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A regenerable flow-through affinity sensor for label-free detection of proteins and DNA☆

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ABSTRACT

Label-free monitoring of biomolecular reactions in real-time is of great interest since it can provide valuable information about binding kinetics and equilibrium constants. In this report, a sensor based on White Light Reflectance Spectroscopy (WLRS) is presented that is capable of real-time monitoring of biomolecular reactions taking place on top of a polymer covered silicon dioxide reflective surface. The optical set-up consists of a visible–near infrared light source, a bifurcated optical fiber and a spectrometer. The outer part of the optical fiber guides the light vertically onto the surface where the biomolecular reactions occur, whereas the reflected light is driven from the central part of the fiber to the spectrometer. A microfluidic module in combination with a pump supplies the reagents at a constant rate. The biomolecular interactions are monitored as shifts of the wavelength of the interference minimum. The proposed methodology was applied for real-time and label-free monitoring mouse gamma-globulins binding onto immobilized anti-mouse IgG antibody. Mouse gamma-globulins at concentrations down to 150 pM were detected in reaction times of 1-min. Regeneration of immobilized antibody was accomplished up to seven times without loss of its activity. In addition, real-time monitoring of hybridization reaction between complementary oligonucleotides was accomplished. The proposed sensor provides a simple, fast, low cost approach for label-free monitoring of biomolecular interactions and therefore it should by suitable for a wide range of analytical applications.

1. Introduction

Immunoaffinity techniques rely on the highly specific and strong interaction between an antibody and its antigen for applications that spread from isolation and/or purification of the immunoreaction components from complex matrices [1,2] to the detection of the antibody or the antigen in a sample through a plethora of immunoanalytical methods [3,4]. These methods usually employ labeling of one of the reaction components and end-point measurements for the determination of antigen or antibody concentration in the sample achieving detection of analytes with high sensitivity and specificity [5]. However, they lack on information about the reaction kinetics, analysis time and cost. Therefore, the current trend is to develop label-free methods that are able to monitor the binding reaction in real-time and provide useful information about the binding kinetics and equilibrium [6].

Label-free detection methods include surface plasmon resonance (SPR) [7,8], grating couplers [9,10], ellipsometry [11], evanescent wave devices [12,13], and reflectometry [14,15]. Most of these methods have detection limits comparable to those achieved with methods involving labeled immunoreagents. However, to date, they have not reached the level of automation, ease of use and cost–benefit factor that can put them to a competitive place against labeled approaches as far as routine analysis is concerned.

To fulfill these requirements, low cost and small size instruments should be available that can be easily adapted to perform different types of analysis with appropriate sensitivity and reliability. To this end, reflectometric approaches seems to be more promising as compared to the equally sensitive and reliable refractometric ones (e.g., SPR) [6]. Our contribution towards this direction consisted in the development of a device and analytical procedures based on White Light Reflectance Spectroscopy (WLRS) [15,16]. The sensing element consisted of reflective silicon (Si) wafer surface with a 1000 nm SiO_2 layer on top, grown by wet oxidation, which acted as interference spacer. The SiO_2 surface was modified with a thin (~40 nm) polymeric film in order to allow biomolecule immobilization through physical adsorption [17]. A fluidic channel made of polydimethysiloxane (PDMS) with attached inlet and outlet

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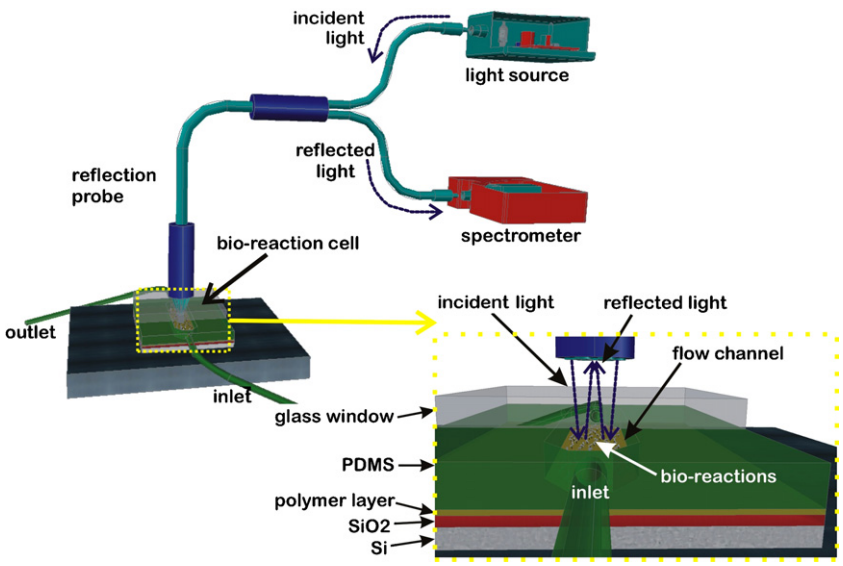

