A Biosensor for the Detection of Triazine and Phenylurea Herbicides Designed Using Photosystem II Coupled to a Screen-Printed Electrode

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Abstract: A biosensor for the detection of triazine- and phenylurea-type herbicides was constructed using isolated Photosystem II (PS II) complexes as a biosensing element. PSII isolated from the thermophilic cyanobacterium *Synechococcus elongatus* was immobilized on the surface of a screen-printed sensor composed of a graphite working electrode and Ag/AgCl reference electrode deposited on a polymeric substrate. The biosensor was mounted in a flow microcell with illumination. The principle of the detection was based on the fact that herbicides selectively block PSII electron transport activity in a concentration-dependent manner. Changes of the activity were registered amperometrically as the rate of photoreduction of an artificial electron acceptor. The setup resulted in a reusable herbicide biosensor with a good stability (half-life of 24 h) and limit of detection of approximately \(10^{-9}\) M for diuron, atrazine and simazine.

INTRODUCTION

Massive use of herbicides in agriculture over the last several decades has become a serious environmental problem. The pollution of soil and water in many agricultural areas represents an important risk factor as a result of the toxicity or carcinogenic nature of some of these compounds.

Herbicides inhibiting photosynthesis via targeting Photosystem II (PSII) function still represent the basic means of weed control. This group consists of several classes of chemicals such as triazines (e.g., atrazine [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine], simazine [2-chloro-4,6-bis(ethylamino)-1,3,5-triazine], cyanazine), phenylureas (linuron, diuron), or phenols (e.g., ioxynil, bromoxynil, Draber et al., 1991). Triazine herbicides continue to be used every year in large quantities. For example, in the United States, approximately about \(35 \times 10^6\) kg of atrazine, \(9 \times 10^6\) kg of cyanazine, and \(3 \times 10^6\) kg of simazine are applied every year (U.S. Environmental Protection Agency http://www.epa.gov/oppbeadl/pestsales/97pestsales/table8.htm).

This practice frequently leads to soil contamination and the subsequent pollution of surface and ground water. Triazines persist in water and represent some of the most frequently detected pesticides in ground water.

These concerns have stimulated research towards development of sensitive methods and technology to detect pesticide residues. Chromatographic methods high-performance liquid chromatography, gas chromatography...
and gas chromatography/mass spectrometry are reliable and are most commonly used to monitor the presence of herbicides (Pacáková et al., 1996). The requirement for expensive equipment, organic solvents, and laborious sample preparation hampers the use of chromatography for rapid screening of a large number of samples. For this reason, attention has been focused on immunological methods for herbicide detection (Bushway et al., 1991) offering high sensitivity and specificity of detection (Giersch, 1993; Schneider et al., 1994; Schneider and Hammock, 1992). The disadvantage of the immunological methods, however, is that the antibodies bind specifically to only one compound or a few structural analogues. Moreover, their preparation is difficult.

Recognition of the fact that a large number of herbicides inhibit PSII activity (Moreland, 1992) has resulted in its use as an analytical tool (bioassay) for designing of new compounds (Good, 1961; Wessels and Van der Veen, 1956; or detecting herbicides (Brewster and Lightfield, 1993; Conrad et al., 1993; Giardi et al., 2000; Loranger and Carpentier, 1994; Merz et al., 1996; Rouillon et al., 1995; Soukupová et al., 1999). Biosensors are analytical devices possessing a biological component that provides high sensitivity and/or specificity and a physicochemical transducer recording the signal (Turner, 2000). Biosensor technology has created new ways for simple analysis in a cost-effective manner.

Previously, we constructed a biosensor by immobilization of PSII complex on a Clark oxygen electrode. The system exhibited a good stability at laboratory temperature as well as high sensitivity to herbicides (Koblížek et al., 1998). However, the use of Clark electrode as the transductor prevents simple and potentially mass production of this system. For this reason, screen printing was chosen as suitable technology offering batch production of electrochemical biosensors with high reproducibility at low price. The electrochemical sensors are made by sequential, multi-layer deposition of metal conductors (Pt, Au, Ag, Pd, Rh, Ru, Ti), dielectric insulators (Al or Zr oxide powders), and polymeric pastes on inorganic (Al2O3 ceramic) or polymeric [polyvinylchloride (PVC)] substrates. The layout drawn on the open mesh of a screen is transferred onto a substrate, providing two-dimensional microcircuits and electrodes (Karlberg and Pacey, 1989; Prudenziati, 1994; Scheller and Schubert, 1992).

