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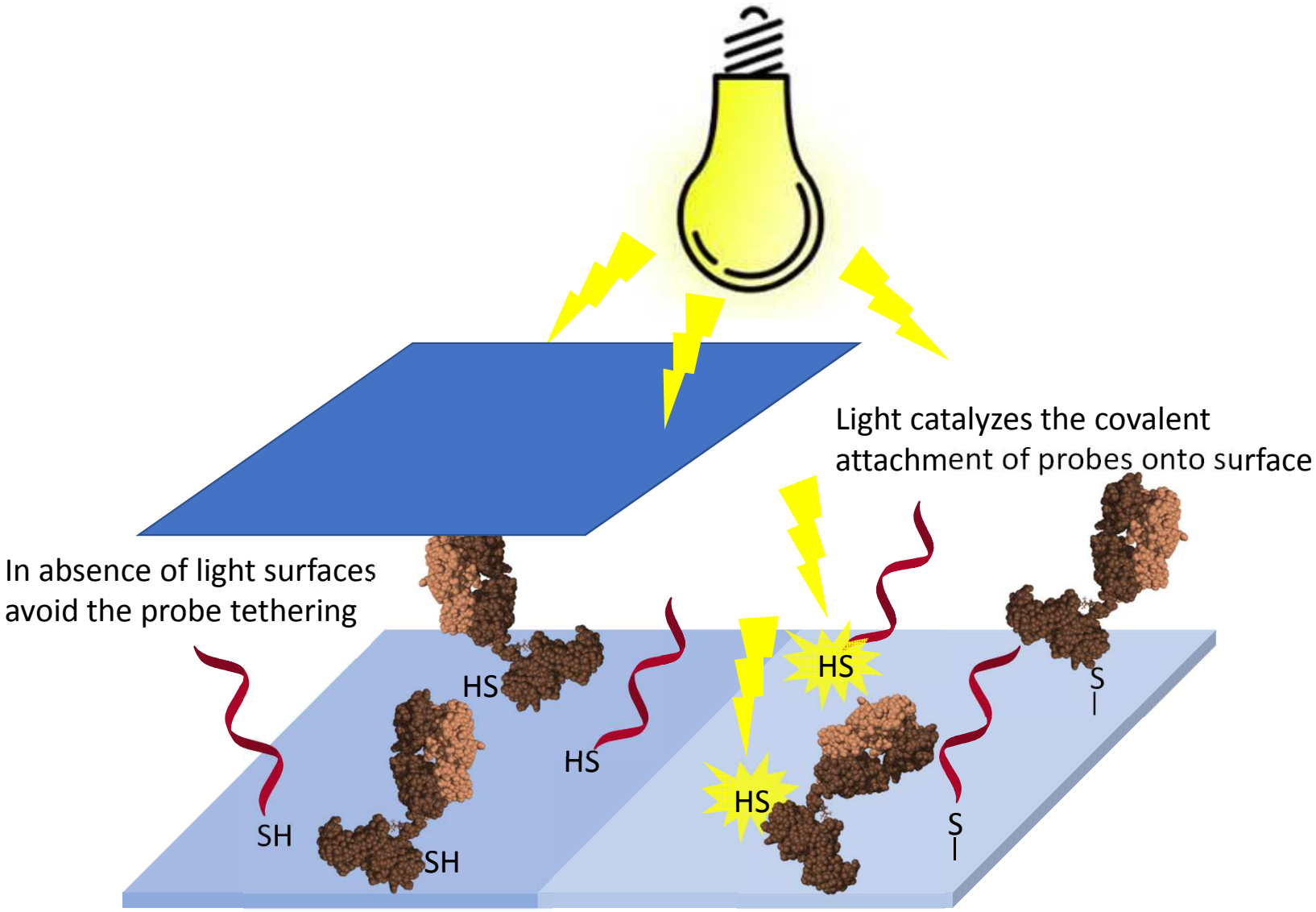
Light catalyzes the covalent attachment of probes onto surface
In absence of light surfaces avoid the probe tethering
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Thiol-*click* photochemistry for surface functionalization applied to optical biosensing

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H I G H L I G H T S

- A pool of photo-click chemistry reactions based on thiol moieties are overviewed for probes attachment in the construction of optical biosensors.
- The role of surface wettability tuning on results quality is discussed.
- High performance fluorescence microarray and label-free nanophotonic biosensing is achieved.

A B S T R A C T

In the field of biosensing, suitable procedures for efficient probes immobilization are of outmost importance. Here we present different light-based strategies to promote the covalent attachment of thiolated capture probes (oligonucleotides and proteins) on different materials and working formats. One strategy employs epoxylated surfaces and uses the light to accomplish the ring opening by a thiol moiety present in a probe. However, most of this work lies on the use of thiol-ene photocoupling chemistry to covalently attach probes to the supports. And thus, both alkenyl and thiol derivatized surfaces are assayed to immobilize thiol or alkene ended probes, respectively, and their performances are compared. Also, the effect of the number of thiols carried by the probe is

analyzed comparing single-point and multi-point attachment. The performance of the analogous tethering, but onto alkynylated surfaces is also carried out, and the sensing response is related to the surfaces hydrophobicity. A newly developed reaction is also discussed where a fluorinated surface undergoes the covalent immobilization of thiolated probes activated by light, creating small hydrophilic areas where the probes are attached, and leaving the rest of the surface highly hydrophobic and repellent against protein unspecific adsorption. These mixed surfaces confine the sample (aqueous) uniquely on the hydrophilic spots lowering the background signal and thus increasing the sensitivity. These probe immobilization approaches are applied to fluorescence microarray and label-free nanophotonic biosensing. All the exposed reactions have in common the photoactivation of the thiol moieties, and give rise to quick, clean, versatile, orthogonal and biocompatible reactions. Water is the only solvent used, and light the only catalyzer applied. Thus, all of them can be considered as having the attributes of *click*-chemistry reactions. For these reasons we named them as thiol-*click* photochemistry, being a very interesting pool of possibilities when building a biosensor.

Keywords: Thiol-ene photocoupling, *click* chemistry, surface functionalization, microarray, biosensing

1. Introduction

Nowadays, biosensors are powerful tools to detect, or monitor, targets related to health, environmental and food, among others [1,2]. In a biosensor, typically, a biological recognition probe is immobilized onto a support that acts as a transduction or inert element. Regarding the transduction mode, optical biosensors are very interesting as they can use many physico-chemical properties, different materials and architectures, and provide multiplexed, simple, fast and direct (no label) detection possibilities. This gives them high versatility [3]. And thus, a pleiad of biosensing designs are reported in the literature, many based on the use of integrated optics [4].

Besides biosensors, microarray is an interesting working format and analytical diagnostic tool. Also known as biochips, they allow multiplexed surface assays comprising tens to thousands of microspots of immobilized capture agents (probes) with binding activity against libraries of target molecules [5,6].

A meeting point in the development of competitive biosensors is the need of searching capture probe immobilization approaches being effective, biocompatible and robust enough [7,8]. Also,

modulating the wettability of the substrate surfaces is key for reducing the unspecific bindings and thus the background signals, dramatically improving sensitivity. In addition, assay reproducibility is closely related to control of the interface between the solid, the attached probe, and the liquid [9]. And thus, chemical tuning of the interface's properties is key for the competitive performance of the final device [6]. Even though many routes are reported on different materials, there is still an interest in developing methods being cleaner, green, efficient in aqueous media and reproducible [10,11].

