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Towards an adrenaline biosensor based on substrate recycling amplification in combination with an enzyme logic gate

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ABSTRACT

An amperometric biosensor using a substrate recycling principle was realized for the detection of low adrenaline concentrations (1 nM) by measurements in phosphate buffer and Ringer's solution at pH 6.5 and pH 7.4, respectively. In proof-of-concept experiments, a Boolean logic-gate principle has been applied to develop a digital adrenaline biosensor based on an enzyme **AND** logic gate. The obtained results demonstrate that the developed digital biosensor is capable for a rapid qualitative determination of the presence/absence of adrenaline in a YES/NO statement. Such digital biosensor could be used in clinical diagnostics for the control of a correct insertion of a catheter in the adrenal veins during adrenal venous-sampling procedure.

1. Introduction

Inadequate high aldosterone secretion by an aldosterone-producing adenoma is one of the most frequent causes of hypertension [1]. Patients with primary aldosteronism (PA) have considerably higher cardiovascular morbidity and mortality than patients with essential hypertension [2,3]. Because of the therapeutical aspects, patients with PA undergo an adrenal venous-sampling (AVS) procedure for aldosteronoma localization and differential diagnosis. The technique used is invasive and complicated because adrenal veins are in general difficult to cannulate [4]. Since adrenaline concentration in adrenal veins is much higher ($\gtrsim$100 nM) than in the periphery ($\lesssim$1.2 nM) [5,6], the concentration difference of adrenaline can be used as an indicator for the correct insertion and positioning of the catheter in the adrenal veins and successful AVS procedure [7,8]. This requires a fast adrenaline detection method with a high sensitivity and low detection limit in the nanomolar concentration range.

Adrenaline belongs to the substance group of catecholamines. Several methods have been developed for the determination of catecholamines, mainly high-performance liquid chromatography, fluorescence spectroscopy, capillary electrophoresis, chemiluminescence [9–11] and electrochemical detection [12,13]. Electrochemical detection using biosensors offer faster and more versatile analytical methods for clinical or biomedical applications. However, for very low analyte concentrations sensitivity is often not sufficient; consequently, different amplifications methods have been developed. Electrochemical recycling of the analyte between two closely arranged electrodes (preferentially interdigitated electrodes) allows a repeated participation of analyte molecules in the signal generation [14]. An alternative is chemical recycling by coupling a chemical reaction to the electrochemical detection reaction [15]. With the application of a proper biological molecule the sensor's selectivity and sensitivity can be further improved. This can be reached by combining the enzymatic reaction with an electrochemical conversion [16] or most efficiently by using two enzymes [17]. In biochemical recycling approach, the analyte is converted

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by one enzyme in a product which can be converted back to the original substrate by a second enzyme and thus amplifying the response by several orders of magnitude, as described in Refs. [18,19]. Other examples are ultrasensitive sensors for the detection of phenolic substances ranging from micromolar to nanomolar levels which were developed by combining oxidase enzymes with a pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) [20,21]. However, maximum sensitivity with the applied enzymes used in Refs. [20,21] was obtained at pH 6.0, which limits the application of these biosensors for the detection of adrenaline in biological liquids such as blood.

The present study describes an adrenaline biosensor based on a laccase/PQQ-GDH bienzyme system and the substrate recycling principle with optimal working characteristics in the pH range relevant for blood samples. In contrast to Refs. [20,21], we utilize a genetically modified laccase variant, which is active in a broad pH range between pH 3.5 to pH 8.0 and stable in phosphate buffer solution (PBS) [22]. The sensor has been tested in PBS and Ringer's solution (RS), a substitute for blood plasma or other physiological liquids. In addition, in "proof-of-principle" experiments, the possibility of construction of a digital adrenaline biosensor based on a Boolean **AND** logic gate with YES/NO output has been demonstrated for the first time.

2. Experimental

2.1. Materials

Glutaraldehyde, bovine serum albumin (BSA), glycerol, $CaCl_2$ and the buffer components (monosodium phosphate and disodium phosphate) were purchased from Sigma-Aldrich (USA). The laccase was provided by AB Enzymes GmbH (Germany). Glucose dehydrogenase (from *Acinetobacter calcoaceticus*) was provided by Roche Diagnostics (Germany). Cellulose acetate filter with a pore size of 0.2 μm was obtained from Sartorius Stedim Biotech GmbH (Germany). Adrenaline solution (1 mg/mL) was purchased from Sanofi-Aventis GmbH (Germany). PQQ was bought from Wako (Japan) and RS (8.6 g/L NaCl, 0.3 g/L KCl, 0.33 g/L $CaCl_2{\cdot}2H_2O$) was purchased from Bernburg (Germany).

