Bioelectrocatalyzed Amperometric Transduction of Recorded Optical Signals Using Monolayer-Modified Au-Electrodes[1]

Itamar Willner,* Mazzi Lion-Dagan, Sharon Marx-Tibbon, and Eugenii Katz

Contribution from the Institute of Chemistry and Farkas Center for Light-Induced Processes, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

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Abstract: Three different methods to control by light electron-transfer communication between redox-proteins and electrodes are discussed. The systems provide a means for the amperometric transduction and amplification of recorded optical signals. A mixed monolayer of thiol pyridine/nitrospiropyran immobilized onto a Au-electrode provides an active interface for controlling electrical communication between cytochrome *c*, cyt *c*, and the electrode by means of electrostatic interactions. The mixed monolayer exhibits reversible photoisomerizable properties across the nitrospiropyran state, SP, and protonated nitromerocyanine, MRH^+. In the pyridine-SP monolayer state cyt *c* exhibits effective electrical communication with the electrode due to association of the redox-protein to the monolayer interface. Electron-transfer communication between cyt *c* and the electrode is blocked in the pyridine-MRH^+ monolayer state due to electrostatic repulsion of the redox-protein. The reversible "ON−OFF" light-regulated electrical communication of cyt *c* with the monolayer-electrode was coupled to cyt *c* electron-transfer mediated reduction of O_2, in the presence of cytochrome *c* oxidase, COX, and oxidation of lactate, in the presence of lactate dehydrogenase, LDH. Light controlled electrical communication between electrodes and redox-enzyme monolayers associated with the electrodes was established by the application of photoisomerizable diffusional electron mediators. The enzymes glucose oxidase, GOD, and glutathione reductase, GR, were photoregulated in the presence of photoisomerizable ferrocene-nitrospiropyran, Fc-SP (**3**), and N,N′-bipyridiniumnitrospiropyran, V^{2+}-SP, (**6**). Electrical communication between the enzymes GOD and GR and the electrodes was effective in the presence of Fc-SP (**3a**) and V^{2+}-SP (**6a**), respectively, and electron-transfer was blocked in the presence of the electron mediators, Fc-MR (**3b**) and V^{2+}-MR (**6b**), respectively. GOD modified by nitrospiropyran and assembled as monolayer on a Au-electrode provides an active interface for the photoregulated bioelectrocatalyzed oxidation of glucose. The SP-GOD acts as an effective biocatalyst for oxidation of the substrate and for the stimulation of an electrocatalytic anodic current. Biocatalyzed oxidation of glucose is inhibited in the presence of MR-GOD. The photoregulated electrical interactions between the various redox proteins and the electrode interfaces provide a means for the amperometric transduction and amperometric amplification of recorded optical signals. This is an essential fundamental feature for the future development of bioelectronic devices.

Application of photoswitchable biological substances in bioelectronics is a rapidly progressing research subject.[2,3] Reversible activation and deactivation of biomaterial activities by external light signals represent a means to record optical signals. Since the biomaterials exhibit chemical functions, the recorded optical signals can be transduced as chemical events, and these substances could provide basic matrices for information storage. Different methods to photostimulate the activities of biomaterials have recently been developed. These include the chemical modification of enzymes[4,5] or receptor proteins[6] by reversibly photoisomerizable components, immobilization of enzymes in photoisomerizable polymer matrices,[7] that control the substrate permeabilities, and by the application of reversibly photoisomerizable inhibitors.[8,9] In all of these systems, the photoswitchable activities of the biomaterials are monitored by assaying the products generated by the enzymes. Application of these light-triggered biomaterials in bioelectronic systems requires, however, their organization as an assembly where the recorded optical signals are instantaneously transduced in the form of a physical signal, such as an electrochemical response. Furthermore, of particular interest are bioelectronic devices where weak optical signals are transduced as *amplified* physical responses.

The organization of biomaterials as monolayers on electrode surfaces and the application of these monolayer electrodes as amperometric biosensors was recently reported.[10−12] Also, photoisomerizable monolayers were assembled onto electrodes and glass surfaces, and photoisomerizable antigenic monolayers

* Author to whom correspondence should be addressed. FAX no. 972-2-6527715 or 972-2-6585345.

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were utilized to tailor reversible (reusable) amperometric immunosensors[13] and to pattern microstructures of antibodies on surfaces.[14] Fabrication of nano-structured monolayers of biomaterials and photoisomerizable units on electrode surfaces opens up the possibility of combining photoswitchable biomaterials and nanotechnology concepts to organize novel bioelectronic devices. Design of photoswitchable redox proteins and their communication with electrodes could lead to electrochemical transduction of recorded optical signals. Furthermore, light activation of an enzyme or coupling of a photoactivated redox-protein to an enzymatic cycle could enable the amplification of the transduced amperometric signal as a consequence of the biocatalyzed process. Here we report a detailed study on three different approaches for the bioelectrocatalyzed amperometric transduction of recorded optical signals using monolayer-modified gold electrodes. These include the following: (i) the application of photoisomerizable monolayer electrodes for photoswitchable electrical communication of cytochrome *c*, Cyt. c, with the electrode surface; (ii) application of enzyme-modified electrodes consisting of glucose oxidase, GOD, or glutathione reductase, GR, and photoisomerizable electron mediators that control the electrical communication between the redox enzymes and electrode surface; and (iii) organization of a chemically modified photoisomerizable glucose-oxidase as monolayer on Au-electrode and the application of the light-active interface for amperometric transduction of the recorded optical signals.

Experimental Section

Absorption spectra were recorded on a Uvikon-860 (Kontron) spectrophotometer. Electrochemical measurements were performed with a potentiostat (EG & G Versa Stat) interfaced to a personal computer (EG & G research electrochemistry software model 270/250). NMR spectra were recorded with a Brucker AMX 400 spectrometer or a Brucker WP-200 spectrometer. Chemical shifts were referenced to TMS, and coupling constants are reported in Hz.

Materials. The redox proteins cytochrome *c* (cyt *c*) (from horse heart), cytochrome *c* oxidase, COX (from bovine heart, E.C. 1.9.3.1), glucose oxidase, GOD (Type X-S from *Aspergillus niger*, E.C. 1.1.3.4), L-lactate dehydrogenase, LDH (Type IV-SS from bakers yeast, E.C. 1.1.2.3), and glutathione reductase, GR (Type III from bakers yeast, E.C. 1.6.4.2) were from a commercial source (Sigma). All other chemicals, unless otherwise stated, were of commercial source (Aldrich) and were used without further purification. Ultrapure water from a Nanopure (Barnstead) source was used in all electrochemical experiments. The nitrospiropyran active ester (**1**) 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran], was prepared by coupling of the parent carboxylic acid 1-(β-carboxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**2**), with *N*-hydroxysuccinimide according to the general reported method.[15] The acid (**2**) was prepared according to the literature.[16]

The ferrocene-nitrospiropyran electron mediator, Fc-SP (**3**) (see Scheme 6 in the text), 1-(β-ethyl ferrocene carboxylate)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran], was prepared by coupling of 1-(β-hydroxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (4) with ferrocene carboxylic acid (**5**). A solution of (**4**) (0.968 g, 2.75 mmol), ferrocene carboxylic acid (**5**) (0.575 g, 2.5 mmol), *N,N*-dicyclohexylcarbodiimide, DCC (0.567 g, 2.75 mmol), and 4-pyrrolidinopyridine (0.037 g, 0.25 mmol) in 10 mL of dried dichloromethane was stirred at room temperature for 4 h. The resulting precipitate of *N,N*′-dicyclohexylurea was filtered off, washed with water (3 × 15 mL), 5% acetic acid (3 × 15 mL), and again with water (3 × 15 mL), and the filtrate was dried and evaporated. The crude product was purified by a two-step chromatography on silica gel (Merck). The first chromatography was performed on a 20 cm column (diameter 2 cm) using 5% methanol in chloroform as eluent. The fractions containing **3** were combined and evaporated, and a second chromatography was performed on the product using a column 15 cm long (diameter 1.5 cm) and 0.5% methanol in chloroform as eluent. The fractions containing pure **3** were combined, dried with $MgSO_4$, and evaporated: ^{1}H-NMR (400.13 MHz, $CDCl_3$) δ = 1.19 (s, 3H), 1.29 (s, 3H), 3.5 (m, 2H), 4.1 (s, 5H), 4.3−4.4 (m, 4H), 4.75 (m, 2H), 5.91 (d, 1H, J = 9 Hz), 6.74 (d, 1H, J = 9 Hz), 6.82 (d, 1H, J = 7.8 Hz); 6.9 (m, 2H), 7.1 (d, 1H, 6.7 Hz), 8.02 (m, 2H). 1-(β-Hydroxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran], (4) was prepared according to the literature.[17]

The *N,N*′-bipyridinium-nitrospiropyran electron mediator, V^{2+}-SP (**6**) (see Scheme 6 in the text), was prepared by alkylation of *N*-methylpyridinium-4-pyridine (**7**) with 1-(4′-iodobutyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**8**). The synthesis of **8** was accomplished[18] by initial synthesis of the respective Fischer base by heating to reflux a mixture of 1,4-diiodobutane (2.4 g, 7.7 mmol) with 2,3,3-trimethylindolenine (3.2 g, 20 mmol) for 3 h. The resulting dark oil was solidified by the addition of acetone. The solid was filtered and washed with ether to yield 2.5 g (94%) of a tan powder of the Fischer base that was used without further purification: ^{1}H-NMR (200 MHz, DMSO-d_6) δ = 1.59 (s, 6H), 2.0−2.1 (m, 2H), 2.91 (s, 3H), 4.53 (m, 2H), 7.67 (d, 2H, J = 3.1 Hz), 7.83 (d, 2H, J = 3.0 Hz), 7.9 (t, 2H, J = 3.1 Hz), 8.08 (t, 2H, J = 3.3 Hz); mp >240 °C dec.

The Fischer base (2.0 g, 5.8 mmol) was reacted with 5-nitrosalicylaldehyde (0.84 g, 5 mmol) in hot butanone, 15 mL, that contained 0.5 mL of piperidine. The mixture was heated for 10 min and left to cool. Brown crystals separated upon cooling, yielding 1.5 g of **8** (30% yield): mp 180 °C; ^{1}H-NMR (400.13 MHz, $CDCl_3$) δ (ppm) = 1.2 (s, 3H), 1.24 (s, 3H), 1.7 (m, 2H), 1.97 (m, 2H), 3.1−3.3 (m, 4H), 5.75 (t, 1H, J = 10 Hz), 6.45 (m, 1H), 6.6−6.7 (m, 1H), 6.8−6.9 (m, 2H), 7.07 (d, 1H, J = 9.9 Hz), 7.15 (t, 1H, J = 7.5 Hz), 7.9−8.0 (m, 2H).

N-Methylpyridinium-4-pyridine (**7**) was prepared by modification of the reported method.[19] 4,4′-Bipyridine (5 g, 32 mmol) was dissolved in methanol (120 mL), and methyliodide (2 mL, 6 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 4 days. The resulting mixture was cooled, and the precipitate (bipyridinium salt) was filtered off. The filtrate was evaporated, and the orange solid obtained was refluxed in chloroform for 15 min. The resulting solid was filtered to yield 3.7 g (40% yield) of **7**: ^{1}H-NMR (200 MHz, DMSO-d_6) δ (ppm) = 4.37 (s, 3H), 8.03 (d, 2H, J = 6.1 Hz), 8.6 (d, 2H, J = 6 Hz), 8.8 (d, 2H, J = 6.2 Hz), 9.15 (d, 2H, J = 6.2 Hz).

