Label-free quantitative detection using porous silicon as optical biosensor*

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The single-layer porous silicon is prepared by electrochemistry etching method, which is used as an immunosensor for determining recombinant mouse zona pellucida 3 fusion protein (r-mZP3) by Raman spectroscopy analysis at room temperature. The molecule binding increases the effective optical thickness (EOT), and thus the Raman spectrum intensity decreases. The concentration and variation of Raman intensity show a good linear quantitative relation. The excellent sensing performance could open the way to a new family of optical sensors for biological standardization.

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Porous silicon (PS), which is often obtained by electrochemical etching of crystalline silicon wafers[1], is one of the most interesting materials in biosensing applications, because it has large internal surface area and biocompatibility and its optical properties can be changed by infiltrating material into the pores[2]. Many sensing methods based on PS were reported. Huimin Ouyang et al[3] used the shift of the optical reflectance spectrum for detecting protein, Guoguang Rong et al[4] used porous silicon waveguide for detecting DNA hybridization, and Gabriela Palestino et al[5] used fluorescence microscopy for detecting DNA hybridization protein. We have already used PS as a kind of immunosensor for the detection of biomolecule in previous work[6,7]. But the relation between the Raman intensity and the concentration of the surface protective antigen protein needs further analysis.

Fourier transform Raman (FT-Raman) spectroscopy is based on near infrared radiation and scattering, which originates from the interaction between the incident light and the phonons (normal-mode energy quantum of lattice vibration) in the sample. Compared with Fourier transform infrared (FTIR) reflectance, the Raman spectrum technology has higher sensitivity and faster response. Recently, as a low cost, rapid and non-destructive detecting technique, FT-Raman method has been widely used in protein and sugar detection, etc[8-10]. But the sample components are already known in these detections. In most cases, the sample component is unclear. Therefore, in this paper, we report a PS biosensor using Raman spectrometer for detecting the antigen-antibody reaction between the artificial immunogen of recombinant mouse zona pellucida 3 (r-mZP3) and complementary (positive) serum.

In this paper, the method of PS preparation process is the same as our previous work[11]. The porous silicon substrate was prepared by electrochemical etching method under 60 mA/cm^2 for 2.5 min, illuminated under halogen lamp during the electrochemical deposition process. The N-type and <100> orientation silicon wafer with the resistivity of 0.02 $\Omega\cdot cm^{-1}$ and the thickness of ~380 μm was used as the substrate material. A mixture of ethanoic and hydrofluoric acid with 1:1 ratio was used as electrolyte. After anodization, the samples were rinsed in ethanol and then de-ionized water, and air dried. Single-layer porous thin films were thermally oxidized to impart greater stability in biological solutions containing salt and to create hydrophilic pore channels. Dry thermal oxidation was conducted using a three-zone Lindberg tube furnace at 1000 °C, and then cooled to room temperature.

PS samples were immersed in antibody solution with a dilution of 1:400, except that one was immersed in negative serum for comparison. Next, all chips were incubated at 37 °C for 2 h for the immobilization of antibody. To prevent

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nonspecific adsorption, the chips were exposed to 3% bovine serum albumin (BSA) for 2 h. Finally, each sensor was immersed in a series of concentrations of target for 2 h at 37 °C respectively and rinsed with phosphate-buffered saline and Tween-20 (PBST) buffer for three times, and each lasts for 5 min. Raman spectra measurements were performed on the PS layers by a Nicolet FT-Raman spectrometer using liquid nitrogen to cool Ge detector. The Raman spectra were recorded in the backscattering configuration at room temperature employing an Nd/VO_4 laser at 1064 nm.

Fig.1 shows the scanning electron microscope (SEM) image of the freshly prepared PS and its Raman spectrum. It can be seen that the surface of PS has a sponge structure as shown in Fig.1(a), and the pore size is about 200 nm. In Fig.1 (b), a single peak is observed at 521 cm^{-1}, which corresponds to the single crystal structure of silicon. The spectrum of PS is different from that of crystalline silicon, because disorder or finite size may result in a relaxation of the momentum conservation rule, leading to a downshift and an asymmetric broadening of the first order Raman peak[12].

