Relative Specificity of the Hybridization Process on DNA Chips: Direct Comparison of Four Interfacial Architectures Investigated by Surface Plasmon Resonance*

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In the field of DNA based sensors, it is crucial to identify a suitable interfacial chemistry providing DNA chips that exhibit a high efficiency with respect to the hybridization process. In this study, we performed a systematic investigation on various chemical architectures by Surface Plasmon Resonance (SPR), which is an optical technique able to follow a molecular recognition process in-situ and in real time. We analyzed four different preparation methods that produce DNA based sensors on gold surfaces. For each one, we monitored by SPR the amount of molecules deposited on the chip when this latter is exposed to different target DNA solutions. For those solutions, we used either complementary or non-complementary target single stranded DNA in order to check either the sensitivity or the selectivity of the sensor. As shown by the results, the interfacial chemistry of the DNA based chips is relevant to the quality of the hybridization process, i.e., a high deposition rate with only a few unspecific adsorptions. Moreover, the importance of adding a protective layer on the gold surface in order to prevent non-specific physisorption of DNA strands is discussed. [DOI: 10.1380/ejssnt.2009.777]

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I. INTRODUCTION

The detection of a specific oligonucleotide sequence from a solution by hybridization adsorption can be achieved by using surface-bound single stranded DNA (ssDNA) sensors. These latter appeared to be powerful tools for a large range of biological and medical applications such as diagnosis of genetic diseases, DNA sequencing or gene expression analysis [1–5]. Because of that popularity, substantial efforts were made over the last decade to understand the fundamentals of the immobilization of ssDNA onto a surface [6–9] and various attachment methods of the probes were investigated [10, 11]. Controlling the physico-chemical properties of liquid-solid interfaces is therefore a prerequisite for the biosensors technology [12, 13]. The purpose of most of these studies was to improve the sensitivity and the selectivity of the DNA based sensors to allow a target sequence detection involving the fewest number of modifications of the original sample [14]. To achieve this, it is crucial to construct the sensor chip so as to maximize the hybridization of complementary target ssDNA and to minimize the non-specific adsorptions of non-complementary ones. Many parameters can affect these binding performances such as the probe density [15], the availability for target bindings [16], the nature and complexity of the surrounding medium [17] and of course the physico-chemical structure of the sensor itself where the interactions take place. It is therefore critical to use an effective surface chemistry appropriate to the binding reactions studied, e.g., the DNA hybridization process. There are various chemical architectures suitable for the sensor functionalization described in the literature. On gold chips particularly, the ssDNA attachment is most often achieved through thiolate-gold bonds relying on the assembly of oligonucleotides via a covalent thiol linker [18, 19]. However, other immobilization strategies are also used involving more complex chemical architectures and/or larger anchoring molecules [20–22].

In this paper, we used Surface Plasmon Resonance (SPR) in order to perform a systematic investigation of various chemical architectures of DNA based sensors constructed on gold chips in order to evaluate for each system, the quality of the hybridization process. For this purpose, we monitored the amount of molecules deposited on the chip when it is exposed to various target ssDNA solutions. The efficiency of the recognition is estimated by comparing the molecular depositions obtained when we used either complementary or non-complementary ssDNA as target. As the SPR technique can detect the molecular adsorption onto a surface through a local change of refractive index, we were able to monitor both specific and non-specific contributions without the need for labeling.

Since the development, over a decade ago, of the first chip achieved by SPR, the use of this tool has steadily increased. Because of its ability to investigate biosensors...
in situ and in real time without labeling requirements. SPR quickly became a key technique for studying molecular interactions in such systems. Basically, SPR-based instruments use an optical method to measure the refractive index near the sensor surface. If an adsorbate is deposited on the chip, it can lead to a measurable modification of the local refractive index at the interface. This variation is measured in situ and plotted in a sensorgram as a function of time. SPR-based sensors can be used to study the interactions of many biological systems such as oligonucleotides, proteins, lipids, cells, and various other molecules presenting interest in the biological field [23, 24]. Particularly, this technique was widely used to provide quantification of DNA hybridization [25–27]. Practically in SPR experiments, a polarized laser beam is focused on a metal/dielectric interface through a prism in an attenuated total reflection (ATR) configuration. A CCD then monitors the intensity of the reflected beam as a function of angle. For a particular angle, a surface plasmon can be excited at the metal/solution interface and a minimum in the reflectance is observed. The value of that given angle is sensitive to variations of the refractive index in the vicinity of the metal surface. The evanescent nature of plasmonic waves in ATR configuration makes that technique intrinsically sensitive to the interface. It means that a molecular deposition on the metallic layer will result in small shifts of the resonance angle values. By monitoring the reflected light intensity minimum as a function of time, we can thus follow the deposition of a molecular adsorbate onto the chip [28–30].

