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Electrochemical Oxidation of Glucose Using Mutant Glucose Oxidase from Directed Protein Evolution for Biosensor and Biofuel Cell Applications

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Abstract In this study, electrochemical characterisation of glucose oxidation has been carried out in solution and using enzyme polymer electrodes prepared by mutant glucose oxidase (B11-GOx) obtained from directed protein evolution and wild-type enzymes. Higher glucose oxidation currents were obtained from B11-GOx both in solution and polymer electrodes compared to wt-GOx. This demonstrates an improved electrocatalytic activity towards electrochemical oxidation of glucose from the mutant enzyme. The enzyme electrode with B11-GOx also showed a faster electron transfer indicating a better electronic interaction with the polymer mediator. These encouraging results have shown a promising application of enzymes developed by directed evolution tailored for the applications of biosensors and biofuel cells.

Keywords Directed protein evolution · Glucose oxidase · Glucose oxidation · Electron transfer · Biofuel cells · Biosensors

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Introduction

The increasing aging population requires better healthcare system for monitoring and therapeutical applications. Progress in medical science has led to increasing demands for implantable electronic devices. Enzymatic biofuel cells and biosensors could offer specific advantages for these purposes [3, 8]. However, current developments are limited by enzyme stabilities and activities in complicated physiological environment and reactions. The power output of such biofuel cells and the sensitivity of biosensors depend on the activities of enzyme biocatalysts and their life time [6]. It is important to have the enzymes with specific properties required for such applications. In the past decade, directed protein evolution has become a powerful method for biocatalyst engineering to tailor enzyme properties to application demands, which could provide a promising solution for the current problems for enzymatic bioelectronics [1, 5].

Directed evolution of glucose oxidase from *Aspergillus niger* was developed in a medium-throughput screening system to improve the properties of glucose oxidase (GOx) [17, 18]. A directed protein evolution experiment comprises two main steps: generating diverse mutant libraries [14] and screening for improved protein variants [13, 15]. The glucose oxidase detection assay [9] is based on NADPH formation in coupled enzyme reactions and was validated by improving the activity of GOx by directed protein evolution. Glucose oxidase detection assay (GODA) medium-throughput screening protocol is especially useful for evolving mediated or direct electron transfer properties of GOx in which oxygen is not used as electron acceptor and reduced to hydrogen peroxide [17]. A mutant GOx (B11-GOx) designed for ferrocenemethanol-mediated electron transfer process cloned with two propeptide sequences has been developed by directed evolution. In the previous studies, characterisation of biological catalytic activities was examined [18]. Improved V_{max} and K_m values from GOx mutants were achieved indicating increased activity and affinity between enzymes and glucose. The catalytic constant k_{cat} towards glucose oxidation was increased by 1.5-fold with the mutant GOx. Improved pH stability in the pH range 8–11 was also obtained from the mutant GOx. In addition, the half-life of enzymes at 57°C increased from 84 min for wild-type GOx to 152 min for mutant GOx showing an improved thermal resistance for mutant enzyme. With the increase in k_{cat} and reduction in K_m values from mutant GOx, it is possible to boost the power output of a biofuel cell and reduce detection limits of a glucose biosensor.

In order to examine the electrochemical activity of the mutant enzymes on the application for bioelectronics, characterisation of the electrochemical property of mutant GOx enzymes on glucose oxidation was carried out in this study for the first time. Electrochemical tests on glucose oxidation using cyclic voltammetry, liner sweep voltammetry and chronoamperometry with wild-type and mutant GOx enzymes were carried out both in solution and by enzyme polymer electrodes. Activities and reaction kinetics were compared for all three enzymes.

Experimental

Isolation of GOx From Recombinant *Pichia pastoris*

Materials

Chemicals All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany) and Applichem (Darmstadt, Germany).

Strains, Growth Media and Enzymes *Escherichia coli* strain XL10-Gold Kan^R was used for plasmid construction and propagation, and *P. pastoris* X-33 was used for expression of GOx. Yeast growth media were prepared according to the *Pichia* expression system manual from Invitrogen (Karlsruhe, Germany).

P. pastoris cells were cultivated in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose). Transformants were grown on YPD, containing Zeocin™ (100 μg/ml), selective plates (2% agar).

