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RESEARCH ARTICLE

Detection of *Brucella abortus* by a platform functionalized with protein A and specific antibodies IgG

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Abstract

Oriented immobilization of antibodies on a sensor surface is critical for enhancing both the antigen-binding capacity and the sensitivity of immunosensors. In this study, we describe a strategy to adsorb immunoglobulin G (IgG) anti-*Brucella* antibodies onto a silicon surface, oriented by protein A obtained from *Staphylococcus aureus* (SpA). X-ray photoelectron spectroscopy and atomic force microscopy were used to characterize topographically, morphologically, and chemical changes of the sensor functionalization. The activity of the biosensor was assessed by confocal microscopy, scanning electronic microscopy, and bacteria capture assays (BCA). According to the BCA, the efficiency of *Brucella abortus* detection with the SpA-IgG anti *Brucella* biosensor was three-fold higher than that of the random orientated IgG anti *Brucella* biosensor. The limit of detection was 1×10^6 CFU/ml. These data show that the orientation of antibodies immobilization is crucial to developing immunosensors for bacterial antigen detection as *Brucella* spp and improve its sensibility level. Functionalization with protein A increases *Brucella* detection by an antibody-coated surface. Functionalized silicon surface for *Brucella* detection was characterized by atomic force microscopy, X-ray photoelectron spectroscopy and confocal microscopy.

KEYWORDS

antibody orientation, atomic force microscopy, brucellosis, immunosensors, protein A

1 | INTRODUCTION

Brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella* and is transmitted to humans through the ingestion of contaminated dairy products or by direct contact with infected animals. *Brucella* spp. are Gram-negative bacilli measuring 0.6–1.5 μm by 0.5–0.7 μm; they are non-spore-forming, non-motile, and aerobic. Their optimal growth may require the addition of CO_2 (5%–10%) at 37°C (Alton & Forsyth, 1996). Brucellosis is highly prevalent in Latin American countries, western Asia, India, the Middle East and Southern Europe (Pappas, Papadimitriou, Akritidis, Christou, & Tsianos, 2006), with an annual incidence of a half million cases globally. *Brucella* spp. isolation in culture is still the gold standard method for the diagnosis of the disease. However, it may take several days of incubation before *Brucella* spp. may take several days of incubation prior to identify the *Brucella* species can be identified. Bacterial isolation from several

tissues or body fluids may be influenced by culture methods, low number of bacteria, and previous use of antibiotics, time of sample collection, and stage of the disease (Espinosa et al., 2009). Instead, serologic and molecular techniques are used as supporting methods of diagnosis (Araj, 2010; Çiftci et al., 2017; Geresu & Kassa, 2016; Gómez et al., 2008). Most of the immunologic diagnostic methods have poor specificity since many antigenic structures of *Brucella* spp. are shared with other Gram-negative bacteria (Seleem, Boyle, & Sriranganathan, 2010).

Given these difficulties, the development of strategies for direct detection of pathogenic *Brucella* spp. bacteria is critical. Novel methodologies for *Brucella* detection and identification are still limited since most methods detect the bacteria indirectly using label-dependent biosensors (Fu-Chun, De-Si, Zhong, Shu-Zhen, & Ya-Fei, 2007; Liebes, Amir, Marks, & Banai, 2009; Wu et al., 2013) or sensors that require sample pretreatment (Pal et al., 2017; Rahi, Sattarahmady, & Heli, 2016). Sensitivity of the immunoassays depends on the immobilization of the antibody on a solid support of sufficient surface density in an orientation that maximizes their antigen capture capability (Holford, Davis, & Higson, 2012; Moina & Ybarra, 2012). The use of protein A extracted from *Staphylococcus aureus* (SpA) has been used to increase the efficiency of recognition site detection of soluble antigens (Tan et al., 2010; Trilling, Beekwilder, & Zuilhof, 2013; Yoon, 2011). SpA orientates the binding of at least two molecules of antibody simultaneously, which increases the amount of antibody molecules attached to the surface and consequently, increases the sensitivity of the antigen detection (Moks et al., 1986).

In the design of a biosensor it is important to assess the proper assembly of the layers of reagents. The aim of this work was to develop a more efficient platform for the detection of *Brucella* by using protein A on silicon surfaces to immobilize and orientate the anti-*Brucella* antibodies. The efficiency of bacterial capture by the antibodies on functionalized SpA surfaces was determined by confocal laser scanning microscopy (Demirel, Çalayan, Garipcan, Duman, & Pişkin, 2007; Souiri et al., 2014) and scanning electron microscopy (SEM) (Mendoza-Madrigal et al., 2013). X-ray photoelectron spectroscopy (XPS) was used to evaluate the composition of each monolayer to ensure the binding of the reagents to the surface or to the previous layer. Layers were also assessed at each step of functionalization by atomic force microscopy (AFM). As AFM provided a resolution at nanometric level, this technique allowed us to characterize the orientation of the antibodies onto functionalized surface.

The current study demonstrates that the adsorption of anti-*Brucella* antibodies on a protein A-covered surface controls the orientation of the fragment of antigen binding (Fab) of the antibodies and thus enables the recognition of *Brucella*. We find protein A functionalization is a critical step for *Brucella* immunosensor efficiency and potentially for other bacterial pathogen detection schemes.

2 | MATERIALS AND METHODS

2.1 | Production of anti-*Brucella* antibodies

A young adult New Zealand white rabbit was used for the production of the anti-*Brucella* antibodies. The rabbit was injected intravenously with 0.1 ml *B. abortus* 2,308 heat-killed suspension at 4.5×10^8, 9×10^8, 1.8×10^9 and 2.7×10^9 colony forming units (CFU)/ml in 0.1 M phosphate-buffered saline (PBS) on days 0, 4, 8, and 12, respectively. The antiserum was collected 1 week after the last booster. Animals were maintained under standard hygiene conditions and food and water were given ad libitum, in accordance with national guidelines (Norma Oficial Mexicana, NOM-062-ZOO 1999). The immunoglobulin G (IgG) fraction was purified by ammonium sulfate precipitation as described by Harlow and Lane (1988).

