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A novel fluorescence-based array biosensor: Principle and application to DNA hybridization assays

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Abstract

A novel fluorescence-based array biosensor targeted for field applications, such as environmental monitoring, has been developed, and successfully applied to DNA hybridization assays. The purpose was to meet the demand for automated, portable but easy-to-maintain systems allowing continuous flow monitoring of surface reactions. The biosensor presented here can be distinguished from the existing systems by the optical method used, which provides an enhanced simplicity and robustness, and enables a simple maintenance by potentially unskilled personnel. The system is based on a conventional microscope slide which acts both as transducer and biological array sensor. The excited fluorescence is guided by total internal reflection into the slide to the detector which is directly interfaced to the slide. Each region of the sensor array is successively optically interrogated, and the detection of the corresponding fluorescent emission synchronized. A real-time three-analyte analysis is thus feasible without any mechanical scanning movement or optical imaging systems as generally used in the existing instruments. The ability of the biosensor to operate in continuous flow for several tens of hours has been demonstrated. The biosensor has been assessed in terms of stability, and slide-to-slide reproducibility, which is found to be less than 3.7%, thus far below the standard biological reproducibility. DNA hybridization assays were performed to estimate a limit of detection, which was found to be 16 mol/μm^2, and to determine the reaction kinetics associated to the DNA model used. The developed biosensor is thus shown to be able to predict reaction kinetics, and to monitor in real time surface reactions between targets and probes.

Keywords: Optical biosensor; Total internal reflection; Real-time detection; Surface reaction monitoring

1. Introduction

Array biosensors are small multisensor systems designed for biomedical analysis (Giannetti et al., 2006), environmental monitoring (Rodriguez-Mozaz et al., 2004), or for the detection of biological warfare agents (Rowe-Taitt et al., 2000). To meet the demands of the field of the environmental monitoring, the biosensor described here has been targeted for being cost-effective, compact (200 mm × 280 mm × 170 mm, 2 kg), automated, capable of continuous multi-analyte detection, robust, and compatible with a use outside the laboratory by potentially unskilled personnel. The demand of such portable identification systems has led in the recent years to the development of two major classes of integrated systems: in the first one fall the truly integrated microdevices which combine integrated circuit elements, electro-optic excitation/detection systems, and bio-receptor probes (Misiakos et al., 2004); in the second one, the systems implement conventional instrumentation (charge-coupled devices, photo-multiplier tube, laser diodes, optical components, etc.) assembled around the central element which is the sensor medium or transducer (Tschmelak et al., 2005). Fluorescence transduction schemes predominate this micro-array field since they provide a better sensitivity and specificity, as well as decreased background signals over free-label arrays (Baldini and Giannetti, 2005). The array biosensor presented here clearly falls in the latter category, differing though from the existing systems by its reduced number of elements – thus its simplicity, combined with continuous flow operation and real-time monitoring.

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Let us first detail the assets and drawbacks of some of the most advanced systems of the first approach. A technique involving in situ hybridization of biological probes on a sensor element was first introduced by Karpf et al. (1988), and then applied by Eggers et al. (1994) to a CCD chip with radio-active or fluorescent labelling of target molecules. Although promising, the system is far from being compatible with out of laboratory testing due to radioactive labelling which is restricted to research applications. Mallard et al. (2005) investigated the use of CMOS elements in biochip. A photoelectrical detection system compatible with decentralised testing and able to detect as low as pM target concentrations on dense DNA arrays has been developed. The system is based on the use of a low cost CMOS photodetector array as a solid support for DNA chip, coupled with revelation by enzyme-catalysed chemiluminescence. This method however prevents any operation in continuous flow, and is thus not adapted to environmental monitoring applications. Vo-dinh et al. (1999) demonstrated the usefulness and feasibility of a DNA biochip using a phototransistor integrated circuit. The system has sensors, amplifiers, discriminators and logic circuitry on board and is shown to be able to include also light-emitting diodes for fluorescence excitation. Up to now, the only sensing device having the light source monolithically integrated with the waveguide and the detector has been presented by Misiakos et al. (2004). In this sensor, the signal transducer is based on an evanescent wave excitation scheme. This approach allows measurements to be confined to the waveguide surface rather than in bulk solution, thus enabling low limits of detection and high signal-to-noise ratios. Detection limits as low as 3.8 pM of gold-labelled streptavidin and 20 fM after a silver revelation step have been reported. Real-time detection without any washing step can be performed using this sensor, thus allowing a simple fluidic and instrumental design. However, the method requires a labelled target, which is not possible with haptens.