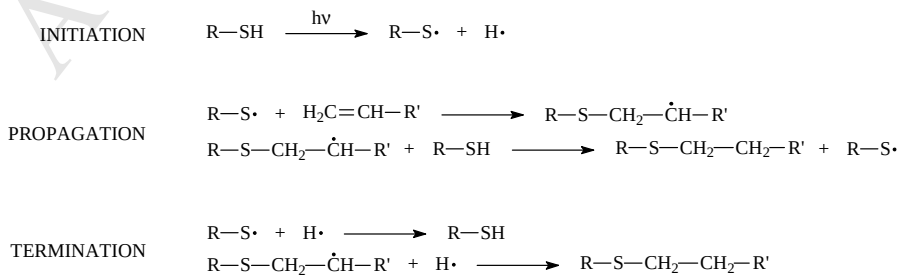
Most of the chemistries employed in the new reported biosensors rely on the "classical" probe coupling methods, and there are few examples using advanced approaches. An interesting via for the probe immobilization is to use the pool of reactions known as the *click*-chemistry ones [12]. These reactions meet the features of proceeding under mild conditions in the presence of oxygen, being regioselective, tolerating many functional groups, performing in neat or benign solvents such as water, and providing quantitative or near-quantitative yields. If this is combined with the use of light as a catalyzer for the coupling reaction, interesting possibilities of probe immobilization arise where site-selective attaching is needed.

The basic concept of using light-induced chemical reactions for attaching bioreagents on a support comes from the 90's, the main idea being polymer photo-crosslinking and entrapment of the active biomolecules [13]. In the 2000 decade, new methodologies were investigated, for instance the use of photoactive cross-linkers such as a photoreactive benzophenone derivative [14-16] or an azido-functionalized succinimidyl ester for proteins [17]. Carbohydrates were also anchored on different surfaces by means of azide photochemistry [18-20]. Nucleic acids were immobilized using anthraquinone as bridge [21] and a psoralen derivative [22]. All these anchoring methods lead to good results regarding immobilization yield and the possibility of micropatterning, but require relatively complex chemical processes because in general a cross-linker bridge between the support and the bioreceptor is needed.

An interesting photo-induced linking reaction is the so-called thiol-ol one [23], that can be employed for derivatizing the common hydroxy-ended surfaces such as poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate) and cellulose. Several *click* metal-free reactions, employed for modifying surfaces, have been reviewed by Escorihuela and coworkers [24].

Other approaches, analogous to that shown in the present paper, make use of more direct photochemical reactions, avoiding the use of auxiliary reagents, in order to achieve a really *click* anchoring process. In this sense, work carried out by the group of Petersen and Neves-Petersen is devoted to the immobilization of antibodies and other receptor proteins containing disulfide bridges with aromatic amino acid residues (e.g. tryptophan) physically near them. UV (280 nm) photons excite the aromatic rings and energy is transferred to the disulfide bridges, so that they break into two free thiol groups able to bind to thiol-reactive surfaces. This approach, employing laser pulses (200 fs, average power 1.1 mW), was applied to prepare arrays and other patterns of a set of different proteins [25,26], and the established basis were further used for nanobiomedical applications [27-29]. In general, the methodology has shown to be effective and allows to create nanomicroarrays without the need of dispensing devices, but a laser source, rather than photomasks, is recommended for achieving those sub-micron resolutions.

Within the possibilities of photochemically anchoring biomolecules on supports for developing biosensor approaches, the coupling of a -SH thiol functional group to a C=C double bond, best known as the thiol-ene photocoupling chemistry (TEC), is a really promising one. The reaction, shown in Scheme 1, consists of the radical addition of the sulfur atom to the unsaturation, generating a C-S bond, and it is activated by UV photons. So, the reaction can be considered as a *click* one, because it takes place in few minutes or even seconds with high yields, it does not require additional reagents, solvents or harsh conditions, and it is clean because no sub-products are generated. Furthermore, it is applicable to molecules such as proteins having thiol groups (or disulfide bridges that can be activated) and oligonucleotides derivatized with this functional group, being commercially available, in order to be attached on vinyl-ended surfaces. On the other hand, it is also possible to couple a vinyl-derivative moiety to the capture probe for covalent immobilization on thiolated supports, this option being more laborious but sometimes preferred [30].



Scheme 1. Thiol-ene photochemical *click* reaction steps.

The thiol-ene photochemical reaction has been known for very long [31], but its exploitation as a *click* process has increased interest in the last decade. As a representative application of this approach for reagent immobilization, the work developed by Jonkheim et al [32] makes use of alkenylated biotin anchored to thiolated silicon dioxide support, and the employment of the biotin-streptavidin binding for creating patterns of biomolecules such as enzymes and other binding proteins. Further, a deeper study of the reaction performance, on glass support and using the biotin-streptavidin recognition, was carried out in 2010 by the same research team [33]. The variables assayed were the surface derivatization, the inclusion of spacers on the surface and the modes of obtaining patterns, among others. Interestingly, the insertion of a dendrimer spacer between the native surface and the final reactive group improved the performance of the arrays obtained. The same group studied also the anchoring of a protein on a thiol-ended surface by attaching an alkene-derivatized protein [34].

Thiol-ene reaction has also been applied to create whole cell microarrays, on the basis on attaching a vinyl derivative of biotin to the cell surface, previously thiolated, and the biotinylated cells bound to a streptavidin arrayed on a support [35]. The whole procedure is complex, but it is an effective way of selectively immobilizing active non-adherent cells.

Also, in the past few years, microfluidics has also been combined with the photochemical thiol chemistry for reagent immobilization with micrometer sized patterns. Thus, the research team headed by Lafleur and Kutter built thiol-derivatized microfluidic chips [36] and waveguides [37], and tested them by anchoring biotin-alkyne conjugate (thiol-yne attachment), with further Alexa Fluor-streptavidin recognition and detection by microscope-based evanescent-wave induced fluorescence. Two years later, members of the same research team [38] developed enzyme microreactors using thiolene-click anchoring strategies.

The immobilization of antibodies by means of the thiol-ene coupling chemistry, without interfering in its biorecognition capability, was achieved for the first time in our group by creating free thiols in the immunoglobulin structure [39]. This was accomplished by means of a selective reduction of the antibodies hinge region using tris(2-carboxyethyl)phosphine. Those half immunoglobulins were then covalently attached to alkenylated glass by irradiation at both 254 nm and 365 nm. The comparison against the whole antibody showed that half immunoglobulins provided a fluorescence signal 7-fold that

obtained for the whole antibody when the microarray was incubated with the labeled target. The procedure was first optimized for a polyclonal antiBSA antibody from rabbit, but then it was successfully employed for mouse monoclonal antibodies against different biomarkers such as troponin, CRP or myoglobin, providing very good results as well.Application of photochemical attaching to label-free biosensing is scarce, although this kind of immobilization can be used with the supports compatible with established label-free detectors such as dual-polarization interferometry (silicon oxynitride), surface plasmon resonance (SPR, gold) and quartz crystal microbalance (QCM, gold and other materials). Only in recent years, probe photoimmobilization for label-free monitoring of targets has been described. For instance, López-Paz et al. [40] successfully applied the thiol-epoxy reaction to anchor thiolated oligonucleotides to a glycidoxypropyl-silanized chip for the dual-polarization interferometry monitoring of DNA hybridization. More recently, protein immobilization through disulfide activation by near aromatic ring irradiation (254 nm) was applied to anchor whole antibodies on gold, for QCM immunosensing of a small molecule such as parathion [41] and as well for determining the α-amilase protein [42]. In a different work [43], antibodies were immobilized on aminated glass via cross-linking with a diazirine reagent and further light (365 nm) activation, to be used with a novel label-free detection based on optical microbubble microresonators.