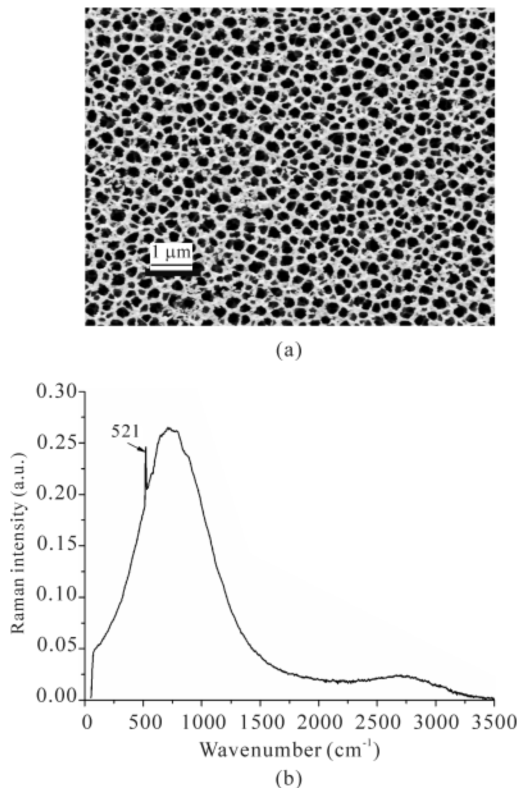

Fig.1 (a) SEM image and (b) Raman spectrum of the freshly prepared PS

Fig.2 shows the variation of FT-Raman spectra of the samples before and after exposure. In Fig.2(b), there is a detectable decrease of 0.0188 after exposure to r-mZP3 of 0.5 μg·ml^{-1} with negative serum. But the FT-Raman intensity is obviously reduced by about 0.2641 when the chip is exposed to r-mZP3 of 0.5 μg•ml^{-1} with the positive serum as shown in Fig.2(a). This indicates that the decrease of the FT-Raman peak intensity is due to the selective antigen-antibody binding. The variation shown in Fig.2(b) may be because of the introduced impurities or operating error. Different concentrations result in different decreases of the peak intensity. Hence it can be a method for the detection of concentration and the identification of biological molecules.

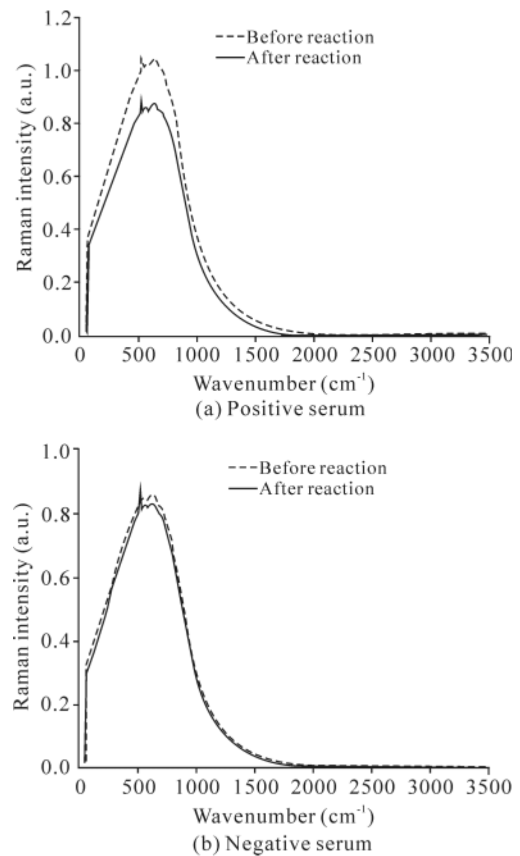

Fig.2 Raman spectra for single-layer PS immunosensor before and after the chip is exposed to r-mZP3 of 0.5 μg·ml^{-1} with the positive serum and the negative serum

In order to investigate the relationship between Raman scattered signal and concentration, we configure four standard serums from 0.125 μg/ml to 1 μg/ml, and assay the Raman spectra. The Raman peak at 521 cm^{-1} is the characteristic peak of PS, and there is no interferential peak nearby. Fig.3(a) shows the relationship between immobilized concentration and absolute deviation of Raman intensity before and after exposure, which shows a good linear quantitative relation. From 0.125 μg/ml to 1 μg/ml, the linear correlation coefficient is R=0.99084, and the linear regression equation is Y=0.00597+0.2424X, where Y is the deviation value of $A2$ and $A1$, and $A2$ and $A1$ are the Raman intensities before and after adding the positive serum, respectively. Fig.3(a) is the

absolute intensity variation, and Fig.3(b) shows the linear relationship between relative intensity variation and sample concentration, with the linear regression equation of $Y = 0.97122 + 0.23721X$ (R=0.99068), where Y and X stand for $A1/A2$ and sample concentration, respectively.

