Direct mediatorless electron transport between the monolayer of
photosystem II and poly(mercapto-p-benzoquinone) modified gold
electrode—new design of biosensor for herbicide detection

J. Maly a,b,∗, J. Masojidek b, A. Masci e, M. Ilie c, E. Cianci d, V. Foglietti d,
W. Vastarella e, R. Pilloton e

a Department of Biology, University of J.E. Purkyne, CZ-40096 Usti nad Labem, Czech Republic
b Institute of Microbiology, Academy of Sciences, CZ-379 81 Trebon, Czech Republic
c University Politehnica of Bucharest, LAPI-Group, P.O. Box 15-135, 02133, Romania
d CNR/IFN, Via Cineto Romano 42, Rome 00156, Italy
e ENEA-CR-Casaccia, Via Anguillarese 301, I-00060 S. Maria di Galeria, Rome, Italy

Received 10 December 2004; received in revised form 2 February 2005; accepted 21 February 2005
Available online 14 March 2005

Abstract
Photosystem II (PSII) modified gold electrodes have been prepared providing mediatorless electron transport on the basis of electrodeposited conductive layer poly-mercapto-p-benzoquinone (polySBQ). Such electrodes are suitable in construction of biosensors for PSII inhibiting herbicides. PolySBQ layer was synthesized on
(i) screen-printed gold electrodes and
(ii) gold microelectrodes in an array on silicon substrate,
by electrochemical-oxidation of sulpho-p-benzoquinone (SBQ) at +650 mV versus Ag/AgCl. The basic properties of polySBQ layer were characterized using linear sweep voltammetry and atomic force microscopy (AFM). The typical redox response for quinones was observed.
The optimal length of the polymer providing direct electron transfer (DET) was found to be very close to 30 nm.
PSII particles isolated from the thermophilic cyanobacteria Synechococcus bigranulatus were physically adsorbed on the polySBQ covered gold electrodes. The generation of photocurrent was observed at E = +250 mV (versus Ag/AgCl) without addition of any mediator. The basic properties of DET were studied. We concluded that: (i) PSII active in DET is immobilized in form of monolayer; (ii) the charge transport from PSII to gold working electrode (AuWE) is fast and dominated by the rate of the enzymatic reaction; (iii) polySBQ layer drains electrons from the QA pocket of the photosystem since the electrode activity is inhibited by specific inhibitor, i.e. diuron (DCMU); (iv) the stability of the photosystem immobilized on gold electrodes by using polySBQ is comparable to the stability of PSII in solution under the same experimental conditions; (v) the inhibition of the photosystem by herbicide DCMU follows the sigmoid dependence; (vi) I50 as well as limit of detection (LOD) show an improved sensitivity compared to other published biosensing systems using PSII as bioactive part.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Photosystem II; Biosensor; Herbicides; Molecular wire; Poly(mercapto-p-benzoquinone)
genic photosynthesis (higher plants, algae, and cyanobacteria). The photosynthetic herbicides selectively bind the reducing side of the PSII complex to the D1 reaction centre protein where they replace the secondary electron acceptor Qb (Draher et al., 1991; Mattoo et al., 1989; Sobolev and Edelman, 1995).

A biosensor for detection of triazine-, urea- and phenolic herbicides was already developed. Isolated PSII particles entrapped between the Teflon and dialysis membrane on the surface of the Clark oxygen electrode, were used as biosensing element (Koblížek et al., 1999). Oxygen evolution due to PSII activity decreases proportionally to herbicide concentration in the medium.

A more advanced type of PSI-based biosensor was developed recently in our laboratories using thick-film printed electrodes with immobilized PSII (Koblížek et al., 2002). These electrodes were used for amperometric detection of PSII activity in the presence of artificial electron acceptor. The performance of this set-up is restricted by cross-linking of PSII in a gel matrix of bovine serum albumin (BSA) and glutaraldehyde (GA). Gel entrapment results in slow media-tor diffusion and a decreased sensitivity to herbicides. The three-electrode system consisting of AuWE (effective surface typically 6.2 mm²), Au auxiliary and Ag/AgCl pseudo reference electrode was used in experiments. The screen-printed ceramic electrodes (BVT Tisnov, Czech Republic) were mechanically polished and cleaned in Piranha solution (30 s) prior to use. The electrodeposition of polySBQ layer was done from SBQ in nitrogen bubbled phosphate buffer solution (PB, 25 mM, pH = 7.4) at E = +650 mV using a potentiostat (Ecochemie, Autolab PGST-A). The amperometric detection curves were recorded. The slightly basic pH of the solution proved to be a critical condition since SBQ is practically insoluble at the neutral pH.

