Biosensors and Bioelectronics 74 (2015) 637–643

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Development of L-lactate dehydrogenase biosensor based on porous silicon resonant microcavities as fluorescence enhancers

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ARTICLE INFO

Article history:
Received 22 May 2015
Received in revised form
10 July 2015
Accepted 11 July 2015
Available online 13 July 2015

Keywords:
Lactate dehydrogenase
Porous silicon
Microcavity
Fluorescence enhancement
Biosensor

ABSTRACT

The up-regulation of L-lactate dehydrogenase (LDH), an intracellular enzyme present in most of all body tissues, is indicative of several pathological conditions and cellular death. Herein, we demonstrate LDH detection using porous silicon (pSi) microcavities as a luminescence-enhancing optical biosensing platform. Non-fluorescent resazurin was covalently attached onto the pSi surface *via* thermal hydrocarbonisation, thermal hydrosylilation and acylation. Each surface modification step was confirmed by means of FTIR and the optical shifts of the resonance wavelength of the microcavity. Thermal hydrocarbonisation also afforded excellent surface stability, ensuring that the resazurin was not reduced on the pSi surface. Using a pSi microcavity biosensor, the fluorescence signal upon detection of LDH was amplified by 10 and 5-fold compared to that of a single layer and a detuned microcavity, respectively, giving a limit of detection of 0.08 U/ml. The biosensor showed a linear response between 0.16 and 6.5 U/ml, covering the concentration range of LDH in normal as well as damaged tissues. The biosensor was selective for LDH and did not produce a signal upon incubation with another NAD-dependant enzyme L-glutamic dehydrogenase. The use of the pSi microcavity as a sensing platform reduced reagent usage by 30% and analysis time threefold compared to the standard LDH assay in solution.

1. Introduction

Enzymatic sensors have received a great deal of attention over the past decades. These biosensors possess high selectivity: the enzyme reacts with its enzymatic substrate under specific reaction conditions, such as pH, temperature and ionic strength (Bisswanger, 2014). Besides being known for high selectivity and specificity, enzyme-based sensors are also sensitive, rapid and can be used for real-time monitoring of target analytes of interest in clinical analysis (Ispas et al., 2012). One of the most common physiological markers in disease diagnosis is the enzyme L-lactate dehydrogenase (LDH) (Chen et al., 2006; Drent et al., 1996; Kato et al., 2006; Moran and Schnellmann, 1996). LDH is an intracellular enzyme present in most of all body tissues (Drent et al., 1996). The enzyme is built upon four peptide chains either forming from the heart or from the muscle. The main biochemical property of LDH is as a hydrogen transfer enzyme, catalysing the reversible reaction of L-lactate to pyruvate through the reduction of the coenzyme nicotinamide-adenine dinucleotide (NAD^+). NAD^+ acts as a hydrogen acceptor forming the reduced form, NADH. LDH is released from damaged tissues, hence the increase of LDH levels in the blood is indicative of a pathological condition, making it one of the most important markers of injury or disease. The up-regulation of this enzyme has been reported to serve as an aid for early detection of cell death levels (Moran and Schnellmann, 1996) and is correlated with conditions such as pulmonary cancer (Hou et al., 2009), leukaemia (Kastritis et al., 2010), melanoma (Ho et al., 2012) and chronic wounds (James et al., 2000). Due to its significance in the early stage of disease diagnosis, sensitive biosensors capable of monitoring LDH activity have recently grown in importance (Kastritis et al., 2010; Kato et al., 2006).

Currently LDH assay kits for cytotoxicity and cell damage evaluations are commercially available. As described in the protocols, these assay kits rely on the monitoring of the absorbance peak of a tetrazolium dye (Decker and Lohmann-Matthes, 1988). When compared to the absorbance-based detection, fluorescence-based detection has been reported to be more sensitive since the fluorescence intensity is proportional to the excitation intensity, hence the system is capable to detect weak signals (Bosch et al., 2007). The release of LDH *via* the distinctly different fluorescence properties of NADH and NAD^+ has been reported (Moran and Schnellmann, 1996; Zhu et al., 2010; Zhuang et al., 2007). NADH itself is a natural fluorophore coenzyme owing to its reduced nicotinamide ring, with an excitation wavelength of 340 nm, a maximum emission at 460 nm and a fluorescence lifetime of 0.4 ns

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http://dx.doi.org/10.1016/j.bios.2015.07.025
0956-5663/

in aqueous buffer. Therefore, when LDH catalyses pyruvate into L-lactate, NADH is oxidised into NAD^+ and consequently the fluorescence is attenuated (Drent et al., 1996; Wang et al., 2008). However, the drawback of this detection system is that the low excitation wavelength at 340 nm may damage the cells when the assay is conducted *in vivo*.

Recent improvements of the fluorescence-based detection involves coupling of the above reaction with the reduction reaction of resazurin (7-hydroxy-3H-phenoxazin-3-one-10-dioxide) into resorufin (7-hydroxy-3H-phenoxazin-3-one) (Larsen, 2005). Resazurin, known as Alamar Blue (O'Brien et al., 2000), is only weakly fluorescent in its pure form. The compound consists of a phenoxazine group which contains a heterocylic N-oxide group that loses its oxygen upon reduction and forms the strongly fluorescent product, resorufin (ex/em 545 nm/583 nm). The reduction of the dye into resorufin procedes by accepting electrons from NADH and NADPH (Das et al., 2013; O'Brien et al., 2000; Candeias et al., 1998). This indirect detection is advantageous as the excitation and maximum emission wavelengths of resorufin are higher than that of NADH, thus avoiding damaging of cells and background autofluorescence. Moreover, a relatively quick response within 4–7 min after the initiation of the reaction has been reported (Larsen, 2005) as compared to the 30 min incubation time in standard LDH assays. The use of fluorescent nanoparticles, such as the ones based on quantum dots (QDs), has also been applied for LDH sensing (He et al., 2012; Ren et al., 2010; Yang et al., 2011). When LDH and its substrate, L-lactate, are present in a solution of NAD^+-modified QDs, the enzymatic reaction reduces NAD^+ into NADH followed by an increase in the luminescence intensity of the QDs. The increase of emission is a linear function of the activity of LDH.