2.2 | Antibody specificity and reactivity

The specificity of anti-*Brucella* antibodies for the detection of *Brucella* was assessed by the microagglutination tube test with 2-mercaptoethanol (Alton, Jones, Angus, & Verger, 1998; Bettelheim, Maskill, & Pearce, 1983). Briefly, microagglutination was carried out on doubling dilutions of purified IgG from 1:2 to 1:2048 with 100 μl heat-killed *B. abortus* 19. The plate was incubated at 37°C for 24 hr. The IgG anti-*B. abortus* titer was determined to be 1:1024. Antibodies were then incubated for 30 min at 37°C with *Escherichia coli* O:157, *Vibrio cholera* 01 and *Ochrobactrum anthropi* in order to adsorb cross-reacting antibodies. Tubes with antibodies and bacteria were centrifuged at 1,000*g* and the supernatant was collected and stored at −20°C. After the absorption, it was verified that the remnant IgG anti-*B. abortus* antibodies did not recognize the cross-reactive bacteria species.

2.3 | Covalent immobilization of protein A and anti-*Brucella* antibodies

Single-side polished silicon (Si) wafers (Sigma-Aldrich, St. Louis, MO) were cut into square chips of ~4 × 4 mm using a diamond-tipped glasscutter. Si surfaces were functionalized in three stages. The chips were cleaned with piranha solution, a mixture of concentrated H_2SO_4 and 30% aq. H_2O_2 (50:50 v/v) for 20 min, rinsed with distilled water, and dried. The cleaned Si surfaces were then immersed in a freshly prepared 20% 3-glycidoxypropyltrimethoxysilane (GOPTS, Sigma-Aldrich) solution in dry toluene for 1 hr. The silicon surfaces were rinsed thoroughly with dry toluene to remove the excess reagent and dried under a N_2 atmosphere. The GOPTS-activated surface was immersed in a solution of protein A from SpA (Sigma-Aldrich; 10 μg/ml in PBS, pH 8.0) for 1 hr at room temperature. The surfaces were rinsed thoroughly three times with PBS and treated with 2.5% (w/v) bovine serum albumin (BSA) in PBS, pH 7.0, for 30 min to block nonspecific binding. Finally, Si surfaces functionalized with protein A were immersed in a solution of anti-*Brucella* antibodies (3 μg/ml) in a phosphate buffer, pH 8.0, for 1 hr at room temperature.

2.4 | Bacterial capture by antibodies coupled to protein A

The functional integrity of the antibodies after completion of the functionalization procedure was tested by a capture assay. A *B. abortus* 2,308 strain transformed with a derivative of the broad host-range stable cloning vector pBBR1-*gfp* Ouahrani-Bettache, Porte, Teyssier, Liautard, & Kohler, 1999) expressing the green

fluorescent protein (GFP) was used. The GFP-*B. abortus* 2,308 strain was cultured and maintained in trypticase soy agar (BD-Dixon, Franklin Lakes, NJ) with 200 μg/ml kanamycin. For the bacteria capture assays, 1×10^8 CFU of heat-inactivated *B. abortus* 2,308-GFP strain (70°C for 30 min) were incubated with the functionalized Si surfaces at 37°C for 1 hr. After the incubation, the Si surfaces were washed three times with PBS mixed with 0.05% Tween 20 (PBS-T) to remove any non-adhered bacteria. The absence of cross reactivity of the IgG anti-*Brucella* antibody was assessed using 1×10^8 CFU/ml of *E. coli* O:157 stained with 4′,6-diamidino-2-phenylindole. The functionalized Si surfaces were mounted onto glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed using a confocal laser scanning microscope (LSM5 Pascal, Zeiss, Jena, Germany). The Si surface was excited with laser lines of wavelength 488 and 555 nm. Under these conditions the Si surface emitted red light (LP 420 nm), GFP-*B. abortus* 2,308 strain emitted green light (LP 505 nm), and *E. coli* O:157 emitted dark blue light (LP 405 nm). Field areas of 142.86 × 142.86 μm were examined at 10 different locations and the images were taken using a confocal microscope with Plan Apochromat 63×/1.4NA oil objective (Zeiss). Bacteria captured by antibodies were counted manually to quantify binding on the Si wafer surface. The results were expressed as the number of bacteria/1×10^4 μm^2.

2.5 | Atomic force microscopy

The topography of functionalized Si surfaces was analyzed by atomic force microscopy (AFM) in tapping mode (diMultimode V, Veeco, Somerset, NJ). Square chips were dried before testing in air and silicon cantilevers model RTESP (Bruker, Billerica, MA) were used. At least five areas of 2.5 × 2.5 μm and 500 × 500 nm were scanned on each Si wafer at a speed of 1 Hz and a resolution of 256 × 256 pixels. Section analyses were conducted to depict sectional profiles of the adsorbed protein. The roughness was calculated using the root square deviation of the heights (Rq) and the arithmetic average of the absolute values of the height deviations of the images, using Nano Scope Analysis 1.5 (Veeco) and applying flattening of third order.

2.6 | X-ray photoelectron spectroscopy analysis

X-ray photoelectron spectra (XPS) from different positions on at least three replicates of each functionalized Si surface were analyzed and collected on a K-α Thermo Scientific spectrometer (Waltham, MA). General spectra were obtained from each Si wafer using Al Kα radiation at 1.49 eV.

