INTRODUCTION

The development of nanoscience has allowed to propose new ways of clinical treatment and diagnosis. Specifically, there has been a development of nano-optical devices based on plasmonic effects in metal nanoparticles. Plasmons in nanostructures are highly environmentally sensitive due to localized electric field enhancements, which have been employed, among other devices, in chemical sensors and biosensors. Nanostructured metals have overpassed the sensitivity performance of traditional surface plasmon resonance systems (surface plasmon polaritons travelling on continuous metallic films) (Haes & Van Duyne, 2004; Mauriz, Garcia-Fernandez, & Lechuga, 2016). The sensitivity of the localized surface plasmon approach was emergetly exploited with triangular silver nanoparticles in a generic biotin–streptavidin immobilization system (Haes & Van Duyne, 2002). The principle was then transferred to technological substrates (such as optical fibres; Chau, Lin, Cheng, & Lin, 2006) using different metals (mainly gold; Hiep et al., 2007) and nanoscale geometries (such as disks; Hanarp, Kall, & Sutherland, 2003) to solid substrates incorporating more complex immobilization strategies (such as the peptide nucleic acid–DNA binding; Endo, Kerman, Nagatani, Takamura, & Tamiya, 2005).

Among the diversity of metallic nanoparticles, gallium nanoparticles (GaNPs) have the advantage that they can be deposited in a fast, simple and cheap way on a wide variety of substrates, that is by applying Joule-effect thermal evaporation. As result of their...
deposition, hemispherical particles are obtained over the substrate. Consequently, one can detect two resonant modes with a broad energy splitting, one in-plane (vibrations parallel to the substrate) and the other out-of-plane (resonance direction perpendicular to the substrate surface). Furthermore, by evaporating different amounts of Ga one can change the GaNPs size in order to tune the plasmon resonances to a desired region of the electromagnetic spectrum from the IR to the UV range (Catalán-Gómez, Redondo-Cubero, Palomares, Nucciarelli, & Pau, 2017). Several GaNP biosensors have been designed previously aiming at the detection of glutathione (Marín et al., 2015) or nucleotide polymorphisms in label-free DNA (Marín et al., 2016).

For the development of a biosensing platform, a conjugation layer conforming the interface between the inorganic nanoparticles and the selective biomolecules is required. In particular, aminopropyltriethoxysilane (APTS) is a silane agent bearing primary amino groups that can create stable covalent bonds with biomolecules. APTS molecules can serve as trapping layer of immunoglobulins (IgGs) through different approaches such as self-assembly (Kim, Cho, Seidler, Kurland, & Yadavalli, 2010; Meskini et al., 2007), plasma-enhanced chemical vapour deposition (Arroyo-Hernández et al., 2007) or hybrid approaches (Manso-Silvan et al., 2007). In the latter case, a condensing molecule such as titanium isopropoxide (TIPT) ensures the stability of the union between the active substrate and the trapping APTS molecules.

Fibrinogen (Fbg) is a relevant blood plasma protein whose unusual concentration can be associated to, not only fibrinogenemia disorders (Casini, Brungs, Lavenu-Bombled, Vilar, & Neerman-Arbez, 2017), but also to cardiovascular diseases (Stec et al., 2000; Wolberg, 2016), acute inflammation (Wolberg, 2016), pregnancy (Abbassi-Ghanavati, Greer, & Cunningham, 2009) or several kinds of cancer (Repetto & De Re, 2017; Sheng et al., 2013). Fbg plays a role in all of these diseases as a result of its decisive structural role in inflammation and coagulation (Hennigs et al., 2014). The healthy levels of Fbg in blood plasma range from 4–10 µM and, although there is controversy about the pathological levels, extended blood tests are strongly suggested for Fbg concentrations below 1 µM or over 20 µM (Oswald, Hunt, & Lazarchick, 1983). Consequently, the monitoring of Fbg concentration has become a reference method, not only to help in the diagnosis or prognosis of a disease (Sheng et al., 2013), but also to monitor the progress of a therapy or the potential interfering effects of new pharmaceuticals (Ogston & Ogston, 1966). There are different techniques to measure the Fbg concentrations, such as the Clauss Fbg assay, enzyme-linked immunosorbent assays (ELISAs) or the clot formation assay (Mackie, Kitchen, Machin, Lowe, & Brit, 2003). These methods should be considerably simplified on the way to user-friendly devices, manageable by the patient and with diagnostic significance, which could become useful for personalized medicine (Ahmed, Saaem, Wu, & Brown, 2014). The colour shift is in fact one of the key phenomena induced upon plasmonic biorecognition through clustering that could facilitate bioanalytical friendliness (Kim et al., 2019; Thaxton, Rosi, & Mirkin, 2005).