In the second approach, efforts have been made on reducing the size of the instrument while increasing the number of analyte to be screened. The portable semi-automated fibre optic immunosensor RAPTOR (Anderson et al., 2000) enables the detection of four bacterial agents, viruses, and toxins. The parallel affinity sensor array (PASA) (Weller et al., 1999) based on chemiluminescence labels showed a high potential for multi-analyte detection and a good regenerability of the indirect assay chips. The RIANA biosensor based on total internal reflection fluorescence has been validated for simultaneous detection of three contaminants (atrazine, isoproturon, and estrone) in natural waters (Rodriguez-Mozaz et al., 2004). The novel analytical system AWACSS (Tschmelak et al., 2005; Hua et al., 2005) enables to extend the number of compounds to be screened up to 32. These instrumentations are based on an evanescent wave technology: laser or led light is coupled into a waveguide, and the surface bound labelled areas are excited in the evanescent field. An imaging system consisting of a CCD camera equipped with imaging optics (Feldstein et al., 1999; Anderson et al., 2000; Weller et al., 1999), or with a fibre-coupled array (Rodriguez-Mozaz et al., 2004; Tschmelak et al., 2005) enables the analyte identification by pattern recognition. On the contrary, the novel biosensor described in this paper uses the following transducing method: (1) the sensing areas are interrogated normally to the surface similarly as in an epi-fluorescence scheme, which allows the totality of the fluorescent molecules present at the interface of the substrate to be excited. (2) The evanescent modes of the fluorescence emission are coupled into the substrate and (3) these are guided by total internal reflection to a mono-detector positioned at one edge of the substrate. The chosen substrate is a standard microscope slide whose large dimensions compared to the sensing areas enables to collect the energy of all evanescent modes with a good tolerance to excitation misalignment. Moreover, the fluorescence collection efficiency on the detector is good enough to avoid the use of any imaging optics interfacing the detector to the substrate edge, which, thus, enhances the simplicity of the biosensor and its robustness to optical or mechanical misalignments. The principle of this signalling method has been first reported as “solar concentrator” in the 1970s for photovoltaic applications (Weber and Lambe, 1976). This principle was then used for capillary gas sensors (Kieslinger et al., 1997; Gouin et al., 1998), yet for single analyte detection only. In 1987, a novel type of optical biosensor called “Fluorescent Capillary Filled Device (FCFD)” using the low-cost technology of liquid crystal display (LCD) to produce the capillary device has been presented (Bradley et al., 1987). The fluorescence signal is collected at the edge of the device, yet through custom optics aimed at extracting the light in the angular range of interest, and at discriminating the excitation and output light. The development of an assay for human chorionic gonadotrophin (Deacon et al., 1991) demonstrated the performances of this device, which has been later improved for multi-analyte identification (Daniels et al., 1998).

The purpose of this work is to describe and characterize a novel array biosensor in which the FCFD signalling method has been adapted to glass slide devices and automated continuous flow assays. The applicability of this biosensor is demonstrated trough a run of DNA hybridization assays.

2. Materials and methods

2.1. Biosensor set-up

A schematic view of the set up is depicted in Fig. 1.

The central element is a conventional microscope slide (size 3×1 in., cat No3010 Gold Seal® Products) which acts both as the transducer and the biological array sensor. A typical device is composed of four sensing areas of 1.4 mm^2, as illustrated in Fig. 2. Each sensing area is successively orthogonally excited using focused led emission, and the detection of the corresponding fluorescent emission synchronized. This configuration makes a real-time four-analyte analysis feasible without any mechanical scanning movement. The device is mounted into a custom monolithic fluidic chamber allowing continuous flow operation. The software implemented enables the automation of the assays, as well as the real time monitoring of the measurements.