For the purpose of the AFM topographic measurement, a microarray of gold electrodes on silicon substrate (De 2. Materials and methods

2.1. Synthesis of the sulpho-p-benzoquinone

The SBQ was obtained according the procedure previously described by Alcalay (1947). Briefly, the acetic benzoquinone (Sigma p.a.) solution was cooled down and mixed in 1:1 molar ratio with concentrated sodium thiosulfate (Sigma p.a.) solution. The reaction results in the bleached solution of hydroquinone. The yellow solution of mercaptohydroquinone is further obtained after reduction of hydroquinone by addition of zinc in the presence of hydrochloric acid. Yellow crystals are isolated and subsequently dissolved in 10 parts of concentrated ethanol solution. After addition of 2 M FeCl₃ (Sigma p.a.) the mercaptohydroquinone is oxidized and benzoquinone disulfide is obtained. Following the crystallisation, the yellowish crystals are refined in glacial acetic acid.

Benzoquinone disulfide was reduced to SBQ prior to electropolymerization by using the immobilized TCEP disul-fide reducing gel (Pierce) following the producer instructions.

2.2. Electrodeposition of SBQ in screen printed gold electrodes and silicon based microarray

The three-electrode system consisting of AuWE (effective surface typically 6.2 mm²), Au auxiliary and Ag/AgCl pseudo reference electrode was used in experiments. The screen-printed ceramic electrodes (BVT Tisnov, Czech Republic) were mechanically polished and cleaned in Piranha solution (30 s) prior to use. The electrodeposition of polySBQ layer was done from SBQ in nitrogen bubbled phosphate buffer solution (PB, 25 mM, pH = 7.4) at E = +650 mV using a potentiostat (Ecochemie, Autolab PGST-A). The amperometric detection curves were recorded. The slightly basic pH of the solution proved to be a critical condition since SBQ is practically insoluble at the neutral pH.

For the purpose of the AFM topographic measurement, a microarray of gold electrodes on silicon substrate (De
Bellis et al., 2003) was also used for immobilization of polySBQ layer and PSII. Here, the WEs have been manufactured using optical lithography and chemical vapour deposition techniques and placed in a μ-array of 7 × 7 with a period of 160 μm and diameter of 70 μm. Each electrode is individually addressable allowing thus to direct the electrochemical synthesis on one selected electrode in the array.

2.3. Characterization of polySBQ-AuWE redox properties by linear sweep voltammetry

Linear sweep voltammetry (CV) was used for determination of the redox properties of polySBQ film. Gold screen-printed electrodes with deposited polySBQ layer were immersed in the PB solution (25 mM, 100 mM KCl) bubbled with pure nitrogen and cycled between −1 and +1 V versus Ag/AgCl. Typically, scanning was repeated 10 times and average scan was used for further evaluation.

2.4. Characterization of polySBQ-AuWE and PSII-polySBQ-AuWE by AFM

In order to characterize the surface topography of the immobilized deposited layers a Digital Instruments D3100 AFM operating in ambient air was used. Imaging was performed in contact-mode using commercial silicon nitride triangular probes with pyramidal tip.

The sample was mounted stationary on the x–y table while the tip mounted in a standard AFM holder was scanned over it. One sample has been dedicated to this investigation and scanning probe measurements (contact mode) have been performed in air on: (a) the bare gold surface, (b) the polySBQ-AuWE, (c) the PSII-polySBQ-AuWE. The scanned surface was of 1 μm × 1 μm.

2.5. Isolation of photosystem II

The PSII complex preparations used for immobilization of polySBQ layer and PSII. Here, the WEs have been manufactured using optical lithography and chemical vapour deposition techniques and placed in a μ-array of 7 × 7 with a period of 160 μm and diameter of 70 μm. Each electrode is individually addressable allowing thus to direct the electrochemical synthesis on one selected electrode in the array.

2.5. Isolation of photosystem II

The PSII complex preparations used for immobilization of polySBQ layer and PSII. Here, the WEs have been manufactured using optical lithography and chemical vapour deposition techniques and placed in a μ-array of 7 × 7 with a period of 160 μm and diameter of 70 μm. Each electrode is individually addressable allowing thus to direct the electrochemical synthesis on one selected electrode in the array.

