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High sensitivity biosensor measurement based on synchronous detection

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ABSTRACT

The principle of synchronous detection (SD) has been applied to biosensor measurement. SD principle achieves significant increases in the signal to noise ratio, limit of detection and overall measurement robustness. Application of SD in biosensor measurement improves the analysis of the response and avoids the influence of interference/noise produced by stirring, electromagnetic effects and influence of parasitic currents. SD also enables the decomposition of signal to stimulation response and phenomena with long time of response. Second-order phenomena are identifiable in the signal. Linear statistical model was used to develop software for identification of the stimulation signal in the output current. SD was applied to the response signal of a Photosystem II complex (PSII) biosensor. PSII response to light stimulation follows first order kinetics. The inhibition kinetics of PSII has been studied. Kinetic constants of herbicide binding to PSII depend linearly on herbicide concentration and enable measurement of its concentration at low concentrations (linear range for diuron is 10^{-6} to 10^{-4} mM).

1. Introduction

In many experiments it is possible to use some "a priori" knowledge about measurement. An example of such a priori knowledge is frequency of stimulation, for example "chopper" speed in optical applications. It is possible to average the signal due to its synchronization with stimulation. The superimposed white noise decreases its amplitude as well as the signal drift is removed. The general source of noise are external electromagnetic fields. In case of electrochemical measurements, which are the prime objective of this article, the main source of noise is instability of mass transport between electrode surface and bulk of solution. The role of mass transport is generally underestimated in electrochemical sensors use [1].

Such application of the synchronous detection (SD) method in electronics and signal evaluation has inspired a similar approach in this work. The "a priori" knowledge is enforced by external stimulation of bioactive part of sensor. This knowledge enables to build sophisticated methods of signal analysis. The new SD method of signal analysis is demonstrated on signals of PSII biosensor.

The output signal of the biosensor is modulated by kinetics of PSII reaction with light both in linear and nonlinear manner. The demodulation of the signal enables the true response of the biosensor. Starting from the 1980s, microscale tests using photosynthetic biosensors based on cellular or subcellular material (e.g. algal cells, chloroplasts, thylakoid membranes or the PSII complex, core or reaction center) have been developed for herbicide detection (for overview see Refs. [2,3]). The binding of the photosynthetic herbicides to the PSII core offers good possibilities to use these preparations as biosensing elements in biosensors with various methods of detection, e.g. measurement of electron transport rate, oxygen evolution, or chlorophyll fluorescence [4–13]. The published signal was improved by analog filtration [7]. This method is implicitly involved in all standard measuring devices used in sensor experiments.

2. Theory

Let us suppose we have an enzymatic electrochemical biosensor with a working electrode of diameter d. On the electrode there is an immobilized layer of bioactive compounds of thickness h. The bioactive layer contains n bioactive centers, that catalyze the reaction of the biosensor with the analyte. The response of each bioactive center is f_j, where $j = 1, \ldots, n$. If phenomena on the working electrode are measured with a time resolution lower than $t_0 = h^2/2/D$ (see [14]), where D stands for the diffusion coefficient of the analyte in the membrane, then all the bioactive centers can be assumed to be in similar conditions – the concentration of the analyte in the membrane will be nearly at equilibrium. The diffusion coefficient of small molecules, such as a mediator or inhibitor, is lower than 1000 Da. The diffusion coefficient in polymeric membranes with standard thickness of a bioactive membrane about 1–10 μm is of the order from 10×10^{-12} to

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100×10^{-12} m^2 s^{-1} (see [15]). Under such conditions, t_0 is typically 5–5000 ms. For the measurements with a time resolution of 100 ms, it can be assumed that all the bioactive centers are under the same conditions, while as the thickness of the immobilized membrane is about 1 μm and a high diffusion coefficient 10^{-10} m^2 s^{-1} of analyte in the immobilized layer is achieved.

