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Integrated Oligoaniline-Cross-Linked Composites of Au Nanoparticles/Glucose Oxidase Electrodes: A Generic Paradigm for Electrically Contacted Enzyme Systems

Omer Yehezkeli, Yi-Ming Yan, Ilina Baravik, Ran Tel-Vered, and Itamar Willner*[a]

Abstract: Electropolymerizable glucose oxidase (GOx) was synthesized by the tethering of thioaniline units to the protein. The electropolymerization of the thioaniline-modified GOx in the presence of thioaniline-functionalized Au nanoparticles (NPs) yielded a three-dimensional bis-aniline-cross-linked Au NPs/GOx network. The bis-aniline bridging units are redox active and mediate the electron transfer between the enzyme redox sites and the electrode, and the Au NPs provide the three-dimensional conductivity for transporting the electrons to the electrode. The covalently linked GOx displays an effective electrical contact with the electrode (turnover rate of ca. $500\,s^{-1}$), which results in a sensitive and selective glucose-sensing electrode. The preparation of the electrode by means of electropolymerization, and the bioelectrocatalytic features of the electrode, suggest its feasibility as a miniaturized electrode with a sensing matrix and as an implantable glucose sensor in future applications.

Keywords: biosensors · electropolymerization · glucose oxidases · gold · nanostructures

Introduction

The electrical contact between enzymes and electrodes has been a subject of extensive research over the past two decades.[1–4] Diffusional electron mediators,[5–7] functionalization of the enzymes with redox-relay units,[8–10] and the incorporation of the enzymes in redox polymer matrices[11–16] are common methods to establish electron-transfer communication between the redox sites in the enzymes and the electrodes. However, the mediated electrical contact of redox enzymes by using these different means reveals relatively low turnover electron-transfer values between the redox centers of the enzymes and the electrodes. Also, the mediated bioelectrocatalytic activation of the enzymes often requires relatively positive oxidation potentials that perturb the electrode performance due to the nonspecific oxidation of interfering substances. The structural alignment, and the electrical contact of redox enzymes on electron-relay-functionalized electrodes by using the reconstitution method has been reported as an effective means of electrical communication between the redox centers of enzymes and electrodes.[17,18] Specifically, the reconstitution of enzymes on gold nanoparticles was proven to be an effective means to electrically activate the biocatalysts.[19,20] Although the surface reconstitution of enzymes leads to an effective electrical contact between the enzymes and the electrodes, the practical applicability of this method is limited due to the synthetic complexity involved in constructing the electrodes. Any further development of electrically contacted enzyme electrodes, and particularly the development of implantable glucose-sensing electrodes for the analysis of subcutaneous fluids, requires several fundamental challenges to be addressed: 1) The electrical contact should be efficient to enhance the sensitivity of the enzyme electrode. This is particularly important since low-surface-area miniaturized electrodes are needed for implantable applications. 2) The bioelectrocatalytic functions of the electrode should be activated at relatively low potentials to prevent nonspecific oxidation of interfering substances. 3) The turnover rate of electrons between the enzyme and the electrode should be high, to compete with any nonspecific oxidation of redox-active interfering substances. Also, high turnover rates are essential to eliminate the competitive reaction of the enzyme, glucose oxidase (GOx), with oxygen. 4) The enzyme (working) electrode with a biocatalytic matrix in a miniaturized three-elec-

[a] O. Yehezkeli, Dr. Y.-M. Yan, I. Baravik, Dr. R. Tel-Vered, Prof. I. Willner
Institute of Chemistry
The Hebrew University of Jerusalem
Jerusalem, 91904 (Israel)
Fax: (+972) 2-652-7715
E-mail: willnea@vms.huji.ac.il

trode configuration should be constructed by using a high-throughput method.

