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TECHNICAL NOTE

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Application of anodized aluminum in fluorescence detection of biological species

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Abstract Thin nanoporous alumina obtained by anodization of aluminum films offers promising advantages for application in fluorescence-based biological sensors including convenient preparation, increased density of binding sites, and improved collection efficiency of fluorescence. These advantages are illustrated in the detection of streptavidin using biotin covalently bound to the surface of alumina nanopores. Fluorescence intensity enhancement as high as 7 times is observed in nanopores in comparison to flat glass surface.

Keywords Biosensors · Fluorescence/Luminescence · Nanoporous Aluminum Oxide · Biotin

Introduction

Detection of biological molecules using radioactive or fluorescence labeling has become ubiquitous in modern biochemistry since the invention of Southern blotting [1]. This detection technique has very effectively been applied in microarray chips, in which multiple spots with specific affinity to analyte species of interest are simultaneously immobilized on the surface [2]. Such assays allow synchronized detection of thousands of DNA and RNA fragments as well as proteins [3] and even their interaction with drugs [4].

Numerous improvements were introduced since launching practical applications of these techniques. Recently nanoporous aluminum oxide films were proposed as substrates for fluorescence detection in microarray DNA chips [5, 6].

Anodized aluminum can be produced in a regularly arranged hexagonal pattern of cylindrical pores with nanometer diameters (nanopores). Such nanoporous oxide films have high surface area densities and allow binding enhanced amounts of target molecules on each spot of the chip and thus afford a substantial increase in the fluorescence signal intensity. The increase in the signal intensity, I/I_0, can be estimated from the surface ratio of a cylinder, πdL, and its cross section, $\pi d^2/4$:

$$\frac{I}{I_0} = \rho \frac{4L}{d} \tag{1}$$

Here ρ is the surface porosity (a portion of the surface area covered by the pores), and L and d are the pores' depth and diameter, respectively. Equation 1 suggests that the signal enhancement can be raised to infinity by increasing the ratio L/d but the limit in length is set by self-absorption of the dye's fluorescence, and the minimum pore diameter should be larger than the size of the analytes.

In this paper we demonstrate the use of anodized aluminum as a convenient substrate for fluorescence-based detection of biological molecules. This template has a number of advantages. First, the porous structure can dramatically increase the effective surface area in relatively thin films, where self-absorption of dye's fluorescence is insignificant. Second, the aluminum underlayer increases the fluorescence collection yield by reflecting most of the light towards the excitation source. Third, the use of reliable aminosilane chemistry for immobilization [3] instead of metal–thiol linkage [7] assists in making stable covalent binding. Additionally, the aluminum underlayer can also serve as an electrode for electric-field-directed specific modification of desired spots [8] or as an electrode for electrochemical DNA detection [9]. All these benefits and straightforward integration with CMOS technology offer an attractive possibility for employing anodized aluminum in microarray biosensor chips.

We demonstrate these advantages using a well-known biotin–streptavidin pair. Biotin is covalently immobilized on the walls of nanoporous alumina sensor through aminosilane–succinimide chemistry, and binding of tagged streptavidin molecules was investigated using fluorescence microscopy. Stability of the immobilized biotin on the aluminum oxide surface was demonstrated using commer-

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cial nanoporous alumina membrane by means of FTIR spectroscopy.

Materials and methods

The scheme used for the sensor preparation is shown in Fig. 1. Polished glass slides (Clini-Slides) were cut into 2.5 × 2.5 cm^2 pieces using a diamond saw. The slides were cleaned by sonicating in acetone followed by soaking in piranha solution (1:1 30% H_2O_2/98 % H_2SO_4) for 30 min (*CAUTION: piranha is very dangerous and should be handled with great care!*) and another sonication in DI water, finished by drying in an oven.

Aluminum film was deposited on the glass slide through a mask using a Gatan-681 ion beam coater. The mask outlined a desired shape of aluminum film pads on the glass. The film thickness was 600 nm as measured by the thickness monitor.

