-phenyl-3H-1,2,4-triazole-3,5(4H)-diones (PTAD) anchors obtained after Ph-Ur oxidation were also shown to be stable in acetonitrile but to decompose in the presence of water.[21] Phenylisocyanate (Ph-NCO) side-product resulting from PTAD decomposition leads to the unwanted modification of lysines.[20–22] The isocyanate could be scavenged in high concentrations of Tris (*tris*-(hydroxymethyl)aminomethane) buffer (100mM) to prevent the lysine conjugation,[21] but these conditions were not always fully effective and amino groups modifications were observed.[17] This cross-reactivity limited the scope of the Y-click reaction that should be performed with acetonitrile as co-solvent or in Tris buffer with excess Ph-Ur.

To date, only a few studies reported the electrochemical activation of urazols,[23] and all of them deal with the electrosynthesis of chemicals or the functionalization of electrode surfaces.[24,25,26] In this work, we explored the possibility of electrochemically activating Ph-Ur species in the presence of proteins such as a traceless Y-labeling method. We applied an appropriate electrochemical potential to activate the Ph-Ur dormant species *in situ*, on demand, without oxidizing the sensitive amino-acids from the protein or the ligands linked to the Ph-Ur (Figure 1A).

RESULTS AND DISCUSSION

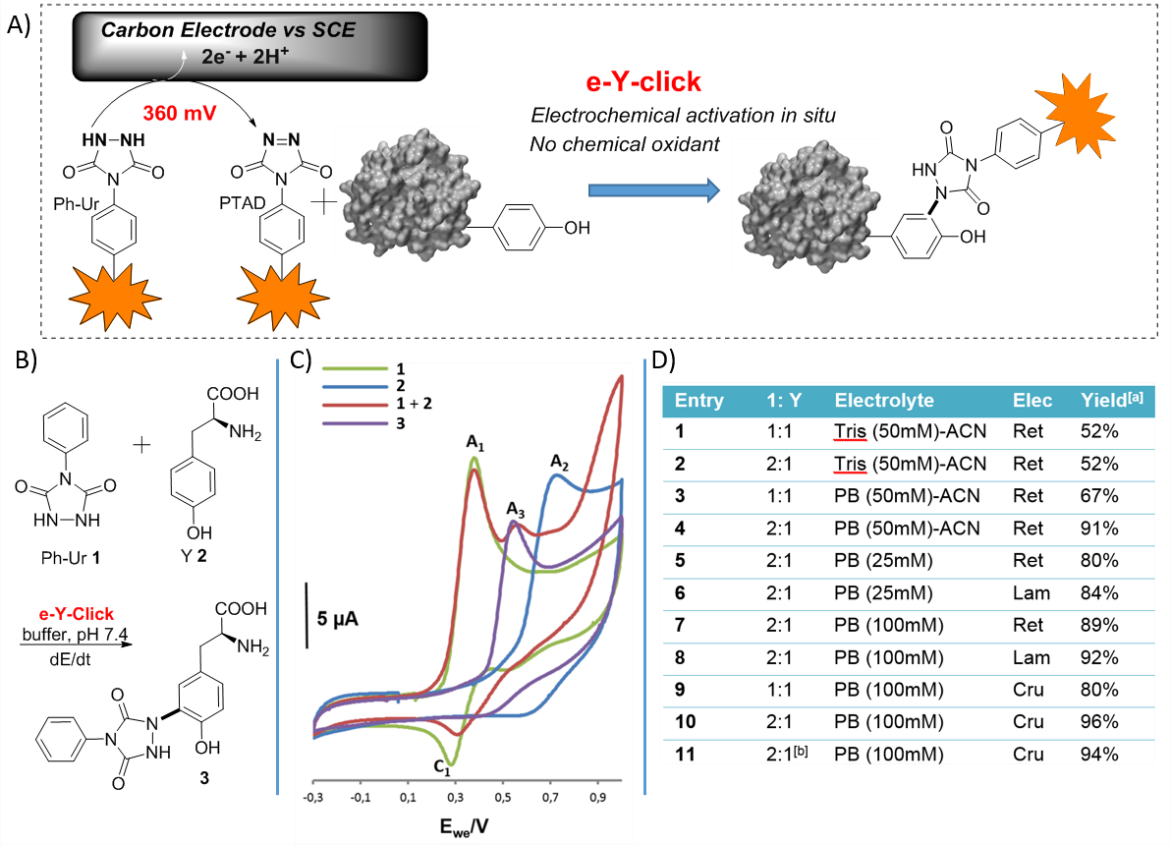
In a first assay (Figure 1B), we studied the electrochemical oxidation behavior of Ph-Ur analytically, in the presence of L-tyrosine (Y). The study was performed in a neutral media (pH = 7.4) for physiological relevance and in a mixture of Tris-acetonitrile (ACN) buffer as the electrolyte. As previously mentioned, ACN was shown to limit the degradation of chemically formed PTAD into isocyanate, and Tris is a scavenger of this side-product which limits lysine modification.[21]

