Enzyme–Electrode Interfaces Hot Paper

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S-Click Reaction for Isotropic Orientation of Oxidases on Electrodes to Promote Electron Transfer at Low Potentials

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Abstract: *Electrochemical sensors are essential for point-of-care testing (POCT) and wearable sensing devices. Establishing an efficient electron transfer route between redox enzymes and electrodes is key for converting enzyme-catalyzed reactions into electrochemical signals, and for the development of robust, sensitive, and selective biosensors. We demonstrate that the site-specific incorporation of a novel synthetic amino acid (2-amino-3-(4-mercaptophenyl)propanoic acid) into redox enzymes, followed by an S-click reaction to wire the enzyme to the electrode, facilitates electron transfer. The fabricated biosensor demonstrated real-time and selective monitoring of tryptophan (Trp) in blood and sweat samples, with a linear range of 0.02–0.8 mM. Further developments along this route may result in dramatic expansion of portable electrochemical sensors for diverse health-determination molecules.*

Amino acids are essential metabolic intermediates and cellular signaling molecules.[1] Aberrant amino acid metabolism leads to many severe diseases, therefore real-time amino acid analysis is of great importance for diagnosis and medicine.[2] For example, tumor cells over-express indoleamine 2,3-dioxygenase (IDO), which mediates tryptophan (Trp, required for T cell activation) depletion to suppress immune response and the effectiveness of cancer immune therapy.[2] Conventional analytical procedures for measuring amino acid concentration are based on non-portable spectroscopic or chromatographic[3] instruments, which are not suitable for POCT and healthcare-monitoring biosensors that are wearable.[4] Interest is growing in personalized medicine through continuous health monitoring, and the use of wearable sensors for monitoring of sweat components was recently reported.[4] However, important metabolites present in blood and sweat, such as amino acids, are rarely studied in the POCT field because real-time sensitive analysis techniques are lacking.

Enzymatic electrochemical biosensors provide a convenient, low cost, and real-time approach for measuring analyte concentration, and they are essential for diabetes management.[5] An efficient enzymatic electrochemical biosensor has several key requirements: high surface density, long-term enzymatic stability, and perhaps most challenging, an efficient electron transfer pathway between the enzyme active site and the electrode. Although great effort has been devoted to improving the robustness, selectivity, and activity of the enzyme to meet the primary requirements for practical sensing applications, highly selective and sensitive electrochemical sensors for most health-determining biomolecules are still underdeveloped.[6,7] To achieve this goal, it is important to improve the electron transfer between the enzyme and the electrodes. Efficient electron transfer between the enzyme's redox active cofactor and the electrode requires a short distance (typically less than 14 Å),[8] redox mediators,[9] and electrode-modifying redox polymers.[10] Using mediators in enzymatic electrochemical biosensors is a robust approach for improving electron transfer, and yet, the use of mediators usually leads to an increased overpotential relative to the original redox potential of the enzyme. Moreover, the redox mediators are generally nonselective, as they facilitate electron transfer between electrode and protein, as well as various interfering molecules.[11]

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Besides, for in vivo applications, mediator immobilization is required to ensure biocompatibility. Enhancing the direct electrical communication across the enzyme–electrode interface with nanomaterials has made great contributions to the realization of third generation biosensors;[12] however, the random orientation of the enzyme with respect to the electrode surface leads to large variations in the electron transfer efficiency. Attachment of enzyme cofactors to conductive nanoparticles can achieve higher catalytic turnover rate than the unmodified system,[13] yet this approach cannot be applied to an enzyme that has its cofactor completely buried within a protein. Site-specific wiring of an enzyme to the electrode through cysteine-based surface ligation requires one unique cysteine to be present in its surface.[14] Genetic code expansion[15] has been used to introduce synthetic amino acids into enzymes to facilitate enzyme wiring[16] to electrodes; typically using carcinogenic linkers or expensive gold electrodes. Therefore, a fully biocompatible and cost-effective approach is highly desirable for making broadly applicable POCT biosensors.

