**Review**

**Design of Amperometric Biosensors and Biofuel Cells by the Reconstitution of Electrically Contacted Enzyme Electrodes**

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Received: August 18, 2007
Accepted: October 3, 2007

**Abstract**

The electrical contacting of redox enzymes with electrodes is the most fundamental requirement for the development of amperometric biosensors and biofuel cell elements. For the effective electrical communication of redox enzymes with electrodes the use of electron relay units that transport the electrons between the enzyme redox center and the conducting surface is essential. Also, the structural alignment of the redox enzyme units in respect to the electrode in a configuration where the enzyme redox center is in close proximity to the conductive surface is needed. The present report summarizes the reconstitution paradigm developed by our laboratory in the last decade as a versatile method to electrically contact redox enzymes with electrodes, and as a generic approach to develop amperometric biosensors and biofuel cell elements. The process is based on the reconstitution of the apo-enzyme on a relay-cofactor monolayer and on thin film-functionalized electrode. Different relay units were used to electrically communicate flavin adenine dinucleotide (FAD)-containing enzymes (flavoenzymes) or pyrroloquinoine quinone (PQQ)-containing enzymes with electrodes. This included molecular redox-active relays, molecular redox-active ‘shuttles’, redox-active polymers (e.g., polyaniline), Au nanoparticles, and carbon nanotubes. The reconstitution of different apo-enzymes on these relay-cofactor-functionalized electrodes led to unprecedented efficient electrical contacting between the redox centers of the enzymes and the electrodes. Besides very sensitive amperometric biosensors that emerged from this method, the resulting amperometric biosensors revealed high selectivity and specificity. A related approach to establish electrical contact between redox enzymes dependent on diffusional cofactors and electrodes and to develop an integrated bioelectrocatalytically active enzyme electrode was developed. The method involved assembly of a relay-cofactor diad on the electrode, and the surface crosslinking of an affinity complex generated between the enzyme and the surface-confined cofactor units. This method was successfully applied to electrically contact nicotinamide adenine dinucleotide (phosphate) NAD(P)*-dependent enzymes and cytochrome c-dependent enzymes. For example, enzyme-modified electrodes for the bioelectrocatalyzed oxidation of alcohol, lactate and malate were fabricated by the electrical contacting of the respective NAD(P)*-dependent dehydrogenases. Similarly, the bioelectrocatalytic reduction of O₂ was accomplished by an integrated cytochrome c/cytochrome oxidase-functionalized electrode. The electrically contacted enzyme electrodes were also used to develop noncompartmentalized biofuel cell elements. Biofuel cell elements consisting of electrically contacted reconstituted enzyme electrodes were constructed. Glucose or alcohol were used in these systems as fuel substrates and O₂ as oxidizer.

**Keywords:** Biosensor, Biofuel cell, Monolayer, Bioelectronics, Enzyme, Nanobiotechnology

DOI: 10.1002/elan.200704128

1. Introduction

Electrical contacting of redox enzymes with electrodes is a fundamental function required to develop amperometric biosensors or biofuel cell elements [1–7]. Redox proteins usually lack, however, electrical communication with electrodes, and the redox centers of the proteins cannot be activated by the potential applied on the electrode. This barrier for electron transfer between the redox site of enzymes and the electrode is easily explained by the Marcus theory [8]. It formulates the electron transfer rate-constant between a donor and acceptor pair, k_{ET}, according to Equation 1, where \( d_r \) and d are the van der Waals and actual distances separating the donor–acceptor pair, respectively, and \( \Delta G^* \) and \( \lambda \) are the free energy change and the reorganization energy that are accompanying the electron-transfer process, respectively.

\[
k_{ET} = \alpha \exp[-\beta(d - d_r)] \exp[-(\Delta G^* + \lambda)^2/(4RT\lambda)]
\]  

(1)

Realizing that the redox center in proteins is embedded in protein matrices exhibiting diameters in the range of 70 Å to 150 Å, it was concluded that the spatial, steric separation of the centers from the electrode surface by the protein shell introduces the barrier for electron-transfer (ET) communication [9–11]. Substantial research efforts were directed in the past two decades to develop methods to establish electrical contact between redox proteins and electrodes, and to overcome the steric barrier for ET introduced by the protein matrices. All of these methods introduce electron-
transfer relays as mediators that shorten the ET distances and shuttle the electrons between the redox centers of the proteins and the electrodes. Among the methods, diffusional ET mediators that diffuse in and out the protein matrices and shuttle the electrons, were applied [12–15]. Similarly, the covalent tethering of electron relays to proteins as a means to shorten the ET distances [16–20], and the incorporation of the enzymes into shape-flexible redox-active polymeric hydrogels were used as general paradigms for the electrical communication of redox enzymes with electrodes and their biocatalytic activation [21, 22]. Although substantial progress was accomplished in the fabrication of electrically contacted enzyme electrodes, and practical biosensor devices based on this concept appeared on the market, several challenging topics need further basic research insight: i) Albeit the different strategies to electrically contact redox enzymes with electrodes were successful, the effectiveness of electronic communication was far lower than the ET exchange between the redox enzymes and their native electron acceptors (or electron donors). This inefficient electrical communication was attributed to the random substitution of the enzymes by the electron relay units in non-optimal steric positions and to the random orientation of the enzymes upon their immobilization on electrodes or their incorporation in polymers associated with electrodes. ii) Redox proteins may include tightly bound redox-centers to the protein shell, e.g., flavin adenine dinucleotide, FAD-, pyrroloquinoline quinone, PQQ-, or heme-containing enzymes. While the tethering of relay units to these enzymes exhibits some structural and steric information, numerous redox enzymes operate in nature with the aid of diffusional redox-active cofactors, e.g., nicotinamide adenine dinucleotide (phosphate), NAD(P)+, or cytochrome cofactors. The enzymes operating with diffusional cofactors generate labile cofactor-biocatalyst complexes with optimized orientation for ET. The electrical contacting of these latter enzymes with electrodes does not only require the incorporation of relay units for shortening ET distances, but the development of methods to integrate the diffusional cofactor units in rigid enzyme scaffolds associated with electrodes is essential.