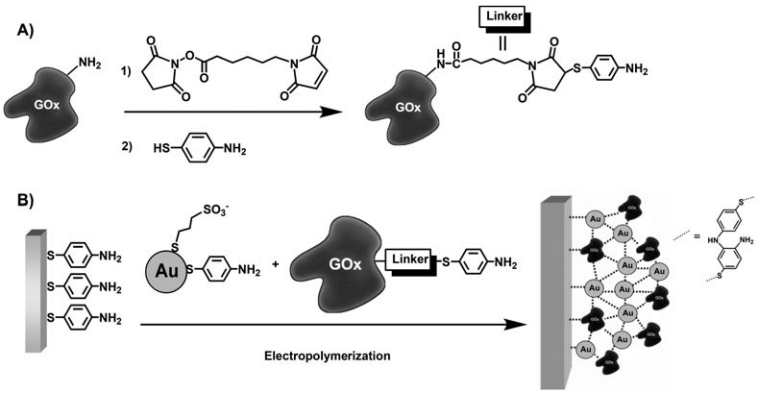
Herein we wish to report on an electropolymerization process that yields integrated, electrically contacted, enzyme electrodes consisting of Au nanoparticles (NPs)/GOx composites, which are cross-linked by redox-active bis-aniline bridging units. Besides the specific addressing of the enzyme to the working electrode, the electropolymerization process yields effectively electrically contacted electrodes that reveal selectivity. The amperometric responses of the electrodes are controlled by the electropolymerization process (number of electropolymerization cycles and ratio of the NPs to electropolymerizable enzyme), and the method permits the miniaturization of the resulting devices. Furthermore, we introduce a method to functionalize enzyme units with electropolymerizable thioaniline groups, and demonstrate the three-dimensional assembly of a Au NPs/enzyme network.

Results and Discussion

Scheme 1 outlines the method used to assemble the biosensor electrodes. Au NPs (diameter ca. 3.5 nm) were functionalized with a mixed capping layer consisting of 4-mercaptoaniline and mercaptoethane sulfonic acid. The enzyme glucose oxidase (GOx) was functionalized with 4-mercaptoaniline by the primary modification of the lysine residues of the protein with the bifunctional reagent *N*-(maleimidocaproyloxy)sulfosuccinimide ester, followed by the Michael addition of mercaptoaniline to the maleimide sites (Scheme 1A). The activity of the thioaniline-modified enzyme was determined, by the O_2-mediated oxidation of glucose and the colorimetric detection of the resulting H_2O_2, to be approximately 95% of the activity of the native enzyme. The enzyme was stored in a lyophilized state (under nitrogen, at −20°C) for at least three months with no observable loss in its activity. Previous studies in our laboratory indicated that the thioaniline-functionalized Au NPs may be electropolymerized onto a mercaptoaniline-monolayer-modified electrode through the cross-linking of the particles with oligoaniline bridges.[21] Accordingly, the functionalized Au NPs were electropolymerized in the presence of the thioaniline-modified GOx and mercaptoaniline-functionalized electrode, by cycling the electrode potential between −0.1 and +1.1 V versus a saturated calomel electrode (SCE), to yield the bis-aniline-cross-linked Au NPs/GOx composite. The electropolymerization yielded a conductive, three-dimensional Au NPs/GOx composite (Scheme 1B).

The Au NPs provide the three-dimensional conductivity needed to assemble the enzyme-cross-linked structure. Figure 1 shows the electrocatalytic anodic currents observed upon analyzing different concentrations of glucose by using the Au NPs/GOx-functionalized electrode. In this experiment the electrode was constructed by applying 60 electropolymerization cycles, and the molar ratio of the thioaniline-modified Au NPs to GOx in the electropolymerization solution was 3.6:1 (for the selection of the number of electropolymerization cycles, and the respective ratio of the thioaniline-Au NPs/GOx, vide infra). As the concentration of the glucose increases, the electrocatalytic anodic currents intensify. Figure 1 (inset) shows the derived calibration curve, in which the current responses at $E=+0.3$ V (versus SCE) are plotted as a function of the concentration of glucose. The bioelectrocatalyzed oxidation of glucose by the bis-aniline-cross-linked Au NPs/GOx-functionalized electrode is attributed to mediated electron transfer in the matrix. The bis-aniline bridges exhibit a quasireversible redox wave in the region of −0.15 to +0.15 V (versus SCE) at pH 7.4. The bioelectrocatalytic currents appear at the oxidation potential of the bis-aniline units, which suggests that the bridging units mediate the bioelectrocatalytic process.

