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Research Article

Comparative Assessment of Affinity-Based Techniques for Oriented Antibody Immobilization towards Immunosensor Performance Optimization

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Immunosensor sensitivity and stability depend on a number of parameters such as the orientation, the surface density, and the antigen-binding efficiency of antibodies following their immobilization onto functionalized surfaces. A number of techniques have been developed to improve the performance of an immunosensor that targets one or both of the parameters mentioned above. Herein, two widely employed techniques are compared for the first time, which do not require any complex engineering of neither the antibodies nor the surfaces onto which the former get immobilized. To optimize the different surface functionalization protocols and compare their efficiency, a model antibody-antigen system was employed that resembles the complex matrices immunosensors are frequently faced with in real conditions. The obtained results reveal that protein A/G is much more efficient in increasing antibody loading onto the surfaces in comparison to boronate ester chemistry. Despite the fact, therefore, that both contribute towards the orientation-specific immobilization of antibodies and hence enhance their antigen-binding efficiency, it is the increased antibody surface density attained with the use of protein A/G that plays a critical role in achieving maximal antigen recognition.

1. Introduction

Immunosensors are affinity-based assays that feature prominently as effective tools for the quantification of the amount of antibodies or antigens in complex samples. Irrespectively of whether it is the antibody or the antigen that is immobilized on the transducer surface, the strong binding forces that develop allow the detection of the target analyte with high sensitivity and specificity [1], making them very attractive for applications in a diverse range of fields such as food safety [2, 3], medical diagnostics [4, 5], and environmental monitoring [6, 7].

One of the main issues in the development of an immunosensor platform is the way in which the recognition element and the surface of the transducer are configured, as both will greatly affect the performance of the final device in terms of its sensitivity and selectivity [8]. A considerable amount of scientific effort has been put towards increasing the sensitivity of an immunosensor through the optimization of either the way the recognition elements is immobilized onto the transducer surface or the morphology of the surface itself or both. As far as the latter is concerned, carbon nanotubes, noble metal nanoparticles, polymers, quantum dots, and graphene are some of the numerous nanomaterials that have been applied in the design of an immunosensor to amplify signal detection, enrich and concentrate trace analytes, and immobilize the recognition elements with enhanced stability [9–12].

With regard to the way the recognition elements are immobilized and for antibody-based sensors in specific, sensor performance will depend on a number of critical factors: (i) antibody immobilization without significant loss of its native state; (ii) accessibility and the relative proportion of the antibody-binding sites for the antigen in solution; (iii)

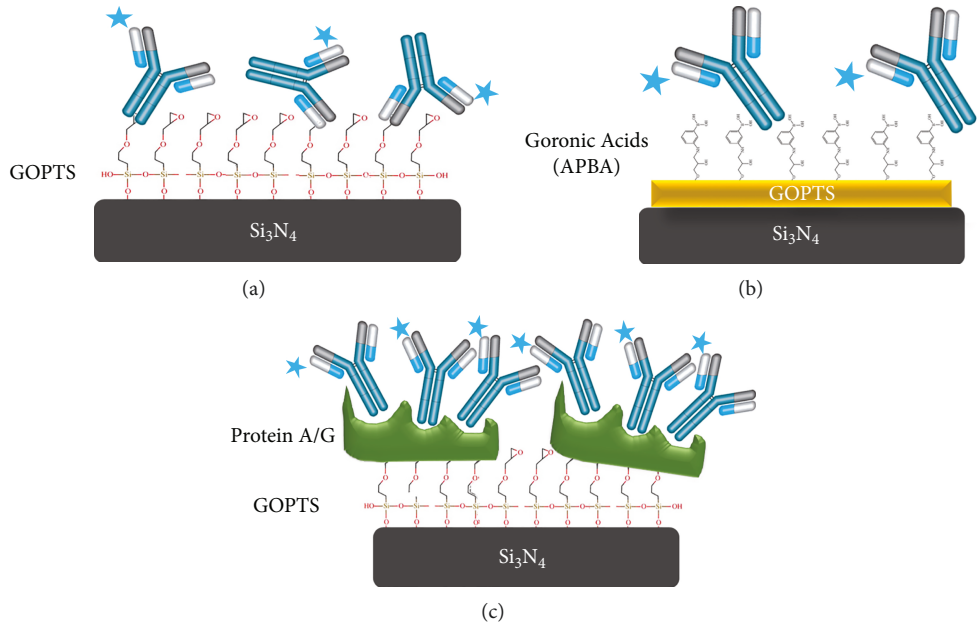


Figure 1: Schematic representation of the random antibody immobilization on GOPTS-functionalized Si_3N_4 surfaces (a) and the two techniques followed to achieve the oriented immobilization of antibodies onto the surfaces: boronate ester chemistry (b) and protein A/G (c).