In this study, we seek to determine Fbg concentrations within the relevant clinical range using a GaNPs plasmonic platform biofunctionalized through a simplified direct immune assay that implies the immobilization of a Fbg antibody. The aim is to follow the cascade of enabling steps with the appropriate structural, morphological and spectroscopic techniques that lead to the evaluation of the bioanalytical performance by ellipsometric interrogation.

### 2 | EXPERIMENTAL

The development of the biosensing platform consists in a cascade of 5 steps that is illustrated in Figure 1: deposition of GaNPs (1), hybrid titanate-aminosilanization (2), biofunctionalization by surface immobilization of the IgGs (3), surface blocking with bovine serum albumin (BSA) (4) and direct label-free immune detection (5). The following section provides the experimental details followed in each of the processing steps.

#### 2.1 | Preparation of the GaNPs platform

GaNPs were deposited by Joule-effect thermal evaporation on one-side polished <100> Si substrates using an Edwards E306 vertical system. The deposition time was 1 min, under constant power of 50 W and working pressure of 1.5 $10^{-5}$ mbar (initial base pressure of 2.0 $10^{-7}$ mbar). The substrate-metal-source distance was kept constant at 200 mm to ensure a homogeneous distribution of circa 55 mg Ga (99.9999% purity).

The aminosilanization of the GaNPs was carried out by deposition of a hybrid APTS-TIPT silanization film prepared by a sol-gel route. The APTS-TIPT solution was prepared by mixing identical volumes of a 0.4 M TIPT silanization film in ethanol, with 0.46% HCl and 0.24% H$_2$O (v/v) and a 0.01 M APTS solution in ethanol. The sol was diluted to 25% in ethanol (v/v) prior to dispersion (50 µl) by spin-coating at 2000 rpm during 1 min (Moreno-Cerrada et al., 2019).
2.2 | Biofunctionalization and biosensing

For the biofunctionalization, Fbg IgGs (polyclonal, FITC labelled, Thermo scientific) were immobilized on the silanized surface. We prepared IgG solutions in 2-(N-morpholino) ethanesulphonic acid (MES) with N’-ethylcarbodiimide hydrochloride (EDC; Oswald et al., 1983). To optimize the solution for IgG cross-linking, we considered four IgG concentrations in the 0.5–100 µg/mL (3.33–667 nM) range. After 120 min of promoted binding, the IgG rich medium was removed and the samples were rinsed in phosphate buffer saline (PBS) once and Milli-Q water twice. After drying, the relative IgG surface coverage was evaluated by spectroscopic ellipsometry. For the characterization of the complete immune reaction by ellipsometry, the GaNP surfaces with immobilized IgGs were blocked with bovine serum albumin (BSA, 1%, w/v in PBS) at 37°C during 30 min to prevent unspecific interactions of Fbg with the surface. Fbg solutions were prepared in PBS within the clinical range of interest. Six different Fbg concentrations in the 0.1–23 µM range were considered. The detection reactions were promoted by incubation of the biofunctionalized GaNPs with the Fbg solutions for 30 min at 37°C. After removal of the Fbg solutions, the samples were rinsed once with PBS and twice with Milli-Q water. After drying in atmospheric conditions, the samples were measured in the ellipsometer.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of the GaNPs platform