The ratio of Au NPs to GOx in the electropolymerization solution is expected to affect the performance of the resulting electrode. A higher content of biocatalyst in the Au NPs/GOx composite is anticipated to increase the sensitivity of the modified electrode and to increase its amperometric response. On the other hand, one should realize that the covalent linkage of the protein to the Au NPs perturbs the conductivity of the NPs. That is, in the presence of high enzyme concentrations in the electropolymerization solution the effective coverage of primary NPs linked to the electrode is low, which insulates the particles towards further electropolymerization, and leads to a low total content of the enzyme in the resulting composite. Thus, we anticipate that the Au NPs/GOx composite should reveal a peak bioelectrocatalytic activity at a certain Au NPs/GOx ratio. Figure 2 depicts the ampero-



Scheme 1. A) Modification of glucose oxidase with the polymerizable thioaniline functionality. B) Synthesis of the three-dimensional oligoaniline-cross-linked Au NPs/GOx composite by the electropolymerization of the thioaniline-modified GOx and thioaniline-modified Au nanoparticles on the thioaniline-monolayer-functionalized Au electrode.

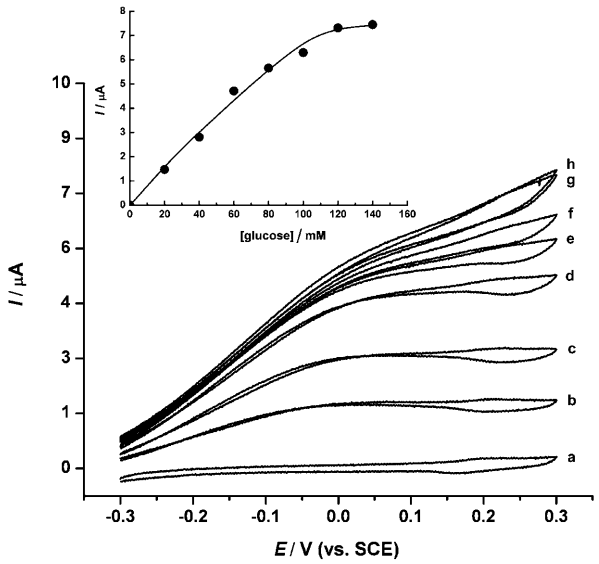


Figure 1. Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrode in the presence of the following concentrations of glucose: a) 0, b) 20, c) 40, d) 60, e) 80, f) 100, g) 120, and h) 140 mM. Scan rate: 5 mV s^{-1}. Inset: calibration curve corresponding to the electrocatalytic currents, measured at $E=+0.3$ V versus SCE for different concentrations of glucose. The electrodes were prepared by the application of 60 cyclic voltammetry scans between -0.1 and $+1.1$ V (versus SCE) at 100 mV s^{-1}, using thioaniline-modified Au NPs and thioaniline-modified GOx at a molar ratio of 3.6:1. All data were recorded in a 0.1 M phosphate buffer solution, pH 7.4, under N_2.

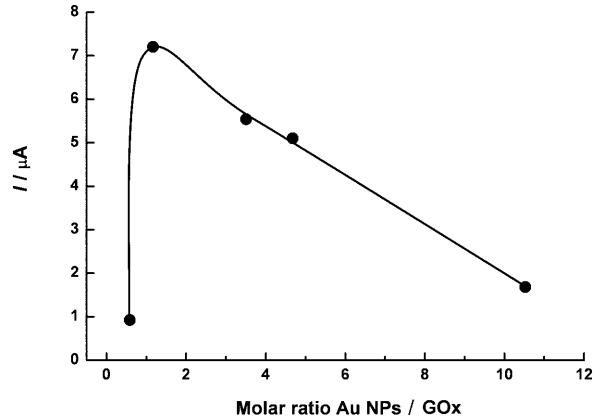


Figure 2. Electrocatalytic currents corresponding to the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrodes, prepared by the application of 60 cyclic voltammetry scans between -0.1 and $+1.1$ V (versus SCE) at 100 mV s^{-1}, in the presence of 0.5 mg mL^{-1} thioaniline-modified GOx and variable concentrations of thioaniline-modified Au NPs. Cyclic voltammograms were performed in a 0.1 M phosphate buffer solution (pH 7.4) that included 60 mM glucose, under N_2. Scan rate: 5 mV s^{-1}. The indicated currents were measured at $E=0.3$ V versus SCE.

metric responses of the Au NPs/GOx composite electrodes electropolymerized in the presence of different ratios of Au NPs to GOx. At a molar ratio of Au NPs/GOx corresponding to 1.2:1, the highest bioelectrocatalytic activity of the electrode is observed.

