Electrochemical Tryptophan-Selective Bioconjugation

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Abstract

Bioconjugation reactions are a fundamental synthetic method for generating artificial peptides and proteins. Despite the potentially superior properties of bioconjugates at hydrophobic amino acid residues compared with those at hydrophilic amino acids, methods to target hydrophobic amino acids with moderate reactivity under mild and metal-free conditions are limited. Here we report the first electrochemically-promoted tryptophan (Trp)-selective bioconjugation of peptides and proteins in neutral aqueous media. The unique electrochemical cooperation of two radicals, keto-ABNO and 4-oxo-TEMPO, was critical to suppress both anodic overoxidation of the products and cross reactivity. Systematic cyclic voltammetry analysis suggested that these two radicals, containing similar redox potentials but contrasting steric demands, had distinct electrochemical roles (reactant and electrochemical mediator). This new protocol will be an important advance toward clean and scalable syntheses of chemically modified biologics.

Main Text

Bioconjugation reactions targeting proteinogenic amino acids are particularly important for conferring supernatural functions to peptide/protein biomolecules, facilitating the development of novel therapeutics, diagnostics, and biomaterials.[1] In recent years, bioconjugation methods targeting poorly nucleophilic, less surface-exposed hydrophobic amino acid residues (e.g., tyrosine,[2-4] tryptophan,[5,6] and methionine[7]) have attracted attention. Applying such synthetic methods to bioconjugates will enhance their homogeneity and chemical space, thereby improving their properties/efficacies and expanding their application scopes. The requirements for practical bioconjugation reactions are as follows: (1) selectivity toward a targeted functional group of a specific

proteinogenic amino acid, (2) selective activation of the bioconjugation reagents over numerous reactive functional groups in biomacromolecule substrates, (3) feasibility under physiologic conditions (mild pH and temperature, aqueous media, and low concentration), and (4) avoidance of excess reagents and toxic metallic reagents that would cause undesired cross reactivities and cumbersome purifications. Satisfying these requirements is far more challenging when targeting less reactive hydrophobic amino acid residues than when targeting nucleophilic amino acid residues such as lysine and cysteine.

Merging electrochemical organic synthesis[8] and bioconjugation chemistry is an attractive strategy toward achieving these aims. Traditionally, electrochemical protein modifications in preparative scales have been limited to direct electrolysis for cysteine-cystine interconversions, site-selective cleavage, and oxidative functionalizations.[9] In 2018, Gouin and co-workers reported the first electrochemical protein bioconjugation by targeting tyrosine (Tyr) residues with a urazole reagent.[3] The electrochemical conditions significantly improved the chemical yield and selectivity of Barbas' original conditions using a chemically preactivated reagent.[2] We report herein an electrochemical tryptophan (Trp)-selective bioconjugation. The hybrid use of two N-oxyl radicals, keto-ABNO and 4-oxo-TEMPO, was critical for the facile and high-yielding bioconjugation. In contrast to our original report using chemical activation,[5] the electrochemical reaction developed here proceeded under neutral pH with remarkably few side reactions.

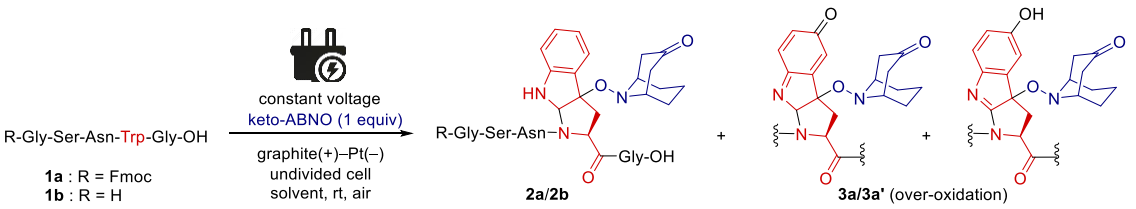
We previously reported a transition metal-free, Trp-selective bioconjugation using a sterically less-demanding organoradical, 9-azabicyclo[3.3.1]nonan-3-one-*N*-oxyl (keto-ABNO), in the presence of substoichiometric $NaNO_2$ in mildly acidic (pH ~ 3) aqueous media.[5] The active species of this bioconjugation is oxoammonium cation **A** (see Scheme 3) generated in situ through single-electron aerobic oxidation of keto-ABNO. Nitrogen oxide (NO_x) produced from $NaNO_2$ and AcOH under air chemically activated keto-ABNO and promoted single-electron oxidation. Despite the easy operation and broad substrate generality, the following features of the previous chemical activation conditions are potentially problematic: (1) an acidic media that potentially hampered the application to pH-sensitive proteins, and (2) NO_x-induced *S*- and *N*-nitrosation side reactions. Encouraged by the rich chemistry of *N*-oxyl radicals as electrochemical mediators,[10] we anticipated that anodic electrochemical oxidation would promote the activation of keto-ABNO, leading to Trp-selective bioconjugation under neutral pH without the use of any external oxidants (e.g., NO_x) that could cause undesired side reactions.