2.6. Amperometric measurement of the photosystem II activity

Amperometric measurements of PSII activity on the PSII-polySBQ-AuWE and the PSII–BSA–GA gel electrode were done in the home-made flow-cell system already described (Koblížek et al., 2002). It consists of electrode chamber with diagonally positioned inlet and outlet and the LED source of light located in front of it. Electrodes were continuously fed with the measuring buffer (40 mM MES, 0.1 M NaCl, 5 mM MgCl₂) with the typical flow rate 0.25 mL min⁻¹. Potential E = +250 mV was set between the PSII-polySBQ-AuWE and Ag/AgCl reference. An illumination was typically set to 10 × (180 s dark period) and the light intensity to about 100 μmol photon m⁻² s⁻¹. Red LEDs (650 nm) were used as the source of light. The photocurrent intensity on the working electrode was registered with a potentiostat (PG STAT-Autolab).

3. Results and discussion

3.1. Synthesis and characterisation of polySBQ on gold electrodes (polySBQ-AuWE)

In order to back up the electrochemical data with electrode surface observation by AFM, the presented experiments were done on the silicon based gold microarray (see Section 2.2). Similar results of electrochemical measurements were obtained on gold screen-printed electrodes, which were also used during the experiments with PSII. The time dependence of the electrooxidation process of SBQ on gold WE is shown in Fig. 1. The typical initial increase of oxidation current (up to about 2 min) is followed by the oxidation current decline which continues with the oxidation process. As we have already shown (Maly et al., 2004a) such electrooxidation process is typical for any free sulphydryl containing molecules. During the initial phase, the fast creation of monolayer is observed. With the prolonging time of oxidation, the complicated multilayer structure
Fig. 1. The amperometric curve of the SBQ electrooxidation process. After stabilization of electrode in pure PB the measurement cell was fed with saturated SBQ solution (20 mM PB, pH = 7.4, \( E = +650 \text{ mV vs. } \text{Ag/AgCl} \), temperature = 25 °C, nitrogen atmosphere). Temporary oxidation current increase (up to 2 min) is followed by its drop and stabilization.

as well as polymerization process (in the case of quinones) occurs.

The immobilization of the quinone group as well as the ability to exchange the electrons between the redox groups and electrode surface was controlled by CV. In Fig. 2 quasi-reversible redox peak pair of quinone moieties was observed at \( E = -126 \text{ mV} \) with anodic peak centred at \( E_{pa} = -107 \text{ mV} \) and cathodic at \( E_{pc} = -146 \text{ mV} \) versus Ag/AgCl (pH = 7.4). The position of peak pair is in agreement with other observations (Sato et al., 1996; Chan et al., 2000). The \( \Delta E_p = 39 \text{ mV} \) does not fulfill the criteria of reversibility. Such behaviour is also usually observed in case of chemically deposited quinones on gold electrodes (Chan et al., 2000) and is due to the protons transfer between quinone and hydroquinone. A linear relation between the sweep rate (up to 160 mV s\(^{-1}\)) and the peak currents was observed confirming the surface coupled redox process (immobilized quinones). The peak position as well as the charge transferred over the oxidation or reduction peak remained unchanged up to 2 h of continuous cycling showing the stability of immobilized layer. The linear dependence between the redox potential and pH was observed at pH interval 5.4–8.4 (20 mM PB, 100 mM KCl). The slope of the linear dependence was \(-59 \text{ mV per pH unit} \) and the redox potential was shifted to more negative values as the pH become higher (data not shown). Such an observation is in agreement with related works (Sato et al., 1996; Chan et al., 2000; Arai et al., 1996). As was already described, we expect the electropolymerization of SBQ by alternate electro-oxidation and addition reactions (Arai et al., 1996). Since the diameter of one electrode in array is 70 μm, the charge transferred over oxidation peak (obtained after 20 min of SBQ electro-oxidation) was \( Q = 7.7 \text{ mC cm}^{-2} \). Taking into account two electrons for redox reaction, then equivalent number of immobilized quinone moieties is \( 2.4 \times 10^{16} \text{ molecules cm}^{-2} \) which is \( 3.98 \times 10^{-8} \text{ mol cm}^{-2} \). Almost identical value was described by Arai et al. (1996), as an ideal value for DET from xanthine oxidase. Furthermore, as already shown elsewhere (Sato et al., 1996; Chan et al., 2000; Hong et al., 1999), the typical experimental values obtained for self-assembled monolayers of SBQ on gold are about two orders lower, close to the theoretical calculated value \( 5.7 \times 10^{-10} \text{ mol cm}^{-2} \) for perpendicularly bound SBQ through the S–Au bond (Stern et al., 1988). Therefore, the quantity of quinone moieties obtained by 20 min of electro-oxidation overcomes the number necessary for an ideal monolayer by a factor of around 68. Accordingly, assuming the polymerization process under ideal conditions, where linear polymerization from each SBQ molecule immobilized on gold is expected, we obtain chain containing 68 monomers which is about 30 nm long (the SBQ is approx 4.4 Å long according to Chan et al., 2000).
3.2. Atomic force microscopy of polySBQ layer

