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Real-time polarimetric biosensing using macroporous alumina membranes

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ABSTRACT

We report the first demonstration of real-time biosensing in free standing macroporous alumina membranes. The membranes with their 200 nm diameter pores are ideal candidates for biosensing applications where fast response times for small sample volumes are needed as they allow analytes to flow through the pores close to the bioreceptors immobilized on the pores walls. A bulk refractive index sensitivity of 5.2×10^{-6} refractive index units was obtained from signal responses to different concentrations of NaCl solutions flowing through the pores. Finally, after functionalizing the alumina pore surfaces with an epoxysilane and then spotting it with β-Lactoglobulin protein, the interactions between the β-lactoglobulin and rabbit anti-β-lactoglobulin, as well as the interaction between the rabbit anti-β-lactoglobulin and a secondary antibody anti-rabbit Immunoglobulin G were monitored in real-time.

Keywords: Porous alumina, Form birefringence, Polarimetry, Optical sensing and sensors, Biological sensing and sensors.

1. INTRODUCTION

Refractive index (RI) sensing is a powerful technique widely used for real-time monitoring of chemical and biological process [1]. This technique forms the basis of many label-free photonic biosensors, where the refractive index of the biosensor surface is modified by the presence of a target analyte [2]. For the development of photonic biosensors nanostructured materials like porous silicon (PSi) or porous alumina (AAO) have gained special attention as they have higher surface areas than planar biosensors for capturing analytes permitting lower detection limits [3]. To date, several label-free photonic biosensors have been successfully developed on both PSi [4, 5] and AAO [6, 7] and are exclusively based on measuring the optical thickness of the porous layer by reflectometric interference spectroscopy (RIfS). The RIfS method involves measuring the reflectance interference pattern that arises from the light reflected from the air-porous and the porous-substrate interfaces. In order to accurately measure the optical thickness change due to presence of the target analyte the interference fringes need to be well resolved, which limits the porous layer to just the top layer of the substrate, and the pore diameters to less than 100 nm in order to avoid light scattering [8]. With that structure, the delivery of the analytes into the pores is therefore mainly governed by the stationary flux produced by electrostatic interactions resulting in slow responses and so long sensing times.

To overcome those limitations we propose an approach that employs free standing macroporous AAO membranes with pore diameters of 200 nm. The membranes allow the analytes to flow-through the pores no more than 100 nm from the sensor surface, breaking the mass transport limitations and so effectively targeting the delivery of the analyte to the sensor surfaces, for fast sensor response [9, 10]. With the pores of the macroporous AAO membranes being perpendicular to their planar surface they can be used for sensing applications as their birefringence is highly dependent on the refractive index of the material within the pores [11, 12].

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2. MATERIALS AND METHODS

2.1 Materials

The free-standing macroporous alumina membranes were acquired from Whatman (Anodisc™ membranes, 13 mm diameter, 200 nm pores, 60 μm thickness and 0.5 porosity). BSA (Bovine serum albumin), Tween 20, PBS (Phosphate buffered saline) tablets, toluene, epoxysilane compound (3-glycidoxypropyl)trimethoxysilane were acquired from Aldrich (St. Louis, MO, USA). The primary antibody used, rabbit anti-β-lactoglobulin, was purchased from Bethyl Laboratories (Montgomery, TX, USA) and both the β-lactoglobulin B protein and secondary antibody, anti-rabbit IgG, from Aldrich (St. Louis, MO, USA). Strepavidin coated CdSe quantum dots (Qdot® 800 Streptavidin Conjugate) were supplied by Invitrogen (Life technologies, NY, USA).

2.2 Epoxysilane surface functionalization

A self-assembled epoxysilane monolayer was chemisorbed on the porous alumina pore walls through a reaction of alumina hydroxyl groups with the silane groups so that later receptors biomolecules could be attached.

In doing so, first the hydroxyl groups of the alumina membranes surface were activated by an oxygen plasma and then the alumina membranes were immersed into a 1% solution (in volume) of (3-glycidoxypropyl) trimethoxy silane in dry toluene over night at room temperature. This was followed by a curing step at 80°C under vacuum to ensure a high degree of coverage of the surface. Finally the membranes were rinsed with toluene and ethanol and dried under a stream of nitrogen in order to remove any physisorbed silane groups.