1-(4′-Iodobutyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**8**) (1.0 g, 2.04 mmol) and **7**, (0.55 g, 1.84 mmol) were dissolved in dry DMF (2 mL) and heated to 110 °C under nitrogen, for 24 h. DMF (2 mL) was added to the mixture, and the precipitate formed was filtered. The solid product was crystallized from methanol−acetone to yield (**6**): 0.8 g (55% yield); ^{1}H-NMR (200 MHz, DMSO-d_6) δ (ppm) = 1.13 (s, 3H), 1.22 (s, 3H), 1.5−2.2 (m, 4H), 4.48 (s, 3H), 4.7 (m, 2H), 6.02 (d, 2H, J = 11 Hz), 6.67 (d, 1H, J = 8 Hz), 6.89 (t, 2H, J = 8.4 Hz), 7.18 (m, 2H), 7.96 (dd, 1H, J = 9 Hz), 8.23 (d, 1H, J = 2.6 Hz), 8.79−8.88 (m, 4H), 9.31−9.45 (m, 4H).

Glucose oxidase modified by nitrospiropyran units, SP-GOD, was prepared according to the following procedure: GOD (100 mg) was dissolved in an aqueous $NaHCO_3$ solution, 0.5 M (12 mL). A solution of 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**1**) (50 mg, 0.1 mmol) in 500 μL of THF was added to the aqueous enzyme solution (4 °C). The resulting mixture was allowed to react at 4 °C for 48 h. The mixture

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was then passed through a Sephadex G-25 column (22 cm length, diameter 2 cm) that was pre-equilibrated with a 0.085 M Na_2HPO_4 buffer solution (pH = 7.0). The modified enzyme was eluted in the first orange 4 mL fraction. The orange solution of SP-GOD was dialyzed overnight and lyophilized to dryness. The average loading of the enzyme by photoisomerizable units that correspond to 21 was determined by following the absorption spectrum of the MR-GOD aqueous solution (λ = 536 nm, ϵ = 2300 M^{-1} cm^{-1})[20] that contained 1.86 mg mL^{-1} of the protein. The activity of the SP-GOD was determined by following the oxygenic biocatalyzed oxidation of glucose by SP-GOD in a 0.05 M sodium acetate buffer solution. The generated H_2O_2 was analyzed colorimetrically with *o*-dianisidine, λ = 500 nm. The activity of SP-GOD corresponds to 70% of the activity of native GOD.

All compounds gave satisfactory elementary analyses.

Modifications of Electrodes. Au-wire electrodes (diameter 0.5 mm, geometrical area ca. 0.2 cm^2, roughness factor ca. 1.2) and Au-foil electrodes (0.2 mm thickness, geometrical area ca. 0.4 cm^2, roughness factor ca. 20) were applied in the different systems. Rough Au-electrodes were prepared by amalgamation of the Au-electrodes by contact of the Au-surfaces with mercury, followed by dissolution of the amalgam layer with concentrated nitric acid.[21] The real surface areas of the electrodes were determined by following the cyclic voltammogram of the respective electrodes in 0.5 M H_2SO_4.[22] The Au-electrodes were cleaned by boiling in 2 M KOH, 1 h, followed by rinsing with water. The electrodes were stored in concentrated sulfuric acid. Prior to modification, the electrodes were rinsed with water, soaked for 10 min in concentrated nitric acid, and rinsed again with water.

Thiol Pyridine-Nitrospiropyran Mixed Monolayer Electrodes. A mixture of a solution of 0.01 M 2,2′-diaminodiethyl disulfide (cystamine) in water, 1 mL, and 0.01 M 4,4′-dipyridyldisulfide in ethanol, 10 mL, was prepared. The Au electrodes were soaked in this solution for 2 h and then rinsed five times with ethanol and water to remove any physically adsorbed compound. Attachment of the photoisomerizable nitrospiropyran component was accomplished by covalent linkage of 1-(β-carboxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**2**) to the amino components of the monolayer. Due to the low solubility of (**2**) in ethanol, the compound was transformed to the merocyanine state, MR, prior to the modification by heating the solution to 70 °C. The Au-modified electrodes were treated with a 1 mM solution of the MR-state of **2** in ethanol in the presence of 10 mM 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) for 1 h in the dark at room temperature. The resulting electrodes were then rinsed with ethanol and water. The surface density of the photoisomerizable units was determined by cyclic voltammetry (see text) and corresponded to ca. 5 × 10^{-12} mol·cm^{-2}.

Glucose Oxidase, GOD, and Glutathione Reductase, GR, Multilayer Electrodes. A rough Au-foil electrode was soaked in 0.05 M aqueous cystamine solution for 2 h, rinsed ten times with water, and treated with a 5% (v/v) aqueous solution of glutaric dialdehyde for 10 min. The resulting electrode was rinsed with water and incubated in the respective enzyme solution (GOD, 830 U or GR, 1.2 U, in 1 mL of 0.1 M phosphate buffer, pH = 7.3) for 20 min. The enzyme-modified electrodes were rinsed with the phosphate buffer solution, and the layers of the respective enzyme were constructed by a repeated two-step procedure that included the treatment of the electrode with the glutaric dialdehyde solution and the respective enzyme solution as described above.[10c] The electrodes employed in our studies included five layers of GOD or GR. The modified enzyme electrodes were used in the electrochemical studies immediately after preparation.

Nitrospiropyran Glucose Oxidase, SP-GOD, Monolayer Electrode. A Au-wire electrode was soaked in 0.02 M DMSO solution of dithiobis(succinimidylpropionate) (DSP, Fluka) for 2 h. The electrode was rinsed three times with DMSO and once with water. The resulting active ester self-assembled monolayer modified-electrode[23] was incubated overnight in 1 mL of 0.1 M phosphate buffer solution, pH = 7.5, that included 5 mg of SP-GOD. The resulting enzyme monolayer-modified electrode was rinsed several times with a phosphate buffer solution and used immediately in the electrochemical measurements.

Photoisomerization of Modified Electrodes and Relay Solution. Photoisomerization to the respective nitrospiropyran, SP, or nitromerocyanine, MR, was achieved by irradiation at appropriate wavelengths. Photoisomerization of the modified electrodes was accomplished by illumination in air, where the electron relay solutions were photoisomerized in a quartz cuvette. A 150 W xenon arc lamp (Oriel), equipped with a Schott filter, λ > 475 nm, and a $CuSO_4$ solution as IR filter, was used as the light source for the generation of the nitrospiropyran, SP, state. A 18W mercury pencil lamp (Oriel-6042) equipped with a long-wave filter, (320 nm < λ < 380 nm) was used as light source for the generation of the nitromerocyanine, MR, state. The electrodes and solutions were protected from room light during all electrochemical measurements.

Electrochemical Measurements. All measurements were carried out at ambient temperature (22 ± 2 °C) in a three-compartment electrochemical cell, consisting of the chemically modified electrode as working electrode, a glassy carbon auxiliary electrode isolated by a glass frit and a saturated calomel electrode (SCE) connected to the working volume with a Luggin capillary as reference electrode. All potentials are reported with respect to this reference electrode. Ar bubbling was used to remove oxygen from the electrochemical cell.

Photostimulation of Cytochrome *c*, cyt *c*, Cytochrome Oxidase, COX, or Lactate Dehydrogenase, LDH, by Electrical Interactions with Photoisomerizable Electrodes. The mixed monolayer consisting of thio pyridine and nitrospiropyran, SP, or merocyanine, MRH^+, units was used as the working electrode. The electrolyte solution, 2.2 mL, consisted of 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0. Cyclic voltammograms were recorded at the specified scan rates (see text) in the presence of cyt *c*, 1 × 10^{-4} M, and in the appropriate systems in the presence of added COX, 10 U, in the absence and presence of air or with added LDH, 1.8 U, in the absence and presence of lactate, 1 × 10^{-2} M. In the latter system the electrolyte consisted of 0.1 M phosphate buffer, pH = 7.5.

Photostimulation of GOD Multilayer Electrode by Photoisomerizable Ferrocene Electron Mediator (3). A rough Au-electrode that included five layers of GOD was used as the working electrode. The electrolyte solution consisted of 2 mL of 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0, containing 10% (v/v) ethanol and the photoisomerizable ferrocene electron mediator Fc-SP or Fc-MRH^+, 1 × 10^{-4} M. The steady-state anodic currents in the cell at a constant potential of +0.5 V on the working electrode were recorded upon sequential addition of glucose portions, 4 × 10^{-3} M, to the cell.

Photostimulation of GR-Multilayer Electrode by Photoisomerizable Bipyridinium Electron Mediator (6). A rough Au-electrode that included five layers of GR was used as working electrode. The electrolyte solution consisted of 2 mL of 0.1 M K_2HPO_4 buffer solution, pH = 7.2, and the respective photoisomer state of the electron mediator V^{2+}-SP or V^{2+}-MRH^+ as electron mediators. The rate of reduced glutathione, GSH, formation was assayed according to the literature.[24] Aliquots of 50 μL were taken from the electrolyte cell every 10 min. These samples were mixed with 50 μL of 5,5′-dithiobis(2-nitrobenzoic acid), 1.6 mg·mL^{-1}, and 0.9 mL of 0.1 M K_2HPO_4 buffer solution. The mixtures were allowed to react for 15 min, and the developed absorbance at λ = 412 nm (ϵ = 13 600 M^{-1}·cm^{-1}) was recorded.

Amperometric Responses of Photoisomerizable GOD Monolayer Electrode. The SP-GOD or MRH^+-GOD monolayer electrodes were prepared by appropriate illumination and used as working electrodes. The electrolyte solution consisted of 0.1 M Na_2SO_4 and 0.01 M

(20) The molar extinction coefficient of nitromerocyanine units associated to proteins was determined as described previously (cf. Zahavy, E.; Rubin, S.; Willner, I. *J. Chem. Soc., Chem. Commun.*, **1993**, 1753). Specifically, this value was estimated for nitromerocyanine attached to concanavalin A, Con A. The Con A and nitrospiropyran-Con A were reacted with trinitrobenzene sulfonic acid that binds to free lysine residues (cf. Habeeb, A. F. S. A. *Anal. Biochem.* **1966**, *14*, 328). The absorbance of the two proteins was recorded at λ = 335 nm, and from the difference in the absorbance of the two proteins the loading of nitrospiropyran on Con A was determined. Photoisomerization of the nitrospiropyran-Con A, of known loading and protein content, to the nitro-merocyanine-Con A then enabled the estimation the ϵ value of nitromerocyanine units.