2.7 | Scanning electron microscopy

Si surfaces functionalized with protein A were immersed in a solution of anti-*Brucella* antibodies (3 μg/ml) in PBS, pH 8.0, for 1 hr at room temperature. The surfaces were then incubated with 1×10^5 CFU *B. abortus* 2,308 for 1 hr at 37°C. The Si surfaces were then rinsed three times with PBS-T to remove any non-adhered bacteria and fixed with 2.5% glutaraldehyde solution. The fixed cells were dehydrated with ethanol, subjected to critical point drying with liquid CO_2, and mounted on aluminum stubs. Finally, the samples were covered with gold in a Denton Vacuum Desk II sputter coater and examined using a scanning electron microscope (SEM; JSM 7800 – JEOL, Tokyo, Japan).

2.8 | Statistical analysis

The experimental values of the bacteria captured by confocal microscopy are shown as the mean ± SD. The data were analyzed using the Kruskal–Wallis test. The differences between the albumin control and test values were considered to be statistically significant when $p < .05$.

3 | RESULTS

3.1 | Characterization of functionalized Si wafers by XPS

The functionalization procedure was performed to generate chemically active surfaces on Si wafers that would enable the covalent immobilization of proteins. The procedure utilized for coupling the protein A or anti-*Brucella* antibodies to the GOPTS-activated surface is shown in Figure 1. Si wafer surfaces were functionalized in three stages: first, cleaned Si wafers were silanized by incorporating GOPTS; secondly, the GOPTS-activated surface was coated with protein A; and finally, the anti-*Brucella* antibody was added for recognition by protein A. In some cases, the Si wafer surface was directly coated with the antibody.

The reaction and bonding of the chemical elements onto the functionalized surface was assessed by XPS (Figure 2). XPS detects elements from the first few atomic layers of a surface and provides information on the chemical compounds bound to the surface, as well as the stoichiometric ratios among the elements. (Baer & Engelhard, 2010; Brundle, Conti, & Mack, 2010; Gouget-Laemmel et al., 2013). Peaks of oxygen (544 eV), silicon (112 eV), and carbon (300 eV) were observed in the XPS spectrum of the clean Si wafer (Figure 2a). The atomic percentage of these elements was previously determined in the functionalization procedure (Table 1). The spectrum of the GOPTS-activated Si wafer surface showed a decrease in the intensity of the silicon peak and an increase in the carbon signal peak (Figure 2b). After the adsorption of protein A and IgG anti-*Brucella* antibody to the GOPTS-activated surface, a strong nitrogen signal of 3.2 (atomic percentage) was observed (Figure 2c and Table 1), corresponding to the bonded proteins. The atomic percentage of silicon increased ~10%, and signals corresponding to the Na, Cl, and Ca present in the buffer solutions were detected. The XPS spectra confirmed that the silicon surface activated with GOPTS was correctly functionalized with protein A and IgG anti-*Brucella*.

3.2 | *B. abortus* recognition on Si wafer surface functionalized with anti-*Brucella* antibodies

To demonstrate proper functionalization by the specific recognition of *B. abortus*, wafers were incubated with *B. abortus* for 1 hr, washed to remove non-attached bacteria. *B. abortus*-GFP strain, and transformed to express the green fluorescent protein reporter, which allowed for the tracking of bacteria by confocal microscopy (Figure 3a). Results