The present article will review the advances in the construction of electrically contacted reconstituted enzyme electrodes and their application as amperometric electrodes or as functional electrodes in biofuel cells. Particular emphasis will be given to the emerging field of nanobioelectronics, where nanomaterials provide exciting opportunities for bioelectronics.

2. Electrically Contacted Reconstituted Enzyme Electrodes in Monolayer Configurations

The electrically contacting of enzymes, e.g., glucose oxidase, was accomplished in monolayer configurations, where a relay unit was used to bridge the reconstituted enzyme with the electrode, Figure 2. In one configuration [24], Figure 2A, pyrroloquinoline quinone, POQ, (1), was assembled on an Au electrode, and N6-(2aminoethyl)–flavin adenine dinucleotide, amino-FAD, (2), was covalently linked to the POQ sites. The reconstitution of apo-glucose oxidase on the surface-bound FAD site led to a structurally aligned enzyme monolayer with a surface coverage of $1.5 \times 10^{-12}$ mol cm$^{-2}$. The POQ electron relay units mediated ET between the FAD sites and the electrode, while activating the bioelectrocatalyzed oxidation of glucose. The electron transfer turnover rate was estimated to be ca. 900 s$^{-1}$, a value that is similar to the exchange rate between the enzyme redox center and its native electron acceptor, oxygen [25]. This efficient electrical communication between the enzyme redox center and the electrode led to an oxygen-insensitive amperometric glucose sensing electrode. Similarly, the effective ET communication between the biocatalyst and
the electrode generated an amperometric biosensor device for glucose that was insensitive to common glucose sensing interferents such as ascorbic acid or uric acid. A modified synthetic route for the assembly of the electrically contacted enzyme electrode is depicted in Figure 2B. This method substitutes the scarce amino-FAD cofactors, (2), with the natural FAD cofactor by applying ‘click chemistry’ principles to construct the enzyme monolayer [26]. The pyrroloquinoline quinone-monolayer-functionalized electrode was reacted with 3-aminophenyl-boronic acid, (3), and the native FAD cofactor, (4), was linked through the vicinal hydroxyl group to the boronic acid ligand. The reconstitution of apo-glucose oxidase on the FAD cofactor sites led to an electrically contacted enzyme electrode. Figure 2C depicts the cyclic voltammograms observed upon the bioelectrocatalyzed oxidation of different concentrations of glucose by the reconstituted enzyme electrode, and the derived calibration curve. Knowing the surface coverage of the enzyme electrode, $2 \times 10^{-12}$ mol cm$^{-2}$, and the saturation current generated by the electrode, the turnover rate of electrons between the redox center and the electrode was estimated to be $700 \text{ s}^{-1}$.

A different approach to electrically contact redox enzymes with electrodes in monolayer structures involved the design of ‘molecular charge transport wires’. Charge transport through molecular wires is a subject of extensive theoretical [27] and experimental [28] research. Oligophenylacetylenes were used as stiff linear conjugated molecular wires for charge transport, and a monolayer consisting of the thiolated diethylaniline oligo-phenylacetylene, (5), was used to electrically contact amine oxidase with an Au electrode (Scheme 1) [29]. The enzyme includes a topaququinone/Cu$^{2+}$ redox center, and it catalyzes the oxidation of amines to aldehydes. The diethylamine group tethered to the thiolated wire (5) linked to the electrode binds to the active site of the enzyme. The association of the wire ‘headgroup’ to the enzyme aligned the redox protein on the electrode surface and the ‘molecular wire’ was found to electrically couple the redox protein with the conducting surface.

An alternative approach to electrically communicate redox enzymes with electrodes included the application of supramolecular architectures that consisted of the dynamic shuttling of the charge along molecular wires [30]. The electrochemical shuttling of molecular units along molecular wires associated with semiotaxane configurations attract scientific interest as a means for information storage and for the development of ‘smart surfaces’ of controllable hydrophobicity [31]. A monolayer consisting of a ‘molecular shuttle’ in a semiotaxane configuration was used to electrically contact glucose oxidase with the electrode [30], Figure 3A. The bis-bipyridinium cyclophane, (6), was threaded on a monolayer unit consisting of molecular wires that included the bis-iminobenzene $\pi$-donor sites. The supramolecular $\pi$-donor-acceptor complex between (6) and the bis-iminobenzene components were stoppered with amino-FAD cofactor, (2), and apo-glucose oxidase was reconstituted on the FAD units. The resulting integrated enzyme electrode revealed electrical contact between the enzyme and the electrode. The redox potential of the threaded cyclophane is $-0.43 \text{ V vs. SCE}$, ca. 100 mV more positive than that of the FAD cofactor associated with the enzyme. Upon the biocatalyzed oxidation of glucose, the cyclophane unit oxidizes the cofactor site, thereby transforming the cyclophane to the respective radical cation that lacks $\pi$-acceptor properties. The potential applied on the electrode, $-0.43 \text{ V vs. SCE}$, attracts the reduced cyclophane, and the electrically shuttled cyclophane is being oxidized at the electrode to the tetracationic cyclophane that favors its dynamic shuttling to the bis-iminobenzene site, where a stable $\pi$-donor–acceptor complex is formed. As a result, a second electrical communication cycle of the enzyme is initiated. Thus, the dynamic shuttling of the cyclophane by the respective redox transformation at the enzyme and the electrode surface, leads to the electrical ‘wiring’ of the enzyme on the electrode. Figure 3B depicts the electrocatalytic anodic currents observed upon the bioelectrocatalyzed oxidation of glucose, and the respective derived calibration curve. The biocatalyzed oxidation of glucose occurs at an onset potential of $-0.4 \text{ V vs. SCE}$. This is the lowest potential ever observed for the bioelectrocatalyzed oxidation of glucose in an electrically wired assembly. The bioelectrocatalyzed oxidation of glucose at such low potentials has the advantage that common glucose interfering compounds, such as ascorbate or uric acid, are not oxidized at these potentials, and thus, these compounds do not perturb the amperometric analysis of glucose. Furthermore, the oxidation of glucose at a negative potential, close to the thermodynamic potential of the redox center of glucose oxidase is invaluable for the design of biofuel cells with high power outputs (vide infra).

