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Nuclear track-based biosensors with the enzyme laccase

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ABSTRACT

A new type of biosensors for detecting phenolic compounds is presented here. These sensors consist of thin polymer foils with laccase-clad etched nuclear tracks. The presence of suitable phenolic compounds in the sensors leads to the formation of enzymatic reaction products in the tracks, which differ in their electrical conductivities from their precursor materials. These differences correlate with the concentrations of the phenolic compounds. Corresponding calibration curves have been established for a number of compounds. The sensors thus produced are capable to cover between 5 and 9 orders of magnitude in concentration – in the best case down to some picomoles. The sensor's detection sensitivity strongly depends on the specific compound. It is highest for caffeic acid and acid blue 74, followed by ABTS and ferulic acid.

Introduction

Micro-or nanopores, as produced by e.g. swift heavy ion irradiation in thin polymer foils and subsequent etching, have been found to be useful for the construction of biosensors. Meanwhile there exist more than half a dozen different strategies for ion track-based biosensing, which were summarized and compared with each other in Ref. [1]. Our group concentrated essentially on the development of the "Product Enrichment Strategy", PES [2,3]. In this concept, a selected enzyme is covalently bound to the walls of etched tracks of energetic heavy ions in thin polymer foils, and thereafter the current transmitted through the tracks upon application of an external voltage is determined. Differences in current magnitude emerge that scale with the corresponding analyte concentration, so that these structures may serve as amperometric sensors.

Application of the 'Product Enrichment Strategy' to sensors with laccase

The basic criterium for the emergence of such conductivity differences in PES is that the conductivities of both the analyte solution (to which the enzyme is specific), and of the corresponding enzymatic reaction product solution must be different. This signifies that this strategy is applicable if:

(a) the analyte is non-ionic in aqueous solution, but the reaction product is
(b) the analyte is ionic, and the reaction product is not
(c) both the analyte and reaction products are ionic, but differ in their charge states.

The strategy is not applicable, however, if both the analyte and the reaction products do not exhibit any change in their charge states. Three examples for case (a) are glucose detection by means of the enzyme glucose oxidase [2], urea detection by urease [3] and oxidation of ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) by laccase [4]. The detection of phenolic reaction products by the enzyme laccase also covers both the cases (b) and (c), depending on the individual phenolic analyte. The oxidation of ferulic acid (a phenolic acid that is negatively charged at acidic pH)

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with laccase results in the formation of at least two dimeric products, in one of which the carboxyl group is implicated in the dimer formation through a β–β linkage [5] producing a non-ionizable molecule. On the other hand, the degradation of the anionic dye indigo carmine by laccase produces anthranilic acid [6], a molecule that exhibits one carboxylic and one amino group both of which may be ionized depending on the pH of the reaction media.

Cases (a) and (b) exhibit most pronounced and clear-cut results whereas cases (c), with both the analyte and reaction products being ionic, often impose some challenge, due to the eventually strong background currents existing. An example where this strategy completely fails is the attempt to detect peroxides by the enzyme horseradish peroxidase, in which case both the analyte H_2O_2 and the enzymatic reaction product H_2O do not differ markedly in their conductivities and hence do not yield any detectable electronic signal. Another example is given in this work; it is the attempt to detect vanillin by the enzyme laccase. Vanillin is an uncharged phenolic aldehyde that is commonly used as a mediator in oxidations catalyzed by laccase. The products of this reaction are mainly phenoxyl radicals that spontaneously react to form dimers and oligomers. In this case, both vanillin and their products are uncharged molecules and hence no difference in conductivities would be expected.

In order to obtain an optimum sensor sensitivity, these differences in conductivity should be enhanced as much as possible. One possibility to accomplish this is to perform the enzymatic reaction in close confinement, e.g. by using very long nanopores as reaction chambers. The large distances between the nanopore's interior and their surfaces, hence the long migration paths of the enzymatic reaction products, largely hinder their rapid escape by out diffusion through the pore openings. Furthermore, as the product's diffusion coefficients decrease with decreasing pore diameters [7], diffusional losses can be reduced even more by selecting very narrow pore diameters. This results in a concentration enrichment of the reaction products by factors between 50 and 10^5 [8], representing a detectable track conductivity enhancement. Etched nuclear tracks in thin (~10 μm) polymer foils are favored for that purpose due to their well-defined pore geometries and large aspect ratios, of the order of 100–1000.