On the basis of this hypothesis, we initiated optimization studies of electrochemical bioconjugation of Fmoc-protected peptide 1a as a model substrate using an ElectraSyn 2.0 kit (Table 1). Product **2a** was produced in only 33% yield when a mixture of **1a** (3 mM) and keto-ABNO (1 equiv) in an electrolyte solution (50 mM TBAP in CH_3CN-H_2O,1:1) was stirred for 1 h without electric current (entry 1).[11] The application of constant voltage electrolysis (working electrode: graphite,

counter electrode: platinum) at 1.2 V *vs* Ag/AgCl to this solution for 1 F/mol (ca. 1 h), however, markedly increased the yield of **2a** to 75% with 18% of **1a** recovered (entry 2). As the amount of electric charge increased, **1a** was completely consumed, but the amounts of overoxidation products **3a**/**3a'** increased (entries 3 and 4). The adduct of Trp–keto-ABNO had a lower oxidation potential than that of keto-ABNO.[12] Thus, electrolysis at a lower potential (1.0 V) successfully suppressed the overoxidation, but the yield of **2a** decreased (55%, entry 5). Application of 2.0 F/mol electric charge increased the yield (78%, entry 6), but further application of electricity (>2 F/mol) at 1.0 V was not possible due to increased resistance. When the amount of keto-ABNO was increased to 2 equiv, the yield of **2a** improved to 84%, but overoxidized products **3a**/**3a'** were still generated (entry 7).

For future application to electrochemical protein bioconjugation using precious payloads, the stoichiometry of the reagent must be minimized. Therefore, we screened additives to facilitate the electron-transfer processes. Interestingly, the addition of sterically-demanding TEMPO derivatives, which themselves are inert in Trp bioconjugation under NO_x conditions,[5] significantly increased the yield of **2a** (entries 8–12 vs entries 5 and 6). In all of these experiments, adducts of the TEMPO derivatives with **1a** were not detected at all. Among the conditions tested, the addition of 20 mol % 4-oxo-TEMPO afforded the best result with complete consumption of 1a and minimum contamination by overoxidized **3a**/**3a'** (97% yield of **2a**, entry 12). The efficiency of the additives correlated with their redox potentials, and TEMPO derivatives with higher oxidation potentials afforded higher yields of **2a**. The current efficiency (i.e., the slope of the graph depicting yield of **2a** vs charge) was approximately 1.5 times greater in the presence of 4-oxo-TEMPO than in its absence.[12]

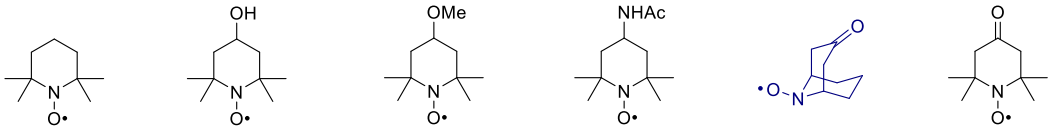
Table 1. Optimization of Electrochemical Trp-Selective Bioconjugation



