Biosensors and Bioelectronics 25 (2010) 1056–1063

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

FTRIFS biosensor based on double layer porous silicon as a LC detector for target molecule screening from complex samples

Yunling Shang[a], Weijie Zhao[a], Erchao Xu[a], Changlun Tong[b,**], Jianmin Wu[a,*]

[a] Institution Microanalytical System, Department of Chemistry, Zhejiang University, Hangzhou, 310027, China
[b] College of Environmental and Resources science, Zhejiang University, Hangzhou, 310029, China

ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form
13 September 2009
Accepted 18 September 2009
Available online 1 October 2009

Keywords:
Double layer porous silicon
Fourier transformed reflectometric interference spectroscopy
Trypsin inhibitor
Biosensor
Screening

ABSTRACT

Post-column identification of target compounds in complex samples is one of the major tasks in drug screening and discovery. In this work, we demonstrated that double layer porous silicon (PSi) attached with affinity ligand could serve as a sensing element for post-column detection of target molecule by Fourier transformed reflectometric interference spectroscopy (FTRIFS), in which trypsin and its inhibitor were used as the model probe–target system. The double layer porous silicon was prepared by electrical etching with a current density of 500 mA/cm^2, followed by 167 mA/cm^2. Optical measurements indicated that trypsin could infiltrate into the outer porous layer (porosity 83.6%), but was excluded by the bottom layer (porosity 52%). The outer layer, attached with trypsin by standard amino-silane and glutaraldehyde chemistry, could specifically bind with the trypsin inhibitor, acting as a sample channel, while the bottom layer served as a reference signal channel. The binding event between the attached trypsin and trypsin inhibitor samples could be detected by FTRIFS in real-time through monitoring the optical thickness change of the porous silicon layer. The baseline drift caused by sample matrix variation could be effectively eliminated by a signal correction method. Optical signals had a linear relationship with the concentration of trypsin inhibitor in the range of 10–200 ng mL^{-1}. The FTRIFS biosensor based on double layer porous silicon could be combined with a UV detector for screening the target molecule from complex component mixtures separated by a LC column. Using an LC–UV–FTRIFS system, a fraction containing a trypsin inhibitor could be separated from a soybean extract sample and identified in real-time.

1. Introduction

Over the last two decades, drug screening has made remarkable progress due mostly to the development of affinity biosensors (Ho and Leclerc, 2004). An affinity biosensor consists of an electrochemical (Ghindilis et al., 1998), piezoelectric (Chu et al., 1995), or optical transducer (G, 1996) and a biological recognition element that is able to monitor the interaction between biomolecules. A variety of optical methods has been exploited in biosensors including fluorescence spectroscopy (Rowe-Taitt et al., 2000), interferometry (Heideman and Greve, 1993; Piehler and Gauglitz, 1996), and surface plasmon resonance (SPR) (Homola and Gauglitz, 1999; Homola and Myszka, 2002). Fluorescence-based techniques such as fluorescence polarization and time-resolved fluorescence have also become available as commercial products for enzyme inhibitor screening. However, assays incorporating fluorometric methods sometimes suffer from interference caused by autofluorescence or from fluorescence quenching of the probe (Pernelle, 2000). Label-free sensors, such as optical interferometers, grating couplers, resonant mirrors, and SPR based on the measurement of binding-induced refractive index changes, can be tailored for detection of any analyte, as these do not have to exhibit any special properties, such as fluorescence or characteristic absorption and scattering bands. An analyte binding event between a biomolecular recognition element and the analyte can be observed directly, in real-time, without the use of radioactive or fluorescent labels. Among various label-free techniques, SPR has been one of the most widely applied technologies for detection of biomolecular interactions (Borch and Roepstorff, 2004). However, screening and identification of target molecules in a complex sample needs more powerful technology.

Usually, a combination of analytical methods often results in improved analytical performance. For example, a SPR sensor combined with a separation technology such as HPLC or CE has been reported (Du and Zhou, 2008; Waseda et al., 1999). An HPLC–SPR or CE–SPR system not only separates the target analytes from a complex sample, but also is able to capture the target analyte and monitor its binding event with the recognition element attached

* Corresponding authors. Tel.: +86 571 88273496; fax: +86 571 88273572.
** Co-corresponding authors. Tel.: +86 571 86968415; fax: +86 571 86968415.
E-mail addresses: cltong@zju.edu.cn (C. Tong), wjm-st1@zju.edu.cn (J. Wu).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.09.029

to the sensing surface. Similar to other affinity biosensors that rely on measurement of refractive index changes, the signal produced by the SPR biosensor might encounter significant interference due to the non-specific interaction between the sensor surface and the sample (adsorption of non-target molecules by the sensor surface), as well as baseline drift due to the refractive index variations of sample matrices. The latter factor is especially obvious when the samples are eluted from LC with a gradient elution mode. To remove the effect of variation in background refractive index, dual channel systems, which include a sample channel and a reference channel, have been developed. However, dual channel SPR systems become more complex and also too expensive.

Porous silicon (PSi) is an attractive material for label-free sensing due to its high surface area, convenient surface chemistry, and optical signal transduction capability (Lin et al., 1997; Van Noort et al., 1998). Biomolecules penetrating into the pores of porous Si layers, driven either by non-specific adsorption or by a specific binding force, could be observed based on the changes in the spectral interference pattern that would result from the reflection of white light at the interfaces above and below the porous Si layer. The spectral positions of the Fabry–Perot fringes shift as a function of the refractive index of the material filling the pores (Collins et al., 2002; Janshoff et al., 1998). Thus, the refractive index change of the porous film could be monitored as target analyte enters and displaces the aqueous solution in the pores.

Although the variation in the optical signal in this kind of biosensor could also be induced by sample matrix or buffer component changes, this interference could be effectively diminished with the use of double-stacked layered porous silicon structures (Pacholski et al., 2005). In this case, the top porous layer would serve as the sample channel while the bottom layer would act as the internal reference channel. Light reflected from the double layer structure could produce optical fringes that would provide information on processes occurring in each of the porous layers.