The choice of the optimal support and probe anchoring method has to regard several important factors leading to maximize all the performance of the final biosensing device. Of course, probe activity must be unaltered, and compatibility with transduction is also to be kept, but there are other factors [44]. Moving the analyte towards the points where probe is located and avoiding the unspecific binding, can be favored with an adequate choice of support wettability [45,46]. This property can also affect the conformations of the biomolecules responsible of the recognition, proteins and nucleic acids [9], thus affecting binding performance.

Wettability depends on the substrate material and the functionalization applied, so it is possible to modulate surface hydrophobicity so as to meet the best requirements, which can be different for each individual system. High hydrophilicity or hydrophobicity for many materials, especially microstructured/nanostructured ones [47], is easy to obtain with the adequate chemical treatment. In this sense, achieving a very wide range of wettability values is affordable by changing the surface functional group, as it was carried out employing photochemical *click* reactions such as thiol-yne by Feng et al. [48], as well as thiol-ene by Zhang et al. [49], over a polymeric substrate in both cases. However, the

further bioreagent anchoring is not possible with most of these functionalities. Hence, the immobilization chemistry should be highly versatile in order to work properly with different polarity surface functional groups.

In this paper we select a pool of reactions for the efficient immobilization of probes onto silicon-based solid supports, which are materials widely use in optics and microarray. The reactions are based on the photochemical activation of thiol groups present in the probe, which in turn react rapidly with different functionalities on the chemically derivatized surface, one of them being the thiol-ene one previously described in Scheme 1. The different surface chemistries modulate the hydrophobicity of the support and influence the background signal and the sensitivity of the assay. Application of some of these methodologies for label-free optical biosensing is also shown.

2. Materials and methods

2.1. Chemicals, reagents and buffers

The glass microscope slides used as substrates for the microarrays were obtained from Labbox Labware, S.L. (Spain). The Silicon-On-Insulator (SOI) substrates (2×2 cm) were purchased to Shin-Etsu Group (Japan). Immobilon-P PVDF membranes were acquired from Merck (Spain). 2-[Methoxy(polyethyleneoxy)$_{6-9}$propyl]trimethoxysilane was purchased from Gelest (Germany). 1H,1H,2H,2H-perfluorodecyltriethoxysilane, vinyltrimethoxysilane, vinyltriethoxysilane, tris(2-carboxyethyl)phosphine (TCEP), bovine serum albumin (BSA), human C-reactive protein (CRP) and anti-bovine serum albumin polyclonal antibody (IgG αBSA) were purchased from Sigma Aldrich (Spain). Human C-reactive protein monoclonal antibody (IgG αCRP), Alexa Fluor 647 NHS ester, and NuPAGE Bis-Tris Welcome Pack, 4-12%, for SDS electrophoresis were purchased from ThermoFisher Scientific (Spain). Toluene was from Scharlau (Spain). 3,3′,5,5′-Tetramethylbenzidine liquid substrate was acquired from SDT (Germany). Note: all the chemicals were handled following the corresponding material safety data sheets and were used without further purification.

Milli-Q water, with a resistivity above 18 mΩ, was used to prepare the aqueous solutions. The employed buffers, phosphate buffer saline PBS1× (0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 0.003 M potassium chloride, pH 7.5), PBS-T (PBS1× containing 0.05% Tween 20) and saline sodium citrate SSC1× (0.15 M sodium chloride, 0.02 M sodium

citrate, pH 7) were filtered through a 0.45-mm pore size nitrocellulose membrane from Fisher (Germany) before being used.

The oligonucleotides in Table 1 were acquired from Eurofins Genomic (Germany) or Sigma-Aldrich (Spain)**.**

Table 1
Sequences and modifications for the oligonucleotides employed throughout the study

Name	Sequence (5' to 3')	5' end	3' end
Probe1*	CCCGATTGACCAGCTAGCATT	1 SH	Cy5
Probe 1	CCCGATTGACCAGCTAGCATT	1 SH	
Target 1*	AATGCTAGCTGGTCAATCGGG	Cy5	
Target 1	AATGCTAGCTGGTCAATCGGG		

2.2. Instrumental methods

Surface activation was carried out with an UV−ozone cleaning system UVOH150 LAB from FHR (Germany). Microarrays were printed with a low-volume non-contact dispensing system from Biodot (USA), model AD1500. Probe photoattachment was done with the same UV-ozone cleaning system described above. Water contact angle (WCA) measurements were taken with a system from Biolin Scientific (Sweden) model Attension Theta Lite and images were processed with OneAttension v3.1. Measurements were taken in triplicate at room temperature with a volume drop of 5 μL employing 18 mΩ water quality. The fluorescence signal of the spots in the microarrays was recorded with a homemade surface fluorescence reader (SFR) [50], with a high-sensitivity charge-coupled device camera Retiga EXi from Qimaging, Inc. (Burnaby, Canada), equipped with light-emitting diodes Toshiba TLOH157P as light source or with a GenePix 4000B Microarray Scanner from Axon instruments (USA). Microarray image treatment and quantification were done using the GenePix Pro 4.0 software from Molecular Devices, Inc. (USA). Antibody concentrations were determined by a NanoDrop 2000 spectrophotometer from Thermo Fisher (Spain).

2.3. Surface silanization

Glass or SOI substrates were activated with the ozone surface cleaner for 5 min and then immersed in a toluene solution containing 2% (w/v) of the corresponding organosilane. After 2 h stirring at room temperature, the substrates were cleaned with toluene, isopropanol and cured at 80 ºC for 30 min. Successful functionalization was assessed by measuring WCA on surfaces before and after silanization. As well, X-ray photoelectronic spectroscopy (XPS), atomic force microscopy (AFM) and infrared reflection-absorption spectroscopy (IRRAS) were applied (see Supplementary data) in order to monitor changes in surface chemistry.

2.4. Microarray probe immobilization and hybridization assays

Thiolated nucleic acid probes were immobilized by dispensing the aqueous solution (from 0.1 to 10 µM) creating arrays of 4 spots/row (40 nL/spot) and irradiating for several minutes with the lamp placed at 0.5 cm from the surface in the case of 365 and 254 nm lamps having a power of 6 mW cm^{-2}. In the case of irradiation with the lamp at 254 nm having a power of 50 mW cm^{-2}, the distance between the surface and the lamp was 1.0 cm, and the irradiation time was 5 s. Arrays were further washed with PBS-T and water, and air dried.

Hybridization assays were carried out in SSC 1×, incubating the labeled complementary target for 1 h at 37 ºC in a humid chamber. After that, the chips were washed with SSC 0.1× and air dried.

