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Signal enhancement in a protein chip array using a 3-D nanosurface

So Yeon Kim [a], Jaeeun Yu [b], Sang Jun Son [a], Junhong Min [a,*]

[a] Department of BioNano Technology, Kyungwon University, Gyeonggi-Do 461-701, Republic of Korea
[d] Department of Chemistry, Seoul National University, Seoul 151-747, Republic of Korea

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ABSTRACT

A silica based 3-D nanosurface was developed to enhance the signal intensity of a protein chip by increasing the surface density and reducing the aggregation of captured proteins immobilized on the nanosurface. The 3-D nanosurface was composed of silica nanopillar bundles formed from a nanoporous alumina template using the sol–gel method. The signal intensity of a protein spot increased exponentially when the capture probe was immobilized on a nanosurface with higher roughness and the amount of protein immobilized on the surface was proportional to the roughness of the nanosurface. To further investigate this nanosurface effect, changes in the nanosurface roughness before and after protein immobilization were investigated by AFM. The surface roughness was shown to increase after protein immobilization when the nanosurface initially had a relatively low surface roughness (Rq: 30–40 nm); however, the surface roughness decreased after protein immobilization when it initially had a high roughness (Rq: 60–130 nm). These results imply that a high nanosurface roughness decreases the overall aggregation of proteins on the surface. These findings were also confirmed by comparing the level of protein aggregation on nanosurfaces with high roughness and low roughness using AFM.

1. Introduction

The importance of early diagnosis has led to the development and commercialization of various detection systems for the detection of diseases such as cancer and infectious diseases [1–3]. Microarrays based on nucleic acids and proteins, such as antibodies, have been rapidly developed as diagnostic tools for early disease detection because these types of detection methods have distinct advantages such as high throughput screening, specific and sensitive detection, and well established protocols [4–6]. Particularly, the DNA microarray has been developed for early diagnosis because highly stable DNA can specifically hybridize with its complimentary sequence. Because genotype diagnosis for diseases requires defined gene functions and a high level of mRNA corresponding to specific proteins, protein arrays have attracted significant attention as a potential tool for disease diagnosis by directly measuring gene products [7–10].

Protein arrays that utilize specific protein–protein interactions have been widely studied with regard to protein immobilization methods, their stability, and signal enhancement for high sensitivity. Particularly, methods to detect low amounts of analyte have emerged as a highly important component in disease diagnosis. To increase the sensitivity of detecting an analyte using protein arrays, various studies have been conducted to improve protein orientation, increase the amount of protein immobilized on a surface, and develop methods to enhance the signal intensity.

Protein A or protein G has been utilized to immobilize antibodies on the surface with high orientation by exploiting its specificity to the Fc fragment of the antibody [11]. However, these methods require a sophisticated process to immobilize protein A or protein G and their orientation is also not well defined. Nanoparticles [12] and liposomes that contain a high concentration of a fluorescent dye or electrochemical reagents [13,14] have been used to amplify the detection signal. However, signal amplification methods require a long signal enhancing time to amplify the signal. In addition, the noise as well as the signal may be increased when nonspecific binding to the surface occurs. 3-Dimensional (D) branched chemical linkers such as dendrimers, which have a tree-like structure, have been developed to increase the density of the capture probe immobilized on the surface [15–17]. These 3-D linkers can dramatically increase the amount of probe that is captured on a limited area and, thus, can increase the detection sensitivity. This 3-D protein immobilization method has led to the development of 3-D surfaces to simplify and normalize the protein immobilization process [18–21].

A 3-D surface can increase the amount of protein immobilized on the surface due to its increased surface area relative to a 2-D surface and it can prevent the denaturation of antibodies by retaining moisture within the 3-D structure. This denaturation

* Corresponding author. Tel./fax: +82 31 750 8553.
E-mail address: jmin@kyungwon.ac.kr (J. Min).

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prevention is a unique property of 3-D protein arrays [22,23]. The 3-D microwell protein array has been used as the representative 3-D surface protein array for enhancing the detection signal [24]. It was developed to achieve a several fold higher signal at a given analyte concentration relative to a 2-D array. The 3-D microwell surface was fabricated by depositing silica on an alumina pore template. It has been shown that adsorption of protein to the silica was stronger than to the aluminum wells because of silane binding and the signal was enhanced to a greater degree due to a higher immobilization density, which was dependant on the depth of the well [24]. However, there are limitations in using this nanoporous silica coated template to detect relatively large proteins due to the time needed for larger sized proteins to insert into the nano-sized wells, which have a high aspect ratio. Even though the protein is immobilized at a higher density, the signal will not significantly increase if the reporter probe cannot penetrate the nanoporous structure. Moreover, protein immobilization onto nanoporous surfaces will be highly limited if protein aggregation occurs.