Optical constants can be extracted by fast Fourier transform (FFT) of the reflectometric interference spectrum. The position of the FFT peaks depends on the thickness and refractive index of the porous layers (Anglin et al., 2004). If these two layers are appropriately designed, one of the layers can act as a blank (or reference) for the other layer. In the present study, we attached a biomolecular recognition element on the top of a porous silicon layer with high porosity and larger pore size, while another layer was designed to be low porosity with a smaller pore size. When the structure was exposed to a sample solution containing the target analyte, the outer layer selectively captured and enriched the target compound in the outer porous layer, leading to a change in the refractive index of this layer. In contrast, small molecules such as salt components in the buffer solution can penetrate the two layers, causing refractive index changes in both layers. As a result, the FFT peak position of both layers will shift together. If the shift of the FFT peak position is used as the detection signal, then the signal from the sample channel, in this case the outer porous layer, can be corrected by the signal produced by the reference channel (bottom porous layer). Selective detection can be achieved by appropriately subtracting the signal values of the outer layer with those of the bottom layer. This method provides an effective means for removing signals arising from buffers and other small molecules in the sample matrix. Thus, the Fourier transformed reflectometric interference spectroscopy (FTRIFS) biosensor based on double layer porous silicon will show advantages over other refractive index detective technologies, when it is used as a LC detector for screening of target molecules from separated fractions.

Enzyme inhibitors are an important class of drugs that target various diseases, such as cancer, AIDS, diabetes, and hypertension (Johnson et al., 2002; Noble et al., 2004). In the early stages of a drug discovery associated with enzyme inhibitors, the major task is to identify the inhibitors for a given enzyme target from a library of compounds (von Ahsen and Bomer, 2005). Therefore, the development of rapid, low-cost, and effective screening techniques for enzyme inhibitors has received much attention (von Ahsen and Bomer, 2005). Trypsin inhibitors are currently applied in pharmacological and medical fields, owing to their ability to prevent or suppress carcinogen-induced transformations, as detected in various *in vitro* and *in vivo* model systems (McBride, 2001). In this paper, trypsin inhibitor was selected as a model target molecule for evaluation of the feasibility of using the FTRIFS biosensor as a LC detector for screening of target molecule from complex sample. Trypsin was attached to a double layer porous silicon biosensor and was able to selectively capture its inhibitor from a fraction of soybean extract sample separated by an ion exchange liquid chromatographic (IEC) column. The binding event between trypsin and trypsin inhibitor was then simultaneously detected by the FTRIFS biosensor. The hyphenated LC–FTRIFS system described in this work may serve as a versatile platform for screening of target molecules from complex samples.

2. Materials and methods

2.1. Silicon etching procedure

Porous Si samples were prepared by anodization of the heavy doped p-type Si wafers with resistivity of 0.0019 Ω cm in ethanolic HF solution (3:1, v/v 48% aqueous HF:ethanol) in a two-electrode configuration using a platinum mesh counter-electrode. Si wafers with an exposed area of 1.2 cm^2 were contacted on the backside with a strip of aluminum foil and mounted in a Teflon etching cell. Double layer samples were prepared by application of 500 mA/cm^2 for 20 s followed immediately by application of 167 mA/cm^2 for 55 s. After etching, the samples were rinsed thoroughly with ethanol and then dried in a nitrogen gas flow. The porous Si samples were thermally oxidized by heat treatment at 800 °C for 1 h in a tube furnace (Fisher Blue M), and then allowed to cool to room temperature.

2.2. Scanning electron microscopy

The porous Si samples were sputter-coated with a thin layer of gold. Scanning electron microscopy (SEM) images were obtained with a SIRION (FEI, USA) electron microscope using an accelerating voltage of 25 keV.

2.3. Interferometric reflectance spectra acquisition

Interferometric reflectance spectra of porous Si were collected using an Ocean Optics CCD USB 4000 spectrometer coupled to a bifurcated fiber optic cable. A tungsten light source was focused onto the center of a porous Si surface with a spot size of approximately 1–2 mm^2. Reflective data were recorded with a CCD detector in the wavelength range of 400–1000 nm, with a spectral integration time of 100 ms. Both the illumination of the surface and the detection of the reflected light were performed at a 90° angle to the silicon surface. Spectra were not corrected for spectral response of the instrument or the lamp. The quantity, $2nL$, referred to as the effective optical thickness (EOT) in this work, was determined from the Fabry–Perot relationship:

$$2nL = m\lambda \quad (1)$$

where λ is the wavelength of maximum constructive interference for spectral fringe of order m, n is the average refractive index of the porous layer and its contents, and L is the thickness of the porous layer. The value of EOT was determined by Fourier transformation of the reflectivity spectrum. The wavelength axis of the

spectrum from the USB4000 spectrometer was inverted and a linear interpolation applied, such that the data were spaced evenly in units proportional to frequency (nm^{-1}). A Hanning window was applied to the spectrum, which was re-dimensioned to 4096 data points and then zero padded to the power of two. A discrete Fourier transformation using a multidimensional fast prime factor decomposition algorithm from the Wavemetrics, Inc. IGOR program library (FFT) was applied. Fourier transformation resulted in a sharp peak whose position was equal to the quantity ($2nL$) of Eq. (1).

2.4. Enzyme immobilization and optical measurement methods

Two oxidized double layer porous silicon chips were functionalized by immersion for 1 h in a 2 mL solution of (3-aminopropyl)-trimethoxyl silane (APTMS, purchased from Alfa Aesar) dissolved in toluene. The chips were then extensively rinsed with toluene, methanol, then acetone, and dried in stream of nitrogen. An 80 μL volume of 2.5% glutaraldehyde in 20 mM HEPES buffer (pH 7.3) was then applied to the surface of the APTMS treated porous silicon. After 30 min, the silicon samples were washed with HEPES buffer and dried with nitrogen. The trypsin attachment was carried out by immersing the glutaraldehyde-activated chip in a solution of trypsin (from bovine panceans, purchased from BBI, activity > 2500 U/mg, MW = 25.4 kDa, dissolved in the HEPES buffer at a concentration of 1 mg mL^{-1}) for 8 h in 4 °C. The residues of the aldehyde group were blocked with glycine. Another activated chip without trypsin was used as a blank sample. Before the interferometric reflectance spectra were measured in air, both porous silicon chips were washed with deionized water and dried at room temperature for 6 h.