2.5. Microarray of hIgG immobilization and biorecognition assays

First, the whole antibody was subjected to selective reduction by treating the purified antibody in acetate buffer (0.15 M sodium acetate, 0.01 M EDTA, 0.1 M sodium chloride, pH 4.5) at 4 mg mL^{-1} concentration, in the presence of 25 mM TCEP for 90 min at 37 ºC. Purification of hIgG was achieved by centrifuge cycles with cut-off filters of 50 kDa. The concentrations of the solutions were determined by means of a NanoDrop 2000 spectrophotometer (Thermo Scientific). Ellman's assay and SDS-PAGE electrophoresis were used to characterize hIgG.

hIgG microarrays were printed over the previously alkene-functionalized chips with the low-volume non-contact dispensing system. The buffer employed was acetate buffer (0.15 M sodium acetate,

0.01 M EDTA, 0.1 M sodium chloride, pH 4.5), and 25 nL per spot were employed for the microarrays read with the microarray scanner, while 50 nL per spot were dispensed for the microarrays read with the SFR. The microarray had 4 spots per row and in both cases only one drop was printed on a single spot.

Five min after printing, chips were irradiated for 5 s with UV light (λ = 254 nm) with the UV-ozone cleaner. Afterwards, chips were stored in the dark for 10 min and then washed with PBS-T, rinsed with water and dried. They were subsequently incubated in the dark with the labeled target dissolved in 10% human serum for 30 min at ambient temperature. After washing with PBS-T and water, the fluorescence of the dried chips was measured by either the SFR or the microarray scanner. With the sandwich immunoassays, when the analyte could not be labeled, an additional 30 min incubation step was run with the corresponding labeled detection antibody solution.

2.6. Optical sensing structures biofunctionalization

In order to introduce alkene groups on the optical sensing structures, the sensing chip was rinsed with ethanol and water and immediately activated with ultraviolet (UV)-vis irradiation at 254 nm (50 mW cm^{-2}) for 10 min. The chip was then immersed for 2 h in vinyltriethoxysilane 2% (v/v) in toluene. Then the chip was washed with acetone and air dried. Finally, it was cured for 30 min at 90 ºC. WCA was measured to ensure the right chemical surface derivatization.

For the experiments with nucleic acid probes, thiolated nucleic acid probe solutions (30 nL, 10 μM in ultrapure water) were drop casted on the sensors and let to dry at room temperature. The chip was then irradiated at 254 nm (50 mW cm^{-2}) for 30 s. Once the probes were immobilized, the sensing chip was thoroughly washed with PBS-T, distilled water and air dried.

The optical measurement set-up used is described elsewhere [51]. For the hybridization, the complementary labeled target in SSC 5× was flowed over the sensor by using a PDMS microfluidic flow cell for 10 min, at 10 μL min^{-1}, then SSC 5× was flowed again to remove the non-hybridized target.

To test the photoimmobilization of hIgGs, the sensing chip was functionalized as before, and the same optical set-up was used. In this case, the immobilization of the hIgG was carried out in flow. To this aim, freshly prepared hIgG in PBS 1× was flowed over the sensor for 10 min without irradiating with UV light, and then it was flowed for 20 min more with the lamp (254 nm, 6 mW cm^{-2}) switched on.

3. Results and discussion

3.1. Surface derivatization and oligonucleotide photocoupling click *reactions*

Here, we discuss a pool of reactions developed by our group which have in common the use of thiolated probes that are activated by UV light (see Scheme 1, initiation step) to promote their covalent attachment to different functionalities chemically provided on the surface of a solid support [30,52-54]. The procedures have been developed for silicon-based materials, which include glass and SOI supports, due to their extended use in optical biosensing.

The employed methodology makes use of fluorescence microarray to set up the optimal conditions and to compare the performance reached in each case. Then, the protocols are easily transferred to the construction of label-free nanobiosensors, as it will be shown below.

Thus, employing the organosilane chemistry, glass and SOI surfaces were modified with epoxy, alkene, and perfluorinated chains. For silanization, chips were treated for 2h with the corresponding silane at 2% in toluene. Further treatment of the epoxylated surface with propargylamine provided an alkynylated surface. The success of the functionalization and its reproducibility was assessed measuring WCA. The contact angles also showed different hydrophobicity on the surfaces which in turn will affect the performance of the biosensor as it will be discussed later. The support modification was also confirmed by applying XPS, AFM and IRRAS to the surfaces (Supplementary data, Figures S1 to S5), which demonstrated clearly the changes in surface chemistry according to the described derivatization. The four routes are shown in Fig. 1, as well as the resulting WCA values for each.

In order to test the immobilization capability of these functionalized surfaces, different concentrations of a labeled thiol-ended oligonucleotide probe were spotted on the surface and exposed to UV light to photoinduce the attachment. The amount of tethered probe was obtained registering the fluorescence before and after washings. The irradiation conditions, as well as the immobilization densities obtained, are shown in Table 2. For all the cases, the maximal immobilization was obtained for 1 to 5 µM, depending on the surface. These and all the following quantitative data collected in further tables were obtained using fluorescence microarray format, employing labeled probes and targets specified in Table 1.

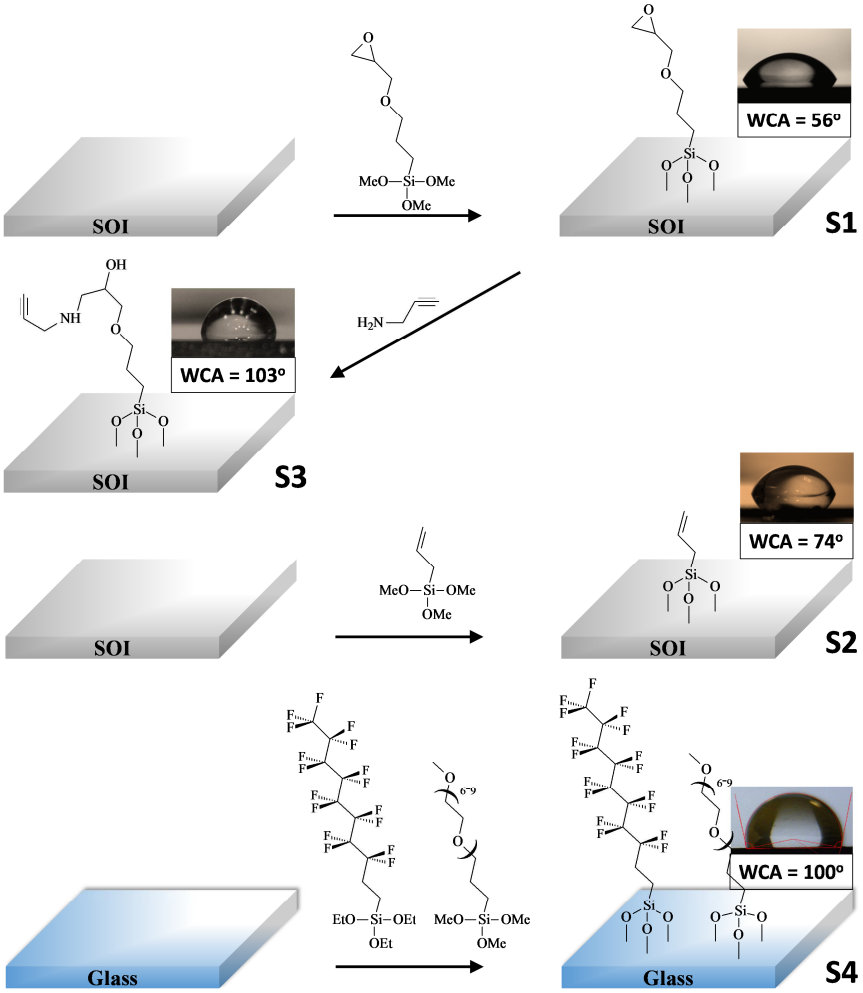


Fig. 1. Reaction scheme for the four support derivatizations, showing WCA values of the resulting functionalized surfaces.