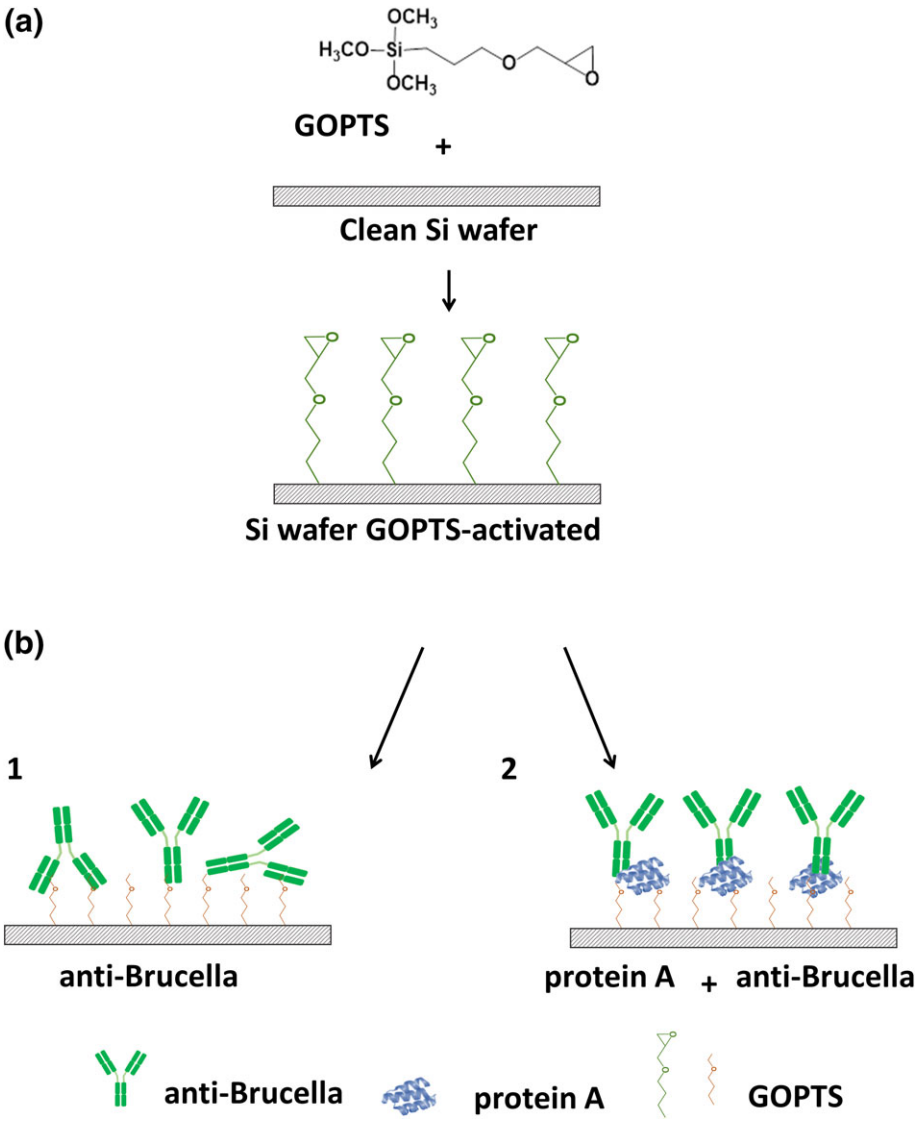

FIGURE 1 Scheme of functionalization of the silicon (Si) wafer surface with protein A and anti-*Brucella* antibodies. (a) Silanization of Si wafer with 3-glycidoxypropyltrimethoxysilane (GOPTS). (b) Covalent bonding of proteins to the surface. 1. Surface functionalized with antibodies without protein A. 2. Functionalization with antibodies and protein A [Color figure can be viewed at wileyonlinelibrary.com]

were confirmed by SEM. The images were compatible with the expected figures of the bacterium, that is, bacterial bodies from 0.5 to 1.0 μm with a short bacillus shape (Figure 3b; Alton & Forsyth, 1996). The bacterium was also identified by atomic force microscopy, which confirmed observations made by CLSM and SEM (Figure 3c).

3.3 | Immobilization of anti-*Brucella* antibodies by protein A maintained the functional conformation of the antibody

Through analysis of roughness parameters it is possible to profile the shape of the molecules as they are assembled (Trilling, Beekwilder, & Zuilhof, 2013). AFM images were obtained to topographically visualize the conformation and the height of the layers of reagents on Si wafer surface after the functionalization process. First, the silicon surface was characterized before its activation with GOPTS. An apparently flat surface was observed, with a mean roughness value (Rq) of 0.656 ± 0.17 nm (Figure 4a). The cross-section profile obtained with the NanoScope Analysis software confirmed the flatness of the surface.

Protein A left a uniform layer with a peculiar honeycomb-like shape formed by polygonal substructures. The surface roughness (Rq) was determined to be 2.02 ± 0.03 nm (Figure 4b). A Magnification of this image showed the apparent pentagonal shape. Analysis of cross-section profiles at 10 different locations showed peaks with a height of 4.99 ± 1.3 nm (Figure 4c). A top view of GOPTS-activated surfaces after adsorption of antibodies plus protein A showed two different peak regions, one with a mean height of 7.7 ± 1.4 nm and one with a mean height of 2.51 ± 0.75 nm (Figure 5a). In contrast, the image of the GOPTS-activated surfaces after adsorption of the antibodies without protein A exhibited a quite different character. The image showed round globular particles and the formation of aggregates on the functionalized surface that might correspond to the randomly immobilized antibodies (Figure 5b). Cross-section profiles of the surface coated with protein A plus anti-*Brucella* antibodies (Figure 5a) compared with the surface coated only with anti-*Brucella*

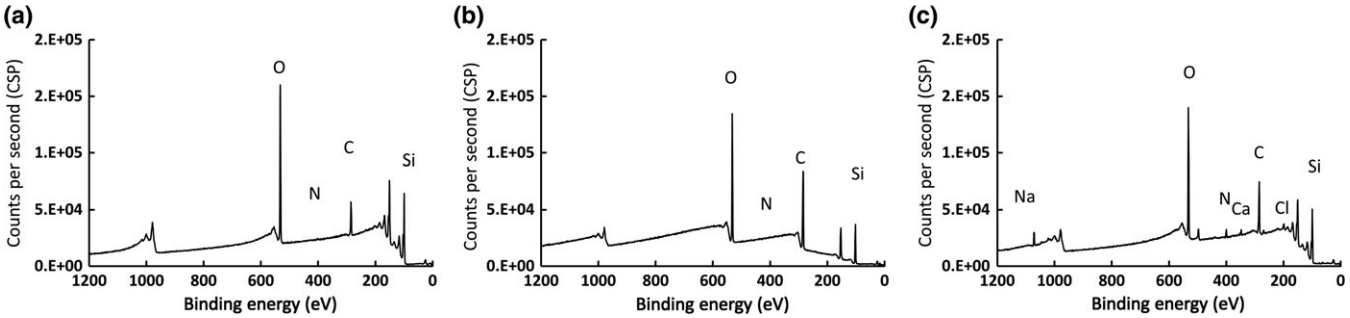

FIGURE 2 X-ray photoelectron spectroscopy (XPS) general spectra and elemental composition of functionalized silicon (Si) wafer surface. (a) XPS spectrum of a cleaned Si wafer. (b) 3-Glycidoxypropyltrimethoxysilane (GOPTS)-activated Si wafer surface. (c) GOPTS-activated surface coated with protein A, bovine serum albumin (BSA) and immunoglobulin G (IgG) anti-*Brucella* antibodies

TABLE 1 Elemental composition of the functionalized surface (% atomic concentration)

	Si wafers		
Element at %	Cleaned	GOPTS-activated	GOPTS-activated and proteins
Si	42.8	19.7	31.9
O	20.4	26.0	28.6
C	36.0	53.9	37.5
N	0.8	0.4	3.2
Others (Cl, Na, Ca)	0	0	4.8

Abbreviations: GOPTS, 3-glycidoxypropyltrimethoxysilane; Si, silicon.

antibodies (Figure 5b) showed a very different pattern of peak size and distribution. The surface coated with protein A plus anti-*Brucella* antibodies exhibited a more regular pattern, with regularly distributed peaks with a mean height of 7.7 nm ± 1.4 (Figure 5a). On the other hand, the antibodies directly bound to the surface activated with GOPTS were irregularly distributed and peaks with a height of 11 ± 1.1 nm were found to have an apparent concentration of antibodies in some regions while in others no bound antibodies were observed (Figure 5b). The honeycomb shape adopted by protein A on the silicon surface was reflected in the way the antibodies were assembled (Figure 5c). A schematic view of the antibodies adsorbed on the surface without the protein A shows the random and non-oriented arrangement of the antibodies (Figure 5d).