The excitation module (Fig. 1) is composed of four low-cost light emitting diodes (Luxeon Emitter III, Lumiled) delivering

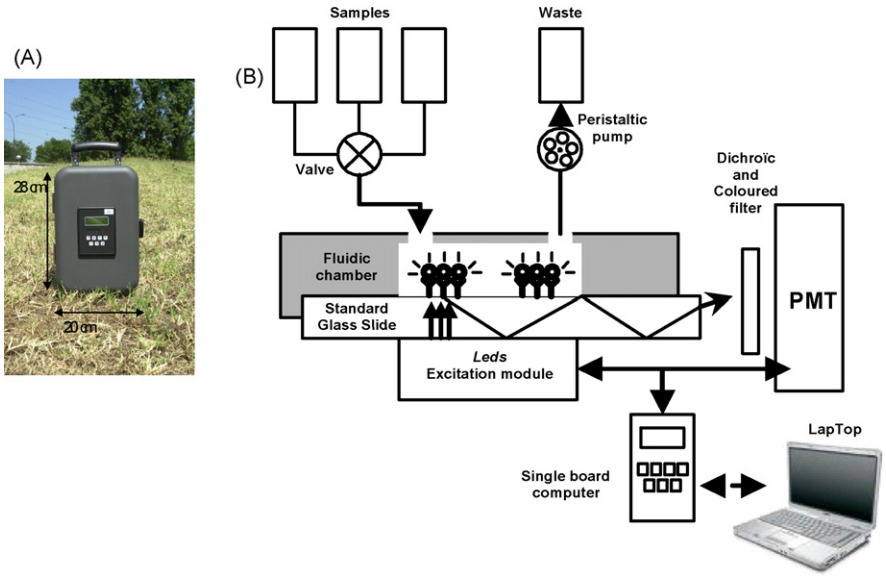

Fig. 1. (A) Pictures of the portable box (20 cm × 28 cm × 17 cm) which contains the whole instrument, and (B) a schematic set-up of the biosensor with details of the transducer surface. Fluorescence excited by focused and filtered light emitting diodes @ 532 nm (Leds excitation Module) is guided by total internal reflection in the glass slide up to the detector, a photo-multiplier tube (PMT) equipped with filters. The glass slide surface is prepared using a four-spot geometry to enable on-line immobilization of biochemical elements, such as antigens or oligonucleotides using the fluidic reaction chamber. Both excitation and detection modules and fluid handling are automatically controlled using a stand-alone smart card.

an optical power of 4.5 mW at a wavelength of 532 nm. Each area of the sensor chip is then excited by a dedicated led. Light is focused on the sensor areas through two ball lenses (uncoated, 5 mm diameter, LaSFN9, Melles Griot) with a spot diameter of 1.47 mm (full-width half maximum). A single band pass filter (525AF45, Omega Optical) is incorporated between the two series of lenses to reject wavelengths below 480 nm and above 560 nm. The measured optical power impinging the biological areas is about 1.8 mW. The average consumption of the module is 0.3 A (@max). Typical illumination time is 200 ms, and the delay between two acquisitions can be adjusted from 6 s to 1 h or more. The influence of the illumination of one spot on the

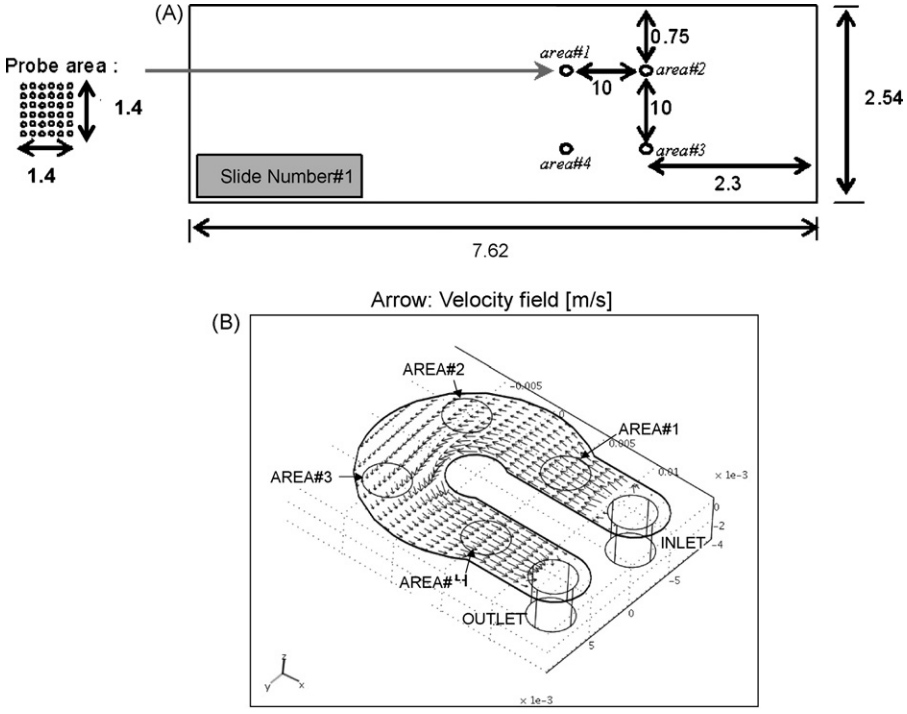

Fig. 2. (A) Schematic of the sensor surfaces on the waveguide. Distances are expressed in centimeters. The detector is interfaced with the right side of the slide. In the DNA hybridization assays, the sensor areas are made of an array of 36 micro-spots (636 pL each). (B) A schematic 2D view of the flow cell is shown, and the flow direction and flow vectors small arrows) over the four spots are indicated.