The number of applied electropolymerization cycles is also anticipated to control the amount of the biocatalyst in the resulting Au NPs/GOx composite. Figure 3 shows the

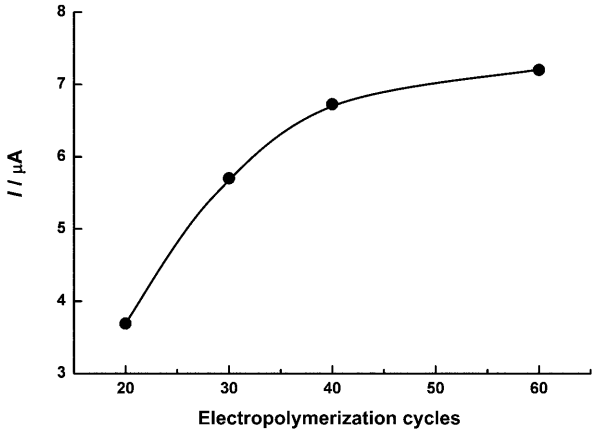


Figure 3. Electrocatalytic currents corresponding to the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrodes, prepared by the application of variable number of cyclic voltammetry scans between -0.1 and $+1.1$ V (versus SCE) at 100 mV s^{-1}, in the presence of thioaniline-modified Au NPs and thioaniline-modified GOx at a molar ratio of 1.2:1. Measurements were performed in a 0.1 M phosphate buffer solution (pH 7.4) that included 60 mM glucose, under N_2. Scan rate: 5 mV s^{-1}. The indicated currents were measured at $E=+0.3$ V versus SCE.

electrocatalytic anodic currents generated by the Au NPs/GOx composite electrodes, prepared by applying a variable number of electropolymerization cycles, and upon using an optimized Au NPs/GOx ratio of 1.2:1 in the electropolymerization solution. The bioelectrocatalytic anodic currents were recorded in the presence of a fixed concentration of glucose (60 mM). As the number of electropolymerization cycles increases, the bioelectrocatalytic currents intensify and then level off after approximately 60 cycles. The increase in the amperometric response is attributed to the higher content of the enzyme in the resulting conducting Au NPs/GOx composite electrodes, whereas the saturation of the current is attributed to the gradually increasing perturbations in the conductivity paths of the Au NPs attaching the enzyme units, as electropolymerization proceeds. Alternatively, inner parts of the Au NPs/GOx composite might become inaccessible to glucose as electropolymerization proceeds. Thus, we find that the application of 60 electropolymerization cycles yields the composite electrode with optimal bioelectrocatalytic function.

Figure 4A demonstrates the electrocatalytic currents observed upon analyzing different concentrations of glucose by using the Au NPs/GOx-functionalized electrodes, assembled under the optimized conditions. As the concentration of glucose is increased, the electrocatalytic anodic current increases. The respective calibration curve is depicted in the inset