3.4 | Protein A functionalization increases the efficiency of *Brucella* recognition

Confocal microscopy images allowed visualization and quantification of the bacteria captured on the silicon surface (Figure 6). Si wafers functionalized with BSA as a passivation control showed very low reactivity. Less than one bacteria/10,000 μm^2 was detected on BSA functionalized Si wafers (Figure 6a) compared to ~10 bacteria/10,000 μm^2 on Si wafers functionalized with anti-*Brucella* antibodies (Figure 6b). On wafers functionalized with protein A and IgG anti-*Brucella* antibodies, an average of 30 bacteria/10,000 μm^2 were counted (Figure 6c). A three-fold increase in the capacity of antigen uptake was observed due to the addition of sequential layers of protein A and IgG anti-*Brucella* antibodies (Figure 6d).

These results confirmed the specific recognition of *Brucella* by the Si surface functionalized with the anti-*Brucella* antibody. Moreover, these results showed the increased efficiency in the detection of *Brucella* by using protein A.

3.5 | Limit of detection and specificity

The limit of detection (LOD) is defined as a signal measuring three-fold of the background (at least 3 bacteria/10,000 μm^2). The LOD of

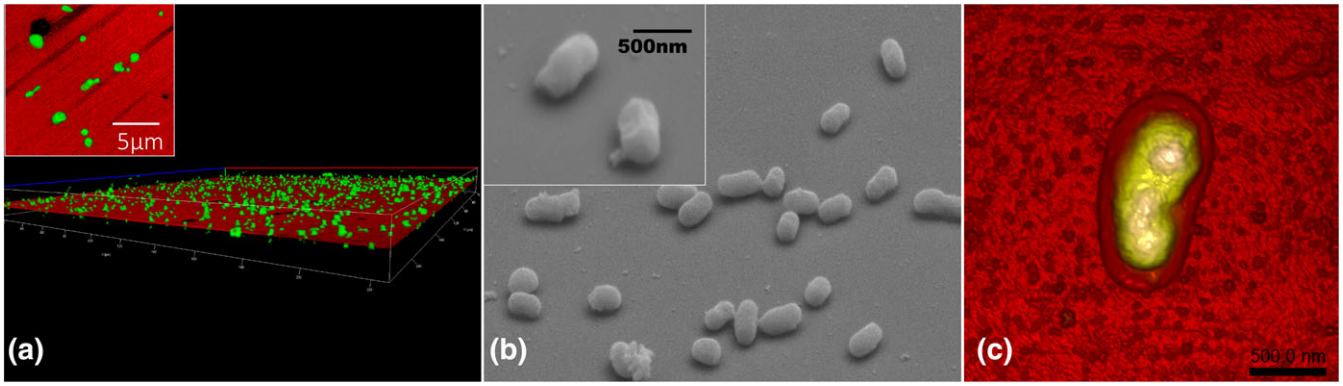

FIGURE 3 *Brucella abortus* on silicon (Si) surfaces functionalized with anti-*Brucella* antibodies. (a) 3D reconstruction of the confocal image of a Si surface (red) and green fluorescent protein (GFP)-*B. abortus* (green; 60× magnification). (b) Scanning electron microscopy (5,000× magnification). (c) Topographic image obtained by atomic force microscopy (AFM), *B. abortus* (yellow) and silicon wafer (red) [Color figure can be viewed at wileyonlinelibrary.com]