The gold electrodes microarray on silicon substrate already described (De Bellis et al., 2003) was selected for experiments since each electrode is individually addressable for electrochemical experiments and the surface roughness of gold surface is substantially low (average roughness 3.5 nm, Fig. 3, Panel A) in order to provide topographic imaging of synthesized polymer. This condition is not fulfilled in case of screen-printed electrodes although they were widely used during the experiments with PSII. As shown in Fig. 3 (Panel B), the surface appearance of electrode is markedly changed after electro-deposition of polySBQ layer. Measurements have been performed in different positions of the electrode, on the border of the deposited area, on a single needle as well as in the middle of the deposited area where the needles formed crowded clusters. We found out an average width of about 1 nm or less (taking into account the curvature radius of the probe that was about 20 nm and the needle measured width of about 40 nm). The average length varied from 7 nm, in case of individual needle, up to 34 nm in the case of clusters of needles, most probably because of their stratification one over the other to compose the multilayer. Interestingly, the resulting value is quite close to 30 nm which was previously obtained by electrochemical measurements. The repeated scans on the same place were stable, showing a strong attachment of polymers on gold electrode.

The appearance of the same electrode after loading the PSII from solution is shown in Fig. 3 (Panel C) (see Section 2.6). We suppose the needle-like structures representing the polySBQ layer diminished after the protein deposition; the bigger protein molecules are hiding the needles under them. Round shaped forms have been noticed with relative average height in the range of 10 nm. They should be the immobilized PSII molecules trapped by each needle-shape polymer chain. In contrast, no such features were obtained on unmodified gold electrode immersed in the same solution of PSII which shows that change in polySBQ modified electrode is due to immobilized protein. The AFM scans were reproducible and no material was removed from the surface. It indicates a strong adherence of protein to polySBQ layer. PSII itself is a big membrane protein, with X–Y dimer core dimensions 17.2 nm × 9.7 nm (Bacon, 2001), surrounded by all the remaining thylakoid membrane as well as the detergent micelle when isolated. No strict homogeneity in size of immobilized PSII particles could be expected as is usually possible to achieve in case of water soluble proteins. Therefore, in case of densely packed monolayer, we are not able to observe AFM topography consisting of individually recognizable protein unit. Instead, the superimposing effect caused by hydrophilic adherence for polySBQ layer, rests of thylakoid membranes as well as the detergent and PSII itself creates the irregular hill-like structures which were observed. As will be shown later, the presumption of densely packed PSII layer was confirmed independently by electrochemical measurements.

Fig. 3. The atomic force microscopy images of electrodeposited polySBQ layer (20 min) using contact mode (Panel A: pure gold, Panel B: polySBQ layer, Panel C: polySBQ layer with adsorbed PSII). Standard silicon nitride tip with the following characteristics: spring constant = 0.6 N m⁻¹, resonant frequency = 40 kHz, curvature radius = 30 nm, height = 3 μm, tip angle = 45°; cantilever configuration: cantilever length = 115 μm, cantilever width = 122 μm, cantilever thickness = 0.5 μm.

