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# DNA-Directed Immobilization of Horseradish Peroxidase–DNA Conjugates on Microelectrode Arrays: Towards Electrochemical Screening of Enzyme Libraries

**Ljiljana Fruk,[a] Joachim Müller,[a] Günther Weber,[b] Arántzazu Narváez,[c] Elena Domínguez,[c] and Christof M. Niemeyer*[a, b]**

**Abstract:** This work is aimed towards the generation of enzyme arrays on electrochemically active surfaces by taking advantage of the DNA-directed immobilization (DDI) technique. To this end, two different types of horseradish peroxidase (HRP)–DNA conjugates were prepared, either by covalent coupling with a bifunctional cross-linker or by the reconstitution of apo-HRP, that is, HRP lacking its prosthetic heme (protoporphyrin IX) group, with a covalently DNA-modified heme cofactor. Both conjugates were characterized in bulk and also subsequent to their immobilization on gold electrodes through specific DNA hybridization. Electrochemical measurements by using the phenolic mediator *ortho*-phenylendiamine indicated that, due to the high degree of conformational orientation, the apparent Michaelis–Menten constants of the reconstituted HRP conjugate were lower than those of the covalent conjugate. Due to the reversible nature of DDI, both conjugates could be readily removed from the electrode surface by simple washing and, subsequently, the electrodes could be reloaded with fresh enzymes, thereby restoring the initial amperometric-response activity. Moreover, the specific DNA hybridization allowed us to direct the two conjugates to distinct sites on a microelectrode array. Therefore, the self-assembly and regeneration capabilities of this approach should open the door to the generation of arrays of redox-enzyme devices for the screening of enzymes and their effectors.

**Keywords:** DNA · immobilization · microelectrode arrays · proteins · redox enzymes

## Introduction

Electrochemical biosensors consisting of biological recognition elements, such as enzymes,[1–3] antibodies,[4] or whole cells[5] are very valuable analytical tools for studying complex biomolecular interactions,[6–9] to quantitatively investigate catalytic processes,[10–12] and to detect analytes[13,14] in, for instance, toxicological screenings and cancer research.[15–17] Redox-enzyme-modified electrodes are particularly useful for a broad range of applications because they allow a direct electrochemical read-out of the biomolecular recognition event.[18] The immobilization of redox enzymes on the surface of electrodes comprises a crucial step in the production of effective sensors, and it is usually achieved by physical adsorption,[19] chemical cross-linking,[20] sol–gel,[21] and polymer[22] encapsulation, biospecific recognition,[23,24] or covalent attachment strategies.[25,26] Immobilization methods should be based on mild chemical procedures to allow for the immobilization of intrinsically instable enzymes, involve a regiospecific linkage to control the enzyme's orientation with respect to the electrode surface, and lead to high local concentrations of the enzyme at the sensing surface. Nowadays, electrodes are often modified with self-assembled monolayers (SAMs) bearing appropriate functional groups to which the enzyme of interest can be specifically attached.[27–29] This approach even allows the control of the orientation of the immobilized redox enzymes by employing

[a] Dr. L. Fruk, Dipl.-Chem. J. Müller, Prof. Dr. C. M. Niemeyer
Universität Dortmund, Fachbereich Chemie
Biologisch-Chemische Mikrostrukturtechnik
Otto-Hahn Strasse 6, 44227 Dortmund (Germany)
Fax: (+49) 231-755-7082
E-mail: christof.niemeyer@uni-dortmund.de

[b] Dr. G. Weber, Prof. Dr. C. M. Niemeyer
ISAS–Institute for Analytical Sciences
Bunsen-Kirchhoff-Strasse 11, 44139 Dortmund (Germany)

[c] Dr. A. Narváez, Prof. Dr. E. Domínguez
University of Alcalá
Department of Analytical Chemistry
28871 Alcalá de Henares (Spain)

in situ reconstitution of apoenzymes with electrode-bound cofactors. For example, Willner et al. used gold electrodes modified with flavin adenine dinucleotide (FAD) cofactor groups for the reconstitution of apo-glucose oxidase,[30–32] and the same method was also applied to horseradish peroxidase (HRP) immobilization by reconstitution of apo-HRP on electrodes bearing SAMs with terminal heme-cofactor moieties.[25] Such in situ reconstitution is suitable for controlling the enzyme's orientation in such a way that the prosthetic group and the active-site point towards the electrode, thereby enabling a more direct electron transfer. In this respect, Armstrong and Corbett[33] and Bowden and co-workers[34] have demonstrated that the direct electron-transfer rates were significantly enhanced when the prosthetic heme groups of cytochrome c were oriented towards the electrode rather than being randomly immobilized at the surface.

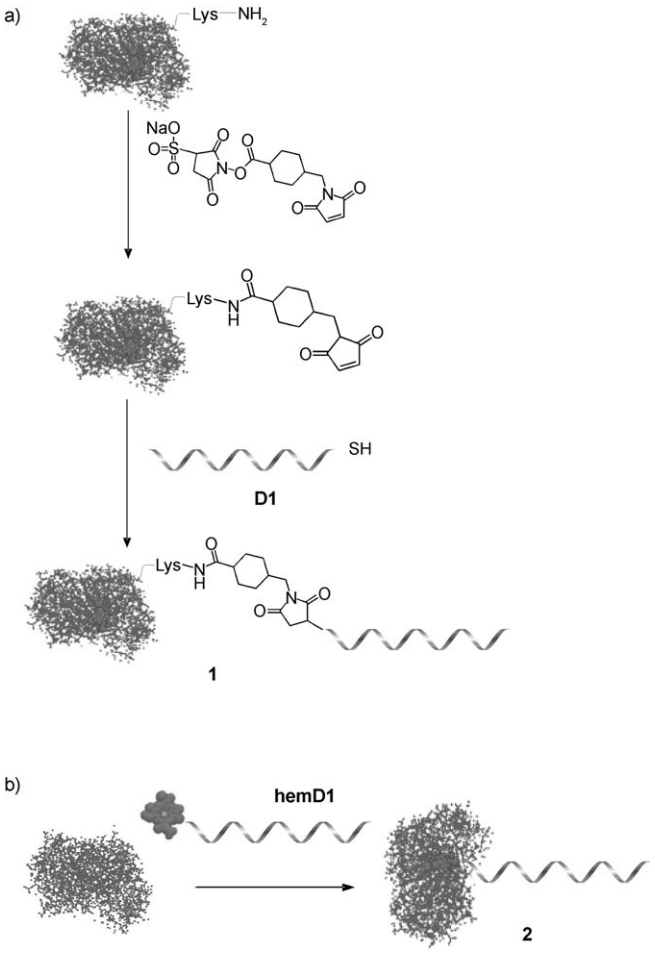
When libraries of enzymes need to be studied, for instance, in a microarray format, a reliable and efficient immobilization technique is needed which also enables control of the enzyme orientation. Additionally, such applications require a high reproducibility of the immobilization method that leads to a long shelf life of the immobilized biomolecules and the possibility of the sensor-array regeneration.