Fig. 1. Schematic of the experimental setup. The arrows indicate the incident and reflected light routes.

tubing along with a glass top cover formed the measurement unit (Fig. 1). The optical part of the device included a bifurcated optical fiber (reflection probe) which directed the light from a white-light source vertically to sensing surface and sent the reflected light to a spectrometer. The reflectance spectra were recorded every second and the extremum shift was monitored in real-time by the software [15]. This shift can be directly correlated with the built-up of biomolecular films onto the reflective surface either by physical adsorption or through specific binding reactions. Thus, biomolecular reactions can be monitored in real-time with high detection sensitivity and quantitative results could be obtained though appropriate calibration curves [16].

In our previous work, a beam splitter and a double spectrophotometer were employed in order to receive the reference and the reflectance spectrum simultaneously. In the present work, a single channel spectrometer was used and the reference spectrum was recorded only at the beginning of each experiment and used for the normalization of the reflectance spectrum throughout it. This device simplification was possible through further development of the wavelength shift monitoring methodology and it is significant since it suppress the required instrumentation size and cost, thus moving forward the objective of a compact small-size instrument adapted to routine use. The detection capabilities of the novel set-up are demonstrated through a mouse gamma-globulins/anti-mouse IgG immunoassay. In addition, here, for the first time, we provide experimental evidence of real-time monitoring of hybridization reactions between complementary DNA probes.

2. Experimental

2.1. Chemicals

Mouse gamma-globulins were from OEM Concepts Inc. (Toms River, NJ). Goat anti-mouse IgG antibody was purchased by Fitzgerald Industries International (Concord, MA). Bovine serum albumin (BSA; Cohn fraction V, RIA grade) and bovine gamma-globulins (Cohn fractions II & III) were from Sigma (St. Louis, MO, USA). Thiol terminated oligonucleotide corresponding to the mutant sequence of 3099delT mutation in BRCA1 gene (5′-thiol-C6-TTTTCTTACATTTAGTTT-3′) as well as the complementary sequence (5′-AAAACTAAAGTAAGAAAAAT-3′) was obtained from Sigma Genosys (Steinheim, Germany). Sulfo-succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was obtained by Pierce (Rockford, IL). The water used throughout the study was doubly distilled. All other chemicals and reagents were from Merck (Germany). Four-inch silicon wafers were from Wacker Chemie AG (Germany). An approximately 1000-nm-thick silicon dioxide film was created on top of the wafers by wet oxidation at 1100 °C for 2 h in the clean room facility of the Institute of Microelectronics of NCSR "Demokritos." The surface was further processed to create a thin film (35–40 nm) of polymeric material appropriate for biomolecule immobilization through adsorption. For this purpose, AZ-5214 photoresist (Clariant, Charlotte, NC) was applied by spin-coating and baked at 180 °C for 60 min to create a surface with high protein-binding capacity.

2.2. Instrumentation

The WLRS experimental setup used in this study, which is schematically depicted in Fig. 1, consisted of a visible–near infrared (VIS–NIR) light source (AvaLight, Avantes BV, The Netherlands), a VIS–NIR spectrometer (QE65000, Ocean Optics; Dunedin, FL, USA; 400–780 nm), a reflection probe, and the measurement cell. The outer part of the reflection probe guides the light vertically onto the surface on which the affinity reactions take place, while the reflected light is collected by the central part of the reflection probe and guided to the spectrometer. The measurement cell was assembled by combining the polymer-modified SiO_2/Si wafer with an open fluid compartment made of PDMS, to which the fluid inlet and outlet tubing had been embedded, and a flat glass plate as cover. The whole structure was secured in place by two aluminum holders. The delivery of fluids to the measurement cell at a constant rate was achieved by employing a precision microsyringe pump (Cole-Palmer, Vernon Hills, IL). All measurements were performed at room temperature using software developed in the LabView environment (National Instruments Corp., Austin, TX). The reflectance spectrum was recorded every second.