Cyclic voltammograms of the isolated reagent phenylurazol **1** (Ph-Ur, quasi-reversible system $\mathbf{A_1/C_1}$ 0.36V/0.27V), Y **2** (non-

reversible system $\mathbf{A_2}$) and clicked compound **3** (chemically prepared, non-reversible system $\mathbf{A_3}$ (0.55V) were recorded (Figure 1C). When the same experiment was performed mixing equimolar quantities of Ph-Ur/Y the new anodic peak $\mathbf{A_3}$ corresponding to the conjugated oxidation product **3** appears at a more positive potential of 0.55V and the cathodic peak $\mathbf{C_1}$ at 0.27V, corresponding to the PTAD reduction, disappears during the reverse scan, indicating a chemical reaction on the surface of the electrode. The anodic peak $\mathbf{A_2}$ for Y **2** also disappeared in the presence of Ph-Ur **1**.

Fortunately, the oxidation of both the phenol moiety present on the Y ($\mathbf{A_2}$) and the clicked product **3** ($\mathbf{A_3}$) appear at even more positive potential from Ph-Ur oxidation ($\mathbf{A_1}$), making the selective electrochemically activation of the PTAD possible. Thus, this analytical study shows that the selection of an appropriate potential of 0.36V (**A1** peak), accomplishes the e-Y-Click reaction without oxidizing the Y reagent or the clicked product **3**.

The electrosynthesis experiments were optimized and carried out using coulometry technique (Figure 1D) at controlled-potential (0.36 V *vs* SCE). A conventional three-electrode system was employed under stirring conditions. A graphite (reticulated, laminar or crucible) electrode (see Table S1) was used as the working electrode, a platinum as the counter-electrode and a saturated calomel electrode (SCE) as the reference electrode. The e-Y-click was stopped after the effective consumptions of 2 mol e- per mol of PTAD (around 2h, Figure S1B). Phosphate buffer (PB), PB-ACN and Tris-ACN mixtures were used as electrolytes (pH = 7.4). We were pleased to see that the highest conversion yields were obtained in pure PB without the need for a Ph-NCO scavenger. The reaction yields were even higher in this medium compared to Tris-ACN. This could be explained by a better transfer of charge with the phosphate ions. The Ph-NCO side-product was not observed, even when conducting the e-Y-click with one equivalent of L-lysine in the media as a nucleophilic trap (Entry 11). The influence of the Y:Ph-Ur stoichiometry and of the carbon working electrode (reticulated, laminar or crucible, see details in Table S1) were also evaluated, with the best yields (96%) obtained for the crucible with Y: Ph-Ur = 1:2 (Entries 7, 8 and 10). The small yield improvement observed with the crucible electrode may be the result of a higher available area.



Entry	1: Y	Electrolyte	Elec	Yield[a]
1	1:1	Tris (50mM)-ACN	Ret	52%
2	2:1	Tris (50mM)-ACN	Ret	52%
3	1:1	PB (50mM)-ACN	Ret	67%
4	2:1	PB (50mM)-ACN	Ret	91%
5	2:1	PB (25mM)	Ret	80%
6	2:1	PB (25mM)	Lam	84%
7	2:1	PB (100mM)	Ret	89%
8	2:1	PB (100mM)	Lam	92%
9	1:1	PB (100mM)	Cru	80%
10	2:1	PB (100mM)	Cru	96%
11	2:1[b]	PB (100mM)	Cru	94%

