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Engineering of Glucose Oxidase for Direct Electron Transfer via Site-Specific Gold Nanoparticle Conjugation

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Supporting Information

ABSTRACT: Optimizing the electrical communication between enzymes and electrodes is critical in the development of biosensors, enzymatic biofuel cells, and other bioelectrocatalytic applications. One approach to address this limitation is the attachment of redox mediators or relays to the enzymes. Here we report a simple genetic modification of a glucose oxidase enzyme to display a free thiol group near its active site. This facilitates the site-specific attachment of a maleimide-modified gold nanoparticle to the enzyme, which enables direct electrical communication between the conjugated enzyme and an electrode. Glucose oxidase is of particular interest in biofuel cell and biosensor applications, and the approach of "prewiring" enzyme conjugates in a site-specific manner will be valuable in the continued development of these systems.

Electron transfer (ET) in enzymes generally occurs through metal centers or tunneling events that are largely insulated by the surrounding globular protein structure. There is great interest in developing devices and advancing applications that include an enzyme−electrode interface, but efficient electrical communciation between enzymes and electrodes is often hampered by this insulating effect.

Enzymatic biofuel cells are one area where efficient electrical contacts between electrodes and enzymes is critical.[1] Since glucose is ubiquitous and abundant in most living organisms, much of the research in enzymatic biofuel cells has focused on the use of glucose as the fuel source. The glucose-based enzymatic fuel cells developed to date have used either glucose oxidase (GOx)[2,3] or glucose dehydrogenase (GDH)[4] at the anode, while laccase, bilirubin oxidase, and other three-copper oxidases have been utilized to reduce oxygen at the cathode.

Achieving direct ET (DET) between a redox enzyme and an electrode is advantageous because it allows one to avoid the problems associated with the use of redox mediators, such as high cost, potential toxicity, and limited stability. Achieving DET depends significantly on the distance between the redox-active cofactor and the electrode surface. Several recent publications have reviewed enzymatic DET processess and approaches.[5−11] Many attempts, with varying degrees of success, to create or modify electrode materials that promote DET with GOx have been reported.[12−23] The challenge in the development of this approach is overcoming the long electron-tunneling distance. In GOx, as with many redox proteins, the redox-active cofactor, flavin adenine dinucleotide (FAD),[24] is buried deeply within the protein core, rendering it inaccessible for direct communication with electrode surfaces. A typical way to overcome this is to add small, mobile redox mediators, which can diffuse into and out of the enzyme active site, ferrying reducing or oxidizing equivalents with them.[25] This approach can have several disadvantages including high cost, potential toxicity, and diffusion of untethered mediators away from the enzyme.

Attachment of such mediators to the enzymatic surface[12,27−29] or to a surrounding redox polymer hydrogel[30−32] can potentially solve the diffusion problem. The attachment of conductive nanoparticles (NPs) to the enzyme cofactor has also proven to be a successful strategy, with the resultant enzyme−NP complex reported to have a higher catalytic turnover rate than the unmodified system.[33] However, this approach involves complex chemical synthesis and reconstitution of native enzymes around modifed cofactors, and none of the above approaches have proven to be amenable to scale-up and practical use.

We sought to bypass such difficulties through a protein-engineering approach in which the enzyme was genetically modified to make it more amenable to simple, site-specific attachment of gold NPs (AuNPs). Instead of having to make extensive chemical modifications to the protein or its cofactor or rely upon random placement of possible attachment points for the mediators, why not modify the gene and let cheap and efficient biological expression systems produce "wire-ready" proteins? Here we report the production of a GOx mutant with a single free sulfhydryl group (cysteine) engineered onto its surface (Figure 1) and the attachment of a single maleimide-labeled AuNP to it as a redox relay (Figure 1, right).