2.3. Preparation of bovine gamma-globulins/oligonucleotide conjugate

The bovine gamma-globulins/oligonucleotide conjugate was synthesized using a modification of the procedure described by Niemeyer et al. [18]. Briefly, a 10 μM bovine gamma-globulins solution in phosphate buffered saline (100 mM phosphate, 150 mM

NaCl, pH 7.4; PBS) was reacted with a 40 molar excess of the sulfo-SMCC cross-linker in the same buffer for 60 min at room temperature (22 °C). The unreacted cross-linker molecules were removed by filtration in a D-Salt Dextran Desalting Columns (Pierce) equilibrated with PBS containing 5 mM EDTA. The fractions containing the purified product were combined and reacted at a 1:1 molar ratio with the thiol-modified oligonucleotide for 30 min at room temperature. The protein oligonucleotides conjugate was then purified using an YM-100 Amicon microconcentrator (100 kDa MW cut-off; Millipore, Bedford, MA). During this purification step, the buffer was switched to PBS. The purified conjugates were stored at 4 °C. The protein concentration was determined by Bradford assay (Pierce) according to manufacturer's instructions.

2.4. Label-free detection of anti-mouse IgG/mouse gamma-globulins binding reaction

A baseline was obtained by running through the measurement cell 50 mM phosphate buffer, pH 7.4 (washing buffer), at a constant rate of 20 μL/min. Then, a 10 μg/mL solution of anti-mouse IgG antibody in washing buffer was injected into the measurement cell, and the antibody adsorption onto the surface was monitored in real-time. The antibody solution was left in the flow cell for at least 2 h in order to achieve stable binding to the surface. The antibody solution was then removed, and washing buffer was allowed to flow into the cell. The free protein-binding sites on the surface of the coated wafer were blocked by incubation with a 50 mM phosphate buffer, pH 7.4, containing 10 mg/mL BSA (assay buffer). As soon as a steady state was achieved, the surface was washed to remove loosely bound protein. Solutions of mouse gamma-globulins (ranging from 0.1 to 100 nM) in assay buffer were then introduced into measurement cell and the immunoreaction was monitored. After completion of the immunoreaction, the measurement cell was washed with assay and washing buffer and the binding activity of the immobilized antibody was regenerated by a 1-min wash with 0.1 M glycine–HCl buffer, pH 2.5. By flushing with washing buffer a new baseline was obtained and the binding reaction was repeated. The initial binding rate, calculated as the signal increase within the first 60 s of injection, was fitted by linear regression against the mouse gamma-globulins concentration to obtain the calibration curve.

2.5. Label-free detection of hybridization reactions

The bottom surface of the measurement cell was coated with bovine gamma-globulins/oligonucleotides conjugate using a 10 μg/mL solution in washing buffer, overnight at room temperature. The surface was then blocked and excessively washed to remove any unbound protein molecules, prior to the introduction of hybridization buffer (0.1 M HEPES, 0.5 NaCl, pH 8.0) into the measurement cell. As soon as a baseline was obtained, a solution of complementary oligonucleotide in hybridization buffer was introduced to the measurement cell and the hybridization reaction was monitored in real-time.