Methods for GOx Expression and Purification

The GOx DNA sequence from pYES2 vector used in directed evolution experiments was amplified by PCR as described previously [18] using oligonucleotides 5′-ATCGGTACCATG CAGACTCTCCTTG-3′ as forward primer and 5-TGTTCTAGATCACTGCATGGAAGC-3′ (Operon, Germany) as reverse primer. PCR product was purified and cloned into pGAPZαA expression vector between *Kpn*I and *Xba*I restriction sites, and *Pichia* X-33 cells were transformed (Invitrogen).

Transformants expressing the highest amount of GOx were grown in BMDY medium (1% yeast extract, 0.34% yeast nitrogen base extract, 2% peptone, 1% ammonium sulfate, 2% glucose, 100 mM sodium phosphate buffer [pH 6.0], and 0.4 mg/l biotin). After 2 days of expression cells were separated from fermentation broth by cross-flow ultrafiltration, and GOx was concentrated using same device with membrane cutoff of 30 kDa (Amicon).

GOx was further concentrated by ultrafiltration using 50-kDa cut-off membranes (Amicon) and equilibrated in 10 mM sodium phosphate buffer, pH 6.0. Enzyme was eluded by chromatography with DEAE TSK 650S column (1.0 cm×18 cm) equilibrated with the same buffer. The gradient program was as follows: washing with 50 ml of the equilibrating buffer, linear gradient (100 ml) of 0.01–1 M sodium phosphate buffer, and pH 6.0.

Fractions containing GOx activity were pooled, dialyzed against distilled water, and lyophilized. Purity of GOx was confirmed by SDS–PAGE and UV–vis absorbance measurements.

Enzyme Activity and Kinetic Characterization

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was used for measurement of GOx activity [11]. Maximum reaction rate (V_{max}), Michaelis–Menten constant (K_m), and turnover number (k_{cat}) were determined for β-D-glucose for kinetic characterisation of enzyme. Obtained data were fitted directly into the Michaelis–Menten equation, and kinetic constants were determined.

The K_m value for β-D-glucose was obtained from kinetic measurements of purified wild-type GOx and mutant performed with final concentrations of β-D-glucose of 100, 50, 25, 10, 5, and 2.5 mM in acetate buffer (100 mM, pH 5.5, ionic strength 158 mM). The reactions were recorded at 414 nm by UV spectra at ambient temperature for 5 min using a FLASHScan S12 microplate reader (Analytik Jena AG).

Turnover number (k_{cat}) was calculated based on

$$k_{cat} = V_{max}/[E]_T,$$

where V_{max} (maximum rate of reaction) was obtained from K_m determination curve, $[E]_T$ is molar concentration of enzyme which was calculated from the result of bicinchoninic acid assay (BCA) protein determination and the molecular weight of enzyme.

Further details of the enzyme kinetic characterisation can be found in a previous study [16]

Electrochemical Study of Glucose Oxidation Using Wild-Type and Mutant B11-GOx

Electrochemical Tests in Solutions

The electrochemical tests were performed in a three-electrode cell using BASi Rotating Disk 2 system. The working electrode was a gold electrode with a surface area of 0.071 cm^2. A platinum coil was used as the counter electrode, and the reference electrode was an Ag/AgCl electrode (0.208 V vs NHE). The potentials described in this study are all against Ag/AgCl reference electrode, unless otherwise specified. Enzymes (1 mg/ml) and ferrocenecarboxylic acid (0.5 mM) (Sigma Aldrich) were dissolved in 0.1 M phosphate buffer solutions (pH 7.0). Cyclic voltammetry, linear sweep voltammetry, and chronoamperometry were used to characterise the enzyme containing solutions and with various glucose concentrations. The solution was deaerated with nitrogen prior to the electrochemical tests, and nitrogen was used to blanket the solution throughout the tests.