Figure 1 A) The electrochemical activation of urazol species *in situ* (e-Y-click). B) e-Y-click protocol with Ph-Ur **1** and L-Tyr **2** C) Cyclic voltammograms of 1.0 mM Ph-Ur (**1**) in the absence (green) and in the presence (red) of 1 mM Y (blue) and of 1 mM isolate conjugated product **3** (purple) at a glassy carbon electrode, in 50 mM TRIS / ACN (50/50), pH 7.4. Scan rate: 100 mVs^{-1}, reference electrode SCE. D) Conversion yields after 2h of controlled-potential coulometry as determined by 1H NMR (Figure S1C). The electrolysis was performed with 1.0 mM of Y in the presence of 1.0 or 2.0 mM Ph-Ur (**1**) using different graphite forms as the working electrode. [b] 1.0 mM of L-lysine was added in the media to potentially scavenge the Ph-NCS side product (not observed). Elec = Electrode, Ret = Reticular, Lam = Laminar, Cru = Crucible.

Next we investigated the chemoselectivity of the reaction with regards to other potentially oxidizable amino-acids bearing aromatic or heteroaromatic residues (Figure 2A). Cyclic voltammograms of Ph-Ur **1** were recorded in the presence of a stoichiometric amount of commercially available protected

(p) L-tyrosine, L-phenylalanine, L-tryptophan, L-histidine or L-lysine. Only L-tyrosine ($\mathbf{A_Y}$ = 0.6 V) and L-tryptophan ($\mathbf{A_W}$ = 0.7 V) are electroactive at the studied potential range (-0.3 V – 1 V). As seen in Figure S1F the two amino-acids start to oxidize at around 0.45 and 0.5V, respectively, which is not problematic considering the lower potential of 0.36 V (Ph-Ur oxidation peak) applied to generate the PTAD species. Notably, all aminoacids except L-tyrosine were inert with PTAD and no electrochemical-chemical mechanisms were detected. The occurrence of a chemical reaction would spawn the disappearance of the cathodic peak $\mathbf{C_1}$, indicating that the PTAD formed on the surface of the electrode is consumed. This result opens perspectives for the chemoselective *in situ* electrochemical Y-labeling of peptides and proteins.

The e-Y-click protocol was subsequently evaluated on the neuropeptide hormone oxytocin (Figure 2B) in various biological roles in the childbirth process, human behaviors and social bonding. Oxytocin was selected as a nonapeptide bearing a single Y and a disulfide bridge.

The electrolysis was performed in the presence of 2 eq Ph-Ur **1** in PB using a crucible carbon electrode at 0.36V and monitored by cyclic voltammetry showing the complete Y conversion in less than 2 hours (a decrease in the anodic peak). The kinetic reaction was also followed by HPLC-MS (Figure S2) and the labeled peptide was obtained as a pure compound in approximately 99% conversion yield after 2h.

The e-Y-click was applied in optimized conditions to label angiotensin II, a hormone regulating blood pressure (Figure 2C). When commercially available Ph-Ur (**1**) was used, the conjugated compound was obtained in 83% conversion, with 17% of starting material recovered, and no degradation during the reaction (see kinetics by HPLC, Figure S3). More interestingly, when the e-Y-click reaction was carried out using 4-(4-(2-Azidoethoxy)phenyl)-3H-1,2,4-triazole-3,5(4H)-dione (**4**),[20] the conjugated peptide was obtained in 73% conversion. This azide-armed peptide can be later functionalized by copper-free click cycloaddition reactions (SPAAC), giving other perspectives to biorthogonal tagging.