To address these issues, we have recently introduced a strategy which utilizes DNA-oligonucleotide-modified heme groups for the reconstitution of apo redox enzymes, such as apo-myoglobin[35] (aMb) and apo-HRP[36] (aHRP). The resulting conjugates contain a programmable DNA tag which allows their facile and specific immobilization at solid surfaces through the DNA-directed immobilization (DDI) technique. As the specificity of Watson–Crick base pairing can be used to produce laterally microstructured arrays of highly functional proteins by means of DNA-driven self-assembly,[37–39] the DDI method should offer a feasible approach to the generation of spatially ordered arrays of DNA-tagged redox enzymes. However, the challenge of implementing the DDI concept in an array format to enable enzyme characterization by means of electrochemical transduction schemes has not yet been addressed.[40]

Towards this goal, we report here initial steps in the fabrication of microstructured self-assembling arrays of redox enzymes. To this end, two different types of HRP–DNA conjugates were prepared either by using covalent coupling with a bifunctional cross-linker or by the reconstitution of apo-HRP with a DNA-modified cofactor. Hybridization with complementary capture oligonucleotides was employed to anchor the HRP–DNA conjugates on gold electrodes, and the amperometric currents were measured by using the diffusional mediator *ortho*-phenylendiamine. Both HRP–DNA conjugates remained catalytically active after DDI at the electrode; the resulting sensors showed high stability and could be successfully regenerated by denaturation of the oligonucleotide linkage. To demonstrate that our DDI strategy is suitable for the production of enzyme arrays within electroactive sensing devices, the two HRP conjugates, equipped with different DNA sequences, were immobilized on a chip containing four microelectrodes, each of which was functionalized with an individual capture oligomer.

## Results and Discussion

**Preparation and bulk characterization of DNA–HRP conjugates**: Horseradish peroxidase labeled with oligonucleotides has previously been used for the electrocatalytic detection of nucleic acid hybridization on carbon electrodes.[41,42] There, HRP–DNA probes were prepared by treating hydrazine-modified oligonucleotides with aldehydes introduced by oxidation of the HRP's oligosaccharides.[43] This coupling strategy suffers from lack of specificity, and the extensive treatment of HRP even led to complete loss of the HRP activity.[41] Here, we synthesized two different types of HRP–DNA conjugates, both based on a less aggressive chemical treatment of the protein. The first one (**1** in Scheme 1) was synthesized by chemical cross-linking by using a thiolated oligonucleotide and the heterobifunctional cross-linker sulfo-succinimidyl-4-(*N*-maleimido-methyl)-cyclohexan-1-carboxylate (sSMCC), as previously described for other pro-




Scheme 1. Reaction pathway of the synthesis of HRP–DNA conjugates by a) covalent cross-linking and b) reconstitution of apo-HRP with a DNA-modified heme group **hemD1**.

teins.[44,45] The second conjugate (**2** in Scheme 1) was prepared by reconstitution of apo-HRP with a DNA-modified heme group.[35,36] Both HRP–DNA conjugates contained DNA oligonucleotides of identical length and sequence (5′-AAG ACC ATC CTG-3′, referred to as **D1**). According to the reaction mechanisms, the chemically linked DNA–HRP **1** contained DNA oligomers coupled at random positions on the surface of the enzyme, while in the case of the DNA–heme-reconstituted HRP conjugate **2**, both the number of DNA oligomers per enzyme (only one) as well as the linkage position (namely, the HRP active site) were well defined.

The conjugates were purified by fast protein liquid chromatography (FPLC) and quantified by spectrophotometry. Similarly to recently reported results,[36] the chromatographic purification of the reconstituted conjugate **2** allowed the facile preparation of the mono-DNA adduct (data not shown). However, two major peaks were observed in the chromatogram of the cross-linked conjugate **1** (Figure 1). This suggested that at least two different conjugates were produced; these conjugates differed in their coupling stoichiometry with respect to the number of DNA oligomers per HRP molecule.

The determination of the crystal structure of HRP has shown four accessible lysine residues at its surface,[46] thereby enabling, in principle, the coupling of up to four oligonucleotides per enzyme molecule. Thus, further characterization of HRP–DNA conjugate **1** was aimed at the determination of the number of DNA oligomers conjugated to the products observed in the FPLC analysis. To this end, a calibration curve was produced by plotting the absorbance ratio values ($A_{260}/A_{402}$), determined from stoichiometric mixtures of the two components, versus the DNA/native HRP ratio (not shown). From the measurements of the $A_{260}/A_{402}$ ratio of the two major peaks isolated by FPLC (Figure 1), it was determined that peak 1 corresponds to the 1:1 DNA–HRP conjugate **1**, while peak 2 represents adducts with a higher ratio of DNA to HRP. The latter conjugates were not further evaluated in this study.