3. Electrical Contacting of NAD(P)$^+$-Dependent Enzymes Using Monolayer-Modified Electrodes

While the electrical contacting of flavoenzymes or PQQ-dependent enzymes with electrodes included the reconstitution of the apo-enzymes on the respective cofactors to yield structurally fixed biocatalysts, numerous redox enzymes use diffusional cofactors, such as nicotinamide adenine dinucleotide (phosphate), NAD(P)$^+$, or hemoproteins. These cofactors form labile complexes with the respective redox enzymes that adapt the appropriate structural alignment for ET. The electrical wiring of such redox enzymes requires the rigidification of the cofactor–enzyme complexes and the electrical contacting of the cofactors with the electrodes. Nonetheless, the electron-
transfer communication between the NAD(P)⁺/NAD(P)H cofactors with electrodes suffers from kinetic barriers and unfavorable side reactions [32, 33]. This originates from the fact that the reduction of the NAD(P)⁺ or the oxidation of
the NAD(P)H cofactors are \(2e^-/H^+\) processes. Accordingly, catalysts have to be integrated with the electrode to stimulate the redox reactions of the NAD(P)\(^+\)/NAD(P)H cofactors. For example, different organic dyes, quinones or transition metal complexes were reported to electrocatalyze the oxidation of the NAD(P)H cofactors \([34 – 40]\). Specifically, pyrroloquinoline quinone, PQQ, (1), associated as a monolayer on electrodes was reported to act as an effective electrocatalyst for the oxidation of the NAD(P)H cofactors \([41, 42]\). This enables the tailoring of integrated, monolayer-functionalized electrode of NAD(P)\(^+\)-dependent enzymes \([26]\). A monolayer of pyrroloquinoline quinone, PQQ, was assembled on Au electrodes, and the 3-aminophenyl boronic acid, (3), was covalently linked to the PQQ monolayer. (For the different coupling reactivities of the three carboxylic acid residues of PQQ, see \([42]\)). The formation of the boronate complex between the boronic acid ligand and the ribose site associated with nicotinamide adenine dinucleotide phosphate, NADP\(^+\); (7), was followed by the generation of the labile affinity complex between the NADP\(^+\) cofactor and malate dehydrogenase, MalD. The subsequent cross-linking of the surface-confined cofactor-enzyme complex with glutaric dialdehyde yielded then an integrated rigid electrically contacted enzyme electrode, where the bioelectrocatalyzed oxidation of malate to pyruvate occurred, Figure 4A. Figure 4B shows the cyclic voltammograms observed upon the oxidation of variable concentrations of malate, and the respective calibration curve. Knowing the surface coverage of the enzyme in the monolayer film, and the saturation current generated by the biocatalytic film, the turnover rate of electrons between the enzyme and the electrode was estimated to be \(190 \text{s}^{-1}\).

A similar approach was undertaken to electrically activate the NAD\(^+\)-dependent enzyme, lactate dehydrogenase, LDH, Figure 5 \([26]\). As before, the nicotinamide adenine dinucleotide, NAD\(^+\); (8), was linked to the PQQ/boronic acid monolayer-functionalized electrode. The availability of two ribose units in the cofactor enables, however,
two binding modes of the cofactor to the boronic acid ligand (cf. I or II in Fig. 5). The formation of the affinity complex between the NAD$^+$ cofactor and LDH, followed by the crosslinking of the labile complex with glutaric dialdehyde yielded a rigid electrically contacted enzyme film that mediated the bioelectrocatalyzed oxidation of lactate to pyruvate. Detailed chronoamperometric studies revealed that, indeed, the NAD$^+$ cofactor binds to the boronic acid ligand by the two modes, I and II, and that the binding mode II is ca. 10-fold more efficient as compared to I in mediating the bioelectrocatalyzed oxidation of lactate [26]. It should be noted that the bioelectrocatalyzed oxidation of lactate to...
pyruvate in the presence of LDH and of malate to pyruvate in the presence of MalD are activated at ca. \(-0.15\) V vs. SCE. As the redox potential of the PQQ is \(-0.15\) V at pH 7.0, the experimental onset potentials for the bioelectrocatalyzed oxidation of lactate or malate are consistent with the fact that PQQ electrocatalyzes the oxidation of the NAD(P)H cofactors in the systems.

