Use of Magnetoresistive Biochips for Monitoring of Pathogenic Microorganisms in Water through Bioprobes: Oligonucleotides and Antibodies

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ABSTRACT

Magnetoresistive spin valve sensors have been integrated in a micro-engineered biochip and applied to the detection of pathogenic microorganisms for water biological quality management.

Two different strategies of biomolecular recognition events, involving 16S rDNA oligonucleotide sequences and antibodies as recognition agents, are suggested for the detection of *Escherichia coli* and *Salmonella sp.* as model microorganisms.

The development of the platform involves the immobilization of specific probes for target capture, as well as the labeling of the target species with nanometer-sized paramagnetic markers. The molecular interaction between probe and target is monitored almost in real-time through the measurement of the variation of the sensor resistance with the magnetic stray fields created by the labels.

Preliminary results for the detection of Salmonella whole cells using this immunosensor platform are presented.

Keywords: magnetoresistive sensors, biochips, biosensors, biomolecular recognition, paramagnetic particles

1 INTRODUCTION

Microbial pathogen detection is of utmost importance for clinical diagnosis, food safety, water quality control and bioterrorism issues. Nevertheless, traditional methods, mostly based on plate counting, are slow, relatively expensive and are unable to provide high throughput screening.

A magnetoresistive biosensor is being developed as an immunosensor for pathogen detection. This system combines state-of-the-art thin-film magnetic field sensors with specific functionalised surfaces and functionalised magnetic nanoparticles or labels, enabling a very fast, highly sensitive and specific detection of biomolecular recognition [1, 2].

Capture or probe molecules, such as single stranded oligonucleotides or antibodies, specific to the molecule of interest are chemically attached to the biochip surface. The sample fluid is brought into contact with the surface, and the capture molecule recognizes specifically the target analyte. Then, the captured target molecules bind to magnetic labels, such as small paramagnetic beads or particles coated with a chemical or biological species (e.g., streptavidin or antibodies) that selectively binds to the target analyte.

Alternatively, by applying magnetic field gradients, the transport and manipulation of magnetic beads already carrying the target biomolecules can be controlled over the chip's surface, addressing it towards specific binding sites on sensing areas [3, 4].

Thorough washing ensures that only specific and strongly bound targets are left over the sensing area.

Finally, spin-valve magnetic field sensors detect the magnetic fields created by the labels, corresponding to the presence of the target molecules.

2 EXPERIMENTALS

2.1 Chemicals

All reagents were of analytical grade. The crosslinker Sulfo-EMCS was purchased from Pierce. Sodium *m*-periodate ($NAIO_4$); sodium borohydride ($NaBH_4$); Tween 20 and bovine serum albumin (BSA) blocking solution were purchased from Sigma. Polyclonal antibodies against *Salmonella sp.* and *E. coli* O157:H7 were obtained from KPL, Guildford, UK. Single stranded oligonucleotides, 23 bases long, were synthesized by Thermo Electron (Ulm, Germany), encoding for a region of 16S rDNA from *E. coli*. Their designation, base sequences and modifications are as follows: probe Coli2, 5' CGG TCC AGA CTC CTA CGG GAG GA-SH 3' and target Coli4, 5' GCC AGG TCA GAG GAT GCC CTC CT-biotin 3'.

Salmonella cells were a gift from Water Analysis Laboratory from IST.

Nanomag®-D magnetic particles of 250 nm in diameter and modified with streptavidin or amino groups, were obtained from Micromod GmbH, Germany. Dynabeads 2.8 μm in diameter and modified with anti-*Salmonella sp.*, were supplied by Dynal Biotech, (Oslo, Norway). The

phosphate buffer (PB) 100 mM consisted of a combination of monobasic sodium phosphate (NaH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4), pH was adjusted to 7. Borate buffer (BB) was prepared from 50 mM Bórax ($Na_2B_4O_7.10H_2O$), pH adjusted to 9 with NaOH. Acetate buffer consisted in 0.1 M of sodium acetate, pH adjusted to 5. Phosphate saline buffer (PBS) was prepared from a stock solution 10X concentrated, which consisted of a combination of Na_2HPO_4, KH_2PO_4, NaCl and KCl, pH 7.4 adjusted with HCl. Hybridization buffer consisted of 5X saline-sodium citrate (SSC), 0,1% N-lauroylsarcosine, 0,02% sodium dodecyl sulfate (SDS) and 1% blocking reagent (% refers to w/v).