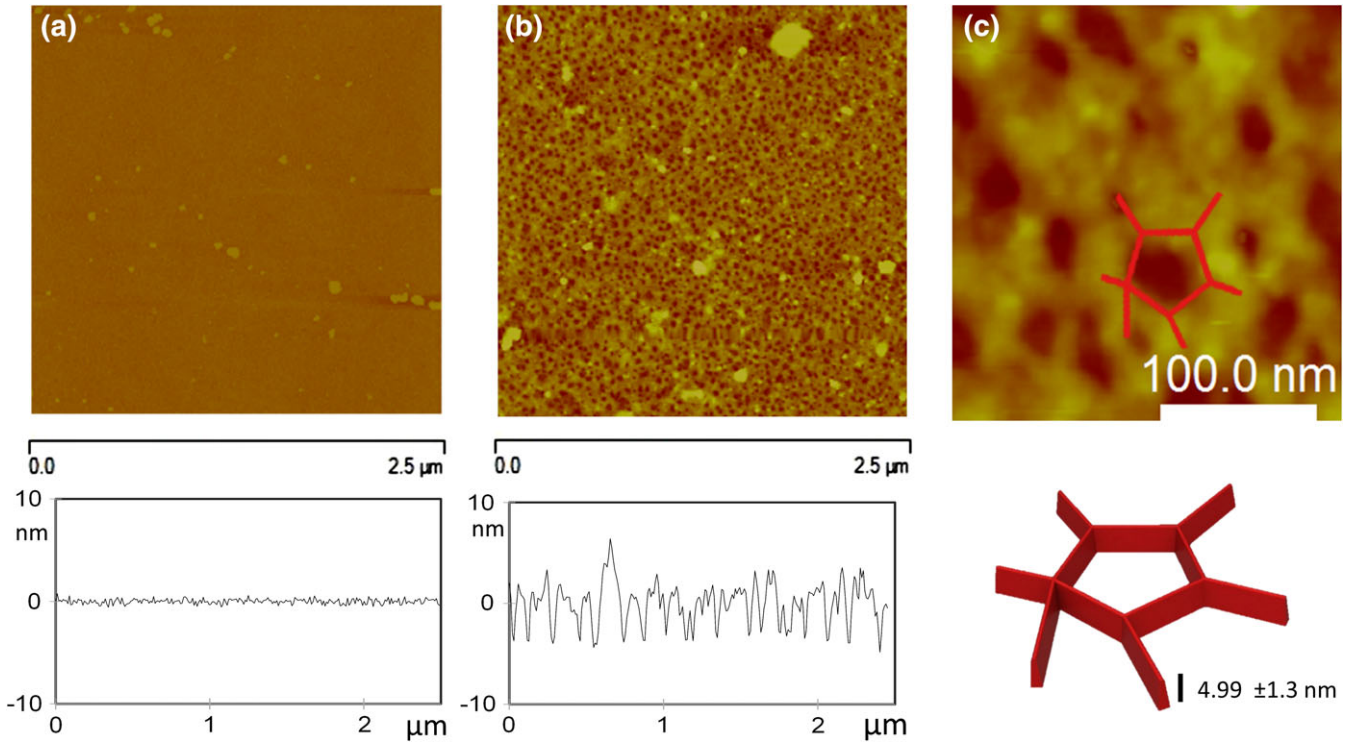

FIGURE 4 Topographic analysis of functionalized silicon (Si) wafers surfaces. (a) Top view and cross-section profiles of Si wafer surfaces before functionalization process. (b) Protein A adsorption on the surface of a silicon wafer. Top view topographical analysis and representative cross-sectional profile. (c) Magnification of protein A bound to the 3-glycidoxypropyltrimethoxysilane (GOTPS)-activated Si surface. Representative analysis of three areas examined at random on the functionalized Si wafer surface [Color figure can be viewed at wileyonlinelibrary.com]

the wafers functionalized with protein A and anti-*Brucella* antibodies was determined after incubation with *B. abortus* suspensions at the concentrations 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 CFU/ml. As a control to determine the background, a wafer without anti-*Brucella* antibodies incubated with a suspension of 1×10^9 CFU/ml of *B. abortus* was included. The results showed that the LOD was 1×10^6 CFU/ml, and the number of bacteria captured on the silicon surface increased with the number of bacteria in suspension (Figure 7).

The cross-reactivity of the antibodies is an important concern in immunodetection. No bacteria were found on the silicon wafer functionalized with anti-*Brucella* and protein A when it was incubated with up to 1×10^9 CFU/ml of *E. coli* O:157 (data not shown).

4 | DISCUSSION

Brucellosis, caused by bacteria of the genus *Brucella*, is a zoonotic disease with socioeconomic impact due to human illness and decreased animal productivity. Isolation of the bacteria is the most specific diagnostic test, but the rate of isolation is generally low (Araj, 2010). Alternative serologic diagnostic tests generally have low sensitivity and specificity, exhibiting cross reactivity with other gram-negative bacteria (Álvarez-Ojeda et al., 2015), therefore, the development of new detection techniques is important. Biosensors are capable of high sensitivity and specificity pathogen detection and are promising tools for the direct detection of *Brucella*.

The aim of this work was to design a platform for developing an immunosensor for direct *Brucella* detection on a surface coated with protein A to increase antibody–bacteria attachment. Silicon, a material used in design of optical, electrochemical, and mechanical sensors was chosen as the platform material (Jenison, La, Haeberli, Ostroff, & Polisky, 2001; Juan-Colás et al., 2016; Mendoza-Madrigal et al., 2013). The functionalization procedure was monitored by XPS at each step to assess the binding of the different chemical components to the silicon. Changes in silicon, carbon, and nitrogen signals that correlated with treatment with GOPTS or proteins were observed, as reported previously (Gao, Traeger, Shekhah, Idriss, & Wöll, 2009; Rasi Ghaemi, Harding, Delalat, Vasani, & Voelcker, 2013). Alkylsilane GOPTS generated chemically active surfaces on Si wafers that enabled the immobilization of proteins through the epoxy group and allowed covalent bonding to the amino groups of the Lys residues of proteins (Piehler, Brecht, Valiokas, Liedberg, & Gauglitz, 2000). This may be taken as evidence of the proper coating of the surface with protein A and antibody, although it cannot be concluded whether the antibody maintained its biological activity.

As described by others, substrates such as gold, hydroxylated silicon, and plastic have been functionalized with protein A in order to adsorb antibodies (Demirel et al., 2007; Lee et al., 2003; Ohnishi, Murata, & Hato, 1998). Cross-section profiles show a uniform height of 4.99 nm with protein A. Coen et al. (2001) reported a protein A cross-section profile height of 2 nm. This discrepancy may be explained by differences in the material used to adsorb the protein. Silicon likely promotes molecular arrangements different

