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The analytical performance of a porous silicon Bloch surface wave biosensors as protease biosensor

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ABSTRACT

We investigate the analytical performance of label free porous silicon Bloch surface wave (BSW) devices as protease sensors. Protease detection is based on the digestion of gelatin, which was covalently immobilized onto the surface modified PSi structure. Exposure to the protease enzyme, subtilisin initiates catalytic degradation of the gelatin gel network. The degradation of this network which is present in the top layer of the sensor, causes a blue shift in the spectral position of the Bloch surface mode. The magnitude of the resonance shift is directly proportional to the concentration of subtilisin and the digestion time. The lowest concentration of subtilisin detected was 370 pM. Secondary spectral features, such as band-edge modes, are relatively insensitive to refractive index changes in the superficial layers and thus can be utilised as an internal reference to exclude any nonspecific adsorption and bonding that may occur through the bulk of the film. The advantages of the system here include fast diffusion of digested product and self-referencing capability.

1. Introduction

Proteases, also known as proteolytic enzymes, are globular, water soluble proteins that function to specifically cleave or degrade proteins by catalysing the hydrolysis of amide bonds in peptide chains, either from the N or C termini and/or in the middle of the molecule [1]. The primary role of proteases was long considered to be protein metabolism relevant to food digestion and intracellular protein turnover. It was also later discovered that precise cleavage of proteins by proteases is also a very subtle means of regulation [2]. Proteases also play an important role in pathologies including cancer [3–5] and cardiovascular disease [6]. In cancer, high levels of protease activity are associated with invasive and metastatic cancers, whilst in cardiovascular disease, proteases are active in apoptosis, tissue remodelling, and modification of cardiac proteins [7]. The activity of enzymes can be indicative of normal or abnormal cellular function and is often used to detect infections [7–9]. Therefore, proteases have been seen as ideal biomarkers for the diagnosis of disease [10] and important pharmaceutical targets [11,12]. Hence the measurement of protease enzymes and their activity is of major importance.

Enzymes are analysed based on an activity assay due to the specificity of action on a certain substrate. An enzyme assay usually involves determining the rate of product formation or substrate depletion during the enzyme catalysed reaction. The method currently used most frequently to assay proteases is the assay technique zymography, where enzyme activity is analysed through electrophoresis of polyacrylamide gels containing copolymerized protease substrate, mainly gelatin. Other common techniques are fluorogenic or calorimetric protease assay kits that can be obtained commercially [13].

Porous silicon (PSi) optical biosensors have several unique advantages such as; large internal surface area, tuneable pore sizes, and, various biomolecules can be immobilized at the pore walls using robust surface chemistry [14–17]. Changes that occur at the porous silicon internal surface can be interrogated through the use of optical reflectivity where the porous silicon scaffold and intervening analytes are seen as an optically uniform substrate with dielectric properties that vary as a function of the reaction event. As an example, Orosco et al. [18] reported a zein-coated multilayer mesoporous silicon rugate filters to monitor enzyme activity. Based

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on the red-shift in the peak spectral resonance of the rugate filter structure, 7.2 pM of pepsin could be detected by visual inspection. Using a similar structure spin-coated with gelatin, Gao et al. [19] demonstrated the detection of gelatinase-2 (also known as matrix metalloproteinase-2 or MMP-2) through the optical response to as little as 1 μL gelatinase solution with concentrations varying from 0.1 to 1000 ng/mL at room temperature. A further example by Kilian et al. [15,20]. showed that by covalently immobilizing gelatin in high quality rugate filters, detection of MMP-2 and MMP-9 secreted from live cells (macrophage), with detection limit of 0.37 pM, was possible. In combination with fluorescence enhancement, Voelcker and co-workers has designed porous silicon resonant microcavity for detection of MMPs [21].

In this work we investigate the performance of gelatin modified silicon photonic structures that support Bloch surface waves (BSW) for the detection and monitoring of enzymatic activity of proteases [22,23]. We have recently introduced this type of optical structure where the sensor arrangement allows open access of large biomolecules to the resonant optical modes whilst supporting optical resonances with sharp features – both features suggest increases in sensitivity and response time of the transducer [24]. The use of BSW modes is chosen due to its intrinsic advantages over other porous silicon photonic structures, such as microcavities [23,25] and rugate filters [26]. This advantage is due to the structural reason that Bloch surface wave structures possess an open sensing space that made it easy for protease molecule to digest and ease of diffusion of the biomolecule fragments to leave the pores [27–29]. The versatility of Bloch surface and sub-surface wave structure PSi was also recently shown by Rodriguez et al. where it could be used for simultaneous detection of small chemical molecules and bacteriophage [29]. We perform protease assays on the BSW sensors using subtilisin as a model protease and study the reaction kinetics of the enzyme acting on the gelatin substrate. We find that the BSW sensor has a large dynamic range giving linear initial reaction velocities for enzyme concentrations in the micro to nano-molar range. Reaction kinetics parameters measured using the BSW sensors are consistent with other reported values indicating the mass transport of the enzyme of digestion products is not the rate-limiting step [30–32].