3.3. Characterization of the photoactivity of immobilized PSII on polySBQ-AuWE

The photoactivity of PSII-polySBQ-AuWE was determined using the flow cell and ceramic screen printed electrode, as described in Section 2.7. Since the most optimal pH for PSII function lies between 6.5 and 6.8 (Bacon, 2001),
no pH dependence of immobilized PSII was followed and the pH = 6.5 was used throughout all experiments. The same is for the time of polySBQ synthesis (20 min) on each electrode. The dependence of intensity of photogenerated signal on electrode potential was screened between 0 and 400 mV versus Ag/AgCl. Therefore, a potential of +250 mV was used throughout all experiments. The same was also possible (i.e. at 0 mV versus Ag/AgCl). Therefore, the highest signal observed started at potential +250 mV although the detection at lower potentials is for the time of polySBQ synthesis (20 min) on each electrode. The dependence of intensity of photogenerated signal is for the time of polySBQ synthesis (20 min) on each electrode. The dependence of intensity of photogenerated signal on electrode potential was screened between 0 and 400 mV versus Ag/AgCl. Therefore, a potential of +250 mV was used throughout all experiments.

The illumination of PSII-polySBQ-AuWE with red LED (100 µmol photon m⁻² s⁻¹, λ = 650 nm, WE potential  

\[ E = +250 \text{ mV versus Ag/AgCl} \]

results in photogenerated electric current (Fig. 4, Panel A). No detectable photogenerated current is observable, when polySQB-AuWE is illuminated (Fig. 4, Panel B). In the former case, the velocity of the electrode reaction is restricted only by velocity of the enzyme reaction and by charge diffusion during propagation along the polySBQ layer. From CV measurements described above it is clear, that the transduction of charge through polymer is very fast, since the separation of  

\[ E_{aq} \] and  

\[ E_{pc} \] is only  \( \Delta E_h = 39 \text{ mV} \). Thus we can assume that the velocity of the electrode reaction will be dominated predominantly by the velocity of the enzymatic reaction itself. On the other hand, in the case of MET (Fig. 4, Panel B), the velocity of the electrode reaction will be greatly influenced by diffusion coefficient of reduced as well as oxidized mediator in gel structure. Therefore, the steady state is achieved under the same conditions within several minutes (Koblížek et al., 2002; Maly et al., 2004b). The fast electrode kinetics is a common phenomenon already described for other kind of enzymes in various systems offering DET (for review see Sheller et al., 2002; Gerard et al., 2002). Since there was not any mediator in loading and measuring solution, we can state that we have achieved a DET from PSII to polySBQ-AuWE.

Another question has to be raised, how the structure of the immobilized PSII layer is organized and how the DET is achieved. The QA pocket located on acceptor side of PSII represents the place where, under natural conditions, plastoquinones are reduced by secondary electron acceptor QA. The redox potential of QA is about  

\[ E_{QA} = -130 \text{ mV} \] (versus pseudo Ag/AgCl) and that of QB is  

\[ E_{QB} = +50 \text{ mV} \] (versus Ag/AgCl) (Bacon, 2001). Since the redox potential of SBQ was measured to be  

\[ E_{SBQ} = -126 \text{ mV} \] (versus Ag/AgCl) it could easily replace the plastoquinone and supply its function as electron acceptor. The overall simplified electron transfer reaction may be then written as follows:

\[
\text{H}_2\text{O} \rightarrow \text{P680} \rightarrow \text{QA} \rightarrow \text{polySBQ} \rightarrow \text{AuWE}
\]

where \( \text{H}_2\text{O} \) is an electron donor, P680 is special chlorophyll pair which offers the charge separation following the photon absorption, QA is a secondary electron acceptor which undergoes one electron oxidation/reduction process under physiological conditions, polySBQ is the conductive polymer film and AuWE represent the gold working electrode.

It is known that the enzymatic turnover rate of PSII is limited mainly by the reaction time of QA and QB reduction (for review see Bacon, 2001). Assuming the average number for reaction time of QA = 100–600 µs (Bacon, 2001), the time required to achieve steady state is very short, not more than 5 s. Here, we can observe a big difference when this is compared with the mediated transport (MET) described in a previous work (Koblížek et al., 2002) and represented by example in Fig. 4 (Panel B). In the former case, the velocity of the electrode reaction itself is restricted mainly by the diffusion of the mediators in gel structure. Therefore, the steady state is achieved under the same conditions within several minutes (Koblížek et al., 2002; Maly et al., 2004b). The fast electrode kinetics is a common phenomenon already described for other kind of enzymes in various systems offering DET (for review see Sheller et al., 2002; Gerard et al., 2002). Since there was not any mediator in loading and measuring solution, we can state that we have achieved a DET from PSII to polySBQ-AuWE.