2.2 Spin Valve Sensors and Chip Design

The biochip in development is composed of a silicon substrate with integrated magnetoresistive sensors (spin-valve type) and aluminum current lines. Spin-valve sensors are U-shaped and have dimensions of 2.5 × 80μm^2 (full sensing length) (see Fig. 1) [4]. The spin valves were deposited by an ion beam system onto 3 in. Si wafers and have the structure Ta 20 Å/NiFe 30 Å/CoFe 25 Å/Cu 26 Å/CoFe 25 Å/MnIr 60 Å/Ta 20 Å/TiW(N_2) 150 Å with an average magnetoresistance of 7%. Each sensor is connected to aluminum leads and associated to a contiguous aluminum U-shaped current line, 3000Å thick, 10μm wide, 120μm full length and with a spacing between the arms of the line of 17μm, corresponding to an area of ~1000μm^2 surrounding the sensing area. Both sensor contacts and current line lead to wire-bonding pads.

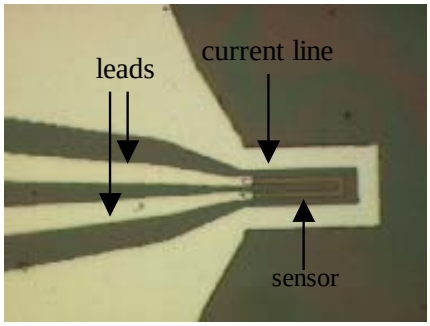

Figure 1. U-shaped spin-valve sensor with contact leads and associated current line.

The whole structure is covered with a double layer of oxide thin films, respectively, 1000Å of alumina (Al_2O_3) and 2000Å of silicon dioxide (SiO_2), which are used as passivation layers and additionally provide a suitable surface for derivatization chemistries and immobilization of bioreceptors.

Approximately 45 (8x8 mm) chips were fabricated per 3 in. silicon wafer and cut using a dicing saw.

2.3 Experimental Set-up

Individual chips were mounted on 40 pin chip carriers and the electrical connections made via wire bonding of the contact pads. Wire bonded areas were protected using a silicon gel. The chip-carrier was mounted in a small breadboard and coaxial cables were used to make the electrical connections to the associated hardware, namely to power sources and to a general purpose interface bus (GPIB)-controlled lock-in amplifier. Measurements were made using an external AC excitation field of 13.5 Oe rms and a frequency of 30 Hz in combination with a DC bias field of 24 Oe [4]. These fields were generated in the plane of the chip by a horseshoe electromagnet positioned over the chip-carrier and were used to induce a magnetic moment within the superparamagnetic labels, enabling lock-in detection, and to bias the sensors for maximizing output. During the experiments, labels were controllably focused to the sensor area using magnetic field gradients created by passing AC currents of 25 mA rms at a frequency of 0.2 Hz in the neighboring U-shaped current lines in combination with the 24 Oe DC bias field [4].

2.4 Surface Functionalization

The adopted procedure for surface derivatization and functionalization is based on a covalent reaction between aminosilane molecules, 3- aminopropiltrietoxisilane (APTES), and silanol groups (-OH) from the support. On SiO_2 surfaces –OH groups were achieved by an initial activation step using a mild solution of 2% cholic acid, overnight. Subsequently, functional amino groups were achieved by silanization with a 10% aqueous solution of APTES for 2 hours at RT. Those aminated surfaces were subjected to different immobilization procedures depending on the nature of the bioreceptor (nucleic acid-based molecules or proteins).

2.5 Antibody Surface Functionalization

For the preparation of immunosensitive surfaces, specific antibodies against common structure antigens (CSA) of *Salmonella sp.* whole cells were covalently immobilized through an oriented process. The natural carbohydrate moieties present in the Fc region of the antibody were oxidized with sodium *m*-periodate 10 mM in acetate buffer and 0.15 M of NaCl in order to originate aldehyde groups that chemically react with free amino groups on the support. This way, the antibody immobilization is site-directed and the antigenic binding sites regions are outside oriented and available for antigen capture. Sodium borohydride 1 mg/mL was used to reduce the (-N=C-) double bond and turn immobilization irreversible.

After antibody immobilization the remaining free binding sites were inactivated using a blocking solution during one hour at room temperature (RT) to prevent non-specific binding. Then, a droplet of 50 μL of *Salmonella* solution was brought into contact with the sensor immunosensitive surface for 30 minutes at RT and the free species, not

specifically bound were washed away by rinsing with a solution of 2% Tween 20.

2.6 DNA Surface Functionalization

On the other hand, for DNA chips preparation, probe immobilization chemistry involved the use of a crosslinker reagent [1, 2]. Probe oligonucleotides were synthesized bearing a thiol group on the 3' end, which made them suitable to react with the sulfo-EMCS crosslinker, making a bridge between the aminated surface and the probe.