the density with which the surfaces have been coated with antibodies; and (iv) low nonspecific adsorption [13]. Different immobilization methods can have markedly different results with respect to the orientation of the antibodies onto the surfaces, which will ultimately affect the performance of an immunosensor. Noncovalent physisorption or chemisorption, for example, are attractive in their simplicity but result in random antibody arrangements on the surfaces and hence in low sensor performance, leaking, and instability issues [14]. This is due to the fact that the immobilization of the asymmetric antibody molecules may take place through the variable antigen-binding sites (Fab), which leads to decreased or entirely eliminated binding activity [15]. Improvements on the stability and density of the immobilized antibodies can be achieved with the use of covalent immobilization techniques, though these methods too are far from ideal since they are often not site-directed [13, 16, 17], with some notable exceptions. Covalent and oriented antibody coupling on the surfaces that does not interfere with the antigen-binding Fab region can be achieved through thiol and glycan coupling as well as through the incorporation of tags such as biotin and single-stranded ssDNA; however, both techniques suffer from major drawbacks. In the first case, reduction of the disulfide bridges to generate thiol groups in the formed antibody fragments or the use of strong oxidizing agents to generate activated diols that disrupt the conformation of the antibody and excessive loss of reactivity has been observed [13, 16]. In the second case, the increased number of processing and purification steps required result in antibody losses [15].

The techniques mentioned above illustrate the fact that there is no single strategy that caters for all of the desired properties, such as orientation, site-specificity, high package density on the surface, and preserved antibody activity [18]. Nevertheless, a compromise can be reached with affinity-based approaches that offer the only relatively straightforward solution to the elusive goal of site-directed decoration of surfaces with antibodies without concomitant losses in their bioreactivity [19]. Out of these, the most popular and widely employed one is immobilization via Fc binding proteins, protein G and protein A, [16, 19]. An interesting alternative is the use of boronic acids to target the oligosaccharide moieties, albeit without the need for their oxidation [19, 20]. Despite the fact that both of these techniques have been extensively employed in numerous immunosensor platforms, the focus has been primarily on the optimization of a single protocol rather than on the effect different functionalization methodologies have on sensor performance [21, 22]. Review papers, on the other hand, are limited in simply listing the different methodologies without permitting a truly valid comparison to be made [16–20].

Herein, a comparative study into the efficiency of the two aforementioned techniques is being attempted for the first time. To this end, a recombinant protein A/G that combines four immunoglobulin-binding domains from protein A and two from protein G was employed to modify silane-functionalized silicon nitride surfaces. Its efficiency in achieving the site-specific orientation of antibodies was compared with that of aminophenyl-boronic acid- (APBA-) modified surfaces, as this is the boronate that has been most widely employed in immunosensor development (Figure 1). For their evaluation, an antibody-antigen model system was employed that consisted in a primary antibody and the secondary antibody against it. This model system was chosen so as to investigate the efficiency of the optimized protocols as to their stability, specificity, and selectivity when they are presented with complex matrices, similar to the ones encountered in real applications. Spotting of the secondary antibody onto the functionalized surfaces was achieved with the use of the laser-induced forward transfer (LIFT)

technique that aids towards the uniform deposition of the biological material as a monolayer and ensures the creation of viable biopatterns with high special resolution (10–100 μm for liquid printing) [23].

2. Materials and Methods

The Donkey anti-Rat IgG Cy5-conjugated (2ary antibody) was purchased from Jackson Immunoresearch, while the rat-raised anti-CD24 PE-conjugated (1ary antibody) from Thermo Fisher Scientific. Recombinant protein A/G and Pierce™ Antibody Clean-up Kit were also purchased from Thermo Fisher Scientific. All other reagents were purchased from Aldrich Chemicals. All solutions were prepared with deionized (DI) water (18 MΩ/cm resistivity, Millipore MilliQ).

2.1. Surface Silanization. For the functionalization of the silicon nitride (Si_3N_4) surfaces (3-glycidyloxypropyl) triethoxysilane (GOPTS) was used, according to a previously published protocol [24]. Briefly, prior to their silanization, the surfaces were cleaned by sonication in acetone and isopropanol and immersed in a solution of 1 M NaOH for 1 hour, rinsed with DI water, and dried with nitrogen gas.

2.2. Preparation of Protein A/G-Modified Surfaces. Protein A/G stock solution was prepared in 50 : 50% (v/v) glycerol : H_2O (concentration 10 mg/ml). Protein A/G at different concentrations was prepared in 10 mM Carbonate-Bicarbonate Buffer (CBB) pH 9.2 and was either drop-casted or spotted to GOPTS-functionalized surfaces with the use of the LIFT technique. It was then left to covalently bind with the epoxy functional groups overnight at 4°C. The surfaces were then rinsed extensively with the same buffer to remove any nonspecifically bound molecules. The surfaces were subsequently incubated with a solution of 10 mM ethanolamine in CBB for 30 minutes to quench the rest of the epoxy functional groups on the surfaces and were then rinsed with the same buffer. They were stored in PBS 1× pH 7.4 until further use.

2.3. Preparation of APBA-Modified Surfaces. A solution of 3% APBA dissolved in DI water was applied to GOPTS-functionalized surfaces by drop-casting and was allowed to covalently bind through its amino moieties overnight. The modified surfaces were extensively rinsed with ethanol and then with DI water and stored until further use.

2.4. Antibody Array Fabrication. Different buffers were employed for the immobilization of the Cy5-labeled secondary antibody onto the surfaces. Prior to their application onto the chemically functionalized surfaces, BSA that acts as a stabilizer was removed with the use of a Pierce™ Antibody Clean-up Kit according to the manufacturer's instructions.