2.5. Flow cell experiment and LC–UV–FTIRFS construction

The experiment for monitoring the binding events on the trypsin attached porous silicon surface were carried out in a flow cell system that has been described previously (Janshoff et al., 1998). Briefly, the flow cell was constructed of Plexiglass and connected via an outlet and inlet to a peristaltic pump. The light beam was focused on the surface of the double layer porous SiO_2 sample through the Plexiglas cover and interference spectra were recorded. Initially, PBS buffer pH 7.4 solution was pumped through the flow cell until equilibrium was reached. The interference optical signal was recorded by Spectrasuite software at a 10 s time interval (Ocean Optics Inc.). Standard sample solution of trypsin inhibitor was prepared by dissolving 1 mg trypsin inhibitor (from soybean, purchased from Shanghai Boao Biotech, Inc. MW = 12.5 kDa, activity > 4000 BAEE U/mg) in 10 mL 50 mM PBS (pH 7.4), and it was diluted to a required concentration with the same buffer solution. The sample solution was then pumped through the surface of the porous silicon. The shift in the EOT value, caused by binding of trypsin inhibitor onto the outer porous silicon layer as well as by the penetration of small molecules into both porous layers, was monitored throughout the entire experimental process. The procedure for screening of each fraction eluted from the ion exchange chromatographic column was essentially as just described, except that the inlet of the flow cell was connected with the outlet of the LC–UV system, as shown in Diagram 1.

2.6. Sample preparation, chromatography separation and trypsin inhibitor peak identification

Commercially available soybean seeds were washed with five volumes of diluted sodium hypochlorite (0.1%) to remove dust and to reduce microbial contamination. Seeds (40 g) were homogenized with 80 mL 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM

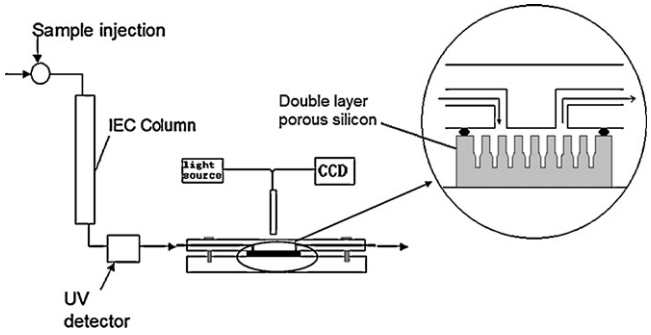

Diagram 1. The LC–UV–FTRIFS system included a chromatographic column hyphenated with a UV detector and FTRIFS biosensor in series. A tungsten light source was focused onto a porous Si surface through optical fiber probe, and the interferometric reflectance spectra of porous Si were collected using a CCD spectrometer coupled to a bifurcated fiber optic cable. The porous silicon chip was placed in the flow cell system as described in the text and the detailed setup was also shown in the zoomed cross sectional view. Mobile phase was driven by a peristaltic pump at a flow rate of 1 mL min^{-1}.

$CaCl_2$ and 0.1 M NaCl. The insoluble substances were removed by centrifugation at 2800 × *g* for 10 min, yielding a clear yellowish supernatant solution. An aliquot was taken for trypsin inhibitory unit (TIU) assessments, while the rest of the solution was dialyzed overnight against buffer B (10 mM Tris-HCl buffer + 1.0 mM EDTA + 0.1 mol L^{-1} NaCl, pH 8.0). The sample was loaded onto a Q-Sepharose fast flow column (Pharmacia), pre-equilibrated with buffer B. The column was eluted with a linear NaCl gradient from 0.2 to 1.0 mol L^{-1} in buffer B within 180 min. The flow rate was controlled at 1.0 mL min^{-1} by a peristaltic pump (Model BT00-300 M.China). A UV detector was employed for post-column detection at 280 nm wavelength. The flow cell for FTRIFS detection was connected to the outlet of the UV detector. The EOT shift of the double porous silicon layer was monitored as described above. Each component eluting from the column was collected with an automatic fraction collector, and fractions of each peak in the elution curve were pooled together. Activity of trypsin inhibitor in each collected fraction was subsequently assayed by a photometric method. Trypsin activity was determined using BAPAN as substrate (Ruckenstein and Guo, 2004), using a UV–vis spectrophotometer (UV1200, Shimadzu Corp., Japan) to monitor the reaction rate. One unit of trypsin activity was defined as a 0.001 increase in optical density at 410 nm min^{-1}. The inhibitory percentage (%*I*) was calculated as $\%I = [1 - (A_1/A_0)] \times 100$, where A_1 and A_0 represented the absorbance in the presence and the absence of the inhibitor, respectively. One unit of inhibitory activity was defined as the amount of inhibitor that reduced half the activity of 2 mg of trypsin (Choi et al., 2002).

3. Results and discussions

3.1. Characterization of double porous Si layers by optical measurement

The porosities and thicknesses of the individual porous Si layers could be determined by optical measurements based on Bruggeman theory (Anglin et al., 2004; Theiss et al., 1995), which has been proven to be in good accordance with the results obtained by gravimetric determination. To obtain the thickness and porosity value of the double layer structure, the reflective spectrum was acquired with the chip held in air and in ethanol. In order to extract the optical thickness value ($2nL$), the reflective spectra were transformed into FFT spectra according to methods described in the experimental section. Fig. 1(a) is the reflectometric interference spectrum of the air dried double layer porous Si chip,