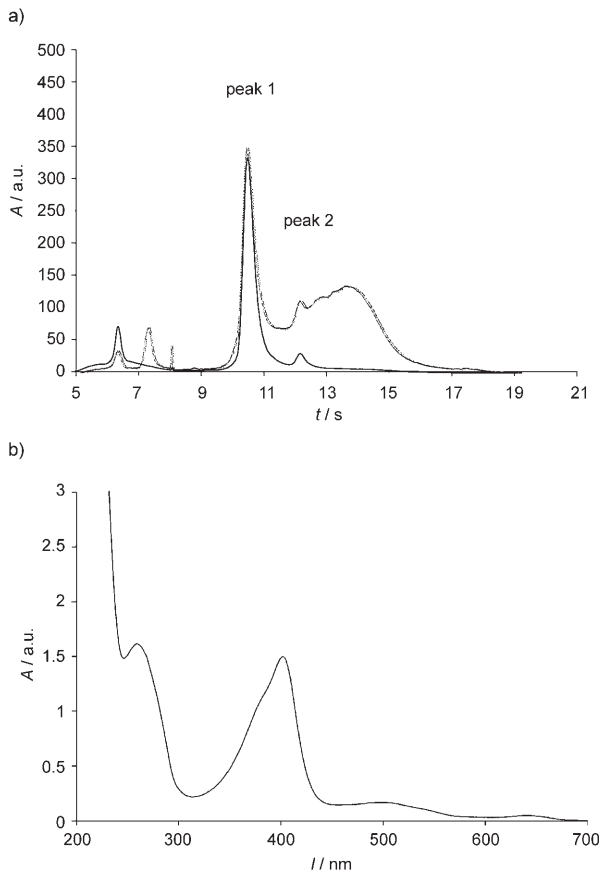



Figure 1. a) FPLC purification and b) UV/Vis spectra of HRP–DNA conjugate **1**. In (a), the absorbances at 402 and 260 nm are indicated by solid and dashed lines, respectively.

The enzymatic activity of the DNA–heme-reconstituted HRP has recently been determined by assuming Michaelis–Menten kinetics and using the soluble fluorogenic substrate Amplex Red.[36] This previous study showed that the peroxidase activity was reduced to about 30% after its reconstitution with the DNA-tethered cofactor. We here used the same methodology to make an initial estimate of the activity of the two DNA–heme conjugates, **1** and **2**, prepared by the two alternative approaches. To this end, the Michaelis–Menten parameters (the Michaelis–Menten constant, $K_M$, and the rate of catalysis, $k_{cat}$) were determined by a simplified model of a multisubstrate reaction, similar to that described earlier.[36] The data obtained are shown in Table 1.

Table 1. Kinetic parameters of HRP–DNA conjugates and native horseradish peroxidase, as determined with Amplex Red and $H_2O_2$ under homogeneous conditions.

| Enzyme | $k_{cat}$ [$s^{-1}$] | $K_M$ [μM] | $k_{cat}/K_M$ [$\mu M^{-1} s^{-1}$] |
|---|---|---|---|
| native HRP[a] | 860 ± 38 | 106 ± 13 | 8 ± 1 |
| HRP–DNA **1** | 232 ± 10 | 131 ± 15 | 1.8 ± 0.2 |
| HRP–DNA **2**[a] | 271 ± 5 | 125 ± 14 | 2.2 ± 0.3 |

[a] Values taken from reference [36].

The values of $K_M$ and $k_{cat}$ determined experimentally allowed us to calculate the catalytic efficiency ($k_{cat}/K_M$) of the two conjugates under investigation and to compare them with those of native HRP. These data show that native HRP is about fourfold more efficient than the two DNA conjugates **1** and **2**, mainly as a consequence of the decreased turnover rates, $k_{cat}$, observed for the synthetic conjugates.

In the case of the reconstituted conjugate **2**, the removal and insertion of the heme may lead to a disturbance of the HRP hydrogen-bonding network and, thus, to a decreased enzymatic activity.[47] In addition, the presence of bulky and charged DNA in close proximity to the heme may also partially hinder the access of the substrate to the reaction pocket.[36] Interestingly, the random attachment of DNA oligomers to the HRP (conjugate **1**) had similar effects on the reactivity of this enzyme conjugate. Apart from the steric hindrance introduced by the DNA strands, which impairs the enzyme activity, the covalent modification of the lysine residues might alter the microenvironment at the pro-

tein surface and, thus, influence the conformation and functionality of the reaction pocket.

Irrespective of the decrease of catalytic efficiency, these DNA adducts should enable site-specific immobilization on solid surfaces through DNA hybridization. Consequently, DDI-based enzyme assays were carried out with hydrogen peroxide and Amplex Red as a fluorogenic substrate. To this end, conjugates **1** and **2** were immobilized in streptavidin-coated microplate wells, previously functionalized with the complementary biotinylated capture oligomer **bcD1** (5′-biotin-CAG GAT GGT CTT-3′), and the peroxidase activity was then measured by addition of the substrate mixture.

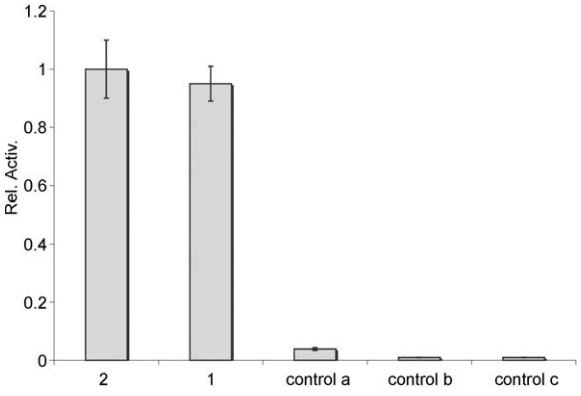



Figure 2. Amplex Red peroxidase activity of HRP–DNA conjugates **1** and **2**, immobilized by specific DNA hybridization to the complementary capture oligomer **bcD1**. Controls a and b refer to reactions carried out by incubation of the HRP–DNA conjugates **1** and **2**, respectively, in wells containing the noncomplementary capture oligomer **bcD2**. Control c involved the incubation of native HRP in wells containing capture oligomer **bcD1**.