In this article, we present a herbicide biosensor based on isolated PSII particles immobilized to a graphite-Ag/AgCl screen-printed electrode. The system exhibits selective sensitivity to phenylurea and triazine herbicides, whereas phenolic herbicides are not registered. The use of screen-printed electrodes allows a cheap, large-scale production of these devices.

**MATERIAL AND METHODS**

**Printed Electrodes**

Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate (Fig. 1). The conducting paths and pads were deposited directly on the PVC sheets using the Ag/Pd ink (DuPont, 5025). The second layer was printed with graphite (DuPont 7101, Acheson 423S) to obtain the working electrode. For the reference electrode, the metallic layer was printed using the same Ag/Pd ink as used for the conducting paths. Finally, an insulator layer was placed over the conducting paths. After each printing step, the pastes were left overnight at laboratory temperature or treated at 80°C for 20 min.

**Preparation and Immobilization of PSII Particles**

The PSII particles were prepared from the thermophilic cyanobacterium *Synechococcus elongatus* (cells grown at 56°C) as described previously (Koblížek et al., 1998; Šetlíková et al., 1999). After cells were digested with lysozyme, the protoplasts were disrupted by osmotic shock. Thylakoid membranes were solubilized with the non-ionic detergent heptylthioglucoside using a detergent/chlorophyll (Chl) ratio of 8. After ultracentrifugation at 250,000 g for 30 min, the supernatant of the PSII particles usually contained between 200 and 400 mg Chl L⁻¹.

To assure a firm coupling of the PSII particles to the electrode surface, several immobilization techniques were tested, the first of which was entrapment into gelatin. Gelatin (10%) in the measuring buffer [15 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, containing 0.5 M mannitol, 0.1 M NaCl, 5 mM MgCl₂, and 5 × 10⁻⁵ M chloramphenicol] was left for several hours to swell. The mixture was warmed up to about 50°C and intensively mixed with an equal volume of the PSII preparation. Then, about 5 μL of the mixture was placed on the surface of the working electrode. The second technique used was entrapment into agarose.
The procedure was the same as the previous one, except that 3% agarose (low melting point; Sigma Chemical Co., St. Louis, MO.) was used instead of gelatin. The third technique used was entrapment into calcium alginate. The immobilization procedure was similar to the one described by Synkova et al. (1990) and Brewster et al. (1995). A 3.5% solution of alginate was dissolved in the measuring buffer and mixed with an equal volume of the PSII preparation. About 5 µL of the mixture was placed on the surface of the working electrode. Then, 5 µL of 50 mM CaCl₂ was added to solidify the mixture. In the case of calcium alginate immobilization, 20 mM CaCl₂ was included into the measuring buffer. The last technique used was crosslinking into bovine serum albumin (BSA)-glutaraldehyde matrix. The immobilization procedure was similar to that described for chloroplasts and thylakoid membranes (Loranger and Carpentier, 1994; Synková and Šesták, 1991; Thomasset et al., 1988). A 10% solution of BSA in the measuring buffer was mixed with equal volume of the PSII preparation. Then, 10% glutaraldehyde was added to a final concentration of 0.3%. The suspension was mixed and approximately 5 µL was placed on the surface of the working electrode. In all the cases, the prepared sensors were stored in a freezer (−20°C) until used.

**Biosensor Assembly, Flow Cell and Apparatus**

The biosensor was prepared by immobilizing the PSII particles (0.5 to 1 µg Chl, ~10⁻¹¹ mol PSII) on the graphite (working) electrode surface as described above. The graphite electrode was polarized at 0.62 V for duroquinone (DQ; 0.2 mM) or 0.36 V for ferricyanide (FeCy; 1 mM) to the reference Ag/AgCl electrode. Changes of the current intensity on the graphite electrode were registered by a potentiostat (Metrohm 641, Herisau, Switzerland). Impurities were removed during the initial stabilization under the constant potential (20–40 min). No other special (electrochemical) treatment of the electrode surface was used.

The biosensor was mounted into a custom-made flow cell with the illumination provided by a single light-emitting diode (ultrabright, peak wavelength at 650 nm) controlled by an electronic timer. The duration of light pulses was set to 5 s at the light intensity of about 100 µmol photons m⁻²·s⁻¹. The volume of the flow-cell was adjusted by a silicon spacer (0.5 mm) as shown in Figure 2. The electrode was continuously washed with the measuring buffer (see above) plus an electron acceptor. The flow of the buffer (0.25 mL·min⁻¹) was driven by a peristaltic pump (type MiniPulse 3, Gilson).

