

Bioelectrochemical systems with oleylamine-stabilized gold nanostructures and horseradish peroxidase for hydrogen peroxide sensor

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ABSTRACT

This paper describes ultrathin gold nanowires (NWs) and nanoparticles (NPs) prepared by oleylamine (OA) synthesis and their assembly with horseradish peroxidase enzyme (HRP) for bioelectrochemical sensing of hydrogen peroxide for the first time. The immobilization of oxidoreductase enzyme HRP on the electrodes modified with OA gold nanostructures (OANSs) is discussed. The HRP-sensor characteristics, namely sensitivity, working concentration range, sensor-to-sensor and measurement-to-measurement reproducibility as well as long-term stability, are improved significantly compared to the planar thin-film sensors by using OANSs. The thin-film gold electrodes modified with OANWs and OANPs exhibit a catalytic activity towards oxidation of hydrogen peroxide with a working concentration range from 20 μM to 500 μM , a sensitivity of $0.031 \text{ A M}^{-1} \text{ cm}^{-2}$ (RSD 0.046) and $0.027 \text{ A M}^{-1} \text{ cm}^{-2}$ (RSD 0.045), and a detection limit of 5 μM and 8 μM , respectively (RSD near the detection limits was 9–12%). Our study shows that ultrathin gold nanowires and nanoparticles prepared by oleylamine synthesis are prospective materials to assemble biomolecules into functional nanoarchitectures for enzyme-based bioelectrochemical sensors, metalloprotein bioelectronics, and energy research.

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1. Introduction

Several investigations have focused on assembling functional biomolecules and nanoarchitected electronic elements into bioelectronic systems (Bertoncello and Forster, 2009; Kisner et al., 2012; Kumar, 2007; Linic et al., 2011; Merkoçi, 2007; Mubeen et al., 2013; Reddy and Gobi, 2012; Sarma et al., 2009; Wang, 2005; Willner, 2002; Willner and Katz, 2005; Xiang et al., 2007). Evolutionary optimized structures and the functions of biomaterials, such as recognition, binding, carrier and catalysis, make them attractive building blocks for sensors, information processing, bioelectrocatalysis, biofuel cells, and solar energy conversion.

It has been proposed that nanostructures can improve the interface between biomolecules and an electronic transducer, such as an electrode (Mena et al., 2005). The advantages of nanostructured surface designs include a reduced distance between the redox

center of proteins and an electrode, and the facilitation of electron transfer (Scognamiglio, 2013; Wang, 2008). Three-dimensional nanostructured materials provide a favorable surface for the immobilization of biomolecules allowing them to retain their biological activity due to the enhanced orientation freedom, thus preventing denaturation of biomolecules and favoring longer stability and higher reproducibility of the metalloprotein functions on the nanostructured electrodes. Furthermore, geometrical signal enhancement can be achieved using nanomaterials, i.e., the effective electrode surface area is increased by three-dimensional nanostructures (Schröper et al., 2008; Kuposova et al., 2013).

Therefore, assembling functional biomolecules, such as redox proteins, receptors, and antibodies on various newly prepared nanostructured materials, e.g. metal and semiconductor nanoparticles, and higher aspect ratio nanostructures (nanowires, nanorods, and nanopillars), carbon nanotubes, graphene, and porous materials has been extensively studied and reviewed (Bertoncello and Forster, 2009; Cui et al., 2008; Jensen et al., 2012; Jiang et al., 2013; Kisner et al., 2012; Kuila et al., 2011; Mena et al., 2005; Merkoçi, 2007; Scognamiglio, 2013; Wang, 2008; Yarman et al., 2011). On some of these electrode materials, particularly on carbonaceous electrodes, direct electron transfer has been reported

