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Thin protein LB films as functional components within biosensors*

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Biomolecules can often be incorporated and immobilized into Langmuir–Blodgett (LB) films by using covalent immobilization of proteins with glutaraldehyde. Laccase from *Cerrena Unicolor* was incorporated into an LB film deposited on a glass substrate. The samples fabricated in such a way can be used for the detection of phenolic compounds. The function of enzyme immobilization was carried out by glutaraldehyde added to a film of stearic acid and laccase. The sensor sensitization was achieved by an amphiphilic *N*-alkyl-bis(thiophene)diphenylamine admixed into the film. The interlaced diphenylamine derivative was expected to facilitate the electron transfer thereby enhancing the sensor sensitivity.

Key words: *Langmuir–Blodgett films; laccase; diphenylamine derivatives; biosensor; AFM*

1. Introduction

For years Langmuir monolayers and Langmuir–Blodgett films of pure molecules or biomolecules embedded into organic matrices have been used to investigate molecular processes as well as to facilitate biosensing [1–5].

An effective use of enzymes widely applied for this purpose may be hampered by some peculiar properties of enzymatic proteins such as their non-reuseability, high sensitivity to denaturating processes, and the presence of agents blocking active centres of proteins. Many of these undesirable constraints may be avoided by the immobilization of enzymes. Immobilized enzymes appeared to be more useful in all catalytic processes than unbounded proteins. In measurements of electrical properties of enzymes exhibiting direct electron transfer to electrode surface, the orientation, organisation are parameters of utmost relevance. Several strategies have been employed to

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immobilize biological compounds such as physical adsorption on graphite or on polymer matrices, covalent or electrostatic attachment to a electrochemically modified carbon surface and to self-assembled monolayers [6]. Here, advantage can be taken of the molecular control provided by the Langmuir–Blodgett (LB) technique, in which suitable orientations of biomolecules are possible to achieve.

The LB technique allows control of the thickness of the film deposited on a surface and is therefore well suited for the fabrication of both mono- and multilayer structured films. Such films could provide a simple foundation for the construction of various types of chemical, electrochemical and biological sensors. Few reports appeared on this subject; an ordered arrangement of glucose oxidase and ferrocene on a platinum electrode has been obtained by the LB technique [7]. In this paper, the fabrication of a biosensing LB layer is described built of laccase, cross-linked with glutaraldehyde.

Laccases (EC 1.10.3.2) are multicopper oxidases widely distributed in plant and fungal species. They have received particular attention due to their rather low substrate specifity and due to ability to oxidise phenols, anilines, benzenethiols, phenothiazines with the concomitant reduction of molecular oxygen to water. Laccases are mainly used in the paper and textile industries, for wastewater treatment, delignification and dye bleaching [8]. They also found application in biofuel cell technology. Laccase-based biosensors, in the absence or in the presence of mediators, have been applied for the determination of a broad range of phenolic species [9].

Generally, a mediator could be a sort of ‘electron shuttle’ that, after being oxidised by the enzyme, diffuses away from an active site to oxidise any molecule that, because of its size, cannot enter the enzymatic pocket directly. In addition, the oxidised form of the mediator, being structurally ‘diverse’ from the enzyme, might undergo a different mechanism of oxidation, thereby extending the range of substrates susceptible to the enzymatic action [10, 11]. Also, another type of the mediating agent, especially easily incorporated into LB film conducting polymers and monomers has been extensively studied due to its improving sensing effect of the sensor. For example, poly-3-dodecyl thiophene which, when mixed with stearic acid, is a suitable matrix for the deposition of glucose oxidase, retains its electroactivity and detects glucose [12].

Continuing our interest in the of precursors of conducting and sensing materials [4], we covalently immobilized laccase from *Cerrena Unicolor* into LB film deposited on a substrate. The system can be used for detection of phenolic compounds. The function of enzyme cross-linking was carried by glutaraldehyde (GA). Moreover, sensor sensitisation was achieved by an amphiphilic, conducting *N*-alkyl-bis(thiophene)-diphenylamine [13] admixed into the film. The interlaced diphenylamine derivative as expected facilitates the electron transfer and therefore enhances the sensor sensitivity.

2. Materials and methods

Materials. Prior to the deposition of LB films, the substrates were washed according to a standard procedure, followed by sonication in detergent (1% solution of DE-

CON 90), rinsing with deionized water, etching in an ethanolic solution of KOH (Yirayama solution) to make them hydrophilic. An amphiphilic 4,4'-bis(thiophene)-*N*-nonyldiphenylamine (DFTA9, Fig. 1) was synthesized according to the procedure described earlier, using a multi step method, in which the main point was the Stille coupling reaction [13]. Laccase (from *Cerrena unicolor*) was isolated and purified by the standard method [14, 15], whereas 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate, ABTS), catechol (all supplied by Aldrich) were used as- received.