The silanized chip was first reacted with a 1 mM sulfo-EMCS solution in borate buffer for one hour and then reacted with thiol-modified oligonucleotides at 4 μM in PB for 3 hours. Non-immobilized molecules were removed by rinsing with buffer.

Before target recognition, a pre-hybridization step with hybridization solution for one hour was required in order to avoid unspecific adsorption of target molecules.

Hybridization with biotinylated target molecules was performed with a 4 μM oligonucleotide solution in hybridization buffer for 3 hours at RT. Weakly and non-hybridized molecules were removed rinsing the surface sequentially in 2X SSC + 0.15% SDS, 1X SSC + 0.075% SDS and 0.2X SSC + 0.015% SDS solutions.

2.7 Magnetic Particles Functionalization

Magnetic labels can suffer different functionalization chemistries and carry diverse molecules (proteins, antibodies, DNA) depending on the target nature or target post-modification.

For the labeling of biotinylated and previously hybridized target oligonucleotides, 250 nm streptavidin-functionalized magnetic particles were used.

Conversely, in *Salmonella* detection chips, aminated magnetic markers were functionalized in-house with antibodies anti-*Salmonella* from KPL, using the same site-specific immobilization chemistry as previously described for antibody functionalized surface preparation.

2.8 Labeling and Setup Measurements

Experiments proceeded as follows: the chip was loaded with a 20 μL concentrated droplet of specifically modified magnetic labels. Particles were focused at sensor sites using the U-shaped current line and left to interact with immobilized probes for 15 minutes. Afterwards, the sensor was washed twice with PB to remove unspecifically and weakly bound particles and to confirm the stability of the binding signal.

Control experiments were performed where a non-specific biorecognition element was immobilized and used to assess for non-specific recognition background signals. For the DNA chips a target non-complementary to the probe oligonucleotide was used, while for the antibody chips a non-specific anti-*E.coli* O157:H7 antibody was used as a control.

3 RESULTS AND DISCUSSION

Salmonella cells were captured by the immunosensitive platform and specifically recognized either by 2.8 μm anti-*Salmonella* Dynabeads (see Fig. 2) or by smaller 250 nm Nanomag-D particles functionalized with antibodies anti-*Salmonella*.

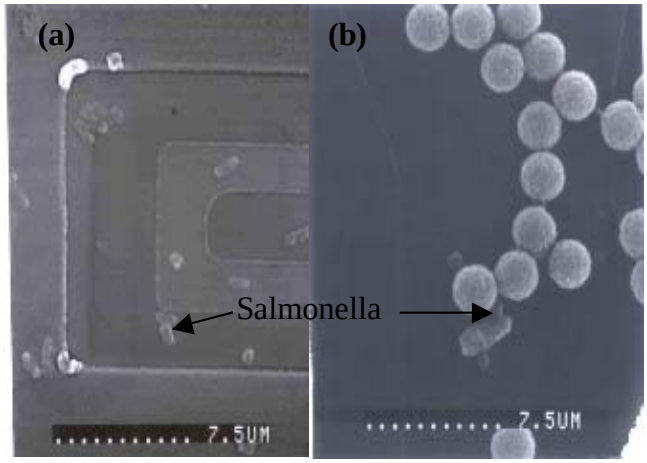

Figure 2. Scanning Electron Microscopy (SEM) images. In (a) *Salmonella* cells were immobilized onto the immuno-modified sensor surface. In (b) *Salmonella* cells were recognized and specifically labeled with 2.8 μm antibody anti-*Salmonella* modified particles.

The saturation signals, corresponding to particle detection signals when a large number of particles were settled over the sensor, were completely dissimilar for both types of particles. An average value of ~0.2 mVrms for the larger labels contrasts with the ~1 mVrms attained to the nanometer-sized ones. This is related to the fact that the larger labels possess a lower percentage of magnetic content (~15%, in weight) in comparison to magnetic nanoparticles which have an higher iron-oxide content (70–85%, in weight), together with the fact that only ~30 larger labels can fit in a monolayer over the sensor against ~3200 smaller labels. It results in the end in a larger magnetic field created, at the sensor, by the nanoparticles than by the larger beads.

Additionally, it was observed that, after the washing steps using an autopipette to remove overloaded free labels, the 2.8μm particles were easily washed away from the surface, even those specifically bound to target cells. The forces implicated in antibody-antigen recognition events are probably not enough to prevent large particles, waving and bending during washes, from getting released. Further studies are in preparation to confirm this possibility.

