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Direct and label-free monitoring oligonucleotide immobilization, non-specific binding and DNA biorecognition

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ABSTRACT

DNA binding chemistry on silicon surface has been investigated. Aminated oligonucleotide probes were immobilized on the chip surface by chemical silanization and further covalent attachment. The chemistries employed were the classical 3-aminopropyltriethoxysilane/glutaraldehyde and, for comparison purposes, the novel 3-isocyanatepropyltriethoxysilane, that allows the direct attachment of the aminated probe. Alternatively, a thiolated oligonucleotide was also photochemically immobilized by means of a thioether linkage. The experiments were followed label-free by Dual Polarization Interferometry. All chemical and photochemical methods gave rise to a probe density immobilization in the order of 1.0-2.5·10^{10} molecules/mm^2, similar to the values reported for other chemistries. The obtained data suggest that DNA strands are anchored in a different conformation depending on the immobilization method employed. In order to avoid non-specific binding of target molecules, ethanolamine and inert proteins were assayed, and successful results were obtained when using small size proteins such as gelatine. Hybridization efficiency was around 20% for aminosilane-based immobilized probes, and more than 4-fold this value when the other immobilization methods were employed. The ability for recognition complementary DNA strands discriminating non-complementary ones was applied for species identification in mixtures.

Keywords: DNA Immobilization; Silicon; Surface chemistry; Dual Polarization Interferometry; Biosensing

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1. Introduction

The studies and uses of nucleic acids have been growing up for decades, and the trend is to keep on in first line. Thus, basic research on nucleic acids provides fundamental knowledge for *in vivo* and *in vitro* applications. DNA-based methods have also been employed in many areas, examples being forensic science [1], environmental control [2] and biosensing [3]. Additionally, new issues addressed to both array-like platforms [4] and lab-on-a-chip arrangements [5] are currently under development.

Nearly all nucleic acid-based methods employ the recognition element immobilized on a surface, so DNA immobilization has been extensively studied in the last years, and many attachment chemistries have been described [6]. Among all the supports used in biosensing, silicon is one of the most popular because this is a highly versatile solid, readily functionalized, biocompatible, and its derivatization for attaching biomolecules has been deeply studied [7].

Many strategies have been developed for detecting DNA hybridization processes. The employment of labels such as fluorophores is well-known, facilitated by the PCR amplification of the target and recommendable to routine high-load analysis. However label-free detection retrieves better information and the processes to be monitored are more similar to what is happening in life or biological systems.

There are several label-free technologies for biosensing and related applications, e.g. Quartz Crystal Microbalance (QCM) [8], Surface Plasmon Resonance (SPR) [9] and interferometry [10]. Among the latter ones, Dual Polarization Interferometry (DPI) is a very interesting option.

DPI is considered a reliable technique for monitoring binding events happening on a silicon oxynitride surface in real time. Basically [11], light from a laser is passed through a sandwiched waveguide structure, and an interference pattern is detected on the opposing side by a CCD camera. The phase shift of transverse magnetic (TM) and transverse electric (TE) modes is recorded in real time, and data are resolved; so that only one value of thickness and absolute refractive index at a given timepoint t will satisfy Maxwell's equations of electromagnetism for both TM and TE polarizations. Differences in the waveguide mode dispersion between the TE and TM modes allow unique solutions for adlayer thickness and refractive index, and these data can be converted to surface mass and density. Thus, this technique provides label-free precise measurements, in real time, enabling details of the structure and function of biomolecules to be elucidated. Hence, high-quality information on the orientation, distortion, and efficiency of immobilization procedures as well as the interaction events can be obtained. It is remarkable that a DPI instrument is a quantitative analytical tool, not a sensor. DPI routinely and reproducibly provides simultaneous quantitative data on real-time changes in dimension (resolution < 0.1 Å) and density (resolution < 0.1 pg/mm^2) of biomolecules immobilized on the chip surface, without recourse to tagging, but it is not intended

as a sensor system for the highly sensitive determination of the agents that provoke these small changes [12].

DPI is frequently compared to SPR [13] because both are based on similar principles. However, SPR utilizes only the TM mode, while DPI takes advantage of measuring both the TM and the TE polarizations [14], which allows the simultaneous determination of both effective refractive index and thickness values. That is clearly a great advantage over SPR and other optical biosensing techniques, which can only report relative changes of refractive index [15]. Combining the thickness, mass and density data allows not only to monitor the binding but also to retrieve structural information at molecular level, and this is the main benefit of DPI.

Another difference between DPI and SPR or QCM relies on the different solid substrate employed. QCM and SPR use gold as platform, while DPI employs silicon-based chips, this material having the advantage of its low cost, versatility and applicability [7]. Nevertheless, both DPI silicon [16] and SPR gold [17] can be coated with a polymer in order to expand and improve the attaching chemistry.

Applications of DPI can be found in any area where microscopic surface properties need to be monitored, provided that silicon-based chips act as a supports. Interesting reports are the monitoring of lipids [18], polyelectrolytes [19] and other multilayers [20], events happening in liposomes [21], as well as the study of induced conformational changes in proteins [22], molecular aggregations [23], basic protein-protein interactions [24,26] and protein crystallization [27].