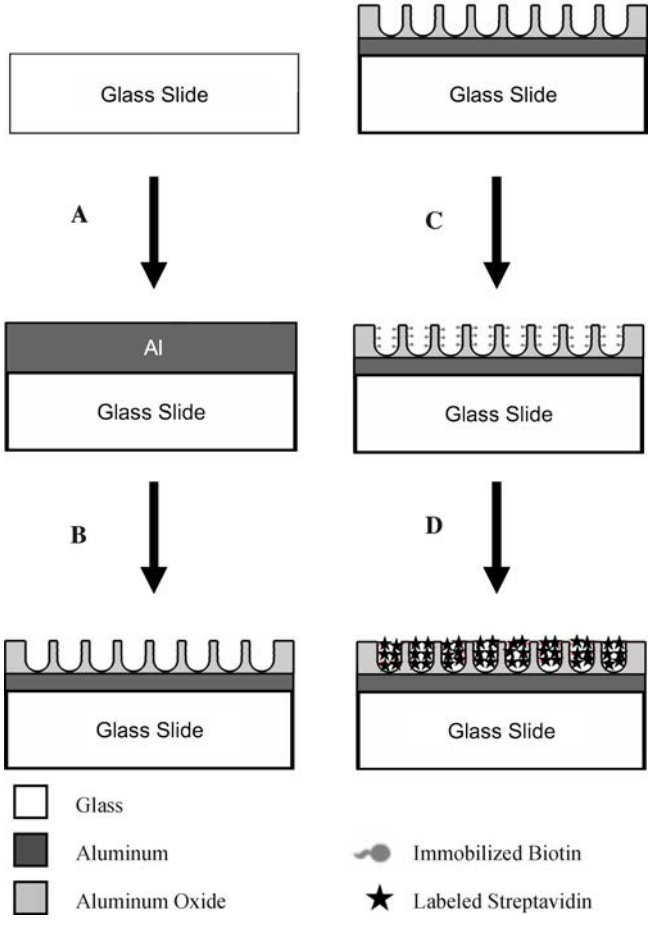

Fig. 1 Sample preparation: (**a**) glass slide was covered with thin aluminum layer. Anodization was then performed (**b**) and biotin was immobilized using aminosilane–succinimide chemistry (**c**). Alexa-modified streptavidin was bound to the surface from buffer solution (**d**)

Anodization of the aluminum film was performed in 3% oxalic acid at 4 °C using 40-V DC applied for 2 min, similar to a previously reported procedure [10]. The process was intentionally stopped before the whole film thickness was anodized, so that an obvious reflective layer of aluminum remained. According to the SEM micrograph of the sample cross section shown in Fig. 2, the apparent thickness of the aluminum oxide layer is approximately 450 nm.

Biotin was immobilized on nanoporous and flat surfaces using aminosilane chemistry [5, 9]. First, a dried sample of nanoporous alumina was "aminated" by soaking in 5% acetone solution of 3-aminopropyltrimethoxysilane (Aldrich) for 1.5 h, washing in acetone, and baking in oven at 120 °C overnight. The substrate was biotinylated by treating with a DMSO solution of biotin *N*-hydroxysuccinimide ester (Aldrich) for 6 h, rinsing with acetone, and drying in air.

The surface concentration of biotin and its stability was investigated using FTIR in transmission mode on commercially available Anodisc (Whatman) aluminum oxide membranes with nominal pore size 20 nm and 60-μm thickness. Anodisc membranes are more convenient for transmission measurements and possess similar properties

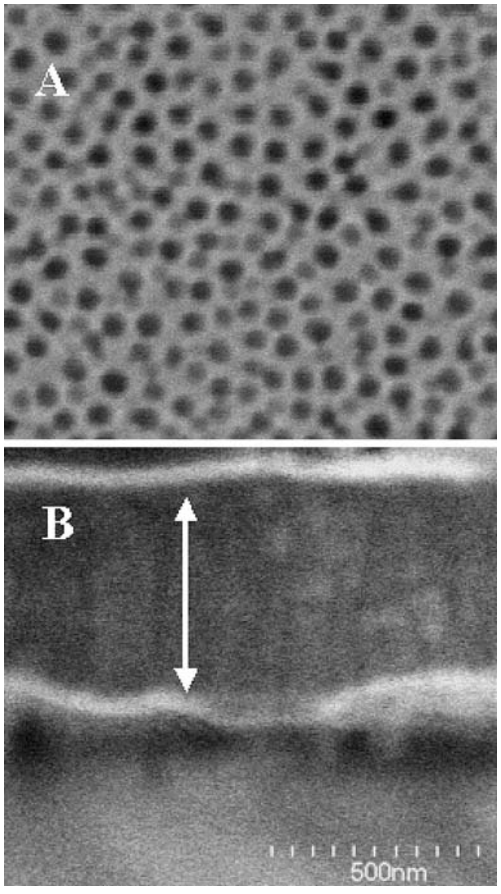

Fig. 2 SEM micrographs of the nanoporous alumina film. The *top view* of typical pores for the given anodization conditions (**a**). The *side view* of the nanoporous alumina on glass (**b**); the *white arrow* indicates the thickness of the nanoporous oxide layer, approximately 450 nm

