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Tackling reproducibility in microcantilever biosensors: a statistical approach for sensitive and specific end-point detection of immunoreactions

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In the last decade, microcantilever biosensors have shown enormous potential for highly sensitive label-free detection of nucleic acid and proteins. Despite the enormous advances, the promise of applications of this technology in the biomedical field has been frustrated because of its low reproducibility. Here we tackle the reproducibility issue in microcantilever biosensors and provide the guidelines to minimize the deviations in the biosensor response between different assays. We use as a model system the label-free end-point detection of horseradish peroxidase. We choose the end-point detection mode because of its suitability for implementation in the clinical field that requires simplicity and point-of-care capability. Our study comprises the analysis of 1012 cantilevers with different antibody surface densities, two blocking strategies based on polyethylene-glycol (PEG) and bovine serum albumin (BSA) and stringent controls. The study reveals that the performance of the assay critically depends on both antibody surface density and blocking strategies. We find that the optimal conditions involve antibody surface densities near but below saturation and blocking with PEG. We find that the surface stress induced by the antibody–antigen binding is significantly correlated with the surface stress generated during the antibody attachment and blocking steps. The statistical correlation is harnessed to identify immobilization failure or success, and thus enhancing the specificity and sensitivity of the assay. This procedure enables achieving rates of true positives and true negatives of 90% and 91% respectively. The detection limit is of 10 ng mL^{−1} (250 pM) that is similar to the detection limit obtained in our enzyme-linked immunosorbent assay (ELISA) and at least two orders of magnitude smaller than that achieved with well-established label-free biosensors such as a quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) sensor.

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1 Introduction

In the biomedical field, end-point detection bioassays such as ELISAs are essential tools because of their simplicity, high-throughput, and suitability for their use at the point-of-care. End-point bioassays are significantly constrained by the need of sample labeling with fluorescent or colorimetric tags for subsequent detection. End-point bioassays require labeling of the samples usually with fluorescent dyes, or colorimetric enzymes. However, the labelling is laborious and lengthy, and limits the number and types of targets that can be studied. Moreover, it can alter the biological activity of the labelled molecule, altering the result of the assay.^{1,2} Label-free bioassays rely on the measurement of an inherent property of the targeted

molecules.^{1–3} The most widespread biosensors based on this concept are the quartz crystal microbalance and the surface plasmon resonance sensor. The first one is based on the measurement of the downshift of the mechanical resonance frequency of the quartz crystal previously sensitized with biomolecular receptors as a consequence of the added mass brought by the binding of the analyte on the crystal surface. The second one is based on the optical measurement of the shift of the plasmon resonance of a thin metallic surface functionalized with biomolecular receptors as a consequence of the increase of the optical thickness brought by the captured molecules. A serious limitation of the label-free techniques is the poor sensitivity in comparison with label-based techniques. In addition, they are prone to high background noise by nonspecific binding.

The need of label-free techniques with higher levels of sensitivity has boosted the development of an increasing number of label-free techniques based on nanotechnology such as optical whispering gallery microcavity sensors, plasmon

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resonance sensors based on nanostructures, electrical nanowire sensors, suspended microchannel resonators and micro- and nanocantilever sensors, to name a few.^{4–9} Whereas these technologies have brought about unprecedented levels of sensitivity, some of them have shown multiple pitfalls and issues regarding specificity, reproducibility and reliability, and hence they are still far from the implementation in the biomedical field.^{10,11} In fact, statistical analyses of the false positive and false negative rates of many of these emerging biosensing techniques are scarce in the literature. Despite the important challenges of attaining reproducible and reliable assays with nanosensors, little attention has been paid to the variability of nanosensor responses in the last decade.

In this work, we examine the capability of micro- and nanocantilever biosensors in the static mode^{12–17} for end point label-free immunoassays. In these devices, molecular recognition between the analyte and the receptor monolayer anchored on one side of a tiny cantilever induces a nanoscale bending as a consequence of the surface stress variation on the functionalized surface.¹⁸ The surface stress variation depends on the intermolecular interactions between the biomolecular complexes anchored on the cantilever surface such as van der Waals forces, electrostatic forces, hydrophobic interactions, hydrogen bonding, steric forces, *etc.* Microcantilever-based biosensors can be produced at the wafer scale by adopting semiconductor technology, which results into a large number of identical devices at relatively low cost. In addition, the size of these devices has been increasingly reduced which leads to higher device density and, more importantly, implies higher sensitivity. In addition, high throughput detection can readily be achieved by fabricating hundreds of cantilevers in arrays and by the fast read-out of their response by optical^{19–22} or electrical techniques.^{12,23}

So far, surface-stress based nanomechanical biosensors have been applied for real-time measurements in the liquid environment. The applications include detection of proteins, DNA, RNA and pesticides with high levels of sensitivity.^{14,19,24–30} Here, we study for the first time the potential of surface-stress-based nanomechanical biosensors for end-point analysis. Our vision is to join the unparallel features of these devices demonstrated in liquid; label-free detection, high sensitivity and high specificity, with the simplicity and high-throughput capability of end-point bioassays that is the preferred choice in the biomedical context. In this study, we place the focus on the variability of the sensor response rather than on the determination of detection limits. In fact, very little is known about the rate of false positives and false negatives of nanomechanical biosensors, while high throughput measurements, including statistical evaluation of acquired data, will be the key to find nanomechanical sensors in every biochemistry laboratory in the near future. In order to obtain quantitative data, our study has involved 1012 cantilevers, in which we measured the surface stress induced during antibody immobilization, blocking steps and antigen recognition. The selected antigen is the horseradish peroxidase (HRP) that exhibits the advantage that it can be subsequently detected by spectrophotometry, in order to accurately quantify the molecular recognition reaction on the

cantilever surface. In addition, the small size of the antigen, about 4 nm, allows assessing the capability of the technique for specific detection of small molecules.