The difference between the activity of individual bioactive centers is caused by differences in their immobilization, ambient conditions, local pH fluctuations and many other influences [16]. On the other hand, the external influences are the same for all bioactive centers. The measurement response f_r is expressed as a sum of each active center contribution

$$f_r = \sum_{j=1}^{n} f_j. \tag{1}$$

The average response of one bioactive center is

$$\bar{f} = \frac{1}{n}\sum_{j=1}^{n} f_j \tag{2}$$

and the measured response can be expressed by the average bioactive center response

$$f_r = n\bar{f}. \tag{3}$$

The inhibition can affect the measured response of the sensor f_r in two possible ways:

Model 1: The number of bioactive centers remains unchanged but the properties of individual centers change. This situation is described by the equation

$$\bar{f} \rightarrow \bar{f}_i. \tag{4}$$

Model 2: The number of free bioactive centers is changed

$$n \rightarrow n_i, \tag{5}$$

but the average response $\bar{f}$ of a single active center is preserved (n_i is number of free active centers in presence of inhibitor). This last process is dynamic. Active sites are inactivated when the active centers are occupied by an inhibitor, and by the release of the inhibitor they can be reactivated. The model 2 is more frequently accepted. The two simplest submodels of model 2 are reversible and irreversible inhibition.

(a) *Irreversible inhibition* n initially free bioactive centers transfer to the final state where n_i centers are free and $(n - n_i)$ are occupied by inhibitor. The irreversible reaction is described by the equation

$$n \xrightarrow{k_1'} n_i, \tag{6}$$

where k_1' is the irreversible inhibition rate constant. This rate constant depends on the number of molecules of inhibitor in the close proximity of an active center. The scheme can be expressed by the differential equation

$$\frac{dn_i}{dt} = -k_1' n = -k_1 i n_i, \tag{7}$$

where i is the number of inhibitor molecules in close proximity of active centers. The initial condition can be written as $n = n_0$, where n_0 is the initial amount of free active centers. The solution of Eq. (7) can be expressed in concentrations as

$$C_{n_i}(t) = e^{-k_1 C_i t}, \tag{8}$$

where $C_{n_i}(t) = \frac{n(t)}{n_0}$ is the concentration of free active centers at time t and C_i is the molar concentration of the inhibitor in the bioactive membrane.

(b) *Reversible inhibition*A change in the number of free bioactive centers is described by the equation

$$n \underset{k_{-1}}{\overset{k_1'}{\rightleftarrows}} n_i. \tag{9}$$

This scheme can be described by the differential equations

$$\frac{dn_i}{dt} = -k_1' n_i + k_{-1}(n_0 - n_i), \tag{10}$$

where k_1' is the rate constant of transition from state n to state n_i and k_{-1} describes the transition of the inactive center to its active state. The rate constant $k_1' = k_1 C_i$ depends on the number of molecules of inhibitor in the close proximity of an active center. The solution can be expressed in concentrations as

$$C_n(t) = \frac{1}{k_1 C_i + k_{-1}}(k_{-1} + k_1 C_i e^{-(k_1 C_i + k_{-1})t}). \tag{11}$$

2.1. SD method implementation

The measured response f_r is influenced by additive a and multiplicative α experimental influences. If the multiplicative influences are not changed by addition of inhibitor, then the typical experiment can be expressed as follows:

$$\begin{aligned} f_r &= n\bar{f}\alpha + a \quad \text{the response without inhibitor,} \\ f_{ri} &= n_i\bar{f}\alpha + a_i \quad \text{the response with inhibitor,} \end{aligned} \tag{12}$$

where n_i is the number of free bioactive centers when the measurement is influenced by the inhibitor.

The unknown average response of signal active center can be eliminated from Eq. (12), if model 2 is assumed to be valid

$$f_{ri} = \frac{n_i}{n} f_r - \frac{n_i}{n} a + a_i. \tag{13}$$

The Eq. (13) can be rewritten in the form

$$f_{ri} = \frac{n_i}{n} f_r - b\left(\frac{n_i}{n}, a, a_i\right) \tag{14}$$

and expressed in concentrations as

$$f_{ri}(t) = C_n(\tilde{t}) f_r(t) - b(C_n(\tilde{t}), a, a_i), \tag{15}$$

where the tilde above the t emphasizes the fact that the time scales for f_r and C_n are different. Eq. (15) is a linear model with respect to f_r. The linear correlation $f_r(t)$ and $f_{ri}(t)$ gives the values of $C_n(\tilde{t})$ and $b(C_n(\tilde{t}), a, a_i)$.

$C_n(\tilde{t})$ in Eq. (15) can be expressed by Eqs. (8) or (11), where time t is replaced by $\tilde{t}$. The model is a general one and independent of Eqs. (7) and (10). The Eq. (15) also helps evaluate more complicated models of reaction kinetics can be evaluated.

If the influence of the inhibitor on an active center is linear, i.e. $\bar{f}_i = \alpha\bar{f}$, it is impossible to distinguish between model 1 and 2. This ambiguity is not important because both models will be isomorphic in this case.