Fabrication and Electrochemical Test of Enzyme Electrodes

Enzyme electrodes were prepared by immobilizing wt-GOx and B11-GOx on the polymeric mediator, poly (vinylferrocene-co-2-hydroxyethylmethacrylate) (poly (VFc-co-HEMA)), synthesised as previously described by Takai et al. [7, 12] on glassy carbon substrates. Before applying polymer and enzyme, the glassy carbon electrode, with a surface area of 0.07 cm^2, was polished and cleaned by cyclic voltammetry in 1 M H_2SO_4. Two microliters of 1 wt% polymer solution in DMF and 0.4 μl HDAM aqueous solution were pipetted on top of the carbon electrode and dried in air. Then 2 μl of enzyme solution (10 mg/ml) was added on the polymer layer and left dried in air. Then the electrode was put into glutaraldehyde vapour for 1 hour and then dried and stored in the refrigerator at 4°C before tests.

The prepared polymer electrode was tested for glucose oxidation using electrochemical methods. The procedure was the same as described in "2.2.1 Electrochemical tests in solutions".

Results and Discussion

Determination of Activities and Kinetic Parameters of GOx

The values of Michaelis–Menten constant (K_m) and catalytic constant (k_{cat}), as well as the specificity constant (k_{cat}/K_m) for wild-type glucose oxidase (wt-GOx) and mutant glucose oxidase B11-GOx (T30V I94V), were calculated from the experimental data [18]. The results are summarised in Table 1.

The K_m value for the mutant GOx was reduced to 16 mM for B11-GOx-1 compared to the wild-type GOx (22 mM), suggesting that the affinity between glucose and mutant enzyme was improved by the modification. The catalytic rate constant k_{cat} for B11-GOx was improved 1.4 times to 80 s^{-1}. The k_{cat}/K_m value for the mutant enzymes was 5.00 mM s^{-1} for B11-GOx, almost double of that for wt-GOx (2.64 mM s^{-1}), implying a higher and faster rate of converting glucose to oxidation product (gluconic acid) with the mutant GOx. In order to determine the electrochemical properties and activity of the enzymes, electrochemical characterisations were carried out in solutions and polymer mediators.

Table 1 K_m, k_{cat}, and k_{cat}/K_m values of glucose oxidase at 25°C

Type of GOx	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM s^{-1})
wt-GOx	22	58	2.64
B11-GOx	16	80	5.00

Electrochemical Characterisation of Enzymes in Buffer Solution

Cyclic voltammograms (Fig. 1) of enzymes with ferrocene mediator in buffer solution showed little difference between wild-type and mutant enzymes, as well as ferrocene solution without enzyme. Redox peaks associated with ferrocene were observed, and the redox property shown here was mainly attributable to mediator redox process in the solution.

Figure 2 demonstrates the linear sweep voltammograms of glucose oxidation with wt-GOx and B11-GOx enzyme electrodes in 1 mM and 5 mM glucose solutions, as well as the voltammograms in blank buffer solution (in absence of glucose). With the increasing glucose concentration, the current response from both enzymes was increased compared to the buffer solution without glucose indicating a dependence of current response with the catalytic activity of the enzyme, and electrochemical communication between the enzymes and electrode surface was also established with ferrocene mediator in the solution. Two potential regions separated by 0.35 V, the ferrocene redox potential, can be observed from the voltammograms in Fig. 2. The first region is for potential $0.15\ V<E<0.35\ V$. In this region, the oxidation current increased rapidly as the potential scanning to a more positive direction. This suggests that the glucose oxidation in this potential region was kinetically controlled by the electron transfer between the enzyme, mediator, and the electrode surface. The second region is for potential $E>0.35$ V; a plateau can be observed from the glucose oxidation current. The current did not change significantly as potential scanning to a more positive direction for the solution with certain concentration of glucose. Nevertheless, the level of the plateau and the oxidation current were increased with elevating glucose concentration. This is clearly implying that in this region, the glucose oxidation with enzymes was controlled by