3. Results and discussion

3.1. Real-time monitoring of antibody–antigen interactions

The device developed provided the ability to monitor in real-time the evolution of different biomolecular interactions taking place on a reflective surface. Thus, as it is shown in Fig. 2, the immobilization of the anti-mouse IgG antibody onto the surface resulted in a net shift of maximum wavelength of about 8 Å (arrow a to b in the graph) as was determined by subtraction of the values determined by running washing solution through the measurement cell prior to and after antibody immobilization. From

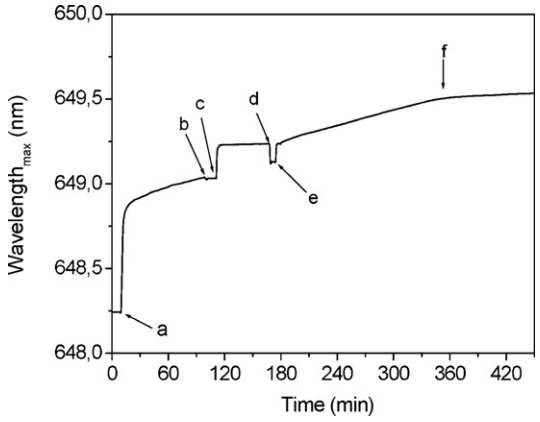

Fig. 2. Real-time signal evolution upon running though the measurement unit: start to arrow a: 50 mM phosphate buffer, pH 7.4 (washing buffer); (arrow a to b): a 10 μg/mL anti-mouse IgG antibody solution in washing buffer; (arrow b to c): washing buffer; (arrow c to d): a 10 mg/mL BSA solution in washing buffer (assay buffer); (arrow d to e): washing buffer; (arrow e to f): a 4 nM mouse gamma-globulins solution in assay buffer; and arrow f to end: washing with assay buffer.

the graph it can be concluded that plateau signal values were obtained in approximately 2 h. The introduction of blocking solution to the measurement cell has as effect an abrupt increase in the signal which, however, is the accumulative result of increase in the solution reflective index due to high protein content plus a moderate increase in the immobilized protein layer thickness as the BSA molecules cover the remaining surface protein-binding sites. The net signal increase, as determined by the values received during flushing washing solution prior to and after the blocking step, is about 1 Å. When the mouse gamma-globulins solution is run through the measurement cell a constant signal increase is observed for at least 3 h of reaction (arrow e to f in Fig. 2). Upon washing with assay buffer, the signal does not retreat but stabilizes in a value of approximately 2.9 Å higher than the initial one. The fact that there is a constant increase rate for the whole reaction time between the immobilized anti-mouse IgG antibody and the mouse gamma-globulins is indicative of the stability of the immobilized antibody. These findings indicated that real-time monitoring of binding reaction and therefore kinetics determinations are feasible with the device developed.

3.2. Mouse gamma-globulins calibration curve

To evaluate the detection sensitivity of the sensor developed, a mouse gamma-globulins calibration curve was established by running through the measurement cell solutions with concentrations ranging from 0.1 to 100 nM at a constant rate of 20 μL/min. As it is shown in Fig. 3A, there is a concentration dependent response that tends to saturation for the higher analyte concentrations as a result of continuous flow of analyte solution on top of the immobilized binding antibody. Therefore, the initial reaction rate, calculated as the signal increase upon the first minute of introducing the analyte solution into the measurement cell, was employed for the creation of calibration curve (Fig. 3B). As it is shown, there is a linear relationship between the initial reaction rate and the analyte concentration for the whole range of concentrations tested. In addition, from this calibration curve, the detection limit of the method was determined as the analyte concentration corresponding to +3SD of the mean value obtained by 12 zero analyte consecutive determinations, and it was 150 pM (Fig. 3B). The working range of the assay was extended from 0.2 to 100 nM. The precision of the assay under continuous flow conditions was satisfactory with intra-assay coefficients of variation (CVs) between 3.5% and 8.9% and inter-assay CVs of 4.8–9.5% over the working range of the calibration