surrounding spots has been measured, and shown to be negligible: when the first area is optically excited with a power of 1.8 mW, the measured optical power on areas #2, #3, and #4 is below 10 μW, which is not sufficient to generate fluorescent emission.

Fluorescence is guided in the slide by total internal reflection and is directly collected at the end-face by a photo-multiplier tube (H8249-101, Hamamatsu). The detector has been interfaced with a bandpass filter (595AF60, Omega Optical) and a high pass coloured filter (OG570, Schott) to suppress the remaining excitation radiation. The high collection efficiency of the transducer makes the use of imaging optics not necessary, thus increasing the robustness, compactness, and simplicity of the system. The transducer is embedded into a home made fluidic reaction chamber made of aluminium and coated with black PTFE. This coating enables aggressive fluids to be used for decontamination steps. The flow channel is 6 mm in width, 0.130 mm in height, and 46 mm in length. The sample goes successively over the areas #1, #2, #3, and #4 with a flow rate adjustable in the range 0.03–2.5 ml/min. The fluidic circuitry is composed of tygon wires (0.51 mm in diameter, R3607, Instech, Almere, The Netherlands), a peristaltic pump (P625/900-143, Instech Almere, The Netherlands), and a three-way electro-mechanical valve (N21172, NJR Corporation, San Jose, CA) enabling the selection of the sample.

The system is controlled using a single board computer (BL2120 Smartcat, Z-World, USA) which has been programmed using Dynamic C® language. This board has a LCD/Keypad window where commands can be entered and measurements displayed. An additional external memory of 16 Mo has been incorporated, which makes the system fully stand-alone and automatic. A computer can also be interfaced using RS232 serial bus, and Microsoft® Office Excel macros have been written to dialog with the single board computer, to program a full experiment, and to display real-time graphics. Fluid handling (flow rate, samples switching), acquisition parameters, and PMT settings (voltage, measurement frequency, number of samples) are some examples of the parameters that are controlled by the software developed.

2.2. Chemicals

Hybridization buffer (200 mg/ml DNA, 10X SSC, 2x denhardt, lot#120k9100, ref#h71400), and cleaning buffer (0.3 M Na_2HPO_4) were obtained from Sigma. Capture probes (20-mers oligonucleotides possessing the 5′ amino group, 15-mers hybridizable), and Cy3-labelled complementary 19-mer oligonucleotides (15-mers hybridizable) were purchased from MWG (Germany). The sequences of the capture and target probes are 5′ *TTT TT* GAT AAA CCC ACT CTA 3′, and 5′*CA* TAG AGT GGG TTT ATC *CA* 3′, respectively.

2.3. Surface preparation

The substrate surface is functionalised by an aldehydic group according to the method described in (Vinet and Hoang, 2002), which consists of three major steps: (1) surface cleaning, (2) surface silanisation, and (3) activation of the sensor areas. Silanisation consists first in treating the glass slides by oxygen plasma using a 5,6-epoxy hexyl-trietoxy-silane (Sigma). Subsequently, the epoxy function is opened in 0.2 N HCl solution to afford the vicinal diol, which is then converted to the aldehyde by sodium periodate oxidation. The aldehydic group enables the covalent binding of 5′ amino oligonucleotides. To activate the sensing areas, the phosphate buffer solution containing the oligonucleotide probes in the concentration of 10 μM is automatically spotted using a BioChip Arrayer (Packard Instrument, USA). The spotting geometry is given in Fig. 2: the four sensing areas are composed of 36 spots of 636 pL in volume and separated by a centre-to-centre distance of 250 μm. The total width of an area is 1.4 mm. This geometry has been preferred to a single sensing spot of the same diameter to achieve a better homogeneity and repeatability. Also, the achievement of different spot geometries (square, rectangular, etc.) is very easy, which is important when studying the influence of the sensing spot profile on detection performances. One of the four areas is spotted with non specific oligonucleotides, and is thus used as an on-line control of potential instrumental deviations. The slide is then kept during 1 h with 0.1 M sodium borohydride (Sigma) in water in order to reduce the amine function formed. After washing and drying steps, the substrate is ready for use in the hybridization assay. To characterize the background signals, three kinds of devices were used: (i) typical devices having one reference area and three test areas as just described, (ii) devices obtained after steps 1 and 2 that have no capture probes attached to the surface, and (iii) reference devices having four non-specifically capture probes attached to the sensing areas.