Figure 2 shows the activity of the immobilized conjugates **1** and **2**. In wells containing the complementary capture oligomer, specific enzyme activity was clearly detectable and indicated that both conjugates were almost equally active after specific immobilization. Control experiments, carried out in wells containing the noncomplementary capture oligomer **bcD2** (5′-biotin-GGT GAA GAG ATC-3′) showed no significant enzymatic activity. This indicated that HRP conjugates **1** and **2** were both immobilized exclusively through the formation of specific Watson–Crick base pairing.

**Electrocatalytic activity of the HRP–DNA conjugates**: The previous experiments clearly showed that both enzyme–DNA conjugates are catalytically active and prone to specific immobilization on solid surfaces. More challenging seems to be the implementation of the DDI concept to electrochemical-transduction schemes and particularly, as in the case of redox enzymes, to amperometric measurements because the redox centers have to communicate efficiently with the electrode. To this end, a mixed monolayer surface comprising the capture DNA **tcD1** (5′-thiol-CAG GAT GGT CTT-3′) and mercaptoethanol was prepared on gold electrodes by using a procedure slightly modified from that of Peterlinz et al.[48] To achieve a high density of surface-bound DNA, a reduction of the disulfide groups of the capture oligomer **tcD1** with 1,4-dithiothreitol (DTT) was performed prior to its chemisorption onto the gold electrodes. Subsequently, the electrodes were incubated in a solution containing mercaptoethanol to minimize nonspecific interactions between the DNA nucleobases and the gold surface.[49]

The DNA surface coverage of the electrodes was estimated by chronocoulometry and by measuring the redox charges of hexamminoruthenium(III) chloride (RuHEX).[49] This electrochemical quantification is based on the electrostatic interaction between the charged soluble redox mediator and DNA in solutions of low ionic strength, an interaction that can be measured over time. By using this method, the immobilization procedure was optimized (see the Experimental Section) to achieve an average surface coverage of $(5.2\pm0.4)\times10^{12}$ DNA molecules per $cm^2$, which corresponds to $0.27\pm0.02$ pmol of DNA on the 2 mm diameter gold electrodes. This figure is in excellent agreement with the recent immobilization studies by Lao and co-workers.[50]

The HRP–DNA conjugates were then immobilized on the gold electrodes through DDI (Figure 3). In addition, the covalent DNA–heme conjugate **hemD1** was also immobilized on the electrode, and apo-HRP was subsequently allowed to reconstitute in situ (Figure 3c).

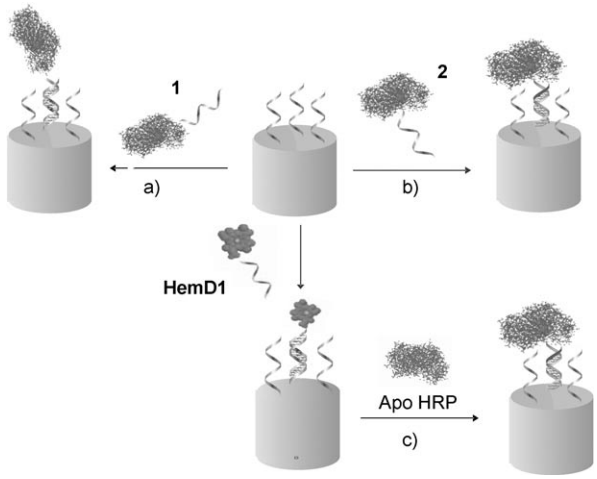
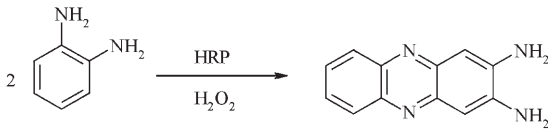



Figure 3. Schematic representation of the immobilization of HRP on Au electrodes through DDI with a) conjugate **1**, b) conjugate **2**, and c) **hemD1** and apo-HRP.

The amperometric currents at −100 mV versus $Ag/AgCl_{sat}$ were then recorded with the various HRP-containing electrodes in the presence of hydrogen peroxide at a saturating concentration (150 μM $H_2O_2$). We observed that direct electrochemical communication of the DNA-directed immobilized HRP conjugates was almost negligible (1.3–2.0 nA) and remained similar to that observed in control experiments when native HRP was assayed with DNA-modified electrodes. This poor communication has previously been

noticed and persisted even in the case of nondiffusional redox mediators.[51,52] Therefore, it was necessary to use a soluble redox mediator to shuttle the electrons between the electrode and the enzyme heme redox center. As it is well established as an efficient electron donor for HRP,[51,52] *ortho*-phenylendiamine (PDA) was used for this purpose (Scheme 2).[53] The resulting response curves for both conjugates are depicted in Figure 4.




Scheme 2. Oxidation of *ortho*-phenylendiamine in the presence of HRP and $H_2O_2$.