The transducing platform for the sensing of fibrinogen consists of the GaNPs evaporated on top of a Si substrate and the aminosilanization layer formed by titanate condensation after spin casting (as illustrated in the two first designs of Figure 1). Figure 2a shows the SEM image of the GaNPs deposited on a Si substrate. A rough surface conformed by spherical particles can be observed in agreement with previous observations. The cross-section view at the bottom of Figure 2a demonstrates that the GaNPs are in fact hemispherical. The dispersion on size of the GaNPs emerges from the coalescence of smaller liquid GaNPs evaporated on the Si surface, which tends to create a bimodal distribution. Their mobility is favoured by the low surface tension at the liquid-Ga/Si substrate, which prevents the formation of a conformal film. The surface of the GaNPs is only stabilized upon formation of a thin GaOx layer on the particles, taking place during atmospheric exposure (Catalan-Gomez et al., 2017). Therefore, the average diameter of the smaller GaNPs obtained for the particular amount of evaporated Ga is 33 ± 5 nm, with bigger particles of 100 ± 15 nm.

The aminosilanization step was achieved by spin casting a 0.0125M:0.05M APTS:TIPT solution at 2000 rpm. The topography
of the systems is not drastically affected by the aminosilanization step. The overall mean particle size is conserved and only a minimal blurring effect is evidenced as an issue of the dielectric character of the APTS-TIPT layer (Figure 2b). Such dielectric character is responsible of an increase in charge accumulation and the interference of the electrostatic charge with secondary (and other) non-elastic electrons used for imaging. The cross-section view confirms the lack of alteration of the hemispherical shape of the particles and only suggests a slightly preferential condensation of the aminosilanization mixture at the base of the GaNPs, which diminishes the apparent contact angle of the hemispherical GaNPs with respect to the Si substrate. The high surface tension of the APTS-TIPT solution and its acidic character, along with the surface oxidation of the GaNPs, suggests a tendency for the GaNPs to get coated by the solution. However, the mentioned preferential condensation on the GaNPs edges suggests that the coating is not perfectly conformal.

The aminosilanization process was further studied by obtaining a spectroscopic evidence of the molecular structures incorporated on the GaNPs/Si structure. FTIR (ATR) spectra of the APTS-TIPT and pure TIPT film on Si showed the presence of the characteristic covalent bonded structures of the precursors, as observed in Figure 3 (Langlet, Jenouvrier, Kim, Manso, & Valdez, 2003; Moreno-Cerrada et al., 2019). In fact, the characteristic bands of TIPT condensates, such as Ti-O-Ti vibrations at circa 615, 730 and 880 cm⁻¹ were identified. Vibrations at 1,110 cm⁻¹, which are related to Ti-O-C bonds, denote the limited degree of condensation of the TIPT platform. The presence of carboxylate COO⁻ related to TIPT condensation is evidenced by the vibration at 1615 cm⁻¹. Finally, the strong band at 3,300 cm⁻¹ related to hydroxyl by-products reinforces the idea of the limited condensation of the pure TIPT film.

The spectrum of the APTS-TIPT hybrid is relatively richer in the diversity of bands as could be expected from the new molecular structures present on the surface. However, most relevant bands overlap with TIPT characteristic bands, such as the δNH₂ deformation band, (also related to imine formation and oxidation of amindicarbonate salts) overlaps with the COO⁻ band at 1615 cm⁻¹. Nevertheless, this band increases considerably when APTS is included in the film. Also, the C-N bond-related band at circa 1,110 cm⁻¹ overlaps partially with the ι(Ti-O-C) of the pure TIPT condensate, but the diversity of bands in the APTS containing film is evident. Finally, also the stretching band of the amino group (NH₂) in APTS at 3,200 cm⁻¹ overlaps with the wide absorption related to hydroxyls. The well-defined presence as a shoulder of the above-mentioned band and the alkyl related bands at circa 2,900 cm⁻¹ are a trustable indication of the integration of the aminopropyl group in the APTS-TIPT film.

We evaluated the integration of the aminosilanization film with the GaNPs structure by microanalytical techniques. We used resonant RBS (non-Rutherford) in order to detect the distribution and amount of elements heavier than the probing ions (4.035 MeV He). The RBS spectra shown in Figure 4a allow comparing the integration of a pure TIPT film on top of the GaNPs with an aminosilanized analogue. Both the spectrum profile and the simulation confirm that the Ga layer is very similar in both samples, with an averaged thickness of 6 nm, including a 1 nm thick Ga₂O₃ film.