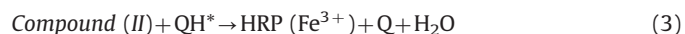
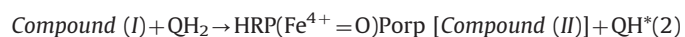
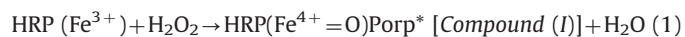
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between an electrode and electrocatalytic center of enzymes (Lindgren et al., 2000; Liu and Ju, 2002; Mao et al., 2013). Nevertheless, redox centers in biocatalysis generally lack electron transfer communication with electrode transducers, because they are deeply buried in an insulating protein matrix. The rate of electron transfer between the redox center and an electrode is negligibly small and drops by a factor of e for each distance of 0.91 Å (Gray and Malström, 1989; Heller, 1992; Marcus and Suttin, 1985). Therefore, artificial redox groups or mediators are employed to mediate electron transfer between redox centers of biomolecules and electrodes. Various approaches have been outlined for incorporating these artificial mediators. These include redox hydrogels and conductive polymers, covalent attachment of sophisticated engineered electron relays to biomolecules, and the use of soluble artificial redox mediators substituting, e.g., a natural physiological acceptor of electrons oxygen (Schuhmann, 2002). Electron transfer pathways in biosensors have been extensively reviewed (Sarma et al., 2009; Schuhmann, 2002).

Metalloproteins, e.g., oxidoreductase enzyme horseradish peroxidase (HRP, EC 1.11.1.7.), are often used to study the properties of the bioelectronic interfaces, because they provide stable and reliable systems and are interesting for fundamental and applied studies. Horseradish peroxidase (HRP) is one of the most widely used enzymes in analytical biochemistry for the construction of biosensors and for immunoassays. HRP-based sensors are also used in a bi-enzymatic approach for the detection of hydrogen peroxide produced in the reaction of a wide spectrum of oxidoreductase enzymes with their substrates. For example, a number of contemporary laboratory glucose analyzers (Heller and Fedman, 2013) and sensors for an important analyte like the neurotransmitter glutamate rely on the measurement of the produced hydrogen peroxide (Hozumi et al., 2011; Tian et al., 2009). Moreover, since HRP is selective for hydrogen peroxide, but unselective for its reducing agents, HRP-based biosensors can also be used for the bioelectrochemical monitoring of phenols, amines, aminophenols, and other donor substrates (Lindgren et al., 2000). The cycle of HRP immobilized on the electrode with hydroquinone (QH₂) as a reducing agent (mediator) can be schematically represented as follows (Gajhede et al., 1997; Ruzgas et al., 1996):



where Q is quinone, an oxidized form of a mediator, and QH* is a reaction intermediate semiquinone radical. Quinone can be reduced at the electrode, producing a cathodic current as a sensor signal in a mediated electron transfer scheme (Ruzgas et al., 1995)



For the creation of functional interfaces, many aspects should be taken into account, such as the geometry and morphology of the nanomaterials, immobilization of biomolecules on surfaces, electron transfer at the interfaces, and additional components like mediators or additives, as well as compatibility with the modern methods of micro- and nanofabrication.

Recent studies by different authors deal with an inexpensive and versatile synthesis of various gold nanostructures employing oleylamine (OA) as a reducing agent and stabilizer (Halder and Ravishankar, 2007; Kisner et al., 2009). Ultrathin nanowires with diameters of 2 nm and an aspect ratio of up to 4000 have been obtained by this method (Kisner et al., 2011). The nanowires present a face-centered cubic crystalline structure with about 70% of the atoms on their surface, suggesting that these new metallic nanostructures could be used to design new electrochemical platforms

with a large surface area. Although this method has been widely used, the nanostructures (NSs) prepared so far have not been exploited for bioelectrochemical systems. One of the reasons for this is that the surface of the nanostructures is insulated by stabilization compounds, which preserve the integrity of nanostructures, but simultaneously form an insulating barrier for the charge transfer reactions (Pud et al., 2013). It is thus challenging to obtain access to the free or chemically functionalized surfaces for bioelectrochemical systems.