4. Electrically Contacted Reconstituted Redox Enzymes in Thin Polymer Films Associated with Electrodes

The physical incorporation of redox proteins in electroactive polymer matrices for establishing electrical contact between the enzymes and the electrodes is a common practice in the development of bioelectronic devices such as amperometric biosensors [21, 22] or biofuel cell elements [43]. The embedded enzymes exist, however, in random configurations in respect to the mediator groups associated with the polymer, thus leading to nonoptimal ET communication with the electrode. The immobilization of enzymes on cofactor-tethered redox polymers by a reconstitution process may then align the biocatalyst in an optimal configuration for mediated ET. This approach was, indeed, materialized by the reconstitution of redox enzymes in polyaniline redox-active films. A composite of polyaniline (PAn) and polyacrylic acid (PAA) was electropolymerized onto electrodes, and the resulting PAn/PAA film revealed quasi-reversible redox activity at neutral pH values due to the doping of the PAn with the negatively charged PAA chains, Figure 6A. The amino-FAD cofactor, (2), was then covalently linked to the PAA chains, and apo-glucose oxidase was reconstituted onto the FAD cofactor sites [44]. The resulting biocatalytic polymer construct revealed electrical communication with the electrode, Figure 6B. The bioelectrocatalytic activation of the reconstituted enzyme was attributed to the alignment of the enzyme in an optimal orientation in respect to the PAn/PAA film that acted as ET mediator between the cofactor site embedded in the enzyme and the electrode. The onset potential for the bioelectrocatalytic oxidation of glucose is observed at \(+0.2\) V vs. SCE. This is the redox potential of the PAn/PAA composite at pH 7.0, implying that the redox polymer is mediating the electron transfer between the redox center of the enzyme and the electrode. In fact, a control experiment in which the enzyme glucose oxidase was randomly covalently linked to the PAA chains revealed a 10²-fold lower bioelectrocatalytic activity as compared to the reconstituted GOx, an observation that highlights the significance of orientation of the enzyme in respect to the redox polymer by the reconstitution method [44]. The bioelectrocatalytic functions of the enzyme/polymer system were characterized in detail. The coverage of the film with GOx was found to be \(3 \times 10^{-12}\) mol cm\(^{-2}\), and from the saturation value of the electrocatalytic anodic current, \(i = 0.3\) mA cm\(^{-2}\), the ET turnover rate between the enzyme redox site and the electrode was estimated to be \(1000\) s\(^{-1}\). This value is comparable to the ET exchange between the redox site of GOx and its native electron acceptor \((O_2)\) [25], \(900\) s\(^{-1}\). A similar approach was
adapted to electrically activate the pyrroloquinoline quinone-dependent enzyme glucose dehydrogenase [45], GDH (Fig. 7A). The PQQ cofactor, (1), was covalently linked to the PAA chains incorporated in PAN using 1,4-diaminobutane as a bridge. The resulting enzyme/polymer composite revealed electrical contact with the electrode, and the PAN polymer mediated the bioelectrocatalyzed oxidation of glucose.

The PAN/PAA film was also used to activate the bioelectrocatalytic oxidation of lactate by the NAD$^+$-dependent lactate dehydrogenase [44], LDH, Figure 7B. The N$^6$-(2-aminoethyl)-nicotinamide adenine dinucleotide, amino-NAD$^+$, (9), semisynthetic cofactor was covalently linked to the PAA chains of the composite polymer. The formation of the affinity complex between LDH and the NAD$^+$ cofactor units, followed by the crosslinking of the associated proteins with glutaric dialdehyde led to an integrated electrically contacted enzyme electrode that bioelectrocatalyzed the oxidation of lactate. The ET cascade involved the biocatalyzed oxidation of lactate to pyruvate, with the concomitant reduction of the NAD$^+$ cofactor to NADH, followed by the oxidation of NADH by the oxidized PAN/PAA film. The turnover rate of the integrated enzyme electrode was estimated to be ca. 350 s$^{-1}$.
5. Electrical Contacting of Enzymes by Means of Metallic Nanoparticles (NPs) and Carbon Nanotubes (CNTs)

The nanoscale dimensions of metallic NPs or CNTs suggest that the integration of redox proteins with these nanoobjects might yield hybrid systems, where the NPs or CNTs provide nanoscale electrodes that communicate the redox center of the enzymes with the electrodes.

Au NPs (1.4 nm in diameter) were used as conducting material to electrically communicate glucose oxidase with electrodes [46]. The amino-FAD cofactor, (2), was covalently tethered to the Au NPs functionalized with a single N-hydroxy-succinimide functionality, Figure 8A. Reconstitution of apo-glucose oxidase on the FAD-functionalized Au NPs yielded the Au NP-enzyme hybrid nanostructure. The resulting Au NP-enzyme conjugate was then assembled on an Au electrode using different dithiol crosslinkers as bridging units, e.g., p-xylene dithiol, (10), 4,4’-dimercapto-biphenyl, (11), and 1,4-benzene dithiol, (12). The resulting enzyme electrode revealed unprecedented electrical contact efficiency between the redox center of the enzyme and