Entry	Substrate (conc.)	Electrolyte solution	Additive (equiv)	Voltage (V *vs* Ag/AgCl)	Charge (F/mol)	**1** (%)	**2** (%)	**3** (%)
1	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	—	—	67	33	0
2	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.2	1.0	18	75	7
3	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.2	2.0	0	73	27

4	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.2	3.0	0	62	38
5	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.0	1.0	41	55	4
6	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.0	2.0	13	78	7
7[a]	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	—	1.0	1.5	12	84	4
8	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	TEMPO (0.2)	1.0	3.0	11	87	2
9	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	4-HO-TEMPO (0.2)	1.0	2.0	8	88	4
10	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	4-MeO-TEMPO (0.2)	1.0	2.5	6	90	3
11	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	4-AcNH-TEMPO (0.2)	1.0	2.0	3	94	2
12	**1a** (3 mM)	50 mM TBAP/CH_3CN-H_2O (1:1)	4-oxo-TEMPO (0.2)	1.0	1.5	0	97	1
13	**1b** (1 mM)	0.1 M $LiClO_4$ aq.	4-oxo-TEMPO (2.0)	0.9	1.0	0	89	3
14[b]	**1b** (1 mM)	0.1% AcOH aq.	$NaNO_2$ (0.6)	—	—	0	70	2

Yield was determined by HPLC analysis (UV trace: 230 nm). TBAP = *n*-tetrabutylammonium perchlorate. [a] keto-ABNO (2 equiv) was used. [b] Nitrosated **2b** was produced in 17% as a byproduct. [c] The half-wave redox potentials ($E_{1/2}$ *vs* ferrocene) are cited from ref 13.



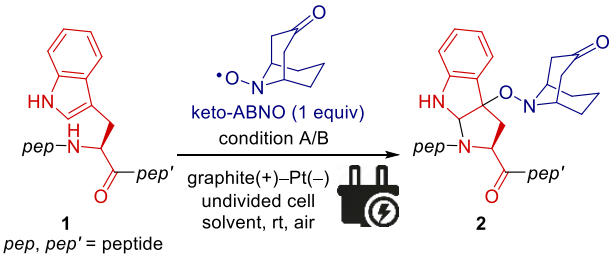
$E_{1/2}$[c] 239 mV 296 mV 299 mV 340 mV 416 mV 426 mV

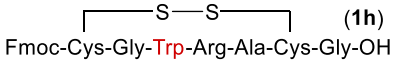
We further optimized the organic solvent-free conditions using water-soluble, N-terminus-unprotected peptide **1b**. The reaction proceeded efficiently with an increased amount (2 equiv) of 4-oxo-TEMPO in a 0.1 M $LiClO_4$ aqueous solution (entry 13).[12] It is noteworthy that the transformation of **1b** under the NO_x activation conditions[5] produced **2b** in a diminished yield (70%), because N-nitrosated **2b** (17%) was produced as a major byproduct (entry 13).

Having determined the optimal conditions, we next surveyed functional group compatibility and scope using peptide substrates containing oxidation-sensitive amino acid residues (Table 2). Constant voltage electrolysis was applied to a mixture of an Fmoc-peptide (**1a**, **1c**–**1g**), keto-ABNO (1 equiv), and 4-oxo-TEMPO (0.2 equiv) in CH_3CN–H_2O (1:1) with 50 mM TBAP (condition A). The reaction proceeded smoothly for Ser- and Lys-containing peptides **1a** and **1c** (entries 1 and 2). For

His-, Tyr-, and Met-containing peptides **1d**–**1f**, the reactions were slow under standard condition A. By increasing the voltage and total charge (entry 3) and/or the amount of 4-oxo-TEMPO (entries 4 and 5), however, the reactions produced satisfactory yields. Although Cys-containing peptide **1g** produced the desired bioconjugation product in moderate yield (entry 6), the reaction proceeded in high yield for disulfide bond-containing peptide **1h** (entry 7). Moderate reactivity with free Cys-containing peptides will not preclude the practical utility because surface-exposed Cys residues form disulfide bonds in most proteins. Water-soluble peptides **1b**, **1i**, and **1j** also produced satisfactory yields without inducing notable side reactions (condition B, entries 8-10). Modification of the commercially available Trp-containing drug leuprorelin acetate (**1k**) was also successful, affording the product in 60% yield (entry 11).