2.2. Analysis software

The implementation of the method needs quite big amount of calculations. All calculations are integrated in a computer program which allows routine use of method. The program consists of following blocks:

- construction of pattern signal f_r;
- the pattern signal is correlated with signals in presence of inhibitor. Unknown signals are synchronized with pattern signal and principle of SD is applied; and
- kinetics of inhibitor reaction $\left(\frac{n_i(\tilde{t})}{n_0}\right)$ is displayed and evaluated.

3. Materials and methods

3.1. Biosensor system

The oxygen evolving PSII-complex preparations (Institute of Microbiology, Trebon, Czech Republic) were isolated from the thermophilic cyanobacterium *Synechococcus elongatus* (strain Kovrov 1972/8 grown at 56 °C) using a non-ionic detergent, heptylthioglucoside, according to the procedure described previously [7,17]. The suspension of isolated PSII particles was thoroughly mixed with a solution of bovine serum albumine and glutaraldehyde. A 1-μl drop of this mixture was placed on the surface of the Pt-working electrode AC1.W2.RS (BVT Technologies, a.s., Czech Republic, www.bvt.cz) and left for polymerization under dimmed green light at 5 °C for 1 h.

The sensors with immobilized PSII particles were inserted in micro flow system MFS equipped with flow cell FC3 (BVT Technologies, a.s., Czech Republic, www.bvt.cz). This device assures reproducible mass transfer between bioactive sensor layer and measured solution. The measuring system is displayed in Fig. 1 and the characteristics of sensor chamber (dependence of conversion rate on flow, dimensions of microflow channel) can be found at FC3 data sheet. The measurements were performed at 25 °C by single device developed in project IBIS. The amperometric analyzer Biosensor Toxicity Analyser BA1.1 (BVT Technologies, a.s., Czech Republic, www.bvt.cz) with integrated stimulation circuits was used for data acquisition.

Typical response to light stimulation can be seen in Fig. 2. It consists of a short pulse of red light (5 s), which stimulates signal increase on the electrode. This increase is due to the reduction of duroquinone by electrons being transported through the PSII complex [18]. The light pulse is followed by a 20 s dark period. When the light is switched off, the signal decreases (due to reoxidation of the duroquinone) and returns close to its initial value.

The electric current corresponding to this stimulation was measured [1]. Measured data were evaluated by SD method (as described above) which is implemented in SynDet program.

3.2. Measuring procedure

The sensor with the immobilized PSII complex was inserted into the MFS chamber filled with 10 ml of MES buffer (15 mM MES, 0.1 M NaCl, 5 mM $MgCl_2$, pH 6.8) containing the artificial electron acceptor duroquinone(2×10^{-4} M). The working electrode of the

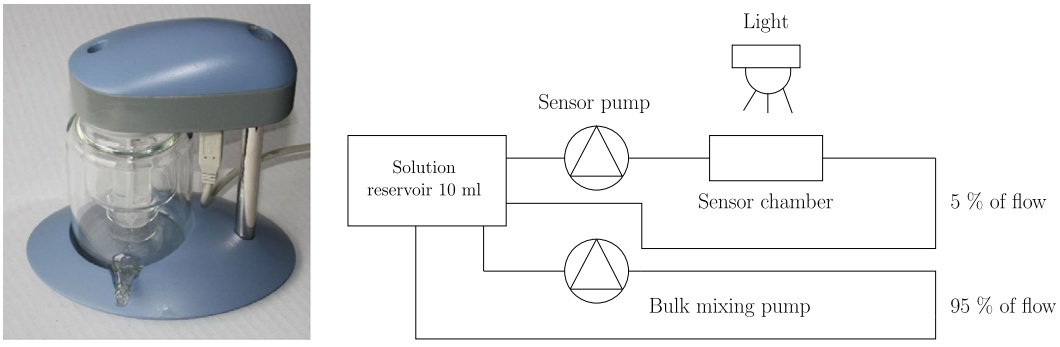


Fig. 1. The microflow system used for experiments and schematics of its functionality.