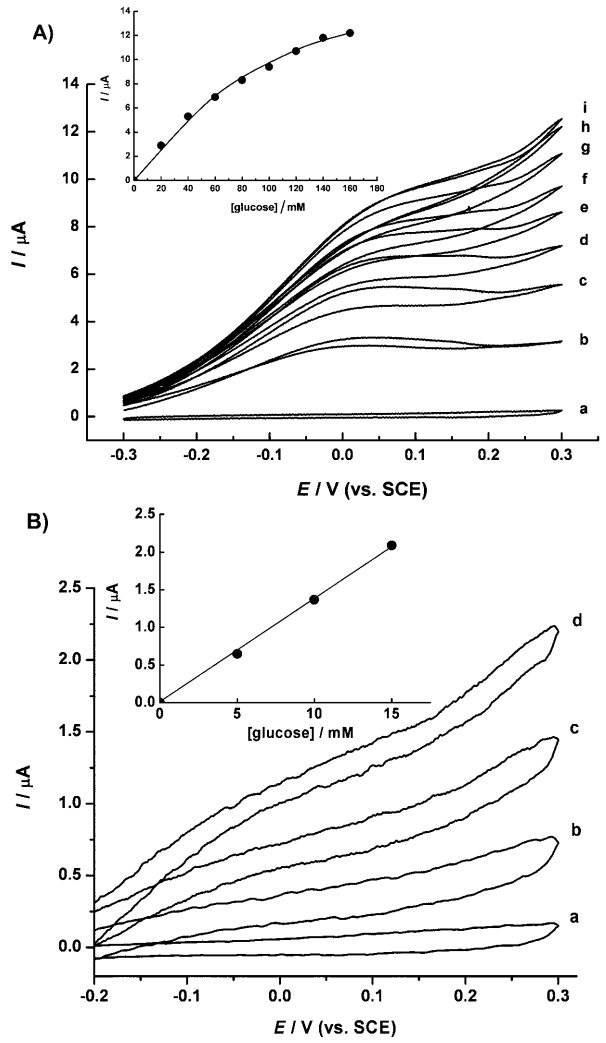


Figure 4. A) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrode in the presence of the following concentrations of glucose: a) 0, b) 20, c) 40, d) 60, e) 80, f) 100, g) 120, and h) 140 mM. Scan rate: 5 mV s^{-1}. Inset: calibration curve corresponding to the electrocatalytic currents measured at $E=+0.3$ V versus SCE for different concentrations of glucose. B) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrode in the range of glucose concentrations relevant for diabetes: a) 0, b) 5, c) 10, d) 15 mM. Inset: calibration curve corresponding to the electrocatalytic currents measured at $E=+0.3$ V versus SCE for the range of glucose concentrations relevant for diabetes. The electrodes were prepared by the application of 60 cyclic voltammetry scans between -0.1 and $+1.1$ V (versus SCE) at 100 mV s^{-1}, using thioaniline-modified Au NPs and thioaniline-modified GOx at a molar ratio of 1.2:1.

of Figure 4A. The electrocatalytic current converges to a saturation value of approximately 23 μA.

By assaying the activity of the GOx linked to the electrode, a surface coverage of approximately 2.0×10^{-12} mol cm^{-2} is revealed. By using the saturation current and the enzyme loading, we estimate the turnover rate of electrons between the enzyme redox center and the electrode to be approximately 500 s^{-1}. Furthermore, gravimetric quartz crystal microbalance (QCM) measurements enabled us to elucidate the surface coverage of the Au NPs, and the Au NPs/GOx molar ratio in the network. The total mass of the Au NPs and GOx on the electrode corresponded to 1.9×10^{-6} g cm^{-2}. Knowing the weight content of the GOx in the array from the activity assay, we estimated the coverage of Au NPs to be 5.7×10^{-12} mol particles cm^{-2}. Thus, the molar ratio of Au NPs/GOx corresponded to 2.8:1 (note that the Au NPs/GOx ratio in the polymerization solution corresponded to 1.2:1). The electrode responses are in the appropriate concentration range for analyzing glucose levels in diabetic patients (Figure 4b).

In a further control experiment we examined the significance of modifying GOx with the electropolymerizable thioaniline units for the assembly of a cross-linked Au NPs/enzyme hybrid system. In this experiment we electropolymerized the functionalized Au NPs in the presence of native GOx that lacked the electropolymerizable units (60 electropolymerization cycles). We found that GOx is incorporated in the cross-linked Au NPs composite, presumably due to electrostatic or hydrogen-bond interactions between the functional capping layer of the NPs and the protein. The content of the enzyme in the electropolymerized composite is, however, substantially lower in comparison with the enzyme-cross-linked configuration (ca. 3×10^{-13} mol cm^{-2}). The enzyme incorporated in the Au NPs matrix by this inefficient route yields electrical contact with the electrode, and stimulates the bioelectrocatalyzed oxidation of glucose (Figure 5). The lower content of the enzyme in the composite results in substantially lower amperometric responses.

