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Development and characterization of a field-effect biosensor for the detection of acetoin

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ABSTRACT

A capacitive electrolyte-insulator-semiconductor (EIS) field-effect biosensor for acetoin detection has been presented for the first time. The EIS sensor consists of a layer structure of Al/p-Si/SiO_2/Ta_2O_5/enzyme acetoin reductase. The enzyme, also referred to as butane-2,3-diol dehydrogenase from *B. clausii* DSM 8716^T, has been recently characterized. The enzyme catalyzes the (*R*)-specific reduction of racemic acetoin to (*R,R*)- and *meso*-butane-2,3-diol, respectively. Two different enzyme immobilization strategies (cross-linking by using glutaraldehyde and adsorption) have been studied. Typical biosensor parameters such as optimal pH working range, sensitivity, hysteresis, linear concentration range and long-term stability have been examined by means of constant-capacitance (ConCap) mode measurements. Furthermore, preliminary experiments have been successfully carried out for the detection of acetoin in diluted white wine samples.

1. Introduction

Acetoin and diacetyl are widely distributed in various beverages, and are also used in foods and cosmetics as flavouring and fragrance, as well as in chemical synthesis (Hahn et al., 1987; Lawson et al., 1995; Vasavada and White, 1977; Xiao et al., 2012). These compounds are products of fermentative metabolism in different microorganisms. Acetoin can be formed, e.g., in most bacteria from pyruvate and is thus a product of carbohydrate metabolism, or from diacetyl while NAD(P)H serves as a cofactor of the enzyme acetoin reductase (Cogan et al., 1981; Huang et al., 1999).

During the fermentation process of alcoholic beverages, such as beer or wine, acetoin plays an important role in their quality due to its buttery-like taste, although acetoin is not pungent smelling (Romano and Suzzi, 1996). Typical acetoin concentrations in alcoholic beverages are in the range of 10–50 µM in beer, ~500 µM in white wine and ~150 µM in red wine (Haukeli and Lie, 1975; Reed and Nagodawithana, 1991; Romano and Suzzi, 1996). The detection of acetoin content during the fermentation process could control the quality of alcoholic beverages due to its involvement in the wine bouquet or its influence in the beer flavor. Furthermore, the acetoin concentration during beer storage is used as a parameter to establish the degree of the beer's maturity (Haukeli and Lie, 1975). Precise detection of the acetoin level can be used to avoid unnecessary maturation time (Romano and Suzzi, 1996; Vann and Sheppard, 2005). Hence, its control of concentration change during the fermentation course could help assess the fermentation processes, as well as the maturation process.

Several methods have already been described for the detection of acetoin, mainly colorimetric techniques, like the Voges-Proskauer test, which is the most commonly applied procedure for the detection of acetoin in analytical microbiology or gas chromatography (Levine, 1916; Speck and Freese, 1973). However, none of these techniques provide the advantages that can be achieved by using a biosensor which offers a faster analytical approach and that does not need additional trained staff.

Capacitive EIS sensors are field-effect devices that are used for the detection of surface potential changes, e.g., due to pH alterations (Poghossian et al., 2004). These changes can also be induced by, e.g., enzymatic reactions (Poghossian et al., 2001b; Schöning et al., 2005b; Siqueira et al., 2014; Thust et al., 1996), and binding of charged molecules, such as DNA (Abouzar et al., 2012; Bronder et al., 2015; Poghossian et al., 2001a; Veigas et al., 2015). These sensors can also be applied for the development of enzyme logic gates (Molinnus et al., 2017b; Poghossian et al., 2011, 2015). Furthermore, EIS sensors have many advantages over conventional analytical methods such as small size, low weight and fast response time, and they are easy and cost-

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effective in fabrication (Poghossian and Schöning, 2014; Schöning et al., 2005a). Furthermore, due to the miniaturized sensor layout, a small sample volume is necessary for the measurement. Additionally, with an array of different modified EIS sensors, several analytes can be detected simultaneously.

In this study, we report on the development of a chip-based biosensor for the acetoin detection for the first time. The sensor is based on a pH-sensitive capacitive EIS field-effect structure consisting of an Al/p-Si/SiO_2/Ta_2O_5 layer set-up. The chip was modified using a recently introduced acetoin reductase from *B. clausii* DSM 8716 ^{T} (Muschallik et al., 2017) accountable for the reduction of acetoin in the presence of NADH as a cofactor (Molinnus et al., 2017a). The local pH shift induced by the enzymatic reaction resulted in the modulation of the flat-band potential of the field-effect biosensor, arising in a shift of the capacitance-voltage (C-V) curve of the EIS structure. Two immobilization strategies, namely adsorptive and cross-linking by forming cross-linkages between the enzyme molecules, have been investigated to attach the acetoin reductase to the pH-sensitive Ta_2O_5 surface of the EIS sensor chip. Characteristic biosensor parameters such as linear concentration range, pH optimum of the field-effect sensor with the immobilized enzyme, sensitivity, lower and upper detection limit, hysteresis and long-term stability will be discussed. In addition, the newly developed acetoin biosensor was applied for measurements in real samples such as white wine.

2. Experimental

2.1. Materials

Acetoin and the cofactor NADH were acquired from Sigma-Aldrich (USA), as well as glutaraldehyde, glycerol and NaCl. TRIS-HCl buffer (0.2 mM) was purchased from Carl Roth (Germany). The respective pH values of the buffer solution were adjusted by addition of 0.1 M NaOH or 0.1 M HCl. The enzyme acetoin reductase from *B. clausii* (~380 U/mL) is produced in our institute as described before (Muschallik et al., 2017).