We report on how we addressed this issue by assembling ultrathin gold nanowires and nanoparticles prepared by OA-based synthesis with an oxidoreductase enzyme HRP into a bioelectrochemically active nanoarchitecture as well as a sensor application for the first time. The synthesis and assembly of the ultrathin nanowires and nanoparticles on the thin film gold electrodes is shown. The immobilization and electrochemical properties of the HRP OANS electrodes are discussed. The HRP-sensor characteristics (sensitivity, concentration range, reproducibility, and lifetime) can be improved significantly compared to the planar thin film electrodes by using OANSs.

2. Experimental section

2.1. Reagents and materials

Horseradish peroxidase (HRP, peroxidase from horseradish type VI-A), cysteamine (Cys, 2-aminoethanethiol), glutaraldehyde solution (GA, 50%, for electron microscopy), triisopropylsilane (TIPS), oleylamine, AuCl₃, HAuCl₄·3H₂O, 2-mercaptoethanol (2-ME), octanethiol (OT) were obtained from Sigma-Aldrich and used as received. Other chemicals were reagent grade. Distilled water was used for the experiments. Synthesis and structural characterization of gold nanoparticles and nanowires are described in SI, Section S1.

2.2. Electrode preparation

2.2.1. Flat thin-film gold electrodes

For the preparation of thin-film gold working electrodes (WE), a silicon oxide layer of 1 μm thickness was grown on a silicon substrate. Thin films of titanium as the adhesion layer (10 nm) and gold (300 nm) were prepared on Si/SiO₂ substrates by sputter deposition. The electrodes were cleaned in acetone, propanol, water, H₂O₂:H₂SO₄ 1:2 v/v and rinsed thoroughly with water. These substrates were subjected to electrochemical cleaning by consecutive potential cycles in 0.1 M H₂SO₄ between 0 V and 1.5 V at 0.05 V s⁻¹ starting and ending at 0 V against Ag/AgCl/KCl 3 M reference electrode. These electrodes are referred to as thin-film flat electrodes (without the immobilized nanostructures). Subsequently, the thin-film electrodes were used to prepare the electrodes with nanostructured surfaces.

2.2.2. Nanostructure immobilization (NS electrodes)

After electrochemical cleaning of the thin-film gold electrodes, the nanostructure samples were dropped onto the electrode surface and left overnight to allow the nanostructures adhere. Subsequently, these electrodes were washed with hexane to remove any nanostructures that had not adhered to the electrode surface.

Oxygen plasma treatment was performed in a plasma oven (diener electronic), 200 W, 0.7 mbar.

2.2.3. Chemisorption of thiols

Electrodes were immersed in 5 mM solutions of a thiol (cysteamine or octanethiol) in a solvent for varied time intervals from 10 min to 24 h. Afterwards, the electrodes were rinsed with a

corresponding solvent and distilled water to eliminate excess alkanethiols. Thiols are chemisorbed at gold surfaces according to the following reaction (Finklea, 1996):



2.3. Enzyme immobilization

The electrodes were immersed in 5 mM solutions of cysteamine in hexane for 1 h. The electrodes were rinsed with hexane and phosphate buffer to eliminate excess alkanethiols. The electrodes were further immersed in glutaraldehyde solution of 1%, 2.5%, or 10% in phosphate buffer (0.1 M, pH 7.0) for 1.5 h at room temperature. The electrodes were rinsed with phosphate buffer and immersed in the solution of HRP (5 mg/ml) for 1 h at room temperature and left at 4 °C overnight. After immobilization, the substrates were thoroughly rinsed with phosphate buffer to remove any excess enzyme from the electrode surface. When not otherwise stated, the concentration of GA in the immobilization procedure was 2.5%.