The parent protein for this effort was a double mutant (T56V/T132S) of *Aspergillus niger* GOx with improved catalytic properties created in the laboratory of Susan Brozik at Sandia Laboratories.[34] This protein has three native cysteines, of which two are involved in a disulfide bond and the third is a free cysteine (Cys 521 in Figure 1). To prevent the attachment of AuNPs to the native free thiol, this was mutated to valine to give the triple mutant (T56V/T132S/C521V), which was the starting point for this project. The C521V mutation did not result in a noticeable decrease in enzymatic activity relative to the double-mutant protein using a horseradish peroxidase (HRP) and ABTS activity assay measuring H_2O_2 production[35,36] [Table S1 in the Supporting Information (SI)].

Site-directed mutagenesis was then used to create additional single mutations in the protein to add cysteine side chains at strategic locations on the surface of the protein near the FAD molecule. Five mutations were made at distances from the FAD cofactor ranging from 13.8 to 28.5 Å based on the crystal structure of the native GOx

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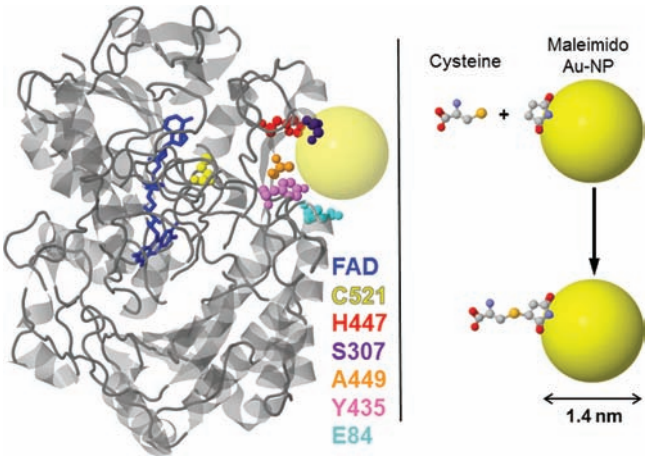

Figure 1. (left) Ribbon diagram of a GOx monomer (from *A. niger*)[26] with the FAD molecule shown in blue. The amino acid residues targeted for mutagenesis are highlighted as space-filling models: cysteine (yellow), histidine (red), serine (purple), alanine (orange), tyrosine (pink), and glutamate (light blue). The yellow sphere represents an idealized AuNP on the same scale as GOx. (right) Schematic drawing of the covalent-binding chemistry of cysteine to a maleimide-modified AuNP. The molecules are displayed as ball-and-stick: carbon (gray), oxygen (red), nitrogen (blue), and sulfur (yellow).

Table 1. Estimated Distances between Surface Cysteine Mutations and the FAD Cofactor

mutation	estimated distance (Å)
H447C	13.8
E84C	15.3
A449C	18.6
Y435C	22.2
S307C	28.5

enzyme (Figure 1 and Table 1). The actual distances could be different because of differences in glycosylation of the recombinant enzyme. The activities of the mutant proteins were relatively unaffected by the mutations, except for the A449C mutation, which resulted in diminished activity (Table S1).

Maleimide-modified AuNPs (1.4 nm) were added to the purified mutant proteins (1:1 ratio) to react with the free thiol groups. Attachment of the AuNPs to the purified proteins led to an immediate and dramatic decrease in activity for all of the mutants investigated, as measured by the HRP/ABTS assay. The activity of the A449C mutant was too low to be measured. The remaining four mutants exhibited residual activities of 25−40% of the original activities, and this was stable for a 16 h time period (Figure S1 in the SI).

These GOx−AuNP conjugates were then tested for DET capability by immobilizing them onto gold electrodes, and control experiments were performed with nonconjugated enzymes (both the mutants and a commercially available wild-type GOx). Upon immobilization of only 5 μL of ca. 1 nM GOx−AuNP solution, a monolayer coverage of the gold electrode was assumed, which should eliminate ET between excess conjugates in the system. The H447C mutant was estimated to have the shortest distance between the newly introduced cysteine group and the FAD center (Table 1), making it the most promising candiate for DET. Indeed, only the H447C mutant exhibited DET activity. Thus, only this mutant was investigated further.