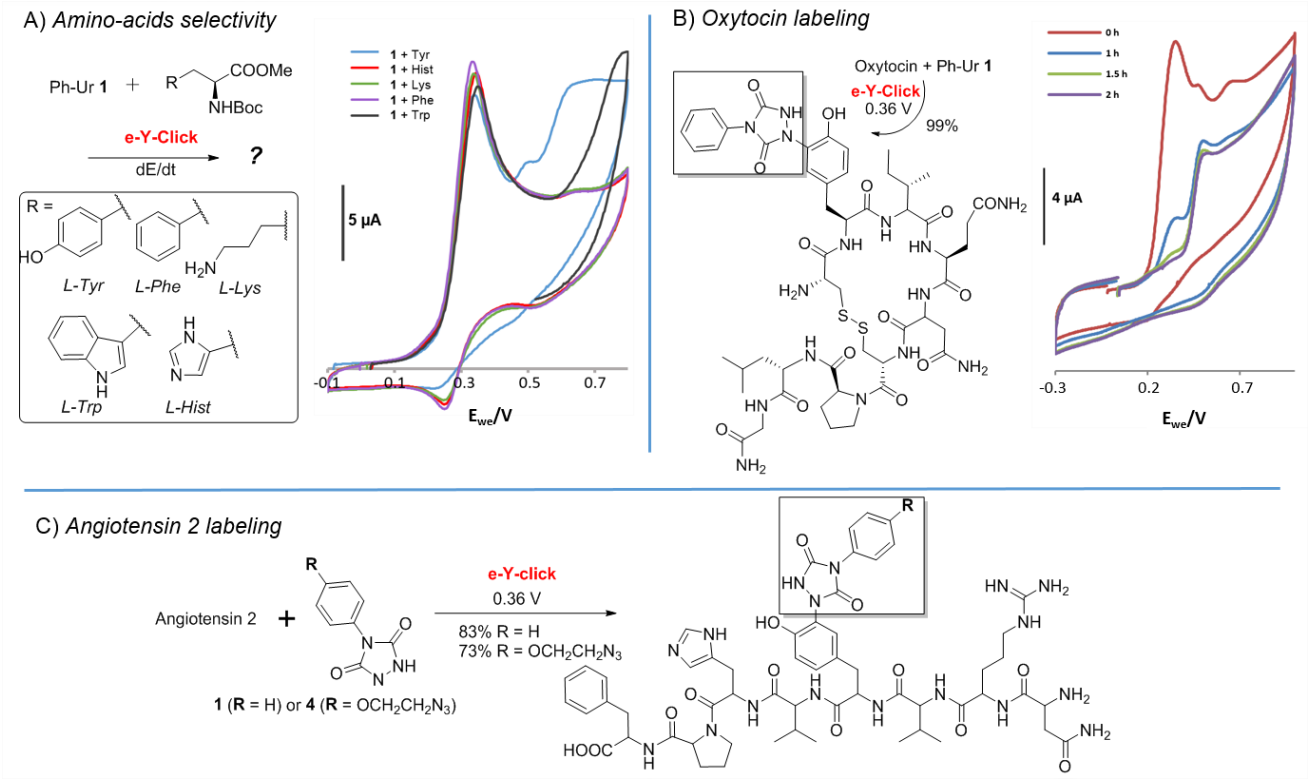


Figure 2 A) L-Tyr chemoselectivity of the e-Y-click. Cyclic voltammogram of 1.0 mM Ph-Ur (**1**) in the presence of 1mM of a) L-tyrosine, b) L-phenylalanine, c) L-tryptophane, d) L-histidine and e) L-lysine, at a glassy carbon electrode, in 50 mM TRIS / ACN (50/50), pH 7.4. Scan rate: 50 mV s-1. B) The e-Y-click protocol for 0.5 mM oxytocin labelling in presence of 1 mM Ph-Ur (**1**). B) Cyclic voltammograms at a glassy carbon electrode during controlled-potential coulometry at 0.36V versus SCE. After 0, 1, 1.5 and 2 h. Scan rate: 100 mV s^{-1}. C) The e-Y-click protocol for angiotensin II labeling. In the presence of 2 equiv of Ph-Ur (**1**) or 2 equiv of N_3-Ph-Ur (**4**) by controlled-potential coulometry at 0.36V versus SCE. c) HPLC analysis of the reaction media after two hours showed 83 and 73% conversion after 2h respectively (Figure S3).