The roughness of the GaNPs introduces some uncertainty on the determination of the TIPT or APTS-TIPT properties. However, the presence of the TiOₓ overlayer is justified by the simulated spectra. Relevantly, the stoichiometry of the APTS-TIPT film is clearly poor in Ti and rich in C with respect to the pure TIPT film. For both situations, the detection of the Ti peak suggests a coating on top of the GaNPs layer, reinforcing the information provided by SEM. In order to correlate this C enrichment with the presence of organic species, we performed also a parallel ERDA analysis, which allows determining the amount of hydrogen. The results shown in Figure 4b illustrate the higher concentration of H in the APTS containing and free TIPT films.

**FIGURE 3** FTIR (ATR) spectra of the APTS-TIPT (black line) and pure TIPT (light grey line) film on Si. The characteristic bands of the aminopropyl group are indicated

**FIGURE 4** a, Non-RBS spectra from the surfaces of GaNPs deposited on Si and coated with TIPT (bottom) and APTS-TIPT (top). The inset highlights the compensation of Ti composition induced by the integration of the C-rich silane APTS. b, ERDA spectrum revealing H retention in the APTS containing and free TIPT films.
indication of retention of protonated molecular structures by FTIR (NH₂ and CH₂).

The APTS-TIPT/GaNPs/Si stacks were further characterized by ellipsometry as a previous step to biofunctionalization. The imaginary part of the pseudo-dielectric function was selected as the most prominent function to reveal the evolution of GaNPs based bointerface (Figure 5). Specifically, the in-plane resonance mode was our peak of reference. The corresponding function for the APTS-TIPT/GaNPs structure showed a clear asymmetric resonant peak at circa 570 nm, which served as starting reference for the biofunctionalization and sensing steps. The width observed in the SPR of the APTS-TIPT/GaNPs platform is due to the multimodal size distribution of the nanoparticles (Marin et al., 2015).

3.2 | Biofunctionalization and sensing

In a first stage, we optimized the immobilization of Fbg-IgGs on the surface of the APTS-TIPT/GaNPs structure by estimating the surface coverage using ellipsometry and considering antibody seeding onto the surface within the 0.5-100 µg/mL concentration range.

The test indicated saturation of the coverage, interpreted as a surface organic layer on top of a flat APTS-TIPT model sample, from concentrations of 50 µg/mL. The overall thickness of the APTS-TIPT layer was estimated in 20 nm, which appears as an overestimation if compared with the SEM results.

In any case, such thickness is compatible with retention of a sensing capability in a nanostructured system exhibiting localized surface plasmons (Pellacani et al., 2019). As we can observe in Figure 6a, the thickness of the silane–protein layer in a flat model gets saturated for concentrations greater than 66.67 µg/mL (444.70 nM). This was considered then as the reference concentration for the biofunctionalization step.

The pseudo-dielectric function corresponding to the IgG biofunctionalized structure is presented in Figure 5, showing a moderate intensity decrease in the resonance with respect to the silanized sample. The next step of the biofunctionalization consisted in the blocking of unspecific interactions. This was achieved by adsorption of BSA, which led again to a new moderate decrease of intensity of the imaginary part of the pseudo-dielectric function (follow the trend in Figure 5). After complete biofunctionalization, it can be outlined that the process induces a screening of the electric field (decay of the intensity) but there was no significant modification of the plasmon resonance frequency. This situation established the starting point for the determination of the biosensing platform performance.