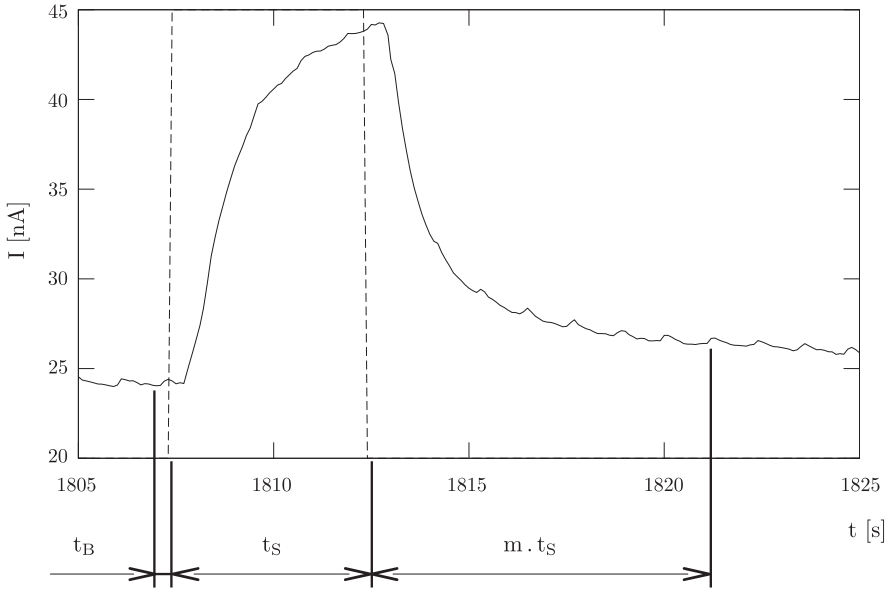


Fig. 2. Response of the PSII biosensor to a light pulse. The model response $f_r(t)$ is constructed from the measured signal (solid line). The current rises after the LED is switched on (dashed line). The response includes the time period before signal t_B, the stimulation signal period defined by the light pulse of length t_S (dashed line) and the period of signal after the stimulation defined by the ratio m to the light-stimulation time t_S. The one-second delay of the current response after the LED signal is due to digital filtration of the measured signal.

sensor was polarized at +620 mV vs. the Ag/AgCl pseudo-reference electrode of the AC1.W2.RS sensor in order to allow the reoxidation of duroquinone [1].

The circulation and sampling pump were switched on for a 10-min dark-stabilisation. After stabilisation, a sequence of m light/dark cycles was applied to the sensor in the solution without herbicide (pattern signal f_r), where m was in the range of 4–25 cycles.

4. Results and discussion

Typical measurement with PSII biosensor is shown in Fig. 3a. SD synchronise the response to light stimulation (Fig. 3b). SD divides the measured signal to fast response corresponding to stimulation Fig. 3d and slow processes connected with PSII particles inhibition Fig. 3c). Fig. 3c shows how the signal with and without inhibitor correlate. It is typically the set of straight lines with decreasing slope (inhibitive).

If Fig. 3c shows set of straight lines, then the model 2 is proved and decomposition to response to stimulation and net inhibition process can be carried out. The more complex lines in Fig. 3c proves that model 2 is insufficient. The response in this particular case consists of a change in response of single free active center and other phenomena such as active center washing out of bioactive membrane.

The normalized response of active center is in Fig. 3d. This data can be exported independently and used to study of mechanisms of stimulation process.

Final dependence of free active centers concentration $\left(\frac{n(t)}{n(0)}\right)$ on time is in Fig. 3f. The method of SD is demonstrated on example of PSII but decomposition procedure is general. SD can be used in