We developed an efficient and broadly applicable wiring strategy to transform redox enzymes into electrochemical sensors. Our strategy involved site-specific incorporation of the synthetic amino acid 2-amino-3-(4-mercaptophenyl) propanoic acid (or *p*-thiolphenylalanine, TF), as a unique enzyme-anchoring point, to two different amino acid oxidases, glycine oxidase (GlyOx, PDB code 1NG4) and L-tryptophan oxidase (TrpOx, PDB code 5G3T). Importantly, TF differs from tyrosine (Tyr) by only one atom, which introduces minimal perturbation to the target enzyme. We then used boron-dipyrromethene (Bodipy373)[17] as an enzyme/electrode wiring linker, which reacts with TF specifically through a thiol–chlorine nucleophilic substitution reaction (S-click reaction, Figure 1). Distinct from other biorthogonal reactions,[16] Bodipy373 undergoes a dramatic bathochromic shift (Supporting Information, Figure S3) after reaction with TF, which facilitates convenient characterization of Bodipy373-labeled redox enzymes. The modified enzyme can be attached onto the carbon electrode surface where it generates an efficient electron transfer biocatalytic current sufficient to transform specific substrates at a potential close to the original redox potential of the enzyme, thus yielding improved selectivity.

The synthesis of TF consists of four steps starting from 4-nitrobenzyl bromide (Supporting Information, Figure S1). To obtain an orthogonal transfer ribonucleic acid (tRNA)/aminoacyl-tRNA synthetase pair that selectively charges TF in response to amber suppressor, three rounds of positive and two rounds of negative selections with a *Methanocaldococcus jannaschii* (*Mj*) tyrosyl-tRNA synthetase (*Mj*TyrRS) library were performed, as described previously.[18] A TF-specific TyrRS mutant, termed TFRS, was identified. To further verify that TF was incorporated into the target protein with high efficiency and fidelity, an amber stop codon was substituted into GlyOx and TrpOx. Mutant GlyOx and TrpOx expression were carried out with TFRS and *Mj* $tRNA_{CUA}^{Tyr}$ in the presence of 1 mM TF, or in the absence of TF as a negative control. Analysis of the purified GlyOx-266TF and TrpOx-395TF by sodium dodecyl sulphate gel electrophoresis

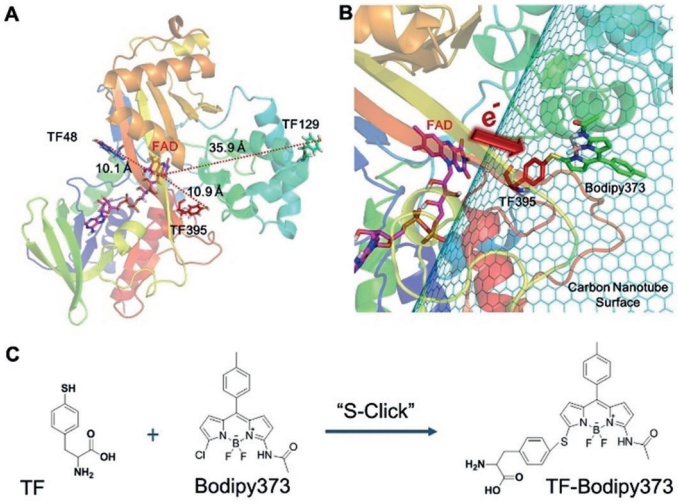

Figure 1. A) The structure of TrpOx and the positions for TF incorporation. The distances between TF and FAD are indicated. B) A schematic depiction of the wiring of TrpOx from the TF395 site through a Bodipy373 linker to the CNT surface. C) A schematic depicting the S-click reaction; sulfur atom in TF (S), chlorine atom in Bodipy373 (Cl).