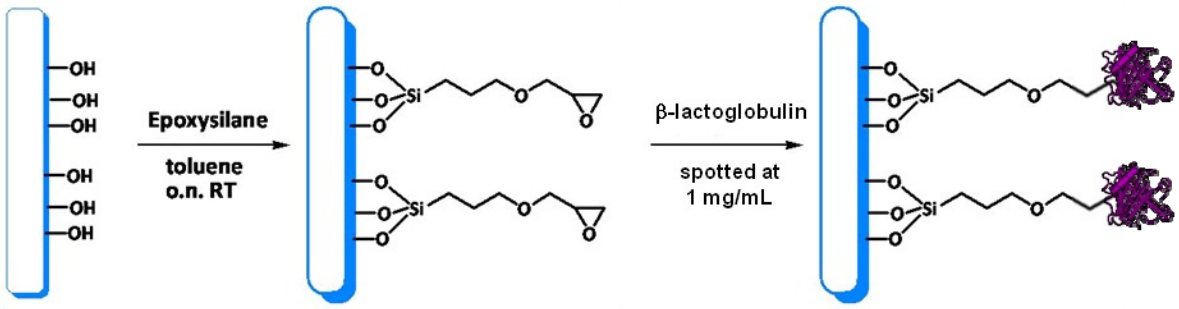

Figure 1. Formation of the epoxysilane monolayer on the alumina surface by (3-glycidoxypropyl)trimethoxysilane and the following immobilization of β-lactoglobulin protein onto the resulting monolayer.

2.3 Membranes transfer to silicon supports

In order to provide mechanical stability to the free-standing alumina membranes they were transferred to silicon substrates after the epoxysilane functionalization. This was done by mounting the alumina membranes onto silicon supports using a 1 μm thick layer of PMMA (Poly(methyl methacrylate)) resist as the adhesion layer. The silicon substrates used were 500 μm thick single side polished square pieces of 15 by 15 millimeters with a CO_2 laser cut 750 μm hole for the laser diode light and the analytes to pass through the membranes.

2.4 Bioreceptor immobilization

Once the free standing membranes had been functionalized and transferred to the silicon supports the β-lactoglobulin protein bioreceptors were immobilized on the silanized surface, via spotting, through the linkage to the active surface epoxy groups. For this purpose a piezoelectric spotter SciFlexArrayer S5 from Scienion was used to spot ten microliters of β-lactoglobulin B protein at 1 mg/mL concentration on the functionalized alumina membrane. Thereafter the spotted membranes were incubated at room temperature in a moisture chamber overnight and rinsed with PBS solution to remove unbound protein. The spotted membranes were then blocked by a BSA solution at 0.1 μg/mL concentration, washed again with PBS and dried under a nitrogen stream.

2.5 Polarimetric readout platform

The polarimetric readout platform for measuring the optical anisotropy of the free standing macroporous AAO membrane is depicted in Fig. 1 (a). Output light from a laser diode is first collimated and then directed to a linear polarizer with the resulting linearly polarized light then enters a photoelastic modulator (PEM; Hinds Instruments PEM-

100). The modulated light exiting the PEM is then incident at 45º to the planar surface of the alumina membrane, which is mounted within a flow-cell. The light exiting the membrane, after passing a second polarizer, is detected by a photodiode which is connected to a lock-in amplifier (SR-830). The lock-in amplifier demodulates the detected signal extracting the amplitudes of its first and second harmonics, which are related to the phase retardation $\Delta\phi$ between the ordinary and extraordinary components of the polarized light traversing the membrane by [13]:

$$\Delta\phi = \arctan\left(\frac{V_{1f}}{V_{2f}} \cdot \frac{J_2(A_0)}{J_1(A_0)}\right) \tag{1}$$

where V_{1f} and V_{2f} are the amplitudes of the first and second harmonics of the modulated signal, $J_1(A_0)$ and $J_2(A_0)$ are the Bessel functions of first and second order respectively, and A_0 is the amplitude of the modulating signal (in radians).

A layout of the flow-cell used for the flow-through sensing is depicted in Fig. 2 (b). The silicon chip mounted alumina membrane is placed between two glass windows with inlet and an outlet ports. The inlet port is connected to a syringe pressure source which operating at 15 psi deliver a constant flow rate of 100 μL/min.