**RESULTS**

**Method of Measurement**

The measurement of the PSII activity was based on potentiostatic (amperometric) registration of the reduced form of artificial electron acceptors, DQ, or FeCy. The acceptor was present in the measuring buffer in its oxidized form. Thus, there was only a small background signal in the dark (about 20 nA), which was more stable (low noise) as compared to the biosensor based on Clark oxygen electrode previously described (Kobližek et al., 1998). Under illumination, the immobilized PSII complex splits water, releases oxygen, and reduces the artificial electron acceptor. The reduced electron acceptor (DQ, FeCy) was re-oxidized on the surface of the graphite working electrode. The resulting increase of the current was proportional to PSII activity. Response signals were about 5 nA when using 0.2 mM DQ or 15 nA for 1 mM FeCy used as electron acceptors.

**Immobilization and Stability Optimization**

Immobilization ensures the spatial coupling of the PSII preparation with the transduction system. We tested four immobilization techniques: entrapment in agarose, alginate, or gelatin gels and cross-linking into the BSA–glutaraldehyde matrix. The immobilization techniques were first characterized by the half-life of the constructed biosensor. The entrapments in agarose and alginate failed in this test because these gels exhibited only poor adhesion to the electrode surface and were therefore washed out rapidly. Better results were obtained
when the PSII particles were entrapped in gelatin or cross-linked into the BSA–glutaraldehyde matrix. Both the latter techniques provided biosensors with a half-life of about 8 h in the presence of DQ (Table I). On the other hand, the half-lives of the biosensors were much shorter than 30 h for the PSII preparation itself (Koblížek et al., 1998). The lower stability of the sensors was probably caused by Ag⁺ ions released from the reference electrode, which are toxic for the PSII activity. This assumption was verified by spatial separation of the working and reference electrodes, placing the latter one down-stream to the flow cell. Thus, the Ag⁺ ions released from the reference electrode do not come into contact with the PSII particles immobilized on the working electrode. Indeed, when set-up this way, the half-life of the biosensor was extended to 24 h, which was similar to that of the PSII preparation (Table I).

Table I. Comparison of immobilization techniques.

<table>
<thead>
<tr>
<th>Immobilization method</th>
<th>Half-life (h)</th>
<th>Herbicide detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate (coupled electrodes)</td>
<td>&lt;1</td>
<td>Not tested</td>
</tr>
<tr>
<td>Agarose (coupled electrodes)</td>
<td>&lt;1</td>
<td>Not tested</td>
</tr>
<tr>
<td>Gelatin (coupled electrodes)</td>
<td>7.5</td>
<td>Not reproducible</td>
</tr>
<tr>
<td>BSA–glutaraldehyde (coupled electrodes)</td>
<td>8</td>
<td>Reproducible</td>
</tr>
<tr>
<td>BSA–glutaraldehyde (separated electrodes)</td>
<td>24</td>
<td>Reproducible</td>
</tr>
</tbody>
</table>

Stability was characterized as half-life of the biosensor activity at laboratory temperature. The measurement was performed at 25°C in the presence of 0.2 mM DQ. The reproducibility of the detection was tested by sequential measurements of a sample containing 10⁻⁷ M of diuron.

Detection of Herbicides

The presence of herbicides in a solution was detected as a decrease of the biosensor signal in the presence of DQ compared to the activity in the absence of the herbicide. Using a three-way valve, it was possible to switch among the buffer and the sample/standard reservoirs. When the sample (about 5 mL) containing herbicide (diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], atrazine, simazine, ioxynil [4-hydroxy-3,5-diiodobezonitrile], bromoxynil [3,5-dibromo-4-hydroxybenzonitrile], or dinoseb [2-secbutyl-4,6-dinitrophenol]) was loaded into the cell, the resulting light-induced activity was measured after 20 min (see Fig. 3). The ratio of the signals (expressed in percent) in the presence and absence of herbicide was plotted against herbicide content in the sample (Figs. 4 and 5). The measured value was corrected for about 1% decline in the sensor activity over the 20 min measurement due to its natural decomposition (24 h half-life). Then, the herbicide was washed out using the measuring buffer and the biosensor used for a new measurement. The repeated use of the biosensor did not affect the reproducibility of the measurement when the BSA–glutaraldehyde immobilization was used. This, however, was not possible in case of the immobilization into gelatin (see Table I). Therefore, only the BSA–glutaraldehyde immobilization was used for further measurements.