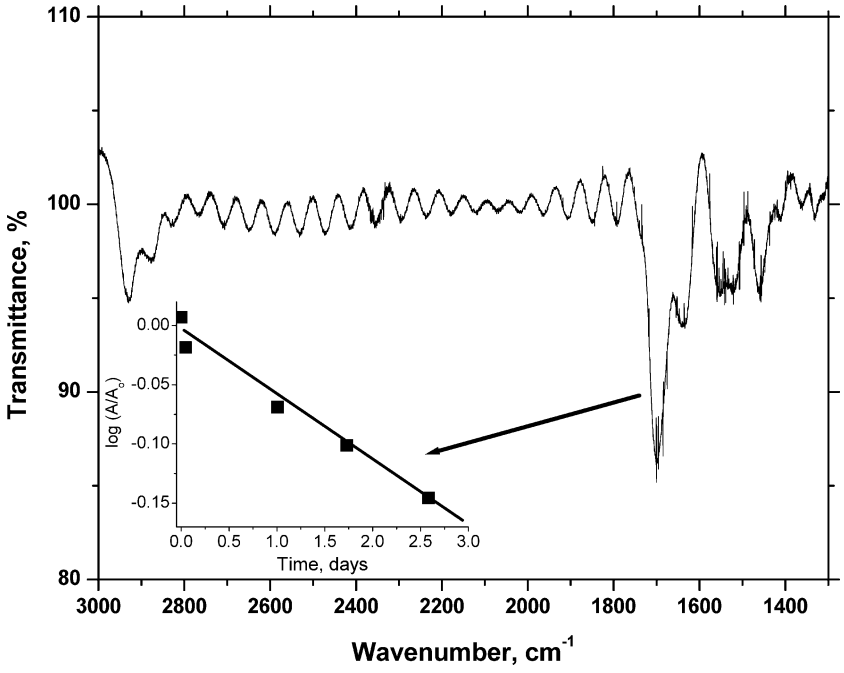

Fig. 3 FTIR spectrum of an Anodisc membrane with immobilized biotin. The *inset* provides a semilogarithmic plot of intensity versus time for the 1,700 cm^{-1} band illustrating the decay time of 8±1 days

to our films. They were modified with biotin in a similar fashion. IR spectra were recorded using Perkin–Elmer Spectrum One FTIR Spectrometer. Unmodified Anodisc membrane was used as a reference.

Efficiency of streptavidin binding to biotin on nanoporous and flat surfaces was investigated using fluorescence of streptavidin modified with Alexa Flour 488 dye (Molecular Probes) by exposing biotinylated substrate for 5 min to 1.5 μM streptavidin solution in PBS buffer (Antibodies Incorporation; pH=7.5). The unbound streptavidin was washed off by rinsing with copious amounts of buffer. Fluorescence of the surface protein was immediately measured on the sample immersed in water using a Carl-Zeiss Axiovert 200M inverted fluorescent microscope and AxioCam MRm monochromic digital camera equipped with a filter assembly for Alexa Flour 488 dye: HQ 470/40-excitation, and HQ 525/50–luminescence to match the dyes' absorption and emission maxima at 495 nm and 519 nm, respectively. The exposure time was set at 10.2 ms for all samples. The images were analyzed using ImageJ NIH open source software.

Results and discussion

FTIR: immobilized biotin stability

An FTIR spectrum of an Anodisc membrane with immobilized biotin is shown in Fig. 3, from which the surface concentration of biotin was estimated to be approximately 2×10^{14} molecules per cm^2 of the surface area. The number was calculated using absorption coeffi-

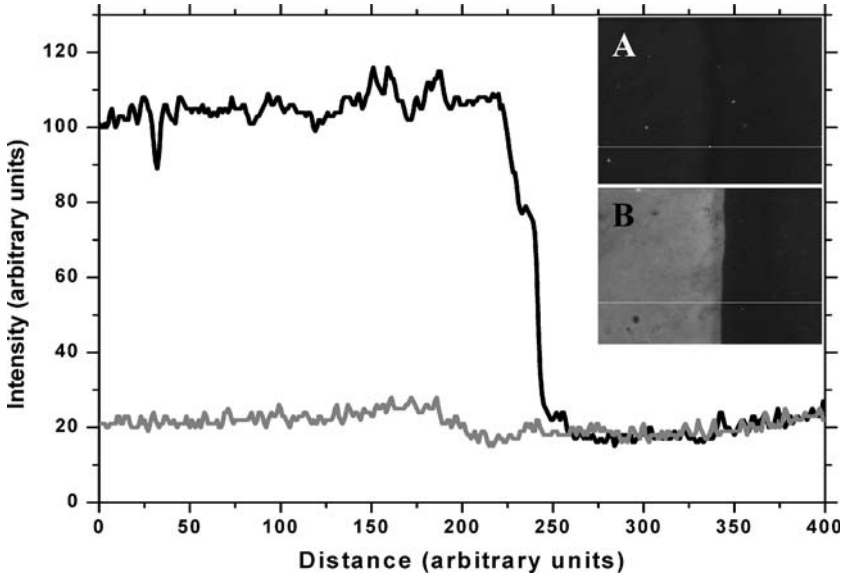

Fig. 4 Overlay of the fluorescence intensity profiles for the images in the inset. *Black line* anodized alumina surface, *gray line* metallic alumina surface. *Inset* shows the fluorescence images of the edges of metallic aluminum (**a**) and anodized aluminum (**b**) films (X100 Zoom). Both films are modified with biotin and incubated with 1.5 μM streptavidin (see text for details)