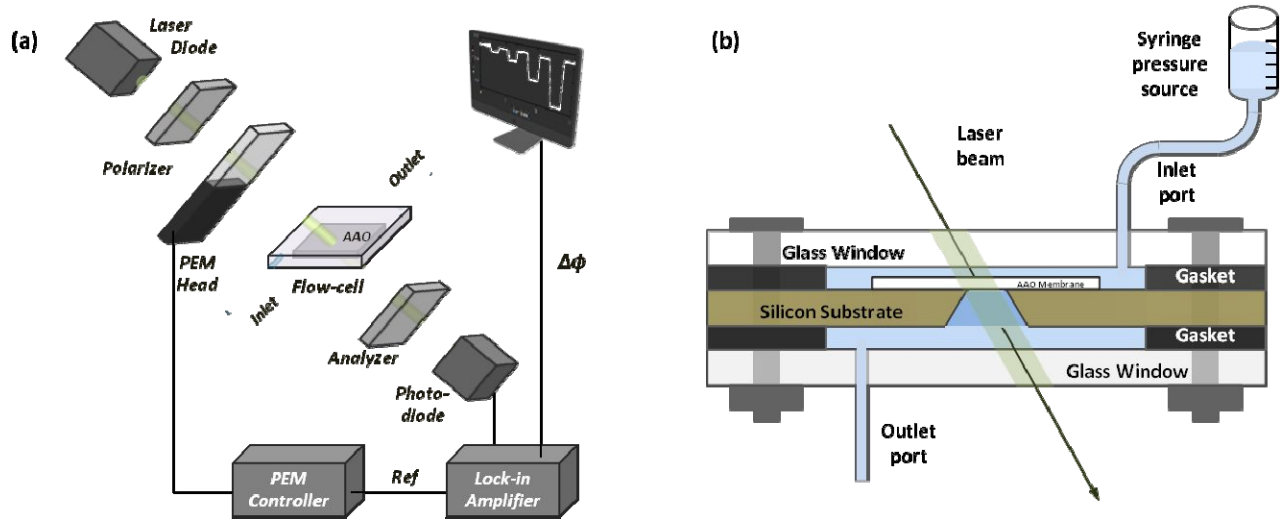

Figure 2. (a) Polarimetric readout platform used for the optical anisotropy measurement of a macroporous free-standing alumina membrane. (b) Detail of the silicon chip mounted free-standing alumina membrane within the flow cell.

3. RESULTS AND DISCUSSION

3.1 Bulk refractive index sensitivity

Real-time sensing experiments were carried out for several epoxysilane coated membranes in order to determine the bulk refractive index sensitivity of the AAO membranes as well as the reproducibility of the sensor device and the measurement platform. To do so different concentrations of NaCl in deionized water (DIW) solutions whose mass concentrations ranged from 0.2% to 2% were flowed during three minutes through the AAO membranes. After each NaCl solution injection DIW was pumped through the membrane in order to prove that phase retardation had returned to its initial value and so the sensing system was reversible [14]. The phase retardation change as a function of time for one such membrane is shown in Fig. 2 (a) where a transitory response of about one minute can be seen immediately after switching, produced by the new solution replacing the old one inside the pores. Fig 2 (b) shows phase retardation changes as a function of the refractive index change for different solutions of NaCl used for ten different membranes. The mean sensitivity is 5.36 rad/RIU (refractive index units) and has a standard deviation of 0.1 rad/RIU and a correlation coefficient up to 0.999.