3. Results

3.1. Robustness, stability, reproducibility

The instrumentation had been operating continuously for 36 h without any bubbles forming or being trapped in the reaction chamber. Twelve glass slides, six of them being chemically prepared but free of capture probes and the other six being typical functionalised devices, were used to quantify the stability and reproducibility of the biosensor. For each area, the background signals obtained from the hybridization buffer flowing at a rate of 1 mL/min in the reaction chamber were measured during 1 h at a frequency of 1 measurement every 2 min. The variability of the signal intensity on 120 consecutive measurements was very low and similar for each interrogated area (coefficient of variation in the range 0.05–0.2%). Similar results were obtained for the 12 slides at several days interval, as confirmed by the interclass coefficient of variation (0.13, 0.06, 0.09, and 0.26% for the areas #1, #2, #3, and #4, respectively). The inter-slide reproducibility was assessed for six sensing slides. The variability of the mean of the signal intensity (calculated using the 120 consecutive measurements) was found far below the usual biological variability, and thus very satisfactory (coefficient of variation for the mean signal intensity in the range 1.4–3.7%). The inter-slide reproducibility was found similar for the two kinds of devices (with prepared surface only, or with probe

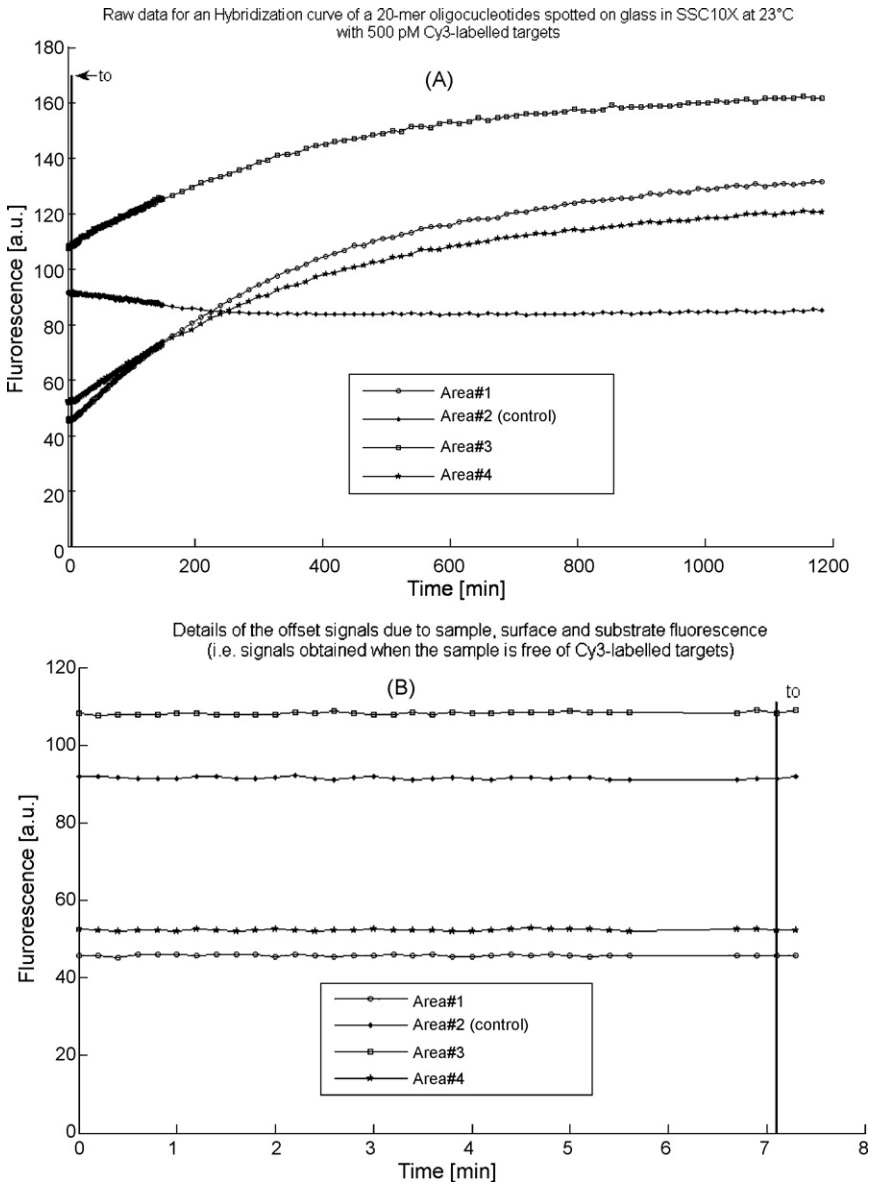

Fig. 3. (A) Real time raw data signals recorded for an hybridization assay of a 20-mers capture probes spotted on glass with a 500 pM cy3-labelled targets. The flow rate is 1 mL/min. The cell is first filled in with the hybridization buffer, leading to the offset values (see (B)). Then, after 7.1 min (labelled as "to"), the hybridization solution containing the labelled targets is loaded and the hybridization reaction occurs on the specifically functionalized areas (#1, #3, and #4). The difference between the background levels of areas #2–3, and #1–4 (B) is mainly due to the vigneting effect: the further the areas are from the detector, the lower is the amount of signal impinging the detector.

areas activated), thus demonstrating that the activation step did not degrade the reproducibility. Also, the manual loading and unloading of the sensing device was proved to be compatible with the measured tolerance of $\pm 150\,\mu m$ required to align the probe area over the excitation source. The resistance of the prepared surface has been evaluated by monitoring the background signal during 25 h, and by performing after this long washing step a DNA hybridization assay. The variation of the background signal during the washing step is very satisfactory (1.36–2.08%), and the kinetics and plateau values of the hybridization assay are of the same order as those measured when the hybridization assay is performed immediately. Therefore, the chemical preparation is shown to be compatible with a continuous flow operation.

3.2. Instrumental response