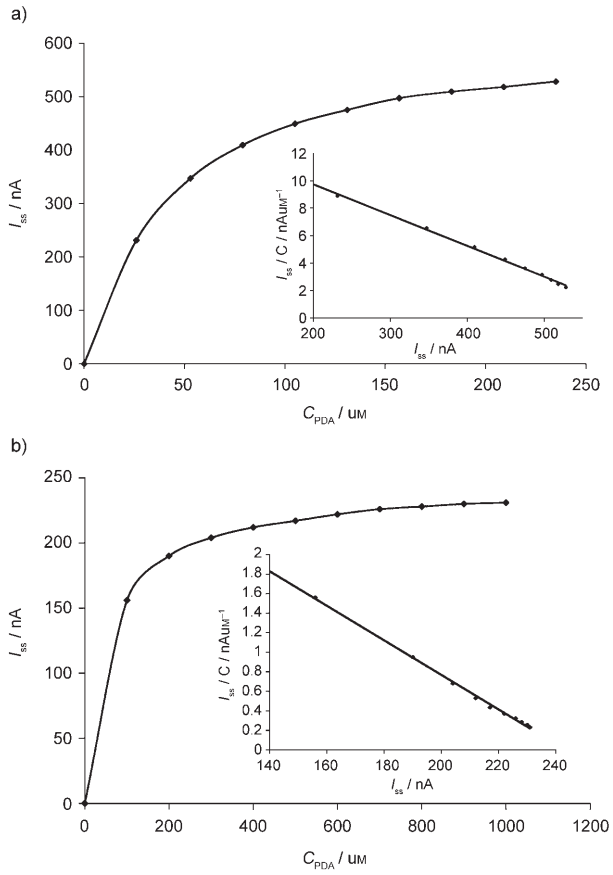



Figure 4. Steady-state cathodic currents of HRP-DNA conjugates a) **1** and b) **2** immobilized on gold electrodes through DDI. The insets show the corresponding Eadie–Hofstee plots for the determination of the apparent kinetic constants.

As previously indicated (see Figure 3c), the in situ reconstitution of apo-HRP at the electrode surface was also investigated. Despite long incubation times (up to 18 h), only very low currents were observed with these electrodes. For instance, maximum currents of 15 nA were obtained from electrodes modified with the same amount of capture oligomers as were used in the case of HRP–DNA conjugate **2**. These currents represent less than one tenth of those obtained with the corresponding HRP–DNA conjugate and reflect the low reconstitution of apo-HRP, most likely due to the limited accessibility of the heme group after attachment to double-stranded DNA.

The steady-state cathodic currents obtained from the DNA-immobilized HRP conjugates **1** and **2** enabled the calculation of the apparent Michaelis–Menten parameters by using the electrochemical Eadie–Hofstee-type Equation (1),[54] in which $I_{MAX}$ and $I_{SS}$ are the currents, measured for the enzymatic reaction under substrate saturation and substrate limitation ($C$), respectively, and $K_M'$ is the apparent Michaelis–Menten constant.

$$\frac{I_{SS}}{C} = \frac{I_{MAX}}{K_M'} - \frac{I_{ss}}{K_M'} \quad (1)$$

Linear regression was applied to the plot of $I_{SS}/C$ against $I_{SS}$ and the $K_M'$ value was determined from the slope of the resulting straight line. The apparent rate of catalysis ($k_{cat}'$) is proportional to the $I_{MAX}$ value and can be determined when the maximum current, $I_{MAX}$, and the electrode area, $A$, are known.[55] The difficulties in accurately estimating the amount of active enzyme on the electrode surface make it difficult to determine the apparent catalytic constants.[55] For comparative purposes of the conjugates and with consideration of the highly reproducible single-stranded-DNA loading ($0.27 \pm 0.02$ pmol DNA) of the modified electrodes, we estimated a similar enzyme loading, because the loading depends on the formation of the specific base pairing. Table 2 shows the numeric values of the determined apparent constants. Additional attempts were undertaken to measure the amount of HRP immobilized on the surface of the gold electrodes. To this end, freshly loaded enzyme electrodes were stripped, by incubation in water at 65 °C for 60 min. Subsequent measurements of the amperometric response proved that most of the enzyme was removed. The supernatant was then analyzed for HRP enzyme activity by using the Amplex Red assay. The typical values obtained indicated that about 0.14 pmol of the covalent conjugate **1** and about 0.20 pmol of the reconstituted conjugate **2**, corresponding to about 50 and 70 % of the hybridized capture oligonucleotides, respectively, were present in the washing solution. These results, however, need to be regarded with caution, because the heating step might affect the activity of the two enzyme conjugates to a different extent.

Table 2. Values of the apparent kinetic constants for DNA-directed immobilization of HRP–DNA conjugates on Au electrodes.

| Conjugate | $k_{cat}'$ [pmol cm$^{-2}$ s$^{-1}$] | $K_M'$ [μM] | $k_{cat}'/K_M'$ [$10^{-4}$ cm s$^{-1}$] |
|---|---|---|---|
| **1** | $104 \pm 1$ | $44.6 \pm 0.4$ | $23 \pm 1$ |
| **2** | $41 \pm 1$ | $56.5 \pm 0.4$ | $7.2 \pm 0.5$ |

The values listed in Table 2 reveal that the turnover rate for the reconstituted conjugate **2** was found to be more than

50% lower than that of the chemical conjugate **1**. On the other hand, the $K_M'$ values differed to a lesser extent, and HRP–DNA **1** showed about 20% higher substrate affinity for PDA than the reconstituted HRP **2**. Contrary to the values found in solution, in which no relevant differences were observed (see Table 1), the reconstituted conjugate **2** showed approximately one third of the catalytic efficiency ($k_{cat}'/K_M'$) for the free diffusional redox mediator, as compared to that of the chemical conjugate **1**. Since this mediator seems to interact with an amino acid residue in the vicinity of the active site,[25] it is tempting to assume that the access of the PDA to conjugate **2** is hindered due to an unfavorable orientation of the active site. In other words, after the DNA-directed immobilization, the redox center of the enzyme is pointing towards the electrode surface which makes it less accessible. In the case of the chemical conjugate **1**, this effect is less significant because the enzymes are oriented randomly at the electrode surface.