Conversely, results obtain with 250 nm particles, confirms that smaller labels were able to recognize the cells and remain attached even after intensive washing. The signal for the sample chip was kept around 200 μVrms. On the other hand, the signal for control sensors has returned near to the baseline (~40 μVrms) meaning that despite the unspecific binding verified it is much lower when related to the signal obtained for the sample (see Fig. 3) and hopefully it can be minimize either using a more efficient blocking step or stringent washes to remove adsorbed particles.

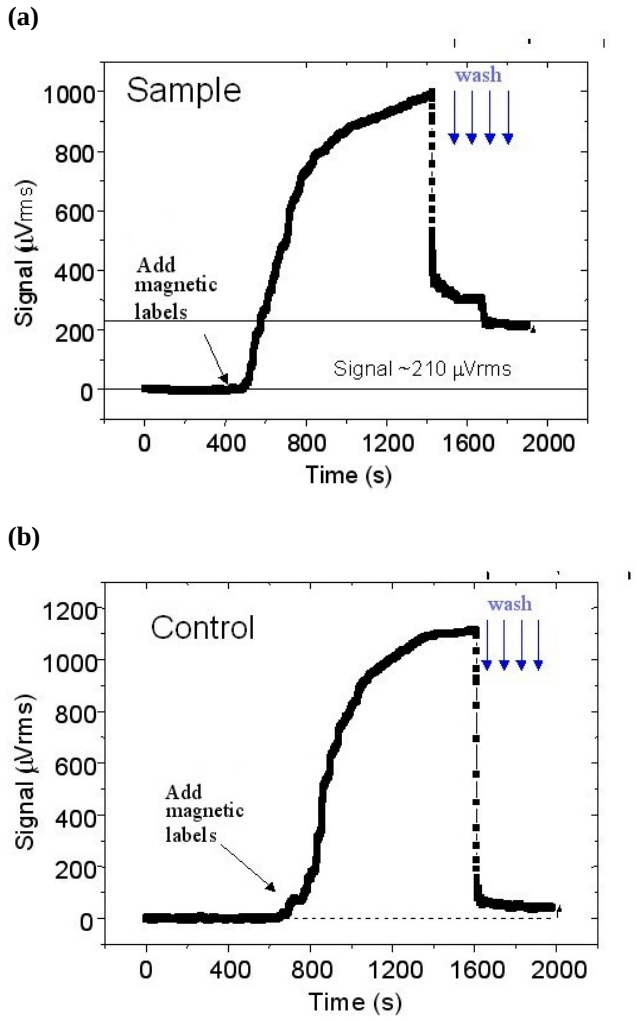

Figure 3. Real time sensing data from a spin valve sensor. (a) Detection of antibody-antigen recognition event between immobilized anti-*Salmonella* antibodies and *Salmonella* using 250 nm antibody anti-*Salmonella* modified particles. (b) Control experiment where the recognition agent was non-specific for *Salmonella* cells.

At the end of the experiments chip surfaces were observed under the optical microscope. It was confirmed that the output signals from the sensors corresponded to the presence of immobilized magnetic labels. Further, it was verified that particles were more or less homogeneously distributed over the surface although some particle clustering was still observed (see Fig. 4).

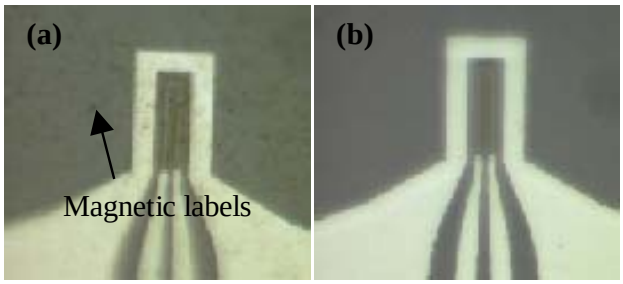

Figure 4. Optical microscope pictures (800X magnification) from sensors after experimentation. (a) and (b) are sample and control sensors, respectively.

Preliminary results using 16S rDNA oligonucleotide probes from *E. coli* and a complementary target were obtained for real time hybridization detection with streptavidin modified 250 nm Nanomag-D magnetic labels (data not shown) and further testing is on-going.

4 CONCLUSIONS

A magnetoresistive immunosensing platform is being developed for monitoring of the microbiological quality of drinking water and hydric resources. Probe site-directed immobilization, specific biomolecular recognition, and magnetic label detection were shown. In particular *Salmonella* cell recognition and detection was achieved, while the detection of hybridization of *E. coli* related oligonucleotides is underway. In the future, a better control of the background and non-specific binding is required together with the assessment of the biological sensitivity of the chip and the use of different microbial cells to test for the system specificity.

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