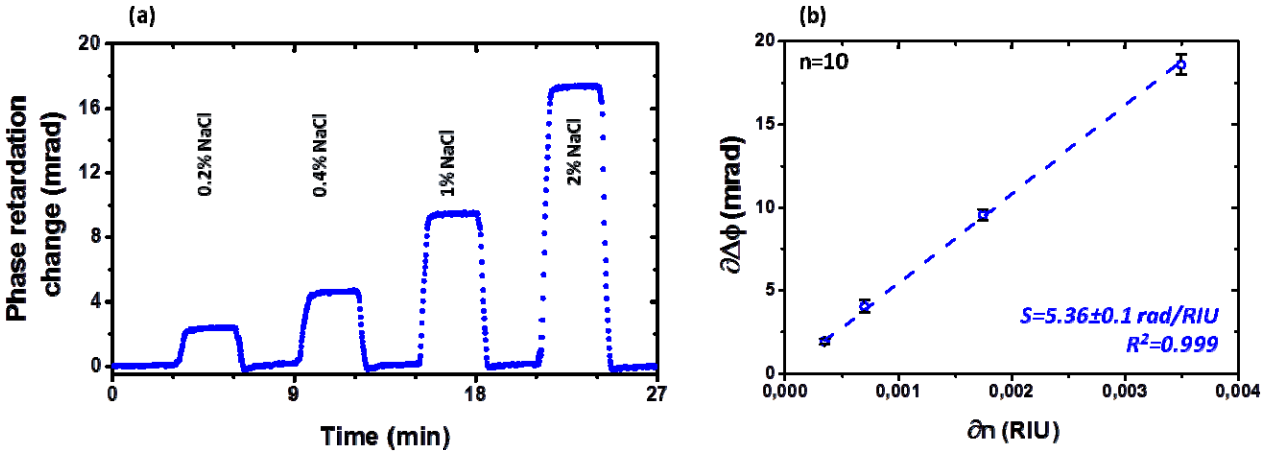

Figure 3 (a) Sensorgram showing the signal response due to flowing several solutions of NaCl in deionized water through a macroporous AAO membrane. (b) Phase retardation change as a function of refractive index change for different NaCl solutions.

From knowing the sensitivity of the alumina membranes, the detection limit of the whole sensing system could then be obtained from the relationship between the sensitivity of the membranes and the resolution of the polarimetric measurement platform:

$$DL[RIU] = \sigma[rad] / S[rad/RIU] \qquad (2)$$

The resolution of the measurement platform was considered to be equal to the standard deviation of the measured phase retardation values, or 2.7×10^{-5} rad, resulting in a detection limit of 5.2×10^{-6} refractive index units.

3.2 Real-time biosensing

After characterizing the reproducibility and the bulk refractive index sensitivity of the coated alumina membranes the immunoassay represented in Fig. 4 (a) was carried out. First a base line for the sensing system was obtained by flowing PBS solution during 10 minutes through a spotted and functionalized membrane. On achieving a stable base line a 1 µg/mL concentration of primary antibody was flown through the membrane to bind specifically to the immobilized β-lactoglobulin protein. A secondary antibody (biotinilated anti-rabbit IgG) and a signal enhancer (strepavidin coated CdSe quantum dots) were used to increase the response produced by the binding between the first antibody and the b-lactoglobulin. Fig. 4 (b) shows the real-time response produced by the binding between the primary and secondary antibodies as well as the signal enhancement produced by the quantum dots.

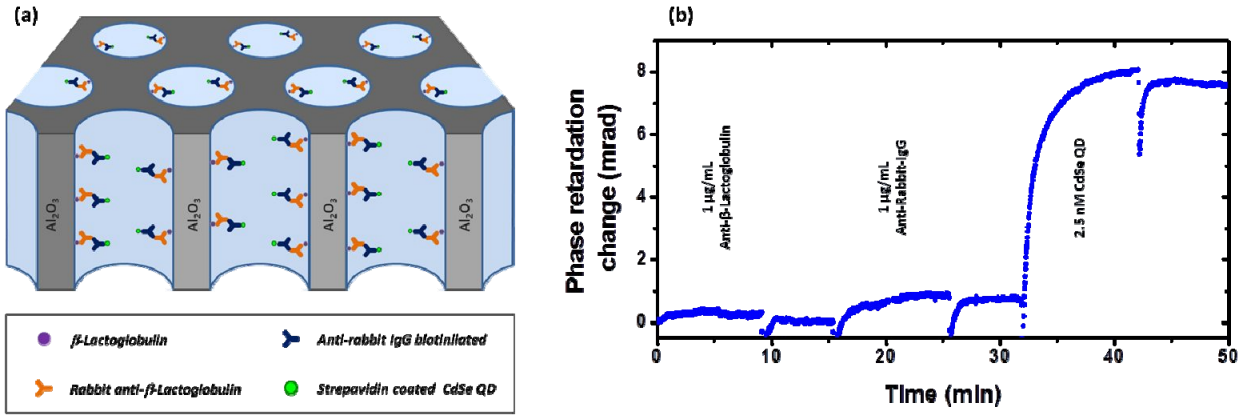

Figure 4 (a) A schematic representation of the immunoassay carried out in a macroporous alumina membrane. β–lactoglobulin protein was used as the immobilized antigen for the detection of rabbit anti-β-lactoglobulin. Secondary antibody biotinilated anti-rabbit-IgG and strepavidin coated CdSe quantum dots were used to increase the signal produced by the primary antibody. (b) A sensorgram showing the signal response due to the binding of the first and secondary antibodies as well as the enhancement produced by the strepavidin coated CdSe quantum dots.