2.4. Electrochemical measurements

Cyclic voltammetry measurements were performed in a three-electrode setup controlled by a potentiostat (AUTOLAB, Eco Chemie, Netherlands). An Ag/AgCl reference electrode (3 M KCl, $E^f = 0.210$ V against NHE), a coiled platinum wire counter electrode, and a gold working electrode composed a three-electrode cell. The values of potentials are reported against Ag/AgCl reference electrode. Electrochemical experiments were performed at room temperature 21 ± 1 °C. Solutions were deaerated with argon and maintained under an argon stream during the measurements. The diameter of the working electrode in the electrochemical cell was 0.5 cm.

3. Results and discussion

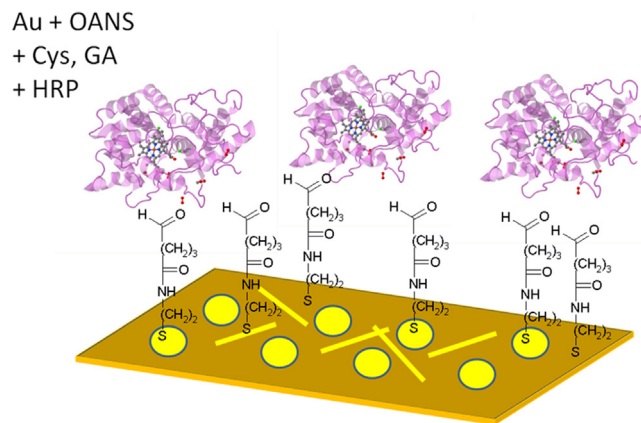
3.1. Characterization of the Au OANs

Prepared electrodes were washed with hexane, and analyzed by electron microscopy (Fig. S1). The nanowires were about 2 nm in diameter and up to several micrometers in length, forming bundles. The nanoparticles were 12 nm (9%) in diameter (Fig. S2).

For the immobilization of proteins, the $-\text{NH}_2$ - and $-\text{COOH}$ -terminated monolayers of thiols on gold were used. In a previous study, we found that Au OANWs exhibit low stability in polar solvents (Koposova et al., 2013). We observed that, e.g., in ethanol (a polar solvent) OA-stabilized gold NWs are partly disaggregated to gold NPs. Thus, an unpolar solvent, e.g. hexane, in which OANWs exhibit higher stability, would be more favorable for the subsequent immobilization of biomolecules. Therefore, we compared the chemisorption of cysteamine (Cys) on gold in ethanol (most often SAMs are produced in ethanol solutions of thiols) and hexane solvents (SI, Fig. S3). According to our studies, the chemisorption of Cys from hexane solutions can be used to provide a favorable surface for the subsequent immobilization of proteins.

3.2. Hydrogen peroxide sensing with horseradish peroxidase (HRP) immobilized on OANS electrodes

HRP was immobilized on flat thin-film and OP-treated OANS electrodes, as described in Section 2.3 and Scheme 1. During the first step, i.e., thiol chemisorption in hexane solutions, the OA molecules on the surface of the gold nanostructures were replaced



Scheme 1. Assembly of oxidoreductase enzyme horseradish peroxidase on electrodes (structure of horseradish peroxidase was adapted from the Protein Data Bank, <http://www.rcsb.org/pdb>).

by thiols after the formation of a covalent gold sulfur bond according to Eq. (5). The energy of the sulfur–gold interaction is in the order of 188 kJ/mol (Dubois and Nuzzo, 1992), forming a stable, semi-covalent bond, which is higher than the energy of interactions of Au(0) and Au(I) with OA of about 44 kJ/mol (Halder and Ravishankar, 2007). XPS surface analysis confirmed the formation of a sulfur–gold bond (SI, Section S2). Thus, OA molecules were replaced by thiols with negatively charged carboxyl and alcohol groups. However, electrochemical and XPS experiments showed that the OA molecules were not completely replaced by thiols. Therefore, we used OP treatment (5 min) to remove any remaining OA from the surface prior to the thiol immobilization.