In this study, a 3-D silica surface consisting of nanopillar bundles was developed to resolve the problems associated with the nanoporous 3-D surface. Silica nanopillar bundles were formed on a PDMS (polydimethylsiloxane) layer through the aggregation of long pillars with a high aspect ratio (length: 1000 nm, diameter: 80 nm). The amount of protein immobilized on the pillar structured 3-D surface of varying roughness was measured using a fluorescently labeled protein. The change in surface roughness after immobilization of protein on the 3-D surfaces was measured using Atomic force microscopy (AFM). The aggregation of protein immobilized on the surface was investigated by comparing the signal enhancement of the 3-D protein array and the amount of capture probe immobilized on the surface. Protein aggregation was also confirmed by examining the surface morphology by AFM.

2. Materials and method

2.1. Materials

(3-Aminopropyl)triethoxysilane (APTS; Gelest) was purchased as a linker for immobilization, rabbit immunoglobulin (IgG) (#4970, Cell Signaling) was purchased as the capture antibody and anti-rabbit-IgG IgG labeled with Texas Red fluorophore (vector) was purchased as the reporter antibody. Protein immobilization was detected and an excitation and emission wavelength of 604 and 615 nm, respectively. Bovine serum albumin (BSA) was obtained from Sigma–Aldrich and used as a blocking agent. Phosphate buffered saline (pH 7.4) was obtained from Sigma–Aldrich and used as the reaction buffer. Silicon tetrachloride ($SiCl_4$, 99.8%, Acros Organics), perchloric acid (70%, DC Chemical), phosphoric acid (85%, DC Chemical), oxalic acid (Dihydrate, DC Chemical), and mercury(II) chloride (99.5%, DAEJUNG) were used as supplied without further purification. Aluminum foils (99.99%) were purchased from Alfa Aesar. Polydimethylsiloxane elastomer kits (Sylgard 184) were purchased from Dow Corning (Midland, MI).

2.2. Preparation of silica-coated AAO templates

Anodic aluminum oxide (AAO) templates were prepared by two-step anodization according to previously published protocols [25,26], except that only one side of the Al plate was anodized and the other side was protected by silicone rubber (Fig. 1(a)). Preannealed aluminum foils (0.25 mm thick) were first degreased

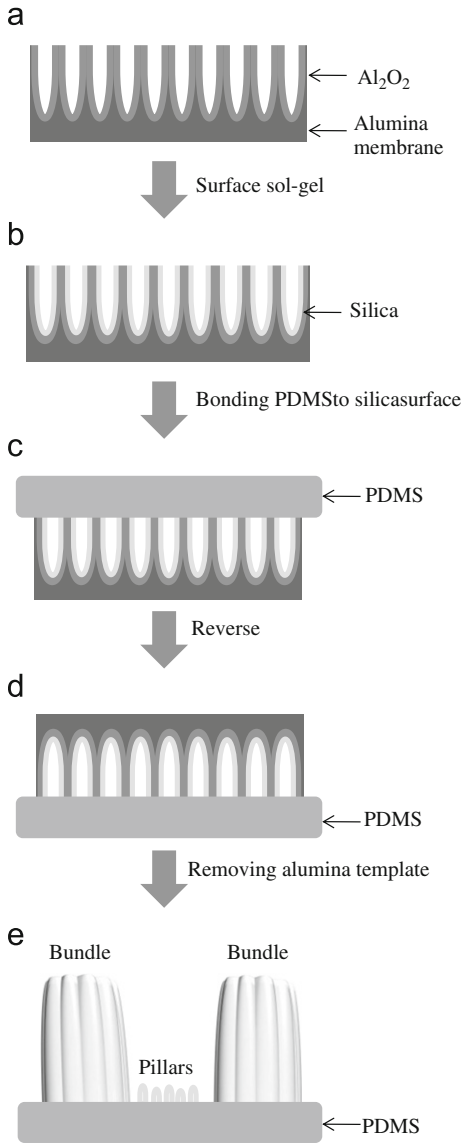

Fig. 1. Schematic procedure used for the fabrication of the silica nanotube surface.

in acetone, and then electropolished with perchloric acid and ethanol (v/v 1:5) at 15 V and 5 °C. The anodization was carried out at 40 V and 10 °C. The irregular oxide film obtained from the first anodization step was etched away in the mixed solution of chromic acid and phosphoric acid. The final oxide film was then prepared by the second anodization step. The pores were widened in phosphoric acid solution (5 wt%) at 30 °C.

The silica-coated alumina templates were obtained using the surface sol–gel method (Fig. 1(b)) [27], as reported previously [28]. The resultant AAO template was dipped in $SiCl_4$, rinsed with hexane, immersed in a mixture of hexane and methanol, then rinsed with ethanol. After being dried with nitrogen gas, the templates were finally immersed in water to finish the reaction. Four deposition cycles were performed in this study.

2.3. Fabrication of nanosurface

The resulting silica-coated AAO templates were placed on the clean glass plate, with the anodized side faced up. A prepolymer of PDMS was poured onto the plate and solidified at 70 °C for 1 h resulting in the formation of a smooth PDMS film that was 2 mm thick (Fig. 1(c)). After PDMS formation, the remaining back-side