The e-Y-click was next evaluated on human insulin, a two chain polypeptide bearing 51 amino acids including four tyrosines, one lysine (putative side reaction) and three disulfide bridges (Figure 3A). The electrochemical reaction was conducted in the presence of a varied amount of Ph-Ur **1** (0, 1, 4, 10 eq per insulin) for 2h. Samples were then reduced, alkylated and trypsin digested prior to UPLC-MS and UPLC-MS/MS analyses (Figures S4, S5). UPLC-MS analyses were

also performed on undigested samples to estimate the number of Ph-Ur grafted to the whole peptide for each condition. Then, UPLC-MS analyses were achieved on samples after proteolysis to confirm and refine observations. Trypsin digestion led to the formation of 3 major peptides (GIVEQCCTSICSL**Y**QLEN**Y**C, GFF**Y**TPK, and NFVNQHLCGSHLVEAL**Y**LVCGER) carrying two Y, one Y and one K, or one Y residues, respectively. Fragmentation patterns obtained in MS/MS mode ascertained which amino-acid residue was modified with Ph-Ur as well as the quantification of the number of Y modified in function of the amount of Ph-Ur (0, 1, 4 or 10 eq). The proportion of the average number of modified Y (0 to 4) in function of Ph-Ur **1** is shown Figure 3B. Insulin proportion was shown to decrease with increasing amounts of **1** to reach less than 10% in the media when using 10 equivalents of **1** (0xY, Figure 3B). The high concentration of mono-clicked peptides (1xY in green) rapidly reached a stable threshold of around 40% suggesting a higher reactivity and availability of a specific Y. In that respect, our MS/MS data suggested that Y108 and Y40 could respectively be the most and the least reactive Y residues (Figure S5). The reaction showed a complete Y chemoselectivity and unspecific labeling of lysine (K53) was not observed.

Bovine Serum Albumin (BSA), a 66 kDa protein was chosen as a more complex system bearing 21 Y. The e-Y-click protocol could easily be scaled to functionalize up to 100 mg of modified BSA in a single batch using the 50 mL electrochemical cell. However, a limitation of the method would be the functionalization of small quantities of material (< 1 mg) which would require specific miniaturized electrochemical cells or flow cells. Previous Y-BSA modifications with luminol derivatives (100 equiv) in the presence of H_2O_2 (100 equiv) and hemin (1 equiv) showed site-specificity in Y labelling.[17] Modifications were shown to occur at 8 specific Y out of the 21 available Y. The authors also observed a double modification at Y400 which is one of the most exposed residues on BSA surface. Here, the BSA : Ph-Ur **1** ratio was varied from 1:0 (reference) to 1:30 The gradual increase in BSA: Ph-Ur **1** ratio led to a higher BSA modification up to an average of 9.1 clicked Y (Table 1). The level of Y modifications can therefore be tuned by adjusting the amount of Ph-Ur **1**. When the labeling experiment was performed in strictly similar conditions using a chemical oxidant (1,3-Dibromo-5,5-dimethylhydantoin) instead of the electrochemical potential, the number of labeled Y on the BSA more than halved (from 9.1 to 3.7). Thus, high levels of Y modification, surpassing the chemical approach, were reached with the e-Y-Click protocol. The faradaic efficiency ef_F was calculated for the Ph-Ur **1** oxidation using the faraday law ($ef_F = \frac{n.N.F}{Q}$) where n is the number of electrons exchanged, N the number of moles for Ph-Ur **1**, F Faraday's constant (96485 C/mol) and Q the charge passed (C). A high ef_F of around 90-100% was observed independently of the Ph-Ur **1** concentration (5 to 100µmol) which is indicative of the absence of surface passivation during the experimental time (two hours, Figure S6A).

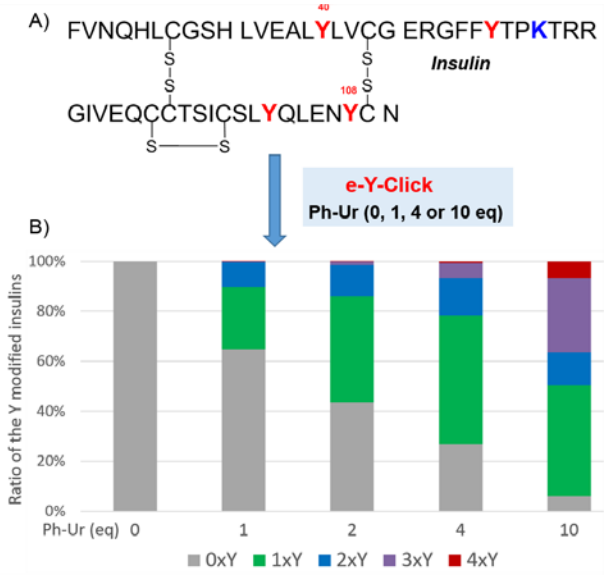


Figure 3 A) Structure of native insulin. B) % of mono- to tetra-clicked PTAD adducts in function of Ph-Ur equiva-lents. A penta-clicked product (4Y + 1K = 5) was not observed further highlighting the e-Y-Click chemoselectivity for the 4 Y.