Table 2. Substrate Scope of Short Peptides.

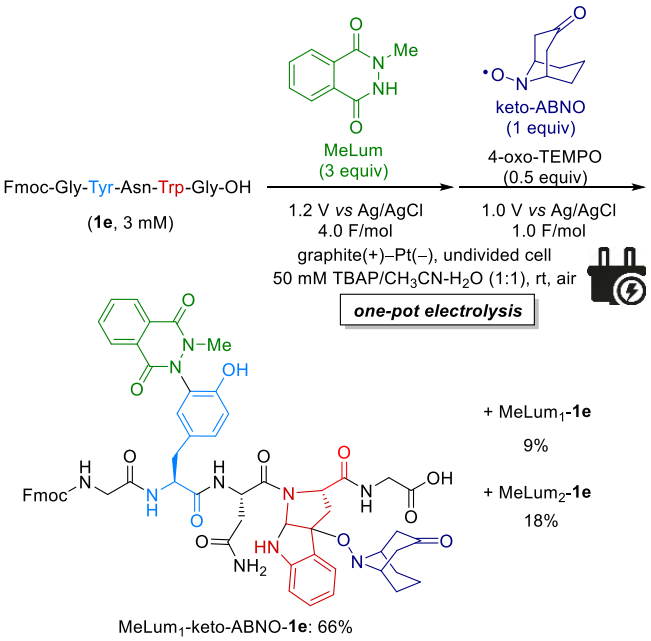


Entry	Substrate	Voltage (V)	Charge (F/mol)	Yield (%)
1	Fmoc-Gly-Ser-Asn-Trp-Gly-OH (1a)	1.0	1.5	97
2	Fmoc-Gly-Lys-Asn-Trp-Gly-OH (**1c**)	1.0	1.5	85
3	Fmoc-Gly-His-Asn-Trp-Gly-OH (**1d**)	1.2	3.0	86
4[a]	Fmoc-Gly-Tyr-Asn-Trp-Gly-OH (**1e**)	1.0	3.0	98
5[b]	Fmoc-Gly-Met-Asn-Trp-Gly-OH (**1f**)	1.0	2.5	85
6	Fmoc-Gly-Cys-Asn-Trp-Gly-OH (**1g**)	1.0	1.0	27
7	 (**1h**)	1.0	2.0	82
8	H-Gly-Ser-Asn-Trp-Gly-OH (**1b**)	0.9	1.0	89
9	H-Gly-Tyr-Asn-Trp-Gly-OH (**1i**)	0.9	1.5	78
10	H-Gly-Met-Asn-Trp-Gly-OH (**1j**)	0.9	1.5	80
11[c]	Leuprorelin acetate: Pyr-His-Trp-Ser-Tyr-$_D$Leu-Leu-Arg-Pro-NHEt·AcOH (**1k**)	0.9	2.0	60

Condition A (entries 1–7): **1** (3 mM) and 4-oxo-TEMPO (0.2 equiv) in 50 mM TBAP/CH_3CN–H_2O (1:1) unless otherwise noted. Condition B (entries 8-11): **1** (1 mM) and 4-oxo-TEMPO (2.0 equiv) in 0.1 M $LiClO_4$ aq. (no organic solvent) unless otherwise noted. The reaction time was 1.0-3.0 h. Voltage was *vs* Ag/AgCl. Yield was determined by HPLC analysis (UV trace: 230 nm). [a] 4-oxo-TEMPO (2 equiv). [b] 4-oxo-TEMPO (1 equiv). [c] $NaHCO_3$ (1 equiv) was added.