The reproducibility of the catalytic currents and the stability of the modified electrodes were also tested. The two conjugates **1** and **2** were removed from the electrode surface by simple incubation in deionized water at 65°C for 30 min. Subsequent electrochemical measurements indicated that more than 95% of reconstituted conjugate **2**, but only about 80% of chemical conjugate **1** was removed (Figure 5). Repetition of the regeneration step led to complete removal of **2**, while the remaining activity indicated the persistence of approximately 10% of the chemical conjugate **1**. The reason for this partly irreversible binding is as yet unclear. However, we assume that the random attachment of oligonucleotide strands and the chemical modification of the enzyme's outer surface contribute to the apparent reduction of the dehybridization capabilities of conjugate **1**.

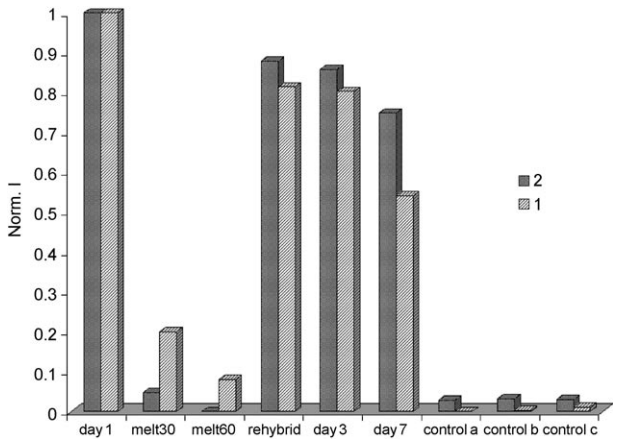



Figure 5. Normalized catalytic currents measured with HRP–DNA conjugates **1** and **2** at different times after the preparation of the electrode (days 1, 3, and 7) and after removal (30 min or 60 min; melt30 and melt60, respectively) of the conjugates and rehybridization (rehybrid). Control measurements were performed with immobilized **hemD1** only (a), HRP conjugates incubated with electrodes containing noncomplementary capture oligonucleotide **tcD2** (b), and immobilized thiolated oligonucleotide **tD1** only (c).

The stripped electrodes were then used for renewed loading with the DNA–HRP conjugates. This led to the recovery of active electrodes with about 90 and 80% of the original enzyme activities in the case of conjugates **2** and **1**, respectively. The DDI-modified electrodes were stable for a week when kept at 4°C in phosphate buffer. However, a significant difference in stability was observed for the two conjugates, with the electrodes functionalized with the reconstituted conjugate **2** being more stable than those containing conjugate **1** (Figure 5). Repeated experiments with different batches of the conjugates also indicated a very high reproducibility of the electrode preparation with a relative standard deviation of 4.6%, measured for 3 independent preparations.

To demonstrate that our DDI strategy is suitable for the production of self-assembling enzyme arrays within electroactive sensing devices, two HRP conjugates, equipped with different DNA sequences, were immobilized on a chip containing four microelectrodes (Figure 6). In this experiment we used two chemically linked HRP–DNA conjugates, because they showed higher catalytic efficiency (Tables 1 and 2), each of which was tagged with an individual DNA sequence (**1** and **3**, containing oligonucleotides **D1** and **D2**, respectively). The four microelectrodes on the chip were functionalized with complementary (**tcD1** and **tcD2**) or noncomplementary (**ncD**) oligomers and one electrode was left unmodified (Figure 6).

The microelectrode arrays were immersed in solutions containing either one or two of the different HRP–DNA conjugates **1** and **3**. Subsequent to DDI, the arrays were washed and the amperometric response was measured in the presence of $H_2O_2$ and PDA at each of the individual microelectrode sites. The results (shown in Figure 6) indicated that both conjugates specifically hybridized with their complement, even when the mixture was applied during immobilization. Moreover, it was evident that electrodes containing **3** yielded higher amperometric responses. This result is in agreement with the slightly higher catalytic activity of **3** in comparison with that of **1** which was determined independently by kinetic studies in solution to be 2.3 and 1.8 $\mu M^{-1} s^{-1}$ for conjugates **3** and **1**, respectively.

## Conclusions

This work describes the DNA-directed immobilization (DDI) of horseradish peroxidase (HRP) on electrochemically active surfaces. Different types of HRP–DNA conjugates were prepared by either covalent linking through a heterobifunctional cross-linker or reconstitution of apo-HRP with a DNA-modified heme group. Kinetic analyses of the peroxidase activity of these conjugates revealed that they exhibited similar catalytic efficiencies, which, however, were decreased in comparison to that of the native HRP. Since both HRP–DNA conjugates retained substantial enzymatic activity, electrodes modified by specific DNA hybridization of the conjugates with electrode-bound capture oligomers yielded