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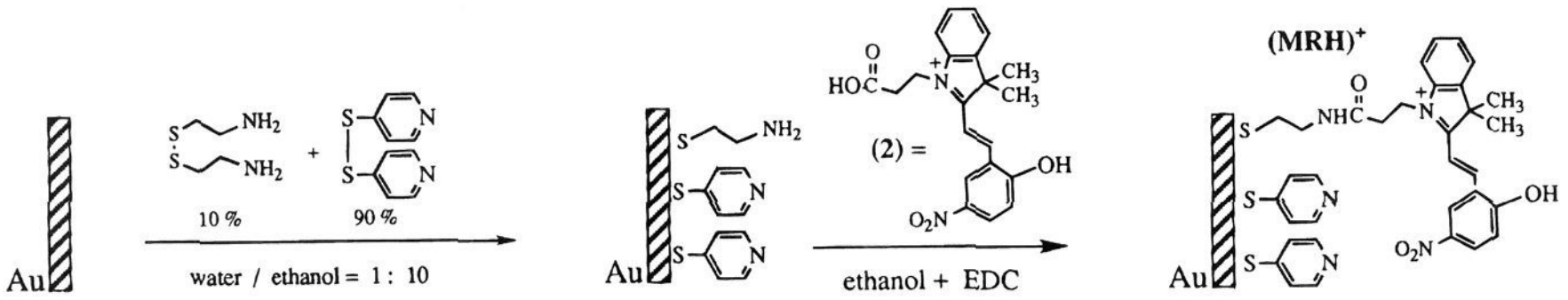
Scheme 1. Organization of Photoisomerizable Nitrospiropyran/Pyridine Mixed Monolayer Electrode



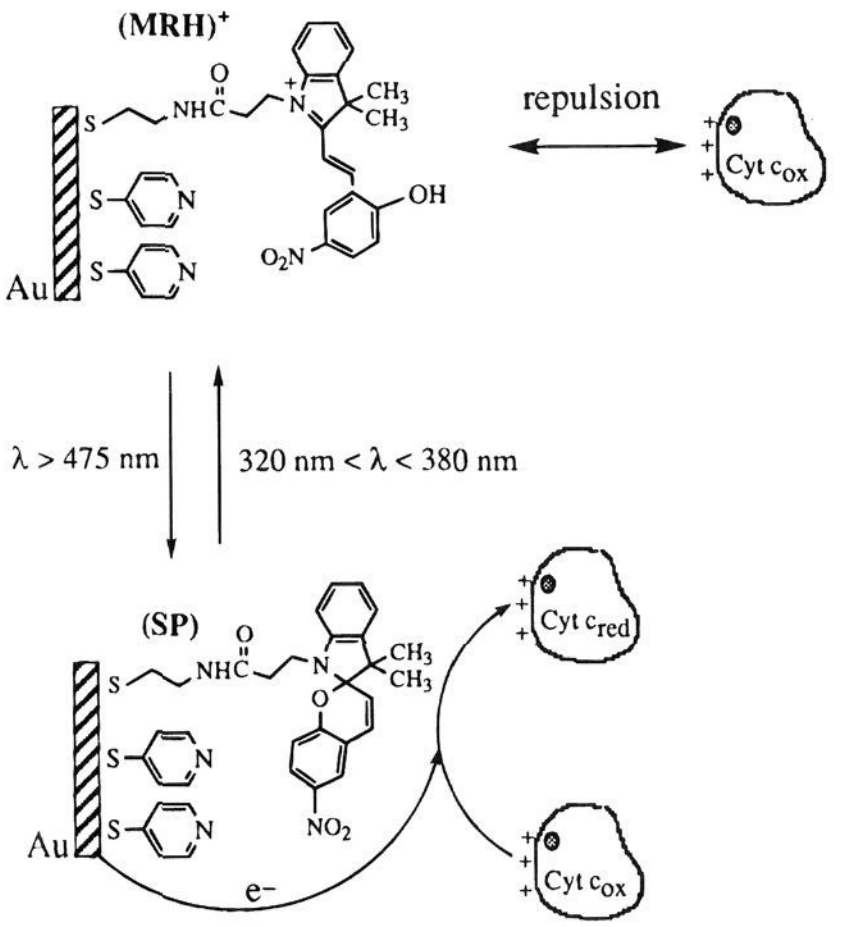
phosphate buffer, pH = 7.0, 2.4 mL, that included ferrocene carboxylic acid, Fc-CO_2H, 5×10^{-3} M, and glucose, 2.5×10^{-2} M. Cyclic voltammograms were scanned in the potential range and conditions specified in the text.

Results and Discussion

Photoswitchable Electrical Communication of Cytochrome *c* Using a Photoisomerizable Monolayer Electrode. The redox protein cytochrome *c*, cyt *c*, lacks electrical communication with conventional electrodes. Modification of electrodes by 4,4′-bipyridine or 4-thiol pyridine effects electrical interaction between the iron-heme redox center of cyt *c* and electrode surfaces.[25–27] It was suggested that modification of electrodes by weakly basic or anionic functional groups could generate active surfaces for promotion of electron transfer between cyt *c* and the electrode. This electrical communication results from the appropriate alignment of the protein onto the electrode surface via hydrogen bonds or salt links between the promoter and the lysine residue associated with the heme crevice. Accordingly, we attempted to photoregulate the electrical communication of cyt *c* with the electrode surface by the organization of a mixed monolayer consisting of a promoter component and a photoisomerizable component capable of perturbing the associative interactions between the promoter and cyt *c*. The selected photoisomerizable counter component of the monolayer was nitrospiropyran since it undergoes reversible photoisomerization to the merocyanine isomer state, which in an aqueous solution, pH < 8, is protonated. The protonated, positively charged merochyanine could then perturb the association of cyt *c* to the promoter by electrostatic repulsion of the positively charged cyt *c*.[25]

The mixed monolayer of the promoter thiolpyridine and the photoisomerizable merocyanine (nitrospiropyran) was constructed onto Au-electrodes according to Scheme 1. The monolayer of thiolpyridine and cystamine was generated by chemisorption of pyridine disulfide and cystamine at a ratio of 10:1. Covalent linkage of *N*-propionyl nitromerocyanine (**2b**) to the cystamine residues resulted in the bifunctional monolayer consisting of thiolpyridine and the photoisomerizable groups. The surface density of the nitrospiropyran units was evaluated electrochemically and corresponded to 5×10^{-12} mol·cm^{-2}, and the ratio of merocyanine units and pyridine components in the mixed monolayer was ca. 1:10.[28] The monolayer exhibits reversible photoisomer-

Scheme 2. Photoinduced Electron Transfer Communication of cyt *c* with the Photoisomerizable Monolayer Electrode



izable properties.[10d,14] Illumination of the resulting electrode by visible light, $\lambda > 475$ nm generates the nitrospiropyran, SP, mixed monolayer, where irradiation of the SP-monolayer, 320 nm $< \lambda <$ 380 nm, resulted in the formation of the merocyanine isomer state in the protonated configuration, MRH^+, at the conditions employed, pH = 7.0, Scheme 2.

Figure 1 shows the cyclic voltammogram of cyt c (from bovine heart) in the presence of the SP-monolayer electrode (curve a) and in the presence of the MRH^+-monolayer electrode (curve b). Effective electrical communication between cyt *c* and the SP-monolayer electrode is observed as reflected by the reversible reduction and oxidation of the protein ($E_{pa} - E_{pc} \sim$ 60 mV). The interfacial electron transfer rate-constant was estimated to be $k_{et} = 1 \times 10^{-3}$ cm·s^{-1}. The interfacial electron-transfer rate constant at the pyridine-nitrospiropyran monolayer electrode is similar to the reported rate constant of cyt *c* using a pyridine-modified monolayer electrode.[26] This suggests that the nitrospiropyran units co-immobilized in the monolayer do not perturb the associative interaction of cyt *c* with the pyridine units. In the presence of the mixed monolayer that included the MRH^+-photoisomer state, electrical communication of cyt *c* with the electrode is perturbed and only the background current of the electrolyte is observed. Further control experiments reveal that the organization of the bifunctional nitrospiropyran/pyridine monolayer on the Au-electrode, at the specified ratio of 1:10, is important in controlling electrical interaction of cyt *c* with the electrode surface. A pure SP-monolayer electrode lacks electrical communication with cyt *c*, where bifunctional

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(25) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. *Quart. Rev. Biophys.* **1986**, *18*, 261.
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(27) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. *Acc. Chem. Res.* **1988**, *21*, 407.
(28) The nitro group of the nitrospiropyran component reveals an irreversible reduction at −0.8 V. The cathodic waves corresponding to the pyridine-nitrospiropyran mixed monolayer electrode and of a fully covered nitrospiropyran monolayer electrode were recorded. The mixed monolayer electrode showed an ca. 10-fold lower coulometric response, suggesting a 1:10 composition of the mixed monolayer. It should be noted that electrodes subjected to this irreversible reduction were discarded and were not used for further measurements.