Table 2. Apparent Steady-State Kinetic Parameters of the H447C Mutant and Commercially Available GOx before and after Covalent Attachment of AuNPs

	$k_{cat,\ app}$ (s^{-1})	$K_{M,app}$ (mM)
H447C	425 ± 50	15.0 ± 4.4
GOx	152 ± 11	96.4 ± 28.3
H447C−AuNP	55.3 ± 0.8	8.2 ± 2.5
GOx−AuNP	22.8 ± 3.3	6.3 ± 0.9

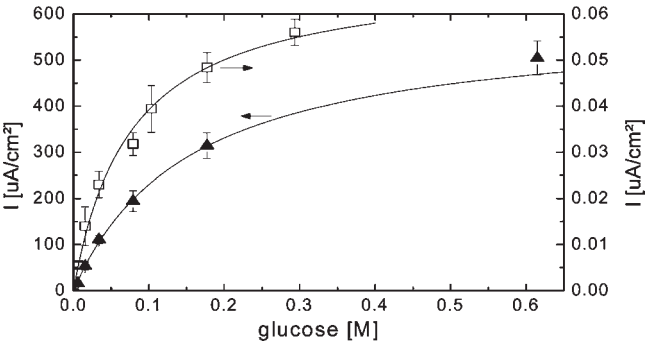

Figure 2. Amperometric responses of (▲, left axis) H447C mutant and (□, right axis) H447C−AuNP conjugate to glucose oxidation under O_2 saturation at an applied potential of +0.6 V.

The apparent steady-state kinetic parameters for the H447C mutant with and without AuNP conjugation were measured using the HRP/ABTS assay with saturating oxygen concentrations, and these values were compared to those obtained using the commercially available GOx with and without AuNP conjugation (Table 2). The increased activity imparted by the parent double mutations in the H447C quadruple mutant relative to the commercially available enzyme is obvious. The addition of the AuNPs decreased the overall apparent saturating enzymatic activity ($k_{cat,app}$) of both enzymes (as measued by H_2O_2 production). Interestingly, the addition of the AuNPs decreased the apparent Michaelis constant ($K_{M,app}$) in each case. Other researchers have observed unexpected decreases in K_M values when enzymes are conjugated with NPs,[37,38] and this could be due to subtle structural changes induced by the attachment of the NPs. Further experiments were performed to verify this effect on the K_M values.

H_2O_2 is the coproduct of glucose oxidation in solution, and as an alternative to the ABTS assay, its concentration can be determined by measuring the current resulting from its direct electrochemical oxidization at 600 mV (vs Ag/AgCl) on gold electrodes. Thus, the non-DET activity of the enzyme can also be measured electrochemically. The resulting amperometric response of H_2O_2 as a coproduct is shown in Figure 2. The $K_{M,app}$ values derived from these measurements were found to follow a trend similar to that in the solution experiments (155 ± 14 mM for H447C and 75 ± 10 mM for H447C−AuNP). Although the kinetic mechanisms of ping-pong enzymes can be complex,[39] these results suggest that the addition of the AuNPs increases the apparent affinity of the enzyme for the glucose substrate.

The GOx enzyme catalyzes the oxidation of glucose to gluconolactone via reduction of the FAD cofactor to $FADH_2$. The reoxidation of $FADH_2$ in the ping-pong mechanism is normally achieved using oxygen as the electron acceptor. Therefore, either the competing direct oxidation of $FADH_2$ on an electrode must be performed under

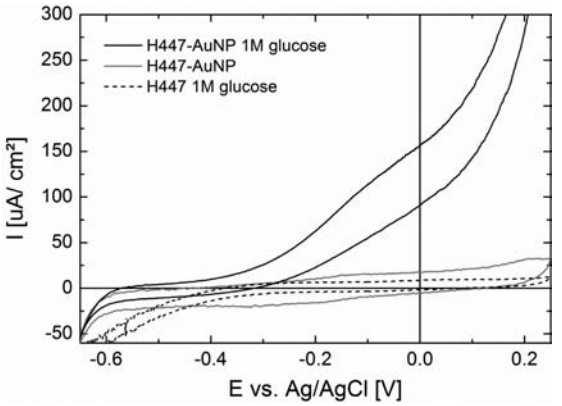

Figure 3. CVs of H447C−AuNP conjugates on a gold electrode in the presence (black line) and absence (gray line) of 1 M glucose (N_2-saturated buffer, pH 7, 10 mV/s). The CV for unconjugated H44C is shown as a dotted line. The H447C−AuNP conjugates in the presence of glucose exhibit enzymatic glucose oxidation starting at ca. −400 mV.