Another advantage of this electrochemical method is its facile applicability to one-pot, orthogonal dual conjugation reactions by simply tuning the applied voltages.[14,15] We found that the electrochemical method also promoted Tyr-selective oxidative bioconjugation with MeLum, originally developed by Nakamura using hydrogen peroxide as a chemical oxidant for preactivation.[4g] Based on this finding, the one-pot, electrochemical orthogonal double modifications of **1e** bearing Tyr and Trp residues were realized (Scheme 1). Thus, after the Tyr-selective bioconjugation of **1e** with MeLum at 1.2 V, the Trp-selective bioconjugation with keto-ABNO and 4-oxo-TEMPO was conducted at 1.0 V. Each bioconjugation reagent reacted one by one selectively with the target residues to produce doubly modified peptide $MeLum_1$-keto-ABNO-**1e** in 66% yield.

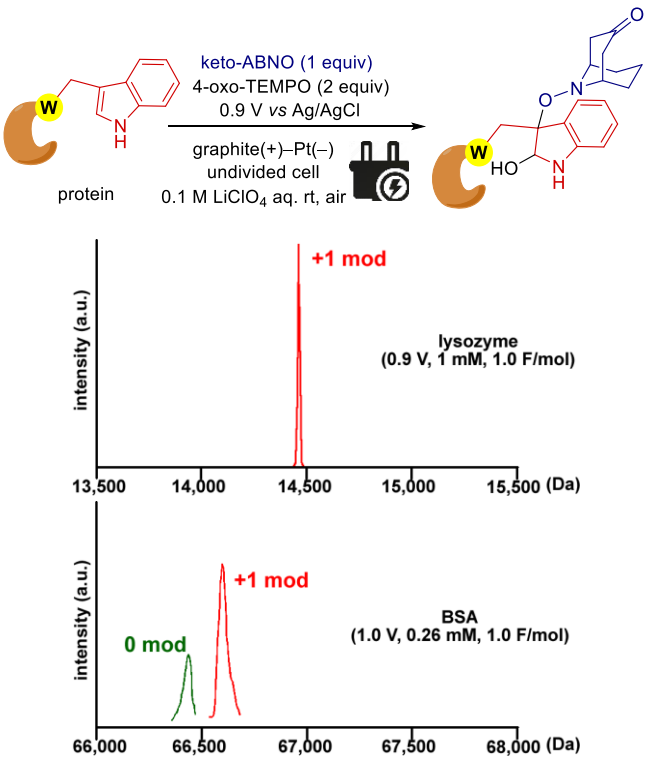
Scheme 1. One-pot, Orthogonal, Dual Electrochemical Modifications



Finally, the optimized reaction conditions for Trp-bioconjugation in an aqueous medium without use of any organic solvents were applied to native proteins, lysozyme and bovine serum albumin (Scheme 2). In the case of lysozyme, applying 1.0 F/mol electric charge at 0.9 V led to complete consumption of the starting material. The corresponding lysozyme–keto-ABNO conjugate was obtained in nearly quantitative yield (MS detection). In the case of bovine serum albumin, 1.0

F/mol electric charge at 1.0 V cleanly led to the mono-adduct as the major product (~70% conversion based on MS intensity), although some of the starting material remained. Therefore, the electrochemical Trp-bioconjugation was generally applicable to peptides as well as protein substrates.

Scheme 2. Electrochemical Trp-Selective Bioconjugation of Proteins



To gain preliminary insight into the reaction mechanism, we conducted systematic cyclic voltammetry (CV) measurements (Figure 1).[12] We used TEMPO as the additive for the CV studies, because keto-ABNO and 4-oxo-TEMPO exhibited indistinguishable redox potentials.[12] First, we assessed the interactions between keto-ABNO or TEMPO and a Trp residue using *N*-Ac-Trp-OEt as a model substrate. A solution of keto-ABNO + *N*-Ac-Trp-OEt afforded new anodic peaks at ca. 0.75, 0.83, and 1.0 V (Figure 1a), suggesting an interaction between keto-ABNO and Trp (tentatively drawn as structure **C** in Scheme 3). Notably, this interaction significantly decreased the oxidation potentials of both keto-ABNO and Trp.[16] The cathodic peak currents disappeared because an irreversible bioconjugation reaction proceeded between oxoammonium cation **A** derived from keto-ABNO and Trp. In the case of TEMPO + *N*-Ac-Trp-OEt, however, the cyclic voltammogram was a simple superposition of the two components (Figure 1b). Therefore, TEMPO and Trp neither interacted nor reacted. The fact that no bioconjugation product was observed between TEMPO or its derivatives and **1a** (Table 1) was consistent with this CV profile. Second, there was no significant electrochemical interaction between two radicals in the absence of Trp; the cyclic voltammogram for keto-ABNO +