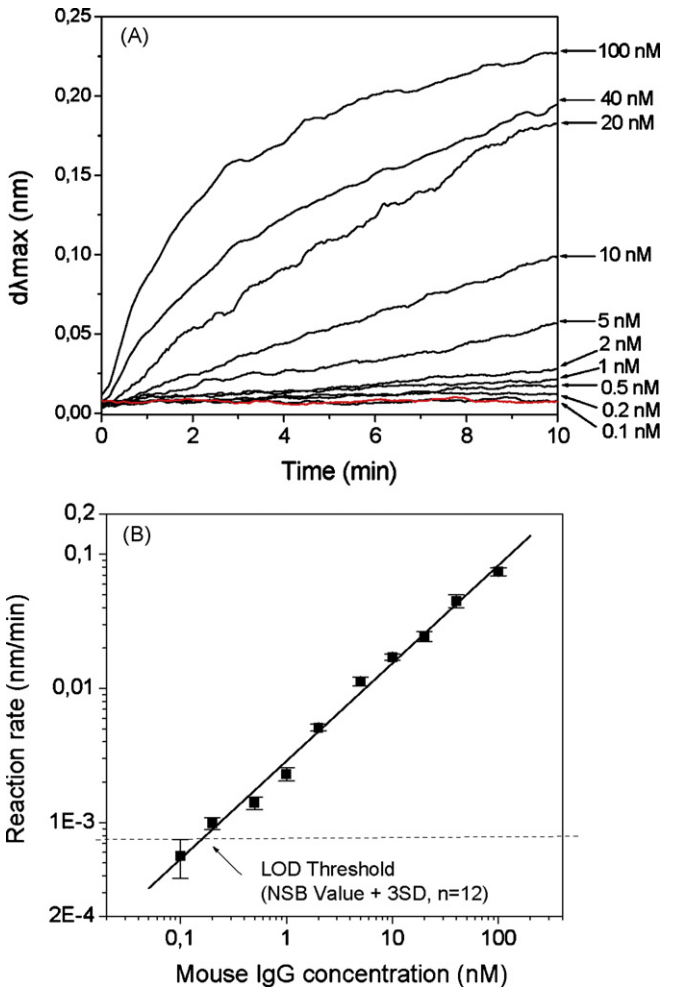

Fig. 3. (A) Signal versus mouse gamma-globulins solution concentration (0.1–100 nM). The baseline obtained by running the assay buffer is also provided (red line). (B) Initial reaction rate versus mouse gamma-globulins solution concentration. Each point is the mean value of three measurements; error bars represent ±1SD. The linear regression equation is: $y = -2.54\ (\pm 0.02) + 0.73\ (\pm 0.02)x$; $R = 0.996$; $p < 0.0001$. The dashed lines indicate the limit of detection value (0.15 nM), which was defined as the concentration corresponding to +3SD of 12 zero analyte concentration replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