Mediated and mediatorless sensor responses to H_2O_2 were investigated in the present study. However, a stable and reproducible direct electron transfer between catalytic center of the enzyme and electrodes was not observed. In previous study, it was observed that the cationic peroxidases, like HRP demonstrate a lower percentage of molecules in direct electron transfer on graphite electrodes than the anionic peroxidases (Lindgren et al., 2000). Hence, the electrocatalytic properties of the sensor were evaluated based on the determination of hydrogen peroxide in the presence of the redox mediator hydroquinone (the choice of hydroquinone as a mediator is discussed in SI, Section S3).

As can be seen from Fig. S4 and Scheme S1, the cathodic current corresponding to the reduction of the oxidized mediator hydroquinone increases after the addition of hydrogen peroxide. At the same time, the anodic current corresponding to the oxidation of hydroquinone decreases. This is in accordance with Eqs. (1)–(4) and the reaction Scheme S1: when hydrogen peroxide is added, the enzyme transfers into an oxidized state, while reducing the substrate. The electron donor hydroquinone reduces the oxidized enzyme, while being oxidized to quinone. Quinone is reduced at the electrode, resulting in an increased cathodic current. Anodic current is reduced, because hydroquinone is oxidized by HRP, thus reducing the concentration of hydroquinone (reduced form) at the electrode surface.

Fig. S5 compares the response of HRP immobilized on a flat thin-film electrode with that of HRP immobilized on the electrodes modified by nanoparticles (OANP electrode) and nanowires (OANW electrode) (A) before and (B) after the addition of hydrogen peroxide. As can be seen in Fig. S5A, cyclic voltammograms for the diffusion-controlled redox mediator show no significant increase in current density for the nanostructured electrodes in comparison with the planar gold electrode. A minimal increase was observed in the electroanalytical signal (current density) as

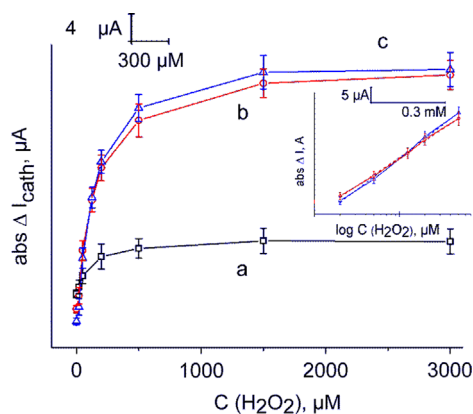


Fig. 1. Calibration curves of HRP sensors (a) HRP-Au, (b) HRP-OANP, and (c) HRP-OANW. Inset in (c) shows the sensor response as a function of $\log C(\text{H}_2\text{O}_2)$. Other conditions: 2.5% GA, deaerated solutions, scan rate 50 mV s^{-1} , phosphate buffer 0.1 M, pH 6.8, and 1.0 mM QH₂. Error bars represent the confidence limits ($p=0.95$, $n=5$) at each concentration point. Relative standard deviations were 3–5% for the thin film HRP-Au sensor and 2–4% for the HRP-OANP and HRP-OANW sensors ($n=5$).

Table 1
Detection limit, sensitivity, and long-term stability of the HRP-sensors^a.

HRP-sensor	Detection limit, $\mu\text{M}^{b,c}$	Sensitivity, $\text{A M}^{-1} \text{cm}^{-2}$	Long-term stability, % ^d
HRP-Au thin-film gold	16 (0.12)	0.012 (0.048)	82
HRP-OANP	8 (0.09)	0.027 (0.046)	91
HRP-OANW	5 (0.09)	0.031 (0.045)	91

^a Relative standard deviations are given in parenthesis.

^b $S/N=3$.

^c Relative standard deviations of the determinations near the detection limit were 9–12%.