Another question has to be raised, how the structure of the immobilized PSII layer is organized and how the DET is achieved. The QA pocket located on acceptor side of PSII represents the place where, under natural conditions, plastoquinones are reduced by secondary electron acceptor QA. The redox potential of QA is about  

\[ E_{QA} = -130 \text{ mV} \] (versus pseudo Ag/AgCl) and that of QB is  

\[ E_{QB} = +50 \text{ mV} \] (versus Ag/AgCl) (Bacon, 2001). Since the redox potential of SBQ was measured to be  

\[ E_{SBQ} = -126 \text{ mV} \] (versus Ag/AgCl) it could easily replace the plastoquinone and supply its function as electron acceptor. The overall simplified electron transfer reaction may be then written as follows:

\[
\text{H}_2\text{O} \rightarrow \text{P680} \rightarrow \text{QA} \rightarrow \text{polySBQ} \rightarrow \text{AuWE}
\]
Comparison between theoretical calculations and experimental data with respect to photocurrent, efficiency and apparent reaction time of an ideal PSII monolayer, PSII-polySBQ-AuWE and cross-linked BSA–GA–PSII

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(x = 17.20 \text{ nm})</td>
<td>X dimension derived from PSII crystal (monolayer, dimer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(y = 9.20 \text{ nm})</td>
<td>Y dimension derived from PSII crystal (monolayer, dimer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(A_{\text{PSII}} = \pi y^2 = 8.34 \times 10^{-15} \text{ cm}^2)</td>
<td>Estimated 2D area occupied by PSII monomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(r = 0.142 \text{ cm})</td>
<td>Electrode radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(A_{e} = \pi r^2 = 0.06 \text{ cm}^2)</td>
<td>Electrode surface area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(f = 1.13)</td>
<td>Roughness factor of the gold surface</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(n = A_{\text{PSII}}/r^2 = 5.86 \times 10^2)</td>
<td>PSII molecules on the gold electrode (theor)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>(t_{Q_{B}} = 350 \mu\text{s})</td>
<td>Reaction time (reduction due to (Q_{B}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>(e^{-n_{t}f} = 2860 \text{ s}^{-1})</td>
<td>Electrons transferred per molecule per second</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(N = 3.025 \times 10^{10} \text{ mol}^{-1})</td>
<td>Avogadro number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>(F = 6.022 \times 10^{23} \text{ mol}^{-1})</td>
<td>Faraday constant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>(i_{\text{PSII}} = nF \times (3.9) \times 10^{-1} \text{ A})</td>
<td>Photocurrent density on the electrode (theor.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>(i_{\text{PSII}} = 8.17 \times 10^{-1} \text{ A})</td>
<td>Experimental photocurrent density measured with PSII-polySBQ-AuWE (mediatorless, Fig. 4A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>(i_{\text{PSII}} = 4.97 \times 10^{-5} \text{ A})</td>
<td>Experimental photocurrent density with cross-linked PSII (DQ as mediator, Fig. 4B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>(k = k_{\text{DQ}})</td>
<td>Linear equation describing photocurrent vs. inverse reaction time (simulation), (k = 1.75 \text{ mA cm}^{-2})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>(t_{\text{app}} = 0.0148 \text{ s})</td>
<td>Calculated (row 15) reaction time (apparent) for PSII-polySBQ-AuWE (mediatorless)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>(t_{\text{app}} = 0.276 \text{ s})</td>
<td>Calculated (row 15) reaction time (apparent) for cross-linked PSII (DQ as mediator)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments: 1 and 2: 2D dimensions of the PSII as a crystal dimer; 6: roughness of the gold electrode surface (experimental data); 7: number of PSII units in an ideal monolayer on the gold electrode; 8: average reaction time of \(Q_{B}\) reduction (Bacon, 2001).