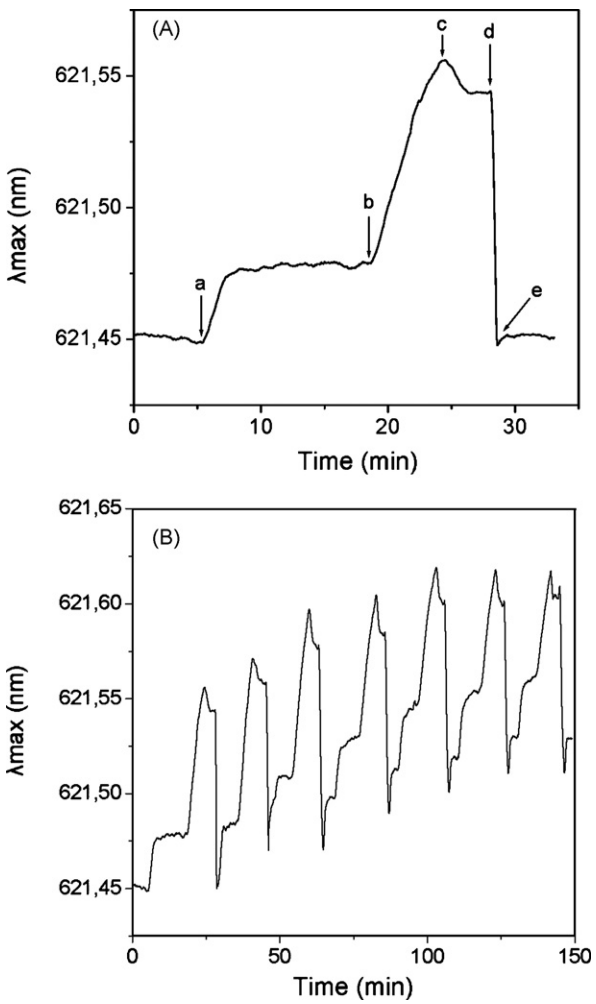

Fig. 4. Regeneration of immobilized antibody. (A) Real-time signal evolution during: start to arrow a: washing with 50 mM phosphate buffer, pH 7.4; (arrow a to b): equilibration with assay buffer; (arrow b to c): reaction with a 10 nM mouse gamma-globulins solution in assay buffer; (arrow c to d): washing with 50 mM phosphate buffer, pH 7.4; (arrow d to e): regeneration buffer; and (arrow e to end): washing with 50 mM phosphate buffer, pH 7.4. (B) Real-time signal evolution during seven consecutive reaction/regeneration cycles.

curve. The accuracy of the assay was evaluated by analysing three control samples (low, medium, and high concentration) before and after the addition of known concentrations of mouse gamma-globulins (recovery study). In all cases, the percent recovery ranged between 84.4% and 112%.

To facilitate interpretation of the results, the subtraction of the zero calibrator signals can be performed automatically by the software at the end of each binding reaction. In fact, the detection limit of the system is mainly defined by the signal variation due to the flow of the analyte solutions during the measurement since, both the incident and reflected light beams are passing through the running liquid phase. Although the performance of binding reaction under stop-flow could improve this signal variation, both the detection limit and the analysis time would be negatively affected. These findings indicate that the sensor developed provided high detection sensitivity and reliability in short analysis time.

3.3. Regeneration of immobilized binding antibody

One of the main advantages of the label-free approaches based on interferometry is the relative low cost of instrumentation involved. When, however, the application to routine analysis is considered, the cost of the consumable parts of the device should be also taken into account. In our device, the only part that should be exchanged between different analyses is the appropriately functionalised reflective surface. This surface is formed on top of a 4-in. silicon wafer by deposition of a thermally grown thick SiO_2 film and then creation of a thin polymeric film (~40 nm) that can readily adsorb protein molecules. For experimental purposes, the surface can be regenerated by short treatment (~30 s) in oxygen plasma up without affecting its quality in relation to light reflection. However, this regeneration would not be possible in a more automated system dedicated to routine analysis. In this case, the reuse of immobilized binding molecules could be beneficial in terms of analysis cost and time. Therefore, the regeneration of immobilized antibody was attempted through treatment with a chaotropic buffer (0.1 M glycine–HCl buffer, pH 2.5) usually employed as elution buffer in affinity chromatography columns for the removal of antibody bound molecules. As shown in Fig. 4A, the introduction of the elution buffer resulted in instantaneous release of bound analyte from the immobilized antibody. Upon washing with phosphate buffer the signal was restored to the initial value indicating successful removal of the bound analyte. This process was repeated

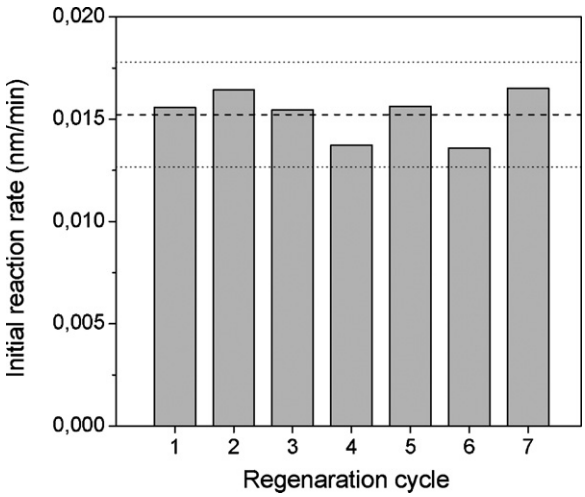

Fig. 5. Initial reaction rates values obtained from the seven successive reaction/regeneration cycles. The dashed line corresponds to the mean value of the initial reaction values obtained from the seven consecutive cycles and the dotted lines correspond to ±2SD ($n = 7$).

six more times. The sensor response over the total seven reaction-regeneration steps is presented in Fig. 4B. The regeneration process affected only slightly the immobilized antibody activity as can be concluded by the comparison of the actual responses obtained during the seven consecutive binding reactions. The overall binding activity loss reached about 35% of the initial one when the signal increase after 4 min of reaction were considered, however, the initial reaction rates deviated by about 8.5% by a mean value of 0.0152 Å/min (Fig. 5). No trend was observed from the first to the last regeneration as far as the reaction rate was concerned, justifying once more the implementation of kinetic measurements as a more appropriate mean for quantitative determinations with the proposed system.