2.4.1. GOPTS- and GOPTS-APBA-Modified Surfaces. For GOPTS- and GOPTS-APBA-modified surfaces, secondary antibody solutions of different concentrations were prepared in CBB and were allowed to bind with the surfaces for 1 hour at room temperature in a humid chamber. The surfaces were then rinsed extensively with the same buffer to remove any nonspecifically bound molecules. Unreacted functional groups for GOPTS-modified surfaces were quenched with ethanolamine as previously described. For GOPTS-APBA-modified surfaces, blocking was achieved by incubating the surfaces with a solution of 0.25% (w/v) dextran (MW~6000) in PBS 1× pH 7.4 for 30 minutes. In all cases, the surfaces were extensively rinsed with the respective buffers and blocked for a further 30 minutes with BSA in PBS 1× pH 7.4 with the addition of 0.5% Tween-20 and 1% BSA (PBST BSA). They were finally rinsed and stored PBST BSA until further use.

2.4.2. GOPTS-Protein A/G-Modified Surfaces. For GOPTS-protein A/G-modified surfaces, the antibodies were dissolved in PBS 1× pH 7.4 with the addition of 0.5% Tween-20 and 1% BSA and they were incubated with the surfaces for 1 hour at room temperature. The surfaces were rinsed extensively with the same buffer. Crosslinking between the antibody and protein A/G was achieved by applying a solution of 0.4 mM DMP (dimethylpimelimidate hydrochloride) in 0.1 M sodium borate buffer, pH 9.2 for 1 hour followed by quenching the reaction with the use of 1 M ethanolamine pH 8.5 for 20 minutes. The surfaces were extensively washed with PBST, blocked for a further 30 minutes with PBST BSA, and stored in the same buffer.

2.5. Microarray Immunoassay and Visualization. The bioreactivity of the secondary antibodies immobilized onto GOPTS-, GOPTS-APBA-, and GOPTS-protein A/G-modified surfaces was examined through their incubation with different concentrations of primary PE-labeled antibody dissolved in PBST BSA 1 hour at 37°C. Unbound primary antibody was removed by extensive washes in the same buffer. Visualization of the surfaces was achieved with the use of a Leica fluorescence microscope. In all cases, the recorded fluorescence from the microarray spots was quantified with the use of the ImageJ software (following subtraction of the background). Towards this a threshold value was set to include all the spot area (drop-casted or LIFT--spotted) and distinguish it from the background. This permitted the minimum, maximum, and mean fluorescent intensities as well as the standard deviations (SD) from the mean to be quantified throughout the threshold area.

2.6. Laser-Induced Forward Transfer Process. LIFT experiments were carried out, using a pulsed Nd:YAG laser (355 nm wavelength, pulse duration of 10 ns) and a high power imaging micromachining system. After the laser beam exits the laser source, it passes through an optical setup to determine the shape and the size of the laser beam which will irradiate a donor substrate that carries the material to be deposited. The donor substrate consists of a layer which will absorb the laser pulse and a transparent carrier. On top of the absorbing layer, a thin film (approximately 5 μm width) of the desired liquid material is coated. During the printing process the donor substrate is placed parallel and in close proximity to a receiver substrate (500 μm donor-receiver distance). Following the irradiation of the donor substrate, a high-pressure air bubble is created in the interface between

the absorbing layer and the liquid to be deposited. As the bubble expands, a liquid jet is created which results to the deposition of a droplet at the receiver substrate. By controlling the irradiation conditions and the characteristics of the laser pulse, the size of the deposited droplets can be tuned. In this work, the optimum laser spot size as it irradiates the donor substrate was fixed at 50 μm and the laser printing pulse fluence was 300 mJ/cm^2. Each laser pulse results to a single droplet and for the formation of the bioarray, a computer-controlled *x-y* translation stage was used.

3. Results and Discussion

3.1. Optimization of GOPTS-APBA-Modified Surfaces. In aqueous solution, boronic acids exist in equilibrium between two forms, a neutral triangular form and a negatively charged tetrahydral hydroxyboronate anion [25]. Although ester formation with 1,2- and 1,3-diols, like the ones found in the oligosaccharide moieties of the Fc domain of antibodies, can proceed with both of these forms, reversible boronate esters formed with the anionic form are significantly more stable [25]. For this reason, antibody immobilization was realized from a carbonate buffer with a pH value above the pKa of boronate, which is 8.9 [26].

However, and despite having optimized antibody immobilization onto GOPTS-APBA-modified surfaces, the reversibility of boronate ester chemistry could compromise the stability of the antibody-decorated surface. The rearrangement of boronate esters in which the esters dissociate by hydrolysis to yield free boronic acids and diols is greatly affected by both pH as well as the presence of other competing saccharides [27]. For example, it has been shown that at alkaline pH values high levels of antibody dissociation are observed upon incubation with sugar-containing media [28]. In the case of immunosensors, the buffering systems usually employed are in the physiological pH range, while exposure to complex sample matrices is highly likely and depends on the application. Immunosensors developed for clinical diagnostics, for example, are bound to be exposed to high concentrations of blood plasma immunoglobulins.