Nucleic acids chemistry and their interactions have also been monitored by DPI, in terms of DNA multilayer films formation [28], interactions of DNA aptamers with small molecules [29,30] and even with metal ions [31]. However the studies of DNA hybridization are scarce. Two interesting pioneer papers describe a basic study about monitoring DNA immobilization and hybridization. In the first one [32], single stranded DNA immobilization by passive adsorption, covalent linkage and avidin-biotin interaction are studied, and hybridization is monitored. In the other [33], authors covalently immobilize aminated oligonucleotides on commercial amine-derivatized chips, then block with BSA the remaining active sites, and finally add a complementary oligonucleotide for hybridization, all the three processes being monitored. Both papers are focused on monitoring DNA immobilization and hybridization, with useful discussions, but other issues such as unspecific binding or practical applications are scarcely addressed, there being little or none good contribution papers on this topic.

The aim of the present paper is to describe a basic study of DNA immobilization and hybridization by means of DPI including variables such as chip derivatization (silanization) as well as different modes of DNA anchoring including photochemical activation [34]. Attention is especially paid to the use of different blocking agents to avoid unspecific binding but maintaining specific hybridization. The applicability of the DPI for DNA screening and other practical issues is also discussed. The paper pretends to show that DPI can be a very useful tool

for understanding and helping in the designing of reliable biosensors, due to the conformational information provided.

2. Materials and Methods

2.1. Materials

Synthetic oligonucleotides for immobilization, hybridization and control are shown in Table 1, and were supplied by Sigma-Aldrich (Madrid, Spain) and TibMolbiol (Berlin, Germany). Silanizing agents: 3-aminopropyltriethoxysilane (APTES), 3-(triethoxysilyl)propyl isocyanate (ICPTS) and 3-glycidoxypropyltrimethoxysilane (GOPTS), as well as blocking proteins: bovine serum albumin (BSA), gelatine, lysozyme and casein, were supplied by Sigma-Aldrich. Ethanolamine, 2-mercaptoethanol and 4,7,10-trioxatridecane-1,13-diamine supplied by Sigma-Aldrich were also used as a blocking agents. All other common chemicals were analytical grade and Milli-Q purified water was employed in all stages.

Phosphate buffered saline (PBS, 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), sodium saline citrate (SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7) and carbonate buffer (CB, 100 mM sodium carbonate, pH 9.12) solutions were used as a carrier or medium in all assays. They were previously filtered through a 0.45 μm PVDF filter and degassed by sonication under vacuum prior to be employed.

Table 1

Sequence of the oligonucleotides used

Name	Sequence (5' to 3')	5' end	3' end	MW
Probe A	$(T)_{10}$TTTGATTACAGCCGGTGTACGACCCT	-NH_2	none	11162
Target A	$(T)_{10}$AGGGTCGTACACCGGCTGTAATCAAA	biotin	none	11618
Probe B	$(T)_{15}$CCCGATTGACCAGCTAGCATT	-SH	none	11115
Target B	AATGCTAGCTAATCAATCGGG	biotin	none	6859
Probe C	$(T)_{12}$AATGCTAGCTGGTCAATCGGG	-NH_2	none	10315
Target C	$(T)_{15}$CCCGATTGACCAGCTAGCATT	biotin	none	11334
Probe D	$(T)_{10}$CAAAGCATAGTATCTCGCAATG	-NH_2	none	9948
Target D	$(T)_{10}$CATTGCGAGATACTATGCTTTG	biotin	none	10351
Probe E	CGCCGATAACTCTGTCTCTGTA	-NH_2	none	6840
Target F	CGCCGATAACTCTGTCTCTGTA	biotin	none	7066

Oligonucleotide target A is a *Salmonella spp* DNA fragment, targets B and C correspond to different *Plum pox virus* serotypes, target D is a *Campylobacter jejuni* strand, while oligonucleotides E and F come from *Escherichia coli*.

2.2. Instrumentation

All experiments were performed at 20 ºC with an Analight Bio200 Dual Polarization Interferometer (Farfield Scientific Ltd, Crewe, UK). The apparatus consisted of a helium neon laser (λ= 632.8 nm), a sensor chip (24 × 5.8 mm) clamped inside a thermostated block (temperature control within 1 mK) and a 1024 x 1024 element-imaging device.

Silicon oxynitride chips (FB 100, Farfield, UK) were used for measurements. The sensor surface has two fluidic interfaces, 2 µL dead volume each, named channel 1 and channel 3, with an additional waveguide reference area, channel 2, having a dielectric cover with constant refractive index.

Carrier and other solutions were flowed using a double-channel precision syringe pump (Harvard Apparatus PHD 2000 Infusion, Kent, UK) and injections were carried out by means of a dual injection valve. The state of polarization of the input beam was switched (50 Hz) between TE and TM using a ferroelectric crystal before passing through the sandwiched waveguide structure, the interference pattern being detected on the opposing side by the element-imaging device. Analight DAQ and Analight Explorer (Farfield) software packages were used for system managing/data acquisition and data treatment, respectively.