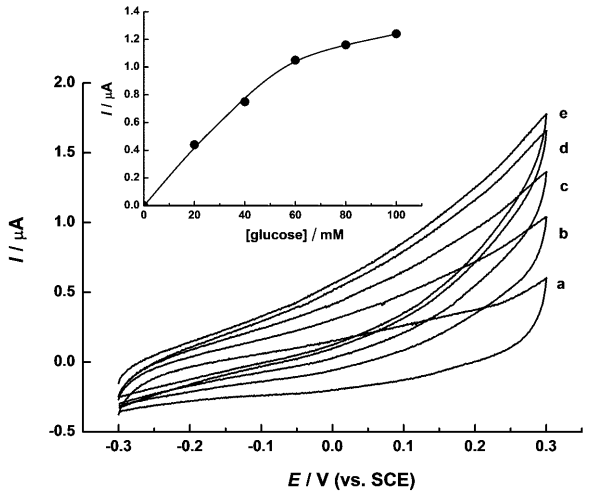


Figure 5. Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of glucose by an oligoaniline-cross-linked Au NPs-modified Au electrode, electropolymerized in the presence of native GOx that lacked the electropolymerizable thioaniline units. Measurements were performed at the following concentrations of glucose: a) 0, b) 20, c) 40, d) 60, and e) 80 mM. Scan rate: 5 mV s^{-1}. Inset: calibration curve corresponding to the electrocatalytic currents measured at $E=+0.3$ V versus SCE for different concentrations of glucose. The electrodes were prepared by the application of 60 cyclic voltammetry scans between -0.1 and $+1.1$ V (versus SCE) at 100 mV s^{-1}, using thioaniline-modified Au NPs and native GOx at a molar ratio of 1.2:1.

A further aspect that needs to be addressed relates to the specificity of the resulting electrodes. Figure 6 depicts the amperometric responses of a GOx/Au NPs electrode in the presence of a common interfering substance in glucose sens-

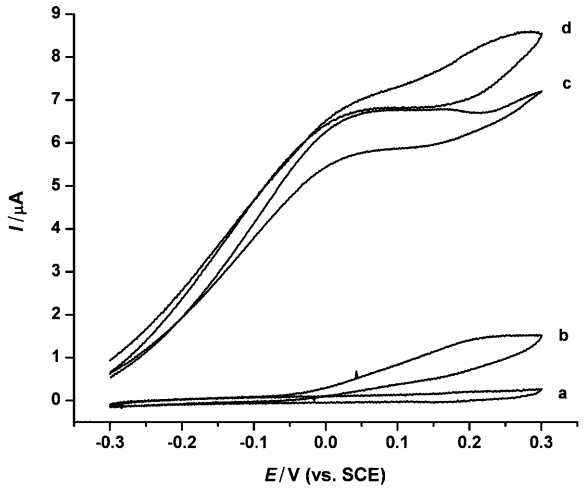


Figure 6. Cyclic voltammograms corresponding to the oligoaniline-cross-linked Au NPs/GOx-composite-modified Au electrode upon treatment with: a) a pure buffer; b) 0.1 mM ascorbic acid; c) 60 mM glucose; d) 60 mM glucose and 0.1 mM ascorbic acid. All measurements were performed in 0.1 M phosphate buffer, pH 7.4, under N_2. Scan rate: 5 $mV s^{-1}$. The electrode was prepared by the application of 60 cyclic voltammetry scans between −0.1 and +1.1 V (versus SCE) at 100 $mV s^{-1}$, using thioaniline-modified Au NPs and thioaniline-modified GOx at a molar ratio of 1.2:1.