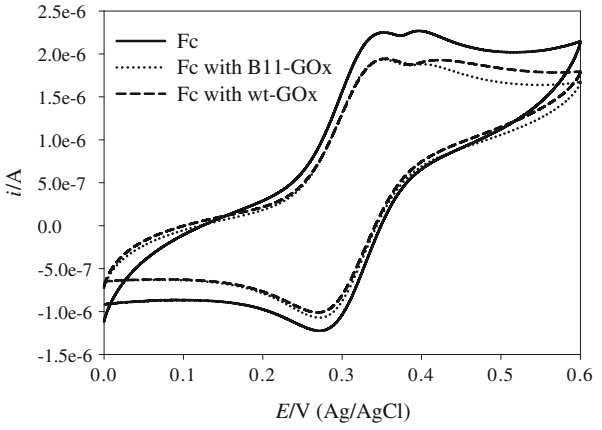

Fig. 1 Cyclic voltammograms with 0.5 mM ferrocenecarboxylic acid and with B11-GOx and wt-GOx in 0.1 M phosphate buffer, pH 7.0, scan rate 5 mV/s, and electrode surface area 0.07 cm^2

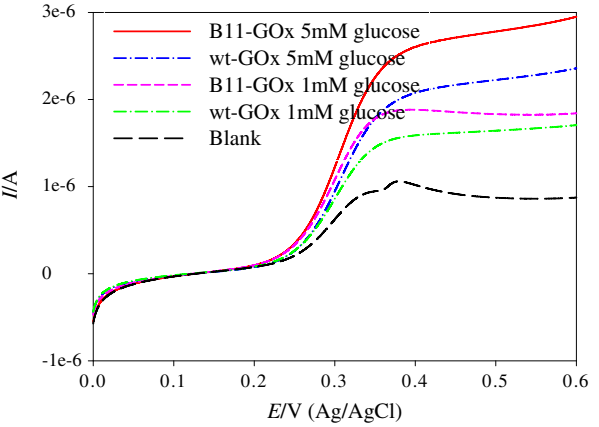

Fig. 2 Linear sweep voltammograms for glucose oxidation with wt-GOx and B11-GOx, scan rate 1 mV/s, and surface area 0.07 cm^2 in 0.1 M phosphate buffer and pH 7.0 with 0 mM, 1 mM, and 5 mM glucose

mass transport of glucose substrate in the solution. In both potential regions, higher glucose oxidation current was achieved with the B11-GOx enzyme in both glucose concentrations. Particularly with the kinetic controlled potential range, potential lower than 0.35 V, the oxidation current obtained by B11 mutant GOx in 1 mM glucose was even higher than that obtained by wt-GOx in 5 mM glucose indicating a much improved glucose oxidation activity from the mutant GOx.

A Tafel slope around 0.091 V/dec was observed for the glucose oxidation with wt and B11 GOx in solution. In solution, enzymes and mediator both exist in the bulk solution freely. Therefore, the similar Tafel slopes suggest the same reaction mechanisms and electron transfer rate for glucose oxidation in solution with both enzymes which is mainly dependent on diffusion of mediator in solution to the electrode surface of the Tafel region. For the potential region E>0.35, the level lines indicating the oxidation currents were not changed according to scanning potential but the glucose concentration suggesting that it is a mass transport-controlled region.

Electrochemical Characterization With Enzyme Electrodes Prepared From wt-GOx and B11-GOx

Enzyme polymer electrodes were prepared by immobilizing wt-GOx and B11-GOx in the polymeric mediator, poly (vinylferrocene-co-2-hydroxyethylmethacrylate) (poly (VFc-co-HEMA)), on glassy carbon substrates. Figure 3a shows the cyclic voltammograms of the polymer electrodes with only polymer and wt-GOx enzyme in pH 7 phosphate buffer solution. The redox peaks appeared at similar potential for both electrodes, around 0.36–0.38 V, which corresponds to the redox potential of the ferrocene containing polymer mediator. The oxidation currents from electrodes with enzymes were suppressed with the immobilisation of enzymes. A higher current was observed from the CVs in buffer solution with mutant B11-GOx compared to wt-GOx suggesting a better interaction between mutant enzyme and the redox polymer (Fig. 3b). Moreover, it was observed that the potential difference between oxidation peaks and reduction peaks was different. For wt-GOx, it was around 82 mV for the peak separation, while for polymer and B11-GOx it was around 68 mV and 50–59 mV, respectively, which indicated a better reversibility for the mutant enzymes with the polymer mediator [2].