Table 2
Surface irradiation conditions for optimal thiol anchoring

Surface	WCA (º)	Irradiation conditions	Probe 1* Immobilization density (pmol cm^{-2})
S1	46	10 min, 365 nm	2.5 ± 0.2
S2-20	74	20 min, 365 nm	3.0 ± 0.3
S2-60	76	60 min, 365 nm	12.7 ± 1.5
S3-20	103	20 min, 365 nm	28-30 ± 3
S3-60	103	60 min, 365 nm	30.4 ± 2.1
S4	110	30 s, 254 nm	41.0 ± 2.4

From results obtained in these experiments, two conclusions were raised. The first one was that a better immobilization density is achieved for surfaces with a higher hydrophobicity. This could be due to the confinement of the spotted drop in a smaller area which increases the number of collisions per cm^2. Secondly, all four routes allowed effective surface patterning by irradiating through a photomask observing no significant unspecific adsorption for any surface, as it is exemplified in Fig. 2.

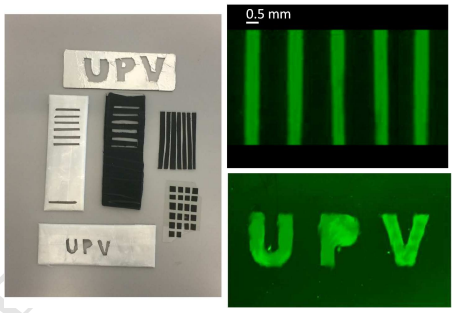


Fig. 2. Image of some of the handmade masks used to demonstrate the photomasking capability of the developed approaches (left) and fluorescence images obtained when irradiating through the photomask an alkynylated (top right) and epoxylated (bottom right) functionalized surfaces with a solution of labeled thiolated oligonucleotide spread out over the surface.

It is worth mentioning that all the photoattachments were performed using aqueous solutions and light as the only catalyzer. The use of thiol-ene type reactions, onto functionalized surfaces, taking place in absence of photoinitiator, has been reported previously by several authors for different purposes [32-34,48,55-57].

3.2. Hybridization assays

It is known that a higher probe coating does not necessarily provide a better performance in terms of hybridization, because crowding effect or charge repulsion can hinder a successful biorecognition. For that, using the supports shown in Figure 1 and irradiation conditions registered in Table 2, microarrays were prepared immobilizing the non-labeled thiolated probes and hybridized with the corresponding solution of labeled target. Target densities, obtained as before, and hybridization yields are summarized in Table 3. In all the cases, the target was incubated for 1 h at 37 ºC and the spotted probe concentrations corresponded to those providing the maximal immobilization density indicated in Table 2. Also, the calculations were done for the intensities provided in the *plateau* of the hybridization curve.

Table 3
Target densities and hybridization yields achieved in microarray format, for different supports and irradiation conditions n.d. not determined.

Surface	**Target density (pmol cm^{-2})**	**Hybridization yield**	**RSD interchip**	**RSD intrachip**
S1	1.4	56%	9-13%	8-13%
S2-20	1.8	60%	8-12%	7-12%
S2-60	5.7	42%	12-15%	5-12%
S3-20	21.7	71%	8-10%	5-8%
S3-60	21.2	70%	12-15%	5-12%
S4	---n.d.	---n.d.	--	--

It was noticed that, again, the higher the hydrophobicity is, the higher hybridization efficiency is achieved. This result was explained on the basis on two facts. Firstly, the studies carried out by Monserud and Schwartz [58] which indicate that hydrophobic surfaces can prevent the natural trend of long DNA chains to internalize their nucleobases, thus being more accessible and easing the hybridization. Secondly, having a highly hydrophobic surface can help to confine the sample solution only in the more hydrophilic areas which are the spots where the nucleic acid probe is immobilized. Anyway, the hydrophobicity may reach an equilibrium, as an excessively hydrophobic surface can result in no hybridization at all, and an extremely low wettability of the surface hinders the proper aqueous

sample distribution, making impossible to carry out the assay. This is, for example, the case of surface S4.

It was evident that the hydrophobicity of the surface played a key role in the final performance of the assay, concluding that contact angles around 100º performed the best. This might be related to the influence of the interaction of the attached probe with the surface over its conformation. For that, a comparison between surfaces S2-60 and S3-60 was made using the same nucleotide sequence but with one, two or four thiols to be multipoint tethered. The results obtained for the immobilization and the hybridization are collected in Table 4. In the light of these results, two conclusions raised. Firstly, the maximal immobilization density was not significantly affected by the multi-point attachment but determined by the surface hydrophobicity. Secondly, the hybridization yield increased for the multipoint attached probes. This did not constitute an important improvement in the case of S3-60, where the single point attachment already provided a 70% of hybridization; however, in the case of S2-60 the hybridization yield increased from 42% to nearly 100%, this being an interesting way to improve the performance of the assay.

Table 4
Comparison between S2-60 and S3-60 surfaces for different number of attaching thiol groups at the probe

No. of thiol residues at the probe	**Surface**	**Immobilization density (pmol cm^{-2})**	**Hybridization density (pmol cm^{-2})**	**Hybridization yield**
1	S2-60	13.7 ± 1.2	5.7 ± 1.3	42%
1	S3-60	30.4 ± 2.1	21.2 ± 0.4	70%
2	S2-60	12.7 ± 1.5	12.2 ± 1.8	96%
2	S3-60	29.5 ± 2.2	23.4 ± 1.1	79%
3	S2-60	13.3 ± 2.5	14.4 ± 1.5	100%
3	S3-60	30.5 ± 0.9	26.0 ± 0.2	85%

Besides the multipoint attachment, other strategies for improvement can be explored. An appealing one based on the results shown above is to chemically or physically modulate the surface hydrophobicity. Regarding chemical modulation, an interesting result was obtained on the basis on surface derivatization with mixtures for tuning wettability in surface S4. Thus, a new S4-2 surface was optimized combining 1H,1H,2H,2H-perfluorodecyltriethoxysilane as an anchoring ligand and 2-

[methoxy(polyethyleneoxy)$_{6-9}$propyl]trimethoxysilane as a wettability modifier. The optimal ratio between them was found at 1:4, providing immobilization density similar to S4 but allowing the hybridization with very good performance. As a matter of fact, the surface was applied to the discrimination of single nucleotide polymorphisms and to determine bacterial PCR products using colorimetric development, in this case 1.7 pM being the lowest concentration detected, which improved the values obtained for the other surfaces [54]. In the case of fluorescence experiments, all the assayed surfaces were able to detect the lowest concentration measurable with our surface fluorescence reader device [50], thus it is difficult to set a comparison. Interestingly, WCA for S4-2 was 100º, which fits with the optimal hydrophobicity previously found. Indeed, when comparing the spots visual quality (Fig. 3), highest intensity and definition, as well as lowest background, can be appreciated for S4-2 surface.