2 Materials and methods

2.1 Microcantilever functionalization

Cleaning. Arrays of eight silicon microcantilevers purchased from Concentris GmbH (Basel, Switzerland) were used. The cantilevers are 500 μm long, 100 μm wide, and 1 μm thick. The arrays were cleaned with piranha solution (H_2SO_4 and 30% H_2O_2 , 3 : 1) (**caution:** piranha solutions are extremely corrosive, reactive, and potentially explosive) for 15 minutes, and then they were rinsed three times with Milli-Q water (18.2 $\text{M}\Omega\text{ cm}$) and dried under a stream of dry nitrogen.

Silanization. The microcantilevers were dipped into a 0.2% solution of (3-glycidyloxypropyl)trimethoxysilane (98%, Sigma-Aldrich) in dry toluene (99.8%, Sigma-Aldrich) overnight at room temperature. After that, the samples were washed with toluene (ACS reagent, Sigma-Aldrich), Milli-Q water and dried under nitrogen. A 100 mM N_α, N_α -bis(carboxymethyl)-L-lysine hydrate (97%, Sigma-Aldrich) solution in 50 mM carbonate buffer (pH 9.5) was prepared and the microcantilevers were incubated overnight at room temperature under gentle agitation. Microcantilevers were then washed with 50 mM carbonate buffer pH 9.5, Milli-Q water and dried under dry nitrogen.

Activation. The carboxyl groups at the microcantilever surface were activated by immersion in a mixed solution of 100 mM N -(3-dimethylamino propyl)- N' -ethylcarbodiimide hydrochloride (EDC) and 150 mM N -hydroxysuccinimide (NHS) both dissolved in 10 mM 2-(N -morpholino)ethanesulfonic acid (MES, pH 5.5). These chemicals were purchased from Sigma-Aldrich. The microcantilevers were incubated for 30 minutes at 37 °C under gentle agitation. The samples were extensively rinsed with 10 mM MES.

Antibody immobilization. The used antibodies, anti-peroxidase antibody produced in rabbit (Sigma-Aldrich) and the control antibody against human chorionic gonadotropin hormone (anti-hCG, clone 5014), were diluted in Milli-Q water at a concentration of 4 mg mL^{-1} , and the solutions were purified by using a dialysis kit with a 8 kDa molecular weight cut-off (GE Healthcare) overnight at 4 °C. The concentration of the antibody solution after the dialysis was determined using the colorimetric Bradford's method (Coomassie Plus Assay Kit from Thermo Scientific).³¹ A calibration curve was made using bovine serum albumin (BSA) as a protein standard. The range of linearity of the assay was from 5 $\mu\text{g mL}^{-1}$ to 2000 $\mu\text{g mL}^{-1}$. Solutions of 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 250 $\mu\text{g mL}^{-1}$ of the antibodies were prepared in 10 mM MES (pH 5.5). The microcantilevers were incubated for 2 hours at 37 °C. The microcantilevers were then washed with 10 mM MES and incubated for 45 minutes at 37 °C with 10 mM sodium phosphate buffer (pH 8.0, 0.3 M NaCl) in order to desorb antibodies which are not covalently attached to the surface.

Blocking. After the antibody immobilization, the microcantilever surface was blocked to prevent non-specific adsorptions. Microcantilevers were dipped into a 1 mg mL^{-1} (aminoethyl)polyethylene glycol (PEG – 5000 Da) or bovine

serum albumin (BSA) in 10 mM MES (pH 5.5) for 1 hour at room temperature under agitation. The samples were washed and dried under a nitrogen stream. PEG and BSA were purchased from Sigma-Aldrich.

Antigen-antibody recognition. Microcantilevers were incubated overnight at 4 °C in 1 $\mu\text{g mL}^{-1}$ peroxidase from horseradish (HRP) or 1 $\mu\text{g mL}^{-1}$ lysozyme from chicken white egg (control) in 10 mM sodium phosphate buffer at pH 7.0. All these compounds were purchased from Sigma-Aldrich. The microcantilevers were then extensively washed with 10 mM sodium phosphate buffer, Milli-Q water and finally dried under a stream of nitrogen. For the limit of detection experiments, HRP solutions of 0.01, 0.1, 1 and 10 $\mu\text{g mL}^{-1}$ were used.

Enzymatic assay of peroxidase. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (98%, TLC) from Sigma-Aldrich was used as a substrate in the enzymatic peroxidase detection assay. The arrays of microcantilevers were dipped into a 1 mL of a solution ABTS-H₂O₂ (100 : 1 v/v) in 50 mM sodium phosphate buffer (pH 6.0). The increase in the absorbance at 405 nm was measured for 10 minutes (BioPhotometer Plus, Eppendorf).

Fluorescently labeled antibody. An aliquot of 1 mL of 0.8 mg mL⁻¹ of anti-peroxidase antibody solution in 0.9 mL of 100 mM sodium bicarbonate (pH 9.5) was incubated with 0.1 mL of 1 $\mu\text{g mL}^{-1}$ of Rhodamine B isothiocyanate (Sigma-Aldrich) for 2 hours at room temperature. The solution was applied to a PD-10 column (GE Healthcare Bio-Science) using 10 mM sodium phosphate buffer (pH 7) as the exchange buffer to eliminate the excess of Rhodamine. In order to check if the isoelectric point of the antibody has not been modified after the fluorescent labeling, the isoelectric point of the modified antibody was measured using the Pharmacia Phast System and PhastGel® IEF 3–9. The isoelectric point of the fluorescently labeled antibody was similar to that of the unmodified antibody, between 5.85 and 6.55.