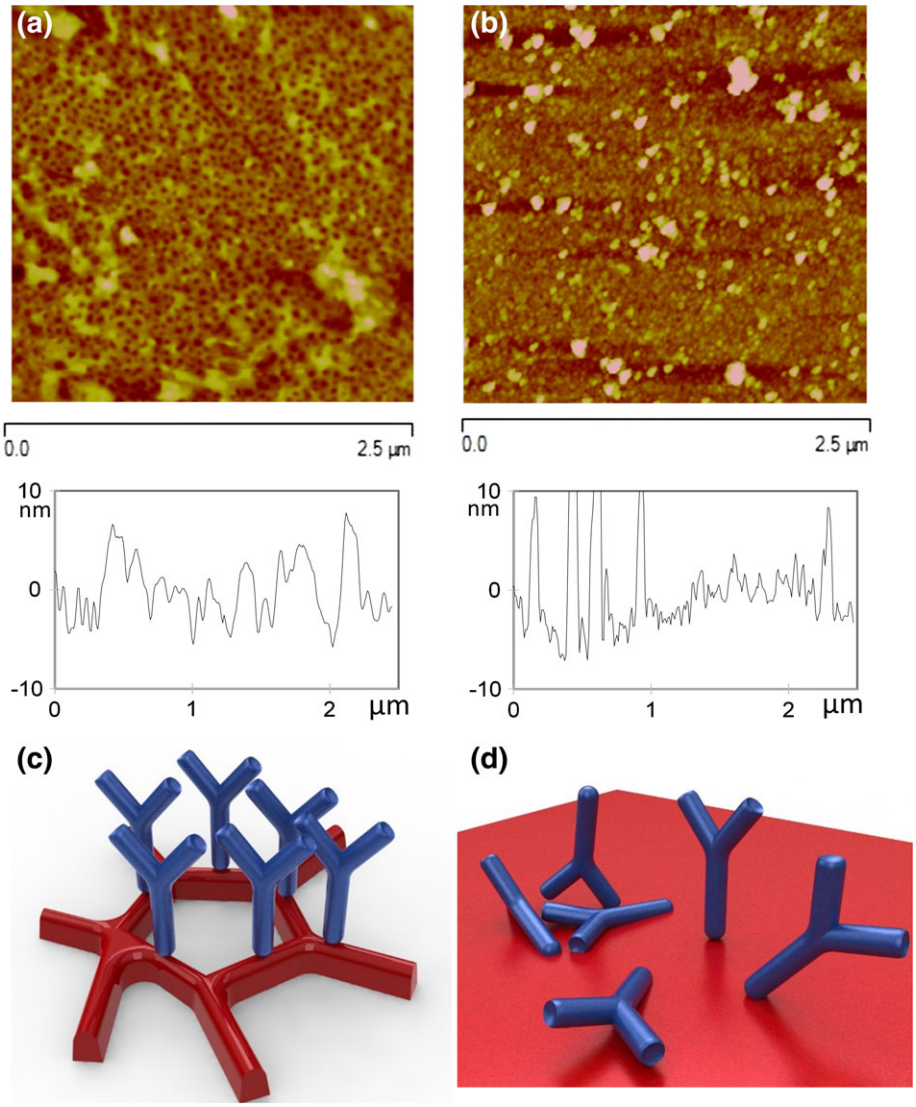

FIGURE 5 Comparative analysis of functionalization with protein A and antibodies. Topographic analysis and cross-sectional profiles of functionalized Si wafers. (a) 6 µg/ml anti-*Brucella* antibodies adsorbed on protein A. (b) 6 µg/ml anti-*Brucella* antibodies without protein A. Scheme of the orientation of the anti-*Brucella* antibodies directed by protein A (c) and without protein A (d) [Color figure can be viewed at wileyonlinelibrary.com]

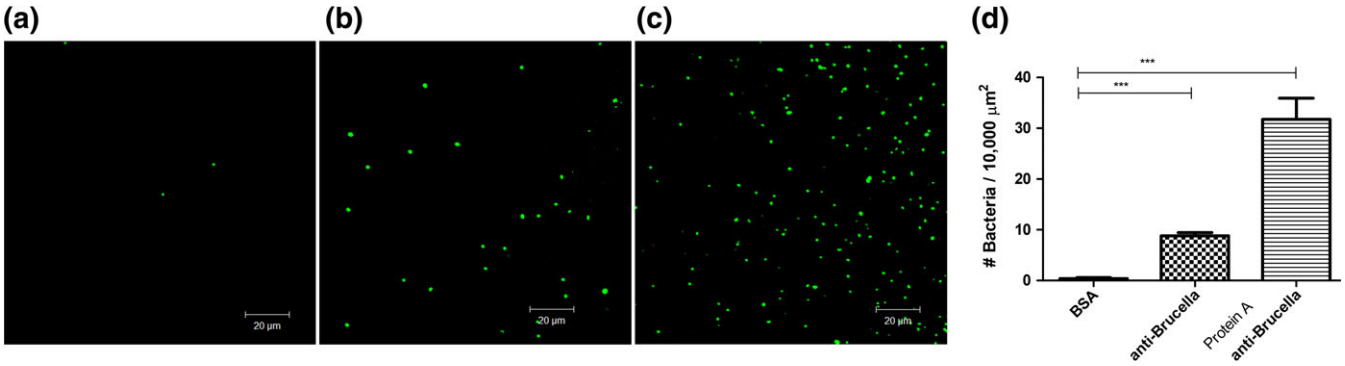

FIGURE 6 Binding of *B. abortus*- green fluorescent protein (GFP) on silicon (Si) wafer surfaces. (a) Surface without antibodies, (b) surface functionalized with antibodies, and (c) surface functionalized with protein A and antibodies. Selected images from three independent experiments. (d) Quantitative analysis of the bacteria attached to the surface (*$p < .05$) [Color figure can be viewed at wileyonlinelibrary.com]

than those induced by other materials. In addition, other factors that may affect the measured height are related with environment conditions. In this case, the sample was let dry at room temperature, which may have reduced the size of the proteins; however, the general structure was preserved. Finally, other factors that may affect are attributed to AFM settings like force set