The instrumental function acts on the amplitude distribution of the four signals (#1, #2, #3, and #4), as seen in Fig. 3. The background levels of areas further away the detector (#1 and #4) are about 2.4 times below the background levels of the closest areas (#2 and #3). This difference is due (i) to the vignetting effect which refers to the fluorescence intensity falloff with distance from the detector, and (ii) to area-to-area differences in illumination power. These effects were clearly identified and found highly reproducible, since the measured coefficient of variation for the background signal differences (area #1–area #2) and (area #3–area #4) was in the range 1–2.8%. The background offset is made of sample fluorescence, surface fluorescence, and

substrate fluorescence. In absence of surface reaction between probes and labelled targets, surface fluorescence arises from unbound molecules present within the excited volume. The level of the background signal depends on the sample (auto) fluorescence properties, and on the nature of the surface which is interrogated: as an example, non-spotted devices led to an offset level about 24% larger than typical devices, a difference due to the increased amount of volume fluorescence measured when there is no capture probes attached to the surface.

In order to quantify the variation of the background signal with the concentration in Cy3-labelled targets of the hybridization solutions, offset signals were recorded using four reference devices (the areas were non specifically activated) and five concentrations (0.05, 0.1, 1, 10, and 20 nM) of labelled target. A linear relation between the background signal of fluorescence and the concentration were found for each area (not shown). Moreover, these four linear equations were linked together by a gain factor, thus enabling the online determination of the background offset of an area using the measurement of a reference area within the device. The contribution of the background fluorescence to the total signal of fluorescence arising from the surface reaction was found low, as shown by a Signal-to-Background ratio of 22 measured at the plateau of an hybridization curve with 10 nM of labelled targets.

3.3. DNA hybridization experiments

After probes immobilization, the micro-array was assembled together with the reaction chamber. A typical hybridization experiment was performed in three steps: the cell was first filled in with the hybridization buffer which is free of the complementary oligonucleotides for a few minutes, then with the hybridization solution until the plateau was reached, and finally with the cleaning buffer. The first step gave the background signal related to each area, whereas the second step led to the hybridization curve. The last step (not shown) is a washing step aimed at removing targets in excess, without though denaturing the hybridization target. The slide was finally scanned using a commercial fluorescent scanner (Scanner GeneTAC® LSIV, Genomic Solutions, US) in order to provide a rough comparison of the fluorescence intensities between the two instruments.