(SDS-PAGE) showed that full-length GlyOx and TrpOx were expressed only in the presence of TF (Figure 2A), indicating that TF was recognized specifically by TFRS. The purified protein was then subjected to trypsin digestion, and mass spectrometry (MS–MS) analysis of the product mixture revealed the site-specific incorporation of TF into the target protein (Figure 2C). Enzymatic assays show that the wild-type and mutant TrpOx have similar activity, indicating that the site-specific incorporation of TF cause minimal perturbation to enzyme function (Supporting Information, Figure S2).

To demonstrate that Bodipy373 selectively reacts with the thiophenol group of TF, wt GlyOx, GlyOx-266TF, wt TrpOx, and TrpOx-395TF were incubated with 50 μM Bodipy373 in pH 8.0 Tris buffer, at 4 °C for 60 minutes. SDS-PAGE analysis revealed fluorescent bands associated with GlyOx-266TF and TrpOx-395TF, but none were observed for wt GlyOx and wt TrpOx (Figure 2B). Notably, wt GlyOx and wt TrpOx both contain four surface cysteine residues, which did not react with Bodipy373 under the condition tested.[17] A titration experiment shows that TF reacts with Bodipy373 at a rate of $5.6\,M^{-1}s^{-1}$. By contrast, Bodipy373 reacts with *N*-acetylcysteine about 1000-fold slower, at a rate of $0.0057\,M^{-1}s^{-1}$ (Supporting Information, Figure S3). The much lower pK_a of TF (6.4) relative to cysteine (8.4) accounts in part for this remarkable reactivity, which allows for the selective modification of TF in the presence of surface exposed cysteines.

Fluorescence measurements demonstrate that Bodipy373 fluorescence is quenched 60-fold (Supporting Information, Figure S4) when incubated with carbon nanotubes (CNTs), which is likely a consequence of strong pi–pi interactions between Bodipy373 and CNT, and photoinduced electron transfer (PET) quenching.[19a] The site-specific attachment of TrpOx to the carbon electrode was further characterized by atomic force microscopy (AFM). In the absence of Bodipy373 modification, the coverage of physically adsorbed TrpOx-395TF enzyme on the highly oriented pyrolytic graphite