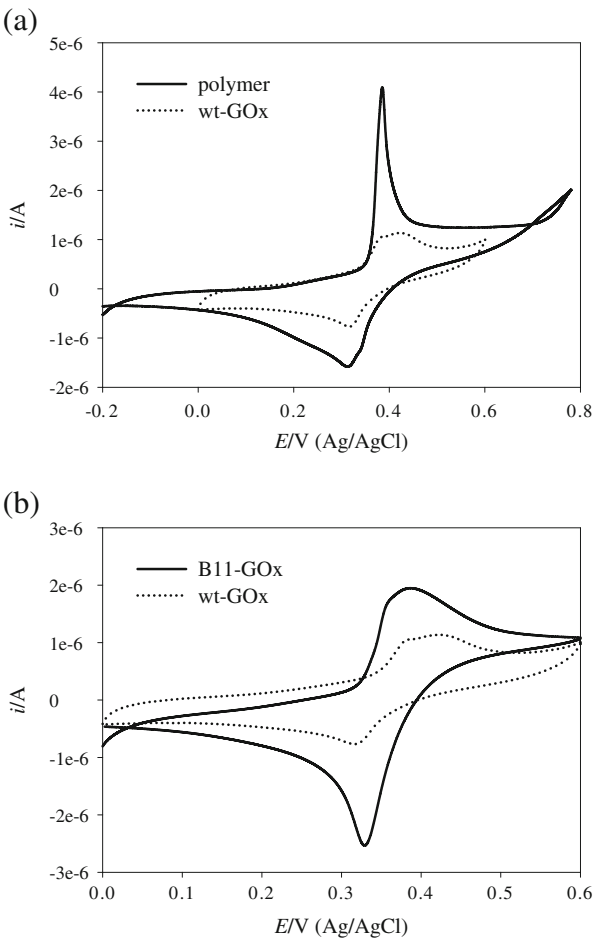

Fig. 3 Cyclic voltammograms for polymer electrodes and electrodes immobilised with wt-GOx and B11-GOx on poly (VFc-co-HEMA), scan rate 5 mV/s, surface area 0.07 cm^2, in 0.1 M phosphate buffer. **a** polymer and wt-GOx. **b** wt-GOx and B11-GOx

Glucose oxidation with glucose oxidase in the presence of ferrocene (FcR) mediator follows the well-studied reaction path [4, 10]

$$\mathrm{FcR} \rightleftharpoons {}^{+}\mathrm{FcR} + \mathrm{e}^{-} \tag{1}$$

$$\mathrm{GOx} - \mathrm{FADH_2} + 2{}^{+}\mathrm{FcR} \rightarrow \mathrm{GOx} - \mathrm{FAD} + 2\mathrm{FcR} \tag{2}$$

$$\mathrm{GOx} - \mathrm{FAD} + \mathrm{glucose} \rightarrow \mathrm{GOx} - \mathrm{FADH_2} + \mathrm{gluconolactone} \tag{3}$$

From this reaction path, the electrochemical activity of the enzyme is largely dependent on the interactions between the mediator and enzyme FAD active centre in polymer matrix.

The CVs for glucose oxidation in 1 mM glucose with B11-GOx and wt-GOx are demonstrated in Fig. 4. With the presence of glucose, the current response from both electrodes was increased indicating that the catalytic activity of the enzymes was retained with the polymer configuration and electrochemical communication between the enzymes

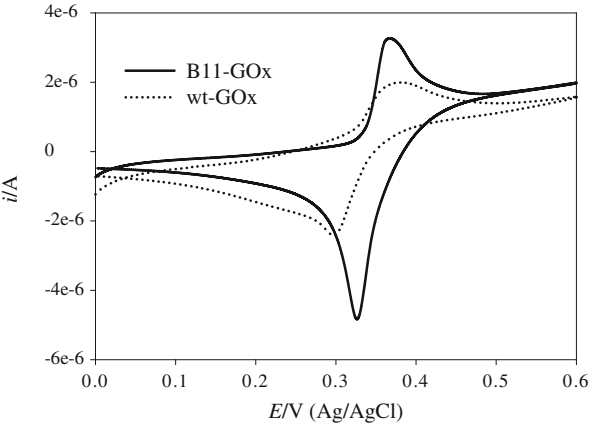

Fig. 4 Cyclic voltammograms for enzyme electrodes immobilised with wt-GOx and B11-GOx on poly(VFc-co-HEMA), scan rate 5 mV/s, surface area 0.07 cm^2, in 0.1 M phosphate buffer with 1 mM glucose, pH 7.0

and electrode surface was also established with the polymer mediator. It was observed that the polymer electrode with wt-GOx showed higher current response in the low potential region, $0.25<E<0.34$ V. A rapid increase of oxidation current occurred on B11-GOx in this potential region, while on wt-GOx, current increased gradually. This could be related to ohmic resistance of the polymer layer. A better interaction between mutant enzyme and ferrocene polymer mediator could lead to a lower resistance in the polymer layer.