Radiolabeling assay. Iodine 125 was used for labeling the antibodies at tyrosine residues. 10 μL of 2 mg mL⁻¹ of anti-HRP solution prepared in 0.1 M sodium phosphate pH 7.5 were incubated for 15 s at 25 °C with 50 μL of 0.5 M sodium phosphate pH 7.5 containing 60 μCi of Na¹²⁵I and 264.0 nmol of Chloramine T as oxidizing agents. The reaction was immediately terminated by the addition of 60 μL of 0.5 M sodium phosphate pH 7.5 containing 148.0 nmol of tyrosine (previously dissolved in acid and neutralized) and 3.6 μmol of potassium iodide. Then, the radiolabeled antibody was purified by Sephadex G-25 gel filtration column equilibrated with 20 mM Tris pH 7.5, 150 mM NaCl and dialyzed against the corresponding buffer that will be used in the immobilization process for 12 h at 4 °C. The labeled antibody was immobilized as previously described. The gamma emission of the functionalized cantilever arrays was measured for 60 s in a LKB Wallac 1282 Compugamma Universal Gamma Counter.

3 Results and discussion

3.1 Microcantilever functionalization

Arrays of eight cantilevers fabricated in monocrystalline silicon were used (Concentris). The cantilevers are 500 μm long,

100 μm wide and 1 μm thick. The nanometer-scale out-of-plane displacements of the cantilevers were measured in air at room temperature by scanning laser beam deflection microscopy (SCALA, MecWins)^{21,22} (Fig. 1(a) and 1(b)). A scheme of the methodology employed for the cantilever functionalization and the immunoassay is shown in Fig. 1(c). The method comprises silanization, antibody binding, blocking, and exposure to the antigen solution (see Materials and methods). The out-of-plane cantilever displacement along the longitudinal axis was measured after each incubation step as shown in Fig. 1(b). The chip surface provides a reference to follow surface stress changes after the successive incubation steps. The surface stress is derived by fitting the curves to a second-order polynomial and applying the Stoney's equation.³² This method requires an accurate determination of the cantilever thickness that can change up to 10% with respect to the nominal value. Here, we measure the resonant frequency of the cantilever in order to calculate the thickness, and thus obtain a more accurate surface stress value.²¹

The cantilever was functionalized by adapting a procedure that does not require previous modification of the antibody and it enables an efficient way to bind the antibodies to the surface without involving the antigen-binding regions.³³ The antibodies are oriented in-plane by harnessing the kinetic differences among the ionic adsorption processes and the covalent reactions. It is expected that the in-plane orientation of the antibodies optimizes the surface stress variation upon the antigen binding due to the surface proximity. Importantly, the immobilization procedure keeps the biological activity of most of the

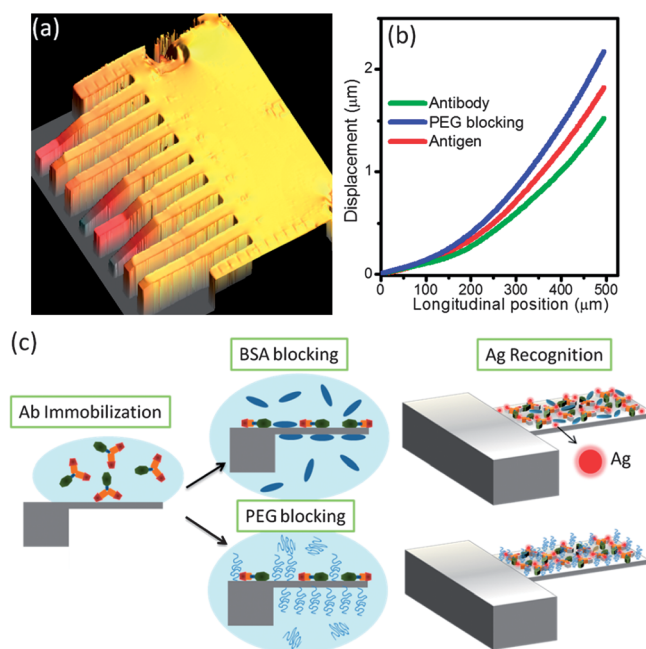


Fig. 1 (a) Static images of the cantilever arrays used in this work obtained by scanning laser beam deflection microscopy. (b) Displacement profiles of a cantilever after each functionalization step: antibody immobilization, blocking (with PEG) and antigen binding. (c) A schematic cartoon of the covalent oriented antibody immobilization, surface blocking with PEG or BSA and antigen (HRP) recognition.

antibodies, as confirmed by our assays of enzymatic activity of the horseradish peroxidase (the antigen used in our work). In order to achieve differential functionalization between the cantilever opposite sides, a droplet of the antibody solution was deposited on the cantilever chip after activation of the carboxyl groups on the surface (Fig. 1(c)). The surface tension of the droplet prevents wetting of the opposite cantilever surface. This was confirmed by fluorescence microscopy by immobilization of fluorescently labelled anti-peroxidase (see the Materials and methods section). Fig. 2(a)–(c) show the bright field and fluorescence images of the front and back sides of the cantilevers. The fluorescence images clearly confirm that the droplet of antibody solution deposited on the front side of the cantilever does not wet the cantilever's opposite side. Importantly, the immobilization method eliminates the widely extended need of a gold-coating on one cantilever side to differentiate opposite cantilever surfaces. The gold coating is a source of non-specific cantilever bending due to variations in the temperature.³⁴ Fig. 2(d) shows the cantilever responsivity to temperature variations in comparison with a cantilever coated with a 25 nm thick gold layer. The insensitivity of the cantilever response to the temperature makes the end-point detection for point-of-care applications simpler and cheaper as specialized equipment for accurate temperature control is not required.