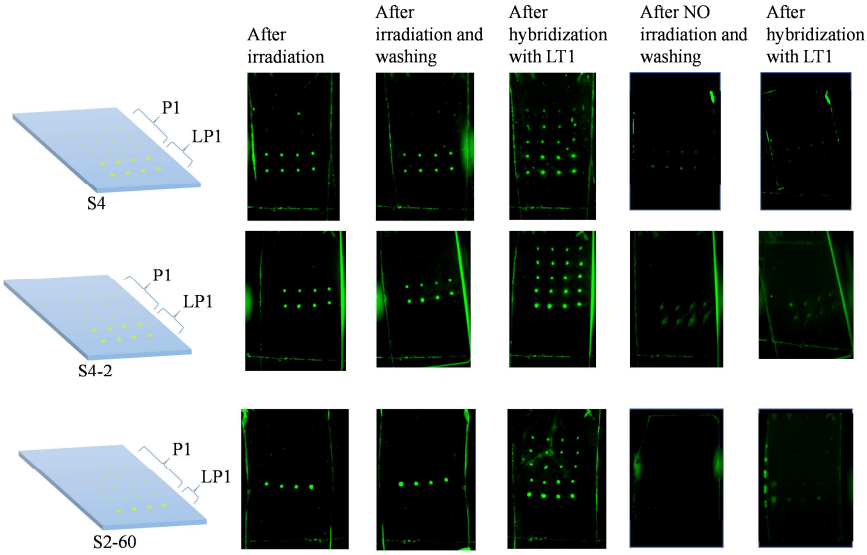


Fig. 3. Visual quality spots related to surface wettability. Comparison of three surfaces: S4, S4-2 and S2-60. LP Labelled probe, P probe, LT1 labelled target. Best spot quality and lowest background are obtained for S4-2. As can be seen, when thiolated probes are not irradiated there are no significant probe immobilization and further hybridization. Additional controls are shown in Figure S1, Supplementary data.

3.3. Hydrophobic and hydrophilic surfaces. Tuning wettability

Other strategy to control the interaction between the probe and the support consists of modifying the surface with the suitable functional group at the end of a hydrocarbon chain of variable length (Fig.

4), so the higher the number of carbon atoms, the more apolar the molecule, and the more hydrophobic the resulting surface [59]. This was carried out at our group by silanizing conventional glass with vinyl-ended silanes of different chain length and further arraying thiol-derivatized biotin, employing thiol-ene reaction, to be recognized by both Cy5-labelled streptavidin and an anti-biotin antibody. Binding events were better performing (smaller array spots, higher SNR) for large-chain silanes (C_{11} and C_{22}) providing mild hydrophobicity to surface (WCA 90-100º), while worse although acceptable results were achieved with short silanes (C_2 to C_8) leading to hydrophilic surfaces (WCA from 45º to 65º). The employment of a fluorine-substituted C_{10} vinyl silane (Fig. 4, bottom) also led to good wettability and improved the signals obtained. Optimal results were also achieved when modifying the vinyl silanes with 1% of 1H,1H,2H,2H-perfluorodecyltriethoxysilane, which caused mild hydrophobicity (WCA 105-110º) for all derivatized glass surfaces regardless the silane employed, and removed unspecific interactions, thus achieving very high values of signal-to-noise ratio.

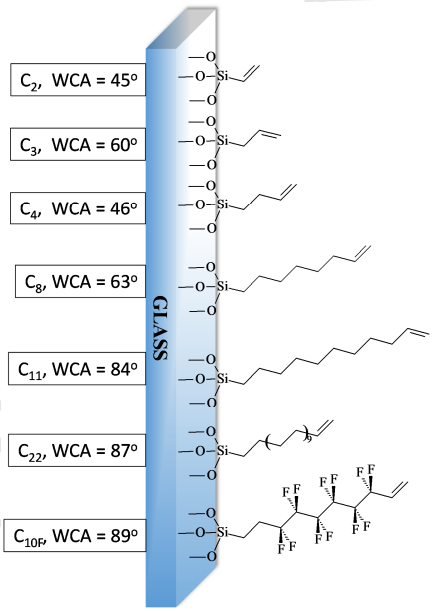


Fig. 4. Glass derivatized with different vinyl-ended silanes for modulating hydrophobicity according to hydrocarbon chain length and the presence of F atoms.

Regarding the use of physical modification, a micro/nanoporous poly(2-hydroxyethyl methacrylate-co-ethylenedimethacrylate) (HEMA-EDMA) polymer derivatized with vinyl groups was fabricated onto glass slides [60]. Thiol-ene coupling of thiolated oligonucleotides was carried out and

compared to the thiol-ene coupling on a planar surface. After probe anchoring, the surface was blocked with a fluorinated compound, providing a WCA of 109º. The performance in the hybridization was better in the case of the structured polymer, what was explained on the basis on two facts. On the first hand, the micro/nanostructuration increased the effective surface where the probes can be attached. And on the second hand, it was experimentally observed that the fluorescence emitted by a fluorophore deposited on the structured polymer was 5-fold the fluorescence signal obtained for the planar surface.

3.4. Antigen-antibody recognition assays. Selectivity

All the above discussed approaches have been applied to oligonucleotides due to their availability and easy handling. However, as stated in the introduction, the thiol-ene coupling reaction was also successfully applied onto IgG antibody fragments, after selective cleavage at the hinge region. Thus, half IgG (hIgG) could be covalently attached to the vinyl-surfaces here explained, performing better biorecognition capability than the immobilized whole antibody. Here, we employed this approach to assess the selectivity of the surface, and to discard that the material surface may immobilize non-specifically other proteins.

Thus, a set of microarrays were created where two different hIgG, specific towards Bovine serum albumin (BSA) and C-reactive protein (CRP), respectively, were immobilized by thiol-ene coupling chemistry onto vinyl-derivatized surface. Then, the chips were incubated with fluorescence labeled BSA, with fluorescence labeled CRP or with a mixture of both. For all the cases the buffer employed was 10% human serum diluted in PBS-T. When the fluorescence of the microarrays was interrogated (Figure 5) the specificity in the recognition was demonstrated.

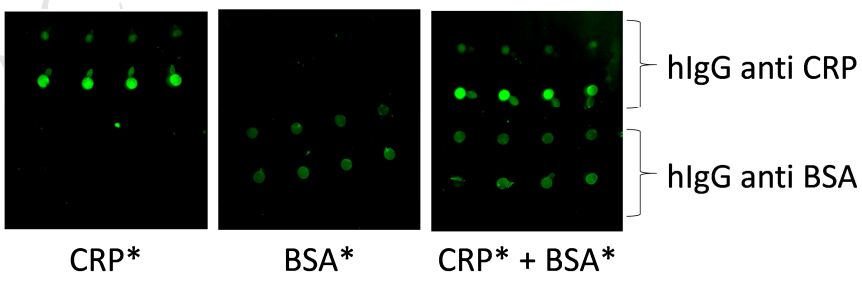


Fig 5. Fluorescence image for a microarray having immobilized hIgGs towards CRP (two top rows) and BSA (two bottom rows) incubated with labeled CRP (left), labeled BSA (middle) and a mixture of both (right). Buffer employed was 10% human serum diluted in PBS-T. The selectivity of the material surface and the bioreceptors is thus demonstrated.