Fig. 7. A) Assembly of the electrically contacted polyaniline/POQ-reconstituted glucose dehydrogenase electrode, GDH. (Reproduced from [45] by permission of The Royal Society of Chemistry). B) Assembly of the integrated lactate dehydrogenase, LDH, biocatalytic interface on the NAD\(^+\)-functionalized polyaniline/poly(acrylic acid) thin film. (Reprinted in part with permission from [44]. Copyright 2002 American Chemical Society).
the electrode, and with benzene dithiol, (12), as linker a turnover rate of 5000 s⁻¹ was observed, Figure 8B. This value should be compared to the ET exchange rate between the enzyme redox center and its native electron acceptor, ca. 900 s⁻¹. The efficient electrical contact between the enzyme redox center leads not only to the effective bioelectrocatalyzed oxidation of glucose and to the sensitive analysis of glucose, but has indispensable significance reflected by the specificity of the electrode that was found to be insensitive to common glucose sensing interferants or to atmospheric O₂. The Au NPs in this system act as a nanorelay that mediates the ET between the redox center of GOx and the electrode. In fact, the molecular bridges that link the Au NPs to the electrode were found to be the rate limiting components that control the ET between the biocatalyst and the electrode. The order of electron transfer mediating effectiveness was determined to be (12) > (11) > (10). This charge transfer order can be explained by the fact that (12) includes fully conjugated molecular wire, whereas the biphenyl linker includes a perturbed π conjugation, due to the tilting of the two benzene rings. The less effective charge mediator is (10), and this is explained by the two sp³ carbon units that ‘break’ the conjugation path of the linker. In fact, theoretical studies [47, 48] on the charge transport behavior of the three molecular wires (10), (11) and (12) confirmed the experimentally observed results. It should be noted that the bioelectrocatalyzed oxidation of glucose is observed at a high overpotential of ca. +0.3 V vs. SCE. This high overpotential may be attributed to the triphenylphosphine capping layer that stabilizes the 1.4 nm Au NPs and to the dithiol bridge that bridges the NPs to the bulk electrode. A similar approach was applied to electrically activate the pyrroloquinoline quinone-dependent glucose dehydrogenase, GDH [49]. The Au NPs (1.4 nm) were linked to a gold electrode, and the PQQ cofactor was covalently linked to the particles. The apo-GDH was then reconstituted on the PQQ cofactor, and the resulting Au NP/GDH conjugate exhibited electrical communication with the electrode.

Fig. 8. A) The assembly of an Au NP (1.4 nm) electrically contacted glucose oxidase electrode by the reconstitution of apo-GOx on the FAD-functionalized Au NP, and the immobilization of the enzyme/nanoparticle hybrid on an electrode surface. B) Cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of variable concentrations of glucose by the electrically contacted Au NP-reconstituted GOx-modified electrode. Glucose concentrations correspond to a) 0 mM, b) 1 mM, c) 2 mM, d) 5 mM, and e) 10 mM. Inset: Calibration curve corresponding to the electrocatalytic currents at different glucose concentrations. (Reproduced from [46]. Reprinted with permission from AAAS).
turnover rate that corresponded to 11800 s⁻¹ between the enzyme and the electrode was observed upon the bioelectrocatalyzed oxidation of glucose.

Carbon nanotubes (CNTs) provide a different conducting nanoobject. The folding modes of the graphite layers that form the CNTs control their electronic properties, and tubes exhibiting semiconductor properties or ballistic conductivity can be formed. Specifically, the ‘armchair’ or ‘zigzag’ folding of the graphitic layer yields CNTs that reveal metallic conductivity [50]. The integration of biomolecules with CNTs attracted substantial research efforts [51, 52], and the hybrid systems found different applications in designing electrical or optical sensors [53 – 57], nanocircuity [58], and nanoscale devices [59 – 62]. Naturally, the diameter of CNTs (ca. 1.8 nm) and their conductivity properties suggested their potential use as nanoconnectors that electrically wire redox proteins with electrodes [63 – 65]. Carbon nanotubes were subjected to oxidative cleavage under harsh acidic conditions, a process that led to shortening CNTs substituted by carboxylic acid functionalities at their edges. The resulting CNTs were then separated into several fractions consisting of nanolength distributions, and fractions of CNT with lengths corresponding to 25 – 30 nm; 40 – 50 nm; 80 – 100 nm; 200 – 230 nm, were isolated. The assembly of the CNT-bridged glucose oxidase, GOx, functionalized electrode is depicted in Figure 9A [66]. The carboxylic acid-modified CNTs were covalently linked to a cystamine-functionalized electrode, and the amino-FAD cofactor (2) was linked to the carboxylic acid functionalities at the ends of the CNTs. The reconstitution of apo-GOx on the FAD units yielded, then, the integrated enzyme CNT electrode. AFM and TEM imaging confirmed the formation of the reconstituted enzyme at the ends of the CNTs. The reconstitution of the standing CNTs on the Au surface generated nanostructures with a height corresponding to 6.5 nm, consistent with the formation of reconstituted protein units on the CNTs, Figure 9B. The AFM and TEM images of solution suspended CNTs modified at their two ends with the FAD cofactor that were reconstituted with apo-GOx confirmed the tethering of the proteins to the ends of the CNTs, Figure 9C and D, respectively. The GOx reconstituted onto the CNTs revealed electrical contacting, and with connecting 25 – 30 nm long CNT, the turnover rate was estimated to be 4100 s⁻¹, Figure 9E. The electrical contacting efficiency was, however, controlled by the length of the CNTs and as the tubes were longer, the turnover rate was lower. This was attributed to defects introduced into the side walls of the CNTs upon the scission of the long unsubstituted CNTs. As the number of defects increase with the length of the CNTs, the barriers for charge transport are enhanced for the enzyme systems bridged to the electrode with longer CNTs. The bioelectrocatalyzed oxidation of glucose proceeds at a high overpotential that corresponds to ca. +0.3 V. The overpotential is affected by the length of the CNTs, and as the CNTs are longer, the overpotential is higher. The overpotential is attributed to structural defects on the walls of the CNTs, generated upon the scission of the CNTs. As the CNTs are longer, the density of the defects increases, and the conductance of the CNTs is perturbed.