Calibration curves for each herbicide were analyzed using the Langmuir adsorption isotherm:

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

where act is residual activity of the biosensor (in percent) after addition of the herbicide, |H| is concentration of the herbicide in the solution, and I₅₀ is the concentration causing a 50% inhibition of the activity (equal to the PSII–herbicide complex dissociation constant). The limit of the detection (LOD) was determined on the basis of 99% confidence interval, which, assuming the normal distribution, corresponds to 2.6 × standard error of the measurement (σ). Then, using the modified relationship for the Langmuir adsorption isotherm LOD was calculated as

\[ \text{LOD} = 2.6 \times \sigma \times I_{50}/(100 - 2.6 \times \sigma) \]

Figure 3. Principle of herbicide detection. The presence of herbicides was detected from a decrease of the biosensor signal in the presence of DQ. Firstly, the dark signal after the LED pulse was recorded (left hand dashed arrow) in the absence of herbicide. Then, the sample (about 5 mL) containing a herbicide (10⁻⁷ M atrazine, indicated by the solid, thick arrow) was loaded into the cell, and the signal induced by the light pulse from the LED was recorded. The ratio of the signals in the presence (right-hand dashed arrow) and absence (left-hand dashed arrow) of the herbicide was calculated as a measure of concentration.
The results of the analysis are shown in Table II.

Using the BSA–glutaraldehyde immobilization system, an important observation was made. The system was found sensitive to classical herbicides (diuron, atrazine, simazine) but it had only low sensitivity to phenolic-type herbicides (dinoseb, ioxynil, bromoxynil). The detection limit for phenolic herbicides was more than an order of magnitude higher when compared with the system using the physical entrapment of the PSII particles (Table II; Kobližek et al., 1998).

The herbicide detection was also tested in the presence of FeCy as an artificial electron acceptor. In this case, the biosensor exhibited about three times higher signals as compared to the signals obtained with DQ. The calibration curve for atrazine showed that about 50% of the biosensor activity could not be blocked even at very high herbicide concentrations. It was found that one half of the activity was herbicide-sensitive and could be characterized by the Langmuir isotherm (I$_{50}$ = $10^{-7}$ M), and the other part of the activity was herbicide insensitive (Fig. 5). Because this feature reduced the amplitude of the signal measured and, in this way affected the sensitivity of the assay, only DQ was considered well suited for the measurements.

**DISCUSSION**

We have developed a sensitive biosensor for detection of classical photosynthetic herbicides. The biosensor was constructed using screen-printed electrodes with immobilized PSII particles as the biosensing element. The use of PSII preparation from *S. elongatus* assured higher stability of the biological part of the biosensor (Kobližek et al., 1998). The PSII activity was measured as a rate of the photoreduction of an artificial electron acceptor.

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**Table II.** I$_{50}$ dose and LOD determined for various herbicides.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Screen-printed sensor</th>
<th>Clark electrode$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I$_{50}$ (M)</td>
<td>LOD (M)</td>
</tr>
<tr>
<td>Classical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuron</td>
<td>$7 \times 10^{-8}$</td>
<td>$1 \times 10^{-9}$</td>
</tr>
<tr>
<td>Atrazine</td>
<td>$9 \times 10^{-8}$</td>
<td>$2 \times 10^{-9}$</td>
</tr>
<tr>
<td>Simazine</td>
<td>$2 \times 10^{-7}$</td>
<td>$4 \times 10^{-9}$</td>
</tr>
<tr>
<td>Phenolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ioxynil</td>
<td>n.d.</td>
<td>$\sim 10^{-7}$</td>
</tr>
<tr>
<td>Bromoxynil</td>
<td>n.d.</td>
<td>$\sim 10^{-6}$</td>
</tr>
<tr>
<td>Dinoseb</td>
<td>n.d.</td>
<td>$\sim 10^{-6}$</td>
</tr>
</tbody>
</table>

$^a$Data from Kobližek et al. 1998.

n.d. = not determined.