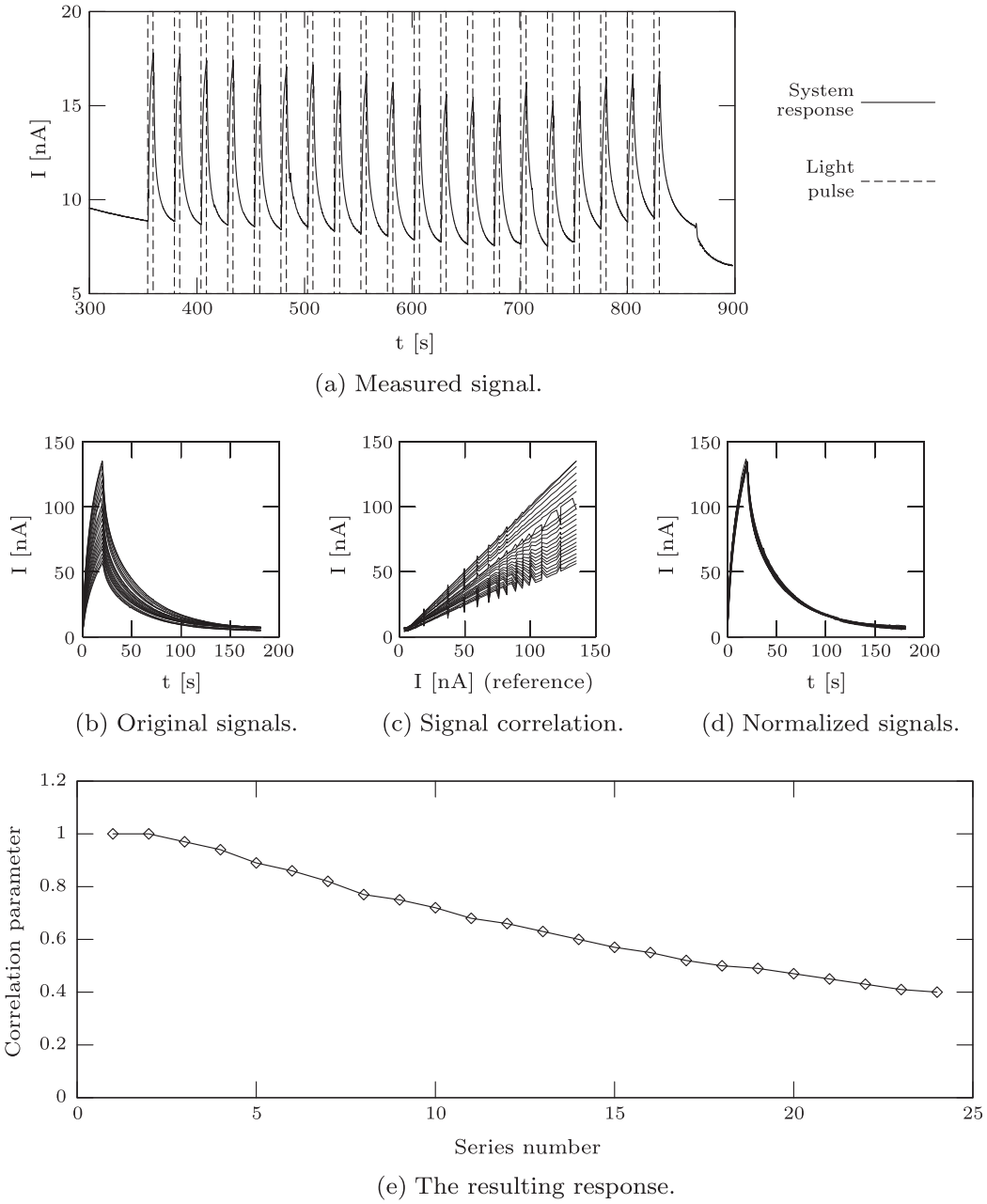


Fig. 3. An example of measured signal with evaluation. Inhibition of PSII causes the signal decrease.

application where two processes are superimposed – the fast response to stimulation and slow response of processes on the background of stimulation.

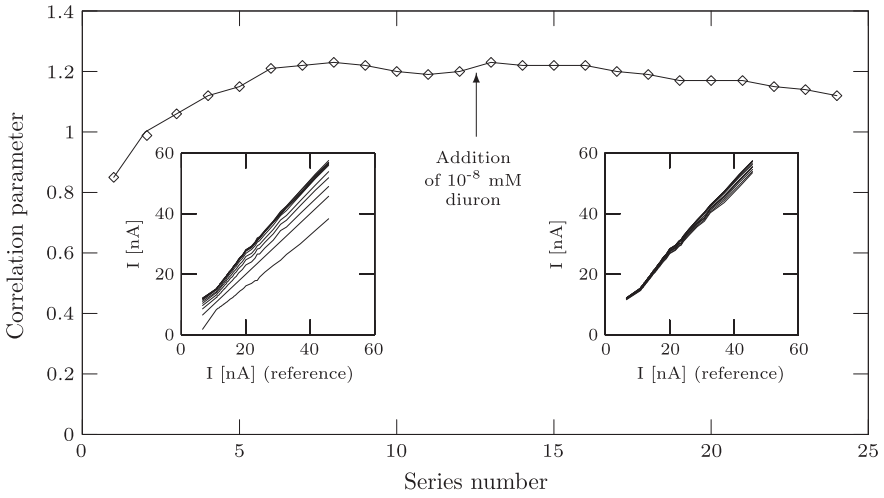
The analysis of the slow process helps to obtain information about the inhibition kinetics. Analysis of the fast process after light stimulation enables a deeper insight into phenomena connected with the average behavior of an PSII active center; these involve diffusion of the mediator, transfer of electrons in the active center, influence of membrane swelling.