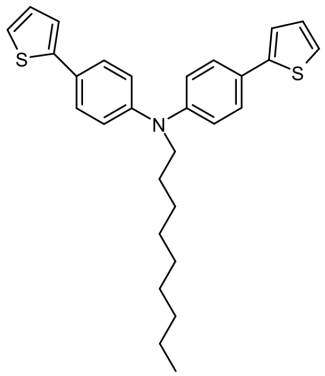

Fig. 1. The structure of 4,4'-bis(thiophene) -*N*-nonyldiphenylamine

Method. The Langmuir and Langmuir–Blodgett films were obtained with a commercial LB trough (KSV, System 5000). The amphiphilic 4,4'-bis(thiophene)-*N*-nonyldiphenylamine (DFTA9) and stearic acid were dissolved in chloroform (Aldrich, HPLC grade) and mixed in equimolar proportions, then laccase was dissolved in the obtained solution. The concentration of each solution was maintained at ca. 1 mg·ml^{-1}. About 50 μl of the mixture was spread on a water subphase (22 °C) of the trough and the monolayer was compressed with a movable barrier at 50 mm·min^{-1}. The deposition was Y-type with the transfer ratio very close to unity, and the π–A isotherms were recorded. The layers were transferred at a dipping rate of 20 mm·min^{-1} and removed at the drainage rate of 3.5 mm·min^{-1} at the transfer pressure of 25 mN·m^{-1}. Between dipping cycles, the samples were dried for 15 min in the open air. All LB films were built up on the hydrophilic quarz microscopic slides.

For a covalent cross-linking of laccase on a modified surface, an obtained LB film was sprinkled with one millilitre of glutaraldehyde (GA) [16]. In each case, immediately after applying the protein to the thin LB layers, the substrates were placed in a desiccator. The process of immobilization was carried out for 12 hours, at 4 °C in a humid environment. Figure 2 shows a simplified scheme for fabricating LB type film with immobilized proteins.

For determination of laccase activity, the substrates were immersed in 25 cm^3 of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate) ABTS (0.228 mmol·dm^{-3}, pH 5.25) or catechol (10 mmol·dm^{-3}, pH 5.25) and incubated at 30 °C, under continuous stirring The laccase catalyzed oxidation of reagent (ABTS or catechol) was carried out for 30 min.

ABTS is commonly used as a standard reagent with a laccase enzyme. This compound has been chosen because the enzyme facilitates the oxidation process, turning it into a green and soluble end-product. The fungal laccase oxidises ABTS to green-coloured radical cation ($ABTS^{+}$) and colour changes of ABTS solution can be measured.

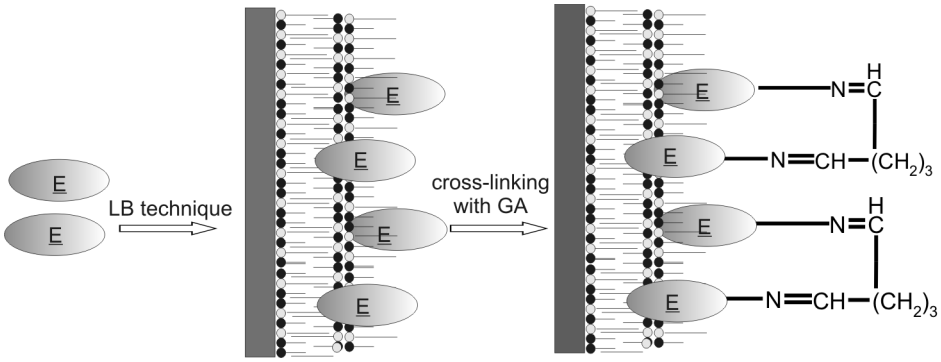

Fig. 2. The possible scheme of fabricating biological active layers; grey elipses – laccase

After washing off the oxidized reagent (with buffer, pH 5.25), the regenerated substrate (immobilized laccase) was ready to react repeatedly, and the reaction was repeated up to 25 times. The activity of the immobilized laccase was monitored by continuous recording changes of the absorbance at the wavelength characteristic for the maximum for the green oxidation product of ABTS – 420 nm or 410 nm for catechol by means of the spectrometer Unicam Helios during 30 min incubating cycle with reagent.