ing, namely, ascorbic acid. It can be seen that ascorbic acid has a minute effect on the resulting current (<3–8% in the region 0 to +0.1 V). Upon testing the optimized electrodes for glucose oxidation in an air-saturated atmosphere, we have also detected a marginal reduction in the intensities of the electrocatalytic currents. This observation is attributed to the efficient electrical contact of the electropolymerized enzyme/Au NPs/bis-aniline relays with the electrode. The bioelectrocatalytic process competes effectively with the O_2-biocatalyzed oxidation of glucose, and this allows the analysis of glucose under aerobic conditions. However, the miniaturization of the bioelectrocatalytic, electrically contacted electrodes requires the development of additional routes to enhance the amperometric signals. To reach this goal, we roughened the electrode surface by the amalgamization of the Au with mercury, and the subsequent removal of the amalgam by concentrated nitric acid. Upon this treatment, the surface area of the roughened electrode increased by approximately 50-fold relative to the nonroughened surface. Figure 7 shows the amperometric responses of a roughened Au NPs/GOx-functionalized electrode, generated by 60 cycles at the optimal Au NPs/GOx ratio, upon analyzing various concentrations of glucose. Clearly, the amperometric responses are up to approximately 20-fold higher than the analogous, nonroughened electrode.

Finally, the stability of the resulting electrodes was examined. Even though no attempts were made to stabilize the

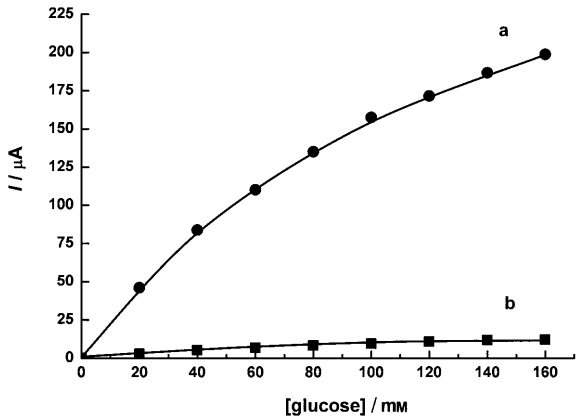


Figure 7. Calibration curves corresponding to the analysis of various concentrations of glucose by the oligoaniline-cross-linked Au NPs/GOx-composite-functionalized structure immobilized on a) a thioaniline-modified roughened Au electrode and b) a thioaniline-functionalized Au electrode (roughness factor 1.3). The calibration curves were extracted from the respective cyclic voltammograms at +0.3 V versus SCE. Electrodes were prepared by applying 60 electropolymerization cycles, using thioaniline-modified Au NPs and thioaniline-functionalized GOx at a molar ratio of 1.2:1. Cyclic voltammograms were recorded at the conditions given in Figure 4.

electrodes by polymer coating or to store the electrodes under sterile conditions, we observed a 20% decrease in the electrode activity after continuous operation at room temperature, and we found that the electrodes lost approximately 15% of their activity upon storage for seven days at 4°C. The further coating of the surfaces with inert polymer films, and handling the electrodes under sterile conditions, are anticipated to enhance their long-term stability.

It should be noted that related glucose-sensing systems have a polyaniline matrix for electrical contact and charge transport. The enzyme GOx was coimmobilized with the polymer on the electrode,[22] physically adsorbed on the polymer,[23] or electrodeposited on the polymer.[24] Our system differs from these electrodes by several key features: 1) The enzyme/polyaniline-based films require a hydrogel configuration to allow the effective permeation of glucose. As the hydrogel state is sensitive to the enzyme content, only limited amounts of the enzyme can be incorporated into the polymer film. The present system provides a nanoporous structure as an intrinsic feature. 2) The charge transport across polyaniline films is controlled by electron exchange between polymer segments.[25] In the present system electrons are transported through the three-dimensional Au NPs network and give rise to the efficient electrical contact of the enzyme with the electrode. The effective charge transport through the Au NPs/enzyme composite makes the electrode insensitive to oxygen and almost unaffected by interfering substances; 3) Electropolymerization of polyaniline proceeds at acidic pH (pH<3), thus limiting the possibility of preparing the enzyme electrode by electropolymerization. The electrodes assembled in the present study are prepared by electropolymerization at a neutral pH, giving rise to covalently linked Au NPs/GOx networks. This enables the directing of

the bioelectrocatalytic composite to the working electrode, and provides the ability for the parallel, high-throughput synthesis of many miniaturized electrodes.