To overcome these difficulties, Adak et al. developed a crosslinker to covalently and irreversibly link the oriented antibodies to the boronate-functionalized surfaces [29]. Nevertheless, this solution relies on the use of complex custom-built linkers and a number of additional steps and hence cannot be widely applicable. To investigate, therefore, whether the reversibility of boronate ester chemistry is an obstacle to the development of stable immunosensors without crosslinking of the antibodies to the surfaces, an investigation into the extent of secondary antibody displacement in the presence of glucose-containing medium was undertaken. Secondary Cy5-labeled antibodies were drop-casted onto GOPTS-APBA-modified surfaces and were subsequently incubated for 1 hour with a solution containing 10 mg/ml glucose in two different buffers: CBB pH 9.2 and PBS 1× pH 7.4.

The results shown in Figure 2 reveal that minimal antibody dissociation is observed when the antibody-spotted surfaces are incubated with the glucose-containing medium

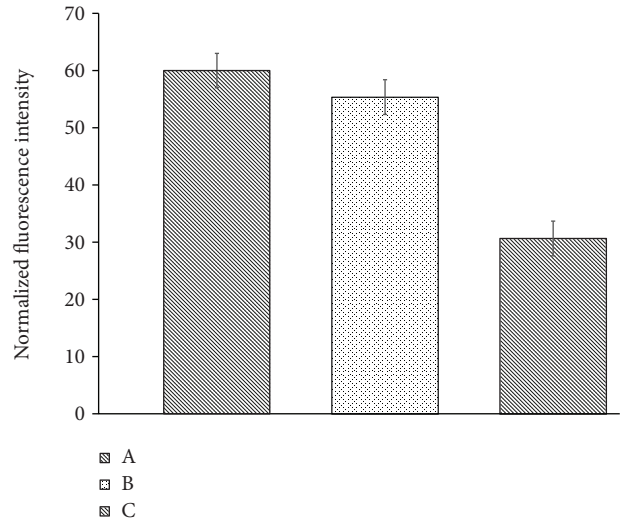


Figure 2: Normalized fluorescence intensities from (A) the immobilized Cy5-labeled secondary antibodies in CBB pH 9.2 for 1 hour and upon incubation with (B) 10 mg/ml glucose in PBS 1× pH 7.4 for 1 hour or (C) 10 mg/ml glucose in CBB pH 9.2 for 1 hour.

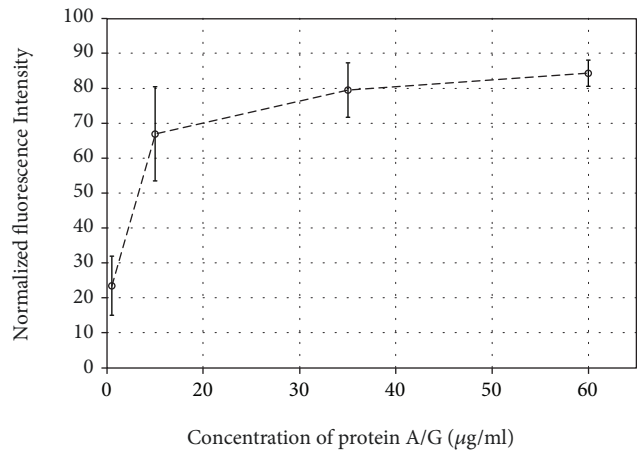
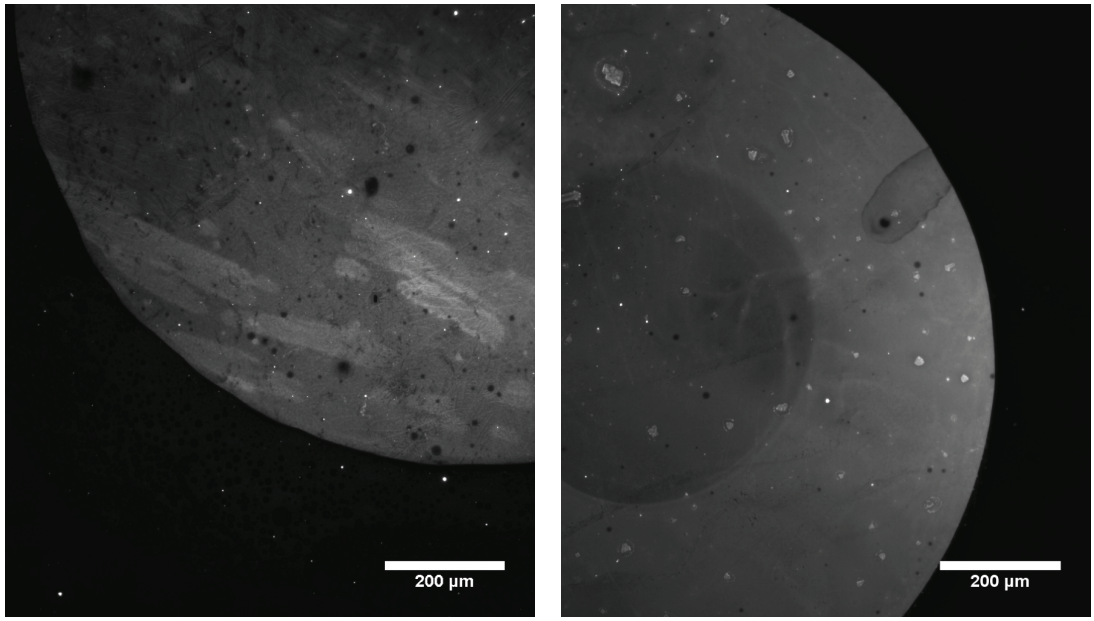