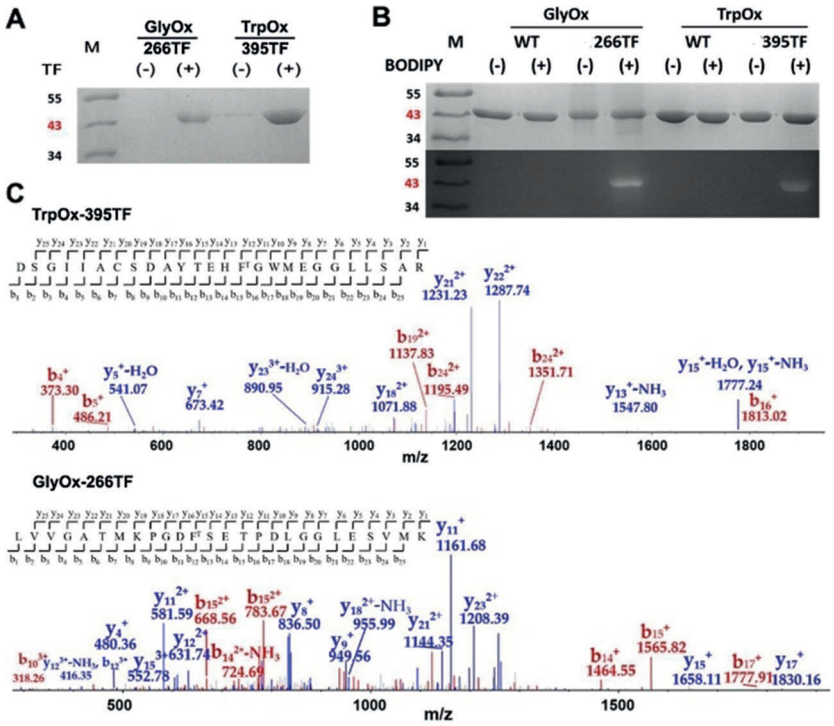

Figure 2. Genetic incorporation of TF into protein and site-specific modification using the S-click reaction. A) A coomassie-blue-stained SDS-PAGE gel of GlyOx and TrpOx mutants expressed in the absence or presence of TF (1 mM). B) A coomassie-blue-stained (top) and fluorescence imagine (bottom) of SDS-PAGE gel of TrpOx-395TF and GlyOx-266TF mutants incubated in the presence or absence of Bodipy373. C) MS spectra of TrpOx-395TF and GlyOx-266TF.

(HOPG) surface (Figure 3A) was much lower and less uniform than that of TrpOx-395TF-Bodipy373 (Figure 3B). Quantitative analysis of the protein coverage was further performed by using the Nanoscope IIIa software function;[19b] the calculated result for physically adsorbed TrpOx-395TF was 12.3%, which is much lower than the calculated value of 51.5% for TrpOx-395TF-Bodipy373. An absorption peak at 570 nm associated with the purified TrpOx-395TF-Bodipy373 complex was used to quantify the protein before and after immobilization. The calculated amount of enzyme immobilized on the HOPG electrode was 2.1 pmol cm^{-2} for TrpOx-395TF-Bodipy373 complex and 0.52 pmol cm^{-2} for physically adsorbed TrpOx-395TF. These results suggest, with the aid of Bodipy373 linker as a "sticker" to the carbon surface, that the modified enzyme has significantly increased surface coverage on the HOPG surface. Molecular dynamics simulations were performed to elucidate the binding mechanism between Bodipy373 and CNT. The binding energy between Bodipy373 and the CNT surface was calculated to be -152 kJ mol^{-1}, which is stronger than that of pyrene (-110 kJ mol^{-1}). The more precise PM6-DH + semiempirical quantum mechanical method gave similar results (Supporting Information, Figures S5 and S6).

Having established site-specific wiring of TrpOx to the electrode, we then performed real-time electrochemical measurement of Trp. While Trp is an electrochemically active amino acid that can be oxidized at an overpotential of 300 mV, it is not practical to perform selective Trp measurement at this potential in biological systems because of strong interference. We demonstrated the detection of Trp at a much lower potential by TrpOx-395TF-Bodipy373-wired CNT-modified glassy carbon electrode (TrpOx-395TF-Bodipy373/CNT/GCE). As shown in Figure 3D, a significant increase in the oxidative current was observed with a half-wave potential of -94 mV upon the addition of 2 mM Trp. To exclude the possibility that Bodipy373 served as a redox mediator, the electrochemical signal of wt TrpOx in the presence of Bodipy373- or Bodipy373-modified electrode without enzyme modification was measured; no catalytic current was observed (Supporting Information, Figure S7). Moreover, the same TrpOx-395TF mutant without Bodipy373 wiring to the electrode (Figure 3C) showed negligible oxidative current upon Trp addition, indicating that Bodipy373 wiring is required for efficient electron transfer.

To investigate the origin of the oxidation current, the absorption spectra of TrpOx-395TF was measured before and after addition of Trp substrate. A new absorption peak at about 320 nm was observed during the oxidation of Trp (Supporting Information, Figure S8A), corresponding to formation of one-electron-reduced radical semiquinones (FAD·) in flavoproteins, as