oxygen-free conditions or the rate of reoxidation of $FADH_2$ by the electrode (the actual DET reaction) must be higher than that of the competing reduction of oxygen to peroxide (the native electron-acceptor process for GOx). All of the GOx mutants and their AuNP-modified analogues listed in Table 1 were tested for DET via cyclic voltammetry in N_2-saturated buffer. Only the H447C−AuNP conjugate exhibited a significant oxidative current starting at −400 mV upon addition of glucose (Figure 3). The steep increase in the oxidative current is due to enzyme-catalyzed glucose oxidation. The starting potential of −400 mV indicates direct contact between the electrode and the FAD center, which has a formal potential of −460 mV vs Ag/AgCl at pH 7.9. In control experiments (Figure 3), the same mutant without the AuNP modification showed no apparent DET upon glucose addition. The observed onset of a reductive process for the unmodified enzyme may have several interpretations, such as the onset of an oxygen reduction reaction at the electrode or a H_2O_2 reduction reaction. The fact that only the modified enzyme exhibited a DET signal leads to the conclusion that electrical contact between the electrode and FAD center is made through the conjugated AuNP.

A very similar approach of bridging the FAD center and an electrode via AuNPs was reported by Willner et al.,[40,41] and this was discussed as an electron-mediating two-electron "relay". The major difference in the approach presented here is that the AuNPs are designed to be attached to the surface of the protein near the FAD center, which should decrease the ET distance. Overall we observed similar results: a catalytic glucose oxidation current without the appearance of the $FAD/FADH_2$ redox wave. Even though the enzymatic oxidation begins at ca. −400 mV, its shape is tilted toward more anodic potentials. Both effects can be explained by a voltage drop caused by the AuNP bridge between the enzyme and the electrode, which can serve as an electron relay while adding additional resistance to the system.

The fact that the H447C mutant was the only one shown in this study to promote DET successfully underlines the importance of the ET distance. Of the five residues targeted for site-directed replacement with cysteine in this work, H447C is the closest to the FAD in the crystal structure (Table 1). The modern Marcus theory[42,43] has been applied to proteins and correctly predicts the exponential decrease in the ET rate constant (k_{ET}) with the ET distance (d): $k_{ET} = k_0 \exp[-\beta(d - d_0)]$, where k_0 is the ET rate constant at the distance of closest contact d_0 and the pre-exponential

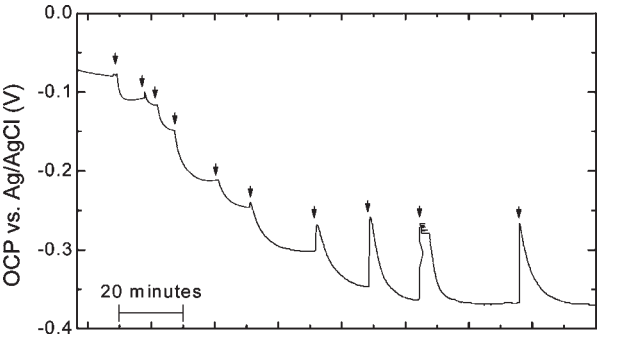

Figure 4. Potentiostatic response of the open-circuit potential with time for the H447C−AuNP conjugate upon addition of glucose (arrows) in N_2-saturated buffer (a plot of E vs log concentration is shown in Figure S3).