2.3. Chip silanization and oligonucleotide immobilization

For silanization using APTES, the chemical vapour deposition or dry method was employed. It consisted of exposing the chip surface to silane vapour at room temperature in a dessicator [35,36], followed by baking at 160 ºC for 30 min. Silanized chip was placed in the DPI device, starting the data collecting, and the surface was activated by injecting glutaraldehyde (5% v/v in PBS, 250 µL at 50 µL/min). Then, on-line oligonucleotide immobilization was carried out by injecting the aminated probes (0.1 µM in PBS, 150 µL at 10 µL/min).

For ICPTS chip derivatization, silanization was accomplished as described for APTES, but changing the silanizing agent. Once the chip was placed in the DPI, glutaraldehyde was not necessary and oligonucleotides were directly immobilized by injecting the aminated probes exactly in the same conditions, using CB as a buffer.

On the other hand, chip silanization with GOPTS for photochemical oligonucleotide anchoring was carried out by immersion in a solution of this compound in toluene at 2% (v/v) at room temperature, followed by rinsing with dichloromethane, air-drying and finally baking at 160 ºC. Immobilization of thiolated probes was performed off-line, according to a procedure already described [34].

The chemical reactions involved in the three protocols can be found in Figure 1. X-ray photoelectron spectroscopy was applied to characterize the chip surface after silanization, in the three protocols.

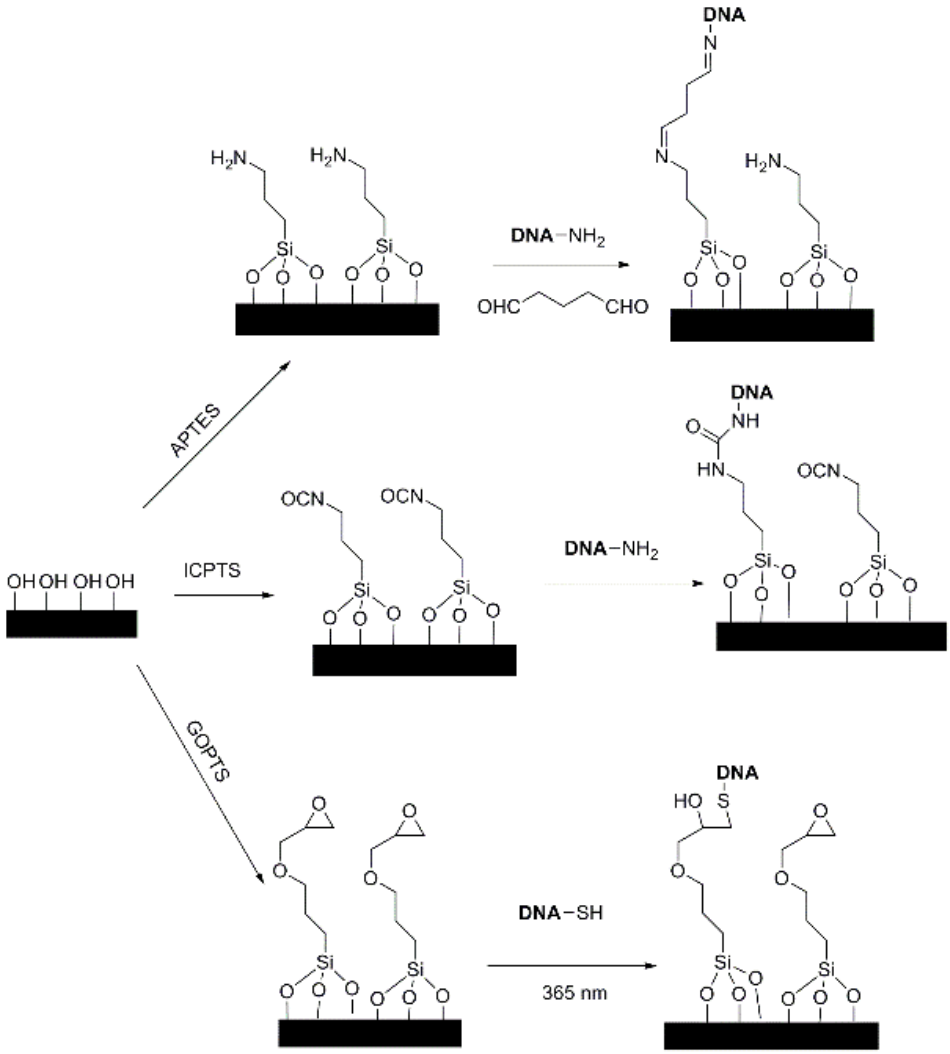

Figure 1. Scheme of the chemical processes taking place on the chip surface.

2.4. DPI experiments

After oligonucleotide immobilization, the remaining binding sites were neutralized by injecting a blocking agent (100 µL at 10 µL/min in all cases). The materials assayed with that purpose were ethanolamine and 4,7,10-trioxatridecane-1,13-diamine (0.1 M in PBS, pH 9.0) and the proteins: BSA, casein, lysozyme and gelatine (1000 µg/mL in carrier buffer).

The hybridization experiments were carried out by injecting a complementary biotin-ended oligonucleotide (0.5 µM in SSC, 250 µL at 10 µL/min).