The BSA samples were further studied by protein mapping to detect the modified Y. Sixteen peptide candidates with at least one Y were identified in silico (web.expasy.org/peptide_mass/) but only two of them (HPEY^{374}AVSVLL R and LGEY^{424}GFQNAL IVR) could be found experimentally by LC-ESI-HRMS analysis after protein proteolysis. The ratio of functionalization for Y^{374} and Y^{424} was 30% and 10%, respectively, which is indicative of site-selectivity in function of Y exposition at the protein surface.

Table 1. e-Y-click optimization during controlled potential coulometry.

Entry	BSA: **1**	Method	MW	Mod Y[a]
1	1:0	e-Y-Click	66443	0
2	1:5	e-Y-Click	66769	1.9
3	1:10	e-Y-Click	66894	2.6
4	1:20	e-Y-Click	67058	3.5
5	1:30	e-Y-Click	68035	9.1
6	1:30	Chemistry	67084	3.7

[a] the average of Y modified was determined by LC-ESI-HRMS analysis.

Next, we investigated the possibility of grafting two different chemical functionalities on the BSA protein in a sequential approach. We carried out the e-Y-click using mannosyl derivative **5** (synthesis in Scheme S1) and/or the azido-functionalized compound **4** (Figure 4). Carbohydrates are of primary importance in the design of conjugated vaccines,[27] and N_3-functionalized proteins (**N_3-BSA** here**)** are interesting platforms for anchoring epitopes, ligands, chelators, or probes by Staudinger reactions,[28] conventional copper-catalyzed cyclization,[29,30] or copper-free SPAAC with cyclooctyne derivatives.[31,32] BSA protein was first efficiently labeled with N_3-

PTAD **4** with an average of x = 3.7 modified Y by **N_3-BSA**. In the same way, Man-PTAD **5** was successfully anchored to the BSA to form the glycoconjugated **Man-BSA** bearing 2.7 mannose residues as evidenced by mass spectrometry and western blotting (indirect detection with labeled concanavalin A, Figure 4). The small difference observed in the grafting levels (x and y) with **4** and **5** may be explained by different diffusion coefficients of the compounds at the electrode surface. The bi-functional conjugate **Man-N_3-BSA** was obtained using a 50/50 ratio of phenylurazol (**4**/**5**) during electrolysis (x+y = 4.4). In all cases, SDS-PAGE followed by silver staining, showed that BSA was modified without inducing protein degradation. Secondly, **N_3-BSA** and **Man-N_3-BSA** could by labeled by a commercially available fluorescent FAM probe (DBCO-PEG4-5/6-FAM) by SPAAC. The formation of the **FAM-BSA** and **FAM-Man-BSA** was unambiguously detected in the western blot using an anti-FAM antibody.