TEMPO was a simple superposition of those of the two radicals (Figure 1c). Third, CV measurements were conducted for a three-component mixture of keto-ABNO + TEMPO + *N*-Ac-Trp-OEt. Two anodic peaks were observed at 0.7 V and 1.0 V (Figure 1d). The peak at 0.7 V is attributed to the overlapping currents arising from the oxidation of TEMPO and the species generated via the interaction between keto-ABNO and Trp (**C** in Scheme 3), and that at 1.0 V corresponds to the oxidation of species **C**. In addition, the cathodic peak current corresponding to oxoammonium cation derived from TEMPO ($TEMPO^+$) at 0.6 V markedly decreased. These results indicate the existence of a reduction pathway from $TEMPO^+$ to TEMPO by species C generated via the interaction between keto-ABNO and Trp.

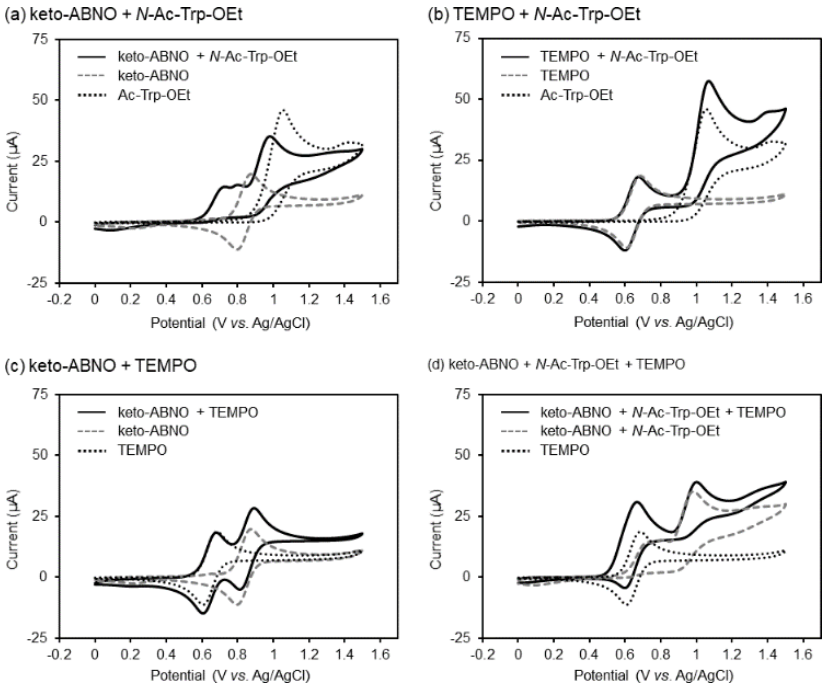
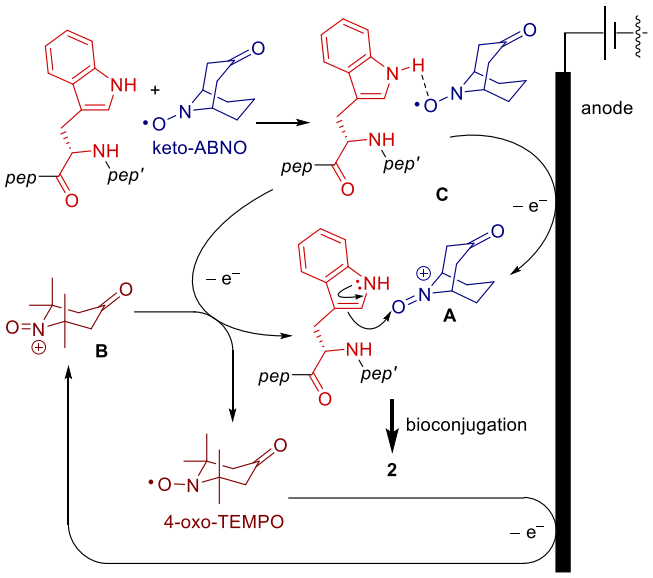