After the antibody immobilization, the cantilever was incubated with a blocking agent to fill the voids between the surface anchored antibodies and minimize the non-specific binding. This step is crucial for optimizing the sensitivity and specificity of the assay, and it is particularly vital in label-free biosensors,

in which the transducer that converts the physical signal into a measurable signal may interpret non-specific adsorption as molecular recognition events (false positive). In addition, HRP is a small molecule (44 174 dalton glycoprotein with a size of about 4 nm) that can easily step into any free space present on the sensor surface, and thus its non-specific adsorption can contribute to the sensor noise. In this work, we use bovine serum albumin (BSA) and poly(ethylene glycol) (PEG) derivatized with an amine linker^{35,36} (Fig. 1(c)) as blocking molecules. Both molecules are covalently bound on the non-coated cantilever regions. BSA is a blocking molecule traditionally used in label-based immunoassays with a reported blocking efficiency of about 90%.³⁷ PEG is a synthetic polymeric molecule that recently has been used for preventing non-specific adsorption and it is considered among blocking agents as one of the most resistant to protein adsorption.^{36,38}

3.2 Control experiments

Two kinds of control assays were tested. In the first control, an antibody nonspecific to HRP was immobilized on the surface. In the second control, the anti-HRP was immobilized on the surface and the sample was exposed to lysozyme. The lysozyme is an enzyme smaller than the HRP with a molecular mass of 14 307 Da, a size of about 3 nm and it is not recognized by the anti-HRP. Its small size and high positive charge makes the sensor exposure to lysozyme a stringent control, as this molecule can easily penetrate in the voids between antibodies on the surface and bind to the negatively charged surface if the blocking step is not effective.

Fig. 3 shows the histogram of the surface stress variation after the control experiments. The cantilever response to non-specific antibody–antigen interactions is of $-2.8 \pm 6.4 \text{ mN m}^{-1}$

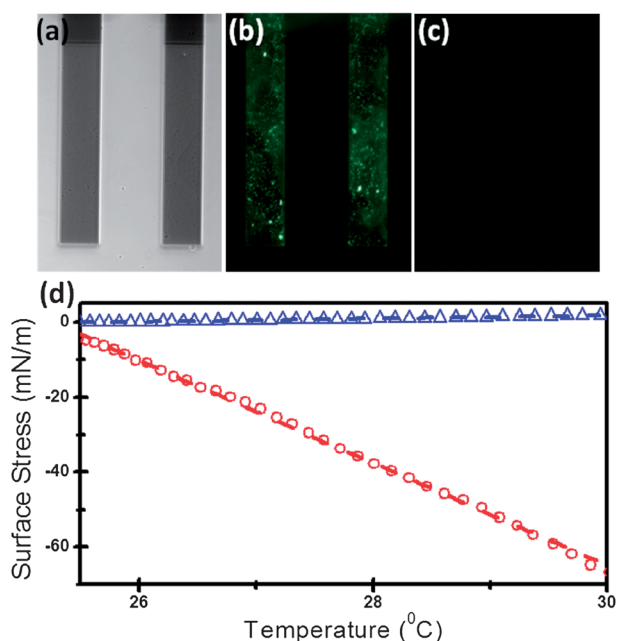


Fig. 2 Bright-field (a) and fluorescence images of the front (b) and back side (c) of two microcantilevers functionalized with the fluorescently labelled antibody. The fluorescence image confirms that the antibodies are only immobilized on the cantilever side where the antibody solution was deposited. (d) Comparison of the temperature effect of a microcantilever with (red circles) and without (blue triangles) a gold layer (25 nm thick).

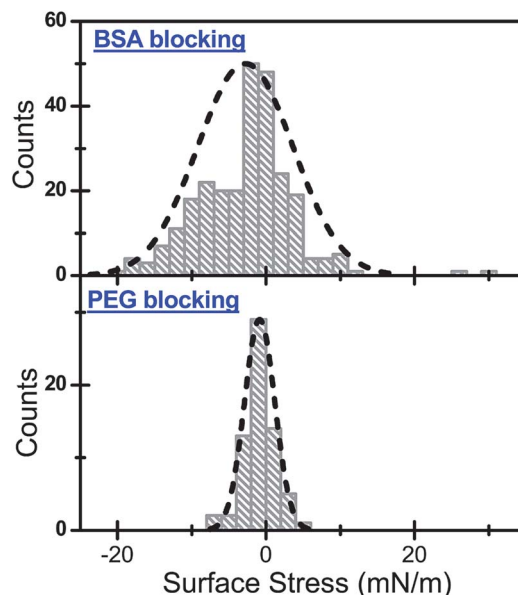


Fig. 3 Statistics of surface stress values obtained in the control experiments for the microcantilevers blocked with BSA and PEG. The control experiments include the exposure of cantilevers functionalized with a nonspecific antibody to HRP, and exposure of the cantilevers functionalized with anti-HRP to lysozyme.

for the blocking based on BSA, whereas the cantilever response is significantly smaller, $-0.86 \pm 2.05 \text{ mN m}^{-1}$, for the cantilevers blocked with PEG. Statistically non-significant differences were found between the two kinds of control. These results indicate a three times better blocking efficiency of the PEG with respect to the BSA. The better antifouling efficiency of PEG can be explained as a result of two effects. Firstly, BSA is a larger molecule (prolate ellipsoid with dimensions of $14 \times 4 \text{ nm}$) than the HRP and lysozyme, with an approximate size of 4 and 3 nm, respectively. Thus, it is expected the generation of gaps of few nanometers during the random chemisorption of BSA on the cantilever surface where small molecules can non-specifically bind. Secondly, PEG achieves a grafting density of the order of 10^{13} cm^{-2} and hence adopts a brush conformation that efficiently repels non-specific adsorption.³⁹

3.3 Effect of the antibody surface density and blocking strategy on the specificity of the cantilever response

The surface density of biomolecular receptors plays a crucial role in the sensitivity and specificity of surface-stress based nanomechanical biosensors.^{26,27,40–42} Here, we characterized the cantilever response for three antibody concentrations: $5 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$ and $250 \mu\text{g mL}^{-1}$. The surface density for each concentration was measured by radiolabelling the antibodies (see Materials and methods) and is shown in Fig. 4 (top graph). For the lowest concentration ($5 \mu\text{g mL}^{-1}$), the antibody surface density is of about 2200 molecules per μm^2 , whereas for $50 \mu\text{g mL}^{-1}$ the surface density increases to 6400 molecules per