^d % of the sensor response to $50 \mu\text{M H}_2\text{O}_2$ after a storage period of one month compared to a fresh prepared sensor.

well as smaller peak separation (probably due to the improved kinetics of the electron transfer) for the OANS electrodes. This is because semi-infinite linear diffusion (Fig. S6) limits the observed current densities, which agrees with previous observations reported (Scanlon et al., 2012; Schröper et al., 2008). Thus, this confirms that redox systems with diffusion (mass-transport) limitation are not suitable for the evaluation of the effective electroactive surface area of electrodes, where immobilized redox probes should be used (Schröper et al., 2008).

However, Fig. S5B shows that significantly higher current densities were observed for HRP-OANW and HRP-OANP electrodes after electrocatalytic reduction of the substrate and oxidation of a mediator hydroquinone compared to the HRP-modified planar thin-film gold electrode. This can be explained by the larger amount of electroactive enzyme HRP on the nanostructured electrodes. Fig. 1 shows the calibration curves for the planar thin film, HRP-OANP, and HRP-OANW electrodes. Sensors based on HRP immobilized on the nanostructured electrodes demonstrate much better performance with respect to the sensitivity and working concentration range (Fig. 1 and Table 1). A working concentration range of 18–500 μM and the detection limits of 5 μM (RSD 0.09) for the HRP-OANW and 8 μM (RSD 0.09) for the HRP-OANP sensors are comparable with the characteristics of the hydrogen peroxide nanostructured electrodes reported recently (Kafi et al., 2008; Mao et al., 2013; Zhao et al., 2008).

Fig. 2 shows the response of the sensors over the substrate concentration range 0–3 mmol l^{-1} for the sensors prepared using a concentration of 1%, 2.5%, and 10% GA. More reproducible results

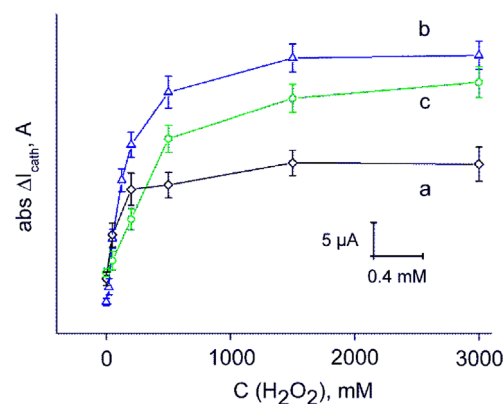


Fig. 2. Dependence of the HRP-OANP sensor response on the concentration of glutaraldehyde: (a) 1%, (b) 2.5%, and (c) 10% GA. Other conditions: deaerated solutions, scan rate 50 mV s^{-1} , 1.0 mM QH₂ in 0.1 M phosphate buffer, and pH 6.8.

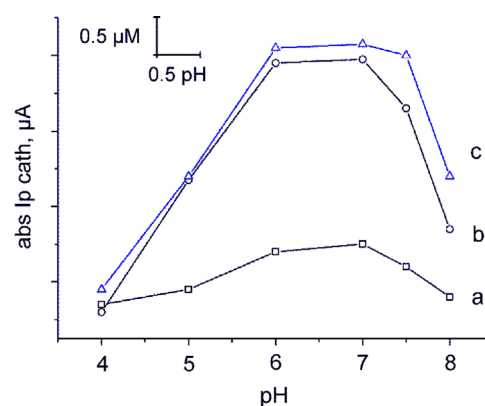


Fig. 3. Influence of the buffer pH on the biosensor response in the presence of 1.5 mM H_2O_2 : (a) HRP-Au planar thin film electrode, (b) HRP-OANP electrode, and (c) HRP-OANW; other conditions: 1 mM QH₂ and scan rate 50 mV s^{-1} .

and a larger interval range of the substrate concentration were obtained when 2.5% GA was used. Relative standard deviations were 2–4% for the sensors prepared with 2.5 and 10% GA and 4–6% for the sensors prepared with 1% GA ($n=5$). We assume that higher concentrations of GA may result in a higher degree of cross-linking of functional amino groups of cysteamine by GA molecules, thus decreasing the number of carbonyl functional groups available for the attachment of HRP in the next step of the immobilization (Section 2.3 and Scheme 1). At a lower concentration of GA, lower amounts of HRP are covalently bound to the electrode surface, resulting in lower sensor sensitivity.