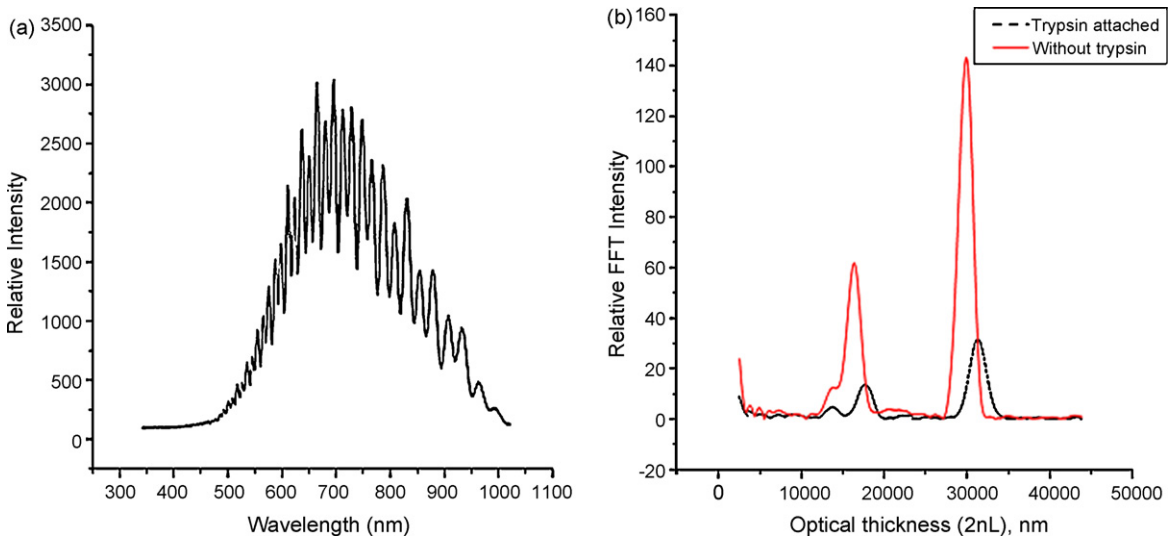

Fig. 1. (a) Interferometric reflectance spectra of thermally oxidized porous Si double layers. A p-type silicon wafer with resistivity of 0.0019 Ω cm was etched for 20 s at 500 mA/cm^2, then immediately at 167 mA/cm^2 for 55 s. Spectra were obtained from a spot ~←1 mm in diameter on the porous Si film. Spectra of samples were measured in air and are not corrected for instrumental spectral response. (b) The solid line trace represents the Fourier transformed spectra of the thermally oxidized p-type porous Si double layer, which has a corresponding reflectometric interference spectrum as shown in (a). The complex interference pattern for the double layer is decomposed into its three components (layer 1, layer 2, and layer 1 + layer2) by the Fourier transform. The dashed line represents the Fourier transformed spectrum of the double layer sample infiltrated with trypsin.

which produced three peaks after Fast Fourier Transformation, as shown in Fig. 1(b). The first and second peaks at positions of 13681 and 16424 nm corresponded to the optical thickness of each porous layer. The third peak, with the position at 30,005 nm, is assumed to be the combination of both porous layers. Since the sum of the optical thickness value of both layers is 30,105 nm, which is approximately equal to the experimental value of the third peak position, this assumption appeared to be correct. The thickness (L) of each porous layer calculated from the measured optical thickness value ($2nL$) is 3.26 and 3.91 μm corresponding to outer layer and bottom layer respectively, assuming the refractive index of the oxidized porous Si is 2.1(Pacholski et al., 2005). The results are in approximately accordance with that of SEM measurement (see Supplementary Information), in which the thickness of outer layer and bottom layer is 3.25 and 4.23 μm, respectively.

Since the peak position in the FFT spectrum is equal to the $2nL$ value, the change in optical thickness could be easily monitored by the peak shift. To measure the porosity of both layers, the FFT peak position of the ethanol infiltrated porous Si sample was detected. The FFT spectrum shows that the positions of the first and second peaks shift to 15450 and 20775 nm, respectively. The difference between the two spectra was caused by the refractive index change after air in the porous structure was replaced by ethanol. Data obtained from the sample in air and in ethanol were fit to the two-component Bruggeman approximation. The average porosity values of both layers, obtained from at least three measurements of FFT spectra and Bruggeman calculation, were 83.6 ± 0.71% and

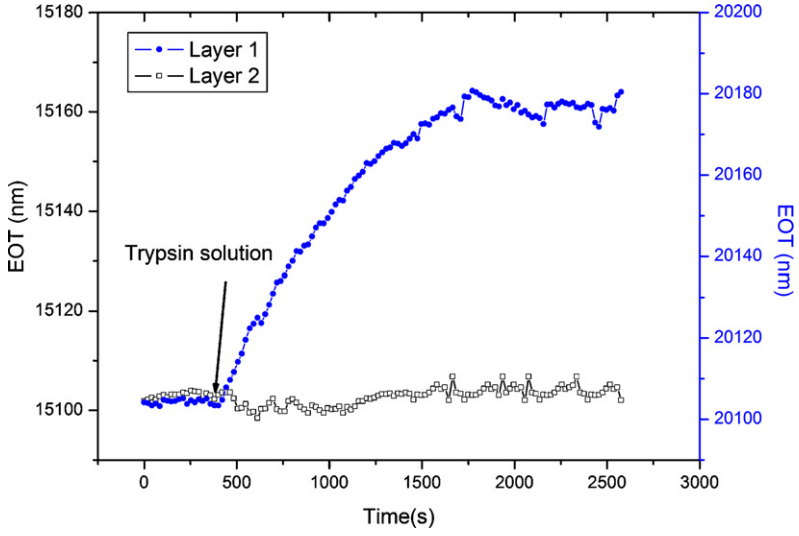

Fig. 2. Time course of the optical response of both porous Si layers during trypsin loading. The y-axis records the value of $2nL$ in the 1st (top, large pore) or the 2nd (bottom, small pore) layers, as indicated. At the beginning of the experiment, the porous Si double layer sample is flushed with 50 mM PBS (pH 7.4), 6 min later, 1 mg mL^{-1} solution of trypsin in the 50 mM PBS is then introduced, and the optical thickness of layer 1 is observed to increase from average value of 20,103 to 20,177 nm. The increase in optical thickness of the 2nd layer upon addition of trypsin indicates accumulation of the enzyme at the high porosity layer. The FFT peak position of layer 2 almost retains its original position, indicating that the enzyme is effectively excluded from layer 2, due to the much smaller pore dimensions of that layer.