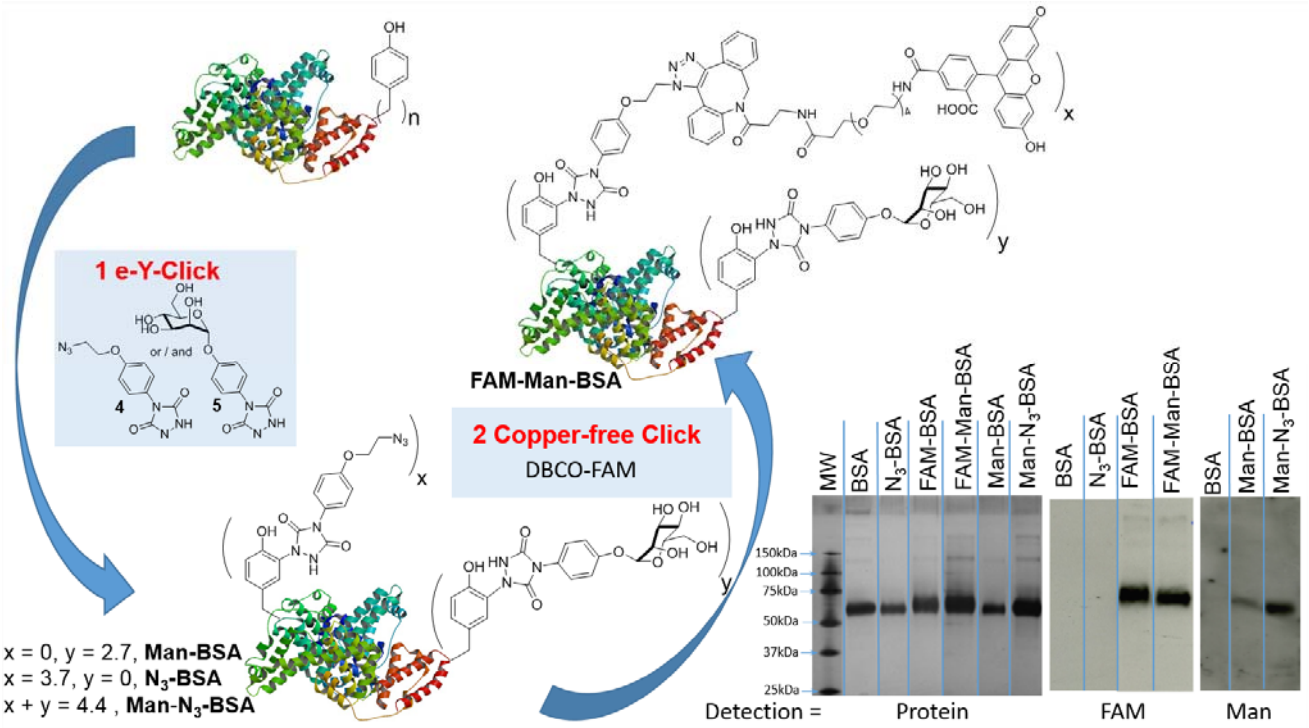


Figure 4 The e-Y-click protocol for BSA labeling. The formation **Man-BSA**, **N_3-BSA** and **Man-N_3-BSA** was successfully achieved using e-Y-click as evidenced by mass spectrometry (SI) and western blot using silver staining, or a labelled mannose-binding lectin (Concanavalin A = ConA). The N_3 group could be further functionalized by the fluorescent FAM probe (detection with anti-FAM antibody) using the commercially available DBCO-PEG4-5/6-FAM probe in a copper-free click-chemistry protocol.

New bioconjugation techniques are being researched to develop more effective antibody-drug conjugates (ADC) for vectorized immunotherapy. ADC combines a specific monoclonal antibody (mAb) acting as a "magic bullet" for the selective delivery of covalently linked cytotoxic compound(s) in the tumoral environment. To assess the applicability of the e-Y-click protocol for mAb functionalization, **4** was reacted with epratuzumab, a humanized anti-CD22 mAb which has previously shown promising clinical activity in patients with non-Hodgkin's lymphoma.[33] Satisfyingly, a shift of the epratuzumab molecular weight, corresponding to an average grafting of 2.3 phenylurazol **4** molecules, was evidenced by ESI-MS (Figure 5).

Finally, we evaluated the softness of the e-Y-Click, by checking if an enzymatic activity could be retained after the electrochemical coupling. Glucose oxidase enzyme (GOx) was selected as a biomedically relevant target used for blood glucose monitoring and in cancer diagnosis and treatment.[34] GOx catalyze the oxidation of D-glucose into D-glucono-1,5-lactone, and oxygen is reduced to hydrogen peroxide. Thus, GOx kinetics can be followed by hydrogen peroxide detection using a well-established cascade with horseradish peroxidase (HRP). The HRP uses H_2O_2 to oxidize the substrate o-dianisidine (3, 3'-dimethoxybenzidine) into an oxidized yellow-orange product which is detected spectrophotometrically at λ= 436 nm (Figure 6A).[35] GOx was electrochemically labeled with **4** (Figure S9) but a strong matrix effect, probably due to the presence of *N*-glycosylated GOx isoforms, prevented the accurate determination of the average number of modified Y.