cient for the in-ring CO stretching frequency band (near 1,700 cm^{-1}) that was separately determined from an FTIR spectrum of biotin solution in DMSO.

When stored in a dry dark environment, biotin shows no noticeable decline in surface concentration, but some gradual decay was observed when the membrane was stored in water or PBS for longer than a week. The kinetics of biotin decay from the membrane was monitored by IR absorption of the 1,700 cm^{-1} band. The characteristic decay time of 7.9±0.7 days appeared to be similar to those of other aminated surfaces [11]. Therefore, biotin covalently immobilized on Al_2O_3 is sufficiently stable during the time frame of experiments that take no more than several hours.

Fluorescence: streptavidin binding

The pore diameter in anodized aluminum is a function of anodization voltage and can be made as small as 10 nm. We chose medium-range-diameter pores of 60 nm to have pores small enough for a sizeable enhancement, on the one hand, and to be large enough to accommodate large streptavidin molecules (d_{strep}≈8 nm) [12] without hindrance, on the another hand. Without such hindrance, the time required to diffuse over the pore length, L, by streptavidin can be estimated by using the hydrodynamic diffusion coefficient of streptavidin, D:

$$\tau \sim \frac{L^2}{D} \sim \frac{3\pi\eta L^2}{k_B T} d_{strep} \qquad (2)$$

where η is the viscosity coefficient of water, and k_B and T are the Boltzmann constant and temperature, respectively. For the given conditions the diffusion time is very short, $\tau \approx 40$ ms.

Fluorescence images of the anodized aluminum and the metallic aluminum films at their edges with underlying glass substrate are shown in the inset in Fig. 4. Both substrates were modified simultaneously with biotin and then exposed to the same solution of streptavidin. Obviously, fluorescence from anodized aluminum is much stronger than that from glass and nonanodized aluminum. Corresponding spatial profiles of fluorescence (obtained with ImageJ software) are given in Fig. 4 and their analysis is given in Table 1. Increasing the streptavidin incubation time from 5 min to 24 h had no effect on the fluorescence intensity, in agreement with the short estimated diffusion time, τ.

For our alumina membranes comprising 450-nm-long pores with 60-nm diameter and 50% surface porosity, the anticipated increase in fluorescence intensity should be around 15, whereas the ratio between the aluminum and glass surfaces should be around 2, due to the increased fluorescence collection on metallic substrate. Experimental values are smaller than that. The background signal was collected on unmodified glass substrate under the same conditions of detection (blank slide column in Table 1).

Table 1 Fluorescence intensities measured for different substrates modified with biotin and exposed to the same solution of streptavidin (1.5 μM Alexa Fluor 488 modified streptavidin in PBS buffer)

	Blank glass slide with no modifications	Glass	Metallic aluminum	Anodized alumina
Absolute intensity	6±3	19±3	24±2	96±7
Net intensity (blank subtracted)	–	13±4	18±4	90±8

After subtracting the background signal, the brightness ratio between the anodized aluminum and metallic aluminum surfaces is 5.0±1.2 and between anodized aluminum and glass is 6.9±2.2, whereas the ratio between the aluminum and glass surface does not exceed 1.4. The discrepancy of these values from the anticipated ones is due to a number of other effects, and their relative contribution requires further investigation. Additional effects that can be responsible for the discrepancy include fluorescence quenching of the fluorophore by underlying metallic Al, interference effects on the membrane thickness, light scattering by nanoporous alumina, incomplete pore wetting, and others. Nevertheless, the enhancement effect in nanoporous alumina is clearly present and can be applied to studies that involve fluorescently labeled analytes.

Conclusions

Application of thin anodized aluminum films as substrates for fluorescence detection of labeled biomolecules is demonstrated. The brightness on 450-nm-deep, 60-nm-diameter anodized alumina increases by a factor of 5.0±1.2 compared to flat aluminum and by a factor of 6.9±2.2 compared to a glass substrate. Concentration of biotin in a monolayer bound to the nanoporous alumina surface using aminosilane–succinimide chemistry is close to 2×10^{14} molecules per cm^2 and is stable for over a week in DI water or PBS buffer.

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