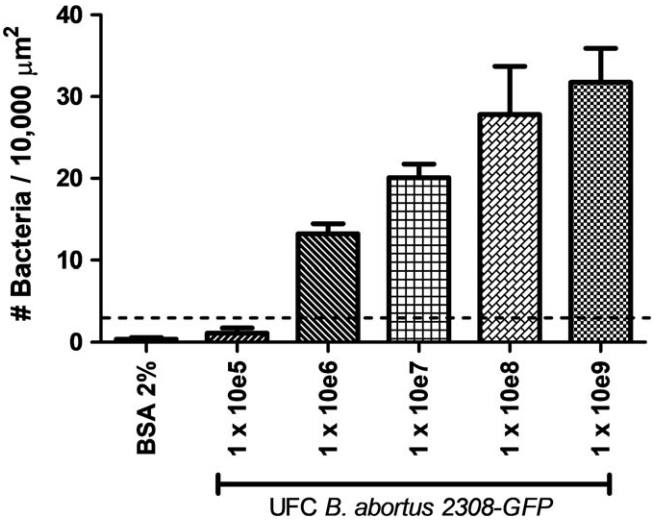

FIGURE 7 Determination of the limit of detection (LOD) of the silicon (Si) wafer coated with protein A plus immunoglobulin G (IgG) anti-*Brucella* antibodies

point, as Sweers, Stöckl, Bennink, and Subramaniam (2014) found in height of α-Synuclein fibril.

It has been reported that drastic variations in the topography of antibody-coated surfaces due to random orientation can negatively affect antigen binding specificity (Firestone, Shank, Sligar, & Bohn, 1996; Parolo et al., 2013). As observed by AFM, there are antibody aggregates on the GOPTS-activated Si wafer without protein A, which may not have the ability to interact with the antigen. Moreover, a decrease was seen in the antigen binding capacity of IgG antibodies immobilized on the surface, with a reduction in some cases >50%. This reduction is caused either by the reactivity of GOPTS and the protein or the random orientation of the antibodies when attached to the surface. In the latter case, some molecules are directed towards the surface by the Fab, so they can no longer recognize the antigen (Buijs, White, & Norde, 1997; Xu et al., 2006; Xu, Zhao, Lu, & Williams, 2007). The functionalized surface showed a peculiar distribution and assembly of the protein A-antibody complex. Furthermore, a symmetrical sectional profile was found to be representative of a unique antibody orientation (Welch, Scoble, Muir, & Pigram, 2017).

Several studies have demonstrated the enhancement of antigen-binding activity by oriented antibody immobilization (Bonroy et al., 2006; Welch et al., 2017). Protein A has a high affinity for IgG and orients the antibody in a position that leaves the recognition portion of the antibody free to bind to the antigen. As expected, antibodies orientation in this way attached up to three times more bacteria, approximately. Antibody orientation using protein A has been previously reported for soluble antigens. Such interaction has been measured by surface plasmon resonance (SPR) techniques, although the effect of the antibody orientation on the measurement of bacterial antigens interaction was not discussed. Previous work showed that antigen recognition increased by a factor of up to 200 by effect of antibody orientation using protein A, immobilized antibody fragment, or photo-cross-linking method without any detectable damage to antigen binding activity. (Alves, Kiziltepe, & Bilgicer, 2012; Mustafaoglu, Alves, & Bilgicer, 2015; Tan et al., 2010). The probable reason for the discrepancy between our results and previous studies is a difference in the SPR signal transduction mechanism used to measure the signals of the interactions between antigen and antibody. These studies utilized amplifiers prior to measurement, which increased the sensitivity of the technique. It is also probable that repulsion forces are enhanced as the bacterial antigens increase in size, so measurements may vary greatly between soluble and particulate antigens. In this work, the differences in recognition generated by the incorporation of protein A are due to the signal transduction, the nature of the antibody, and the size of the antigen (Trilling, Harmsen, Ruigrok, Zuilhof, & Beekwilder, 2013).

The performance of an immunosensor depends on the low non-specific adsorption to the solid support and the capability to immobilize antibodies while maintaining their activity (Sharma, Byrne, & O'Kennedy, 2016). For this reason, the functionalized surface was incubated with BSA in order to saturate the residual bonding sites and reduce non-specific binding to the uncovered surface (Sweryda-Krawiec, Devaraj, Jacob, & Hickman, 2004). Low reactivity was observed on non-functionalized Si surfaces with antibodies, indicating a successful passivation process. Moreover, antibody activity was not affected by the functionalization process on the GOTPS-activated Si surface since the antibody-coated surface attached bacteria.

The process of antibody immobilization can affect immunological activity and, consequently, the detection limit could be modified. Various authors have reported the development of biosensors for the direct detection of *Brucella*, but these biosensors require processing of the sample, including the extraction of genetic material or some other materials (Rahi et al., 2016; Rahi, Sattarahmady, & Heli, 2015). Using an electrochemical sensor for *B. melitensis* detection, Wu et al. (2013) reported a LOD of 1×10^4 CFU/mL in pure cultures of *B. melitensis*, which is two logarithms lower than the LOD reported in the present work. However, our platform is still not coupled with a system of detection. Coupling is expected to noticeably decrease the LOD since the sensitivity depends on the choice of the transducer and signal probe (Ansari, Alhoshan, Alsalhi, & Aldwayyan, 2010; Ju, Zhang, & Wang, 2011). Therefore, the current results could be useful to improve the design of immunosensors for detection of pathogens.

5 | CONCLUSIONS

The work presented here demonstrates that the use of an element such as protein A to direct the orientation of IgG anti-*Brucella* antibodies is an effective strategy that would be useful in designing an immunosensor for the detection of *Brucella* for diagnostic purposes. This strategy provides a versatile platform to be used in the development of biosensors aimed at the detection of bacterial antigens in biological samples as food and beverages or for diagnosis of brucellosis in man and cattle.

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