3.5. Application to optical label-free biosensing

To demonstrate that the new biofunctionalization procedures developed in planar surfaces are applicable not only for microarray assay format but also in optical label-free biosensors, we employed the thiol-ene coupling chemistry for biofunctionalizing a nanophotonic sensor based on corrugated waveguides [51,61]. These substrates are created by periodically introducing several transversal elements into a single mode waveguide, so that this periodicity provokes the appearance of the so-called photonic bandgap (PBG), a spectral region where the light is not propagated. When the target analyte is recognized by the capture probe immobilized on the surface of the optical sensing structure, the PBG is shifted towards longer wavelengths due to the change in the local refractive index. This kind of sensors potentially have a very high sensitivity and their footprint is very small, what permits to integrate a large amount of sensors in a small area for multiplex detection. In this sense, our developed methods are very interesting as they allow the site-specific immobilization by the action of light.

Fig. 6 shows the binding curve of an oligonucleotide detection experiment for a biochip containing four optical sensor pairs. The two central pairs were functionalized with a nucleic acid sequence complementary to the target, while the external pairs remained unfunctionalized or blocked with BSA. The binding curve shows the spectral shift suffered by the upper edge of the PBG when the complementary target is flowed over the sensor. Only those sensors functionalized with the nucleic acid probe provided a shift in the PBG edge. Besides, as the target employed was fluorescently labeled, surface fluorescence was also registered after the sensing experiment and there was evidence of labeled target only on the central sensors.

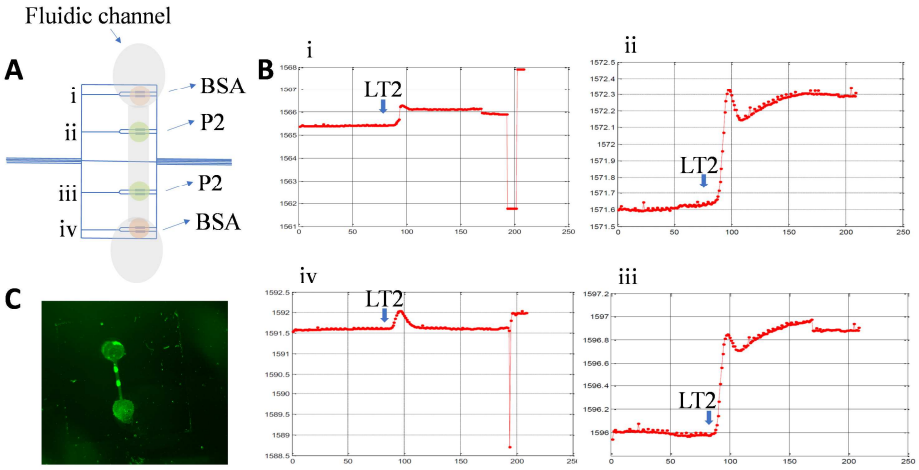


Fig. 6. A) Scheme of the nanophotonic sensing chip consisting of 4 pairs of PBG sensing structures. Outer pairs were biofunctionalized with BSA and inner pairs with thiolated probe 2 (P2); B) Spectral shift of the edge of the PBG observed for one sensor of each pair when labeled target 2 (LT2) was flowed over the sensors at 0.5 µM in SSC 1×; C) Fluorescence image of the chip with the fluidic channel after performing the biosensing experiment.

Further experiments were carried out with the double aim to prove the feasibility of the photo-*click* biofunctionalization in optical label-free biosensors and to demonstrate the role of the light in catalyzing the chemical coupling. In them, the above explained sensors were functionalized with alkene-ended organosilane and then the hIgG was flowed over the sensor for several minutes without any UV illumination. After that time, the lamp at 254 nm was switched on to illuminate the sensors through the microfluidic flow cell used to deliver the samples over the chip. Only when the light was acting it could be observed hIgG accumulation on the top of the sensor, as depicted in Fig. 7.

The described experiments, carried out with the photonic sensors, are a notable contribution of the use of this pool of easy, clean and site-specific photo-*click* reactions, for the development of biosensors, also in label-free mode.

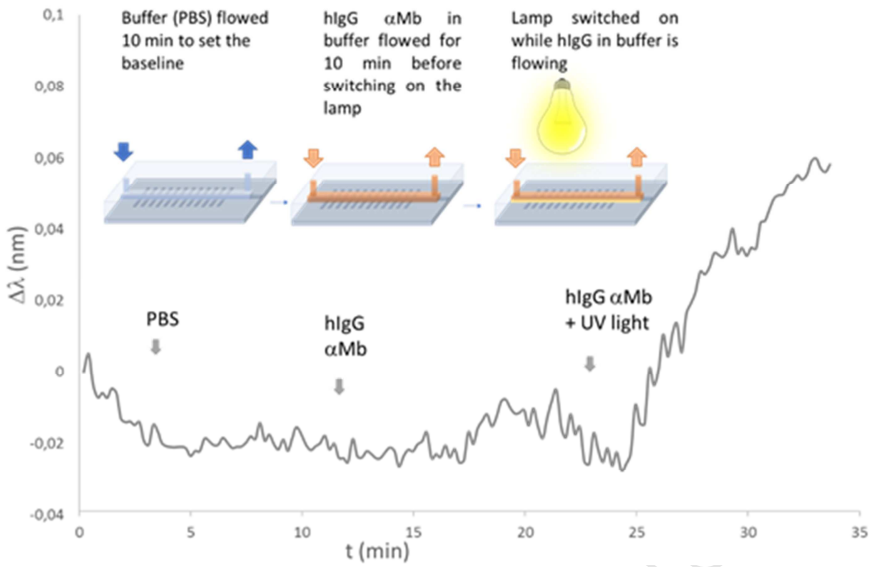


Fig. 7. Photochemical binding of hIgG antiMyoglobin (αMb) antibody fragments detected in a label-free format by means of PBG optical sensor. Signal (Δλ) remains constant as long as buffer or hIgG flows over the alkene-derivatized optical chip, but a clear signal increase is registered when irradiating at 254 nm, what indicated the attachment of the thiolated probes to the surface.

4. Conclusions

Photochemical *click* reactions are an interesting option in probe attachment for biosensing and other related techniques. All of the studied approaches have shown to be simple, rapid, effective, clean and well performing. The methodology is applicable to thiolated nucleic acids, cysteine-bearing proteins such as antibodies, and in general to any thiol-derivatized probe.

Apart from the direct benefits of these anchoring methodologies, and the same as other methods, they can be combined with strategies for surface wettability setting, addressed to improve the analytical results. It is to be noted that in all the described cases, for both protein and nucleic acid biointeractions, hydrophilic surfaces are poor performing because they lead to high size array spots and also to unspecific binding events. Superhydrophobicity is also to be avoided, because the interaction is hindered in this ambient. However, mild hydrophobicity, corresponding to WCA between 90 and 110, is traduced in small spots, good specific recognition and low unspecific unions, thus improving the biosensing performances.

The combined use of the thiol-click photocoupling and the surface wettability chemical tuning is an promising field to be exploited in the search of the achievement of high sensitivity, selectivity, reproducibility, and minimal unspecific signal, necessary in many critical fields.

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Conflict of Interest

None.