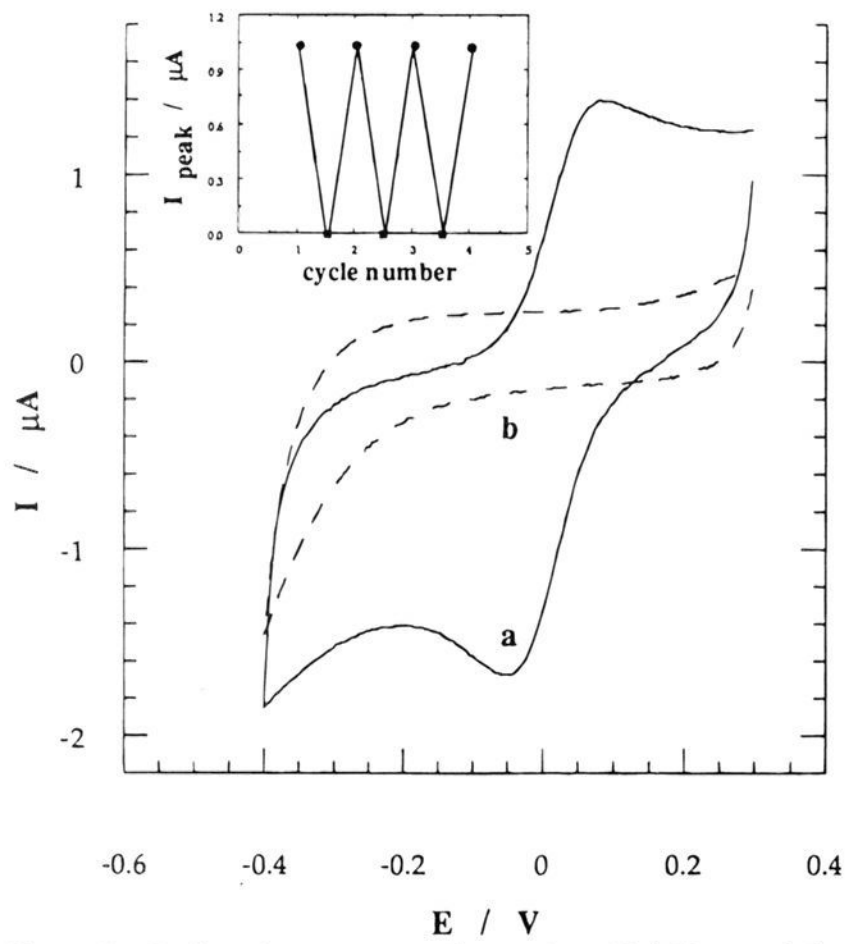


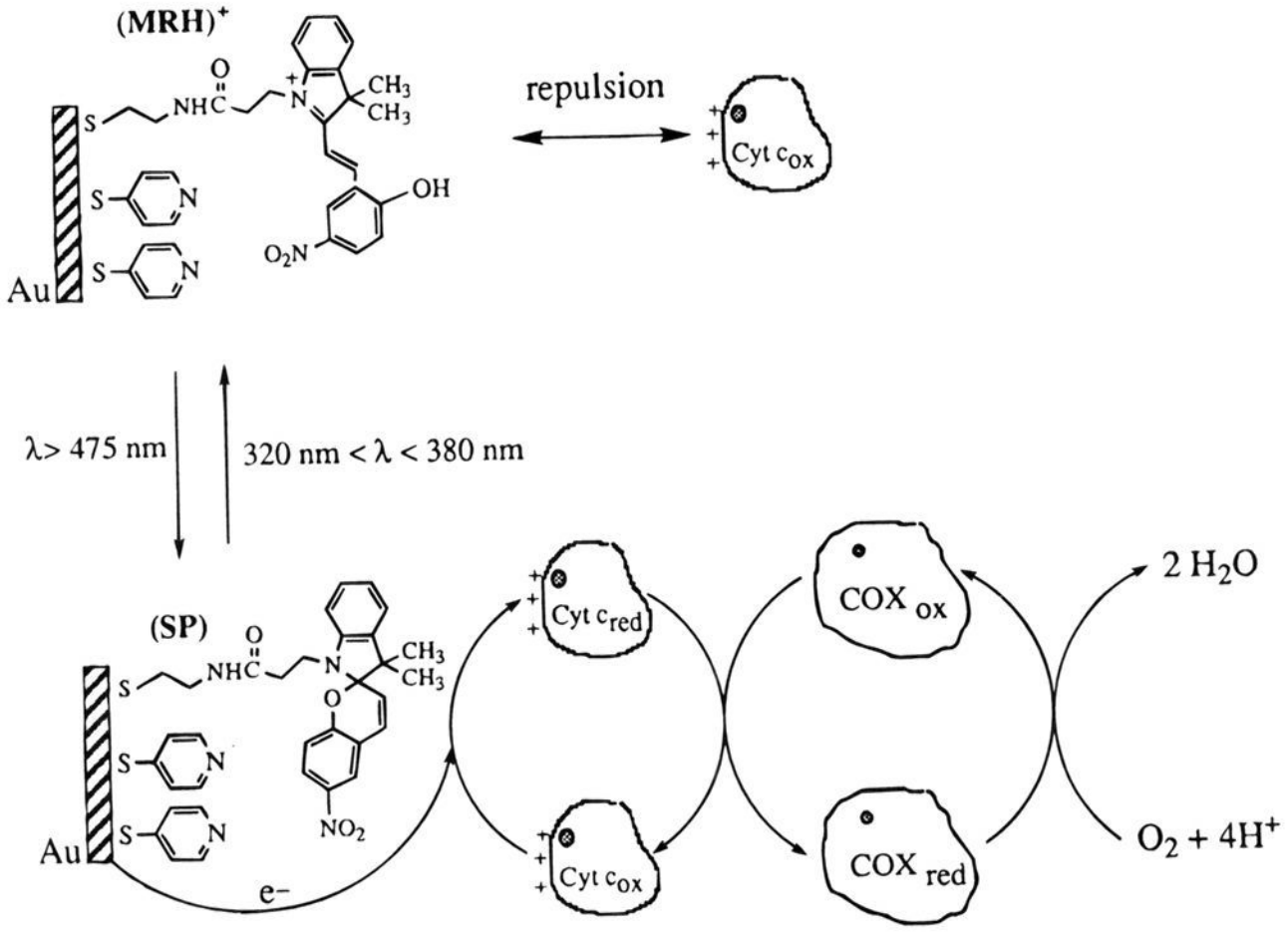
Figure 1. Cyclic voltammograms of cyt *c*, 1×10^{-4} M, recorded at scan rate of 50 mV·s^{-1} in 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0. (a) In the presence of the SP-pyridine monolayer electrode. (b) In the presence of the MRH^+-pyridine monolayer electrode. Inset: Cyclic amperometric responses of cyt *c* in the presence of different photoisomer states of the monolayer-electrode.

electrodes, consisting of a higher nitrospiropyran/pyridine ratio adversely affect the interfacial electron-transfer rates. Bifunctional monolayer electrodes consisting of a lower ratio of nitrospiropyran/pyridine revealed reversible redox reactions of cyt *c*, but the respective MRH^+ monolayer electrodes were not completely blocked toward electron transfer with cyt *c*. Thus, the selected ratio of the bifunctional monolayer composition (nitrospiropyran/pyridine 1:10) represents the appropriate balance for effective electrical communication of cyt *c* with the SP-monolayer electrode and blocking of the electrical interaction of the protein with the MRH^+-electrode. Furthermore, the results indicate that the pyridine component in the mixed monolayer is an essential promoter to facilitate electron transfer between cyt *c* and the electrode. The photostimulated electrical communication of cyt *c* with the monolayer-modified Au-electrode are thus attributed to light-controlled interactions of the protein with the bifunctional monolayer, Scheme 2. In the MRH^+-monolayer state, electrostatic repulsion of cyt *c* exhibiting positive charges perturbs the associative interaction of the protein with the pyridine units, and electrical interaction with the electrode is prevented. Upon photoisomerization to the neutral pyridine-SP mixed monolayer state, cyt *c* binds to the pyridine units and reversible interfacial electron transfer is observed. The reversible photoisomerizable properties of the monolayer enable the cyclic "ON−OFF" photostimulation of the electron transfer communication between cyt *c* and the electrode interface, Figure 1 (inset).

The photostimulated redox interactions of cyt *c* with the monolayer electrode represent a means for amperometric transduction of optical signals recorded by the photosensitive monolayer assembly. Since cyt *c* acts as an electron transfer mediator in various biochemical transformations, one could couple the photostimulated redox activity of cyt *c* to secondary enzyme-catalyzed reactions. The turnover of the biocatalyzed reaction could then recycle the electron transfer between cyt *c* and the electrode surface, thereby leading to the amplification of the amperometric response of the electrode.

Cytochrome *c* acts as electron transfer mediator to cyt *c* oxidase, COX, that catalyzes the reduction of O_2 to water. The COX enzyme includes the dinuclear redox centers, where one Cu_A and low-spin heme Fe_a site acts as acceptor of electrons from cyt *c* and the second Fe_{a3}/Cu_B dinuclear center catalyzed the four-electron reduction of oxygen (Scheme 3).[29] Figure 2 shows the photostimulated amperometric responses of the cyt *c*−COX system in the presence of the photoisomerizable monolayer electrode. The lack of electrical communication of

Scheme 3. Coupling of Photostimulated Electrical Interactions of cyt *c* with the Photoisomerizable Monolayer to the Biocatalyzed Reduction of O_2 by COX



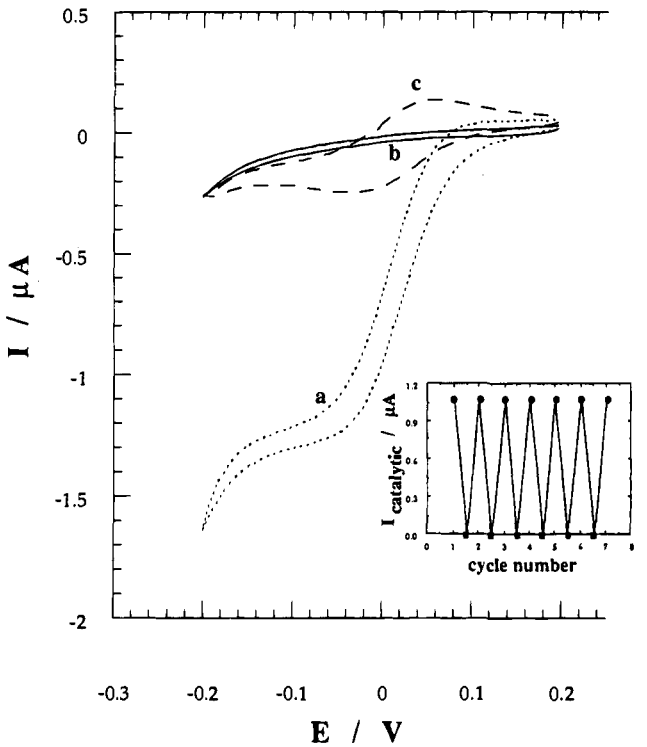


Figure 2. Cyclic voltammograms of cyt *c*, 1×10^{-4} M, COX, 1×10^{-6} M, recorded at scan rate of 2 $mV \cdot s^{-1}$, in 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0. (a) In the presence of O_2 and the SP-pyridine mixed monolayer electrode. (b) In the presence of O_2 (air) and the MRH^+-pyridine mixed monolayer electrode. (c) In the absence of COX and presence of O_2 with the SP-pyridine monolayer electrode. Inset: Cyclic amperometric responses of the photoisomerizable monolayer electrode in the presence of cyt *c*/COX/O_2.

cyt *c* with the pyridine-MRH^+ monolayer electrode yields only the background current in the presence of cyt *c*/COX/O_2. Irradiation of the electrode with visible light, $\lambda > 475$ nm, generates the pyridine-SP monolayer electrode. This electrode yields an electrocatalytic cathodic current as a result of the reduction of O_2. For comparison, the reversible cyclic voltammogram of cyt *c*, recorded under the same conditions (similar scan rate) in a system lacking COX, is also provided (curve c). This redox wave is not affected in the presence of O_2 and does not yield any catalytic current, implying that reduced cyt *c* does not mediate directly the reduction of O_2. These results clearly indicate that electroreduced cyt *c*, formed in the presence of the SP-monolayer electrode, mediates electron transfer to COX that biocatalyzes the reduction of O_2. Blocking of the electrical communication between cyt *c* and the pyridine-MRH^+-monolayer electrode results in the background current only. Thus, the photostimulated electrical communication of cyt *c* with the photoisomerizable electrode controls the inter-protein cyt *c*—COX electron transfer and subsequent biocatalyzed reduction of O_2, Scheme 3. Figure 2 (inset) shows the cyclic photostimulated catalytic cathodic currents developed in the electrochemical cell upon repeated photoisomerization of the mixed monolayer electrode between the SP-state ("ON") and the MRH^+-isomer ("OFF").

The photostimulated redox activities of cyt *c* were further coupled to an oxidative bioelectrocatalyzed transformation. Cytochrome *c* acts as electron mediator for lactate dehydrogenase, LDH, that contains a cytochrome b_2 redox center.[30] Thus, electrobiocatalyzed oxidation of lactate to pyruvate by the LDH (cyt b_2) that is oxidized by cyt *c* could provide an oxidative biocatalytic pathway to amplify the photostimulated electrical communication of cyt *c* with the photoisomerizable electrode. Figure 3 shows the cyclic voltammogram of cyt *c* with added LDH (cyt b_2) in the presence of the pyridine-SP mixed monolayer electrode (curve a). A quasi reversible oxidation—reduction wave for the redox process of cyt *c* is observed. The quasi-reversible, nonideal, cyclic voltammogram of cyt *c* is attributed to the coadsorption of LDH onto the modified electrode surface. Addition of lactate to the systems generates an electrocatalytic anodic current (curve b). Irradiation of the electrode, 320 nm $< \lambda <$ 380 nm, and isomerization of the monolayer to the pyridine-MRH^+ state, in the presence of cyt *c*/LDH/lactate, blocks electrical communication with the electrode, and only the background current is detected (curve c). Figure 3 (inset) shows the cyclic photochemically induced bioelectrocatalyzed oxidation of lactate in the presence of the pyridine-SP-monolayer electrode and the two proteins, cyt *c* and LDH (cyt b_2). In the spiropyran photoisomer state of the monolayer (produced by illumination, $\lambda > 475$ nm) the electrical communication between cyt *c* and the electrode is attained. The oxidized cyt *c* (Fe^{III}) mediates oxidation of LDH that effects