The integration of redox proteins with CNTs may proceed also by the assembly of the enzymes on the side walls of the CNTs [67]. Polycyclic aromatic compounds associate strongly to the graphite side walls of the CNTs via π-π stacking [68, 69]. Accordingly, the redox-active Nile blue, NB, (13), was assembled on CNTs, and it exhibited a quasireversible 2-electron redox process, \( E^\prime = –0.35 \text{ V at pH 7} \). The modified electrodes were deposited onto a glassy carbon electrode, and 4-carboxyphenyl boronic acid, (14), was covalently linked to the NB units. The boronic acid ligand acted as anchor site for the \( \text{NAD(P)}^+ \) cofactors [26] (Fig. 10A). The binding of the \( \text{NADP}^+ \) cofactor was followed by the formation of an affinity complex with glucose dehydrogenase, GDH. The resulting affinity complex was crosslinked with glutaric dialdehyde to yield the integrated electrically contacted enzyme electrode. Similarly, the \( \text{NAD}^+ \) cofactor was linked to the boronic acid ligand, and the affinity complex between the enzyme alcohol dehydrogenase, AlcDH, and the cofactor sites were generated, and subsequently crosslinked with glutaric dialdehyde (Fig. 10B). Two binding modes, I and II, of the two different ribose units to the boronic acid ligand were identified by chronomperometry. The cofactor consisting of the boronate complex with the ribose adjacent to adenine group was found to be ca. 6-fold more efficient in electrically contacting AlcDH with the electrode as compared to the \( \text{NAD}^+ \) coordinated to the boronic acid ligand by the ribose unit close to the nicotinamide site.

6. Reconstituted Enzyme Electrode for Biofuel Cell Applications

Besides the application of electrically contacted enzyme electrodes for the development of amperometric biosensors, the functionalized electrodes find growing interest in the development of biofuel cell elements [1 – 7, 70]. The biofuel cell consists of two electrodes, Figure 11, where the biocatalyzed oxidation of a fueled substrate (e.g., glucose, alcohol, \( \alpha \)-hydroxy acids) occurs at the anode, while the biocatalyzed reduction of an oxidizer proceeds at the cathode. While \( \text{O}_2 \) is the most abundant oxidizer, other compounds such as \( \text{H}_2\text{O}_2 \) were employed [71]. The power output of the biofuel cell is given by Equation 2, where the potential difference between the anode and the cathode, whereas \( I_w \) is the current through the external circuit. For maximum power output, the anodic oxidation of the fuel, and the reduction of the oxidizer, should proceed as close as possible to the thermodynamic redox potentials of the fuel/oxidizer. The current generated by the biofuel cell is controlled by the turnover rates at the anode/cathode or by the effectiveness of the electrical contacting between the biocatalysts associated with the electrode.

Different potential applications of biofuel cells were suggested. Besides the possibility to generate electrical energy from biomass substances, implantable biofuel cells
might use biological fluids, e.g., glucose in blood, to generate electrical power for the activation of mechanical units such as pacemakers or prosthetic elements. Alternatively, the generation of electrical power from biomass or plants could provide new means to operate computers, communications, or imaging facilities in remote isolated regions.

\[ P = \Delta V_{ac} I_{ac} \]  

(2)

Fig. 9. A) Assembly of the CNT electrically contacted GOx electrode. B) Assembly of the CNT electrically contacted GDH-functionalized electrode by the surface crosslinking of an affinity complex of GDH with Nile blue-NADP\(^+\) associated with CNTs. C) Assembly of an integrated alcohol dehydrogenase, AlcDH, electrode by the surface crosslinking of an affinity complex between AlcDH and Nile blue-NADP\(^+\) linked to CNTs. (Note that two modes of binding of NAD\(^+\) to the Nile blue units by the boronic acid ligand are possible, structure I or II). (Reproduced with permission from [67]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA).