The specific activity was calculated from the measured absorbance value and one activity unit (1 U) was defined as the quantity of protein, which in test conditions changes the absorbance by 0.0001 per minute. This specific activity is related to the surface area of LB film, its unit of dimension is ($U \cdot cm^{-2}$), and it expresses the activity of an immobilized enzyme. Enzyme activity could be also expressed in relative units (%); 100% activity could be defined as the change of absorbance (compared with the absorbance at the first measurement), equivalent to 1 U.

AFM studies of SA/DFTA9/laccase cross-linked LB films were carried out using an AFM Dimension V Veeco.

3. Results and discussion

3.1. The surface pressure–area isotherms of laccase

The surface pressure–area isotherms of films a and b on pure water, at 22 °C, are presented in Fig. 3. The isotherms indicate that both, a and b films, at high surface pressure behave as a 2D solids. The surface per molecule area (calculated for a mole-

cule of SA) for the film a is 33 Å^2,whereas for the film b it is 183 Å^2. The low area per molecule for film a, only a little higher than for pure SA and steepy isotherm, suggests that protein molecules may be partly squeezed off the film. This situation is even more

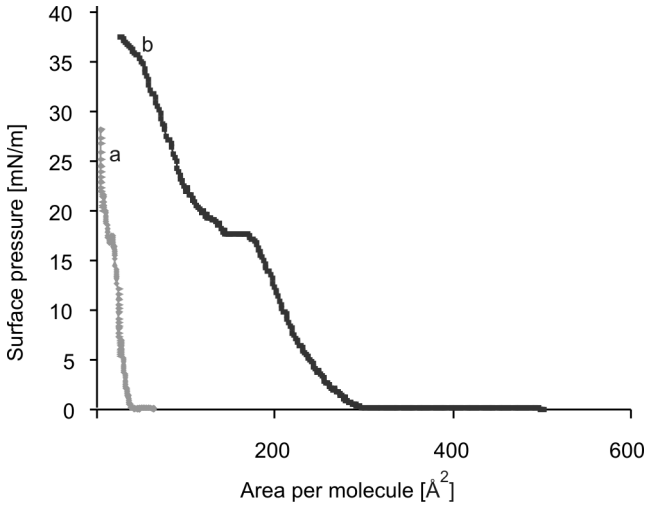

Fig. 3. Surface pressure – area isotherms of layer built of laccase – SA with (a) or without (b) DFTA9 incorporated into the film, at 22 °C on pure water

pronounced in the case of the film of laccase mixed with SA and DFTA9. A short plateaux visible in an isotherm b above 18 mN·m^{-1} does not provide evidence of the collapse of the film but rather of the process of partial squeezing because the transfer ratio observed above 18 mN·m^{-1} was close to unity and the obtained films were tougher and more elastic.

3.2. Characterization of immobilized protein

Since the immobilization of laccase in LB films occurs through the cross-linking reaction with glutaraldehyde, we use it in an excess amount. In our case, the laccase incorporated into the obtained film had an initial enzyme activity of 743 U·cm^{-2} (Fig. 4). This activity, however, makes merely 10% of the activity of the native laccase.

As one can see from Fig. 5, the sensing activity of the laccase, covalently immobilized onto LB films via glutaraldehyde, is rather stable up to 25 incubation cycles (repeated reaction of oxidizing reagent catalyzed by immobilized laccase). The observed decrease in the enzyme activity is rather small, and the cross-linked protein is active for a few months. If the DFTA9 molecules, acting as an electron mediator are present in the system they significantly enhance an ABTS mediating efficiency. We have found the effect of equimolar addition of DFTA9 into protein; an enzyme activity increased almost three times, and it retained ca. 70% of its initial activity for as long as 4 months (stored in the buffer, at 4 °C).

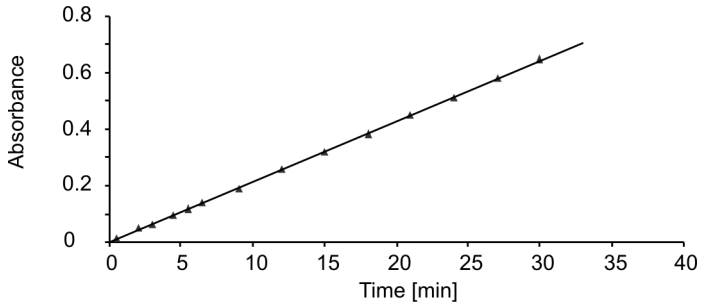

Fig. 4. Time dependence of absorbance (A) at 420 nm as a measure of enzyme activity (U) during the first oxidation of ABTS catalyzed by immobilized laccase