Figure 1. Cyclic voltammograms in 0.1 M TBAP/CH_3CN-H_2O (1:1) on a glassy carbon working electrode at a scan rate of 10 mV s^{-1}. The concentration of each compound was 3 mM. (a) dashed: keto-ABNO, dot: *N*-Ac-Trp-OEt, and solid: keto-ABNO + *N*-Ac-Trp-OEt. (b) dashed: TEMPO, dot: *N*-Ac-Trp-OEt, and solid: TEMPO + *N*-Ac-Trp-OEt. (c) dashed: TEMPO, dot: keto-ABNO, and solid: keto-ABNO + TEMPO. (d) gray dashed: keto-ABNO, black dashed: TEMPO, dot: *N*-Ac-Trp-OEt, and solid: keto-ABNO + TEMPO + *N*-Ac-Trp-OEt.

On the basis of the experimental results, we propose a plausible reaction mechanism (Scheme 3) The initial step is the formation of pre-conjugation complex **C** involving a keto-ABNO/Trp interaction.[16] This type of pre-conjugation complex does not form from 4-oxo-TEMPO, possibly due to steric hindrance. The oxidation potentials of **C** are significantly lower than those of either keto-ABNO or Trp. The activated bioconjugation reagent, oxoammonium cation **A**, can be generated from

C through two pathways: direct anodic oxidation or indirect oxidation by 4-oxo-TEMPO$^+$ (reagent B), which is generated through anodic oxidation of 4-oxo-TEMPO. Therefore, the marked acceleration effects of additive 4-oxo-TEMPO are likely due to efficient electrochemical coupling between the single-electron oxidation of **C** to **A** and the anodic oxidation of 4-oxo-TEMPO to **B**. The two radicals containing similar redox potentials, but distinct steric demands, have distinct roles (i.e., keto-ABNO = a bioconjugation reagent; 4-oxo-TEMPO = an electron mediator), and this alliance is critically important for the success of the facile and high-yielding electrochemical bioconjugation. It is noteworthy that these two radicals do not interact each other by themselves, but only when Trp exists, the two radicals electronically couple through species **C**.

Scheme 3. Plausible Reaction Mechanism



In conclusion, we developed the first electrochemically promoted Trp-selective bioconjugation by identifying a unique electrochemical cooperation of keto-ABNO and 4-oxo-TEMPO. Compared with the chemical activation method of keto-ABNO using NO_x, implementation with neutral aqueous media was possible with suppressed side reactions. Chemically modified biologics are attracting great attention as novel medicinal modalities.[17] The reaction development described herein is an important step toward clean and scalable syntheses of chemically modified biologics.

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Keywords

Electrochemistry • Bioconjugation • Protein • Organoradical • Tryptophan

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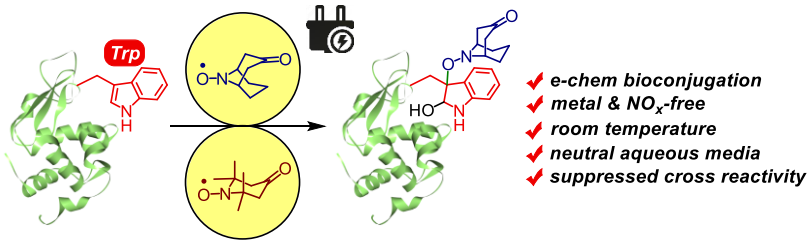
TOC



The first electrochemically-promoted tryptophan-selective bioconjugation in neutral aqueous media was developed. Systematic CV analyses suggested that unique electrochemical alliance of two structurally distinct N-oxyl radicals, keto-ABNO and 4-oxo-TEMPO, was critical for both high yield and suppressed side reactions. This method was successfully applied to water-soluble peptides and proteins, contributing to the progress of clean and scalable synthesis of chemically modified biologics.