3.4. Detection of hybridization reactions

Genetic testing for detection of specific gene mutations that have been correlated with the onset of hereditary diseases and hence can provide information about the disease outcome or even treatment has dramatically increase the last years. Although the established DNA analysis methodologies are characterized by high accuracy and sensitivity, the need for simpler, more rapid and cost-effective methods has led to the development of microarrays and biosensors [19–21]. Here, we employed the developed device for the detection of one deleterious mutation in Breast Cancer 1 (BRCA1) gene (i.e. the 3099delT mutation) [22]. The BRCA1 gene encodes the tumor suppressor protein Breast Cancer 1. Several mutations on this gene have proved of prognostic value for breast/ovarian cancer predisposition in patients with family history of breast/ovarian cancer, including the 3099delT. To detect this mutation with our device, a probe corresponding to mutant sequence of BRCA1 gene was immobilized onto the reflective surface. In order to achieve oligonucleotide immobilization on the reflective surface by physical adsorption, a protein/oligonucleotide conjugate was synthesized and used as solid-phase reagent. Then, a complementary sequence probe was introduced in the measurement cell and its interaction with the immobilized probe was monitored in real-time. Two oligonucleotide concentrations (5 and 10 nM) that fall within the range of PCR products concentration in DNA molecules were tested. The sensor response was concentration dependent with initial reaction values of 0.0472 (±0.0003) and 0.0950 (±0.0012) Å/min for the 5 and 10 nM solutions, respectively. In addition, the response was sequence specific since running a

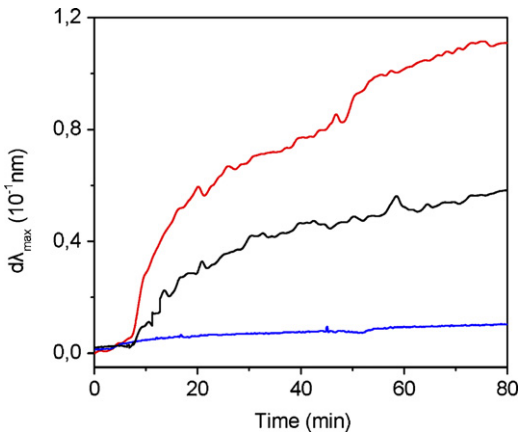

Fig. 6. Real-time signal monitoring during hybridization of 20 bs oligonucleotide corresponding to the mutant sequence of 3099delT mutation at concentration of 5 (black line) and 10 nM (red line), respectively, with a complementary sequence immobilized onto the sensor surface as a conjugate with bovine gamma-globulins. The sensor response when a 10 nM solution of an irrelevant oligonucleotide sequence was run through the measurement cell is also provided (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

10 nM solution of an irrelevant sequence did not cause response that could be statistically distinguished from the reaction buffer baseline (Fig. 6, blue line). Hence, the device developed could be applied for monitoring of DNA hybridization reactions aiming to single nucleotide mutations detection.

4. Conclusion and outlook

A White Light Reflectance Spectroscopy sensor based on a simple and cost-effective instrumentation was evaluated in terms of its application to real-time monitoring of antibody–antigen and oligonucleotide hybridization reactions. The developed device provided the desired sensitivity for such applications with short analysis times that favor its use in routine analysis. In addition, apart from the obvious applications in the quantitative determination of analytes based on specific binding reactions, the device developed could be a valuable tool for comparative studies of reaction kinetics of different binding molecules towards the same ligand or of different ligands towards the same binder. These studies could be greatly facilitated by combining the new sensor with an appropriately structured fluid compartment and appropriate optical setup for multi-channel or multi-analyte determinations. Although the signal monitoring and recording is more complex in such situations, the analytic advantages are considerable. Taking it overall the new simple sensor set-up is a step towards the manufacturing of a small size instrument for both routine and research applications.

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