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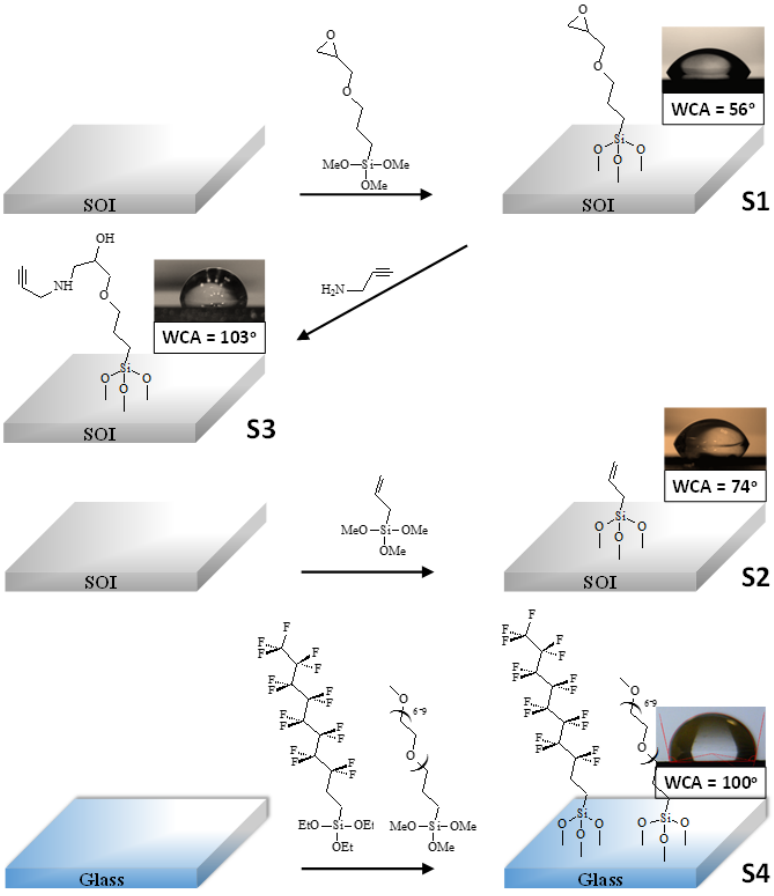

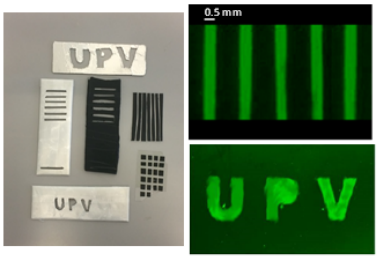

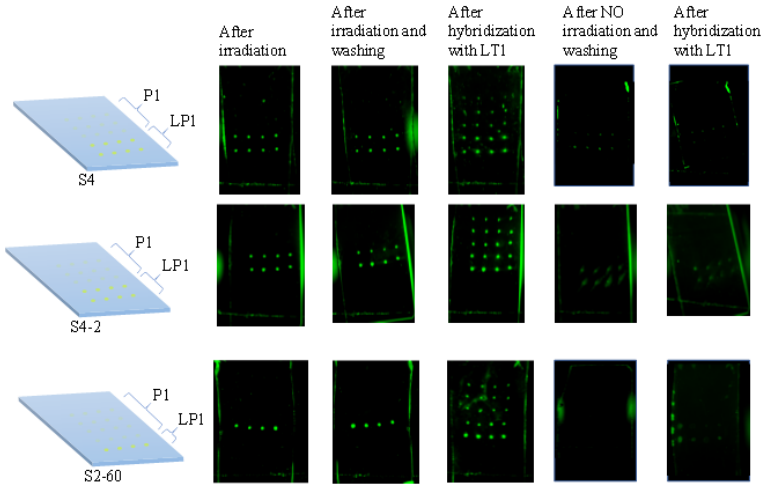

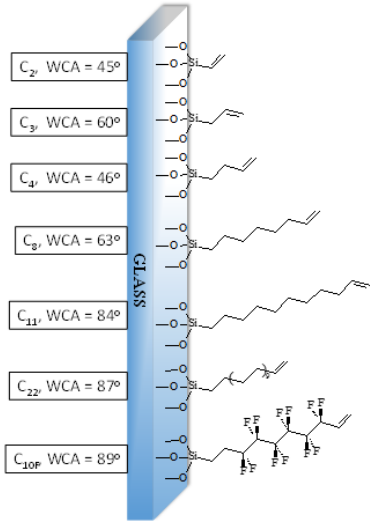

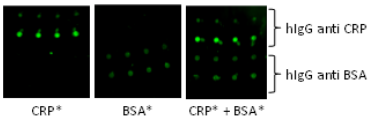

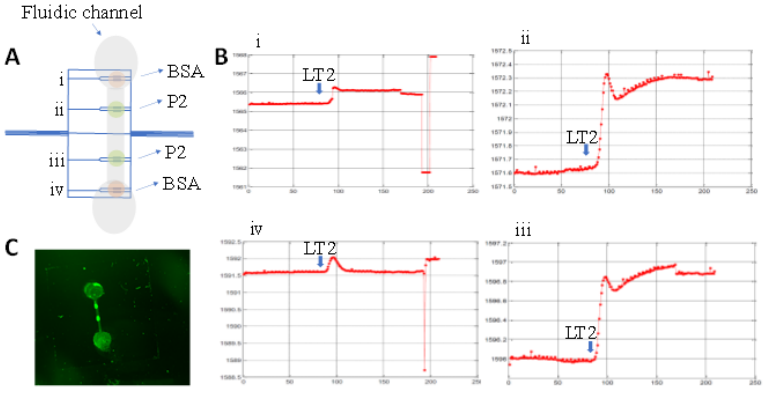

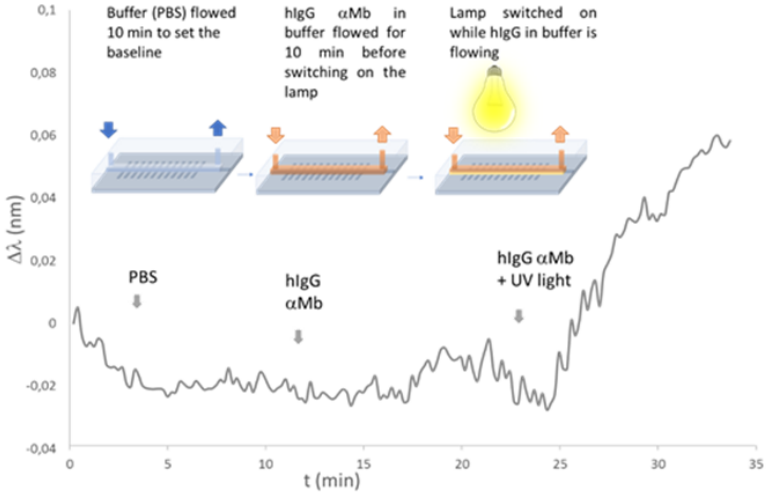

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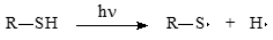
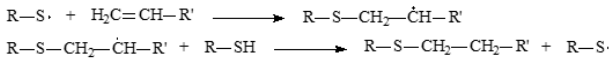
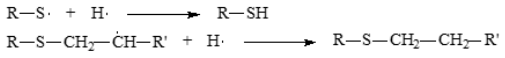


PROPAGATION



TERMINATION



HIGHLIGHTS

- A pool of photo-click chemistry reactions based on thiol moieties are overviewed for the probe attachment in the construction of optical biosensors.
- The role of surface wettability tuning on results quality is discussed.
- High performance fluorescence microarray and label-free nanophotonic biosensing is achieved.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Conflicts of Interest

None.