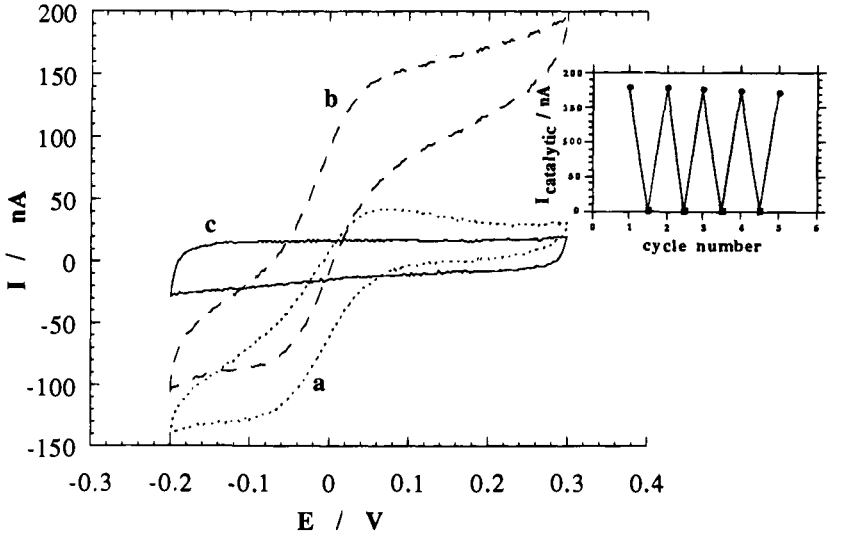
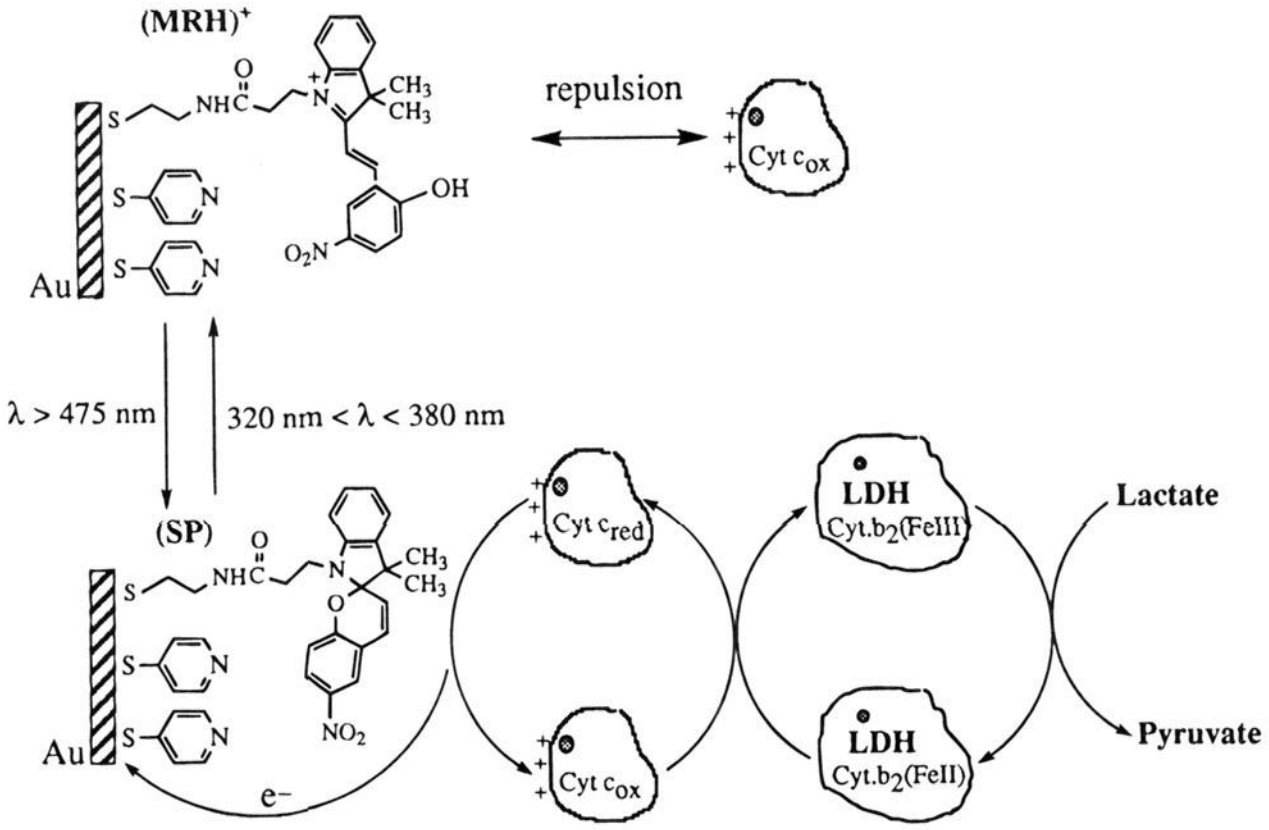


Figure 3. Cyclic voltammograms of cyt *c*, 1×10^{-4} M, LDH, 3.3×10^{-5} M, recorded at scan rate of 2 $mV \cdot s^{-1}$, in 0.1 M phosphate buffer, pH = 7.5. (a) In the presence of cyt *c* and LDH and the SP-pyridine monolayer electrode. (b) In the presence of cyt *c*, LDH, and lactate, 1×10^{-2} M, in the SP-pyridine monolayer electrode. (c) In the presence of cyt *c*, LDH, and lactate, 1×10^{-2} M, and the MRH^+-pyridine monolayer electrode. Inset: Cyclic amperometric responses of the photoisomerizable monolayer electrode in the presence of cyt *c*/LDH/lactate.

Scheme 4. Coupling of Photostimulated Electrical Interactions of cyt *c* with the Photoisomerizable Monolayer-Electrode to the Biocatalyzed Oxidation of Lactate



the oxidation of lactate to pyruvate, Scheme 4. Upon photoisomerization of the mixed monolayer to the pyridine-merocyanine state, 320 nm < λ < 380 nm, electrostatic repulsion of cyt *c* from the electrode occurs. This perturbs the electrical interactions of cyt *c* with the electrode and blocks the subsequent chain of electron transfer. It should be noted that no direct electron-transfer between LDH, and the electrode interface takes place in the absence of cyt *c*.

It should be noted that in the MRH^+-state of the mixed monolayer electrode cyt *c* lacks any electron transfer communication and represents a fully switched-off redox behavior of the protein, with zero-value amperometric response. Usually, photoswitchable biomaterials retain a residual activity even in the "OFF" state. The present photoisomerizable mixed monolayer electrode and cyt *c* represent a unique assembly where the amperometric responses of the system are activated by an external optical signal from a base zero-value. The photoswitchable redox interactions of cyt *c* with the photoresponsive electrode were coupled to bioelectrocatalyzed reduction of O_2 (using COX) and oxidation of lactate (using LDH). These coupled bioelectrocatalyzed transformations enabled the amperometric amplification of the primary event of the activation of electrical interactions of cyt *c* with the electrode.

Amplification of the transduced amperometric signal as a result of optical recording and the operation of the system through a zero-value "OFF" state represent two fundamental features of bioelectronic devices.

It is suggested that such photosensitive monolayer electrodes and the respective biomaterials could be applied as actinometric interfaces for the detection of weak light-signals. Furthermore, the light-stimulated bioelectrocatalyzed reduction of O_2 (by COX) or oxidation of lactate (by LDH) reveal a novel means to control chemical or biochemical ingredients in bioreactors. That is, coupling of a sensing unit that triggers "ON" and "OFF" a photoactive electrode could allow the electrochemical control of the concentration of a specific ingredient in bioreactors to desired values.

Photostimulation of Redox Enzymes by Photoisomerizable Electron-Relays. A further method for amperometric transduction and amplification of recorded optical signals could involve the integration of redox enzymes and electrode surfaces as photoresponsive assemblies. Redox enzymes in biological environments accept (or donate) electrons by inter-protein electron transfer (i.e., cytochrome *c*) or by the participation of low molecular weight cofactors (such as $NAD(P)^+/NAD(P)H$). Usually, no direct electrical communication of redox enzymes with electrode surfaces exists due to the insulation of the redox active-site by the protein.[31] Electron mediators exhibiting reversible redox properties,[32] such as ferrocene,[33] ferricyanide,[34] quinones,[35] dichlorophenol indophenol,[36] and *N,N'*-bipyridinium salts[37] have been widely applied to electrically communicate redox enzymes and electrodes. In these systems the electron mediator acts as diffusional shuttle to carry the electrons to or from the active site of the protein. The electron mediator must penetrate the protein and retain sufficiently short distances in respect to the redox active site to achieve effective electron transfer. Thus, the chemical modification of an electron mediator by a photoisomerizable component could lead to light-controlled electrical communication of a redox enzyme with electrodes. In one photoisomer state the electron mediator can penetrate the protein and establish electrical communication, where in the complementary state the diffusion of the electron mediator into the protein is perturbed (by steric or electrostatic interactions) and electrical communication is blocked.

We have applied this method to photoregulate the electrical communication of the enzymes glucose oxidase, GOD, and glutathione reductase, GR, with electrodes, and used the systems for the amperometric transduction of recorded optical signals. The enzyme electrode assemblies were constructed by the immobilization of enzyme layers on a functionalized self-

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(30) (a) Amine, A.; Deni, J.; Kauffmann, J.-M. *Bioelectrochem. Bioenerg.* **1994**, *34*, 123. (b) Cass, A. E. G.; Davis, G.; Hill, H. A. O.; Nancarrow, D. J. *Biochim. Biophys. Acta* **1985**, *828*, 51.

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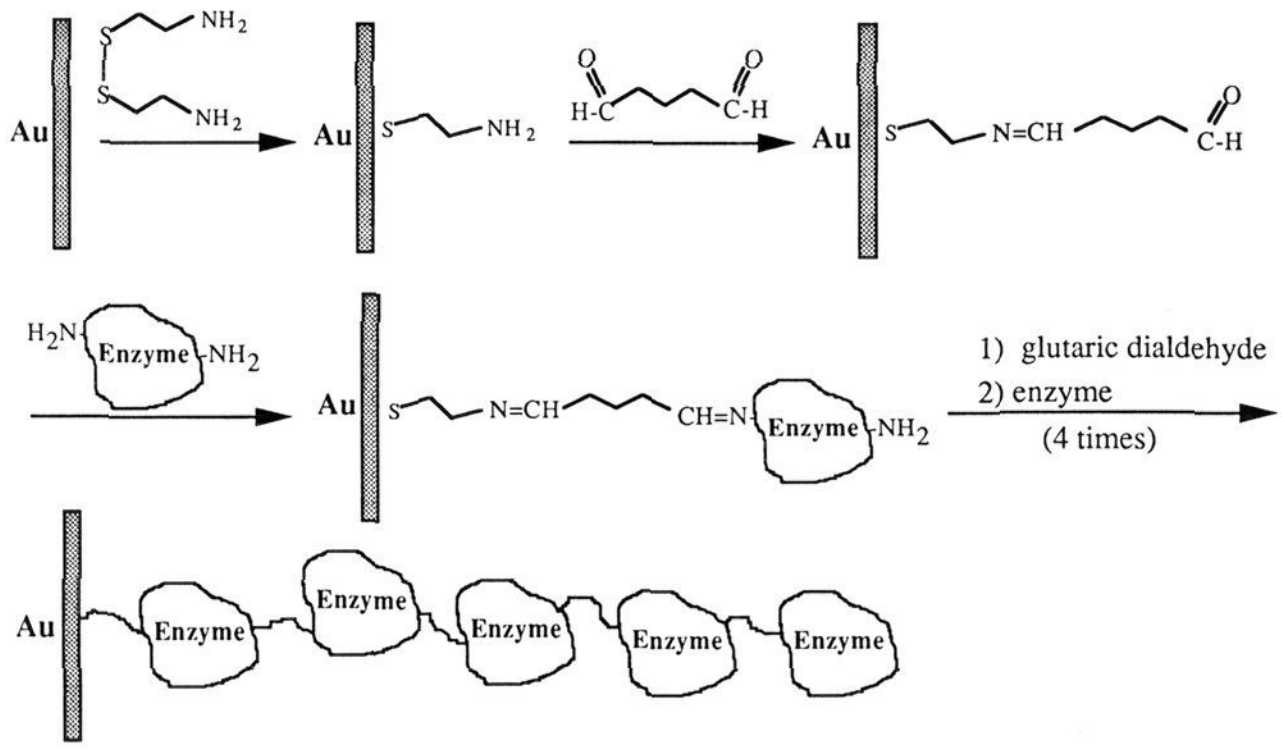
(32) Bartlett, P. N.; Tebbutt, P.; Whitaker, R. G. *Prog. Reaction Kinet.* **1991**, *16*, 55.