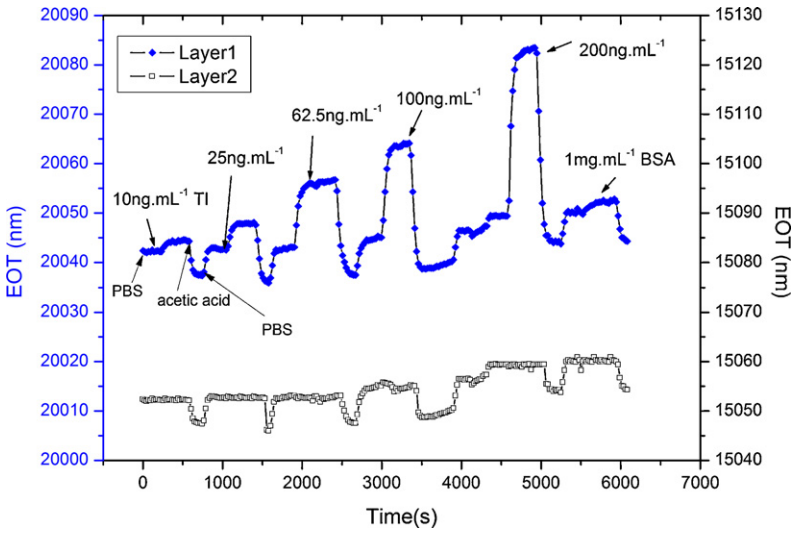

Fig. 3. The optical response of both porous Si layers to different concentrations of trypsin inhibitor. The left and right *y*-axes record the value of 2*nL* in layer 1 (top, trypsin attached) and layer 2 (bottom, without trypsin), respectively. At the beginning of the experiment, the porous Si double layer sample is flushed with 50 mM PBS (pH 7.4), 6 min later, 10 ng mL^{-1} solution of trypsin inhibitor is introduced, and the optical thickness of layer 1 is observed to increase due to the binding of trypsin inhibitor on this layer. Solutions of trypsin inhibitor with the concentration of 25, 62.5, 100, or 200 ng mL^{-1} are sequentially introduced into the flow cell system. Between each step of trypsin inhibitor introduction, the porous Si chip was washed with 0.1 mol L^{-1} acetic acid to renew the sensor surface and re-equilibrated with the pH 7.4 PBS solution. Significant fluctuation of the baseline signal shown was due to the alternations between PBS buffer in the binding step and acetic acid solution in washing step. For correcting the baseline fluctuation, the EOT shift of the layer 2 was also simultaneously recorded.

52 ± 0.43% corresponding to the outer layer (layer 1) and the bottom layer (layer 2), respectively. These results are in accordance with earlier work, in which increases of porosity of porous silicon with increasing current density was found for highly doped p-type samples (Janshoff et al., 1998). The designed double layer structure, with a high porosity layer stacked on a low porosity layer, is exactly what was required for the further experiments.

3.2. Optical characterization of trypsin immobilization on the porous Si layer

Attachment of biomolecules, such as DNA or enzymes and other proteins, on a porous silicon layer has been reported previously (Lin et al., 1997; Strother et al., 2000). To successfully attach biomolecules onto the porous layer, the pore size must be modulated to be accessible to the biomolecules. For example, bovine serum albumin (BSA) with a molecular weight of 68 kDa (dimensions on the order of 3 nm × 8nm × 8 nm) could penetrate into a porous silicon layer that had an 88% porosity. In the present study, the pore size of outer layer measured from SEM image (see Supplementary Information) is about 40–50 nm, but the pore diameters of the bottom layer are too small to be resolved by the current SEM image. Since the molecular weight of bovine trypsin is about 25.4 kDa, the outer porous layer, with 83.6% porosity and pore diameter of 40–50 nm, should also be able to accommodate the size of the trypsin molecules. To confirm this speculation, double layer porous Si was placed in the flow cell for a real-time optical measurement. If the trypsin molecules could enter into the outer layer, the EOT shift of this layer should be observed due to the change in average refractive index of this layer. On the other hand, if the pore size of the bottom layer is small enough, it should prevent the trypsin molecules from infiltrating into this layer, thus leaving the refractive index of this bottom layer unchanged.

Initially, the chip placed in a flow cell was equilibrated with 50 mM PBS. After 6 min, the PBS solution containing 1 mg mL^{-1} trypsin was cycled through the flow cell. The positional shift of the first FFT peak (layer 2) and second FFT peak (layer 1) were monitored in real-time. Fig. 2 illustrates that the 2*nL* value of the second FFT peak increased from an average value of 20,103 to 20,177 nm after the trypsin solution was introduced into the flow cell. The first FFT peak corresponding to the bottom layer remained essentially in its original state. Thus, it was confirmed that the pore size of the outer layer was large enough to admit the trypsin, whereas the bottom layer was too small to host the enzyme.

The trypsin attachment was carried out by immersing the glutaraldehyde-activated chip in a trypsin solution for 8 h and subject to spectrum acquisition. As shown in the dash trace of Fig. 1(b), the position of the second peak had shifted to a larger value, while the first peak remained in its original position. This indicated that trypsin was successfully attached onto the outer layer. Previously, pepsin, another type of protease, was entrapped on the outer layer of a similar double layer porous silicon chip through a physical adsorption process (Orosco et al., 2009), However, a covalently attached protease could be more stable and therefore more suitable for use as an affinity ligand in a biosensor.

3.3. Binding of trypsin inhibitor onto the trypsin attached double layer Porous Si

Binding of trypsin inhibitor onto the trypsin attached double layer porous Si was observed by FTRIFS. Different concentrations of trypsin inhibitor were sequentially introduced into the flow cell system, and the optical response was monitored in real-time. The binding event of trypsin inhibitor on the porous Si chip is illustrated in the line trace in Fig. 3. The optical response, the EOT_1 value, of the outer layer (layer 1) was clearly correlated with the concentration of trypsin inhibitor that was flowed over the chip surface. Since the trypsin inhibitor was specifically captured by the immobilized trypsin, the porous Si chip had to be washed with 0.1 mol L^{-1} acetic acid to renew the sensor surface between each binding step. As a result, severe fluctuations of baseline signal was observed due to alternatively changing the solution from PBS buffer in the binding step to the acetic acid solution in the washing step.

