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Influence of acetylcholinesterase immobilization on the photoluminescence properties of mesoporous silicon surface

Muhammad Saleem [a], Muhammad Rafiq [b], Sung-Yum Seo [b], Ki Hwan Lee [a,*]

[a] Department of Chemistry, Kongju National University, Gongju, Chungnam 314-701, Republic of Korea
[b] Department of Biology, Kongju National University, Gongju, Chungnam 314-701, Republic of Korea

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ABSTRACT

Acetylcholinesterase immobilized p-type porous silicon surface was prepared by covalent attachment. The immobilization procedure was based on support surface chemical oxidation, silanization, surface activation with cyanuric chloride and finally covalent attachment of free enzyme on the cyanuric chloride activated porous silicon surface. Different pore diameter of porous silicon samples were prepared by electrochemical etching in HF based electrolyte solution and appropriate sample was selected suitable for enzyme immobilization with maximum trapping ability. The surface modification was studied through field emission scanning electron microscope, EDS, FT-IR analysis, and photoluminescence measurement by utilizing the fluctuation in the photoluminescence of virgin and enzyme immobilized porous silicon surface. Porous silicon showed strong photoluminescence with maximum emission at 643 nm and immobilization of acetylcholinesterase on porous silicon surface cause considerable increment on the photoluminescence of porous silicon material while acetylcholinesterase free counterpart did not exhibit any fluorescence in the range of 635–670 nm. The activities of the free and immobilized enzymes were evaluated by spectrophotometric method by using neostigmine methylsulfate as standard enzyme inhibitor. The immobilized enzyme exhibited considerable response toward neostigmine methylsulfate in a dose dependent manner comparable with that of its free counterpart alongside enhanced stability, easy separation from the reaction media and significant saving of enzyme. It was believed that immobilized enzyme can be exploited in organic and biomolecule synthesis possessing technical and economical prestige over free enzyme and prominence of easy separation from the reaction mixture.

1. Introduction

Porous silicon is a material obtained by electrochemical dissolution of a single-crystal silicon wafer in HF containing electrolyte which has been intensively investigated for a variety of applications such as optical interferometer, microelectronic circuit [1], Bragg reflector, Rugate filters [2], integration of signal processing circuitry, photonic devices [3,4], immunotherapy [5], biocompatible material [6] and bone tissue engineering [7], as well as *in vivo* gene and drug delivery payload due to its biodegradable nature [8–10]. Consequently, porous silicon can serve as a versatile platform for a biosensor [11,12], interferrometric biosensor [13] and biomolecular screening [14] by modifying the surface with biomolecules. Porous silicon can be used for immobilization of biomolecules such as protein [15], hemoglobin [16], DNA [17,18], and enzymes [19] like β-glucuronidase [20], urease [21], tyrosinase [22], glucose-oxidase, ascorbate-oxidase [23], gluthathion-s-transferase [24], azurine and laccase [25] proved be a promising material for optoelectronics, photonics and biosensors applications [26]. The changes in photoluminescence and refractive index are used as a tool for determination of analytes on the porous silicon surface [27,28].

The porosity of a growing porous silicon layer is proportional to the current density being applied and etching time. However, the porosity of a growing layer can be altered by changing the applied current as well as etching time. The ability to easily tune the pore sizes and volumes during the electrochemical etching is a unique property of porous silicon that is very useful for enzyme immobilization, biosensing and drug delivery applications [29]. Porous silicon can be classified according to its pore sizes as macroporous (pore diameter greater than 50 nm), mesoporous (pore diameter in-between 2 and 50 nm) and microporous (pore diameter less than 2 nm) [30]. For porous silicon to be amenable for enzyme immobilization, the size of the pores must allow infiltration of

* Corresponding author. Tel.: +82 41 850 8494; fax: +82 41 856 8613.
E-mail addresses: khlee@kongju.ac.kr, kihwanlee2610@gmail.com (K.H. Lee).

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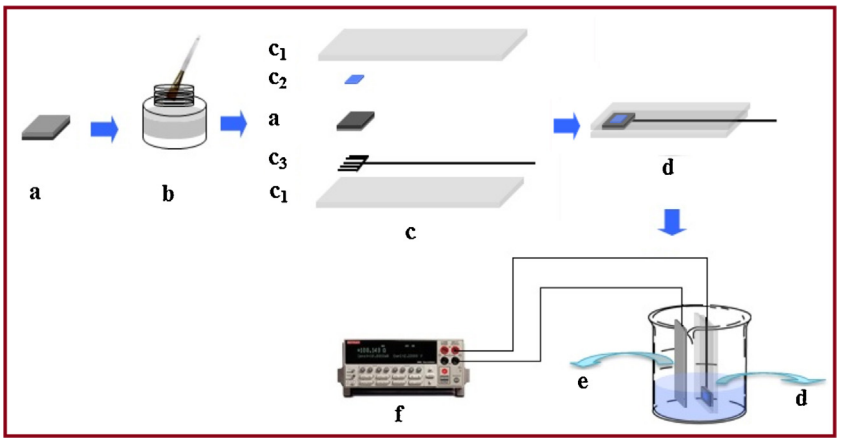

