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Parallel generation and detection of superoxide and hydrogen peroxide in a fluidic chip

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Abstract

A fluidic chip system was developed, which combines a stable generation of superoxide radicals and hydrogen peroxide with their sensorial detection. The generation of both reactive oxygen species was achieved by immobilization of xanthine oxidase on controlled pore glass in a reaction chamber. Antioxidants can be introduced into the fluidic chip system by means of mixing chamber. The detection of both species is based on the amperometric principle using a biosensor chip with two working electrodes. As sensing protein for both electrodes cytochrome *c* was used. The novel system was designed for the quantification of the antioxidant efficiency of different potential scavengers of the respective reactive species in an aqueous medium. Several model antioxidants such as ascorbic acid or catalase have been tested under flow conditions.

Keywords: Biosensor; Cytochrome *c*; Flow system; Reactive oxygen species; Antioxidant

1. Introduction

The role of oxygen for life is hardly to overestimate, but in the form of reactive oxygen species (ROS), such as superoxide, hydroxyl radicals or hydrogen peroxide it is highly toxic to cells. Oxidative stress is a marker of many kinds of pathological situations, it occurs in neurodegenerative diseases [1,2], reperfusion injuries [3], inflammatory processes [4] and cancer [5,6]; it also plays a key role in a cell apoptosis [7]. The cell has elaborated a defence and repair mechanisms to protect itself from the overproduction of ROS. Besides chemical agents there is a number of enzymes (e.g. superoxide dismutase, catalase, glutathion peroxidase) that reduce the concentration of superoxide or hydrogen peroxide in organisms [8].

Antioxidants have also found widespread use in industry. They are applied for the production of cosmetics to protect the skin from the ROS, which are produced under UV-irradiation [9–12]. For the preservation of products antioxidants are applied in food industry: either as single substances (ascorbic acid, tocopherol) or as plant extracts [13–16]. The introduction of antioxidants in food products and beverages requires the strong control of their amount and monitoring of their properties. Particularly their interaction with each other and other constituents of the product mixture can result in a strong modification of the antioxidant potential.

For the analysis of antioxidants in cosmetics and foodstuffs a variety of techniques has been developed. A colorimetric approach provides the advantage, that the "total antioxidant content" of foods could be determined [17,18]. However the specificity of this method needs to be improved. A concentration level of several mg/l was achieved using flow-injection spectroscopy for the determination of single antioxidants (butylated hydroxyanisole and *n*-propylgallate) in fatty foods and cosmetics [19]. A flow-injection chemiluminescence method gave the possibility to measure the scavenging effect of gallic acid on hydroxyl radicals [20]. Very promising in this tech-

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nique was the analysis time: one measurement lasted for only 25 s.

Chromatographic methods combine liquid chromatography for the separation of antioxidants and mass spectrometry for their detection [21]. This tool makes it possible to analyse the antioxidant concentration in complex mixtures such as serum or wine [22,23], but does not allow the determination of its scavenging activity. For this purpose a spectrophotometrical approach is more straight forward. As a rule azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or (1,1-diphenyl-2-picrylhydrazyl) (DPPH) radicals are generated, and than their interaction with antioxidants is monitored photometrically [24,25].

Electrochemical methods provide the advantage of rather simple equipment and thus have found a considerable development during the last decade. Both cyclic voltammetric [26–28] and amperometric [29,30] techniques have been applied. Another strong benefit of electrochemical techniques is that they are suited for integrated analytical systems. In the last years there has been a rapid progress in the development of lab-on-the-chip systems. They can be used for the determination of a wide variety of substances: carbohydrates, aminoacids, hormones [31–33], providing a high throughput, cost-effectiveness and portability of the analytical system.

Following the concept of "lab-on-the-chip" we propose here a fluidic chip for the analysis of the antioxidant efficiency using an electrochemical detection principle. For this purpose the generation and detection of two ROS – hydrogen peroxide and superoxide – are integrated in a flow-through chip system, which allows the introduction of potential antioxidative samples. The influence of these antioxidants on the ROS signals is evaluated allowing a quantification of the scavenging effect within a complex sample. As biosensor for the detection of hydrogen peroxide and superoxide cytochrome *c*-based electrodes were used [34].