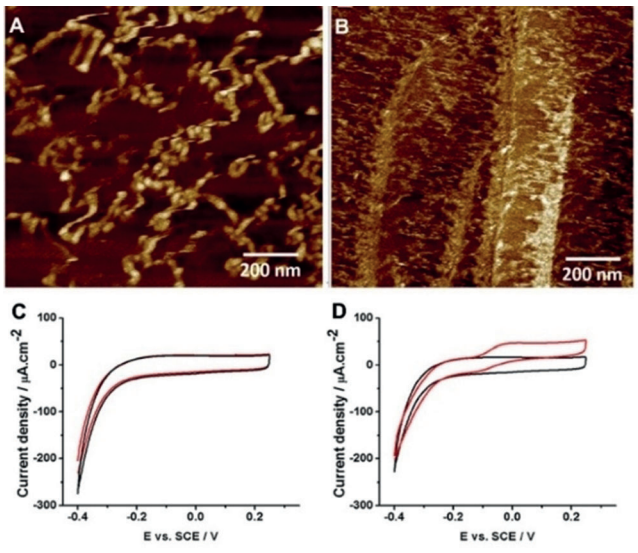

Figure 3. AFM images of A) TrpOx-395TF absorbed on a HOPG surface. B) TrpOx-395TF-Bodipy373 absorbed on a HOPG surface. Cyclic voltammetry (CV) of C) TrpOx-395TF absorbed on CNT/GCE and D) TrpOx-395TF-Bodipy373 absorbed on CNT/GCE before (red) and after (black) the addition of Trp (2 mM). CV conditions: phosphate-buffered saline (PBS), pH 7.4, scan rate 5 mV s^{-1}.

previously reported.[20] Low-temperature electron paramagnetic resonance (EPR) measurements were also performed to verify the presence of FAD·. The EPR spectra of TrpOx during reaction with its substrate are presented in Figure S8B (Supporting Information). The signal was centered at $g = 2.0046$ with a linewidth (peak-to-peak) of 11.2 Gauss, in agreement with those reported for FAD·.[21] The reported oxidation potential of FAD· was around −200 mv (vs. standard calomel electrode (SCE)), which is close to the observed oxidation potential in this study.[20] Therefore, considering the inevitable protein barrier, and also the voltage drop caused by the molecular bridge between the enzyme and the electrode, the half-wave potential of −94 mV indicates an efficient communication between the electrode and the FAD center, which arises from the re-oxidation of FAD·. Further electron transfer rate analysis and kinetic study of the wired TrpOx mutants indicates the importance of proper enzyme alignment on the electrode to favor electron transfer (Supporting Information, Table S3). To illustrate the generality of the approach, we show that Bodipy373 wiring is required for efficient electron transfer between GlyOx-266TF-Bodipy373 and the electrode (Supporting Information, Figure S9D).

We then measured the amperometric response of the TrpOx-395TF-Bodipy373/CNT/GCE for Trp, at an applied potential of −0.1 V (Figure 4A). The dynamic range was 0.02–2.5 mM, and the linear range was 0.02–0.8 mM (Figure 4B). Compared with the current response to 30 μM of Trp, signals corresponding to physiological levels of dopamine, ascorbic acid, and uric acid (0.1 mM, respectively) were negligible (Supporting Information, Figure S10). This anti-interference ability was attributed to the low operating potential that was applied for the selective detection of Trp. Subsequently, we tested whether the TrpOx-395TF-Bodipy373/CNT/GCE is capable of selective real-time monitoring of Trp concentrations in blood. As shown in Figure 4C, after adding 0.03 mM Trp to mice blood, a rapid current increase was observed within 5 seconds. After injection of IDO (a Trp degradation enzyme, 0.1 mM in PBS buffer) a fast drop of steady-state current was observed, indicating that the Trp concentration decreased. We then used TrpOx-395TF-Bodipy373/CNT/GCE electrode to monitor the Trp concentration in the HeLa cell culture. As shown in Figure 4D, in the absence of IFN-γ, the Trp concentration dropped slowly during the growth stage from 48–54 h. However, in the presence of 50 ng mL^{-1} IFN-γ, more IDO was expressed in HeLa cells and a significant decrease in Trp concentration was observed.