3.3 Control assays

Two control assays were carried out in order to demonstrate that the signal responses shown in Fig. 4 (b) are due to specific antigen-antibody interactions having taken place. The results of a first control assay run on an unspotted membrane shows the absence of a binding response when the β-lactoglobulin protein is not immobilized on the functionalized pores surface (Fig. 5 (a)). A second experiment run on a β-lactoglobulin spotted membrane shows the absence of a binding response when the first antibody (rabbit anti-b-lactoglobulin) is not present, demonstrating that the response enhancement produced by the secondary antibody (anti-rabbit IgG biotinilated) as well as the enhancement produced by the strepavidin coated quantum dots are due to the presence of the first antibody.

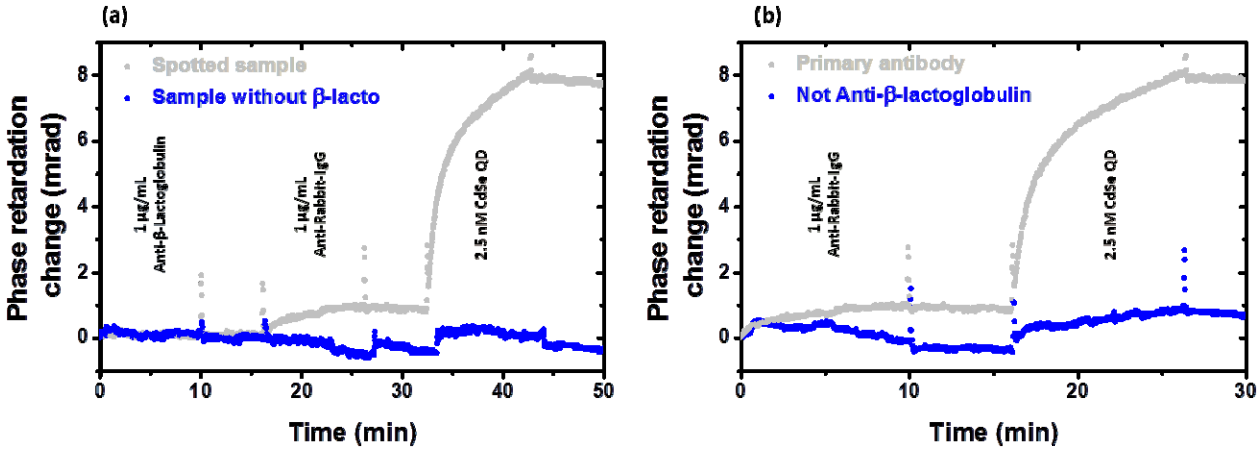

Figure 5. (a) Sensorgram showing a comparison between the responses of a membrane with β-lactoglobulin immobilized on its surface (gray line) and a membrane containing no β-lactoglobulin immobilized (blue line). (b) Sensorgram showing a comparison between the responses produced by the secondary antibody and the quantum dots on a membrane with the anti-β-lactoglobulin (gray line) and a membrane without the anti-β-lactoglobulin (blue line).

4. CONCLUSIONS

In summary, we have presented a biosensing device based on a free standing macroporous alumina membrane with flow-through properties that allows analytes to be targeted delivered for fast sensing response time and therefore real-time measurements for small sample volumes as it is. The interrogation mechanism of the system is based on a polarimetric readout setup for the measurement of the optical anisotropy of a macroporous alumina membrane, whose birefringence is highly sensitive to the refractive index of the material that fills its pores. A bulk refractive index sensing experiment using several solutions of NaCl in deionized water showed a volumetric detection limit below 5.2×10^{-6} refractive index units. Biosensing experiments carried out using β-lactoglobulin as an immobilized antigen provided a specific binding response between the rabbit anti-β-lactoglobulin and the antigen as well as a response from the recognition between the secondary antibody anti-rabbit IgG and the primary antibody rabbit anti-β-lactoglobulin.

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