Biosensors and Bioelectronics 65 (2015) 354–359

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Superoxide microsensor integrated into a Sensing Cell Culture Flask microsystem using direct oxidation for cell culture application

H. Flamm [a,*], J. Kieninger [a], A. Weltin [a], G.A. Urban [a]

[a] Laboratory for Sensors, Department of Microsystems Engineering, IMTEK, University of Freiburg, 79110 Freiburg, Germany

ARTICLE INFO

Article history:
Received 3 July 2014
Received in revised form
24 October 2014
Accepted 27 October 2014
Available online 30 October 2014

Keywords:
Superoxide
In vitro
Electrochemical sensor
Direct oxidation
Cancer cell culture

ABSTRACT

A new electrochemical sensor system for reliable and continuous detection of superoxide radical release from cell culture was developed utilizing direct oxidation of superoxide on polymer covered gold microelectrodes. Direct superoxide oxidation was demonstrated to provide robust measurement principle for sensitive and selective reactive oxygen species (ROS) quantification without the need for biocomponent supported conversion. Sensor performance was investigated by using artificial enzymatic superoxide production revealing a sensitivity of 2235 A M^{-1} m^{-2}. An electrode protection layer with molecular weight cut-off property from adsorbed linear branched polyethylenimine was successfully introduced for long term and selectivity improvement. Thin-film based sensor chip fabrication with implemented three-electrode setup and full integration into the technological platform Sensing Cell Culture Flask was described. Cell culturing directly on-chip and free radical release by phorbol-12-myristate-13-acetate (PMA) stimulation was demonstrated using T-47D human breast cancer carcinoma cell model. Transient extracellular superoxide production upon stimulation was successfully observed from amperometric monitoring. Signal inhibition from scavenging of extracellular superoxide by specific superoxide dismutase (SOD) showed the applicability for selective *in vitro* ROS determination. The results confirm the possibility of direct superoxide oxidation, with exclusion of the main interfering substances uric acid and hydrogen peroxide. This offers new insights into the development of reliable and robust ROS sensors.

1. Introduction

A common attribute of aerobic cell metabolism is the formation of reactive oxygen species (ROS), whereas superoxide anions often represent the first species which are produced directly from one electron reduction of molecular oxygen in mitochondrial respiration chain (St-Pierre et al., 2002). Cellular superoxide production is further linked to the activation of oxidoreductase enzymes and metal catalyzed oxidation (Ebbesen et al., 2009). Superoxide release is shown with inflammatory tissue (Guice et al., 1989) and cell culture (Boyce et al., 1989) as well as response to oscillatory mechanical stress in culture model (Pimentel et al., 2001). The overproduction of superoxide anions is also observed in different malignant tumors and cancer cell cultures. The increased energy demand of cancer cells promotes superoxide formation. Further, specific cancer cells show adaptation to oxidative stress resulting in inhibited apoptosis, malignant transformation and promoted metastasis (Chen et al., 2007; Schneider and Kulesz-Martin, 2004). Thus, efficiency of ROS regulating cancer cell drugs may be dramatically antagonized. The regulation of cellular redox homeostasis is an essential feature of aerobic cells, whereas its deficiency causes severe oxidative stress and often unrecoverable damage (Goetz and Luch, 2008; Penta et al., 2001). ROS detoxification strategies are based on highly efficient enzymatic conversion and scavenging ability of specific biomolecules. The detection or even quantitative measurement of cellular superoxide production is conducted by spectrophotometric or electron spin resonance based techniques (Dikalov et al., 2002) with the inherent drawback of extensive cell culture treatment influencing the standardized environmental conditions. Further, long term monitoring of dynamic changes in cellular microenvironment is a disadvantage using cumulative techniques. Electrochemical sensing of short lived superoxide release from cell culture and tissue probes was presented as sensitive monitoring tool using Cytochrome C (Cyt C) coupled to gold electrodes (Cooper et al., 1993; Scheller et al., 1999; Tammeveski et al., 1998) or superoxide dismutase (SOD) based biosensors (Campanella et al., 1999; Song et al., 1995; Tian et al., 2002). Recent investigations reveal a possible direct oxidation of superoxide anions on gold electrodes

* Correspondence to: Laboratory for Sensors, Department of Microsystems Engineering, IMTEK, University of Freiburg, Georges-Koehler Allee 103, 79110 Freiburg, Germany. Fax: +49 761 203 7262.
E-mail address: hubert.flamm@imtek.uni-freiburg.de (H. Flamm).

http://dx.doi.org/10.1016/j.bios.2014.10.062
0956-5663/

(Chen et al., 2008; Gáspár et al., 2013). For measurement in biological probes or cell culture environment, a protection of the electrodes is indispensable to avoid unspecific adsorption and electrode fouling with time. A fully integrated sensor system for reliable and sensitive pericellular superoxide detection is presented, using direct oxidation on gold electrodes in operating potential region where oxidation of interfering uric acid can be excluded. A polyethylenimine layer was introduced as semipermeable membrane, to enhance both, the selectivity and long term stability of the sensing device. This new enzymeless approach for superoxide measurement in cell culture utilizes the integration in conventional tissue culture flask, allowing direct cell cultivation on chip. The technological platform is based on the concept of Sensing Cell Culture Flask (Kieninger et al., 2014), which enables long term cultivation over days. This concept allows cell cultivation and measurement at the same place, avoiding the transfer to a separate measurement setup, which might induce generation of reactive oxygen species. The SCCF system allows a continuous monitoring of pericellular parameter and direct stimulation at all stages of cell growth. In this work we present the SCCF system, equipped with superoxide sensors and conducted basic cell culture experiments, to demonstrate the applicability of our sensor approach.