GlyOx-266TF-Bodipy373/CNT/GCE and TrpOx-395TF-Bodipy373/CNT/GCE sensors were then used to measure glycine and Trp concentrations in human sweat and blood samples. As shown in Tables S4 and S5 (Supporting Information), all the measured values from amperometry have a good linear correlation to the reference value obtained by LC-MS (Supporting Information, Figure S12). Considering the simplicity and rapidity of amperometry measurement, our approach demonstrates great potential in human sweat and blood sample analysis and real-time health status monitoring.

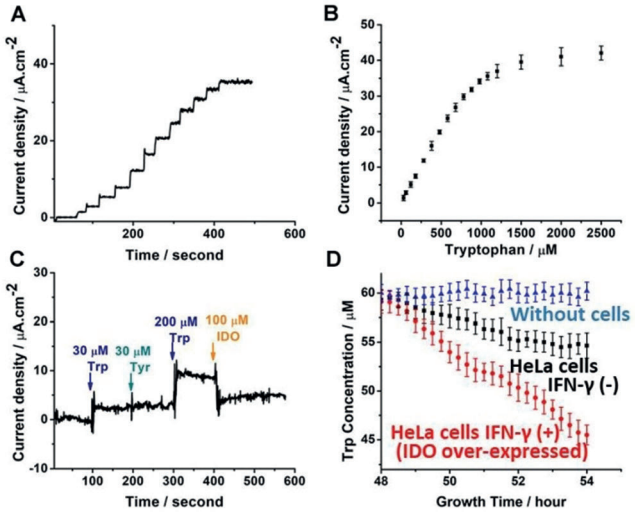

Figure 4. A) Amperometric response of TrpOx-395TF-Bodipy373/CNT/GCE upon addition of Trp; applied potential −0.05 V in pH 7.4 PBS buffer. B) The steady-state currents versus Trp concentration. C) The Trp concentration measurement in vitro, and after the injection of Trp, Tyr, and IDO. D) Real-time and on-line Trp concentration measurement during HeLa cell growth, from 48–54 h; blank culture media (blue dot), HeLa cells in the absence of IFN-γ stimulation (black dot), HeLa cells after 50 ng mL^{-1} IFN-γ stimulation for 24 h (red dot). Prior to measurement, the culture media were renewed by a RPMI 1640 growth medium with a Trp concentration adjusted to 60 μM.

In conclusion, we have demonstrated an efficient and biocompatible approach for wiring redox enzymes site-specifically to carbon-based electrodes using an S-click reaction. This approach can be applied to a wide range of redox enzymes, thereby leading to a much improved enzyme–electrode communication. This method will bring bioelectrochemical systems closer to real-world applications and facilitate a deeper understanding of redox enzyme/electrode interactions.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: amino acids · biosensors · electrochemistry · electron transfer · genetic code expansion

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Communications

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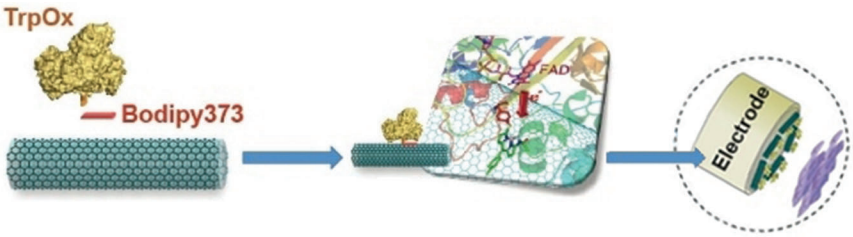

Blood, sweat & Trp: Electrochemical sensors are essential for point-of-care testing and wearable sensing devices. Efficient electron transfer from a redox enzyme to an electrode is established by wiring the two together with S-click chemistry. These biosensors are sensitive and selective for tryptophan and glycine in blood and sweat. Key: L-tryptophan oxidase (TrpOx), boron-dipyrromethene (Bodipy), reduced semiquinone in flavoprotein (FAD·).