Finally, a DPI calibration procedure was carried out, consisting of three stages [26,28]: *linearisation*, performed by injecting an 80% v/v ethanol/water mixture over the chip followed by stabilization back to the buffer baseline; *chip calibration*, by registering absolute signal of the ethanol/water mixture; and *bulk calibration*, performed by recording signals when flushing pure water.

2.5. Chip regeneration and reuse

In order to employ the same chip for more than one experiment, a cleaning-regeneration procedure was performed. It consisted of immersion in chromic mixture (100 g/L $K_2Cr_2O_7$ in 85% H_2SO_4) for 12 h, followed by rinsing with water, cleaning thoroughly the surface with isopropanol, and immersion in chromic mixture again for another 12 hours. Finally, the chip was rinsed with isopropanol and water, and air-dried. This protocol was useful for more than 90 % of used chips, and allowed them to be reused up to four times with no loss of performance. Figure 2 shows a brand new chip, one regenerated three times with the chromic mixture protocol, and a badly regenerated one (only rinsed with water and isopropanol).

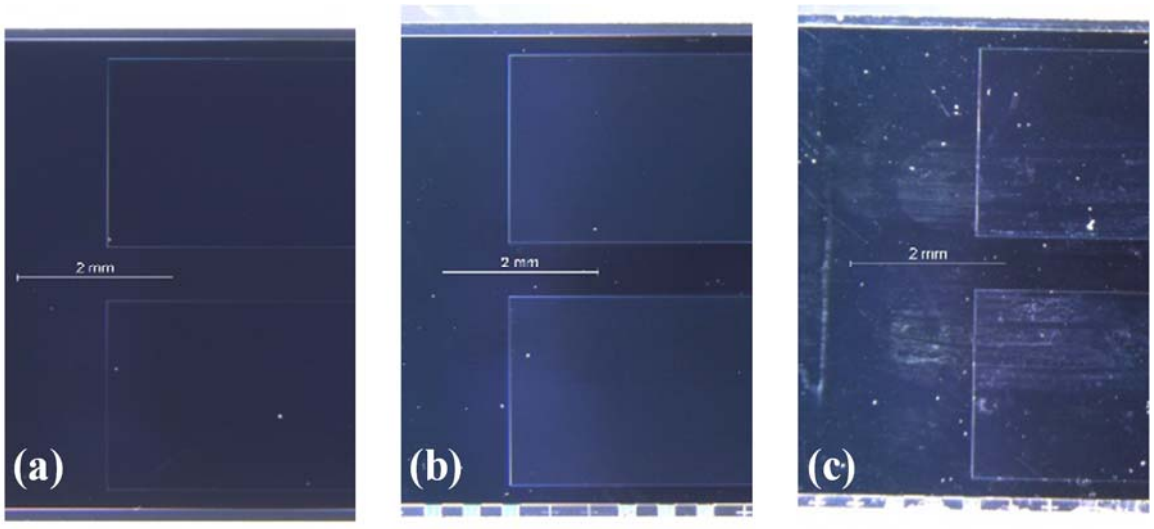

Figure 2. Magnified photograph of a brand new chip (a), a reused chip applying the optimized regeneration protocol (b) and a chip rinsed with water and isopropanol (c).

3. Results and discussion

DPI was used to investigate biorecognition on a silicon-based surface. The events studied were: covalent attachment of a DNA probe, application of a blocking agent for saturating unspecific binding sites and hybridization.

3.1. APTES and ICPTS chemical probe immobilization

Organosilanes are widely used to covalently attach promoters or cross-linkers on silicon-based materials, and the dry chemical vapour deposition is an easy, clean and effective methodology for relatively volatile silanes such as APTES and ICPTS. When applying the former, an amine-derivatized surface is formed, and the binding of the aminated oligonucleotide

is carried out using glutaraldehyde as a cross-linking agent prior to probe injection. For ICPTS, surface ends in accessible isocyanate groups that can react with the aminated oligonucleotides forming a urea linkage. In both cases, two silanization times were applied: 2 h as common procedure and 12 h for ensuring maximal silane coating. The results of chip surface characterization achieved by X-ray photoelectron spectroscopy (see Supplementary material, Figure 1A) show that silicon was correctly functionalized with both silanes.

Probes A and E (Table 1) were injected for recognition and control, respectively. The immobilization was monitored by recording thickness and mass (See curve plot in Supplementary material Figure 2A). Table 2 shows the increment in these parameters for the different silanization chemistries and times assayed.

Table 2

Oligonucleotide chemical immobilization assays

		APTES 2 h	APTES 12 h	ICPTS 2 h	ICPTS 12 h
Probe A (36-mer)	Thickness (nm)	0.53 ± 0.14	0.44 ± 0.09	0.64 ± 0.18	0.61 ± 0.15
	Mass (pg/mm^2)	350 ± 100	330 ± 80	260 ± 40	340 ± 80
Probe E (22-mer)	Thickness (nm)	0.28 ± 0.08	0.24 ± 0.07	0.63 ± 0.06	0.54 ± 0.10
	Mass (pg/mm^2)	160 ± 40	120 ± 40	170 ± 10	290 ± 90

Each value is the mean ± S.D. of three replicates

In all cases, the immobilization process seems to be effective, although there are some significant observations. First of all, regarding inter-assay reproducibility, standard deviation values are high, achieving variation coefficients greater than 25% in some instances, and variability being higher in thickness than in mass. Thus, results of Table 2 should be considered as semi-quantitative tendencies.