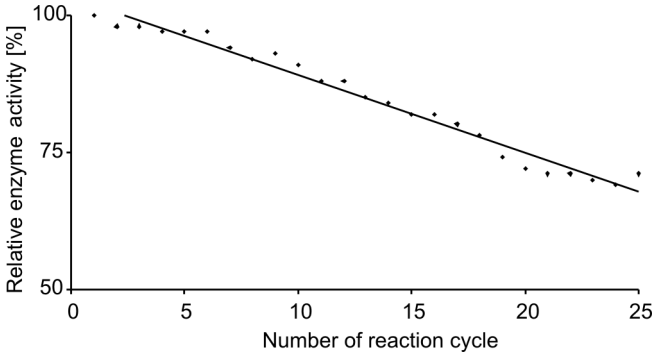

Fig. 5. Immobilized laccase activity during repeated incubation of immobilized laccase with ABTS dependent on number of reaction cycle. The line serves to guide the eye only

In the case of natural reagents like catechol, the laccase efficiency in the film followed the same number of measurement runs, proving the system was stable and reproducible, although the activity itself was as low as 20–30% of the activity of laccase in solution.

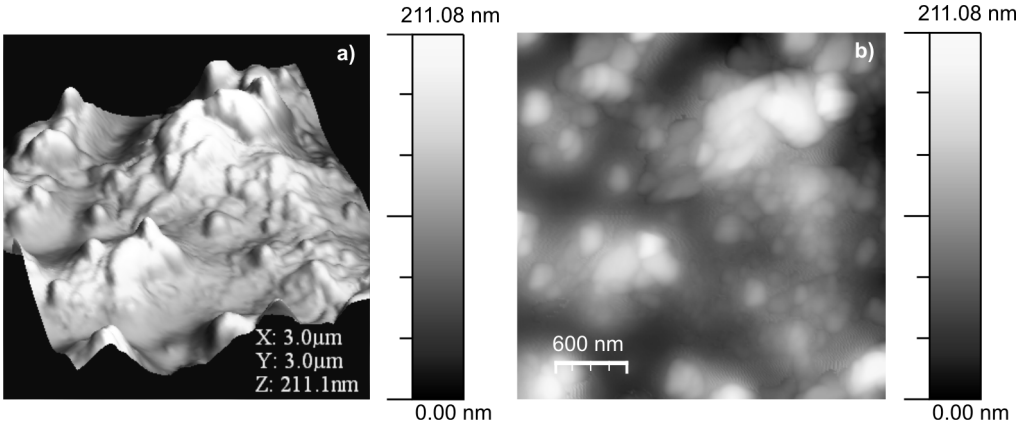

Fig. 6. AFM images of SA/DFTA9/laccase LB film. All images are $3\times3\mu m^2$

ABTS as a standard enzyme activity indicator use for reaction catalyzed by laccase causes much higher activity of the protein compared with natural reagents like catechol. The enzyme activity in the presence of catechol (24.65 $U \cdot cm^{-2}$) as a reagent is a few times lower than in the case of ABTS (195.95 $U \cdot cm^{-2}$) but the results are stable and the sensing film is able to catalyze catechol oxidation for a few months.

Atomic force microscopy was used for the topographic characterisation of the laccase LB films deposited on the quartz microscopic slides (Fig. 6). Laccase was observed as characteristic islands (aggregates, grain size 10–200 nm). The roughness of the film SA/DFTA9/laccase (38.2 nm) confirms the presence of protein, at least partly, out of the film in the form of comparatively large aggregates of immobilized enzyme, cross-linked by glutaraldehyde molecules Moreover, one cannot also exclude the changes of the surface density of during the formation of the film of the aggregates of laccase or the enzyme conformation.

4. Conclusions

It is possible to achieve successful immobilization of laccase by using heterogeneous LB film consisting of laccase cross-linked with glutaraldehyde, amphiphilic *N*-nonyl-bis(thiophene)diphenylamine (DFTA9) and stearic acid. This type of sensing system retains specific enzyme activity equal to 10% of that of native laccase. Enzyme immobilized by this technique is active and stable for at least 25 reaction cycles (ABTS). Catalytical activity of immobilized laccase was observed also for the catechol potentially present in most wastewaters.

The use of this electroactive nanohybrid material as a host matrix for enzyme immobilization prevents the protein leaking and enhances specific interactions. This biosensor offers a fast and a sensitive response in the presence of dissolved oxygen and can be used to detect phenolic compounds. Moreover, based on its good electrocatalysis for oxygen reduction, this system can be applied as the cathodic catalyst to fabrication of biofuel cells.

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The AFM data were visualized by WSxM software (Horcas I., Fernandez R., Gomez-Rodriguez J. M., Colchero J., Gomez-Herrero J., Baro A. M., Rev. Sci. Instrum., 78 (2007), 013705).

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