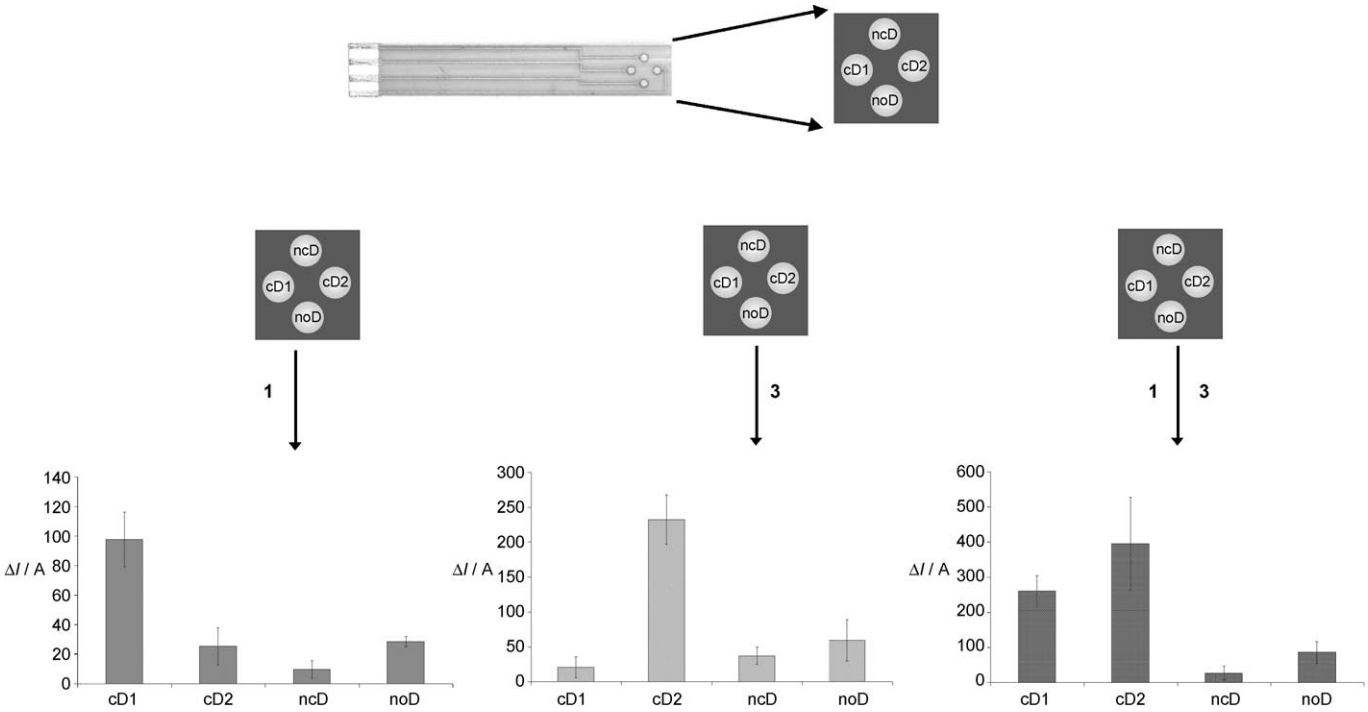



Figure 6. A chip containing four microelectrodes. Each chip was modified with **tcD1**, **tcD2**, and noncomplementary oligomer (**ncD**) as shown at the top. One electode was left unmodified (noD). After immobilization of HRP–DNA conjugates **1** and/or **3**, the change in current upon addition of $H_2O_2$ and PDA was measured.

highly active devices. Amperometric measurements in the presence of *ortho*-phenylendiamine as a diffusional electron donor were used to determine the apparent Michaelis–Menten constants. Comparison of the catalytic efficiencies ($k_{cat}'/K_M'$) showed that the immobilized reconstituted HRP–DNA conjugate is less active than the covalent HRP–DNA conjugate, most likely due to differences in the relative orientation of enzyme active sites at the electrode surface.

We further demonstrated that the DNA-modified electrodes can be efficiently regenerated by dehybridization of the double-stranded DNA connector, and the reconstituted DNA–heme conjugate showed a better performance with respect to this regeneration step, as well as with respect to long-term stability of the functionalized electrodes. Moreover, to demonstrate that our DDI strategy is suitable for the production of arrays of enzymes within electroactive sensing devices, two HRP conjugates were immobilized on a microelectrode chip by means of specific DNA hybridization. Although only two proteins were immobilized in this proof-of-principle demonstration, previous work suggests that DDI is well suited to produce much larger arrays of proteins.[56] Thus, this method offers a powerful tool for generating laterally microstructured arrays of redox enzymes, useful for the screening of libraries of enzymes and small-molecule drugs which inhibit or affect such enzymes. Moreover, applications of such arrays in the analysis of biomarkers and the sensing of environmental pollutants and chemical or biological warfare agents might be foreseen.

## Experimental Section

**Chemicals**: Horseradish peroxidase (HRP; purity number of 3) was purchased from Sigma. Sulfo-succinimidyl-4-(*N*-maleimido-methyl)cyclohexan-1-carboxylate (sSMCC) was obtained from Pierce, 1,4-dithiothreitol (DTT), 2-mercaptoethanol, $H_2O_2$, 2-butanone, and *ortho*-phenylenediamine from Fluka, Amplex Red from Molecular Probes, and hexamminoruthenium(III) chloride from Acros Organics. Buffer salts ($K_2HPO_4{\cdot}3H_2O$ and $KH_2PO_4$) were analytical grade and were obtained from Roth. 5′-Thiolated and -biotinylated oligonucleotides were purchased from ThermoElectron (Ulm, Germany). The 5′-amino-modified oligonucleotide (C6-amino linker) for **hemD1** synthesis was obtained from Tib-Molbiol (Berlin, Germany) in an unprotected form bound on a solid support.

**Preparation of DNA–HRP conjugates**: To prepare the chemical conjugates **1** and **3**, a 100 μM solution (100 μL) of 5′-thiol-modified oligonucleotide **tD1** (5′-thiol(C6)-AAG ACC ATC CTG) or **tD2** (5′-thiol(C6)-GGT GAA GAG ATC) in 10 mM tris-Cl, 1 mM EDTA (TE) buffer was mixed with 1 M DTT (60 μL) and incubated overnight at 37 °C to reduce any disulfide bonds formed upon storage of the oligonucleotide. HRP (0.92 mg) was dissolved in phosphate buffer (200 μL, pH 7.2) and incubated for 1 h at 37 °C with sSMCC (2 mg in 60 μL of *N,N*-dimethylformamide (DMF)). Both the DNA and the protein reaction mixtures were purified by two consecutive gel-filtration chromatography steps with NAP5 and NAP10 columns (Pharmacia). The purified DNA and protein solutions, each of which had a volume of 1.5 mL, were combined and incubated in the dark at room temperature for 3 h. The reaction mixture was concentrated to approx 300 μL by ultrafiltration (Centricon 30, Millipore) and the buffer was exchanged with tris(hydroxymethyl)aminomethane (Tris; 20 mM, pH 8.3) during this step. The conjugate was purified by anion-exchange chromatography on a MonoQ HR 5/5 column (Pharmacia) by using a linear gradient over 25 min (AKTA purifier, Amersham Bioscience; buffer A: 20 mM Tris at pH 8.3; buffer B: 20 mM Tris and 1.5 M NaCl). The concentration was determined spectrophotometrically and was additionally confirmed by using a Quant-It protein quantifica-

tion kit (Molecular Probes). The reconstituted conjugate **2** was prepared as previously described by reconstitution of apo-HRP, with the covalent adduct **hemD1** prepared from amine modified D1 and heme.[35,36]