Regarding silanization time, APTES and ICPTS behave different. Thickness and mass after 2 h APTES silanization are similar to those for 12 h. For ICPTS, 12 h reaction leads to a higher mass than 2 h, although thickness results to be similar. Thus, 12 h vapour deposition time was selected for further experiments so as to ensure the best probe coating.

The effect of the immobilized DNA size is to be remarked. Clearly, the larger the DNA strand is, the higher mass increment, but quantification is not easy. Regarding mean values for the two silanization procedures, anchoring probe A gives rise to a mass increment around 300 pg/mm^2, while the shorter probe E increases the mass by approx. 150 pg/mm^2 (except ICPTS 12 h achieving a higher value). Mass increment values are slightly lower to those reported in the literature, although differences are low (for 18 and 20-mer probes, around 200 pg/mm^2 mass values were achieved [32,33]), and could be attributed to the chemistry employed.

Immobilization yield could be estimated from mass increment data. As an example, 330-340 pg/mm^2 probe A is equivalent to $1.8 \cdot 10^{10}$ molecules/mm^2 and assuming a maximum packaging density of $3 \cdot 10^{11}$ molecules/mm^2 [33], coverage value results to be around 6%.

Similarly, for probe E, 120 pg/mm^2 is equivalent to $1.05 \cdot 10^{10}$ molecules/mm^2, while 290 pg/mm^2 corresponds to $2.54 \cdot 10^{10}$, so the coating varies from 3.5 to 8.5%. The values are similar to that reported for other DPI chips [32,33], but lower than those achieved for other silicon-based supports [5]. If comparing with SPR, immobilization of thiolated probes on gold surface results in a probe density from $5 \cdot 10^{10}$ to $4 \cdot 10^{11}$ molecules/mm^2. These values are higher than those obtained in our experiments. Considering that a high probe density can lead to a decrease in hybridization efficiency and selectivity [37], our results would allow obtaining better hybridization yields.

Thickness data have also to be commented. For 12 h APTES silanization, 330 pg/mm^2 immobilization produces a thickness increase of 0.440 nm. As the measured thickness is a mean value, and considering the contour length (~12.2 nm for probe A) and the estimated 6% coverage, the maximal thickness increment should be 0.72 nm. Experimental values suggest that the oligonucleotides are not straight perpendicular to the surface, but partly horizontal and folded. This statement has been discussed previously with the same conclusion [5,33]. In a hypothetical situation with oligonucleotides placed only in vertical (12.2 nm height) or horizontal (2 nm height) position, it is estimated that 52% of strands are placed vertical.

Clearly, thickness increment is higher for 12 h ICPTS silanization (0.610 nm) than for APTES immobilization (0.440 nm for nearly the same mass), which indicates that the position of oligonucleotides on the surface is different. Consequently, it can be concluded that oligonucleotides are placed more "vertically" when immobilized via ICPTS, so the hybridization should be boosted due to the better availability. Indeed, registered density values for ICPTS immobilization (around 0.5 g/cm^3) were lower than for APTES anchoring (0.7 g/cm^3), indicating that the DNA strand orientation is more erect for ICPTS. The obtained data suggest an 80% of standing oligonucleotides, clearly higher than the result obtained with APTES.

The difference in orientation can be interpreted in terms of surface treatment. APTES silanization leads to an amine-ended surface, and further glutaraldehyde treatment changes amine to aldehyde. If this transformation is not complete, residual surface amine groups are protonated at the neutral pH of carrier buffer, and the resulting positive charge attracts the oligonucleotide phosphate backbone. ICPTS results in isocyanate groups on the surface, which are not protonated, so the surface has not positive charge, allowing the standing of the probes. Hydrolysis of isocyanate groups could transform them in terminal amine ones, but this process is slow on the chip surface, and it should not to be taken into account along a single experiment.

3.2. Blocking and hybridization for chemical probe immobilization

After immobilization of probes A and E (see above), the unspecific binding sites were saturated by injecting a blocking agent. Ethanolamine and 4,7,10-trioxatridecane-1,13-diamine (0.1 M in PBS at pH 9.0), as well as the inert proteins BSA, casein, lysozyme and gelatine

(1000 μg/mL in PBS) were tested. Following, target A was injected in both channels in order to monitor the specific hybridization and non-specific binding, simultaneously assessing the efficacy of the blocking treatment. Hybridization step was carried out in SSC medium, pH 7.0, which is the typical buffer for DNA hybridization assays in microarray and other formats [34]. Table 3 shows the increment in thickness and mass for both channels after assaying the most representative blocking agents: ethanolamine (ETA), BSA and gelatine.

Table 3

Hybridization assays. Comparison between specific and non-specific binding after blocking step

Blocking agent	APTES				ICPTS			
	Thickness (nm)		Mass (pg/mm^2)		Thickness (nm)		Mass (pg/mm^2)	
	Specific	Control	Specific	Control	Specific	Control	Specific	Control
None	0.440	0.450	210	190	0.730	1.130	280	280
ETA	0.290	0.460	150	140	0.390	0.370	150	120
BSA	0.120	0.130	77	64	0.240	-0.06	39	25
Gelatine	0.130	0.010	17	-33	0.730	0.060	340	50

ETA ethanolamine

Data from Table 3 show clearly that blocking step is necessary for both APTES and ICPTS silanization, because target A binding is nearly the same in control and specific channel.