II. EXPERIMENTAL

For this study, we selected from the literature four different chemical architectures leading to the formation of a DNA based biosensor on gold substrate as depicted in Fig. 1 [18, 31, 32]. For all those preparation methods, we used bare gold chips (Biacore) as substrates. Those latter are composed of a ~48 nm thick gold film and of a few-nm thick titanium dioxide layer evaporated onto glass slides. All chips were cleaned by chemical etching within Piranha solution (H₂SO₄:H₂O₂, 3:1) for at least 15 minutes in order to remove any organic contamination and then abundantly rinsed several times with ethanol and pure water (18 MΩ cm). After this cleaning step, the chip is ready to be chemically conditioned according to one of the following methods. All oligonucleotides used further in this work were purchased from Jbios (Japan) and purified by column prior to use. Moreover, all DNA concentrations were checked by UV adsorption measurements performed on a BioSpec 1600 (Shimadzu). Table I summarizes all the DNA sequences that we used in this work. Phosphate buffered saline (PBS) 10 mM (150 mM NaCl - pH 7.4) was employed as running buffer solution in all experiments except for method 3 (as explained below). In addition, all tips, tubes and buffer solutions were previously sterilized in an autoclave device at 120°C in order to avoid any biological impurities.

Method 1. For this procedure, we used thiolated single-stranded 25-mer DNA oligonucleotides (ssDNA-SH). Those probes were adsorbed onto the gold chip by spotting 2 μM ssDNA-SH solution in PBS buffer on the substrate overnight at room temperature leading to the formation of a self-assembled ssDNA monolayer (SAM) anchored through the sulfur-gold bond. Prior to analysis, the sensor was rinsed with pure water and dried under nitrogen flow.

Method 2. This method is mainly the same as the previous one but we add one step by intercalating mercaptohexanol molecules (MCH) between the adsorbed DNA strands. By this way, those hydroxy-terminated alkanes prevent contacts between the substrate and the DNA backbones. Practically, we can adjust the relative coverage ratio of MCH regarding to ssDNA-SH by varying the concentrations of the solutions and the duration of the substrate immersion. In our case, we exposed our gold surface to a 2 μM ssDNA-SH solution in PBS buffer for 90 minutes according to [16]. The sequence of the ssDNA-SH was the same as previously. After rinsing and drying with pure water and nitrogen, we spotted on the chip a 1 mM MCH solution in pure autoclaved water for 5 minutes. Once again, the sample is rinsed with pure water and finally dried under nitrogen flow.

Method 3. This preparation method was reported by Sakao et al. [31]. The probe molecules are composed of two different single stranded DNA parts: a 20-mer oligonucleotide functionalized with a thiol group in order to realize the self-assembly process on the gold substrate, and a 50-mer oligonucleotide containing three remarkable sections. The first one is complementary to the ssDNA-SH allowing the formation of a 50-mer/20-mer thiolated complex. The second part is a “junk” spacer of 10 thymine bases, which increases the mobility of the probe. Finally, the last one will be used as single stranded DNA probe in the SPR experiments. Practically, the probe complexes formation was achieved by mixing the 20-mer ssDNA-SH and the 50-mer ssDNA (~1:2 in mass) in a 500 mM SPB solution (pH 7.4) annealed up to 90°C for 5 minutes. After progressive cooling down, a hydroxy-apatite purification step was performed in order to keep only the hybridized complexes (ds-DNA-SH) for the adsorption on the chip. Note that in this case, we preferred to use 20 mM MgCl₂·6H₂O solution (MgCl₂ buffer) instead of PBS as running buffer in order to increase the amount of ds-DNA complexes standing up on the chip which result in the formation of a condensed monolayer (see [31]). At this point, the surface was exposed to a 2 μM dsDNA-SH solution in MgCl₂ buffer for 5 minutes and was finally rinsed with running buffer.
TABLE I: Summary of the various ssDNA sequences used in the experiments.

| Method     | ssDNA-SH                  | ssDNA-comp   | ssDNA-neg               |
|------------|---------------------------|--------------|-------------------------|
| Method 1   | 5’-SH-(CH₂)₆-CACGACGTTGTAATAAACGACGGCCAG-3’ | 5’-CTGGCCGTCTGTTTTTACACCTCGTG-3’ | 5’-GACCGGCAGCAAATGTGCGACAC-3’ |
| Method 2   | 5’-SH-(CH₂)₆-CACGACGTTGTAATAAACGACGGCCAG-3’ | 5’-CTGGCCGTCTGTTTTTACACCTCGTG-3’ | 5’-GACCGGCAGCAAATGTGCGACAC-3’ |
| Method 3   | 5’-SH-(CH₂)₆-ATGCATGCATTAGCATGCTA-3’ | 5’-CTGTGTCGATCAGTTCTCCA-3’ | 5’-TGGAGAACTGATCGACACAG-3’ |
| Method 4   | 5’-biotin-ACGCCACGAGCTCCTCAACTCACAAGTTTATTTACATGCTATTTCCTCGACGCCCTCCTCCTCC-3’ | 5’-GGAGAGAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTCCTGCGGTG-3’ | 5’-CCTCTCTCCGGACGACTTTTACTGACTTATATTGAGACACCACATCAACCTCGAGCCACCGA-3’ |