Fig. 10. A) Assembly of the electrically contacted GDH-functionalized electrode by the surface crosslinking of an affinity complex of GDH with Nile blue-NADP\(^+\) associated with CNTs. B) Assembly of an integrated alcohol dehydrogenase, AlcDH, electrode by the surface crosslinking of an affinity complex between AlcDH and Nile blue-NADP\(^+\) linked to CNTs. (Note that two modes of binding of NAD\(^+\) to the Nile blue units by the boronic acid ligand are possible, structure I or II). (Reproduced with permission from [67]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA).
Substantial recent research efforts have employed enzymes embedded in redox-functionalized polymers and associated with electrodes as the active components of biofuel cells [6, 43, 72, 73]. In most of the reported systems, the biocatalyzed oxidation of glucose by glucose oxidase or glucose dehydrogenase was used as the anodic processes. The biocatalysts that were applied for the reduction of O₂ to water usually included one of the copper proteins, laccase or bilirubin oxidase. For example, glucose oxidase and bilirubin oxidase electrically contacted with electrodes by means of redox polymers were used as biocatalytic materials for the development of a glucose/O₂ miniaturized biofuel cell element [6, 74]. Glucose oxidase, GOx, was immobilized in a tris-dialkylated-N,N'-biimidazole Os²⁺/³⁺ complex tethered to a polyvinylpyridinium hydrogel, as an electrical wiring polymer. This electrode acted as an anode for the bioelectrocatalyzed oxidation of glucose at −0.36 V vs. Ag/AgCl. The cathode of the biofuel cell consisted of bilirubin oxidase immobilized in a redox-active polymer composed of Os²⁺/³⁺ bis-(4,4'-dichloro-2,2'-bipyridine) complex tethered to a polyacrylamide-polyvinyl imidazole polymer through ligation of the imidazole to the Os²⁺/³⁺-center. The electrically contacted enzyme reduced O₂ at +0.36 V vs. Ag/AgCl. The integrated biofuel cell was found to operate with a power output of 4.3 μW. The effective electrical contacting of redox enzymes with electrodes by means of the reconstitution process, and the demonstration that the bioelectrocatalyzed oxidation of glucose by the surface-reconstituted glucose oxidase is insensitive to O₂, paved the way to construct the first integrated, noncompartmentalized, biofuel cell element [75]. This cell, Figure 12A, was based on an
anode consisting of apo-glucose oxidase that was reconsti-
tuted on a pyrroloquinoline quinone (PQQ)/FAD mono-
layer linked to the electrode (Cf. the detailed construct of
the electrically contacted enzyme electrode is shown in
Figure 2(A). The cathode was composed of a thiolated
cytochrome c, Cyt.c, monolayer that was assembled on an
Au electrode, Figure 12B. The hemoprotein revealed direct
electrical contact with the electrode and acted as electron
transport mediator for the bioelectrocatalyzed reduction of
O2 to water. Upon interacting the Cyt.c-functionalized
electrode with cytochrome oxidase, COx, the Cyt.c/COx
supramolecular complex was formed, and after the cross-
linking of the complex associated with the electrode, an
integrated electrically contacted enzyme electrode was
generated. Figure 13A shows the cyclic voltammogram of
the Cyt.c/COx integrated electrode in the presence of air.
The observed electrocatalytic cathodic current implies that
the electrode is bioelectrocatalytically active towards the
reduction of O2. Figure 13B depicts the performance of the
monolayer-functionalized GOx and Cyt.c/COx electrodes
as a biofuel cell. Figure 13B shows the cell voltage and
currents at different external resistances. Figure 13B, inset,
shows the respective power output of the resulting biofuel
cell. An optimal power output of 4 µW is observed at an
external load of 0.9 kΩ. The resulting power is certainly very
low, but the result demonstrated the feasibility to assemble
biofuel cells, and paved the grounds to search for improved
systems. In fact, the detailed electrochemical characterization
of the biofuel cell consisting of the reconstituted
GOx and Cyt.c/COx electrodes enabled the tracing of the
reasons for the low power output, and the results indicated
directions to improve the power output: i) The biofuel cell
configuration depicted in Figure 12A consists of monolayer-
functionalized bioelectrocatalytic electrodes. Means to
increase the content of the biocatalysts that communicate
with the electrodes are anticipated to increase the current
generated by the system. The immobilization of the enzymes
in redox polymer films [6, 43, 21] (rather than monolayer
structures), the fabrication of electrically contacted enzyme
multilayers [76–78], or the roughening of the electrode
could all be methods to enhance the content of biocatalysts
associated with electrodes. ii) While the GOx reconstituted
enzyme electrodes revealed effective electrical communica-
tion and a turnover rate of ca. 600 s−1 was identified, the
Cyt.c-mediated bioelectrocatalyzed reduction of O2 was
found to be rather inefficient. The turnover rates between
Cyt.c and the electrode were estimated by chronoampero-
metric experiments to be 20 s−1. Practically, the cathodic
process is the rate-limiting step for charge transport through
the external circuit, and hence the cathode is, at present, the
‘bottle-neck’ for the extractable current from the biofuel
cell. Improvements on the electrical contacting of the
biocatalysts associated with the cathode are anticipated to
significantly enhance the biofuel cells performance. iii) The
redox potentials at which the mediated oxidation of glucose
and the reduction of O2 proceeded, are far from the
thermodynamic potential for these reactions. As a result,
the ΔVe is substantially lower than the theoretical values.

For example, the PQQ-mediated oxidation of glucose
within the GOx monolayer-functionalized electrode pro-
cceeded at −0.15 V vs. SCE while the thermodynamic redox
potential for the process is ca. −0.55 V vs. SCE.

Also, externally controlled bioelectrocatalytic transforma-
tion at electrode surfaces might improve the power
output of biofuel cell elements. The application of an
external magnetic field parallel to electrode surfaces was
found to enhance the electron transfer at the electrode
solution interface. Theoretical studies indicated that under a
parallel applied magnetic field the interfacial electron
transfer is controlled by hydrodynamic convection, rather
than diffusion, resulting in the decrease in the diffusion
double layer at the electrode surface [79]. These theoretical
studies were experimentally supported, and enhanced
power outputs of biofuel cell elements were achieved [80].
For example, the bioelectrocatalyzed oxidation of glucose
by the electrode functionalized with GOx reconstituted on the
PQQ/FAD monolayer was ca. 2-fold enhanced under an
external magnetic field of 0.92 T. The effect of an external
magnetic field on the power output of a biofuel cell element
was demonstrated in a system consisting of an electrically
contacted lactate dehydrogenase-functionalized electrode.
acting as anode and the Cyt.c/COx-functionalized electrode as cathode. The integrated, electrically contacted, lactate dehydrogenase-functionalized electrode was composed of lactate dehydrogenase crosslinked on a PQQ/NAD\(^+\) monolayer, Figure 14A. The Cyt.c/COx integrated electrode served as a biocatalytic cathode for the reduction of O\(_2\) (cf. Fig. 12B). Figure 14B shows the cyclic voltammograms corresponding to the bioelectrocatalyzed oxidation of lactate in the absence and presence of an external magnetic field. Figure 14C depicts the bioelectrocatalytic currents generated at different strengths of external magnetic field. The enhancement of the power output of the lactate/O\(_2\) biofuel cell under an applied field of 0.92 T is shown in Figure 14D.