Figure 3: Normalized fluorescence intensity from bound secondary Cy5 antibodies onto surfaces modified with 1, 10, 25, or 100 μg/ml protein A/G (antibody concentration 10 μg/ml). Mean values ± SD shown for each concentrations ($N = 5$).

in PBS. By contrast, significant amounts of antibody get displaced when then surfaces are exposed to the same concentration of glucose but in alkaline conditions. The results are in agreement with the results obtained by Song et al. in a similar investigation [28], although in this case antibody dissociation was not examined in buffer with a pH at the physiological range. It appears that it is harder for glucose to compete with the existing boronate esters between the surface and the glycan moieties of the immobilized antibodies as boronate ester formation occurs but is not favored near physiologic pH [30]. Another plausible explanation for the low levels of displaced antibody even at alkaline conditions is the fact that the surfaces were modified with APBA following their silanization with the epoxy-containing silane GOPTS. As Abad et al. have shown, a significant amount of epoxy



(a) (b)

Figure 4: Images recorded from GOPTS-protein A/G-modified surfaces upon incubation with 10 μg/ml of Cy5-labeled secondary antibodies. (a) 50 μg/ml concentration of protein A/G (min gray value = 5, max gray value = 235, mean gray value = 75, SD = 22). (b) 100 μg/ml concentration of protein A/G (min = 21, max = 327, mean = 81, SD = 11).

groups remains unmodified with APBA and is available to react with protein nucleophiles, which could further stabilize the structures [31].

To passivate the remaining boronic acid groups in the areas adjacent to the drop-casted antibodies, dextran was employed, due to its large molecular weight and number of vicinal diols [32]. Based on the results obtained previously, PBS 1× pH 7.4 was employed as a buffer system so that it does not compete with the already immobilized secondary antibodies.

3.2. Optimization of GOPTS-Protein A/G-Modified Surfaces. As far as the use of Fc-binding ligands, such as protein A and protein G, to site-specifically immobilize antibodies is concerned, numerous naturally occurring as well as engineered proteins exist that have been extensively reviewed elsewhere [16, 33]. Ligand choice should be based on their species-specific reactivity rather than the number of immunoglobulin-binding domains they have, as the binding capacity attained upon surface immobilization drastically differs from the ideal conditions [34]. To improve region-selective immobilization of the ligands themselves, a number of techniques have been developed, such as the introduction of a gold-binding domain [35] and biotin [36]. However, the sensitivity of the system depends more on the orientation of the antibodies than that of the Fc-binding ligands [19]. In fact, it is preferable that all of the immobilized antibodies are oriented rather than reaching the maximal antibody loading efficiency, since with a lower antibody surface density steric hindrance can be minimized, thus improving response to the antigen [37].

For this reason, randomly oriented covalent immobilization of the Fc-binding ligand was chosen for the purpose of this study. The chosen ligand is the chimeric fusion protein A/G that combines four immunoglobulin-binding domains from protein A and two from protein G [38]. Despite the advantages this ligand possesses, it has rarely been employed in the development of immunosensors [39]. Experimental measurements were subsequently carried out to determine the effect the concentration of immobilized protein A/G onto the GOPTS-modified surfaces has on the amount of bound antibody.

Based on the results in Figure 3, the GOPTS-protein A/G-modified surfaces reach their maximal antibody binding capacity at a concentration of 50 μg/ml protein A/G. Nevertheless, a comparison of the SD values obtained for the threshold areas with the use of ImageJ software reveals that they are smaller for the spots obtained with 100 μg/ml of protein A/G than for those where 50 μg/ml of protein A/G was used (Figure 4). This is also visually evident in the fluorescent images recorded and used to generate the graph in Figure 3, where a much more uniform surface coverage with antibodies is attained when concentrations of protein A/G above 50 μg/ml are employed (Figure 4). The uniformity attained for surfaces modified with a higher concentration of protein A/G directly translates into more reliable measurements. In all subsequent experimental setups, a concentration of 100 μg/ml of protein A/G was thus employed.

The optimal value obtained differs to the values reported by other research groups [40, 41] that did not undertake similar investigations. Interestingly, a review of the available literature reveals that even the values stated by research groups that did try to optimize ligand concentration differs by several orders of magnitude. Melo et al., for example, concluded that optimal results were obtained with the use of 5 mg/ml protein G [42], while de Juan-Franco et al. point to