Higher glucose oxidation current was achieved from the B11-GOx enzyme for potential higher than 0.34 V, indicating an improved activity from B11-GOx in the higher potential region. From the CVs for glucose oxidation with enzymes in solution and with the polymer enzyme electrode, it can be observed that the shapes of voltammograms are quite different. More defined redox peaks were observed with polymer electrode, and no diffusion controlled plateau appeared. This indicates a different mass transport profile in the polymer electrode form that in solution.

Two Tafel regions were observed (Fig. 5) with the polymer enzyme electrodes immobilised with enzymes. The onset potential for glucose oxidation on B11-GOx was slightly shifted by −10 mV, compared to wt-GOx. With the potential lower than 0.34 V, the Tafel slopes were similar to those in solution, c.a. 0.092 V/dec; with the potential around 0.34 V, different Tafle slopes were observed from B11-GOx (0.02 V/dec) and wt-GOx (0.059 V/dec). The second

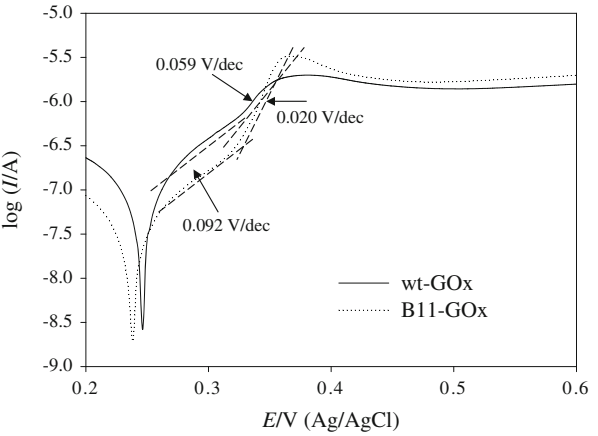

Fig. 5 Tafel plots for enzyme electrodes immobilised with wt-GOx and B11-GOx on poly (VFc-co-HEMA), scan rate 1 mV/s, surface area 0.07 cm^2, in 0.1 M phosphate buffer and with 1 mM glucose, pH 7.0

region is associated with redox process between ferrocene mediator and the enzymes. The smaller Tafel slope obtained from B11-GOx implies a faster electron transfer between enzyme and mediator. This suggests a better interaction between mutant enzymes and polymer mediator than the wt-GOx.

Conclusions

Mutant glucose oxidase (B11-GOx) for ferrocene-mediated electron transfer was obtained by directed evolution from wild-type glucose oxidase (wt-GOx) from *Aspergillus niger*. Electrochemical oxidation of glucose using mutant glucose oxidase (B11-GOx) was carried out in solutions and on the polymer enzyme electrodes by immobilizing enzymes in polymer mediator. B11-GOx showed improved glucose oxidation current both in solution and polymer electrodes compared to wt-GOx indicating an improved electrocatalytic activity. The enzyme electrode with B11-GOx also showed a faster electron transfer indicating a better electronic interaction with the polymer mediator. Higher current response at low glucose concentrations from B11 mutant can lead to developing biosensors with higher sensitivity and biofuel cells with higher power output. New batch of mutant GOx, with similar property of B11-GOx, was cloned with only one propeptide sequence (pro alpha). Catalytic activity assay was carried out, and the kinetic parameters of the new mutant GOx was determined. The k_{cat} value of new mutant enzymes was increased by 2.5 times indicating much improved enzyme catalytic activity. In order to optimally exploit the properties of the improved mutants, further studies are in progress. These encouraging results have shown a promising application of enzymes developed from directed evolution tailored for the applications of biosensors and biofuel cells.

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