Typical raw data obtained for a 500 pM concentration of labelled complementary oligonucleotides are shown in Fig. 3. The control area (area #2) is not contaminated by the targets present in the sample, and thus acts as a reference for instrumental or sample properties deviations. The ability of the biosensor to work in a multiplex mode is also preliminary assessed.

3.4. Data correction prior analysis

The following corrections are applied to the measurements: (1) offset corrections compensate for the baseline current present in the photomultiplier in absence of illumination. Any additional current due to the signal is added to this offset, thus it is necessary to subtract it before making use of the raw data. The offset are measured prior to every acquisition during the same duration (200 ms). (2) Background corrections compensate for the background signal made up of glass fluorescence and sample fluorescence. The background signal takes also into account the vignetting effect. It is measured for each area during the first step of the experiment, and subtracted to the data of the corresponding area. (3) Time rescaling enables to make the kinetics curves starting at the same time. It is particularly convenient when gathering and comparing several experiments together. (4) Reference intensity corrections compensate for fluctuations in the illumination intensity during the hybridization assay. These slight variations due for instance to a variation in temperature are measured for the control area, and the measurements are used to normalize the data of the other areas.

3.5. Data analysis

Fig. 4 illustrates corrected data obtained for an hybridization curve with 500 pM Cy3-labelled targets. The solid lines result from the fitting of the experimental data against a classical Langmuirian model, which allows the determination of the reaction kinetics. The following assumptions have been made: (i) the concentration of targets is in excess compared to the surface density of probes, (ii) the bulk concentration is uniform over the entire sensor areas, and (iii) the dissociation reaction is negligible. The resulting values of the association constant k_{on} are 8.3×10^4, 7.8×10^4, and 7.3×10^4 M^{-1} s^{-1} for areas #1, #2 and #3, respectively. They are in good agreement with the results obtained using another system ($k_{on} = 1.10^5$ M^{-1} s^{-1}, Lion et al., 2001), which preliminary validate the ability of the biosensor developed to record DNA hybridization and to determine kinetic parameters. Several hybridization curves with target concentrations ranging from 0.05 to 10 nM have been performed, allowing the determination of a mean k_{on} value of 8.4×10^4 M^{-1} s^{-1} for the first area in the fluidic path, and of slightly lower values, 7.9 and 7.3×10^4 M^{-1} s^{-1}, for the two following areas. This result

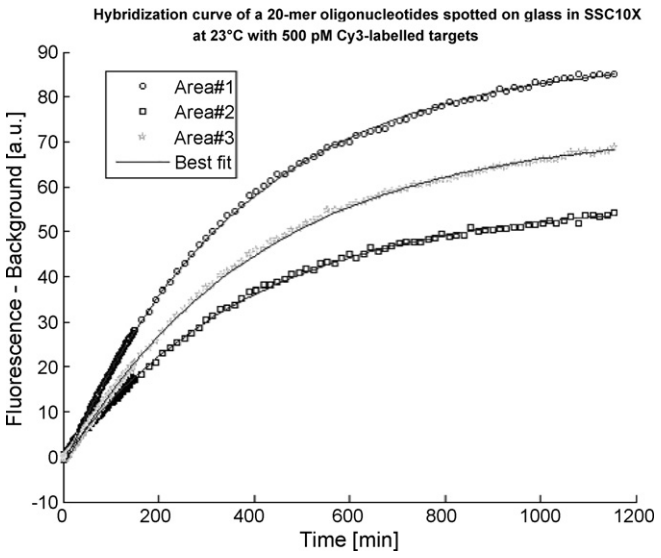

Fig. 4. Real-time corrected hybridization curves with 500 pM labelled targets simultaneously measured for the three specifically spotted areas. The flow rate is 1 mL/min. The solid line is the Langmuirian model which parameters have estimated using non linear model fitting against the measured data.

is due to a decrease of the bulk target concentration as the preceding areas are hybridized, leading to an under-estimated value of k_{on}, in the hypothesis of a Langmuirian model. Models have been developed for years (Karlsson et al., 1993; Christensen, 1997; Myszka et al., 1998; Vijayendran et al., 1999) to take into account such mass transport effects and are even now used as routine procedures to analyse and characterize experimental antigen/antibody complexes interactions (Katsamba et al., 2006; Drake et al., 2004; Säfsten et al., 2006). We are currently implementing such models in our system to make the biosensor well adapted to the determination of reaction kinetics, as well as to the study of more complex surface reactions such as competition immunoassay formats (Lebedev et al., 2006; Holt et al., 2000; Berthier et al., 2004).