The first measurements using this strategy made use of the enzymes glucose oxidase, GOx for glucose sensing [2] and urease for urea sensing [3]. Typical concentration intervals that could be determined with such sensors covered 5–7 orders of magnitude, with minimum concentrations in the order of some 0.1 to 10 μM being obtained. In this paper, sensors using the enzyme laccase were produced according to the same strategy. They were successfully applied to the sensing of various phenolic molecules. As compared with the previous track-based sensors, we succeeded both to expand the measured concentration intervals and to reduce the minimum concentrations considerably.

Laccases (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) are copper containing oxidases that catalyze the one-electron oxidation of phenolic compounds and aromatic amines coupled to the reduction of molecular oxygen to water. Laccases are within the most studied enzymes; they were initially isolated in 1883 from the sap of the lacquer tree (*Rus vernicifera*) and were subsequently characterized as metalloenzymes. Laccases have been found in various organisms, including plants, insects [9] and bacteria [10], but are especially abundant in the large majority of white-rot fungi [11]. Plant laccases plays an important role in the formation of lignin by promoting the oxidative coupling of monolignols. On the other hand, fungal laccases are involved in the degradation of lignin and can therefore be included in the broad category of ligninases. Besides lignin, laccases can degrade complex polymers such as humic acids. Fungal laccases are glycoproteins with molecular weights between 50 and 130 kDa which have degrees of glycosylation between 10% and 45% and isoelectric points between 3.0 and 7.4. Laccases are considered as one of the most important components of the ligninolytic system of the wood degrading fungi [12].

The active site of laccases contains four copper centers, one type 1 (T1), one type 2 (T2) and two type 3 (T3); the centers T2 and T3 forms a trinuclear copper cluster [13,14]. The copper centers are classified according to their spectroscopic and paramagnetic properties. Copper (T1) gives laccases their characteristic blue color. This copper is origin of an intense absorption band centered around 600–610 nm and is coordinated with two histidines and one cysteine forming a trigonal structure. Copper (T2) is paramagnetic and is coordinated by two histidines forming a tetragonal complex. The T2 center does not show characteristic absorption bands. Two copper atoms (T3) form a binuclear center which is coordinated by six histidines and displays a characteristic absorption band at 330 nm. Based on the redox potential of the T1 site of laccases, they can be classified as high- (730–790 mV), medium- (470–710 mV) and low- (430 mV) redox potential laccases [15]. The T1 site is the primary electron acceptor from the reducing analyte and hence laccases only oxidize analytes with redox potentials similar or slightly greater than the redox potential of T1 site. In this way, the ability of laccases to develop oxidation reactions is directly related to the redox potential of the analyte [16]. Once the T1 center has subtracted one electron from reducing analyte the electron are transferred to the T2 center where oxygen reduction to water takes place.

Laccases catalyze the polymerization of phenolic compounds. Polymerization is initiated by the formation of cation radicals which, after proton abstraction generates phenoxyl radicals that react unspecifically to form dimers. Oligomers and polymers are synthesized from dimers when reaction occurs in longer incubation time. The analyte specificity of laccases is broad and includes phenols, polyphenols, methoxy-substituted phenols, aromatic amines, anilines and organic and inorganic metal compounds. Furthermore, the number of analytes that can be oxidized by laccase can be increased using redox mediators. These mediators are low molecular weight compounds which, when oxidized by the enzyme, generate unstable cation radicals which function as diffusible intermediaries. These intermediaries in turn can oxidize other compounds, including some that are not direct analytes of laccases because of their high redox potential. During this process the oxidized mediator is reduced back to its original state to complete the cycle [11]. Some representative analytes and their corresponding enzymatic reactions are listed in Table 1.

The vast majority of possible applications of laccases is based on the enzyme's ability to generate free radicals during oxidation of different analytes, and due to this property, laccases find special applications in the industries of fruits and beverages, paper, textile, cosmetics and waste water treatment, as well as in the preparation of biosensors and in the degradation of xenobiotics and bioremediation [12]. In the latter case, polyphenols – especially flavones, isoflavones, flavonols, flavonoids – e.g. vanillin, catechins and phenolic acids – such as caffeic acid and ferulic acid – have gained importance. Polyphenols are abundant in vegetables, wine, tea, extra virgin olive oil, chocolate and other cocoa products [24]. Phenolic compounds are also interesting in the field of wood decomposition for fuel generation.

Usually, polyphenols have been detected electrochemically, by using voltammetry or amperometry, with the enzyme laccase attached to an electrode [24–27]. In these cases, caffeic acid is often used as a standard. The present state-of-the-art of these biosensors can be described by:

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