Fig. 1. Schematic diagram for electrochemical anodization on silicon wafer to obtain porous surface; (a) 1 cm × 1 cm size silicon wafer. (b) Silver paste. (c) Wire $\mathbf{c}_3$ encapsulated with adhesive tape $\mathbf{c}_1$ on opaque side of silicon wafer and 0.25 cm^2 paper section $\mathbf{c}_2$ tagged on transparent side of silicon wafer surface with adhesive tape $\mathbf{c}_1$ in order to obtained exposed wafer surface for etching. (d) Finally prepared silicon wafer piece for etching. (e) Platinum mesh and (f) Keithley 2400 source meter.

biomolecules. Therefore, biosensing and biomolecule fabrication efforts have focused primarily on mesoporous silicon to accommodate biological species [6]. The critical factor in using porous silicon as an immobilization matrix lies in the choice of its pore size and shape for proper confinement of biomolecules. The pore size has to be large enough to allow biomolecules to enter the pores and also to react with the corresponding analyte freely but be small enough to retain the biomolecules inside the surface. This can be achieved by choosing a proper combination of etching parameters such as current density and etching time. We have prepared porous silicon material with different porosities by changing the etching time keeping constant current density of 20 mA/cm^2. The optimal structural parameters for the porous film were found to be appropriate possess 30–50 nm pore size, 5.06 μm porous film thickness with 88% porosity, respectively, obtained by electrochemical etching in aqueous HF electrolyte solution for 30 min at constant current density of 20 mA/cm^2.

Unlike more traditional organic and inorganic catalysts, enzymes are large and fragile molecules; over the last decade, enzyme immobilization has become more important in industry, medicine, and biotechnology [30–37]. In the present study, unique properties of porous silicon materials, *e.g.*, huge surface area, modifiable surface, and restricted pore nanospaces, were utilized to immobilize acetylcholinesterase on porous silicon surface through covalent attachment *via* ether linkage. The enzyme confinements in the nanochannels of porous silicon materials generate synergistic effects that enhance enzyme stability, improve product selectivity, and facilitate the separation and reusability of enzymes. The immobilized enzymes in the nanospaces of porous silicon can be applied as viable biocatalysts for chemical and pharmaceutical industries, while enzyme entrapment in porous silicon architecture can easily be investigated through photoluminescence measurements, spectrophotometric assay, FE-SEM, FT-IR and EDS analysis.

2. Experimental

2.1. Materials

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7, acetylcholinesterase from human erythrocytes), acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid), neostigmine methylsulphate, 3-aminopropyl triethoxysilane, HNO_3, MgCl, and cyanuric chloride were purchased from Sigma–Aldrich; NaCl (Daejung chemical and metals Co. Ltd., Korea), ethanol, water, toluene and HCl (Samchun chemicals, Korea), HF (48%, w/w; Merck, Germany) and boron doped p-type silicon wafers with resistivity 1–20 Ω cm, thickness 500–550 μm (obtained from Cree Co., USA) were used during experiment.

2.2. Photoluminescence measurements

The photoluminescence spectra and relative photoluminescence intensities were measured on FS-2 fluorescence spectrometer (Scinco, Korea). The enzyme immobilization on the surface of porous silicon was confirmed by photoluminescence measurement by studying the changes of porous silicon photoluminescence intensity before and after enzyme entrapment.

2.3. Morphology characterization

Pore size and porous film thickness were determined by field emission scanning electron microscopic images and elemental analysis was done by recording EDS spectra using (FE SEM, MIRA3 LMH, TESCAN, USA). Cross-sectional views were obtained by scribing the reverse side of the slide and breaking the sample. The FT-IR spectra were recorded on a SHIMADZU FTIR-8400S spectrometer (Kyoto, Japan).

2.4. Enzyme activity measurements

The acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7, acetylcholinesterase from human erythrocytes, 0.03 U/mL) inhibitory activity was determined spectrophotometrically using acetylthiocholine iodide as substrate by using reported method of Ellman [38]. Briefly, the assay solution consisted of 180 μL of 50 mM tris–HCl buffer pH 8.0, containing sodium chloride (0.1 M), magnesium chloride (0.02 M) and 20 μL of enzyme with increasing concentration of neostigmine methylsulfate (0.312–10 μg/mL) and pre-incubated for 30 min at 4 °C. In this reaction mixture 5,5′-dithio-bis(2-nitrobenzoic acid) (0.3 mM, 20 μL), acetylthiocholine iodide (1.8 mM, 20 μL) were added and incubated for 10 min at 37 °C, followed by the measurement of absorbance at 412 nm. The same assay procedure was followed for immobilized enzyme. The assay measurements were carried out by using a micro plate reader (OptiMax, Tunable Micro plate Reader; wavelength range: 340–850 nm; for 96-well plates). The reaction rates of immobilized enzyme was compared with its free counterpart and the percent inhibition was calculated by using the formula $100 - (Abs_{testwell}/Abs_{control}) \times 100$ [39].

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