(33) (a) D'Costa, E. J.; Higgens, I. J.; Turner, A. P. F. *Biosensors* **1986**, *2*, 71. (b) Yokoyama, K.; Sode, K.; Tamiya, E.; Karube, I. *Anal. Chim. Acta* **1989**, *218*, 137.

Scheme 5. Organization of Multilayer Enzyme Electrodes



assembled monolayer associated with a Au-electrode, according to Scheme 5. A cystamine monolayer was modified with glutaric dialdehyde, and the resulting monolayer was reacted with GOD or GR, respectively. By a stepwise procedure using glutaric dialdehyde and the enzyme, five layers of the respective enzymes were attached to the electrodes.

Ferrocene derivatives[33] and *N,N′*-bipyridinium[37] salts are known to act as electron mediators for GOD and GR, respectively. Therefore, the photoisomerizable nitrospiropyran-ferrocene (**3**) and nitrospiropyran-4,4′-bipyridinium salts (**6**) were synthesized. The compounds revealed photoisomerizable properties. Illumination of the nitrospiropyran-ferrocene (**3a**), SP-Fc (in ethanol), or nitrospiropyran-*N,N′*-bipyridinium salt (**6a**), SP-V^{2+} (in water), 320 nm < λ < 380 nm, resulted in the formation of the nitromerocyanine-ferrocene (**3b**), MRH^+-Fc, and nitromerocyanine-*N,N′*-bipyridinium salt (**6b**), MRH^+-V^{2+}. Further irradiation of **3b** or **6b**, λ > 475 nm, restored **3a** and **6a**, respectively, Scheme 6. The absorption spectra of the photogenerated SP-V^{2+} (**6a**) and MRH^+-V^{2+} (**6b**) are shown in Figure 4. The photoisomerizable ferrocene compound shows reversible redox properties, Figure 5. The ferrocene units in SP-Fc and MRH^+-Fc show a reversible redox wave at E° = +0.45 V. The redox potential is identical for the SP-Fc (**3a**) and MRH^+-Fc (**3b**) configurations. The photoisomerizable bipyridinium compound shows a quasi-reversible redox wave at E° = −0.54 V of similar shape in the SP-V^{2+} and MRH^+-V^{2+} isomer state. This reduction potential of the photoisomerizable bipyridinium relay is ca. 180 mV positively shifted as compared to *N,N′*-dimethyl-4,4′-bipyridinium. This positive shift allows to separation of the reduction of the bipyridinium component without reducing the nitrospiropyran unit (vide infra).

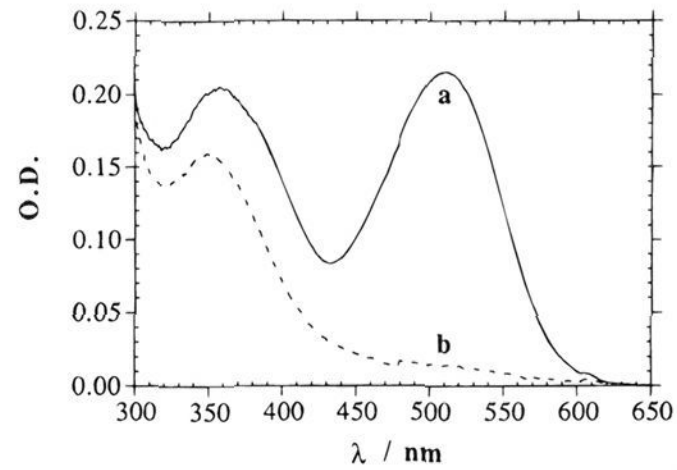


Figure 4. Absorption spectra in phosphate buffer, pH = 7.0, of (a) bipyridinium merocyanine (**6b**), 4 × 10^{-5} M, and (b) bipyridinium-nitrospiropyran (**6a**).

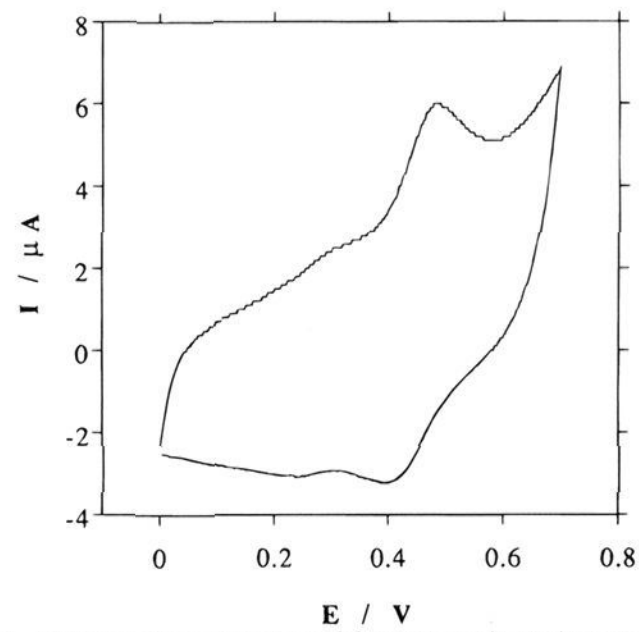


Figure 5. Cyclic voltammogram of ferrocene-nitrospiropyran (**3a**), 1 × 10^{-4} M, in 0.1 Na_2SO_4 and 0.01 phosphate buffer, pH = 7.0, and 10% ethanol. Potential scan rate 100 mV·s^{-1}.

The photoisomerizable electron mediators **3** and **6** strongly affect the effectiveness of electrical communication of the redox enzymes with the electrode. Figure 6 shows the chronoamperometric responses of the GOD multilayer Au-electrode in the presence of the two ferrocene photoisomers, **3a** and **3b**, upon addition of glucose. The experiment is initiated in the presence of the SP-Fc state (**3a**), formed by irradiation of the solution, λ > 475 nm. Injection of glucose (time of injection indicated by arrow) results in the development of a steady-state anodic current (curve a). Isomerization of the electron mediator to the MRH^+-

(34) (a) Mahene, J.; Aussaresses, H. *C. R. Acad. Sci. Paris* **1979**, *289*, 357. (b) Mor, J. R.; Guamaccia, R. *Anal. Biochem.* **1977**, *79*, 319. (c) Taniguchi, I.; Miyamoto, S.; Tomimura, S.; Hawkridge, F. M. *J. Electroanal. Chem.* **1988**, *240*, 333.

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(38) The surface concentration of the enzyme on the electrode surface was estimated by immobilization of a ferrocene-modified GOD, of known loading, on the base active ester monolayer, followed by coulometric analysis of the ferrocene units cyclic voltammogram.

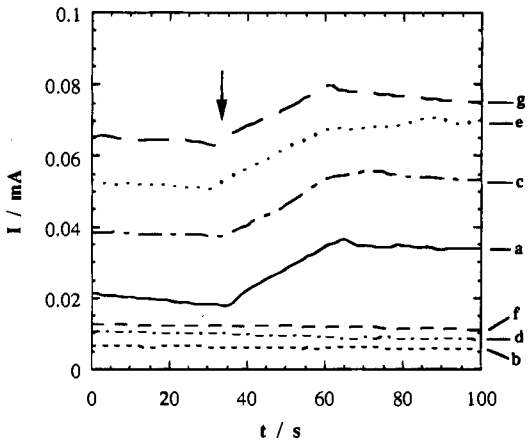


Figure 6. Chronoamperometric responses of a five-layer GOD-modified electrode in the presence of the photoisomerizable ferrocene electron mediator (**3**), 1×10^{-4} M. Curves (a), (c), (e) and (g) correspond to systems that include the ferrocene-nitrospiropyran, Fc-SP (**3a**), as electron mediator. Curves (b), (d), and (f) correspond to systems that include the ferrocene-merocyanine, Fc-MR (**3b**), as electron mediator. Arrow indicates the time of consecutive additions of glucose, 4 mM. All experiments were recorded in 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0, and 10% v/v ethanol. The applied potential corresponded to +0.5 V.

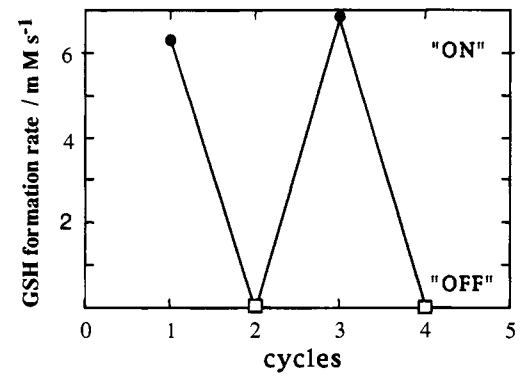


Figure 7. Cyclic photostimulated biocatalyzed formation of GSH by GR using the photoisomerizable electron transfer mediator: (●) V^{2+}-SP, (**6a**) and (□) V^{2+}-MRH^+, (**6b**). In all experiments the working electrode consists of five layers of GR. Electrolyte solution is composed of 0.1 M Na_2SO_4 and 0.01 phosphate buffer, pH = 7.0, **6a** or **6b**, 1×10^{-4} M, and GSSG, 0.01 M. Applied potential was −0.57 V.

Fc state, 320 nm < λ < 380 nm, results in the depletion of the current in the cell (curve b), implying that this photoisomer state does not act as electron mediator for GOD. Further photochemical isomerization of the MRH^+-Fc state to the SP-Fc isomer (**3a**) restores the initial steady-state current, and, upon injection of an additional amount of glucose, a further increase in the steady-state current is observed (curve c). Reversible isomerization of SP-Fc to MRH^+-Fc depletes, again, the current in the system (curve d). Photostimulation of the amperometric response of the system by reversible photoisomerization across the SP-Fc and MRH^+-Fc states is further exemplified in curves (e) and (f). We see that SP-Fc (**3a**) acts as an effective electron mediator for GOD, where the photoisomer MRH^+-Fc lacks electrical communication with GOD. Although MRH^+-Fc is effectively oxidized by the electrode interface, it lacks the ability to penetrate into the protein and establish electrical communication. It should be noted that the background current of the systems in the SP-Fc state prior to addition of glucose is always identical to the amperometric response of the system in the previous cycle. This result is consistent with the fact that for cycles shown in curves (c), (e), and (g) the initial glucose concentration in the system is similar to that present in the preceding cycle. Furthermore, a nonlinear increase in the amperometric responses of the systems, in the SP-Fc state of the electron-mediator, is observed upon addition of glucose. This originates from the saturation of the biocatalytic activity of the multilayer GOD electrode at high glucose concentrations.