μm^2 , approximately. For the highest antibody concentration ($250 \mu\text{g mL}^{-1}$), the surface density slightly increases by 5% indicating that the saturation of the surface was achieved. Fig. 4 (middle and bottom graphs) shows the mean and standard deviation values of the surface stress induced by $1 \mu\text{g mL}^{-1}$ of HRP and the control experiments for the BSA and PEG blocking strategies. The results offer multiple aspects and implications that will be discussed below:

(1) The molecular recognition of HRP is evidenced as a surface stress distribution wider than that of the control experiments. However, the surface stress distribution for the HRP and control experiments partially overlaps. This overlapping is much higher when BSA-based blocking strategy is used, as a consequence of the significant nonspecific adsorption discussed in the previous section.

(2) A second lecture is that the surface stress induced by molecular recognition exhibits a high variability and can be either positive or negative for similar assays. This arises from the short range nature of the non-linear interactions between macromolecular complexes at coverage near saturation. Both the randomness in the adsorption process for a given surface density and the unavoidable fluctuations in the surface density lead to fluctuations in the distribution of the gap separations between complexes that albeit small, $\sim 0.01 \text{ nm}$, lead to significant variability in the surface stress. We illustrate this phenomenon by Monte-Carlo simulations of adsorption of spheres on a flat surface (see Appendix).

(3) The optimal surface density, at which the overlapping between the surface stress induced by the HRP and control experiments is minimized, occurs for the antibody concentration of $50 \mu\text{g mL}^{-1}$. At the lowest antibody concentration of $5 \mu\text{g mL}^{-1}$, the antigen-induced surface stress differs little from the controls, especially when BSA blocking is used. This arises from the small changes in the intermolecular interactions upon the antigen capture at this low surface density of antibodies. Indeed, whereas for $50 \mu\text{g mL}^{-1}$, the gap between antibodies ranges from contact to 2 nm, for $5 \mu\text{g mL}^{-1}$, these gaps are between 10 and 20 nm, for which the interactions on the cantilever surface are very small. Surprisingly, an antibody concentration of $250 \mu\text{g mL}^{-1}$, that only implies an increase in the surface density of about 5% with respect to the antibody concentration of $50 \mu\text{g mL}^{-1}$, brings about a significant decay in the specificity of the nanomechanical response. This phenomenon was recently reported in an analysis of the effect of antibody concentration in planar configuration in the binding capability of a human prostate-specific antigen.⁴³ It was found that 6000 antibodies per μm^2 was the surface density in which the amount of antigen bound to the interfacial immobilized antibody reached a maximum. At higher densities, binding efficiency showed a steady decline due to the crowding or overlapping of antibody fragments. This work is in agreement with the optimal antibody concentration found here, of $50 \mu\text{g mL}^{-1}$, that corresponds to 6400 molecules per μm^2 .

In conclusion, the specificity of the end-point nanomechanical immunodetection critically depends on the surface density of receptors and blocking strategy. The highest specificity to the HRP is found in cantilevers functionalized with an

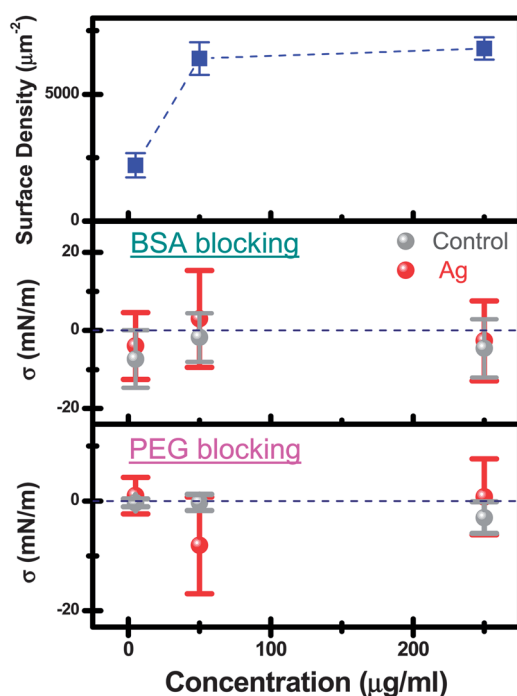


Fig. 4 Top: antibody surface density as a function of the antibody concentration used for the immobilization. The measurements were performed by the radiolabelling technique. Middle and bottom: mean and standard deviation of the surface stress induced by the specific HRP binding (red) and control experiments (grey) for cantilevers blocked with BSA and PEG, respectively.

antibody concentration of $50 \mu\text{g mL}^{-1}$ and subsequently blocked with PEG. Hereinafter, we restrict our study to these optimized functionalization conditions. We believe that the ability of PEG to fill the voids between the antibodies generating a high osmotic pressure assists in the required “elastic” connectivity between the molecular receptors on the cantilever. This mechanism enables a significant cantilever bending upon molecular recognition as a consequence of the collective and cooperative deformation of the biological layer formed on the cantilever surface.^{27,40–42}

3.4 Reliability of the immunodetection

Fig. 5 shows the histograms of the surface stress variation due to the HRP binding and control assays. The cantilever response to non-specific antibody–antigen interactions is of $-0.36 \pm 1.69 \text{ mN m}^{-1}$, whereas for HRP the surface stress predominantly is compressive (negative), following a probability distribution that approximately follows an exponential distribution $\sim e^{\sigma/\Delta\sigma}$ with $\Delta\sigma \approx -11 \pm 1 \text{ mN m}^{-1}$ (dashed line). The negative sign of the surface stress implies that the antigen–antibody recognition brings about in-plane repulsive forces. This is an expected result as the HRP molecules must penetrate through the PEG polymer brush to achieve the antibody binding sites at the cantilever surface. This increases the osmotic pressure and steric interactions in the biorecognition layer.