3.4. Influence of polySBQ film thickness

The dependence of the sensor activity (effectiveness of DET) on the number of immobilized quinone moieties was determined (Fig. 5). DET starts to be observable at about 0.5 \(\times 10^{-15}\) molecules cm\(^{-2}\) which corresponds to a polymeric length of about 6 nm (see Section 3.1). Assuming the population of PSII oriented on the surface of gold electrode in such a way that \(Q_{A}\) pocket is directed towards the polySBQ layer, then 6 nm long chain could be sufficiently long to reach the active centre. On the other hand, for the other populations of PSII (\(Q_{B}\) pocket oriented parallel or towards the opposite side, out of the electrode surface) such length will not be sufficient. This fact is represented by the increase of photocurrent density when the time of polySBQ synthesis is prolonged as far as the maximum at 2.4 \(\times 10^{-16}\) molecules cm\(^{-2}\) is reached (30 nm). Further increase of polySBQ layer thickness causes decrease of PSII activity (no activity after 2 h of synthesis). Our results are slightly different compared to other ones previously published (Arai et al., 1996, 1998, 1999). Time needed to synthesize the polySBQ layer which offer the maximal PSII in DET is short in our case, since the synthesis was not done on dilute SBQ solution in the presence of enzyme. In related works, the presence of water soluble protein in SBQ solution was required in order to achieve a stable immobilization. Instead, the PSII is stably adsorbed following the polymer synthesis, probably due to its hydrophilic nature.

Fig. 5. The dependence of the sensor activity (effectiveness of DET) on the number of immobilized quinone moieties. The sensor activity was monitored as described in Section 2.7. The current density was taken as the maximal current density obtained in steady state. The number of quinone moieties was calculated using the Nernst equation and considering the charge transported over the CV oxidation peak of polySBQ-AuWE as described in Section 2.3.
3.5. Stability of the electrode

The stability of photogenerated signal produced by PSII-polySBQ-AuWE electrode during the 10 s of illumination was recorded using the same conditions shown in Fig. 4 (Panel A). The interval among each light phase was 180 s. The observed signal decreased exponentially with $t^{1/2}$ within about 3 h (data not shown). Identical value of this parameter was observed for soluble PSII as well as for MET from PSII monolayer at comparable experimental conditions (Maly et al., 2004b). It means that the observed activity decay is just due to the light induced inactivation of PSII and no protein is washed out from the surface during the measurement.

3.6. Inhibition effect of herbicide diuron (DCMU)

In Section 3.3 we have proposed the mechanism of electron transport from PSII to polySBQ layer through the $Q_B$ pocket inside of PSII. Such mechanism was proved using DCMU, which has been the most known and used specific inhibitor of PSII activity in basic research of photosynthesis for many decades (Bacon, 2001). It binds inside of $Q_A$ pocket and in competitive way reduce the electron transport from $Q_A$ to plastoquinone. The inhibition of PSII-polySBQ-AuWE activity by addition of $3 \times 10^{-9}$ M DCMU into the measuring solution is shown in Fig. 6 (Panel A). The decrease of PSII photogenerated current is very fast (less than one measuring cycle). However neither the activity of PSII cannot be turned back when the PSII-polySBQ-AuWE is washed again with solution without DCMU, nor were the electrodes reusable when loading PSII again on previously inhibited electrodes. No increase of activity to normal level was observed in the latter case. This indicates that PSII remains attached to PSBQ surface following the inhibition process. The regeneration of polySBQ-AuWE was only possible after application of strong washing conditions ($2$ M NaCl, $0.1\%$ Triton in PB).

By this method, about $70\%$ of electrode activity was restored after new PSII loading.

The above described experiments showed that: (i) the $Q_B$ pocket of PSII immobilized on polySBQ-AuWE is accessible for DCMU; (ii) the polySBQ layer is reduced by $Q_A$ as was already proposed in Section 3.3; (iii) once the polySBQ is displaced from PSII, it cannot be reassembled when DCMU is added to the solution and the decrease of PSII activity is observed. Final measured value is compared with the approximation obtained from control peaks. Inhibition rate as the percentual decrease of initial activity is calculated. Conditions of experiments were the same as in Fig. 4 (Panel A). Panel B: Sigmoid calibration curve for herbicide DCMU. Calibration curve was fitted using non-linear regression (described in Section 3.6). A new sensor was used for each DCMU concentration. Every point in graph is the average value of $10$ replicates.

DCMU, being the inhibition a relative factor in correlation with the only presence of active enzyme. This observation is in agreement with our previous studies (Koblížek et al., 1998, 2002). Calibration curve for DCMU (Fig. 6, Panel B) has sigmoid shape and was further fitted using non-linear regression:

$$\text{act} = 100 - \frac{100 \times [H]}{I_{50} + [H]}$$

where $\text{act}$ represents residual activity of the PSII-polySBQ-AuWE (in percent) after addition of the herbicide, $[H]$ is the herbicide concentration in the solution, and $I_{50}$ is the herbicide concentration causing a $50\%$ inhibition of the activity. The LOD was calculated as:

$$\text{LOD} = \frac{2.6 \times \sigma \times I_{50}}{100 - 2.6 \times \sigma}$$
where $2.6 \times \sigma$ represents the mean standard error of the measurement increased by factor of 2.6.