Similar results are observed in the system consisting of the GR-multilayer electrode and the photoisomerizable *N,N'*-bipyridinium salt (**6b**) as electron mediator. With this enzyme-electrode, we were unable to detect bioelectrocatalytic amperometric responses in the presence of oxidized glutathione, GSSG, and the electron mediators **6a** or **6b**, due to the low turnover activity of GR. We could, however, monitor the effectiveness of electrical communication of GR with the electrode surface by the two isomer states of the electron mediator, **6a** and **6b**, by assaying the amount (or rate) of reduced glutathione, GSH, formed by electron transfer, eq 1. Figure 7 shows the rate of GSH formation in the electrochemical cell consisting of the GR-electrode and the electron mediator in the two states SP-V^{2+}

$$^1/_2\text{GSSG} + \text{SP-V}^{+\bullet}\,\text{H}^+ \xrightarrow{\text{GR}} \text{GSH} + \text{SP-V}^{2+} \qquad (1)$$

(**6a**) and MRH^+-V^{2+} (**6b**), respectively. Application of a potential corresponding to $E^\circ = -0.57$ V on the GR-electrode results in the reduction of GSSG to GSH at a rate of 6.5 μmol·min^{-1}. Photoisomerization of the electron mediator to the MRH^+-V^{2+} state blocks the formation of GSH, although the electron mediator is effectively reduced by the electrode to MRH^+-$V^{+\bullet}$. Cyclic photoisomerization of the electron-mediator from the SP-V^{2+} state to the MRH^+-V^{2+} state reversibly activates and blocks the formation of GSH, respectively. Thus, the electron relay SP-V^{2+} acts as electron mediator for GR, where MRH^+-V^{2+} lacks electron transfer communication with the protein redox-site, Scheme 6.

For the two enzyme electrodes (GOD and GR) we find that nitrospiropyran relay states, SP-Fc and SP-V^{2+}, act as the active diffusional electron mediators, respectively, where the merocyanine relay configurations, MRH^+-Fc and MRH^+-V^{2+} lack electrical communication with the enzymes, Scheme 6. At present, we do not know the origin for the blocked electrical interaction between the redox sites of the proteins and MRH^+-Fc or MRH^+-V^{2+}. Diffusional barriers of these electron relays into the proteins, posed by steric constraints or electrostatic repulsions, could lead to the lack of electrical interactions. The systems reveal, however, a further means to transduce amperometrically optical signals recorded by a photoresponsive electron mediator.

Amperometric Transduction of Recorded Optical Signals by Photoisomerizable Glucose Oxidase Electrodes. Chemical modification of enzymes by photoisomerizable units was suggested as a general method to control biocatalytic activities of proteins by light.[4-6] It has been suggested that in one photoisomer state, the tertiary structure of the protein is retained and the protein is activated for its biological function (biocatalysis, binding, etc.). In the complementary photoisomer state the protein is distorted and is deactivated toward its biocatalytic function. Till now different hydrolytic enzymes such as papain and α-chymotrypsin were photoregulated using azobenzene, thiophenefulgide, and nitrospiropyran photoisomerizable components. Using a similar approach, modification of redox enzymes by photoisomerizable components could lead to the control of their activities by light. Organization of such photoswitchable redox enzymes onto electrode surfaces could then lead to the amperometric transduction of optical signals

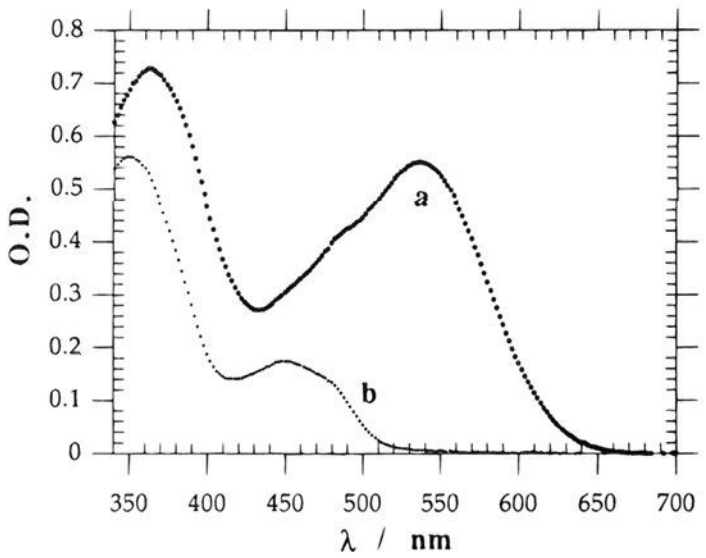


Figure 8. Absorption spectra of photoisomerizable GOD, 1.86 mg·mL^{-1} (cuvette 10 mm path): (a) MR-GOD and (b) SP-GOD.

recorded by the protein. We have developed this method using photoisomerizable glucose oxidase immobilized onto Au-electrodes.

Glucose oxidase, GOD, was modified by nitrospiropyran units by reacting the enzyme with the active ester 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester) nitrospiropyran (**1**). The average *loading of the resulting nitrospiropyran-GOD corresponds to* 21. That is, 21 lysine residues out of the 54 lysines associated with GOD are substituted by nitrospiropyran units. The resulting nitrospiropyran GOD, SP-GOD, exhibits photoisomerizable properties, Figure 8. Irradiation of SP-GOD, 320 nm < λ < 380 nm, yields the merocyanine glucose oxidase, MR-GOD, exhibiting a characteristic absorbance in the visible spectrum, λ_{max} = 536 nm. Illumination of MR-GOD, λ > 475 nm, results in back isomerization to the SP-GOD. Nitrospiropyran glucose oxidase exhibits photoswitchable biocatalytic properties in a homogeneous phase. The activities of SP-GOD and MR-GOD were assayed by monitoring the rates of H_2O_2 formation as a result of oxidation of glucose, eq 2. The rates of H_2O_2 formation were determined spectroscopically via the peroxidase, POD,

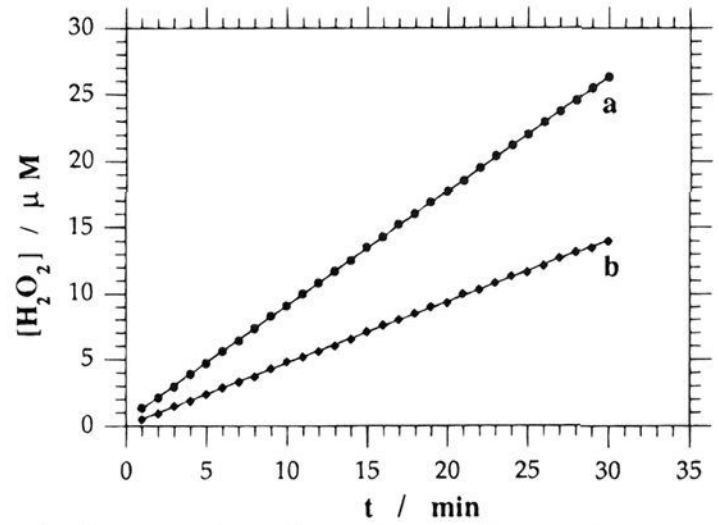


Figure 9. Photostimulated biocatalyzed oxidation of glucose by O_2. The reaction is followed by the rate of H_2O_2 formation: (a) SP-GOD and (b) MR-GOD. All experiments were performed in a 0.05 M sodium acetate buffer, pH = 5.1, that included the biocatalyst 1 × 10^{-4} mg·mL^{-1} and glucose 0.1 M.

catalyzed oxidation of *o*-dianisidine, eq 3. Figure 9 shows the rate of H_2O_2 formation by the SP-GOD and MR-GOD as reflected by the absorbance changes of oxidized *o*-dianisidine

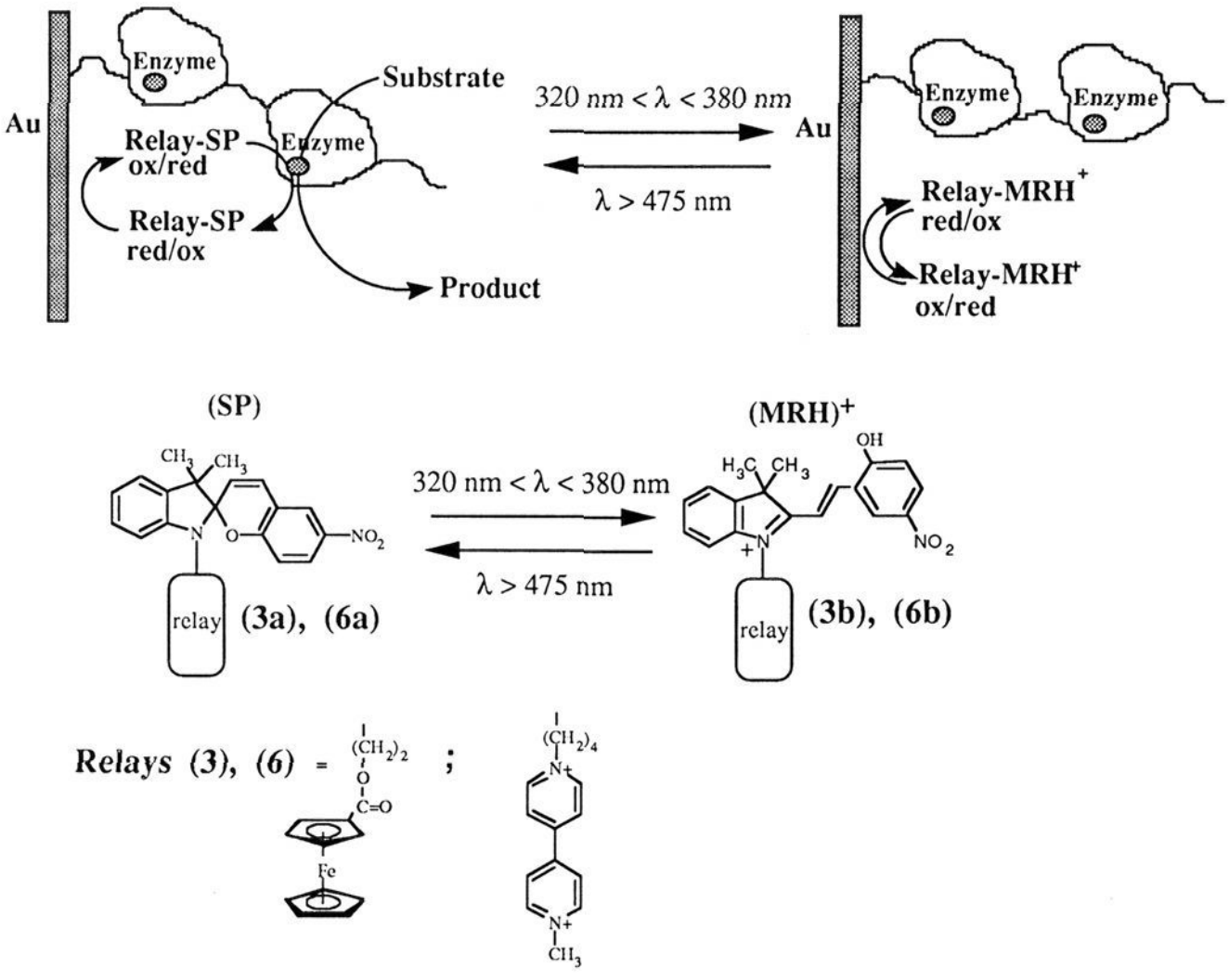
$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\textit{or MR-GOD}]{\text{SP-GOD}} \text{D-gluconic acid} + H_2O_2 \quad (2)$$

$$H_2O_2 + \textit{o}\text{-dianisidine (reduced)} \xrightarrow{\text{POD}} \textit{o}\text{-dianisidine (oxidized)} + H_2O \ (\lambda_{max} = 500\ \text{nm}) \quad (3)$$