The use of ethanolamine led to poor results since strong unspecific binding was also recorded. Similar results (see data in Supplementary material, Table 1A) were achieved with 4,7,10-trioxatridecane-1,13-diamine. These results suggest that unspecific binding depends on physical adsorption phenomena rather than the presence of non-occupied active covalent binding sites (carbonyl for APTES/glutaraldehyde, isocyanate for ICPTS).

The employment of inert proteins provided interesting results. BSA is a commonly used blocking protein, even in DPI experiments involving DNA binding [28]. However, our results with it were not satisfactory; mass and thickness values registered were lower than those obtained without blocking, and nearly the same for the specific and the unspecific binding. This result suggests that BSA covers the whole available surface, including the immobilized DNA binding sites.

Blocking with gelatine afforded better results, since there was a clear difference between hybridization and control for both thickness and mass. Unspecific binding was low, so gelatine was effective as blocking agent. It is worth mentioning that gelatine is a mixture of a wide range size fibrous proteins [38], so peptides having the appropriate size can fill any pore present in the surface. Casein and lysozyme, two relatively small sized proteins, were also assayed (see data in Supplementary material, Table 1A), however gelatine resulted to be the best option.

Considering the difference between specific and control values, net thickness and mass increments were 0.120 nm and 50 pg/mm^2, respectively, for APTES probe immobilization and

optimal blocking with gelatine. The mass values corresponded to $2.6 \cdot 10^9$ molecules/mm^2, equivalent to about 15% hybridization efficiency.

With ICPTS silanization and gelatine blocking, the results obtained were quite different. The absolute mass increment was 290 pg/mm^2, equivalent to $1.5 \cdot 10^{10}$ molecules/mm^2, with an absolute thickness increase of 0.670 nm. The estimated hybridization yield was around 80%, much higher than the reported for APTES silanized chips, and similar or higher than those achieved in previous works (the reported values are 35% for direct attachment and 85% employing a cross-linker [33]). The target amount detected by ICPTS-immobilized probe ($1.5 \cdot 10^{10}$ molecules/mm^2) is also analogous to that obtained in recent SPR approaches ($2 \cdot 10^{10}$ molecules/mm^2 activity [17]). Thus, the higher hybridization yield obtained was expectable, because the ICPTS immobilization led to more vertical DNA strands and therefore more available for hybridization, and the probe coating density on the surface (Table 2) was adequate, in the 10^{10} molecules/mm^2 order [37]. Indeed, the hybridization yield was nearly the same value as the percentage of oligonucleotide probes hypothetically placed in vertical when ICPTS was used (see previous section), although this correspondence is not so clear in the case of APTES.

3.3. Blocking and hybridization for photochemical probe immobilization

The assayed immobilization chemistries provided good results, but surface blocking was critical in both cases. It was interesting to assay a new immobilization method not leading to a reactive (isocyanate) or electrically charged (amine) surface. Silanization with GOPTS was promising because terminal epoxy or its hydrolysis product (alcohol) are neutral. X-ray photoelectron spectroscopy showed that derivatization with GOPTS was effective (see Supplementary material, Figure 1A).

Thiolated oligonucleotides were attached to the GOPTS-derivatized surface, this process being induced by UV radiation, with good efficacy in short time [34].

Thiolated probe B was immobilized off-line in both channels. The rest of steps -blocking with inert proteins and hybridization- were carried out on-line and monitored by DPI, using always SSC as a medium buffer. The specific hybridization was monitored using target B, while unspecific binding was controlled by injecting target F, both being the same size (see Table 1). Results of the target binding are shown in Table 4, for the different blocking agents assayed.

Regarding hybridization data, no blocking or the use of mercaptoethanol led to non-specific binding, but specific values were higher than control ones, so there was a net signal corresponding to hybridization. The net values were 0.190 and 0.220 nm thickness, and 120 and 140 pg/mm^2 mass, for no blocking and mercaptoethanol, respectively. Thus, hybridization was more effective when mercaptoethanol blocking is carried out, but the difference is almost negligible. According to surface chemistry (see Figure 1), no blocking leads to epoxy-finished surface, that can be slowly converted into alcohol by hydrolysis, while reaction with

mercaptoethanol transforms epoxy into terminal alcohol. Both surfaces show neither electrical charge nor reactivity with oligonucleotides and proteins, so the unspecific binding is only due to physisorption on a neutral surface, and a net specific binding of target oligonucleotide can be measured.

Table 4

Blocking and hybridization assays for photochemically immobilized oligo B

	Hybridization			
	Thickness (nm)		Mass (pg/mm^2)	
Blocking agent	Specific	Control	Specific	Control
None	0.320	0.130	240	120
Mercaptoethanol	0.350	0.130	390	250
BSA	0.099	-0.012	47	-3
Casein	0.190	0.020	210	32
Gelatine	0.240	0.072	130	-29

Mercaptoethanol is equivalent to ethanolamine assayed in chemical immobilization experiments, and blocking was carried out off-line and under UV-radiation [35].