2. Materials and methods

2.1. Chip fabrication

Superoxide sensing electrodes are integrated on thin-film processed, transparent glass chips, equipped with on-chip pseudo-reference and counter electrode. The fabrication is based on a standard clean room processing, enhancing the yield and reproducibility of production. Sensor fabrication is done on transparent 100 mm diameter Pyrex® borosilicate glass wafer. First, 500 nm silicon nitride insulation layer is deposited using plasma-enhanced chemical vapor deposition (PECVD). The metal layer consists of 50 nm titanium for adhesion promotion, 100 nm platinum and 20 nm titanium. The deposition is conducted by physical vapor deposition (PVD) onto a sacrificial layer from AZ 5214E (Clariant) for metal structuring by lift-off process. Combined insulation layers for the metal structure consist of PECVD deposited silicon nitride and silicon oxide with a thickness of 800 nm and 200 nm, respectively. Contact pads and electrode openings are structured using reactive ion etching (RIE) down to the platinum layer with patterned AZ 1518 (Clariant) negative photoresist layer, acting as etching mask. Prior to photoresist stripping, the metal layer was treated by ion beam etching, to ensure clean metal surface.

Integration of silver/silver chloride reference electrodes is done on waver-level by electrodeposition process. Silver is cathodically deposited from Arguna S® (Umicore) solution by applying a constant current density of $-4\ mA\ cm^{-2}$ for 560 s. Partial conversion of the silver layer to silver chloride is conducted by anodic oxidation at $1.6\ mA\ cm^{-2}$ for 300 s in 0.1 M KCl solution, resulting in a nominal thickness of silver and silver chloride layer of 2 μm and 1 μm, respectively.

At this fabrication step the chips consist of platinum working and counter electrodes as well as a silver/silver chloride reference. That allows a modular implementation of different chemo- and bio-sensors on the same platform without changing the proven fabrication process.

Gold deposition onto platinum electrodes is done by wafer-level electroplating from Puramet® (Doduco) galvanization bath at constant current density of $2\ mA\ cm^{-2}$ for 18 min to achieve a gold layer thickness of 2 μm.

After full-wafer processing, the wafer was diced into 40 single chips, comprising a Ag/AgCl reference electrode, a gold counter and gold working electrodes with multiple designs as shown in Fig. 1 B. Reference electrodes were covered with UV-curable hydrogel membrane, based on poly-2-hydroxyethyl methacrylate (pHEMA), which acts as diffusion barrier to avoid silver ion release into the cell culture. Precursor solution of the hydrogel was applied by computer numerical control (CNC) dispensing robot, to completely cover the reference electrode. Membrane formation was achieved by UV light crosslinking, resulting in a long term stable hydrogel electrode cover.

The sensor was subsequently modified by a linear-polyethylenimine (LPEI, Fluka) layer as semipermeable membrane, which was applied onto the electrodes in a post process by incubating the chips for 45 min in a 0.1% (w/v) LPEI solution prepared from 0.05 M Na_2CO_3 pH 8.2. Unbound polyethylenimine was removed by washing the chip carefully in 0.1 M PBS at pH 7.4. The sensor was air-dried and stored dry until use.

For cell culture measurements, a sensor chip was mounted into the bottom of a standard 25 cm^2 cell culture flask (BD Biosciences) prior to PEI coating. The opening in the flask was manufactured by CNC milling process. Chips were permanently mounted in the level of the culture flask bottom by UV-curable adhesive Loctite® 3201 (Henkel) as indicated in Fig. 1. Sterilization of the assembly immediately before cell cultivation was achieved by UV exposure (Fluorescent tube Philips actinic BL 18 W) for 15 min. Polyethylenimine solution (0.1% in 2 ml 0.05 M Na_2CO_3, pH 8.2) was applied into the sensor flask for 45 min for membrane formation. After incubation, the flask was rinsed three times with sterilized 0.1 M PBS solution and air-dried before cell seeding.

2.2. Superoxide sensor calibration

For artificial superoxide production, the enzymatic conversion of hypoxanthine with xanthine oxidase (XOD) was used, as described by Ge and Lisdat (Ge and Lisdat, 2002). Briefly, xanthine

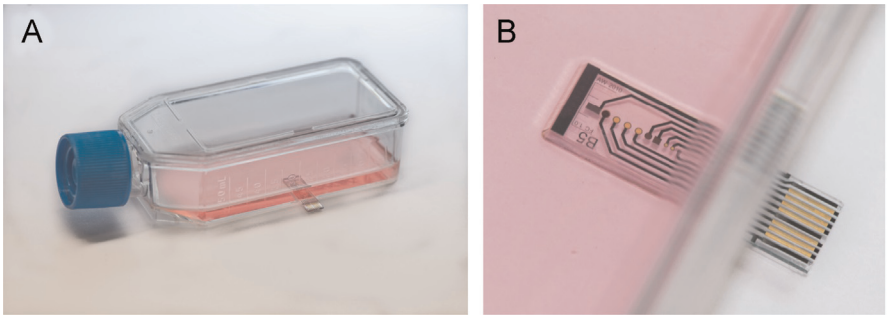

Fig. 1. A: Integrated sensor chip, mounted in bottom of standard 25 cm^2 cell culture flask. B: Detailed view of sensor chip with gold electrodes and Ag/AgCl reference.