In order to quantitatively assess the specificity and sensitivity of the assay, we calculate the receiver operating characteristic (ROC) curve of our data. This statistical tool used in signal detection theory has been implemented in clinical medicine to evaluate the accuracy of the diagnosis tools and classify individuals into healthy and disease groups.⁴⁴ The ROC curve plots

the sensitivity (defined as the true positive rate) versus 1 – specificity (defined as the false positive rate) varying the decision threshold that separates positive from negative detection over the entire range of results observed. We plot the ROC curve for several cut-off values of the sensor response defined as

$$R = \frac{\sigma - \langle\sigma_c\rangle}{\Delta\sigma_c} \quad (1)$$

where σ is the obtained surface stress, $\langle\sigma_c\rangle$ is the mean surface stress in the control experiments and $\Delta\sigma_c$ is the surface stress standard deviation in the control experiments. A sketch of the definition of true and false positives/negatives for a surface stress threshold is illustrated in Fig. 6(a). The ROC curve is shown as circles in Fig. 6(b). The ROC curve ranges from the diagonal line that goes from (0,0) to (1,1) (insensitive biosensors) to the horizontal line that starts at (0,1) (perfect biosensor). The performance of the assay can be quantified by calculating the area under the ROC curve that ranges from 0.5 (insensitive biosensor) to 1 (perfect biosensor). The area under the curve with no previous data rejection is 0.842. The ROC analysis has also been practical for other label free biosensors.^{45,46} It is important to note that ROC plots are usually applied for the assessment of well-established techniques such as ELISA in the clinical practice. However, we notice that there are scarce studies that statistically evaluate the reliability of emerging novel biosensors and nanobiosensors with purified samples, which is the logical first step before pointing out to the implementation in the clinical practice. Here, we propose the use of the ROC curve as a valuable tool to quantify the reliability of nanomechanical biosensors, as a decision tool to set the threshold value for a positive detection and for comparison with other studies and other techniques when the same antigen–antibody pair is used.

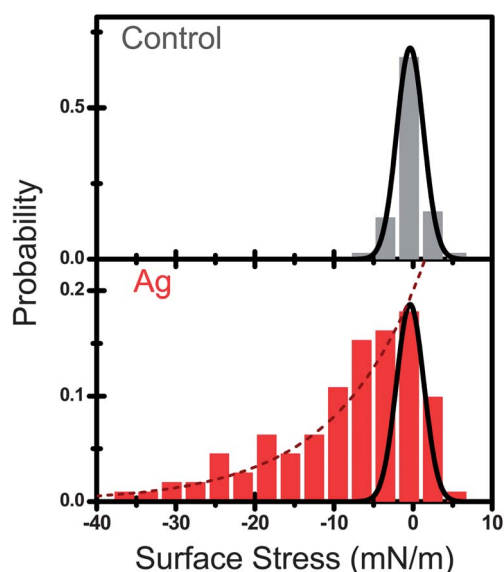


Fig. 5 Histograms of the surface stress values induced by the specific HRP binding and control experiments for cantilevers functionalized with $50 \mu\text{g mL}^{-1}$ of anti-HRP and subsequently blocked with PEG (bars). The continuous line is the Gaussian fit to the surface stress generated in the control experiments. The dashed line in the bottom graph is the fitting of the surface stress induced by HRP to an exponential function (read related text).

3.5 Statistical correlation between the surface stress induced by the analyte and the functionalization steps

The analysis of the pattern of the surface stress generated by the antibody immobilization, PEG blocking step, and antigen binding for each cantilever can provide further insights on how to address the improvement of the immobilization methods to enhance the sensitivity and selectivity of the immunoassays, and alternatively, it can also be used for designing a quality test, or immobilization failure test, that enables the exclusion of cantilevers in which the immobilization features are not satisfactory to obtain a specific antigen response. We have performed bicubic interpolation of the surface stress induced by the antibody (X -coordinate), PEG chemisorption (Y -coordinate) and the antigen response, R , defined in eqn (1) (Z -coordinate) (Fig. 7). The antigen response map follows a well-defined pattern that allows predicting the conditions with higher probability of obtaining a specific and sensitive antigen response. Thus, we can design an immobilization failure rule. Here, we have chosen those cantilevers in which (i) the PEG-induced surface stress was higher than 3 mN m^{-1} , or (ii) the antibody-induced surface stress was higher than 40 mN m^{-1} . The restriction implies to exclude 56 cantilevers out of 111. We