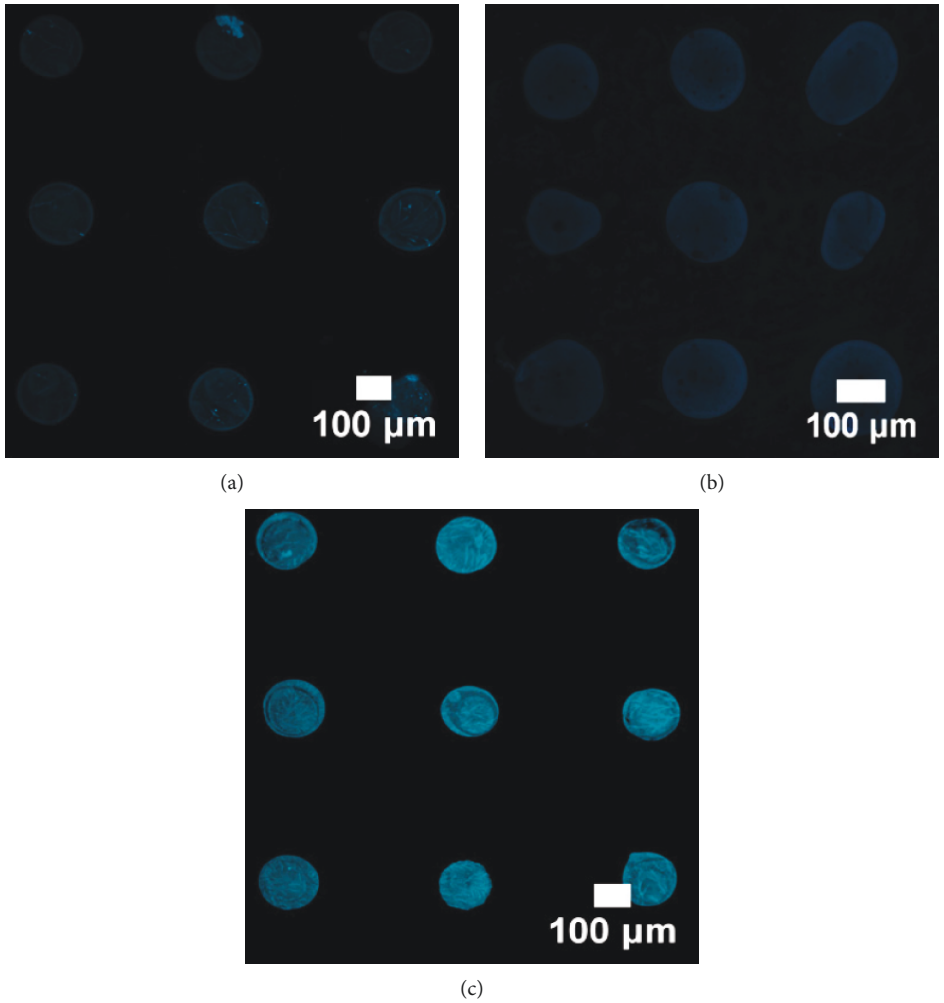


Figure 5: Fluorescent microscopy images of laser-printed secondary antibody tagged with Cy5 (50 μg/ml) on (a) GOPTS-, (b) GOPTS-APBA-, and (c) GOPTS-protein A/G-modified Si_3N_4 surfaces.

a concentration of 5 μg/ml for their fusion protein A-gold-binding domain [35]. The reported values are hard to be justified by the use of different ligands alone and indicate that the method of surface interrogation as well as its nature and the applied functionalization chemistry greatly influence the optimal Fc-binding ligand concentration.

The main challenge posed in the successful application of this immobilization technique is surface instability due to affinity interactions, as antibodies coupled to the surface might get displaced by naturally occurring plasma or serum proteins [43]. One way to address this is the use of various bifunctional crosslinking reagents, most commonly dimethyl pimelimidate (DMP) [43, 44]. Despite the fact that DMP has been recently replaced by BS3 (bis(sulfosuccinimidyl)suberate) in the majority of protocols for antibody crosslinking to Fc-binding ligands, we opted for DMP to crosslink the secondary antibodies to the immobilized protein A/G since it has been shown that it disrupts less the antigen-binding sites of the Fab region of the antibodies [45].