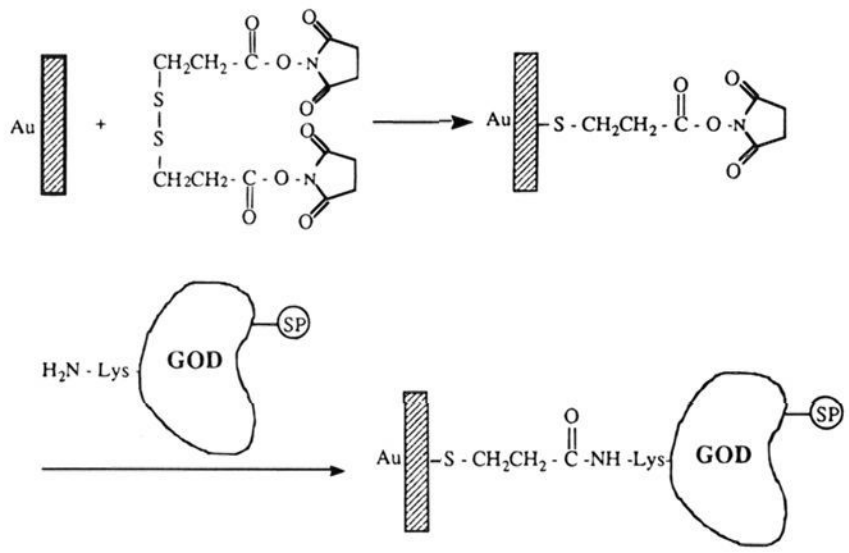
as a function of time. The activity of SP-GOD is ca. 2-fold higher than that of MR-GOD. We find, however, that UV-light irradiation of the SP-GOD to generate MR-GOD significantly degrades the protein. This limits the application of the photoisomerizable enzyme for cyclic "ON−OFF" oxidation of glucose in an aqueous solution.

The chemically modified GOD was organized as a monolayer onto an Au-electrode according to Scheme 7. Rough Au-

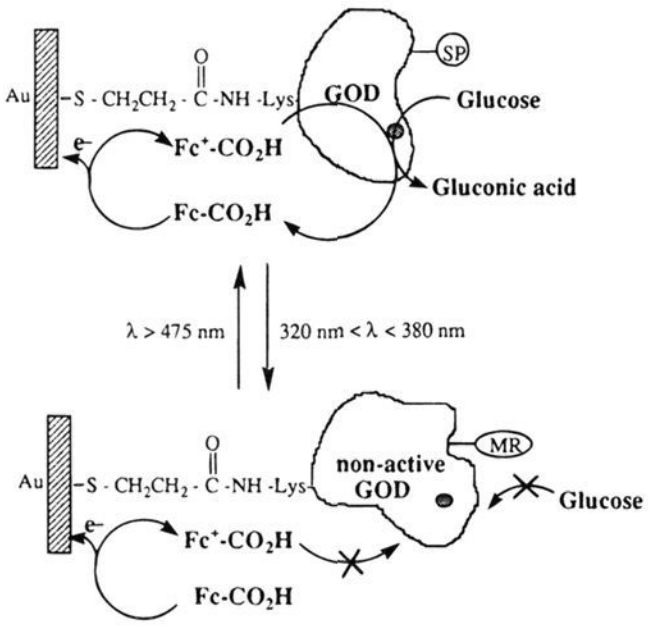
Scheme 6. Photostimulation of Redox-Enzyme Electrodes by Photoisomerizable Electron Relays



Scheme 7. Organization of the Photoisomerizable GOD Monolayer-Electrode



Scheme 8. Photostimulated Control of Electrobiocatalyzed Oxidation of Glucose by the Photoisomerizable GOD Monolayer



electrodes were employed as the substrate in order to generate a high surface density of the protein.

A cysteic acid *N*-hydroxysuccinimide monolayer associated with the Au-electrode was reacted with the SP-GOD. The surface density of the enzyme was estimated[38] to be ca. 1×10^{-12} mol·cm^{-2}. Glucose oxidase immobilized onto the electrode does not exhibit direct electrical communication, but upon addition of the diffusional electron mediator, ferrocene carboxylic acid, Fc-CO_2H, electron transfer between the enzyme and the electrode is facilitated. We thus examined the Fc-CO_2H-mediated electron transfer originating from the oxidation of glucose by the photoisomerizable GOD, SP-GOD, and MR-GOD, respectively, Figure 10. In the presence of the SP-GOD monolayer electrode and Fc-CO_2H as diffusional electron mediator, an electrocatalytic anodic current is observed as a result of bioelectrocatalyzed oxidation of glucose (curve a). Illumination of the monolayer electrode, 320 nm $< \lambda <$ 380 nm, generated the MR-GOD monolayer electrode. Under these conditions the electrocatalytic anodic current is substantially lower (curve b), implying that the active site of the protein is perturbed toward the electron mediator and the bioelectrocatalyzed oxidation of glucose, Scheme 8. Further irradiation of the electrode, $\lambda > 475$ nm, restores the SP-GOD monolayer, and the electrocatalytic current is enhanced. Cyclic modulation of the electrocatalytic anodic current developed in the electrochemical cell is observed upon reversible photoisomerization of the monolayer electrode across the states SP-GOD and MR-GOD, Figure 10 (inset).

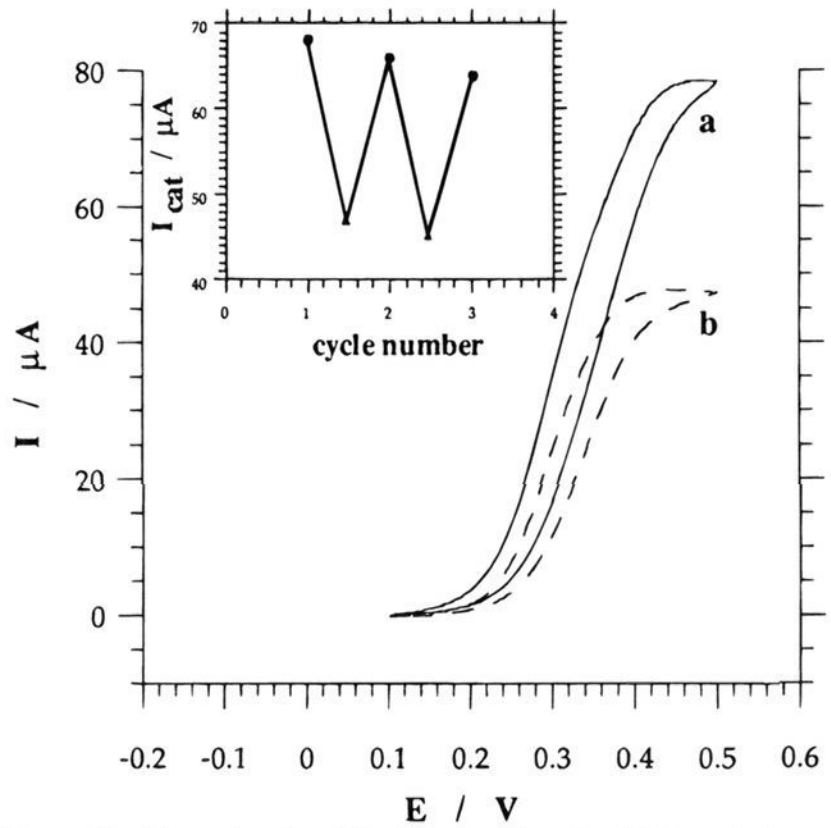


Figure 10. Photostimulated bioelectrocatalyzed oxidation of glucose using a photoisomerizable GOD monolayer electrode: (a) in the presence of the SP-GOD monolayer electrode and (b) in the presence of the MR-GOD monolayer electrode. For all experiments the electrolyte consisted of 0.1 M Na_2SO_4 and 0.01 M phosphate buffer, pH = 7.0, ferrocene carboxylic acid, Fc-CO_2H, 5×10^{-3} M, as electron transfer mediator, and glucose, 2.5×10^{-2} M. The potential scan rate corresponded to 5 mV·s^{-1}. Inset: Cyclic photostimulated amperometric responses of the photoisomerizable GOD monolayer-electrode.

The photomodulated electrocatalytic currents observed upon the cyclic photochemical isomerization of the monolayer electrode across the states SP-GOD and MR-GOD reveal a constant decrease of ca. 4% in their magnitude for each catalytic cycle. Note that this decrease in the anodic currents is observed for both states SP-GOD and MR-GOD. This decrease is attributed to ca. 4% of denaturation of the enzyme upon illumination in the UV-region, 320 nm $< \lambda <$ 380 nm. In a homogeneous phase denaturation of the protein by UV irradiation is even more pronounced (vide supra). Organization of the biocatalyst as a monolayer stabilizes the protein against UV denaturation, although this side reaction is not completely blocked. Thus, the chemical modification of a redox protein by photoisomerizable units and its organization as a monolayer on an electrode surface provides a further method for the amperometric transduction of recorded optical signals. The turnover of the biocatalyst by the oxidation of glucose allows the current amplification of the recorded optical signal. We note, however, that the specific assembly consisting of photoisomerizable GOD suffers from the limitation that the MR-GOD state representing the switched-off enzyme, exhibits a residual biocatalytic activity, and is not fully switched-off.

Conclusions

We have developed three different methodologies for the amperometric transduction and amplification of recorded optical signals using redox proteins as the active components. In all of these systems a functionalized electrode surface was integrated with the biomaterial to form an assembly that translated the optical signal into an electrochemical response. The three methods included the following: (i) The application of a photoisomerizable nitrospiropyran-pyridine mixed monolayer electrode as an active interface that controls the electrical communication of cyt *c*. Coupling of the photochemically controlled redox-interactions of cyt *c* with the electrode interface enabled the bioelectrocatalyzed reduction of O_2 and oxidation

of lactate using COX and LDH as secondary enzymes and provided a means to amplify the electrochemical responses of the systems. (ii) Photoisomerizable electron relays were applied to stimulate the electrical communication of redox enzymes and the electrode surface. (iii) A photoisomerizable redox enzyme immobilized as monolayer on electrode surfaces provided an active interface for controlling electrical communication between the redox-protein and electrode by external light signals.

These integrated assemblies revealed the fundamental features of bioelectronic devices. We envisage the future applications of such systems in the amplification of weak light-signals, their use as electrochemical actinometers, and memory-storage and processing devices. It should be noted, however, that for different applications appropriate configurations for amperometric transduction of recorded optical signals should be employed. The low absorbance of the photoisomerizable monolayer electrodes suggests that amplification of weak light signals is advantageous in a system that applies the diffusional electron mediators. For information-storage more intense light-signals could be used, and the monolayer electrodes might be applied. For some of the practical applications, high turnover numbers of the photoisomerizable components are essential. At this point we are able to perform 10−20 cycles of the systems without noticeable degradation. The long-term activities of the assemblies, however, need further characterization.

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