To evaluate the performance of the biosensing platform, Fbg solutions of different concentrations (with a logarithmic increment) were incubated on the biofunctionalized platforms. In parallel, the plasmonic signal determined by ellipsometric measurements was examined (see the evolution of the response for incremental concentrations in Figure 5). The curves are the representation of one out of the four experiments performed. The general trend agrees with a decrease of the resonance intensity and a simultaneous redshift. However, the platform was observed to have no relevant sensitivity for the lowest Fbg concentrations (0.1–0.3 µM). In fact, a soft reverse behaviour on plasmon intensity could be evidenced, which pointed...
out to a strong competition of Fbg for surface sites, being able to displace BSA from the surface. This possibility suggests also a low specificity of the Fbg binding at low concentration. The fact that the intensity is reversed upon binding of low concentration BSA suggests that the bigger protein (Fbg, circa 350 KDa) changes conformation to displace the smaller protein (BSA, circa 65 KDa). Competitive adsorption conditions and differentiated conformational changes between proteins count in fact among the main aspects leading to unexpected affinity/binding. A full determination of the final adsorption state is viable only in particular cases by performing kinetic studies and applying appropriate theoretical models (Hlady & Buijs, 1996). In fact, models have been already developed to explain adsorption reversal processes due to conformational changes upon protein binding (Brusatori & Van Tassel, 1999), which leads to definition of a rate of exchange between proteins (Wahlgren & Arnebrant, 1991).

Nevertheless, the general trend to lower intensity and redshift for increasing Fbg concentration is confirmed for the samples tested in the high Fbg concentrations range. The wavelength of the plasmon increased versus the Fbg concentration in the range of 1–23 µM (see the trend of the resonance maxima in the magnified inset of Figure 5). The statistical significance is demonstrated in the calibration curve presented in Figure 6b, which integrates the results of the four samples. Although the statistical results have a margin for improvement, we highlight the matching of the signal variation range with the healthy range of Fbg concentration. In fact, the logarithmic scale representation allows confirming that, within the healthy state range (1–10 µM), the curve presents a linear behaviour with respect to the logarithm of the concentration, with a tendency to saturation at higher concentrations (23 µM). As already discussed in the discussion of the pseudo-dielectric function, for concentrations smaller than 1 µM the Fbg concentration changes did not originate a clear shift with respect to the biofunctionalized platform. From the general trend of the calibration curve, we shall highlight that the proposed platform can differentiate between three different states with respect to Fbg concentration, which are in agreement with the low concentration, healthy range and high level. Such three state "logic" levels could serve to design a fast transducing system using this biosensing platform, which could help performing a fast screening of diseases related to high or low Fbg concentration in blood plasma.

4 | CONCLUSIONS

A biosensing platform based on the integration of GaNPs within an aminosilane–titanate trapping layer has been developed. The obtained distribution of GaNPs is narrow enough to produce well-defined plasmonic resonances that were read out by ellipsometry. The aminosilane–titanate trap acts as a transition layer from the inorganic (plasmonics) to the organic (aminopropyl group) interface, which is revealed by both vibrational and ion microprobe techniques. This kind of transition layer could be generalized as first modification step to activate the biofunctionalization cascade of alternative transducing platforms. The final configuration of the biosensing platform was evaluated by ellipsometry and was confirmed to be plasmon active. The optimization of the platform was followed by the evaluation of the bio-sensitization of the device upon immobilization of a Fbg antibody and blocking with BSA.

The biosensing platform was then assayed with different concentrations of Fbg and monitored again through ellipsometry. The pseudo-dielectric function allowed as identifying a trend towards plasmonic screening and resonance redshift for increasing concentrations of Fbg. Concentrating on the resonance wavelength, no significant shift is induced for concentrations below 1 µM, which is followed by a linear increase in the logarithmic scale up to 10 µM. Then, a tendency to saturation has been identified for concentrations of 23 µM. Relevance, the ranges of the response of the developed biosensing platform are related to Fbg concentrations associated with the healthy state and risk of several diseases. This opens the possibility to incorporate the developed platform into portable reading devices to increase the catalogue of personalized medicine devices. The principle of colour shift using plasmonics is currently expanding with a target in proposing platforms with colour shifts that can be identified by the naked eye. To achieve this objective our platform requires further progress, as a labelling of the target with additional plasmonic NPs.

Finally, a margin to improve the platform exists, namely if samples with homogeneous GaNP sizes could be obtained, which would improve the reliability of any device incorporating this biosensing platform and contribute to minimize the statistical errors during biosensing.

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