The influence of inhibitor on the number of active PSII particles $n_i(\tilde{t})$ is expressed as multiplicative factor. This is confirmed by the fact that the correlation between pattern signal and signal with inhibitor are straight lines and their slopes are decreasing. It confirms the Eq. (12) and enables to analyze the fast response to stimulation independently.

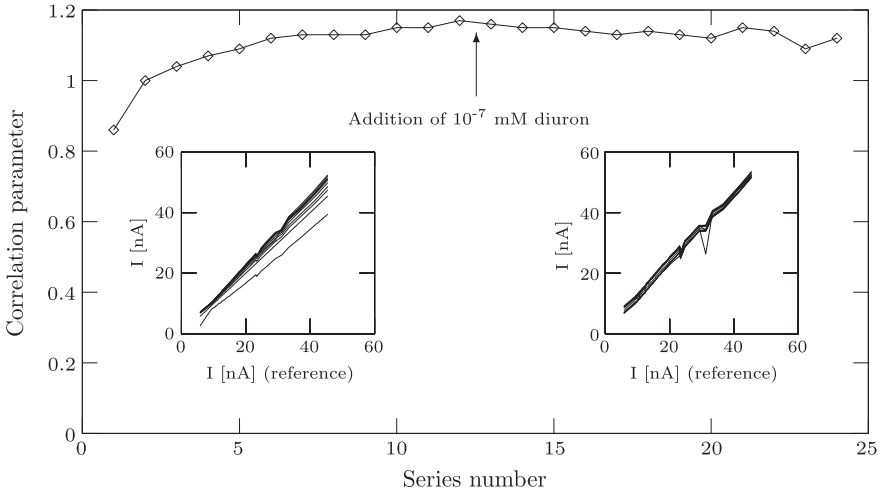
In case of PSII immobilized in thin membrane (1–5 μm), the response to stimulation is 1st order process. The increasing part and decreasing part of response are described by exponential functions. The decline from 1st order process is less than 1%. The linear dependance between pattern and measured signal confirms that process of inhibition is based on a change of number of active centres of PSII.

SD analysis can also help to better understand the details of phenomena influencing the sensor response. As an example, the swelling process of membrane is shown in the left part of Fig. 4a. The output signal rises and the correlation between the reference signal and measured signal are parallel straight lines with a slightly increasing slope. Diffusion coefficient grows due to the membrane swelling. The response of the sensor grows, but the number of active centers remains unchanged as the slope of the curves is almost the same. Small increase of slope is caused by opening the diffusion way to new active centers of PSII.

In Fig. 4b left inserted panel, sensitivity to inhibitor is demonstrated. After addition of 10^{-7} mM diuron the signal decreased. However it decreases as whole at unchanged number of active centers of PSII. The change of signal at low concentration of inhibitor can be caused by other reasons (washing out active centers, their spontaneous inhibition or inhibition by light as can be seen in Fig. 4a right inserted panel addition of 10^{-8} mM diuron).



(a) Decrease in number of active centers caused by inhibition.



(b) Steady number of active centres but other process is involved in reaction.

Fig. 4. The analysis of one PSII measurement. In top part of the Fig. 4a, the left part shows an example of membrane swelling. The right part shows an example of inhibition of PSII active centers by diuron in the concentration 10^{-8} mM. Related correlations for each measurement part are shown in small inserted graphs. Left side of Fig. 4b shows the stabilisation of response without inhibitor. Right inserted panel shows signal decrease after 10^{-7} mM diuron addition. The number of active PSII centers is unchanged. The lines are parallel. The signal however decreases.