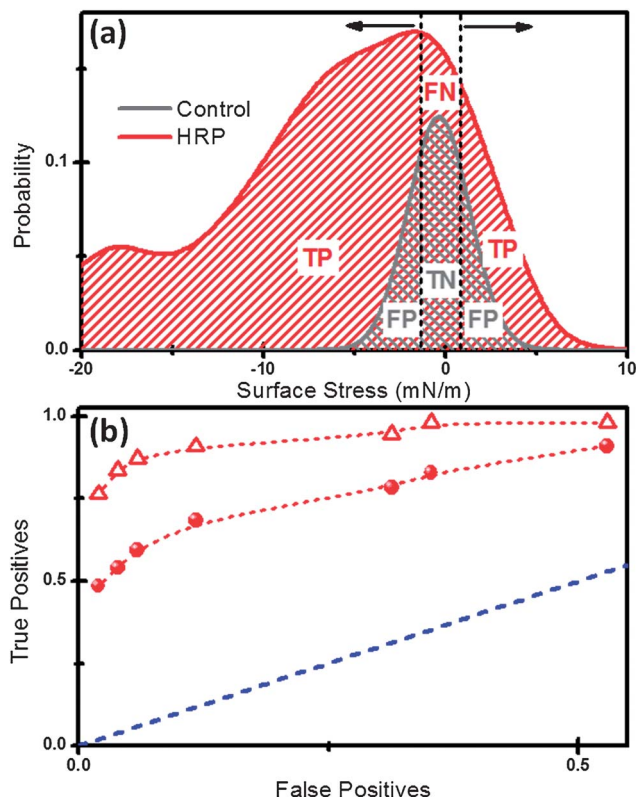


Fig. 6 (a) Histogram of the surface stress induced by the specific HRP binding and control experiments shown in Fig. 4. The vertical lines represent a hypothetical detection threshold that determines the rate of true positives (TP), false negatives (FN), true negatives (TN) and false positives (FP). (b) ROC curve for all the data from cantilevers functionalized with $50 \mu\text{g mL}^{-1}$ of anti-HRP and subsequently blocked with PEG (circles), and for selected data based on the statistical criteria described in the text (triangles). An assay with perfect discrimination of the control and analyte experiments has a ROC curve that passes through the upper left corner, whereas the theoretical plot for a test with no value is a diagonal line from (0,0) to (1,1) (blue dashed line). Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test.

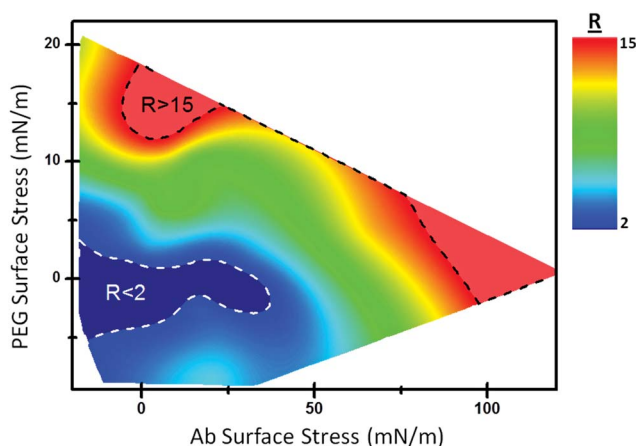


Fig. 7 Colour-intensity 2D map of the bicubic interpolation of the antigen response, R (as defined in the text), as a function of the antibody-induced surface stress and the PEG induced surface stress. The contour lines for $R = 2$ and $R = 15$ are plotted as a white and black dashed lines, respectively.

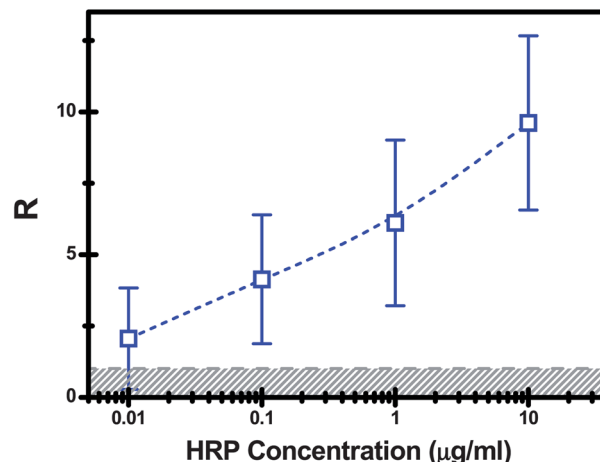


Fig. 8 The cantilever response (R) to the HRP versus the HRP concentration. The dashed region represents the standard deviation of the cantilever response in the control experiments. HRP responses in this grey region cannot be discriminated on statistical basis.

check the validity of this approach by recalculating the ROC curve for the cantilevers that passed this quality test (Fig. 6(b), triangles). The area under the curve of the resulting ROC significantly increases from 0.842 to 0.936.

Adopting the quality control described above, we have measured the detection limit of the current nanomechanical immunoassay. Fig. 8 plots the cantilever response versus the HRP concentration. We achieve a detection limit of 10 ng mL^{-1} (250 pM). Although direct comparison of this detection limit with assays performed with different antigen–antibody pairs may not be taken strictly, the found value is at least two orders of magnitude smaller than that obtained with well-established label-free biosensors, QCM and SPR, with similar samples in direct assays.^{47,48} We have found a detection limit of 5 ng mL^{-1} (125 pM) in our ELISAs with the same antigen–antibody pair. In addition, the achieved sensitivity implies a significant improvement of three orders of magnitude in sensitivity with respect to previously reported immunodetection of proteins (cardiac biomarkers) with nanomechanical biosensors in which reference cantilevers were used to minimize false positives.⁴⁹

4 Conclusions

Here we analyse the potential of nanomechanical biosensors based on the surface stress for the label-free end-point detection of immunoreactions. We have chosen as antigen the horse-radish peroxidase. The study establishes the guidelines to achieve sensitive and specific detection of HRP with a practical protocol including quality control for the standardized decision making process. The read-out and data analysis can be easily automated. We achieve 90% of true positives versus 9.4% of false positives and a sensitivity of 10 ng mL^{-1} (0.25 nM). This demonstrates that nanomechanical biosensors can detect controlled protein samples in purified media with high reliability, sensitivity and specificity, which is the previous and required step before aiming applications in the clinical field,

where the targeted molecules are in complex media such as saliva, urine and serum, and large nonspecific adsorption is expected.

We have addressed the statistical analysis of more than 3000 values of the surface stress generated during the functionalization, detection and control experiments in 1012 cantilevers.