The application of porous silicon (pSi) as a sensitive platform for enzyme monitoring has been addressed in studies (DeLouise et al., 2005; Krismastuti et al., 2013; Martin et al., 2009; Palestino et al., 2009). The excellent performance of pSi-based sensors relies on the advantageous properties of pSi (Bosch et al., 2007; Jane et al., 2009; Janshoff et al., 1998; Lin et al., 1997; Sailor and Wu, 2009; Zhao et al., 2010). Specifically, pSi with a microcavity structure offers signal amplification for fluorescence or luminescence-based detection systems. This is because the microcavity, which is a multilayer consisting of alternating porosities and a spacer layer in between, is able to enhance the emission of a fluorophore confined in the porous structure according to the Purcell effect (DeLouise and Ouyang, 2009; Koenderink, 2010; Poitras et al., 2003; Purcell, 1946; Qiao et al., 2010; Setzu et al., 2000). This particular pSi architecture has therefore been applied to fluorescence biosensor applications (Krismastuti et al., 2014; Palestino et al., 2008, 2009; Sciacca et al., 2009).

Herein, we demonstrate the detection of LDH by monitoring the emission of the reduced form of resazurin, resorufin. Two pathways of surface modification (Scheme 1) were tested on the microcavity to investigate the redox stability of immobilised resazurin on the pSi surface. The resazurin-modified pSi microcavity surface was then used as the detection platform for LDH sensing.

2. Materials and methods

2.1. Preparation of pSi microcavity (MC)

pSi samples were prepared from boron doped, [100]-oriented silicon wafers (0.00055–0.001 Ω cm specific resistivity, 475–525 μm thickness, Siltronix, France). The wafers were electrochemically etched in a 1:1 mixture of 48% (v/v) hydrofluoric acid (HF) (Scharlau, Australia) and ethanol (100%, ChemSupply, Australia) in a custom-built Teflon cell at 25 °C using a source metre (Keithley, USA) as the current source. The cathode used was a platinum mesh and aluminium foil was used to contact the silicon anode. The area of the exposed region of the silicon wafer was 1.76 cm^2. Prior to sample preparation, the parasitic layer of the samples was removed by etching the silicon wafers in a 1:1 HF/EtOH solution at 56.8 mA/cm^2 for 30 s, thorough rinsing with ethanol, drying with N_2 gas and immersing the layer in 1 M sodium hydroxide (NaOH, Merck, Australia) for 2 min. Finally, the wafer was cleaned with deionised water and ethanol consecutively before being dried under N_2 gas (Pace et al., 2013).

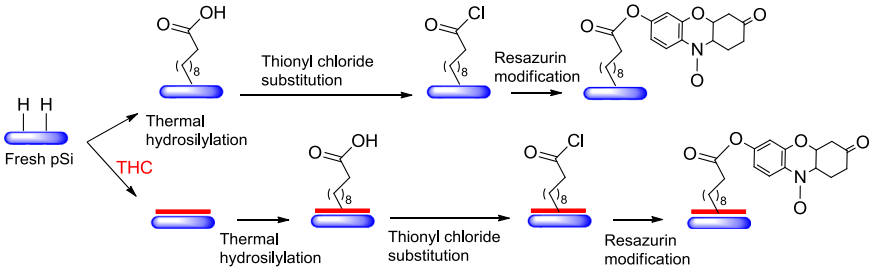
2.2. Modification of the pSi surface

Modification of pSi was accomplished following the steps shown in Scheme 1. In the first pathway, thermal hydrosilylation was conducted for freshly etched samples in neat undecylenic acid (Sigma-Aldrich, Australia) at 120 °C for 3 h. The functionalised surface was rinsed with pure ethanol and dried under a stream of N_2 gas. In the second pathway, freshly etched pSi samples were stabilised using thermal hydrocarbonisation under acetylene gas at 500 °C for 10 min followed by thermal hydrosilylation with neat undecylenic acid at 150 °C for 24 h. The functionalised surface was rinsed with pure ethanol and dried under a stream of N_2 gas. The samples were then immersed in neat thionyl chloride (Sigma-Aldrich, Australia) at room temperature for 10 min, followed by subsequent wash with dichloromethane and drying under a stream of N_2 gas.

pSi modification with resazurin was achieved by incubating the thionyl chloride-modified samples in a 1 mg/ml solution of resazurin (Thermo-Fisher, Australia) prepared in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich, Australia) buffer at room temperature for 10 min and protected from light. Afterwards, the samples were rinsed with HEPES buffer and water, and dried under a stream of N_2 gas.

2.3. LDH detection

The detection of LDH (EC1.1.1.27 from chicken heart, 258 U/mg protein, ProspecBio, USA) *via* fluorescence enhancement was conducted on the pSiMC and the single layer pSi samples. Resazurin-functionalised samples were immersed in a 100 μl solution of β-NAD^+ (1 mM, Sigma-Aldrich Australia), L-lactate (35 mM, Sigma-Aldrich, Australia), PMS^+ (300 μM) and different concentrations of LDH ranging from 0.02 to 16.26 U/ml in Tris-buffer.



Scheme 1. Modification pathways of the pSi surface.