Conclusion

The present study has introduced a new paradigm to construct integrated enzyme electrodes featuring effective electrical contact by the electropolymerization of electropolymerizable Au NPs and electropolymerizable GOx. The resulting bis-aniline-cross-linked Au NPs/GOx composites demonstrate the efficient electron-transfer-mediated bioelectrocatalytic activation of GOx. The effective electrical contact of the enzyme with the electrode enabled the selective analysis of glucose, and facilitated the detection of glucose in the presence of O_2. The method for fabricating the electrodes holds promise for tailoring miniaturized bioelectrocatalytically active electrodes.

Experimental Section

Nanoparticle synthesis: The Au nanoparticles functionalized with 2-mercaptoethane sulfonic acid and *p*-aminothiophenol (Au NPs) were prepared by mixing a solution of $HAuCl_4$ (197 mg) in ethanol (10 mL) with a solution of mercaptoethane sulfonate (42 mg) and *p*-aminothiophenol (8 mg) in methanol (5 mL). The two solutions were stirred in the presence of glacial acetic acid (2.5 mL) in an ice bath for 1 h. Subsequently, an aqueous solution of 1 M sodium borohydride ($NaBH_4$, 7.5 mL) was added dropwise to the solution, resulting in a dark color associated with the presence of the Au NPs. The solution was stirred for one additional hour in an ice bath, and then for 14 h at room temperature. The particles were successively centrifuged and washed (twice in each solvent) with methanol, ethanol, and diethyl ether. The average size of the NPs was estimated by using TEM analyses to be (3.6 ± 0.3) nm.

Modification of the enzyme: Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*, 210000 U g^{-1}, purchased from Sigma; 52 mg) was dissolved in 0.1 M phosphate buffer (3 mL, pH 7.4). The solution was then treated with *N*-(maleimidocaproyloxy)sulfosuccinimide ester (sulfo-EMCS, obtained from PIERCE; 52 μL, 12 mg mL^{-1}). The resulting solution was stirred for 40 min and was then mixed with 4-aminothiophenol (thioaniline, 1.6 mg mL^{-1}) in ethanol (0.8 mL). After the reaction had been left for 2.5 h, the solution was eluted through a G-25 column (GE Healthcare) with 0.1 M phosphate buffer (pH 7.4) as the eluent. The resulting purified, functionalized GOx solution was lyophilized to yield a pale yellow powder that was stored at −20 °C.

Modification of the electrodes and the electrochemical detection of glucose: Clean Au wires (0.3 cm^2) were reacted with a 10 mM solution of thioaniline in ethanol for 2 h. The functionalized electrodes were then assembled by the electropolymerization of the thioaniline-modified Au NPs and the thioaniline-modified GOx in 0.1 M phosphate buffer (pH 7.4) by using a fixed number of repetitive cyclic voltammetry scans, ranging between −0.1 and +1.1 V (versus SCE), at a scan rate of 100 mV s^{-1}. Surface-roughened electrodes were prepared by interacting clean Au wires with Hg for 2 min, with subsequent removal of the amalgamated layer using concentrated nitric acid. All electrochemical measurements were performed by employing a PC-controlled (Autolab GPES software) potentiostat/galvanostat (μAutolab, type III). A graphite rod ($d = 5$ mm) was used as the counter electrode and the reference was a standard calomel electrode (SCE).

Determination of the content of GOx in the electropolymerized film: The content of the GOx in the bis-aniline-cross-linked Au NPs/GOx-modified electrode was determined by reacting the electrodes with 1.5×10^{-4} M 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-\bullet}$) and 4.2×10^{-4} M horseradish peroxidase (1×10^6 U g^{-1}) and monitoring the absorbance of the generated $ABTS^-$ at $\lambda = 416$ nm. A calibration curve was derived by measuring several solutions of 0.1 M phosphate buffer at pH 7.4 that included different concentrations of GOx. Using the derived calibration curve and the color generated by the Au NPs/GOx-cross-linked electrode, the content of the enzyme in the matrix was elucidated.

Acknowledgements

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