2. Experimental part

2.1. Materials

Cytochrome *c* from horse heart (cyt *c*), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), hypoxanthine (HX), 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP), dithiotreithol, superoxide dismutase from bovine erythrocytes (SOD, EC 1.15.1.1), hydrogen peroxide, L-ascorbic acid, 11-mercapto-1-undecanol (MU) and 11-mercaptoundecanoic acid (MUA), aminopropyl-controlled pore glass (CPG) were from Sigma–Aldrich (Steinheim, Germany). Xanthine oxidase from cow milk (XOD, EC 1.17.3.2) and catalase from beef liver (EC 1.11.1.6) were provided by Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade. The buffer solutions were prepared using 18.2 MΩ Millipore water.

2.2. XOD immobilization

XOD was immobilized on CPG according to the modified procedure described earlier [35]. Modification was carried out in 0.1 M sodium phosphate buffer + 50 mM NaCl, pH 7.5. Aminopropyl-CPG (75–125 μm, pore diameter 1400 Å) was rinsed with buffer and incubated with 1 mM SPDP in overhead shaker for 30 min. After removal of the reagent excess 30 mM dithiotreithol was added. Incubation time was 1.5 h. The completeness of the disulfide bond reduction in the introduced 2-pyridyl disulfide group was confirmed by spectroscopic measurements at 343 nm. The extinction coefficient of pyridyne-2-thione was taken to be $8.08 \times 10^3\ M^{-1}\ cm^{-1}$. After that CPG was thoroughly washed and a mixture of 20 U/ml XOD suspension with the equal buffer volume was added. The reaction was left overnight in overhead shaker at +4 °C. The modified CPG was rinsed with buffer and stored in the fridge in 0.1 M sodium phosphate buffer, pH 7.5.

2.3. Activity measurement of immobilized XOD

A sample of 10–15 mg of CPG with immobilized XOD was taken and added to the mixed solution of 100 μM HX and 100 μM cyt *c* in 0.1 M Na-phosphate + 50 mM NaCl buffer, pH 7.5 and stirred continuously. The volume of the sample was 1–2 ml. Portions of the reaction mixture were taken after fixed time periods (60, 120, and 180 s). They were diluted with the same buffer and the absorption of solution was measured at 550 nm ($\varepsilon = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$). The rate of cyt *c* reduction was proportional to the superoxide concentration produced by XOD. CPG-fixed enzyme was compared to known XOD-activities in solution [36,37].

2.4. Sensor preparation

The electrode chips were prepared by Elbau Ltd. (Berlin, Germany). The chip had two pairs of electrodes on a ceramic substrate: two working and two reference electrodes. The working electrode for superoxide detection was made from gold (electrode area 0.8 mm^2). Both the working electrode for hydrogen peroxide (Au) and the reference electrodes (Ag/AgCl) were screen-printed onto the substrate. For the detection of hydrogen peroxide electrodes of different size were used: 0.5 and 1.3 mm^2.

The sensor modification procedure was described in our previous paper [34]. Before the modification the superoxide sensor was cleaned electrochemically by cyclic voltammetry in 0.5 M H_2SO_4 solution between −0.35 and 1.7 V. Cycles were repeated until a sharp stable peak of surface gold oxide reduction was obtained. The H_2O_2-sensor was first incubated in a series of organic solvents: ethylacetate, isopropanol, ethanol (treatment in each solvent 10 min). The cleaned electrodes were placed in a solution of MU:MUA (3.75:1.25 mM) in ethanol, sonicated for 3 min in ultrasound bath and left for 48 h in the ethanolic solution. After a washing step both working electrodes were transferred in a 33 μM cyt *c* solution in 5 mM potassium phosphate buffer, pH 7.0, where 50 cycles of CV were recorded between −0.25 and 0.25 V (50 mV/s). To let cyt *c* adsorb on the promotor layer the electrodes were incubated at room temperature for 2 h. After that EDC was added in 3 mM concentration and the reaction was completed within 30 min. Modification of the hydrogen peroxide sensor included a second step of coupling of SOD to the cyt *c* layer: the sensor was incubated 30 min in the