For correcting the baseline fluctuation, the EOT_2 shift of the bottom porous layer (layer 2) was also simultaneously recorded. Because the bottom porous layer was enzyme-free with low poros-

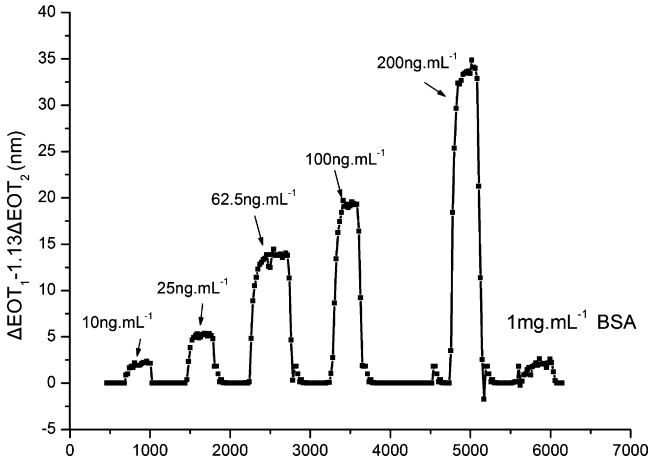

Fig. 4. The corrected optical response of double porous layer Si to different concentrations of trypsin inhibitor. The fluctuation of the baseline is almost eliminated by using the equation of $\Delta EOT_1 - 1.13\Delta EOT_2$, where ΔEOT_1 and ΔEOT_2 represent the net optical thickness shift of the top layer (layer 1) and the bottom layer (layer 2), respectively. The value 1.13 is a weighting factor to compensate for the differences in porosity and thickness between the two layers.

ity, the optical signal fluctuation of this layer (dotted line in Fig. 3) should be mainly caused by the cycling of the different kinds of solutions used in the binding and washing steps. Since small molecules in the buffer and washing solution could easily infiltrate into both layers, the baseline corrections could be realized by subtracting the signal of the bottom layer from the outer layer. Fig. 4 demonstrates that the fluctuation of the baseline could be almost eliminated by using the equation of $\Delta EOT_1 - 1.13\Delta EOT_2$, where ΔEOT_1 and ΔEOT_2 represent the net optical thickness shifts of both layers, and the number of 1.13 is a weighting factor accounting for the differences in porosity and thickness of the two layers. The quantity of weighting factor indicates the degree of difference in relative responses of the two layers, and can be determined approximately by the following equation

$$\delta = \frac{EOT_1(\text{Solution2}) - EOT_1(\text{Solution1})}{EOT_2(\text{Solution2}) - EOT_2(\text{Solution1})} \quad (2)$$

where δ represent the weighting factor. EOT_1 (Solution1) and EOT_1 (Solution 2) are the EOT value measured for layer 1 introduced with two different kinds of solution, respectively, and EOT_2 (Solution1) and EOT_2 (Solution 2) are the EOT value measured for layer 2 infused with the same two kinds of solution as used in EOT_1 measurement. Eq. (2) is only valid when the difference in refractive index (n) between the two kinds of solution is small. Otherwise, the index of a layer is not proportional to its fractional composition (Pacholski et al., 2005). For confirming the value of the weighting factor, 1× PBS buffer (n = 1.3347) and 10× PBS buffer (n = 1.3485) were introduce into the flow cell alternatively for five repeated cycles, and the average EOT shift of both layer caused by buffer changing were measured. A mean value of 1.13 for weighting factor δ was also obtained by application of Eq. (2).

The corrected signal had a linear relationship with the concentration of trypsin inhibitor in the range of 0–200 ng mL^{-1}, with a correlation coefficient of 0.9936. The sensitive of the technique depend on the noise level of EOT measurement, which is decided by the error in reflectance measurement and extracting the effective optical thickness from the spectra data. According to the data shown in the time course trace of Fig. 3, the noise level of EOT measurement in present work is about 0.2 nm. Thus, the LOD of current method for trypsin inhibitor is calculated to be 3.38 ng mL^{-1} (S/N = 3) based on the linear equation: $y = 1.77x$, where x and y represent the concentration of the trypsin inhibitor and corrected signal, respectively.

Control experiments were performed to test the hypothesis that the corrected optical signal was caused by specific binding of trypsin inhibitor with the immobilized trypsin. In the first control experiment, the double layered porous Si without trypsin was used as the sensor chip, which was pre-equilibrated with PBS buffer. After introducing 200 ng mL^{-1} of trypsin inhibitor, the EOT shift of layer 1 and layer 2 was found to be 7.6 and 4.8 nm, respectively. The corrected signal was approximately 2.2 nm calculated by the equation of $\Delta EOT_1 - 1.13\Delta EOT_2$, which only accounted for about 6.4% of the signal produced by the same concentration of trypsin inhibitor on the trypsin immobilized double layered porous Si chip. The molecular weight of trypsin inhibitor used in this experiment is 12.5 kDa, and the measured refractive index of trypsin inhibitor solution (200 ng mL^{-1}) is 1.3348, which is almost the same as the refractive index of PBS (n = 1.3347). The slightly increase in EOT values at both layers might be due to the infiltration and non-specific adsorption of trypsin inhibitor into both porous silicon layers. By using the present methods for baseline correction, the non-specific signal could be reduced.