As we have found multiple sources of variability in the experiments for given immobilization conditions such as differences in: cantilevers (surface, mechanical properties), surface density, elastic coupling of the receptors to the cantilever which critically depends on the features of the silane layer (*e.g.* thickness, mechanical properties, adhesion, defects, *etc.*), quality of the reagents that depends on the quality control of the manufacturer, *etc.* Some of these differences come from human error factors, others are uncontrollable or unavoidable. For instance, as illustrated in Appendix, the surface stress can exponentially vary for similar surface densities of adsorbates with minute variations in the arrangement of the adsorbates. Since this effect arises from the inherent randomness of the adsorption process, this source of surface stress noise cannot be eliminated. This is a peculiar feature of nanomechanical biosensors with respect to most of the biosensors that measure the added mass, directly or indirectly. We found this inherent variability does not preclude the development of a sensitive and specific assay with microcantilever sensors, but it must be accounted for. To do so we propose measuring previous surface stress history during the functionalization steps and calculating the statistical correlation of these data with the target induced surface stress. Given these findings, a statistical analysis of surface stress measurements in a significant number of cantilevers is demanding to attain reliable and reproducible results with cantilever biosensors. The approach that we have adopted here can be extrapolated to other targets or other class of nanosensors.

Appendix

In this Appendix, we raise the fundamental and general question whether the intrinsic randomness in the molecular adsorption processes is a significant source of surface stress noise, here referred to as adsorption surface stress noise. In order to contribute to the answering of this issue, we propose here a simple Monte-Carlo simulation. Notice that it is not the aim of this simulation to obtain the surface stress induced by the interactions involved in biomolecular recognition that poses a formidable complexity and it is still the subject of specialized debate.

Let us adopt the following assumptions: (i) the biomolecules that adsorb on the surface are rigid spheres with a diameter of 10 nm and our surface is a square with a side of 1000 nm, (ii) the interaction between the spheres and the surface is high, so the spheres get stuck to the surface at their first contact, (iii) the interactions between spheres in the liquid are governed by electrostatic double layer repulsion, van der Waals attraction and Born repulsion, and (iv) the interactions in air between the surface grafted molecules are governed by van der Waals, Born repulsion and steric repulsion. The steric interactions play a key

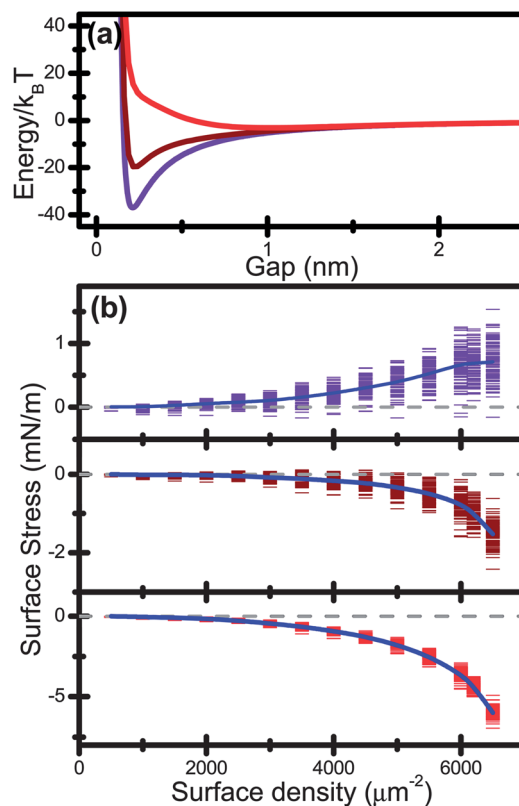


Fig. 9 (a) Interaction energy between spherical particles used for the calculation of the surface stress in the Monte-Carlo simulations. The potential used for modeling the interactions between proteins (particles) include hard sphere repulsion, van der Waals attraction and steric repulsion. The calculations were performed with three strengths of steric interactions, zero (violet line), intermediate (brown line) and significant (red line). (b) Surface stress values obtained in the Monte-Carlo simulations for the three potentials plotted in (a). The simulations are run 100 times for each surface density of particles on the surface. The results are plotted as horizontal dashes. The mean value is plotted as a blue line.

role in the surface stress as shown experimentally.^{26,27,40} Due to the complexity of the interfacial structures, it is difficult to mathematically model the steric potential. In the case of soft matter, steric potentials are referred to as “soft” as they change gradually with interparticle separation. Here we have used the Hayter–Penfold/Yukawa model (HPY) to describe the steric potential.⁵⁰ For each simulation, we calculate the surface energy in air using the above interactions. Then we stretch the surface by an infinitesimal amount, and the variation of the energy per surface unit and strain unit gives the surface stress. For each chosen value of surface density, we run 100 simulations that give rise to different final configurations of the particles on the surface. From these data we calculate the mean and deviation of the surface stress as a function of the particle surface density.

Fig. 9(a) shows the three interparticle potentials in air used in our simulations. All share the van der Waals attraction and “hard” Born repulsion. However, the strength of the “soft” steric potential is gradually increased from zero (violet line) to a value at which the interactions between particles are dominated by steric interactions (red line). The values of the surface stress for each potential as a function of the surface density are shown in Fig. 9(b). In all cases, the absolute value of the surface stress

monotonically increases with the surface density approximately following a functional form of either an exponential or a power law. Thus, the surface stress changes more quickly as the surface density approaches saturation. Notice that the randomness of the adsorption process makes the surface saturate at about 7000 particles per μm^2 that corresponds to a coverage of 56%. This value is below the maximum packing of 91% that can be achieved with a well-ordered close-packing of spheres. The steric interaction strength modulates the coverage-dependence of the surface stress that goes from small positive values (tensile) without steric interactions to significant negative values (compressive) when steric forces dominate the interparticle interactions. The most relevant finding is the broad distribution of surface stress values for a given value of surface density, which confirms our initial hypothesis about the existence of an intrinsic surface stress noise in the adsorption processes. The deviations of the surface stress near saturation coverage ranges from 50% for non-steric interactions to 10% for the potential with the highest strength in the steric interactions. Moreover, since the surface density undergoes fluctuations between experiments, the steep variation of the mean surface stress with the surface density at high-packing (that is the situation that one finds to attain a highly responsive surface stress biosensor) amplifies the noise of the surface stress induced by physisorption and molecular recognition processes.

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