3.6. Limit of detection, speed of detection

Fig. 5 shows the very beginning of the hybridization of 20-mer oligonucleotides with a 300 pM Cy3-labelled complementary target. Temperature was 23 °C, thus not optimized for the hybridization reaction. The instrumental limit of detection expressed in intensity of fluorescence units is superimposed. It is determined as three times the standard deviation calculated for 20 measurements of the background fluorescence. The detection limit in terms of density of molecules (Γ_{LoD} in molecules per μm^2) is calculated using a Langmuirian model: $\Gamma_{LOD} = \Gamma_0(1 - \exp^{-\Delta LoD/\tau})$. Γ_0 is the density of capture probes attached the surface. A value of 10^4 molecules per μm^2 is used, as a result of studies led in the laboratory. Δ_{LoD} is the time interval necessary to detect a signal representative of the hybridization reaction (i.e. equals to the instrumental limit of detection). In the experiment shown in Fig. 5, it is found as short as 70 s. τ is a time constant that depends on the concentration of labelled targets injected in the cell and on the association/dissociation constants. Its value (4.4×10^4 s) is determined by fitting the experimental data against the model. The resulting detection limit is 16 mol/μm^2. If the system was only constrained by the photon noise the limit of detection would decrease to 1 mol/μm^2. To reach this limit it is necessary to identify and overcome the sources of noise.

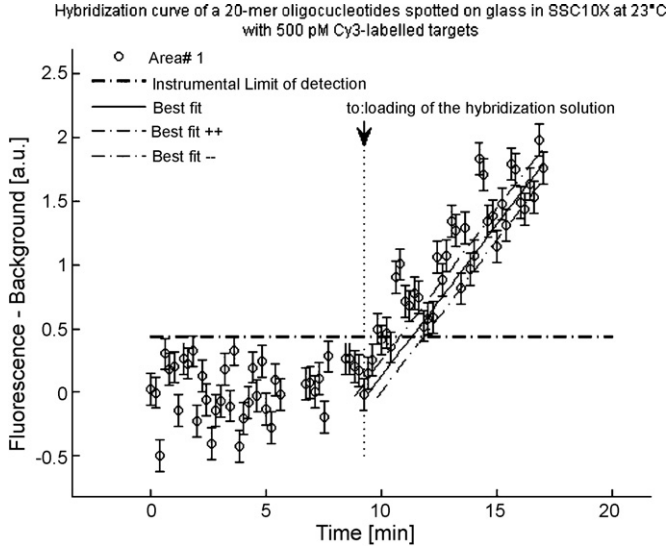

Fig. 5. Details of the fluorescence signal of area #1 at the very beginning of the hybridization reaction with 300 pM Cy3-labelled targets Error bars are defined as three times the standard deviation over the 800 samples, including the offset noise. The dash–dot line represents the instrumental limit of detection determined as 3× the standard deviation (std) over 20 measurements of the background fluorescence. The vertical dotted line shows the time at which the hybridization solution is loaded in the cell. The "Best fit", "Best fit++", and "Best fit−−" curves are Langmuir functions which parameters have been estimated by non linear model fitting against the measured data, (data + 3.std), and (data − 3.std), respectively.

4. Conclusion and outlooks

A portable, automated, easy-to-use and potentially low-cost biosensor has been built up. Its robustness (reproducibility, long-term stability) has been assessed. Its ability to operate in continuous flow for several tens of hours has been demonstrated. The biosensor enables a real time monitoring of DNA hybridization reaction, and is able to detect rapidly the hybridization of low target concentrations. It has been also demonstrated that the biosensor can be applicable to the determination of kinetic parameters, such as association and dissociation constants. Finally, the system is designed to detect up to three different analytes simultaneously, and includes a control signal presently used to monitor the instrumental deviations, but which could also be used as a calibration signal.

Work is now focused on improving the instrumentation: (1) implementing the temperature monitoring of the reaction chamber to improve both the reaction efficiency and the quality of the measurement, and (2) designing a novel reaction chamber with an increased number of capture areas to demonstrate the ability of the system to detect more than four agents.

On going and future work is also focused on achieving sandwich immunoassays, and multiplex competition immunoassays using a FRET method described by Volland et al. (2005). Indeed, this method has the asset of enabling continuous flow immunoassay including the automated regeneration of the solid phase. Further experiments will be conducted using natural samples, and the performances of the system with regard to the sample properties (pH, temperature, etc.) will be studied.

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