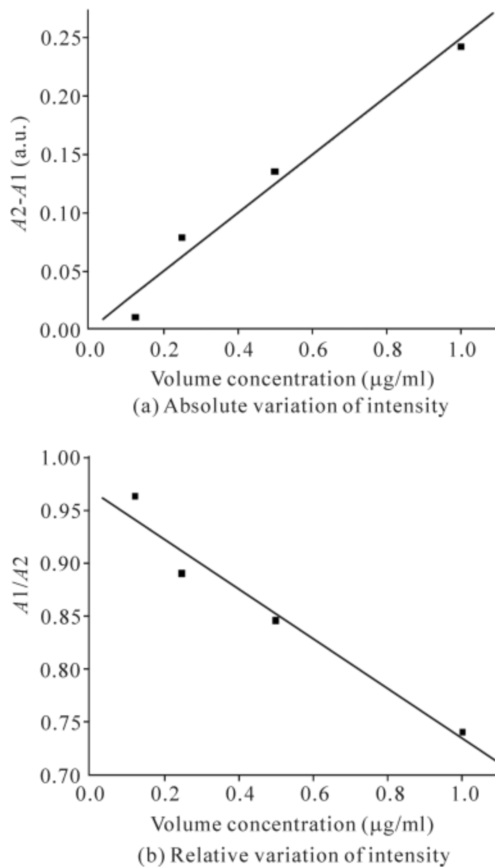

Fig.3 Relationship between immobilized concentration and estimated immobilization capacity

Quantitative spectroscopy is based on the Bouguer Lambert-Beer law, which can be expressed as[13]:

$$A = \lg(1/T) = Kbc , \tag{1}$$

where A is the absorbance, T is transmissivity which is equal to I/I_0, I_0 is the background intensity, I is the intensity measured with the sample present, K is the absorptivity, b is the sample thickness, and c is the sample concentration. When a parallel monochromatic beam propagates vertically through a uniform absorption material, its absorbance A is proportional to its concentration c and thickness b, which also means that the absorbance is proportional to the effective optical thickness (EOT).

The relationship between Raman intensity and the concentration of analyte follows another form of Lambert-Beer law:

$$I = PLCI_0 , \tag{2}$$

where I is the intensity measured with the sample present at given wavelength, P is the parameter of instrument, L is the path length of light, C is the concentration of analyte, and I_0 is the power per unit of incident beam area.

In Eq.(2), LC can be equivalent to EOT, and P and I_0 are constant, so we can get:

$$I/I_0 = PS_{EOT} , \tag{3}$$

where S_{EOT} is the EOT of the samples. So I/I_0 and S_{EOT} are in linear relationship. Since binding of any additional bimolecular can lead to a change in hole wall thickness of PS, the change in EOT is a direct measure of the molecular weight of bound protein. Thus, the reduction of Raman intensity is presumably caused by the antigen-antibody reaction on PS. It can be a method for the detection of dopant and for routine analysis if proper calibration and validation procedures with data acquisition protocols could be established.

Owing to linear relationship between the concentration of samples and EOT[14], the relationship between the intensity and the concentration of samples is also linear. The optical properties of different PS substrates can not be exactly the same. So we can't adopt the method like Fig.3(a), and should consider the changes of relative light intensity. For linear relationship between the concentration of samples and the changes of absolute light intensity, we may draw a conclusion that the prepared PS substrates are almost consistent, and the repeatability is very good. From Fig.2(b), it is found that the absorbance increases due to introducing impurities in the experiments. After exposuring the sample to the negative serum with 1 μg•ml^{-1}, the Raman intensity decreases by about 0.0188. Without regarding the error due to impurity, the function can be modified as (Fig.3(b)):

$$Y = 1.00026 - 0.23721X . \tag{4}$$

The Raman intensity of the PS at 521 nm is reported as a function of the concentration, a good linear response is observed, and a sensitivity of about 0.237 decrease per 1 μg/ml can be estimated. Given that the Raman intensity resolution of the FT-Raman spectrometer is 0.00001 a.u., the ultimate detection limit of the PS immunosensor of ρ=0.00001 a.u./(0.23721 a.u./(μg•ml^{-1})) = 42.2 pg•ml^{-1} can be estimated for this kind of optical sensor[16]. The relationship between the variation of relative Raman intensity and the concentration of the samples is linear, which is similar to the results in Refs.[13] and [15]. If the operation is the norm, although the areas are different or introducing impurity in the experiments, calculating the absorbance on the basis of detected signal can lead to substantial error in quantitative analyses, but the results are still consistent very well with the regression equation.

In conclusion, we describe a new detection method using PS as a quantitative analytical device for optical label-free biosensing. We prove that when the molecule binding events increase, a linear decrease of the Raman intensity corresponding to PS is observed. The results are very interesting, and in fact it could open the way to a new family of optical sensors for biological standardization.

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