2. Experimental methods

2.1. Material

Prime grade single-side polished silicon wafers, 100-oriented (<100> ± 0.05°), p-type (boron), 500–550 μm thick, 0.001–0.0015 Ω cm resistivity as provided by suppliers, were obtained from SILTRONIX (Archamps, France). Hydrofluoric acid (48% aqueous) and ethanol (absolute) were obtained from Ajax Fine Chemicals and used as received. Undecylenic acid, *N*-ethyl, *N*′-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and gelatin were obtained from Sigma–Aldrich and used as received. 1-Amine-hexa-ethylene glycol and PBS buffer (containing NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM) were prepared in house.

2.2. Preparation of silicon photonic structure

Porous silicon based optical BSW sensors were fabricated by anodic etching of highly boron-doped single side polished <100> oriented silicon wafers. The etching solution consisted of 50% v/v hydrofluoric acid diluted in absolute ethanol to a concentration of 25%. Etching was performed in a single-chamber etching cell with the silicon wafer mounted beneath an open aperture at the bottom of the chamber; a 10 mm diameter Viton™ o-ring was used to seal the chamber and define the etched region on the polished side of the wafer. A platinum ring electrode immersed in the ethanolic hydrofluoric acid solution provided the counter electrode and the applied etching currents were controlled by a computer controlled source meter. The etching sequence for the BSW sensor consists of a series of alternately high (158 mA/cm^2 for 1.9 s) and low (5 mA/cm^2 for 18.2 s) current steps that produce alternating layers of high and low porosity silicon in a multi-layered configuration. The porous silicon film is detached from the native silicon substrate by applying a high current pulse in 15% HF ethanolic solution, but is still fixed to the silicon at the periphery of the film.

2.3. Surface modification and functionalization

The hydrogen terminated porous silicon was first stabilised via hydrosilylation with undecylenic acid as previously described [15]. The distal carboxylic group was activated with *N*-hydrosuccinimide (NHS) in the presence of coupling agent *N*-ethyl, *N*′-3-(3′-(dimethylamino) propyl) carbodiimide (EDC). Before the sensing element gelatin is attached, the surface reacts with 1-amino-hexa (ethylene glycol, OEG) in order to form an antifouling layer to eliminate the nonspecific adsorption of proteins and their derivatives in the sensing process, as described previously [33]. The OEG functionalised device was cut off from silicon on the rim and placed on a piece of No. 2 glass coverslip. In order to immobilise gelatin as the sensing element, the ethylene glycol terminated film was activated by immersing in with 0.1 M *N, N*′-disuccinimidyl carbonate (DSC) containing 0.1 M *N, N*-dimethylaminopyridine (DMAP) for 12–24 h. The modified porous silicon optical structure was rinsed with ethyl acetate and CH_2Cl_2 and blown dry under a stream of argon before gelatin coupling by covering the device with 10 mg/mL gelatin aqueous solution in PBS [15]. The completed device is separated from the substrate and transferred to a coverslip, as otherwise the fragility of the device hakes it difficult to handle. The device on the coverslip is then mounted on a prism via refractive index matching oil to facilitate light coupling.

2.4. Enzyme assay

Optical monitoring of enzyme activity was measured using aliquots of the broad based serine protease subtilisin dissolved in phosphate buffered saline (PBS). At the end of reaction, excess buffer was removed, rinsed in water for 5 min and reflectivity spectra measured. Denatured subtilisin was produced by taking 0.005 mg/mL subtilisin, boiling in a water bath and kept in boiling water for 30 min and then cooling down to room temperature before use.

2.5. Characterization

2.5.1. Optical reflectivity measurement

Optical reflectivity spectra were measured in the visible and near-infrared at normal incidence using a custom-built optical arrangement. The setup incorporated a USB2000+ miniature fibre-optic spectrometer (Ocean Optics Inc.) and a fibre-coupled halogen light source (Mikropack GmbH, Germany) and had a spectral resolution of 1 nm and measurement spot size of ~100 μm. Spectra were processed using custom spectroscopy software platform driven by LabVIEW (National Instruments, TA).

2.5.2. Scanning electron microscopy (SEM)

Images were taken using a Hitachi S900 SEM with a cold field emission source (4 kV). PSi samples were cleaved in the centre of the film and mounted on a brass sample base.