3.3. Comparison between GOPTS-, GOPTS-APBA-, and GOPTS-Protein A/G-Modified Surfaces. Having optimized

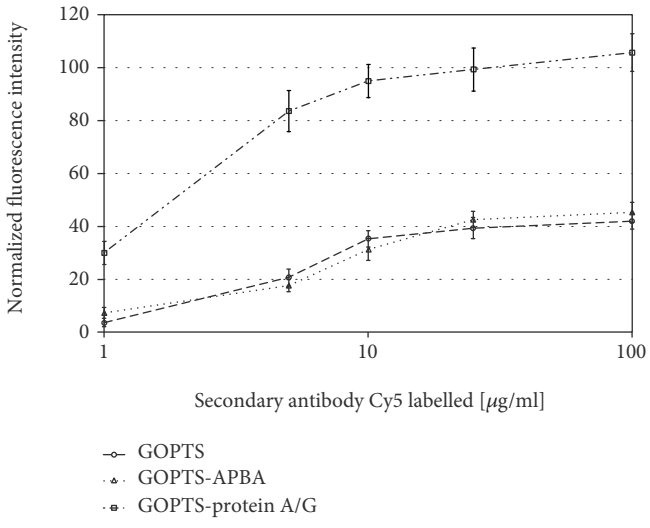
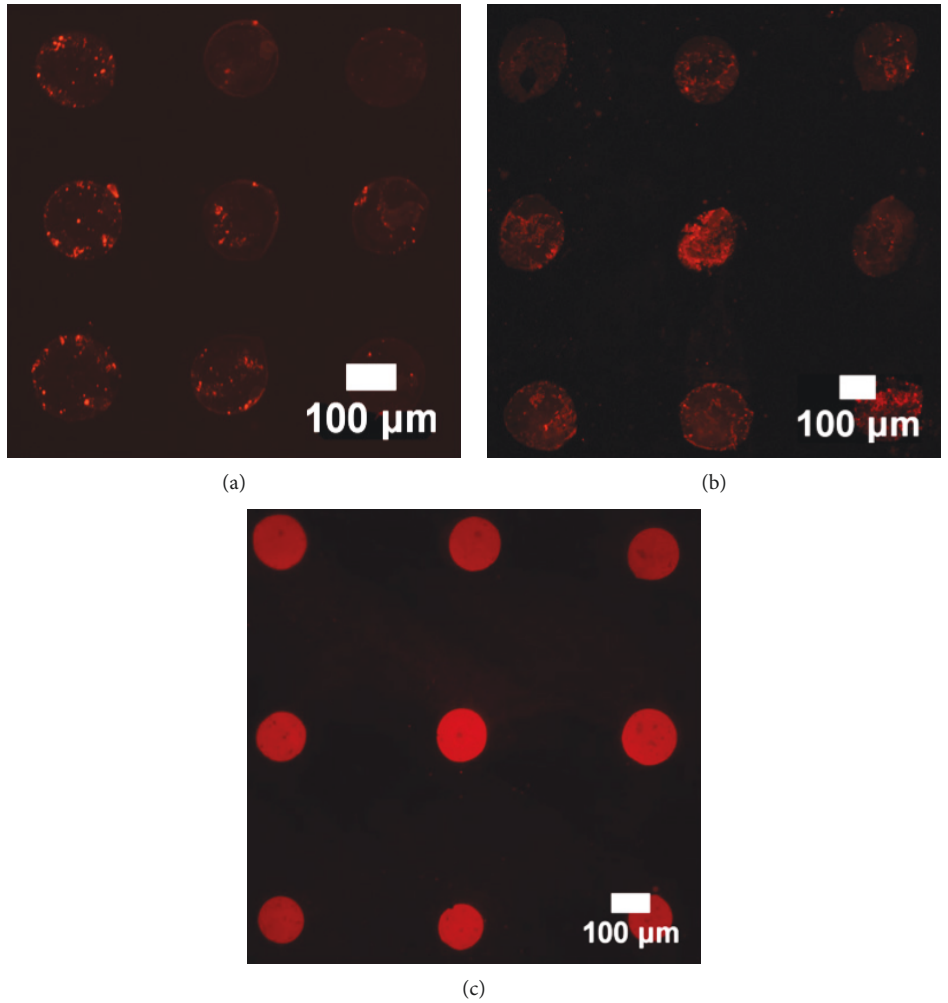


Figure 6: Antibody loading efficiency on GOPTS, GOPTS-APBA, and GOPTS-protein A/G surfaces modified with the optimized protocols. Mean values ± SD shown for each concentrations ($N = 5$).



(a) (b) (c)

Figure 7: Fluorescent microscopy images of the binding efficiency of the laser-printed secondary antibodies (50 μg/ml) upon incubation with its target primary antibody PE-labeled (100 μg/ml) on (a) GOPTS-, (b) GOPTS-APBA-, and (c) GOPTS-protein A/G-modified Si_3N_4 surfaces.

the individual protocols for the different surface functionalization chemistries, their antibody loading capacities as well as their antigen-binding efficiencies were compared for the first time and to the best of our knowledge. In the only similar investigation carried out by Pulido-Tofiño et al., antibody immobilization through the saccharide moieties was achieved through their oxidation instead of their complexation with a boronic acid-modified surface [46]. Microarrays of different concentrations of secondary antibody Cy5-labeled were laser-spotted onto GOPTS-, GOPTS-APBA-, and GOPTS-protein A/G-modified surfaces (Figure 5). The recorded normalized fluorescence intensities were used to plot the graph shown in Figure 6.

Based on the results in Figure 5, comparable quantities of antibody get immobilized onto GOPTS- and GOPTS-APBA-modified surfaces (two-way ANOVA, $p = 0.57$, $N = 5$), while the antibody loading capacity of protein A/G-modified surfaces is significantly higher than that of the GOPTS-modified surfaces (two-way ANOVA, $p < 0.001$, $N = 5$) and GOPTS-APBA-modified surfaces (two-way ANOVA, $p < 0.001$, $N = 5$). As far as the latter is concerned, many research groups have shown that antigen-binding efficiencies are improved with the use of Fc-binding ligands; however, the amount of antibody loaded onto the surfaces was not examined [19], while contradictory results are reported by the few research groups that did look into it. Quinn et al., for example, showed that the use of protein A results in decreases in the amount of antibody immobilized onto the surfaces in comparison to randomly bound ones [47], whereas Song et al. reported a 3-fold increase with the use of protein G [37]. This is probably due to the differences in the antibody binding sites of each ligand but most importantly due to the differences in their immobilization onto the surfaces. The latter propose that antibodies adopt an end-on orientation that allows their close packing in a dense layer [37], which by itself is not sufficient to explain the reduced antibody loading observed onto APBA-modified surface, which is comparable with the unmodified surfaces [48]. There, too, the antibodies adopt an end-on orientation. It seems, therefore, that