Blocking with inert proteins reduced the non-specific binding, because control results are very low or even negative. The net difference signals were 0.170 nm and 180 pg/mm^2 for casein and similar values for gelatine as well as 0.110 nm and 50 pg/mm^2 for BSA. The net values obtained with casein and gelatine were the same order as those achieved with no blocking or mercaptoethanol treatment, suggesting that these proteins do not cover the active centres and allow the hybridization to take place. However, the lower net values obtained with BSA suggest that the higher size of blocking protein hinders the approach of oligonucleotides to the surface, so its use is disapproved. Therefore, it can be concluded that photochemical immobilization is advantageous over APTES and ICPTS anchoring, regarding surface blocking, because this step can be avoided maintaining hybridization net results, although the use of casein or gelatine to totally remove unspecific binding is recommended in order to lower background signals.

Mass increments for hybridization can be traduced to a maximum of $1.56 \cdot 10^{10}$ (casein blocking) and a minimum of $4.4 \cdot 10^{9}$ (BSA blocking) molecules/mm^2. This result is higher than the best achieved with APTES immobilization, but similar to those for ICPTS. The hybridization yield cannot be directly estimated, because no experimental data about probe density on the chip has been measured. On the basis on the value reported in the literature [34] for photochemical immobilization ($1.5 \cdot 10^{10}$ molecules/mm^2), hybridization efficiency would be 70-100%, i.e. similar or higher than the best achieved with ICPTS, except when using BSA for blocking (30%).

According to data shown in Table 4, and target B contour length (~8 nm), a theoretical thickness increment of 0.280 nm can be estimated. The experimental net value was 0.190 nm,

which indicates that hybridization takes place in a partially flattened oligonucleotide conformation. When using blocking agents, the observed trend was the same. This result agrees with the probe placed in part horizontally, as reported [34], and is similar to other immobilization chemistries employed.

Figure 3 compares the efficiency in hybridization achieved employing the three anchoring methods. It can be seen that ICPTS and photochemical DNA probe immobilization show to be better performing that classic APTES/glutaraldehyde, because mass and thickness increments achieved are higher. ICPTS anchoring provides the highest amounts of detected DNA target, improving sensitivity, but a surface blocking step with gelatine is needed. Photochemical immobilization also allows a good detectability of DNA target, and in this case, the surface blocking step can be avoided. The information provided by DPI has been useful to develop high performance DNA microarray sensing systems. In those sensors, the low limits of detection achieved (0.2 nM [34] and 0.02 nM [5]) were competitive with those obtained using SPR methods [37]. In addition, the sensor selectivity was also successfully addressed, and a maximum discrimination ratio of 14.5 was achieved for a single mismatch target [34].

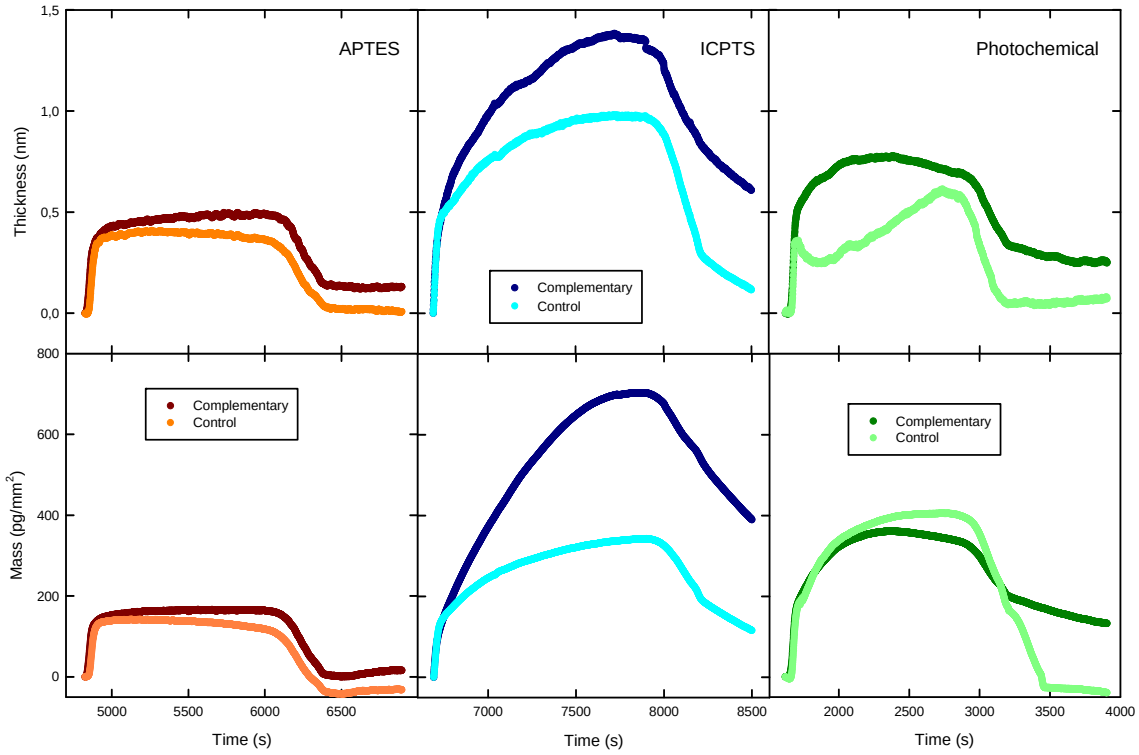

Figure 3. Evolution of thickness (top) and mass (bottom) with time during the hybridization step. Comparison between the different probe immobilization modes. Gelatine was employed as blocking agent in all cases.