The biosensor activity was measured in the presence of 0.2 mM DQ at 25°C. I$_{50}$ represents a herbicide concentration causing a 50% inhibition of the initial biosensor activity (in the absence of herbicide). The data for the screen-printed sensor (this work) were obtained by fitting the experimental points using the Langmuir adsorption isotherm. In this model, the I$_{50}$ value is equal to the dissociation constant of herbicide. The detection limits were determined on basis of the 99% confidence interval calculated from the standard error of the measurement. For comparison, the data reported previously for the Clark electrode sensor are shown in the right part of the table (Kobližek et al., 1998).
(DQ), which was registered as an increase in the response to light pulses.

The close coupling of the biosensing element (PSII) and the transducer (electrode) improved the measured signal and required smaller amount of the biological material in comparison with standard batch detection systems. The use of the flow-through set-up made it possible to re-use the biosensor for several analyses after washing out the inhibitor. Among the four immobilization techniques tested, only cross-linking into the BSA–glutaraldehyde matrix provided a convenient system in terms of stability and reproducibility of the measurement. The system displayed good sensitivity to classical herbicides (diuron, atrazine and simazine) but significantly reduced sensitivity to phenolic herbicides (ioxynil, bromoxynil, and dinoseb). This feature was likely caused by a very low diffusion rate of the phenolic herbicides in the BSA–glutaraldehyde matrix or a steric hindrance and modified specificity of the cross-linked PSII. When the BSA–glutaraldehyde cross-linking was replaced by the gelatin entrapment, the inhibitory effect of phenolic herbicides was restored (data not shown). On the basis of different sensitivity, the classical and phenolic herbicides can be distinguished by this biosensor system.

In general, the sensitivity and the detection limit of the biosensor are given by the binding constant of the sensor system. The binding constant (I50) remained practically unchanged, it seems that the affinity of PSII towards atrazine observed in our case, further modulated by the characteristics of the immobilization system. At low concentrations of herbicides the recorded decrease of the PSII activity is minimal. Thus, the maximal signal-to-noise ratio is necessary. In the system described here, this goal was achieved by two ways. First, we used isolated PSII particles with higher specific activity compared to more frequently used chloroplasts or thylakoid membranes. Second, the PSII particles were immobilized on the surface of the transducer (electrode). This set-up assures a high concentration of PSII complexes in the micro-environment around the electrode, which in turn, results in a good, stable signal. This approach also made it possible to use a very low amount of PSII (∼10⁻¹¹ mol). The Langmuir adsorption isotherm assumes an excess of the free compound with respect to the number of binding places. At very low herbicide concentrations this assumption is not always met and the number of herbicide molecules in the sample starts to be limiting (5 μL of 1 nM herbicide corresponds to 5 × 10⁻¹² mol). In this case, the calibration curve deviates from the model and the signal underestimates the true herbicide concentration. Thus, the amount of PSII used in the assay is critical for detection of very low herbicide concentrations.

Other substances (e.g., heavy metals) can also inhibit the activity of the PSII biosensor; however, their effect is usually found at much higher concentrations than those typical for herbicides. For instance, copper, one of the most phytotoxic heavy metals (Küpper et al., 1996), starts to inhibit the biosensor activity at concentrations above 10⁻⁶ M. This concentration is by two to three orders of magnitude higher than the limit of detection found for herbicides. Moreover, contrary to herbicides, the inhibition of the PSII activity by Cu²⁺ ions is irreversible and therefore, it can be distinguished by its distinct kinetic behavior (Rizzuto and Kobližek, unpublished data).

An interesting finding was the incomplete inhibition of PSII activity of the biosensor by atrazine observed in the presence of FeCy (Fig. 5). Because the binding constant (I50) remained practically unchanged, it seems that the affinity of PSII towards atrazine did not change. Similar phenomenon was observed earlier with isolated thylakoid membranes from a thermophilic cyanobacterium (Yamaoka et al., 1978). A possible explanation is, that, in the presence of FeCy, there exists an alternative herbicide-insensitive pathway of PSII reoxidation bypassing the Qb pocket (herbicide binding site) and operating at 50% of the rate in the absence of herbicides.

In conclusion, the developed biosensor offers the sensitive detection method for triazine and phenylurea herbicides. In comparison to other analytical techniques, such as chromatography and immunological methods, the PSII-based biosensors have opened a possibility to be used for rapid field detection of herbicides, which in addition allows assessment of their biological effect (phytotoxicity). They can be successfully applied for prescreening environmental samples, indicating which samples require detailed analysis by other analytical techniques.

References


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