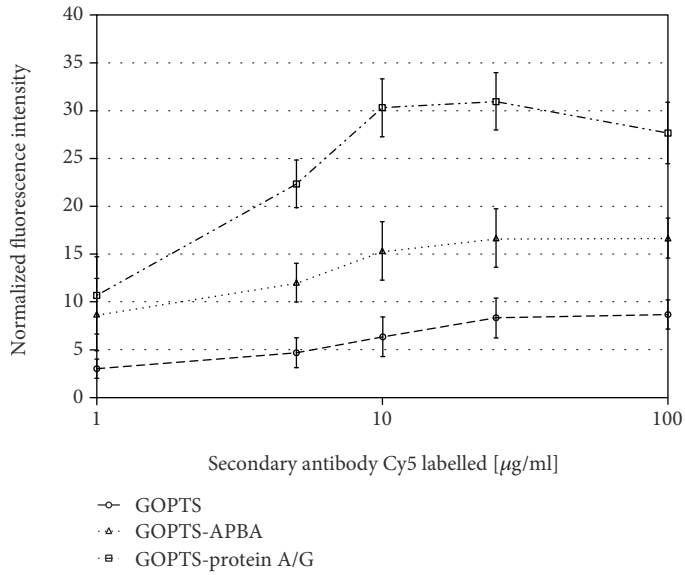


Figure 8: Antigen-binding efficiencies of secondary antibodies Cy5-labeled immobilized onto GOPTS-, GOPTS-APBA-, and GOPTS-protein A/G-modified surfaces upon incubation with 100 μg/ml of primary PE-labeled antibody. Mean values ± SD shown for each concentrations ($N = 5$).

protein A/G increases the active surface onto which antibodies can bind to in three dimensions due to its size, while the boronate chemistry is two-dimensional.

The binding ability of the antibody-modified surfaces was then investigated by incubating the laser-spotted microarrays with the same concentration of primary antibody PE-labeled (100 μg/ml) (Figure 7). As the results in Figure 8 illustrate, an increase in the amount of bound antigen was achieved both on APBA- as well as protein A/G-modified surfaces in comparison to the surfaces that was randomly bound (GOPTS-modified surfaces).

The binding capacity of antigen is mainly correlated to antibody loading, antibody bioactivity, and antibody orientation [20, 28]. For APBA-modified surfaces, the orientation-specific immobilization of antibodies results in an enhanced signal response in comparison to the GOPTS-modified surfaces (two-way ANOVA, $p < 0.001$, $N = 5$) (Figure 6), despite the fact that both have comparable antibody loading capacities (Figure 5). In the case of protein A/G-modified surfaces, the results indicate that further enhancement of the antigen-binding efficiency was achieved in comparison to APBA-modified surfaces (two-way ANOVA, $p < 0.001$, $N = 5$), which can be attributed both to the increased number of antibody molecules immobilized onto the surfaces as well as to the increased number of Fab domains available. No further increases in the amount of bound antigen can be observed above a concentration of 10 μg/ml immobilized secondary antibody in the case of protein A/G-modified surfaces and 25 μg/ml for APBA-modified surfaces. Despite the fact, therefore, that antibody orientation has a weighty influence on the immunosensor response, steric hindrance also plays an important role in determining the optimal antibody concentration to be immobilized on the surfaces [43]. The decrease in the recorded fluorescence at a concentration of 100 μg/ml of immobilized secondary antibody in the case of protein A/G-modified surfaces can be attributed to the inner filter effect, where the amount of fluorophore does not correlate linearly with the emitted fluorescence [48].

4. Conclusions

In summary, in this manuscript two affinity-based techniques for the oriented immobilization of antibodies onto silane-modified surfaces were optimized and compared. Both techniques achieve the ordered and orientation-specific immobilization of antibodies onto the surfaces through their constant regions (Fc) and result in their antigen recognition fragments (Fab) being aligned and facing away from the surfaces, without the need to interfere with the antibody's native structure. As neither of them directly result in the formation of a stable covalent bond between the antibody and the surfaces, an important consideration towards the fabrication of a stable immunosensor platform [49], careful adjustment of the buffer pH in the case of boronic acids, and the use of a crosslinking agent in the case of protein A/G-modified surfaces is required. The obtained results highlight the importance of the antibody's orientation and its surface density in enhancing its antigen-binding capacity. Nevertheless, an immunosensor performance will also depend on the nature of the antigen targeted and its size as well as the constraints imposed by the employed surface functionalization chemistry. Therefore, and despite the fact that improved antibody loading and increased antigen binding were observed for protein A/G-modified surfaces, boronate ester chemistry can still find many applications as it can easily get incorporated in polymer brushes, hydrogel, or complex constructs [25, 32, 50], thus achieving surface loading of

biomolecules in the three dimensions. Furthermore, the tunability of the interactions between boronate esters and the diols on antibodies through the adjustment of the buffer pH value can find numerous applications [51, 52]. The results presented herein can be used as a starting point towards choosing a functionalization method for a particular system under development, while they can be tailored to suit the needs and specific requirements of each immunosensor.

Data Availability

The raw data (images of the deposited spots visualized and captured with the use of a Leica fluorescence microscope) used to generate the graphs in this manuscript and support the findings of this study are available from the corresponding author upon request. Representative fluorescence images from the immobilized microarray spots have been included in the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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