The performance of a biosensor depends on the pH value of electrolyte. The pH range reflects the optimum conditions for both enzymatic and mediated electrochemical reactions on electrodes with covalently immobilized enzymes and can deviate from pH range found in solutions by photometry (Santos et al., 2007; Schomberg et al., 1993). Fig. 3 shows the dependence of the sensor response at different pH in the presence of 1.5 mM H_2O_2 and 10^{-3} M of hydroquinone for the HRP-Au thin film, HRP-OANP, and HRP-OANW electrodes. The absolute value of the cathodic peak current increased with increasing pH from 4.0 to about 7.0. The sensor response decreased when pH increased further to 8.0. Slightly larger pH interval was observed for the HRP-OANW electrodes. This might be due to the fine multilayered structure of the NW electrode surface and the effect of the double layer. At the lower pH values, the current response is low, which might be due to the denaturation of the enzyme. As the maximum current response is achieved between pH 6.0 and 7.0, this pH

interval was considered as the optimized pH for the proposed biosensor.

Studies on the temperature dependence of the sensor response have been performed to optimize the performance of the sensors (SI, Fig. S7). Some of the compounds likely to be present in samples were examined for possible interference effects (SI, Section S4 and Table S2).

The reproducibility of the sensors was evaluated by measuring sensor response in 125 μM H_2O_2 . The relative standard deviation of eight successive measurements was 3%. Sensor-to-sensor reproducibility was evaluated by measuring the response of four sensors in 50 μM and 500 μM solutions of H_2O_2 . The relative standard deviations of the determinations were 6% and 4% for the HRP-OANP and 5% and 4% for the HRP-OANW sensors, respectively. The long-term stability of the sensors was evaluated by measuring the response of the sensors to 50 μM H_2O_2 during one month (Figs. S8, S9, and Table 1). The HRP thin-film, HRP-OANP and HRP-OANW sensors retained about 82, 91 and 91% of their biocatalytic response, respectively, while being stored in a phosphate buffer at 4 °C.

The HRP-OANS sensors demonstrate a lower detection limit, wide working concentration range, higher sensor-to-sensor and measurement-to-measurement reproducibility as well as better long-term stability compared to the planar HRP sensor, thereby improving the sensor response (see Table 1). We anticipate that the studies on the surface chemistry of OANPs and ultrathin NWs as well as protein immobilization may further enhance electrochemical and biosensing properties of the HRP electrodes.

The improved characteristics of the OANS electrodes can be attributed to the following facts: firstly, geometrical signal enhancement due to the higher effective surface area, and as a consequence, a larger amount of the immobilized protein. Secondly, OANSs provide a favorable microenvironment for the enzymes to preserve biocatalytic activity and prevent denaturation of proteins.

4. Conclusions

This paper describes a novel nanostructure platform based on ultrathin gold nanowires and nanoparticles prepared by oleylamine synthesis for bioelectrochemical sensing. Immobilization and electrochemical properties of oxidoreductase enzyme HRP on the OANW and OANP interfaces were detailed. An enzyme electrode for the biocatalytic reduction of H_2O_2 was developed. Higher sensitivity ($0.031 \text{ A M}^{-1} \text{ cm}^{-2}$ and $0.027 \text{ A M}^{-1} \text{ cm}^{-2}$), reproducibility (3%) and long-term stability (sensors retained 91% of their biocatalytic response after one month) were observed for the detection of H_2O_2 in the case of HRP-OANW and HRP-OANP sensors compared to a planar thin-film HRP sensor. Gold OANWs and OANPs were shown to be excellent platforms for designing a variety of bioelectrochemical interfaces for conjugation with metalloproteins and other biomolecules for biosensors, bioelectronic systems, bioelectrocatalysis, and energy research.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2014.01.034>.

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