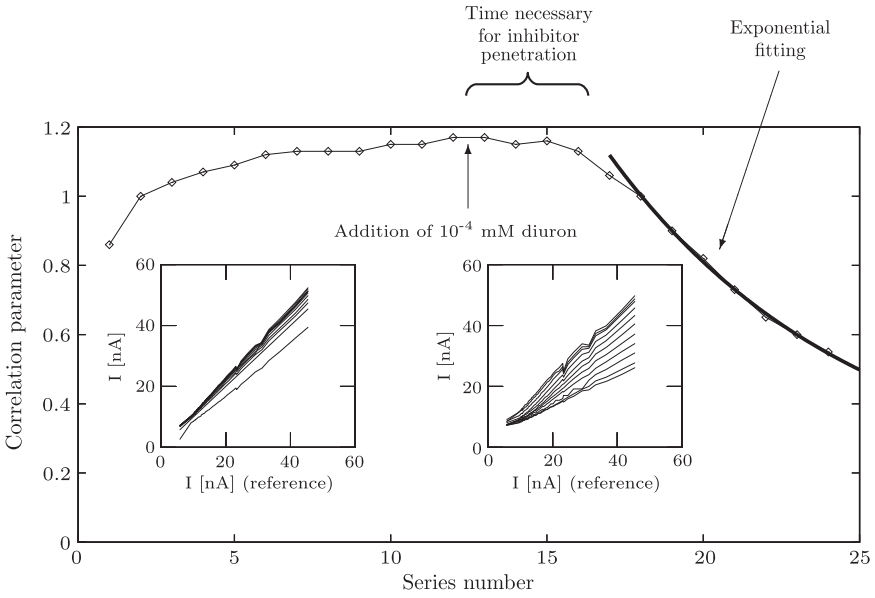


Fig. 5. The analysis of one PSII measurement after addition of diuron. Left part of figure shows stabilisation of membrane without any inhibitor and right part shows a decrease of number of active centers caused by 10^{-4} mM diuron. Related correlations for each measurement part are shown in small graphs at the bottom.

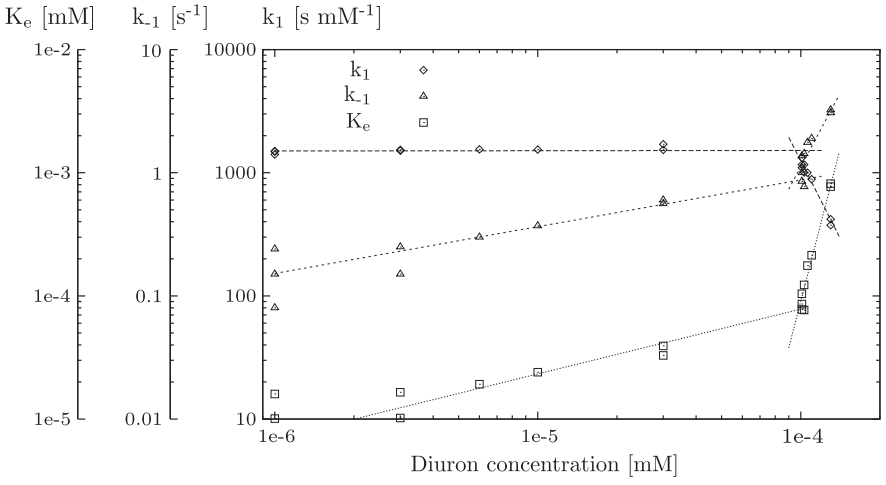


Fig. 6. Dependency of kinetic constants on concentration.

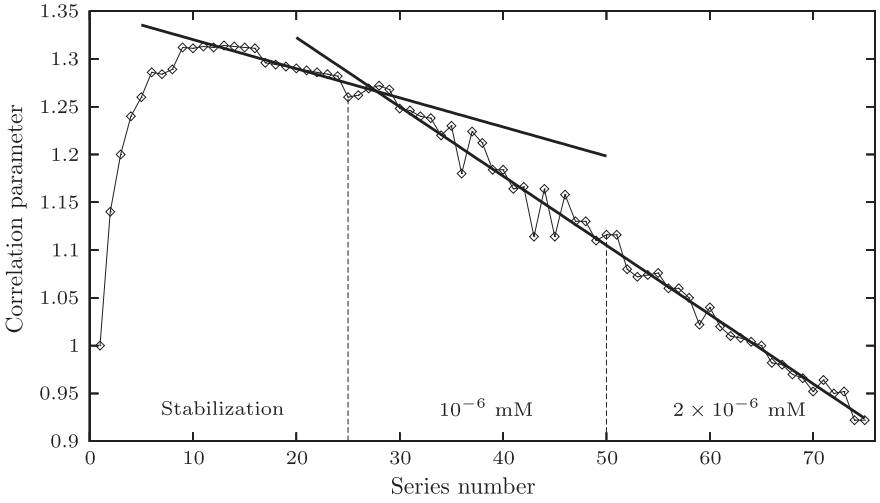


Fig. 7. Experimental results demonstrating the nonlinear response to the inhibitor at its low concentration.

Fig. 5 shows the response to the inhibitor at a concentration of 10^{-4} mM diuron, where the slope has changed significantly. The slope is approximated by exponential function (8) and (11) respectively, and the kinetic coefficients are evaluated. The set of 30 sensors was evaluated at different inhibitor concentrations.