2.2. Preparation of the sensor structures

The applied capacitive EIS sensor consists of the following layer stack: a p-doped silicon substrate with a thickness of ~400 µm and a specific resistance of ρ = 5–10 Ωcm, a 30 nm thermally grown SiO_2 insulating layer and a 60 nm thick Ta_2O_5 gate insulator layer (for that 30 nm Ta is deposited by electron-beam evaporation, followed by a thermal oxidation step). A rear side contact, consisting of a 300 nm thick aluminum layer is deposited by electron-beam evaporation and annealed afterwards. As a final step, the wafer is separated into 1 cm × 1 cm chips with a diamond saw. Fig. 1 shows the EIS-sensor set-up with the different layers. Detailed information about the sensor's fabrication process is described in Ref. (Schöning et al., 2005a). The Ta_2O_5 layer as gate insulator has been selected because of its well-known excellent pH behavior and high permittivity but also because of its chemical stability (Atanassova and Spassov, 1998; Chaneliere et al., 1998).

The acetoin EIS biosensor was developed by modifying the Ta_2O_5 surface with the enzyme acetoin reductase. Immediately before modification, each sensor was cleaned in acetone, isopropanol and deionized water for 5 min, respectively. Two different immobilization strategies have been investigated. As first immobilization method, the enzyme acetoin reductase is adsorptively bound to the sensor surface. For this, 80 µL of acetoin reductase solution was dropped onto the sensor surface. For the second immobilization procedure, cross-linking is performed by formation of cross-linkages between the enzyme molecules, where the membrane cocktail consisting of 48 µL glutaraldehyde (2 vol%) / glycerol (10 vol%) solution and 32 µL of enzyme solution was mixed. 80 µL of the membrane cocktail was pipetted onto the Ta_2O_5 surface. After drying of the different prepared EIS sensors, they were mounted into a homemade measuring cell, sealed by an O-ring to protect the rear side contact and to define the contact area of the EIS sensor with the analyte solution (~0.5 cm^2). Before measurements, the sensors were stored at 4 °C in the dark.

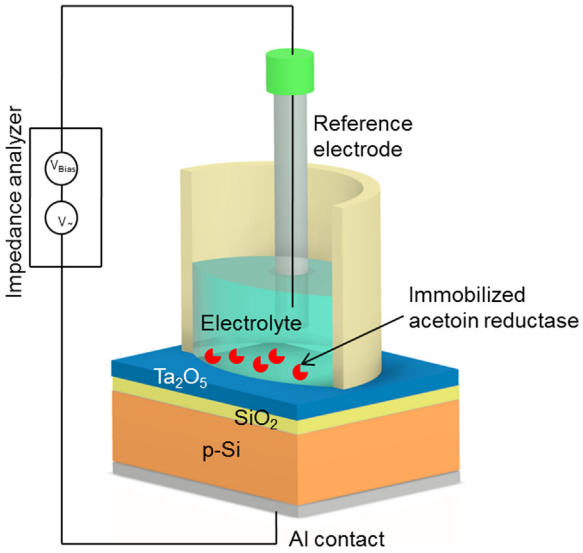

Fig. 1. Schematic of the measurement set-up with the Al-p-Si-SiO_2-Ta_2O_5 EIS sensor modified with the enzyme acetoin reductase for the detection of acetoin.

3. Measurement principle

Fig. 1 illustrates the measurement set-up with the developed acetoin field-effect biosensor. For the electrochemical characterization of the acetoin biosensor chip, C-V (capacitance / voltage) and ConCap (constant capacitance) measurements were performed by connecting the EIS chip with an impedance analyzer IM6 (Zahner Elektrik, Germany). Before performing ConCap measurements, C-V curves of each sensor chip, in a gate voltage range between −2 V and 2 V with steps of 100 mV were recorded to define a fixed capacitance value (in the linear range of the depletion region, ~60% of the maximum capacitance) using a feedback-control circuit. With the help of ConCap measurements, potential and/or charge changes at the Ta_2O_5 surface can be detected in real time. An external liquid-junction Ag/AgCl electrode (Metrohm, Germany) filled with 3 M KCl was applied as the reference electrode. The C-V and ConCap measurements were carried out at a frequency of 120 Hz. A 20 mV ac (alternating current) voltage has been applied between the Ag/AgCl reference electrode and the rear side Al contact, to measure the capacitance.

The measurement principle for the detection of acetoin using the capacitive field-effect sensor is based on the enzymatic reaction as depicted in Eq. (1). (*R*)- and (*S*)-acetoin will be reduced by the R-specific enzyme acetoin reductase to (*R,R*)-2,3-butanediol and *meso*-butanediol, respectively, while NADH serves as a cofactor and will be oxidized to NAD^+.

$$\text{acetoin} + \text{NADH} + H^+ \xrightarrow{\text{acetoin reductase}} 2,3\text{-butanediol} + \text{NAD}^+ \quad (1)$$

As a result of this enzymatic reaction, the hydrogen ion concentration decreases, and this pH change can be detected by the pH-sensitive Ta_2O_5 transducer surface of the field-effect sensor. The resulting change in the flat-band potential of the sensor is recorded and corresponds to the measured acetoin concentration. All measurements were performed in a dark Faraday cage at room temperature. Before starting the measurements, the sensor chip was incubated in 0.2 mM TRIS-HCl buffer solution (pH 7.1) containing 150 mM NaCl for 2 h. All solutions contained 500 µM of the cofactor NADH and all measurements were performed in 1 mL analyte solution containing different acetoin