Another control experiment was conducted by introducing a BSA solution to the trypsin attached porous silicon. As BSA is a relatively larger molecule with MW of 68 kDa, it could infiltrate into the outer layer and was excluded from the bottom porous layer. This caused a significant response even after signal compensation (Pacholski et al., 2005). In the present study, a very small shift of EOT_1 value was observed after introducing a 1 mg mL^{-1} BSA solution, while no significant shift was found at EOT_2, corresponding to the bottom porous layer. The result inferred that some of the BSA molecules could enter into the high porosity outer layer but were excluded from the low porosity bottom layer. As shown in Figs. 3 and 4, addition of 1 mg mL^{-1} of BSA only elicits 2.8 nm of EOT change. Compared with previously reported literature, the small shift in EOT_1 value in this work might be ascribed to the reason that the attached trypsin itself on the outer layer surface blocked the porous layer from admitting large molecules that had no specific affinity for trypsin. The signals produced by BSA could not be fully eliminated by using the equation of $\Delta EOT_1 - 1.13\Delta EOT_2$, as the outer porous layer alone had a substantial response to the large protein. However, the non-specific signal could be easily discriminated from the specific signal, because the responsive trace would go back almost to baseline after the PBS solution was passed over the chip surface, indicating that BSA molecules were not bound onto the trypsin-modified outer layer. These two control experiments supported the conclusions that the signal produced by the outer porous layer arose primarily from the specific binding between trypsin and its inhibitor.

3.4. Chromatographic separation and identification of trypsin inhibitor from soybean seed extracts

Protein or peptide species in soybean seeds are very complex, even though crude protein separated on a Q-Sepharose column only exhibits five major peaks on the elution curve obtained with a UV detector, as shown in Fig. 5(a). Some of the proteins extracted from soybean seeds obviously are co-eluted within these five peaks. For further purification of trypsin inhibitor, the peak containing the target molecules must first be located and identified. Although LC–MS provides a versatile platform for target molecule identification, for bioactive molecules such as peptides or for proteins such as enzymes or their inhibitors, bioassay methods are a more convenient alternative. For example, ELISA has been widely used for the identification of target biomolecules after the separation process (Krämer et al., 1993). However, most bioassay methods cannot be directly coupled to LC systems for real-time detection. Recently, label-free methods such as SPR have been coupled with CE and LC, constituting hyphenated systems that could not only provide the

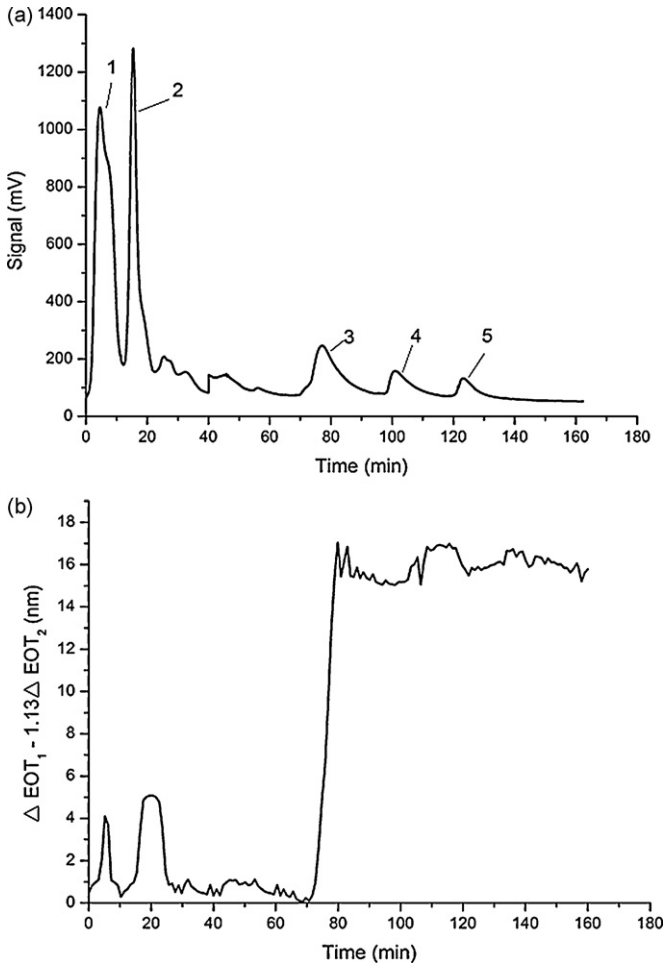

Fig. 5. (a) Chromatogram of a soybean extract separated by a Q-Sepharose column, and eluted with a linear NaCl gradient from 0.2 to 1.0 mol L^{-1} in buffer B solution (10 mM Tris–HCl buffer + 1.0 mM EDTA + 0.1 mol L^{-1} NaCl, pH 8.0). The sample eluted within 180 min at the flow rate of 1.0 mL min^{-1}. The chromatographic trace is recorded with a 280 nm with a UV detector. (b) Chromatographic trace of the same sample recorded by the hyphenated FTRIFS biosensor for trypsin inhibitor screening. Only one fraction corresponding to the peak 3 in UV chromatogram produces a "step-like" trace, indicating that the trypsin inhibitor is eluted within this fraction.

quantitative information of the separated substance, but could also screen and recognize the target molecule in the chromatographic peaks (Jungar et al., 2000).

In this work, a LC–UV–FTRIFS system was constructed. Components eluted from column were first monitored with a UV detector, followed by FTRIFS detection. The reflectometric interference spectroscopy trace was acquired at the interval of 1 min. Fast Fourier transformation was performed simultaneously to real-time monitoring of the 2*nL* value change in both layers throughout the entire chromatographic process. Fig. 5(b) is the optical response curve acquired by the FTRIFS biosensor after the signal was compensated using the EOT correction equation. In fact, the FTRIFS detector could essentially respond to any component eluted from the LC column, provided that the refractive index of that component is different from the mobile phase. The FTRIFS detector based on a double layer porous silicon chip shows advantages over the conventional HPLC refractive index detector (RID), although both of these are refractive index detective technologies. As we have discussed above, the baseline drift cause by mobile phase variation could be effectively eliminated by using the double layer porous silicon as the sensing element for FTRIFS detection, whereas the baseline signal obtained with conventional RID would undergo a serious drift during gradient elution in the LC separation.