2.2. Modification of the oxygen sensor with enzyme membrane

For the realization of the adrenaline biosensor, a commercial galvanic oxygen sensor (Atlas Scientific, USA) was modified by a bienzyme (laccase/GDH) membrane. Unlike the polarographic oxygen sensor, the galvanic sensor does not need a constant voltage applied to it. In the galvanic oxygen sensor, the electrodes are dissimilar enough to self-polarize and reduce oxygen molecules without an applied voltage. The enzyme membrane was prepared from the membrane cocktail consisting of 15 μL of the laccase (1.82 U/μL) solution, 15 μL of GDH (0.03 U/μL) solution combining 20 μM PQQ and 1 mM $CaCl_2$ [23], 60 μL of BSA (10 vol%) and 60 μL mixture of glutaraldehyde (2 vol%) and glycerol (10 vol%) solutions, respectively. All components were mixed with the resulting volumetric ratio of 1/2/2 (enzymes/BSA/glutaraldehyde-glycerol). Detail information for determination of the enzyme activity is described in Refs. [24,25]. A total of 100 μL of the membrane cocktail was then dropped onto a Teflon block. After drying for 24 h at 4 °C, the enzyme membrane with a thickness of approximately 130 μm was fixed with the help of a cellulose acetate filter (dialysis membrane) and silicon rubber (TSE 399C, Momentive Performance Materials, Switzerland) onto the high-density polyethylene (HDPE) layer of the oxygen sensor (see Fig. 1).

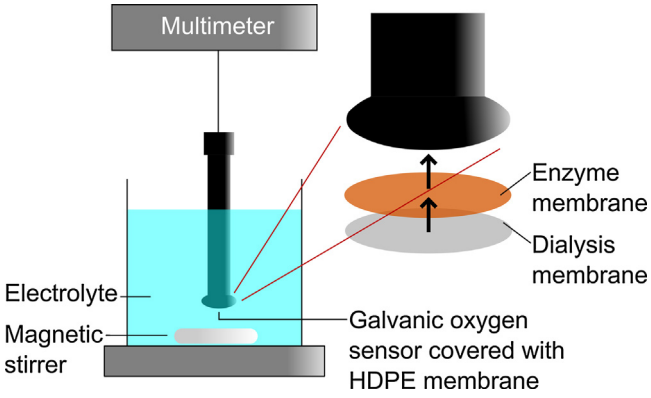

Fig. 1. Measurement setup for the detection of adrenaline (schematically).

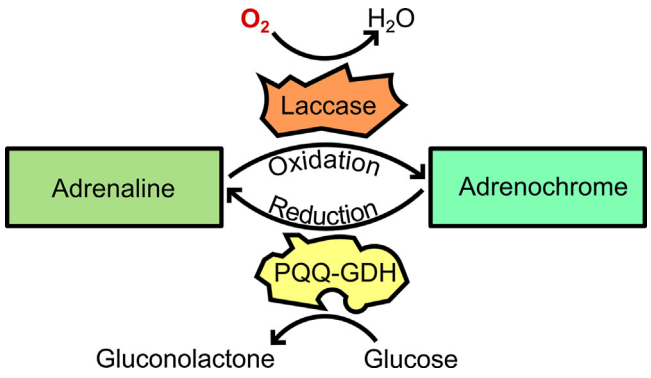

Fig. 2. Scheme of the substrate recycling principle for the amplification of the adrenaline sensor signal.

2.3. Measuring setup

For the electrochemical characterization, the adrenaline biosensor was connected to a potentiometer (2007 Mulitmeter, Keithley Instruments) and exposed to the solution containing different concentrations of adrenaline (see Fig. 1). The sensor measures the oxygen consumption due to the oxidation of adrenaline by the laccase. The produced output voltage (delivered by the galvanic oxygen sensor) is proportional to the oxygen consumption in the solution due to the enzymatic reaction. The sensitivity of the biosensor to adrenaline was investigated in both PBS and RS containing 20 mM glucose. Adrenaline solutions with various concentrations from 1 nM to 1 μM were prepared from a stock solution of 0.1 mM adrenaline, stored at 4 °C in the dark. At each adrenaline concentration, the sensor signal was recorded for about 20 min. All experiments were carried out at room temperature under continuous stirring.

3. Results and discussion

3.1. Substrate recycling principle

In order to measure low adrenaline concentrations (in the nanomolar concentration range), the substrate recycling principle based on a bienzyme system (laccase/PQQ-GDH) has been proposed in Refs. [17,20,21]. PQQ acts here as a prostethic group for the GDH and binds via Ca^{2+} ions to the apoenzyme. Fig. 2 schematically shows the substrate recycling principle for the enzymatic signal amplification of the adrenaline biosensor: In the presence of dissolved oxygen, the enzyme laccase oxidizes adrenaline to adrenochrome; in a second oxidation reaction, GDH transforms glucose into gluconolactone, while adrenochrome is reduced back to