**Method 4.** For this method, we followed the procedure suggested by Sato et al. in [32]. First, a thiolated SAM was deposited on the gold surface by exposing the chip to a 5 mM 1,6 hexanediithiol solution in ethanol overnight at room temperature. After several rinsing steps with ethanol and pure water, the substrate was immersed in ethylene glycol diglycidyl ether (EGDE) for 24 h at 37°C. Those molecules interact with the dithiol layer through a thiol-epoxy reaction and introduce epoxy groups at the surface in order to minimize further unspecific adsorptions. Then, we exposed the substrate to a 10 mM Biotin-PEO-amine solution in sodium phosphate buffer (pH 9.4) for 24 h at 37°C. At this point, the surface was immersed in a solution of PBS buffer and 10 ppm streptavidin (SA) for 5 minutes and, finally, in a 1 μM biotinylated 60-mer ssDNA (ssDNA-biot) solution in PBS for 5 minutes too. Note that between each of those steps, the chip was thoroughly rinsed with pure water or running buffer.

**SPR measurements.** In order to perform the SPR measurements, we used a Biacore 2000 device (Biacore-Sweden). This apparatus allowed us to perform parallel experiments on different channels on the same sensor. By this way, we were able to realize the different measurements involving the different target molecules on each individual sample investigated. All experimental data were analyzed with the BIA evaluation software.

**III. RESULTS AND DISCUSSION**

The main goal of this study was to compare, for each of the above-described methods, the quality of the hybridization process when the probe ssDNA molecules are exposed to a target ssDNA solution. For those latter ones, we used either complementary (ssDNA-comp) or non-complementary (ssDNA-neg) target single stranded DNA molecules in order to check either the sensitivity or the selectivity of the sensor. The deposition of the targets on the chips was then monitored with the SPR setup by following the variation of the plasmonic angle as a function of time. That variation is usually expressed in Resonance Unit (RU). 10⁴ RU represent an angular variation of 1°. For better comparison, we kept all the experimental parameters constant for each measurement. All target molecules were suspended in a PBS solution at a 5 μM concentration and were in contact with the chip for 5 minutes at a flow rate of 5 μl/min. After each deposition, the sensor was regenerated by being exposed to 3 pulses of 1 minute of a 50 mM NaOH solution used to denature the hybridized DNA.

Figure 2 shows a typical sensorgram describing all the steps of one particular experiment. Four main steps can be identified. First, the chip is exposed to the running buffer for at least 5 minutes, which defines a baseline. In the second step, the target ssDNA is injected leading to a molecular deposition at the chip surface and then to a variation of the plasmonic angle. Step 3 is a dissociation step, the injection is stopped and the system is exposed again to the running buffer for at least 7 minutes. At this point, the total angular variation from the baseline is relevant for the molecular adsorption on the chip. The last step is the regeneration one during which the adsorbed material is removed by using the denaturizing solution. Hopefully, the sensorgram plot should reach the baseline level again, meaning that the sensor is fully regenerated and can be reused for another experiment. This methodology was achieved several times for each chemical architecture and target solution. Each time, the total angular variation (ΔRU) reached at the end of the dissociation step was noted.

Figure 3(a) summarizes the values of ΔRU that we obtained for the various preparation methods and target so-
FIG. 2: Typical sensorgram describing the different steps of a particular experiment. Step 1: Baseline. Step 2: Exposure to the target solution. Step 3: Dissociation. Step 4: Regeneration.

FIG. 3: (a) ∆RU obtained for the various preparation methods and target solutions. (b) Values from (a) normalized regarding to the complementary ssDNA adsorption. The percentages are the ratio of the non-specific contributions regarding to the specific ones.

...mments. Moreover, due to the structure of the different chemical architectures, the hybridization sites are not at the same distance from the gold interface. Therefore, because of the evanescent nature of the plasmon wave, a same amount of molecules deposited on the chip can contribute in different ways to the sensorgram. Theoretically, a relevant comparison could be achieved by converting the ∆RU into an absolute molecular surface coverage. This can be realized by fitting the angle-resolved reflectance curves obtained from the SPR device to a multiphase Fresnel optical model [26, 33]. However, the purpose of this study is to evaluate, for each method, the efficiency of the hybridization process. In that respect, it remains possible to perform a relevant comparison between the specific and the non-specific interactions measured for a particular system. Indeed, in this case, the experimental conditions are rigorously identical. Figure 3(b) presents the above-mentioned ∆RU but this time, normalized to the values obtained when we used complementary ssDNA as target molecules.