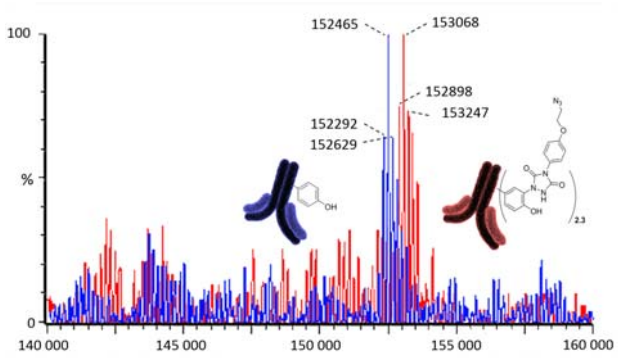


Figure 5 The e-Y-click protocol for antibody labeling. Epratuzumab was efficiently labeled with **4** as evidenced by the mass shift in deconvoluted mass spectra (native epratuzumab in blue, epratuzumab-conjugated in red).

Importantly, the enzyme kinetic of the GOx was not affected by the e-Y-click protocol and native GOx and **4**-GOx showed a virtually identical conversion rate of the glucose substrate (Figure 6B). Thus, the electrochemical protocol can be applied to enzymes without damaging their functionality, even if a redox active coenzyme (flavin adenine dinucleotide FAD for GOx) is buried in the active center. This can be explained by the low accessibility of the coenzyme and by the low coefficient diffusion of proteins at the electrode surfaces.

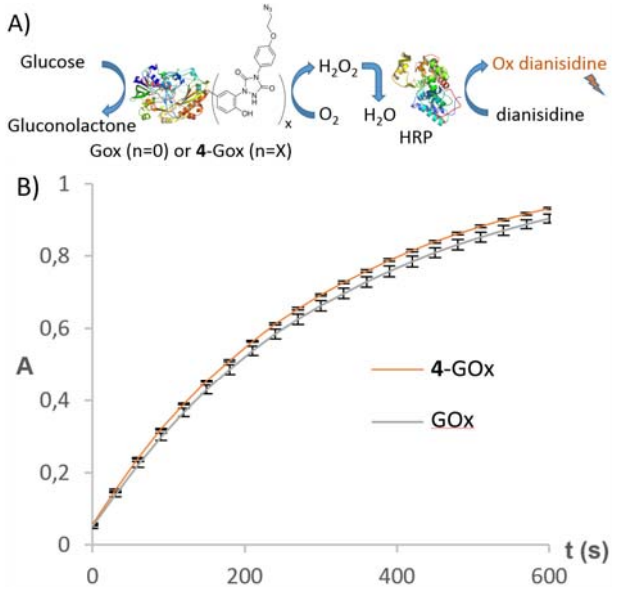


Figure 6 A) Reaction cascade to follow the GOx kinetic by detecting spectrophotometrically the oxidized dianisidine products. B) Absorbance profiles in function of time obtained with the native GOx and e-Y-click modified **4**-Gox. Average of three measurements.

CONCLUSION

As far as we know, this is the first report of a direct protein labelling methodology promoted electrochemically. This represents a valuable complementary approach to the chemical Y-Click methodologies that have recently emerged for the native bioconjugation of proteins. The urazol anchor is now activated *in situ*, in purely aqueous media and without the need of chemical oxidizers. Specific issues including lysine modifications due to PTAD decomposition, double Y modifications and thiols oxidations were not observed. The e-Y-Click protocol may therefore complement the current arsenal of techniques for the traceless preparation of a wide range of peptide and protein conjugates.

ASSOCIATED CONTENT

Supporting Information. "This material is available free of charge via the Internet at http://pubs.acs.org."
Characterization details and general procedures for e-Y-click.

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Notes

The authors declare no competing financial interest.

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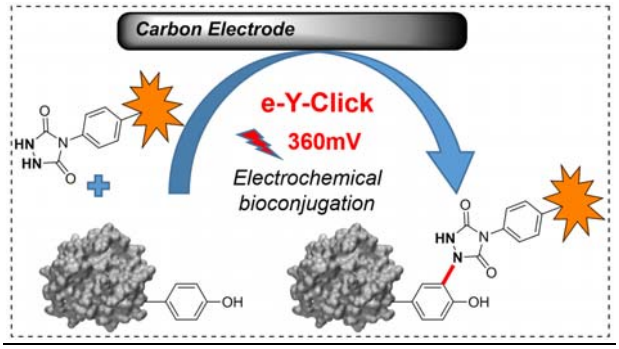
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