3.4. Application to species identification in multi-target mode

Hybridization of complementary DNA strands is widely used with analytical purposes. DPI has shown to be able of monitoring this binding event and providing valuable structural information, this being the main strength of this technique. As a weak point, DPI is able to process simultaneously only two events (two measuring channels), so its applicability as screening methodology is limited. Although the technique is not intended to be used as a sensor [12], the possibility of qualitative identification of specific DNA fragments was planted on the basis on a multiplex format.

For assessing the recognition and discrimination ability in a multi-target mode, probes A, C and D were anchored on both chip channels, in separate and in mixtures. ICPTS immobilization was used due to the higher DPI response, simplicity and ability to monitor on-line the whole process. After blocking with gelatine, combinations of target oligonucleotides, including the non-complementary (F), were passed through, recording the mass and thickness increment in different experiments. SSC buffer was always used as hybridization medium for being adequate in all probe-target combinations. Table 5 shows the mass and thickness display for single and multiple recognition events.

Table 5
Hybridization assays in multiplex modes

Experiment No.	Immobilized Probes[a]	Injected targets[b,c]				
		A+C	A+F	C+F	A+C+D	F
1	Ch 1: A	0.530 160	-	-	-	0.067 11
	Ch 3: C	0.480 88	-	-	-	0.046 12
2	Ch 1: A + C	-	0.340 63	-	-	0.061 14
	Ch 3: A + C	0.500 110	-	-	-	0.054 17
3	Ch 1: A + C	-	-	0.250 69	-	0.043 12
	Ch 3: A + C	0.440 120	-	-	-	0.036 -3
4	Ch 1: A + C + D	-	-	-	0.760 210	0.074 -6
	Ch 3: A[d]	-	-	-	0.160 86	0.090 3

[a]Immobilization is carried out at 0.1 μM for an only probe, 0.075 μM each when two probes are immobilized together, and 0.05 μM for the three probes simultaneously.
[b]Oligonucleotide targets at 0.5 μM are mixed at the same solution and injected.
[c]Numeric values in each cell correspond to thickness increment (top) expressed in nm and mass increment (bottom) in pg/mm^2.
[d]Probe A immobilized at 0.05 μM.

In the first experiment, the ability of each single probe for recognizing its target was checked. High mass and thickness increments for both specific bindings, and negligible values for the non-specific one, were observed.

Further, the recognition of one or two targets simultaneously was carried out using two probes immobilized in each channel and mixtures of strands containing both targets or only one (experiments 2 and 3). The registered mass and thickness increment for the double recognition was always higher (thickness ~0.500, mass ~115) than for a single hybridization (thickness ~0.300, mass ~65), confirming the system potential for a duplex target identification.

Finally, applicability of DPI in a triple analyte sensor was tested by immobilizing three probes, each against a different target, and comparing with the hybridization for only one system (experiment 4). Results showed that the three mixed probes can bind all targets together or in separate. This concept could be used, for instance, in a microorganism survey for food safety; the three-target identification (A+B+C) could be applied for screening a bacteria family e.g. *Salmonella*, while assaying a single organism (A) could differentiate a key microorganism e.g. the dangerous *Salmonella typhimurium*. This application could be also performed by means of a typical DNA array [4], and although DPI philosophy is different from that of the screening analytical methods, this potential application has been also demonstrated.

4. Conclusions

DPI has shown to be a useful tool for monitoring biomolecule binding events and associated conformational dynamics happening on a silicon oxynitride chip, with high accuracy. DPI chips allow employing different immobilization chemistries for covalent anchoring oligonucleotides, the classical APTES/glutaraldehyde method for amine-derivatized biomolecules, and the newer and simpler ICPTS and photochemical protocols. In order to minimize the effect of unspecific binding of oligonucleotide targets, the application of gelatine has proven to be superior to the use of other blocking agents such as ethanolamine, or BSA. With the adequate binding sites blocked, specific hybridization of up to three complementary oligonucleotides can be monitored, allowing the screening of DNA targets in a multiplex format. Chip coverage and binding yields can be determined at real time, and conformational dynamics can be elucidated from mass and thickness data.

The novel oligonucleotide immobilization methods, ICPTS-based and the photochemical procedure, have proven to provide better results of hybridization yields than APTES/glutaraldehyde method, which is an important conclusion for surface genomic analysis. The photochemical method avoids the need of blocking, while using ICPTS it is possible to monitor the probe immobilization and higher DPI responses are achieved. Furthermore, the same chip can be reutilized up to four times before losing its properties. The results obtained indicate the suitability of the methodology applied for the study of surface chemistries in order to be transferred to nanobiosensors development.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version

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