As shown by the results, the behaviors of each sensor regarding the non-specific adsorption are drastically different. From the normalized data, we establish for each method the percentage of non-specific interaction. It means that we do not confront absolute amounts of deposited molecules but relative ratios, normalized to the specific adsorption, which no longer depend on the experimental conditions involved in the different methods. We observed relative unspecific deposition rates of 62%, 8%, 49% and 15% when we consider method 1, 2, 3 or 4, respectively. The second and the fourth method-based sensors reveal the best results by exhibiting a higher specificity, which means a lower adsorption of non-complementary target ssDNA. Actually, we could expect that outcome since those two chemical architectures were initially designed to include a protective layer in order to prevent unspecific target molecules deposition. This aspect is achieved through the MCH layer in method 2 and through the epoxy-terminated one in method 4. Indeed, these kinds of chemical adlayers are widely used in the biosensor field and are known for their bio-resistant abilities [19, 34]. Note that the non-specific interaction ratio is probably overestimated for Method 3. Indeed, because of a short exposition to the probe molecules solution, this sensor is expected to present some defects in the probe layer. Those defects, granting access to the gold surface for the target ssDNA, increase the non-specific contribution. The literature tells us that a higher probe density should lead to a lower ratio, namely around 15-20% [31].

In order to go deeper in the understanding of the role of that preventive layer, we can further compare the results obtained for methods 1 and 2 when the chips are exposed to complementary oligonucleotides. Those two sensors are chemically indistinguishable except for the presence of the MCH molecules. However, a noticeable dissimilarity during the regeneration step can be observed if we examine the complete sensorgrams collected from these experiments, as presented in Figure 4. In the first case, we do not recover the baseline level after exposing the surface to a pulse of NaOH solution. Actually, for method 1-based sensors, the baseline is still not recovered after six regeneration steps of 1 minute using 100 mM NaOH...
FIG. 4: Sensorgrams obtained when method 1- and 2-based sensors are exposed to complementary ssDNA, then to running buffer and finally to regeneration solution.

FIG. 5: Sensorgram obtained when a bare gold surface is exposed to ssDNA solution.

solution (data not shown) while only one regular step is enough to fully regenerate method 2-based chips. Such solutions being known to denature the hybridized DNA [35, 36], it means that most of the deposited molecules are bound to the surface through another kind of interaction. In this particular case, we can easily understand that if the target ssDNA is not hybridized to the probe one, it is actually physisorbed on the gold substrate. Indeed, for method 1, the absence of a protective layer provides an easy access to the metallic layer leading to a strong unspecific adsorption. On the other hand, the MCH molecules used in method 2 prevent that access and thus improve the selectivity of the sensor. We can of course extrapolate that deduction to the other investigated methods and, by this way, explain the better performances of the methods using a substrate protective layer, i.e., methods 2 and 4. Moreover, an additional clue to that can be extracted from the kinetics observed from the shapes of the sensorgrams. They seem dissimilar between both experiments meaning that different phenomena are involved in the adsorption process.

By realizing a simple additional experiment, we can bring another evidence for that trend. For this purpose, we exposed a bare gold surface (cleaned as explained previously) to a target ssDNA solution. As we can see on Figure 5, a non-trivial deposition occurs even for short exposure duration, attesting to an affinity for ssDNA to physisorb on that kind of metallic interface. This observation is consistent with previous works which investigated the interactions between gold and DNA [37, 38]. Note that if we try to perform a regeneration step on such system, we are confronted to the same behavior as observed for method 1-based sensors, i.e., the sensorgram does not reach the baseline again.

IV. CONCLUSIONS

In this paper, we performed a systematic analysis of various ssDNA-based sensors built on gold chips in order to evaluate, for each system, the quality of the hybridization process. The depositions of complementary and non-complementary ssDNA target molecules were monitored by SPR. The results showed that the chemical architecture of the chip is crucial with respect to the sensor selectivity. The observed results were strongly different from one method to the other, highlighting the importance of the interfacial chemistries. Among the investigated methods, the second one exhibited the most convincing performances with a relative unspecific deposition rate of about 8%. Particularly, this work exhibited that the addition of a bio-resistant layer drastically improves the efficiency of the hybridization process. Indeed, by examining the sensorgrams and by comparing the efficiency of the regeneration steps, we showed that this kind of protective layer restrict the target molecules access to the gold substrate, preventing unspecific physisorptions.

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