**Kinetic measurements**: Kinetic measurements with Amplex Red were carried out in black 96-well microplates (Nunc) by using a Synergy HT microplate reader from BioTek (running the software KC4, Version 3.4, Rev. 12). In all experiments, enzyme solution (50 μL), or buffer (50 μL) for use as a blank, was added to the microplate wells. The solutions were thermostated at 25 °C for 5 min. At the same time, the Amplex Red/$H_2O_2$ solutions were prepared, as described below, and thermostated at 25 °C. The reaction was started by adding the substrate solution (50 μL) to the wells containing the enzyme or the blank. The reaction progress was monitored for 20 min and fluorescence values of the reaction product resorufin were recorded at 590 nm, by using an excitation wavelength of 530 nm, every 30 s. KC4 software (BioTek) was used for analysis of the primary data. The initial rate of each experiment was derived by linear-regression analysis of the linear range of the time versus background-corrected fluorescence values. Further calculations and weighted nonlinear regressions were performed by using the Excel and Origin software packages. Amplex Red stock solutions were prepared according to manufacturer's instructions by using potassium phosphate buffer with 300 mM NaCl (pH 7.4). Variable concentrations of Amplex Red were used, while the concentration of $H_2O_2$ was fixed at 1 mM. The final concentration of the horseradish peroxidases was 0.1 nM.

**DNA-directed immobilization (DDI)**: DDI of the DNA–HRP conjugates was carried out by using streptavidin-coated microplates which were previously functionalized with biotinylated capture oligomer **bcD1** (5′-biotin-CAG GAT GGT CTT), as previously described.[57] Controls were carried out in wells functionalized with the noncomplementary oligomer **bcD2** (5′-biotin-GAT CTC TTC ACC).

**Electrode preparation**: Gold electrodes (Metrohm, Germany, 2 mm in diameter) were first polished on a microcloth (Buehler) with Gammma micropolish alumina suspensions (0.3 and 0.05 μm), sonicated in water for 5 min, and cleaned electrochemically in 0.1 M $H_2SO_4$ to remove any remaining impurities. The electrodes were rinsed with ethanol, dried with nitrogen, and immersed in a solution (1 mL) containing the thiolated capture oligonucleotides **tcD1** or **tcD2** (6.7 μM) for 1 h at room temperature. Prior to immobilization, thiolated oligonucleotides (100 μL, 100 μM) were incubated with DTT (1 M, 60 μL) overnight at room temperature, and the residues of DTT as well as any disulfide were removed by two consecutive gel-filtration chromatography steps with NAP5 and NAP10 columns (Pharmacia). The electrodes were washed with phosphate-buffered saline (PBS) and then incubated in a mercarptoethanol solution (1 mM) for 30 min, rinsed with PBS buffer, and dried under a stream of nitrogen. Hybridization of the DNA–HRP conjugates or the DNA–heme conjugate **hemD1** was achieved by incubation of the DNA-modified electrodes in buffer (1 mL, 10 mM Tris (pH 7.4) supplemented with 1.0 M NaCl and 1 mM ethylenediaminetetraacetate (EDTA)) containing the appropriate DNA–HRP or DNA–heme conjugate (0.5 μM) for 60 min at room temperature. The electrodes containing **hemD1** were subsequently immersed in a solution containing apo-HRP (1 mL, 1 μM) overnight at 4 °C. The electrodes were rinsed with hybridization buffer and dried under a stream of nitrogen prior to the electrochemical measurements.

**Amperometric measurements**: Amperometric measurements were performed at −100 mV with a Autolab PGStat30 potentiostat (Deutsche Metrohm, Filderstadt, Germany), an Ag/AgCl reference electrode, and a platinum-wire counterelectrode. Phosphate buffer (pH 7.0) containing 150 mM NaCl was used as the electrolyte. The steady-state current in the presence of 150 μM $H_2O_2$ was determined after each successive addition of aliquots of *ortho*-phenylendiamine (PDA; 2 mM initially) and plotted against the PDA concentration to obtain the apparent Michaelis–Menten parameters. Chronocoulometric data for quantitation of surface-immobilized DNA were obtained by using an Autolab PGStat20 potentionstat with an FI20-Modul instrument (Deutsche Metrohm, Filderstadt, Germany), with a pulse period of 500 ms and a pulse width of 500 mV.

**Microelectrode arrays**: Chips containing four gold microelectrodes (AC8) were purchased from BVT Technologies (Czech Republic), together with the appropriate connector (KA8.1) to be used with the Autolab PGStat 30 potentionstat equipped with an MUX.SCNR8-Module instrument. All measurements were done in a standard electrochemical cell with an Ag/AgCl reference electrode and a platinum-wire counterelectrode. Chips were used as received, washed with ethanol, and dried with nitrogen prior to immobilization. Standard DNA immobilization was used, similar to the method described for the macroscopic gold electrodes with the only difference being that solutions of different oligonucleotides (5 μL, 6 μM) were spotted manually onto the surface of the electrodes and the entire chip was incubated in a humidity chamber for 30 min. Chips were then washed and immersed in the solutions containing the HRP–DNA conjugates (0.5 μM in phosphate (KPI) buffer (pH 7) containing 300 mM NaCl) for 30 min. The amperometric measurements were carried out after careful rinsing and drying under a nitrogen stream.

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