The dependence of kinetic constants of PSII biosensor on concentration is in Fig. 6. If the model (9) describes the function of PSII biosensor, then k_1, k_{-1} and K_e are constants. It was observed that only k_1 is constant in range $10^{-6}, \ldots, 10^{-4}$ mM of diuron. This is in agreement with reaction step $E + I \overset{k_1}{\rightarrow} EI$ where E denotes the PSII concentration. The kinetic constant k_{-1} however depends on the inhibitor concentration. It is in contradiction with simple complex EI decomposition according to $EI \overset{k_{-1}}{\rightarrow} E + I$. The simplest explanation consists of the hypothesis that there is possibility of binding another molecules of inhibitor in the active center or in its proximity and the decomposition of EI_2 complex is faster then EI complex. Then the kinetic scheme of complex PSII + inhibitor can be expressed as follows:

$$\begin{aligned} &E + I \overset{k_1}{\rightarrow} EI, \\ &EI \overset{k_{-1}}{\rightarrow} E + I, \\ &EI + I \overset{k_2}{\rightarrow} EI_2 \overset{k_3}{\rightarrow} E + 2I, \end{aligned} \tag{16}$$

where k_1, k_{-1}, k_2 and k_3 are rate constants.

The fact that the kinetic constant k_1 is independent on inhibitor concentration in range $10^{-6}, \ldots, 10^{-4}$ mM enables to propose regions where the inhibitor can be used for concentration measurement. The constant k_1 can be evaluated by means of inhibitor concentration. Then this constant is used for concentration calculation of unknown sample.

The method was tested on real water samples. Significant improvement of signal to noise ratio enabled precise measurement of inhibitor presence up to concentration of 10^{-8} mM of diuron. However, the mechanics of inhibitor in range of $10^{-8}, \ldots, 10^{-3}$ mM is unstable and unreproducible (not measurement, but the inhibition itself). It seems that main reason of this instability is combination of adsorption and inhibition (see Fig 4b). The signal decreases at unchanged number of active sites. One explanation can be that inhibitor is adsorbed at surface of active layer but it cannot enter inside and create strong binding with the active center. The chemical potential is too low to enable the inhibitor molecule to penetrate into the membrane and occupy the active sites of PSII complex. The typical experimental data are shown in Fig. 7. This confirms the above mentioned range of concentration where the method can be used.

5. Conclusion

The inhibition process which inactivates the active center of enzyme PSII was used to demonstrate the use of synchronous detection (SD). SD enables decomposition of the signal to fast processes connected with electrode transfer, mediator diffusion and substrate reaction and slow processes of inhibition itself.

SD allows to identify the kinetics of the inhibition reaction and the mechanism of the active center response. It is a valuable and powerful tool for exploring the properties of the immobilized active center of enzymes such as PSII. It is possible to study and distinguish between various inhibition mechanisms. SD can also qualitatively distinguish between other processes (swelling of membrane, washing out of active enzyme) and inhibition.

In case of PSII biosensor the dependence of kinetic constant of concentration was found which enables using the inhibition processes for reliable measurement of inhibitor concentration only in range $10^{-6}, \ldots, 10^{-4}$ mM for diuron.

SD offers the possibility of detailed study of immobilization technique influence to reaction kinetics, the temperature influence on reaction kinetics and the deep analysis of inhibitor influence on reaction kinetics. The SD does not depend on the kinetic mechanism. Complicated kinetics of inhibition can be studied by the same signal analysis method. The SD method enables also to study the variance of processes, which can be used for parameters estimation using maximum likelihood method.

There is significant difference between other methods of signal evaluation such as digital filtration regression etc. All these methods are parametric. Their use needs some knowledge about measured object. The SD is analogy of nonparametric methods. The signal without presence of inhibitor is processed as standard signal. This signal is identified in response with inhibitor under the condition, that response is sum of responses of single active centers (the validity of the last condition is tested). This means that mechanism of chemical reaction is implicitly involved in standard signal. The mechanism need not to be described in detail or generally known. This enables (see theory) to split the measured response on two parts. One which can be used for investigation of mechanisms of biosensor reaction, while the other allows analysis of dependence of concentration of active "working" centers in presence of inhibitor $\frac{n_i(t)}{n(0)}$. This enables not only the improvement of signal to noise ratio, but also the set up the measurement process on rigorous basis.

Acknowledgments

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