Using Eqs. (3) and (4), the respective values obtained for DCMU are: $I_{90} = 9 \times 10^{-9} \text{ M}$ and LOD = $7 \times 10^{-10} \text{ M}$.

Interestingly, the value of LOD is comparable to that one, determined by oxygen Clark electrode based biosensor (LOD = $5 \times 10^{-10} \text{ M DCMU}$) (Koblížek et al., 1998) where PSII is free in solution and immobilized only by confinement in dialysis membrane. On the other hand, the LOD is improved compared to biosensor based on immobilized PSII in BSA and GA gel matrix on screen-printed electrodes (LOD = $1 \times 10^{-8} \text{ M DCMU}$) (Koblížek et al., 2002).

Low LOD and $I_{90}$ obtained shows that the Q$_B$ pocket is retained in its natural state without any distortion by the polySBQ layer. Distortion of the protein folding is widely cited to be the main reason for the sensitivity decrease of covalently immobilized proteins, as it is also the case of BSA–GA–PSII (Koblížek et al., 2002). Another advantage of PSII-polySBQ-AuWE electrode is that just a small amount of PSII is immobilized. This advantage becomes clear, when a very low concentration of herbicide is applied, since the high number of PSII centers prevents to achieve a recognizable inhibition. For instance, the average quantity of PSII immobilized in BSA–GA gel (Koblížek et al., 2002) is around $1 \times 10^{-11} \text{ mol}$.

Under such condition, the amount of PSII can be limiting when measuring nanomolar herbicide solutions and can cause the underestimation of the real herbicide concentration. On the contrary, as we have shown in Section 3.3, the amount of PSII present on PSII-polySBQ-AuWE is at least three order lower ($1.8 \times 10^{-14} \text{ mol}$) and so this underestimation should be eliminated. The main disadvantage of the here presented electrodes consists of a lower reusability compared to previously published ones. For example, Koblížek et al. (1998, 2002), showed the possibility to regenerate almost 100% of electrode activity by washing out the DCMU from previously inhibited electrodes, and to use one single electrode in several experimental runs. In our case, this was not possible and only up to 70% of activity can be restored, following the strong washing process and reloading with PSII. On the other hand this disadvantage can be balanced by increased sensitivity of sensor and lower LOD. Moreover, once the DET between PSII and electrode is established, similar systems can be studied with the aim to eliminate such drawback and retain the advantages of fast, sensitive and diffusionless PSII biosensor system.

4. Conclusion

A conductive polymer polySBQ was synthesized on the surface of:

(i) gold screen-printed electrode,
(ii) gold microelectrodes in an array on silicon substrate,

by means of a simple electro-oxidation process. The basic redox and morphology properties of polymer film were determined using respectively electrochemical methods and AFM.

Photosystem II isolated from thermophilic bacteria *Synechococcus bigranulatus* was immobilized on polySBQ-AuWE by means of physical adsorption. Resulting PSII-polySBQ-AuWE showed photoinducible DET at the potential of $E = +250 \text{ mV}$ versus Ag/AgCl. Basic properties of that process were studied. As a result, we have shown that:

(i) PSII active in DET is immobilized in form of a monolayer; (ii) the charge transport from PSII to gold WE is fast and dominated by the rate of the PSII enzymatic reaction; (iii) polySBQ layer drains electrons from the Q$_A$ pocket of the PSII since the electrode activity is inhibited by specific inhibitor, DCMU; (iv) the stability of the PSII-polySBQ-AuWE is comparable to the stability of PSII in solution under the same experimental conditions; (v) the inhibition of PSII-polySBQ-AuWE by herbicide DCMU follows the sigmoid dependence; (vi) $I_{90}$ as well as LOD shows an improved sensitivity compared to other published biosensing systems using PSII as bioactive part; (vii) certain disadvantage is the poor reusability of electrodes which on the other hand is balanced by above mentioned favourable features.

Acknowledgments

This work was supported partly by COSMIC Project (ENEA Target Project on Biosensors and Bioelectronics), by the project 522/00/1274 of Grant Agency of the Czech Republic, by the project IBIS of Czech Ministry of Industry and Trade and by a grant from TRIL Program of the International Centre for Theoretical Physics, Trieste.

References