Fig. 14. A) The assembly of an integrated LDH monolayer electrode by the crosslinking of an affinity complex formed between the LDH and a PQQ/NAD\(^+\) monolayer-functionalized Au-electrode. B) Cyclic voltammograms of the integrated crosslinked PQQ/NAD\(^+\)/LDH electrode: a) in the absence of lactate and in the absence of magnetic field, b) in the presence of lactate, 20 mM, and in the absence of magnetic field, c) in the presence of lactate, 20 mM, and in the presence of magnetic field, \(B = 0.92\) T. C) The dependence of the electrocatalytic current density on the magnetic flux density at lactate concentration of 20 mM. D) The power density output generated by the biofuel cell: a) in the absence of magnetic field, b) in the presence of magnetic field, \(B = 0.92\) T. The biofuel cell operated upon pumping of the solution (flow rate 1 mL·min\(^{-1}\)) composed of 0.1 M Tris-buffer, pH 7.0, containing CaCl\(_2\), 10 mM lactate, 20 mM, and oxygen (the solution equilibrated with air). (Reprinted with permission from [80]. Copyright 2005 American Chemical Society).
The bioelectrocatalytic electrodes consisting of the glucose dehydrogenase, GDH, or alcohol dehydrogenase, AlcDH, reconstituted on the NADP⁺ or NAD⁺ cofactors linked to Nile blue associated with the CNTs, respectively, (cf. Fig. 10) were used as the anodes of biofuel cells that utilized glucose or ethanol as fuels, respectively [67]. The cathode in these biofuel cells, Figure 15A, consisted of bilirubin oxidase, BOD, adsorbed onto the CNTs. The cyclic voltammograms of BOD on the CNTs revealed two redox waves at 0.4 V and 0.2 V vs. Ag/AgCl that were attributed to the Cu-centers of the biocatalyst, Figure 15B. The cyclic voltammograms indicated that the adsorption of BOD onto the CNTs resulted in direct electrical contact between the electrode and the redox centers of BOD. Indeed, the enzyme stimulated the biocatalyzed reduction of O₂ to H₂O. Figure 16A depicts the current-voltage relationship at different external resistances of the glucose/O₂ biofuel cell, and the respective power outputs of the cell. The maximum power extracted from the cell corresponded to 24 μW cm⁻². Similarly, Figure 16B shows the current–voltage curve at different external resistances, for the ethanol/O₂ biofuel cell. The maximum power output for the cell corresponded to 48 μW cm⁻².

Although the power values generated by the two cells are quite low, the results demonstrate that the reconstitution of the enzymes on CNT-functionalized electrodes yield active biocatalytic electrodes that convert the chemical energy stored in the fuels into electrical energy. The detailed analysis of the bioelectrocatalytic functions of the anodes and cathode in the two biofuel cell configurations indicated that the bioelectrocatalyzed reduction of O₂ at the cathode is the power-limiting process. Hence, improving of the cathode material, could significantly improve the performance of these, or related, biofuel cells.

7. Conclusions and Perspectives

The present paper has reviewed the advances in the electrical contacting of redox enzymes with electrodes by means of the reconstitution principle. The reconstitution paradigm represents a major advance in bioelectronics and provides the most efficient means to electrically communicate redox proteins with electrodes. The power of the reconstitution process rests on the fact that all of the enzyme redox centers are aligned in an optimal configuration in respect to the molecular or polymer electron mediator units that transport the electron between the enzyme and the electrode. The effective electrical contact between the enzyme and the electrode enabled the development of sensitive and selective amperometric biosensors, and the construction of noncompartmentalized biofuel cell elements. The high current densities generated by the reconstituted enzyme electrode will enable the design of miniaturized implantable amperometric sensing electrodes for the continuous monitoring of analytes such as glucose or lactate. Also, the resulting biofuel cells could provide implantable devices that provide electrical power extracted from body fluids (e.g., glucose in blood). This electrical power could activate hearing aids, prosthetic elements or, eventually, pace makers.

The alignment of enzymes on electrodes by the reconstitution method was achieved in two general configurations that included either self-assembled monolayers or covalent tethering onto thin polymer films. The limited stability of monolayers and their sensitivity to thermal or photochemical degradation is certainly a disadvantage. While the monolayer configurations may serve as model systems for analogous, stable, polymerized thin films, the surface cross-linking of the enzyme monolayer, and the coating of the biocatalysts monolayer with protecting thin films, might enhance their stability.

The application of the reconstitution principle of redox proteins on electrodes to fabricate bioelectronic devices of enhanced performance could be, however, further developed, and invaluable new bioelectronic systems may be envisaged. The reconstitution principle was applied till now on a limited number of cofactor units. The reconstitution of
redox proteins on new cofactor sites, particularly metallocomplexes, is an exciting path to follow. In this context, the design of new reconstituted O₂-reduction enzyme electrodes would be a significant advance in the fabrication of biofuel cells. Furthermore, recent studies demonstrated superior charge transport functions of metallic NP-redox polymer [81] or CNT-redox polymer [82] systems. The reconstitution of enzymes within such hybrid assemblies could further improve the electrical contacting of the biocatalysts. Also, the electropolymerization of functionalized nanoparticles on electrodes proved to be an effective means to yield an electron relay bridge for transporting electrons between the NPs and the electrode [81]. The reconstitution of enzymes on these nanostructures may lead to new relay structures for the reconstituted enzymes.

8. Acknowledgements

This research is supported by the BioMedNano EC project, and by the EdRox EC training program.

9. References