The porous silicon sensing chip attached with a specific kinds of biomolecules clearly could produce a specific signal when the target molecules present in sample elute from column and flowed over the surface of the porous silicon. Fig. 5(b) shows that, at the same retention time corresponding to peak 1 and peak 2, significant optical response could be observed, which indicated that some protein had penetrated into the high porosity outer layer, and was excluded by the small pore size bottom layer, resulting in a net shift of the corrected optical thickness, calculated by the EOT correction equation. On the other hand, the "peak shape" trace suggested that the components eluted within these fractions were not captured by the trypsin attached the outer porous silicon layer, as the components entering into the pore structure could be readily replaced by the mobile phase. A larger EOT shift could be observed at the retention time corresponding to the peak 3 in Fig. 5(a). The FTRIFS signal trace of this peak fraction is remarkably different from the other fractions. The corrected EOT shift increased steadily until it reached a maximum value, forming a "step shape" trace. Moreover, the signal did not return to the baseline level, even after the fraction passed through the porous silicon chip and the pore was refilled with mobile phase solution. This result demonstrated that at least one of the components eluted within the peak 3 was captured in the pore structure. The retention of this component could be ascribed to the strong affinity between trypsin and its inhibitor. No similar "step shape" response curve was found at the retention time corresponding to peaks 4 and 5. Accordingly, only peak 3 is likely to contain the trypsin inhibitor. To further confirm this conclusion, fractions of each of the five major peaks were collected and subjected to trypsin inhibitor activity assays. As expected, the trypsin inhibitor activity was only found in the peak 3 fraction. Thus, the results obtained by two different methods were in good agreement with each other.

4. Conclusions

We have demonstrated that a label-free optical biosensor for trypsin inhibitor detection could be constructed by attaching trypsin onto a double layered porous silicon chip. Interaction between trypsin and its inhibitor could be monitored in real-time by Fourier transformed reflectometric interference spectroscopy. The double layered structure could effectively correct the baseline drift caused by refractive index variations in the sample during LC separation, which was particularly valuable as it allowed separation of the sample in gradient elution mode. A hyphenated system consisting of LC–UV–FTRIFS was successfully assembled and used to screen for trypsin inhibitor in a complex crude extract of soybean seeds separated by ion exchange chromatography with a gradient elution mode. The LC–UV–FTRIFS system has potential application as a versatile platform for drug screening from complex biological samples.

Acknowledgements

The authors acknowledge financial support from Pao's scholarship of Zhejiang University, and SHUO AN Tech Inc. Some of software used in this work was kindly provided by Professor Michael J. Sailor at University of California, San Diego.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.09.029.

References

Anglin, E.J., Schwartz, M.P., Ng, V.P., Perelman, L.A., Sailor, M.J., 2004. Langmuir 20, 11264–11269.
Borch, J., Roepstorff, P., 2004. Anal. Chem. 76 (18), 5243–5248.
Choi, J.-H., Park, P.-J., Kim, S.-K., 2002. Fish. Sci. 68, 1367–1373.
Chu, X.L.Z., Shen, G.L., Yu, R.Q.M., 1995. Analyst 120, 2829–2832.
Collins, B.E., Dancil, K.-P., Abbi, G., Sailor, M.J., 2002. Adv. Funct. Mater. 12, 187–191.
Du, M., Zhou, F., 2008. Anal. Chem. 80, 4225–4230.
G, G., 1996. Opto-chemical and Opto-immuno Sensors. VCH, Weinheim.
Ghindilis, A.L.A.P., Wilkins, M., Wilkins, E., 1998. Biosens. Bioelectron. 13, 113–131.
Heideman, R.G.K.R., Greve, J., 1993. Sens. Actuators B 10, 209–217.
Ho, H., Leclerc, M., 2004. J. Am. Chem. Soc. 126, 1384.
Homola, J.Y.S., Gauglitz, G., 1999. Sens. Actuators B 54, 3–15.
Homola, J.Y.S., Myszka, D., 2002. Optical Biosensors: Present and Future. Elsevier, Amsterdam.
Janshoff, A., Dancil, K.-P.S., Steinem, C., Greiner, D.P., Lin, V.S.-Y., Gurtner, C., Motesharei, K., Sailor, M.J., Ghadiri, M.R., 1998. J. Am. Chem. Soc. 120, 12108–12116.
Johnson, T.O., Ermolieff, J., Jirousek, M.R., 2002. Nat. Rev. Drug Discov. 1, 696.
Jungar, C., Strandlh, M., Ohlson, S., Mandenius, C.-F., 2000. Anal. Biochem. 281, 151–158.
Krämer, H.J., Stevens, J., Seeger, W., 1993. Anal. Biochem. 214, 535–543.
Lin, V.S.-Y., Motesharei, K., Dancil, K.S., Sailor, M.J., Ghadiri, M.R., 1997. Science 278, 840–843.
McBride, J.D.R.J.L., 2001. Curr. Med. Chem. 8, 909.
Noble, M.E.M., Endicott, J.A., Johnson, L.N., 2004. Science 303, 1800.
Orosco, M.M., Pacholski, C., Sailor, M.J., 2009. Nat. Nanotechnol. 4, 255–258.
Pacholski, C., Sartor, M., Sailor, M.J., Cunin, F.d.r., Miskelly, G.M., 2005. J. Am. Chem. Soc. 127 (33), 11637.
Pernelle, C.G.a.C., 2000. Drug Discov. Today 5, 212.
Piehler, J.B.A., Gauglitz, G., 1996. Anal. Chem. 68, 139–143.
Rowe-Taitt, C.A.H.J., Hoffman, K.E., Cras, J.J., Golden, J.P., Ligler, F.S., 2000. Biosens. Bioelectron. 15, 579–589.
Ruckenstein, E., Guo, W., 2004. Biotechnol. Prog. 20, 13.
Strother, T., Cai, W., Zhao, X.S., Hamers, R.J., Smith, L.M., 2000. J. Am. Chem. Soc. 122, 1205.
Theiss, W., Henkel, S., Arntzen, M., 1995. Thin Solid Films, 255.
Van Noort, D., Welin-Klintstrom, S., Arwin, H., Zangooie, S., Lundstrom, I., Mandenius, C.-F., 1998. Biosens. Bioelectron. 1998, 439–449.
von Ahsen, O., Bomer, U., 2005. Chem. Biochem. 6, 481.
Waseda, S., Shimosaka, T., Uchiyama, K., Hobo, T., 1999. Chem. Lett. 28 (11), 1195.