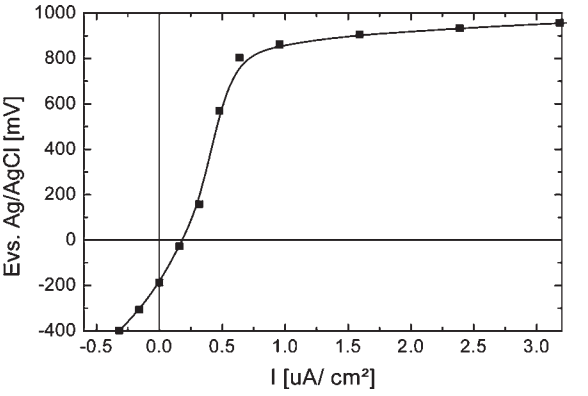

Figure 5. Potentiostatic polarization curve of H447C−AuNP conjugates on a gold electrode with 1 M glucose in N_2-saturated buffer. The current density was calculated using the geometric surface area of the gold electrode.

factor β is typically in the range 8.5−11.5 nm^{-1}.[44] From the location of the mutation and the size of the AuNPs used in this study, we can hypothesize that the distances inferred above have been met, as evidenced by the voltammetric response to glucose (Figure 3).

In previous reports, cyclic voltammograms (CVs) presenting DET of GOx typically show a pair of reversible redox peaks corresponding to the surface-bound prosthetic group FAD.[12,14,16−19,21−23] We can hypothesize that the lack of those peaks in the present work indicates AuNP-bridged contact of the redox center without blocking the active center from substrate access.

The CVs in Figure 3 show an additional reduction process starting at ca. −400 mV. Most likely, small amounts of H_2O_2 were produced by the enzyme because of the presence of some O_2 in the system, and this H_2O_2 can be oxidized or reduced on the gold electrode. The existence of both catalytic reactions is also supported by potentiometric data. Figure 4 shows the dependence of the open-circuit potential (OCP) as a function of increasing glucose concentration. There is a clear negative shift in the redox potential in presence of glucose that reaches a steady-state potential of −370 mV at a glucose concentration of ca. 1 M, which is close to the theoretical redox potential of FAD. According to the Nernst equation, pure catalysis by $FAD/FADH_2$ should show OCPs starting at negative potentials without glucose and decreasing by 60 mV per concentration decade, stabilizing at the formal potential of −450 mV for $FAD/FADH_2$. Since the decrease in OCP with glucose concentration does not follow perfect Nernstian behavior, the existence of a

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competing H_2O_2 reaction is feasible (Figure S2). It should also be noted that there is no direct evidence that all enzymes in the samples are modified with a single AuNP, so the presense of some unmodified enzymes can also explain the observation of such behavior. On the other hand, the closeness of the OCP to the redox potential of FAD/$FADH_2$ indicates that the majority of the sample was modified with AuNPs.

The GOx enzyme has been extensively investigated for use in biosensors and enzymatic biofuel cells. A potentiostatically obtained polarization curve of H447C−AuNP conjugate GOx electrode (Figure 5) shows the benefits of the genetically engineered GOx wired via AuNPs for use in enzymatic biofuel cell applications.

This study has presented the successful site-specific modification of GOx enzymes with AuNPs via protein engineering. The attachment of the NP reduces the apparent catalytic activity of the enzyme while also decreasing the apparent K_M of the enzyme for its substrate. Upon immobilization on an electrode, attachment of the AuNP enables direct electrical communciation across the enzyme−electrode interface. This work demonstrates the achievements that can be made in the engineering of proteins for improvments in the interface between biotechnology and nanotechnology. The new mutant enzyme described here holds great promise for use in third-generation amperometric biosensors (based on DET) or as anodes in microbiofuel cells as a result of the tight electrical contact formed between it and the electrode surface after it is conjugated with off-the-shelf AuNPs.

■ ASSOCIATED CONTENT

Supporting Information. Details of GOx protein engineering, kinetic evaluation of the different mutants, and data analysis using the Nernst equation. This material is available free of charge via the Internet at http://pubs.acs.org.

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